

Citotoxic activity evaluation of essential oils and nanoemulsions of *Drimys angustifolia* and *D. brasiliensis* on human glioblastoma (U-138 MG) and human bladder carcinoma (T24) cell lines *in vitro*

Article

Madson R. F. Gomes,^{*,1} Roselena S. Schuh,¹ Ana L. B. Jacques,¹ Otávio A. Augustin,¹ Sérgio A. L. Bordignon,² Daiane O. Dias,³ Regina G. Kelmann,³ Leticia S. Koester,³ Marina. P. Gehring,⁴ Fernanda B. Morrone,⁴ Maria M. Campos,⁵ Renata P. Limberger¹

Received 4 Jun 2012

Accepted 24 Sep 2012

Available online 22 Nov 2012

¹Laboratório de Toxicologia, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Brazil,

²Laboratório de Botânica, Centro Universitário La Salle, Canoas, Brasil,

³Laboratório de Desenvolvimento Galênico, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Brazil,

⁴Laboratório de Farmacologia Aplicada, Faculdade de Farmácia, Pontifícia Universidade Católica do Rio Grande do Sul, Brazil,

⁵Instituto de Toxicologia e Farmacologia, Pontifícia Universidade Católica do Rio Grande do Sul, Brazil.

Keywords:

bladder carcinoma
Drimys angustifolia
Drimys brasiliensis
essential oils
glioblastoma
nanoemulsions

Abstract: The species *Drimys angustifolia* Miers and *D. brasiliensis* Miers, commonly known as "casca-de-anta", have in their leaves essential oils that can confer cytotoxic effects. In this study, we evaluated the cytotoxic effects of the volatile oils from these two species. We also proposed a nanoemulsion formulation for each of the species and assessed the *in vitro* cytotoxicity on U-138 MG (human glioblastoma) and T24 (human bladder carcinoma) cell lines. The plant chemical composition was evaluated by gas chromatography coupled to mass spectrometer. Furthermore, the nanoemulsions were prepared and characterized. Our results showed that; bicyclogermacrene (19.6%) and cyclocolorenone (18.2%) were the most abundant for the *D. angustifolia* oil and *D. brasiliensis* oil, respectively. Both nanoemulsions, *D. angustifolia* and *D. brasiliensis* appeared macroscopically homogeneous and opalescent bluish liquids, with nanometric mean diameters of 168 nm for *D. brasiliensis* and 181 nm for *D. angustifolia*. The polydispersity indices were below 0.10, with an acid pH of 4.7-6.3, and negative zeta potentials about -34 mV. The results of transmission electron microscopy showed that droplets are present in the nanometer range. Only the *D. brasiliensis* oil was efficient in reducing the cell viability of both U-138 MG (42.5%±7.0 and 67.8%±7.8) and T24 (33.2%±2.8, 60.3%±1.6 and 80.5%±8.8) cell lines, as assessed by MTT assay. Noteworthy, similar results were obtained with cell counting. Finally, *D. brasiliensis* oil incubation caused an increase of annexin-V and propidium iodide population, according to evaluation by cytometry analysis, what is characteristic of late apoptosis. The results presented herein lead us to consider the potential therapeutic effects of the essential oils and nanoformulations as novel strategies to inhibit tumor growth.

ISSN 0102-695X

DOI 10.1590/S0102-695X2012005000136

Introduction

The genus *Drimys* presents the widest geographical distribution of the Winteraceae family includes the several species such as *D. winteri*, *D. granadensis*, *D. brasiliensis* and *D. angustifolia* (Ehrendorfer et al. 1979; Lorenzi & Abreu Matos,

2002). In Brazil, the genus is found from Bahia to Rio Grande do Sul (Backes & Nardino, 1999) and occurs in two species, *D. angustifolia* Miers (DA) and *D. brasiliensis* Miers (DB), commonly known as "casca-de-anta." Brazilians use these plants in folk medicine as antiscorbutic, stimulant, antispasmodic, anti-diarrheal, antipyretic and antibacterial (Almeida, 1993). They are

also employed to treat asthma and bronchitis and it has insecticidal properties (Da Cunha et al., 2001). These plants are characterized by the presence of flavonoids and essential oils (Limberger et al. 2007).

Essential oils are volatile, natural complex compounds characterized by a strong scent, which are formed by aromatic plants as secondary metabolites (Bakkali et al., 2008). Some essential oils appear to exhibit particular medicinal properties that have been claimed to cure some organ dysfunctions or systemic disorders. Many plants containing essential oils present various biological activities, including cytotoxicity effects (Yunes, 2007). However, it is important to develop a better understanding of their biological actions for new applications in human health (Silva et al., 2003).

The cytotoxicity appears to include membrane damage, the leakage of macromolecules and lysis (Lambert et al., 2001; Oussalah et al., 2006). Essential oils seem to have no specific cellular targets, because of the great number of constituents, (Carson et al., 2002). As typical lipophilic substances, they pass through cytoplasmic membrane, breaking the structure of their different layers of polysaccharides, fatty acids and phospholipids and finally permeate them (Di Pasqua et al., 2006; Turina et al., 2006). One way to improve the entry of essential oils into the cells is through formulations that favor the access, as the nanoemulsions.

These formulations are submicron sized emulsions that have been studied as drug carriers for improving the delivery of therapeutic agents and have some advantages as higher surface area, allied to low irritant effects, being suitable for human therapeutic purposes as cancer therapy (Shah, 2010).

Approximately 2% of cancer deaths can be attributed to brain tumors and the complex biology of this tumor, and particularly the ability of tumor cells to invade adjacent normal brain tissue diffusely beyond the surgical resection. It leads to local recurrence and a poor prognosis for the glioma patients (Stylli & Kaye, 2006). In other types of tumor, as human bladder carcinoma, the majority of the patients may present superficial bladder tumors, although 20-40% of the bladder cancers develop invasive tumors (Hinata et al., 2003).

The aim of this study was to evaluate the chemical composition and propose a nanoemulsion formulation for the species (DA and DB), as well as to assess the cytotoxicity of the essential oils and formulations in U-138 MG (human glioblastoma) and T24 (human bladder carcinoma) cell lines. It is worthy to note the uniqueness of the study with these tumor cell lines, as well as the use, for the first time, of a nanoemulsion formulation for species (DA and DB), in order to test anti-cancer actions.

Materials and Methods

Plant material

Drimys angustifolia Miers, Winteraceae, was collected at the Center for Research and Nature Conservation Pró-Mata (CPCN Pró-Mata), São Francisco de Paula, Rio Grande do Sul, Brazil and *D. brasiliensis* Miers was collected in São Jerônimo, Rio Grande do Sul, Brazil. Both were identified by Sérgio Augusto de Loreto Bordignon. Voucher specimens were deposited in the ICN Herbarium (UFRGS, Porto Alegre), under numbers ICN 123644 and ICN167795, respectively.

Extraction of essential oils

The essential oils were obtained from 100 g of DA or DB fresh leaves by hydrodistillation for 4 h using a Clevenger-type apparatus. The yields were calculated to both oils.

Oils constituents

Quantitative and qualitative analyses were performed by capillary gas chromatography (GC) and GC/mass spectrometry (MS), respectively. The GC analysis was performed in a chromatograph (Shimadzu GC-17A) equipped with a Shimadzu GC 10 software, using two fused silica capillary columns (30 m×0.25 mm×0,25 µm) with different polarity, one coated with DB-5. Injector and detector temperatures were set at 220 °C; the oven temperature was programmed from 60-300 °C to DB-5 column. Helium was employed as carrier gas (1 mL/min). The percentage compositions were obtained from electronic integration measurements using flame ionization detection without taking into account relative response factors. The GC-MS analysis was performed in the same apparatus and chromatographic conditions as described above, using a quadrupole MS system (QP 5000) operating at 70 eV. Compound identification was based on a comparison of retention indices (determined relatively to the retention times of a series of n-alkanes) and mass spectra with those of authentic samples and/or with literature data (Limberger et al., 2007).

Nanoemulsions preparation

The nanoemulsions were prepared by high-pressure homogenization method. Firstly, a coarse emulsion was prepared: the oily phase, composed by 4% (w/w) of essential oil (DA or DB), 5% (w/w) of Medium-Chain Triglycerides (MCT)¹ and 1% (w/w) of lipophilic emulsifier Span 80[®] was added into water phase containing Tween 20[®] (1%, w/w) and water (made up to 100%, w/v) and kept under moderate magnetic stirring

for 5 min at room temperature. Afterwards, the coarse emulsions were individually subjected to high-pressure homogenization (EmulsiFlex-C3®, Avestin, Canada) for six cycles at pressure of 750 bars to obtain the final nanoemulsion. The blank nanoemulsions were prepared similarly, but without the addition of essential oils (DA or DB). The nanoemulsions were named as Nano DA or Nano DB, when prepared with DA or DB essential oils, respectively.

Characterization of formulations

Particle size, polydispersity indices and zeta potential analysis

The particle size and polydispersity index were measured by photon correlation spectroscopy after adequate aliquot dilution of the samples in purified water (Zetasizer ZS90 Nanoseries, Malvern Instruments, UK). The zeta potential values were measured using the same instrument at 25 °C, after dilution of the 10 µL in 10 mL of NaCl (1 mM) and ultra-filtration (0.22 µm). All analyses were performed in triplicate.

pH determination

The pH values of the formulations were determined directly in the samples using a calibrated potentiometer (Digimed, São Paulo, Brazil), at room temperature. The analyses were performed in triplicate.

Morphological analysis

Morphological analyses were carried out by transmission electron microscopy (TEM; Jeol, JEM 1200 ExII, Centro de Microscopia-UFRGS), operating at 80 kV. The samples were placed on a carboncoated copper grid (200 mesh) overlaid with 1% formwar in chloroform, stained by 2.0% uranyl acetate aqueous solution.

Cell lines and cell culture

The human bladder carcinoma (T24) and human glioblastoma (U-138 MG) cell lines were obtained from ATCC (Rockville, Maryland, USA). T24 cells were cultured in RPMI/10% FBS and U-138 MG cells in DMEM/10% FBS, at 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5% CO₂ in air.

MTT cell viability assay

The cells were seeded at 6 x 10³ cells per well in 96-well plates and grown for 24 h. The cells were treated with nano DA, nano DB, DA oil and DB oil at the following concentrations 125, 250 and 500 µg mL⁻¹ for

24 h. At the end of this period, the medium was removed, the cells were washed with CMF and 100 µl of MTT solution (MTT 5 mg mL⁻¹ in PBS in 90% DMEM/10% FBS) was added to the cells and incubated for 3 h. The formazan crystals were dissolved with 100 µl of dimethyl sulfoxide (DMSO). The absorbance was quantified in 96-well plates (Spectra Max M2e, Molecular Devices) at 595 nm.

Cell counting

The cells were seeded at 18x10³ cells per well in 24-well plates. On the second day, the cells were treated with DB oil (125, 250 and 500 µg mL⁻¹). After 24 h, the medium was removed and 200 µl of trypsin/EDTA solution was added to detach the cells, which were counted in hemocytometer.

Annexin V/PI flow cytometry staining technique

The cells were seeded at 4x10⁴ cells per well in 24-well plates and grown for 24 h. The cells were treated with DB oil (250 and 500 µg mL⁻¹) for 24 h. Dead cells were quantified by annexin V-FITC-propidium iodide (PI) double staining, using FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen) 24 h after treatment, according to the manufacturer's instructions. Experiments were performed on BD FACSCanto II flow cytometer and the results were analyzed using FlowJo Software (Tree Star).

Results

Composition of essential oils

The hydrodistilled essential oil from *Drimys angustifolia* Miers and *D. brasiliensis* Miers, Winteraceae, leaves yielded 0.4% and 0.3%, respectively. The chemical composition is presented in Table 1 and Table 2. Twenty-nine constituents were identified, accounting for 82.9% of the oil for DA and thirty-three constituents for the 91.9% of the oil for DB. The oil of DA was characterized by monoterpenoids (46.1%) and sesquiterpenoids (28.3%), being the most abundant compound bicyclogermacrene (19.6%), followed by sabinene (9.7%) and mircene (5.2%). The oil of DB was characterized by sesquiterpenoids (41.4%) and monoterpenoids (42.6%), cyclocolorone being the most abundant (18.2%), followed by terpinen-4-ol (8.7%) and alpha-gurjunene (6.9%).

Physicochemical properties of formulation

Both nanoemulsions, DB and DA appeared

macroscopically homogeneous and opalescent bluish liquids. The physicochemical characteristics of the formulations are presented in Table 3. The formulations showed nanometric mean diameters of 168 nm for DB and 181 nm for DA, as well as polydispersity indices below 0.10 indicating an adequate homogeneity of these systems. The values obtained are in agreement with those usually reported for nanoemulsions produced by high-pressure homogenization (Nuchuchua et al., 2009; Sakulku et al., 2009). The formulations demonstrated acid pH (4.7-6.3) and negative zeta potentials (about -34 mV). The negative zeta potential values presented by the samples are related to the presence of hydrophilic emulsifier (Tween 20®), showing a negative surface density of charge due to the presence of oxygen atoms in the molecules (Flores et al., 2011). The results of transmission electron microscopy can be observed in Figure 1. The Figure reveals homogeneous and spherical particles. The results corroborate to the droplet size analysis, showing that droplets are present in the nanometer range, with particle size of less than 200 nm.

Table 1. Composition percentage* of monoterpene hydrocarbons of essential oils from fresh leaves of Southern Brazilian *Drimys angustifolia* and *D. brasiliensis*.

Retention Index		Composition percentage		
DB5	Lit.(Adams, 2007)	<i>D. angustifolia</i>	<i>D. brasiliensis</i>	
Monoterpene hydrocarbons		36.9	25.3	
929	939	α -pinene	3.3	3.2
933	953	camphene	-	0.1
965	976	sabinene	9.7	3.6
968	980	β -pinene	3.9	3.3
985	991	myrcene	5.2	6.5
989	1011	δ -3-carene	-	0.2
1010	1018	α -terpinene	1.9	tr
1014	1031	β -phelandrene	3.9	-
1017	1026	<i>p</i> -cymene	-	2.1
1021	1031	limonene	-	0.6
1031	1040	(<i>Z</i>)- β -ocimene	4.6	0.6
1031	1050	(<i>E</i>)- β -ocimene	tr	0.1
1050	1062	γ -terpinene	3.0	3.2
1080	1088	terpinolene	1.4	1.8

*Expressed by GC/MS analysis on DB5 column.

Cell viability

We tested the effect of nano DA, nano DB, DA oil and DB oil in two different tumor cells. Cell viability was not altered when the human glioma cell line U-138 MG was treated with either nano DA or nano DB in different concentrations (Figure 2A). As shown in Figure 2B, the treatment with DA oil significantly reduced the

viability of this cell type only at the highest concentration of 500 $\mu\text{g mL}^{-1}$ (35.0% \pm 7.7). Of note, DB oil significantly diminished the U-138 MG cell viability when treated at 250 and 500 $\mu\text{g mL}^{-1}$ (42.5% \pm 7.0 and 67.8% \pm 7.8, respectively) when compared to control.

Table 2. Composition percentage* of oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes and phenylpropanoids of essential oils from fresh leaves of Southern Brazilian *Drimys angustifolia* and *D. brasiliensis*.

Retention Index			Composition percentage	
DB5	Lit. (Adams, 2007)		<i>D. angustifolia</i>	<i>D. brasiliensis</i>
Oxygenated monoterpenes			9.2	17.3
1023	1033	1,8-cineole	0.6	4.4
1094	1098	linalool	1.3	1.8
1168	1177	terpinen-4-ol	6.4	8.7
1170	1183	<i>p</i> -cimen-8-ol	-	0.3
1181	1189	α -terpineol	0.9	2.1
Sesquiterpene hydrocarbons			23.5	15.9
1399	1404	isocaryophyllene	-	0.2
1400	1409	α -gurjunene	-	6.9
1406	1418	β -caryophyllene	0.8	0.1
1426	1459	aromadendrene	0.9	0.4
1438	1461	alloaromadendrene	tr	1.4
1452	1477	γ -muurolene	0.4	-
1469	1480	germacrene D	1.5	0.4
1487	1494	bicyclogermacrene	19.6	6.5
1512	1524	δ -cadinene	0.3	-
Oxygenated sesquiterpenes			4.8	25.5
1554	1565	ledol	-	1.7
1566	1576	spathulenol	1.4	2.9
1572	1581	caryophyllene oxide II	1.4	-
1610	1619	10- <i>epi</i> - γ -eudesmol	0.5	1.0
1639	1641	τ -muurolol	tr	0.9
1640	1653	α -cadinol	0.3	-
1745	1759	drimenol	1.2	0.8
1748	1763	cyclocolorone	-	18.2
Phenylpropanoids			8.5	7.9
1278	1285	safrole	3.5	1.4
1511	1520	myristicin	5.0	6.5

*Expressed by GC/MS analysis on DB5 column.

Regarding the human bladder carcinoma T24 cell line (Figure 2B), DA oil reduced T24 cell viability at 250 and 500 $\mu\text{g mL}^{-1}$ (36.7% \pm 6.8 and 73.7% \pm 2.7, respectively), whereas DB oil displayed inhibitory effects in all tested concentrations (33.2% \pm 2.8, 60.3% \pm 1.6 and 80.5% \pm 8.8). Regarding to the treatments with nanoemulsions, DA nano significantly altered the

viability of the T24 lineage in the concentrations of 250 and 500 $\mu\text{g mL}^{-1}$ (26.1% \pm 8.4 and 65.7 \pm 6.2, respectively). However, it did not alter significantly the cell viability of the U-138 MG lineage in any of the concentrations tested. DB nano only altered significantly T24 lineage in the concentration of 500 $\mu\text{g mL}^{-1}$ and it did not altered U-138 MG lineage in the concentrations tested.

Table 3. Particle size, polydispersity index (PDI), zeta potential and pH of nanoemulsions (expressed as mean \pm S.D.).

Formulation	Particle size (nm)	PDI	Zeta potential (mV)	pH
DA	181.0 \pm 1.8	0.08 \pm 0.00	-33.5 \pm 0.5	6.37 \pm 0.1
DB	168.5 \pm 3.0	0.08 \pm 0.04	-34.9 \pm 0.8	4.73 \pm 0.3

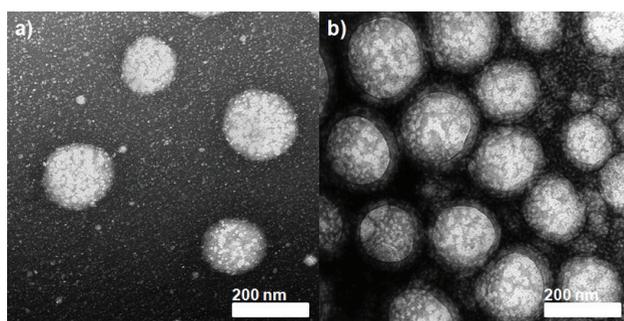


Figure 1. Transmission electron photomicrograph of the nanoemulsions DA at 150 k (b) and DB at 75 k (a).

Cell counting

As shown in Figure 3, the U-138 MG cell number was significantly reduced when the lineage was treated with DB oil in concentrations of 250 or 500 $\mu\text{g mL}^{-1}$ (46.3% \pm 9.1 and 76.0% \pm 2.5, respectively). The T24 lineage had a reduction in the cell number only when

treated with the highest DB oil concentration of 500 $\mu\text{g mL}^{-1}$ (67.1% \pm 3.9).

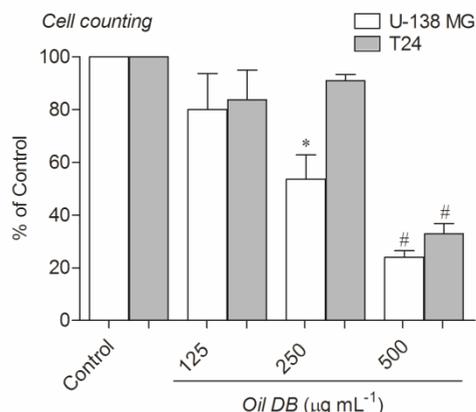


Figure 3. Effect of treatment with DB oil on cell number of U-138 MG and T24 human cells after 24 h. The cell number of the control group (not treated cells) was considered 100%. The experiment was carried out at least three times in triplicate. Each column represents the mean \pm SEM. * p <0.05 and # p <0.001 for comparison versus control, as determined by ANOVA/Tukey-Kramer test.

Annexin V/PI flow cytometry staining technique

As shown in Figure 4, the DB oil at 250 or 500 $\mu\text{g mL}^{-1}$ induced a clear increase in annexin V-FITC/IP positive population on both U-138 MG and T24 cell lines (45.4% \pm 1.9, 53.6% \pm 1.2 and 29.2% \pm 2.4, 38.8% \pm 4.7; respectively).

Discussion

Generally, the major chemical components

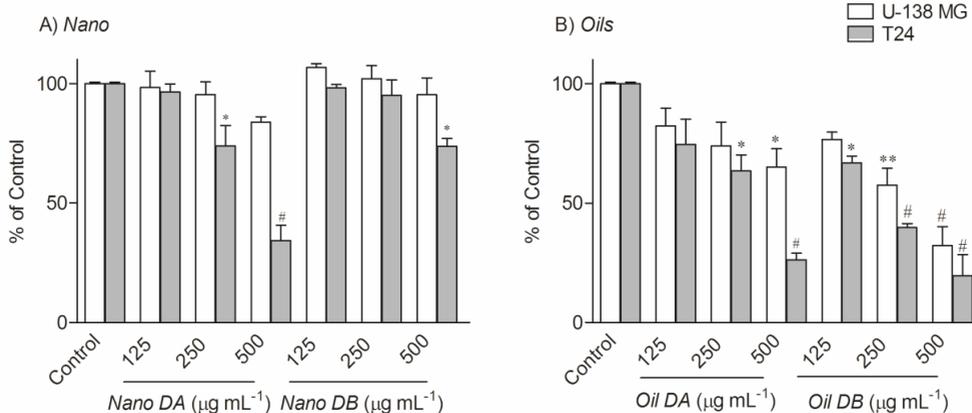


Figure 2. A. Effect of treatment with nano DA or nano DB (125, 250 and 500 $\mu\text{g mL}^{-1}$) on cell viability of U-138 MG and T24 cell lines, after 24 h; B. Effect of treatment with DA oil and DB oil (125, 250 and 500 $\mu\text{g mL}^{-1}$) on cell viability of U-138 MG and T24 cell lines, after 24 h. The experiments were carried out at least three times in triplicate. Each column represents the mean \pm SEM. * p <0.05, ** p <0.01 and # p <0.001 for comparison versus control, as determined by ANOVA/Tukey-Kramer test.

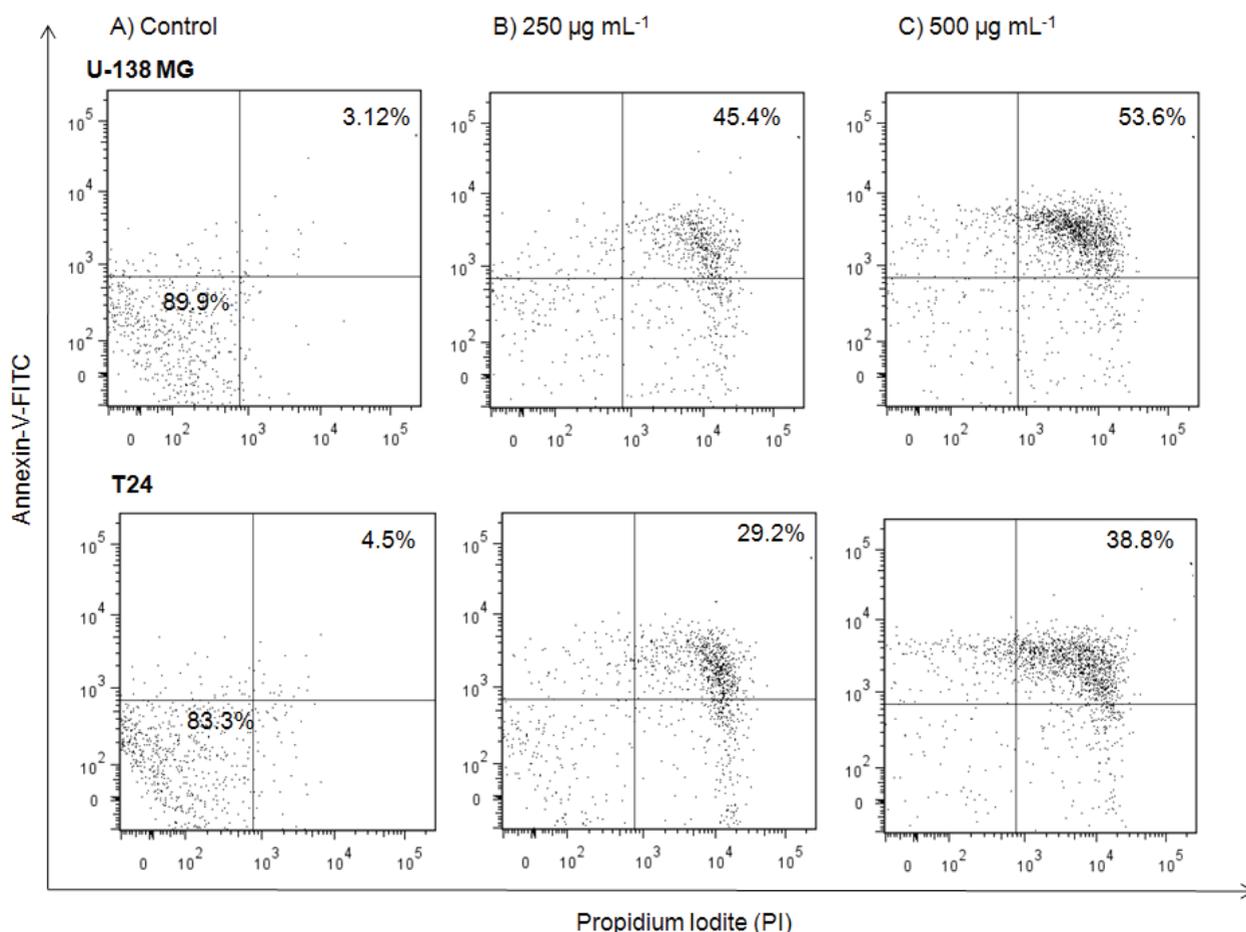


Figure 4. Annexin V-FITC/PI positive U-138 MG or T24 cells 24 h after treatment with DB oil 250 or 500 µg mL⁻¹. Each sample has 20.000 cells. Data shown is representative of at least 2 independent experiments.

determine the biological properties of the essential oils (Croteau et al., 2000; Betts, 2001; Bowles, 2003; Pichersky et al., 2006). Taking into account the composition of the oils studied herein, we can observe that in *Drimys angustifolia* Miers, Winteraceae, oil, there is a prevalence of hydrogenated compounds (bicyclogermacrene, sabinene and myrcene), with a total of 14.0% oxygenates (Table 1 and 2).

Otherwise, *Drimys brasiliensis* Miers oil has oxygenated compounds as major components (cyclocolorenone and terpinen-4-ol), with a total of about 45.5% (Table 1 and 2). It is known that the cytotoxic activity is enhanced by the presence of oxygenated compounds, as they display functional groups of aldehydes, phenols and alcohols (Bruni et al., 2003; Sacchetti et al., 2005). This could explain the evident cytotoxic effects obtained by DB oil in our study (Figure 2B). All components have low molecular weight and high lipophilicity which favors the penetration through membranes, destroying polysaccharides, fatty acids and phospholipids, and disrupting the structure of the membrane, thereby increasing the cell damage (Bakkhali

et al., 2008).

An interesting result obtained in the cell viability analysis was the increased cytotoxic activity of the DB oil when compared to DA oil. The DA oil, was able to reduce U-138 MG cell viability only at the higher tested concentration (500 µg mL⁻¹), while DB oil showed a significant cell viability reduction with half of the effective DA oil concentration, indicating the effectiveness of this oil (Figure 2B). Interestingly, both oils had a cytotoxic activity in the T24 cell line, but DB oil showed a higher reduction in the cell viability when compared to DA oil. It decreased cell viability at the lowest tested concentration of 125 µg mL⁻¹ (Figure 2B). Studies have revealed evidence of the involvement of the purinergic system in bladder tumorigenesis, particularly ecto-5'-NT/CD73, the enzyme responsible for AMP hydrolysis (Rockenbach et al., 2011). Increasing evidence indicates that gastrin-releasing peptide (GRP) acts as an autocrine growth factor for brain tumors. However, it remains unclear whether the cAMP/protein kinase A (PKA) signaling pathway plays a role in mediating the mitogenic effects of GRP. (Farias et al., 2008). Those

features of the tumor cells may explain the different behavior of oils in cytotoxicity.

The nanoemulsions formulated were designed to achieve better penetration associated with low molecular weight and lipophilicity of the components of the oils, increasing the availability within tumor cells (Tiwari & Amiji, 2006). Thus, we observed that the nanoemulsions have not been able to reduce cell viability in the U-138 MG cell line (Figure 2A). However, nanoemulsions were able to reduce cell viability in T24 lineage at 250 and 500 $\mu\text{g mL}^{-1}$ of DA oil or 500 $\mu\text{g mL}^{-1}$ of DB oil. In both nanoemulsions, there was only 4% of DA or DB oils, and the real concentration in the nanoemulsion are 10 and 20 $\mu\text{g mL}^{-1}$, of DA oil and 20 $\mu\text{g mL}^{-1}$ of DB oil, maintaining the same activity with low concentration of oils of both species (Figure 2A). The major components as well as the presence of varying amounts of oxygenated components oils, as described above and the mechanisms involved in genesis and spread of tumor are different for both cell lines and these features may explain the differences between the cytotoxic concentrations for each cell type.

By the fact that only the DB oil was efficient in reducing cell viability on both cell lines on at least two concentrations, we decided to continue the investigation only with this oil (Figure 2B). The data of cell counting followed the same pattern observed in cell viability assay with the two cell lineages, where DB oil reduced cell number in a concentration dependent manner (Figure 3).

Apoptosis and necrosis represent two fundamental types of cell death. Apoptosis cell death is a highly regulated physiological process of programmed cell death and plays an important role in the homeostasis of different tissues in response to various stimuli (Bergantini et al., 2005; Bras et al., 2005) and it is characterized by not showing inflammatory response (Yasuhara et al., 2003). While necrosis is usually viewed as a more or less passive cell rupture caused by excessive exogenous damage, apoptosis is an active process consisting of highly coordinated molecular events leading to a sequence of morphological changes and is accompanied by modifications of the cellular surface. The cell loses its surface anti-phagocytic "don't-eat-me" signals (mediated mostly by CD31 and CD47 glycoproteins) and exposes ligands designating the cell for phagocytosis (e.g. phosphatidylserine) (Ravichandran & Lorenz, 2007; Erwig & Henson, 2008). Moreover, several extracellular molecules bind to the apoptotic cells (e.g. complement factors) facilitating phagocytosis (Ravichandran & Lorenz, 2007; Elliott & Ravichandran, 2010). Importantly, the early apoptotic cells preserve their plasma membrane integrity to retain the potentially harmful cellular contents inside. If not successfully taken up by phagocytes, apoptotic cells proceed to the phase of late apoptosis (termed also secondary necrosis) when the plasma membrane becomes permeable for small

molecules (e.g. propidium iodide (PI)) and subsequently also for macromolecules (proteins) (Silva et al., 2008). The leakage of intracellular molecules during secondary necrosis provokes an inflammatory response (Elliott & Ravichandran, 2010). The establishment of cell death type induced by a treatment is important to suggest which mechanism could be involved in the process.

Therefore, lastly we decided to investigate the type of death induced by DB oil on U-138 MG and T24 cell lines. We have shown that the death induced by DB oil after 24 h has late apoptotic features (Figure 4), what means that the death by apoptosis induced by DB oil occurs before 24 h. Cells on late apoptotic death have both translocation of phosphatidylserin and defragmentation of DNA, thus stain positive for FITC-Annexin V and Propidium iodide (Cornelissen et al., 2002). Several signals modulate the proliferation, survival and cell death (Foster, 2008; Mester & Redeuilh, 2008). In general, toxic or damaging stimuli can trigger cell death by necrosis or apoptosis, which are differentiated by the morphology and cellular pathways (Foster, 2008; Kuwana & Newmeyer, 2003). Accordingly, treatments that induce tumor cells death by apoptosis are considered better, because apoptosis is a natural cell death so did not results in inflammation, which is characteristic of necrosis cell death (Kerr et al., 1995; Boujard et al., 2007).

Conclusions

In the results presented in this study, we showed for the first time the potential citotoxic activity of DA and DB species on human glioma (U-138 MG) and human bladder carcinoma (T24) cell lines. The nanoemulsions of the two oils were not active on the U-138 MG, but had significant cytotoxicity effects towards human bladder carcinoma cell line. Since the DB oil was more cytotoxic than the other oil on both cell lines, it was selected the first for further investigation of its citotoxic activity. It kept, the same standard on cell counting and inducing late apoptosis. Thus, DA and DB oils and nanoemulsions proved to be promising pharmacological tools in cancer treatment, but further studies still are needed to confirm this idea.

Acknowledgements

The authors thank Dr. Cristina Bonorino, Dr. Rafael Zanin for the use of the flow cytometer and CNPq.

Authors contributions

MRFG (PhD student) contributed in collecting plant sample and identification, confection of herbarium, running the laboratory work, analysis of the data and drafted the paper. MRFG, RSS, ALBJ, MPG, FBM,

MMC contributed to biological studies. SB contributed in plant identification and herbarium confection. MRFG, OAA and RPL contributed to chromatographic analysis. RGK, MPG, FBM, MMC and RPL contributed to critical reading of the manuscript. SB and RPL contributed to plant collection. DOD, RGK, LSK, FBM, MMC and RPL designed the study, supervised the laboratory work and contributed to critical reading of the manuscript.

References

- Adams, RP 2007. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy*. Carol Stream, Illinois: Allured Publishing Corporation.
- Almeida ER 1993. *Plantas medicinais brasileiras, conhecimentos populares e científicos*. São Paulo: Hemus.
- Backes A, Nardino M 1999. *Nomes populares e científicos de plantas do Rio Grande do Sul*. São Leopoldo: Unisinos.
- Bakkhali F, Averbeck S, Averbeck D, Idaomar M 2008. Biological effects of essential oils: A review. *Food Chem Toxicol* 46: 446-475.
- Bergantini APF, Castro FA, Souza AM, Feet-Conte AC 2005. Leucemia Mielóide Crônica e o Sistema Fas-FasL. *Rev Bras Hematol Hemoter* 27: 120-125.
- Betts TJ 2001. Chemical characterisation of the different types of volatile oil constituents by various solute retention ratios with the use of conventional and novel commercial gas chromatographic stationary phases. *J Chromatogr A* 936: 33-46.
- Boujrad H, Gubkina O, Robert N, Krantic S, Susin AS 2007. AIF-Mediated Programmed Necrosis: A Highly Regulated Way to Die. *Cell Cycle* 6: 2612-2619.
- Bowles EJ 2003. *Chemistry of Aromatherapeutic Oils*. Australia: Allen & Unwin.
- Bras M, Queenan B, Susin SA 2005. Programmed cell death via mitochondria: different modes of dying. *Biochemistry (Moscow)* 70: 231-239.
- Bruni R, Medici A, Andreotti E, Fantin C, Muzzoli M, Dehesa M 2003. Chemical composition and biological activities of Isphingo essential oil, a traditional Ecuadorian spice from *Ocotea quixos* (Lam.) Kosterm. (Lauraceae) flower calices. *Food Chem* 85: 415-421.
- Carson CF, Mee BJ, Riley TV 2002. Mechanism of action of *Melaleuca alternifolia* (tea tree) oil on *Staphylococcus aureus* determined by time-kill, lysis, leakage and salt tolerance assays and electron microscopy. *Antimicrob Agents Ch* 46: 1914-1920.
- Cornelissen M, Philippé J, De Sitter S, De Ridder L 2002. Annexin V expression in apoptotic peripheral blood lymphocytes: An electron microscopic evaluation. *Apoptosis* 7: 41-47.
- Croteau R, Kutchan TM, Lewis NG 2000. Natural products (secondary metabolites). In: Buchanan B, Grissem W, Jones R (Eds). *Biochemistry and Molecular Biology of Plants*. Rockville: American Society of Plant Physiologists, p. 1250-1318.
- Da Cunha FM, Fröde TS, Mendes GL, Malheiros A, Cechinel FV, Yunes RA, Calixto JB 2001. Additional evidence for the anti-inflammatory and anti-allergic properties of the sesquiterpene polygodial. *Life Sci* 70: 159-169.
- Di Pasqua R, Hoskins N, Betts N, Mauroello G 2006. Changes in membrane fatty acids composition of microbial cells induced by addition of thymol, carvacrol, limonene, cinnamaldehyde, and eugenol in the growing media. *J Agric Food Chem* 54: 2745-2749.
- Ehrendorfer F, Vienna IS, Gerhard GB 1979. Variation on the population, racial, and species level in the primitive relic angiosperm genus *Drimys* (Winteraceae) in South America. *Plant Syst Evol* 132: 53-83.
- Elliott MR, Ravichandran KS 2010. Clearance of apoptotic cells: implications in health and disease. *J Cell Biol* 189: 1059-1070.
- Erwig LP, Henson PM 2008. Clearance of apoptotic cells by phagocytes. *Cell Death Differ* 15: 243-250.
- Farias CB, Lima RC, Lima LO, Flores DG, Meurer L, Brunetto AL, Schwartzmann G, Roesler R 2008. Stimulation of proliferation of U138-MG glioblastoma cells by gastrin-releasing peptide in combination with agents that enhance cAMP signaling. *Oncology* 75: 27-31.
- Flores FC, Ribeiro RF, Ourique AF, Rolim CMB, Silva CB, Pohlmann AR, Beck RCR, Guterres, SS 2011. Nanostructured systems containing an essential oil: protection against volatilization. *Quim Nova* 34: 968-972.
- Foster I 2008. Cancer: A cell cycle defect. *Radiographics* 14: 144-149.
- Hinata N, Shirakawa T, Zhang Z, Matsumoto A, Fujisawa M, Okada H 2003. Radiation induces p53-dependent cell apoptosis in bladder cancer cells with wildtype-p53 but not in p53-mutated bladder cancer cells. *Urol Res* 31: 387-396.
- Kerr JFR, Gobe GC, Winterford CM, Harmon BV 1995. Anatomical methods in cell death. *Method Cell Biol* 46: 1-27.
- Kibbe, AH 2000. *Handbook of Pharmaceutical Excipients*. The American Pharmaceutical Association, Washington.
- Kuwana T, Newmeyer DD 2003. Bcl-2-family proteins and the role of mitochondria in apoptosis. *Curr Opin Cell Biol* 15: 691-699.
- Lambert RJW, Skandamis PN, Coote P, Nychas GJE 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *J Appl Microbiol* 91: 453-462.
- Limberger RP, Scopel M, Sobral M, Henriques AT 2007. Comparative analysis of volatiles from *Drimys brasiliensis* Miers and *D. angustifolia* L. (Winteraceae) from southern Brazil. *Biochem Syst Ecol* 35: 130-137.
- Lorenzi H, Abreu Matos FJM 2002. *Plantas Medicinais do Brasil*. Nova Odessa: Instituto Plantarum.

- Mester J, Redeuilh G 2008. Proliferation of breast cancer cells: regulation, mediators, targets for therapy. *Anticancer Agents Med Chem* 8: 872-885.
- Nuchuchua O, Sakulku U, Uawongyart N, Puttipatkhachorn S, Soottitantawat A, Ruktanonchai U 2009. In vitro characterization and mosquito (*Aedes aegypti*) repellent activity of essential-oils-loaded nanoemulsions. *AAPS PharmSciTech* 10: 1234-1242.
- Oussalah M, Caillet S, Lacroix M 2006. Mechanism of action of Spanish oregano, Chinese cinnamon, and savory essential oils against cell membranes and walls of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *J Food Protect* 69: 1046-1055.
- Pichersky E, Noel JP, Dudareva N 2006. Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science* 311: 808-811.
- Ravichandran KS, Lorenz U 2007. Engulfment of apoptotic cells: signals for a good meal. *Nat Rev Immunol* 7: 964-974.
- Rockenbach L, Bavaresco L, Farias PF, Cappellari AR, Barrios CH, Morrone FB, Battastini AMO 2011. Alterations in the extracellular catabolism of nucleotides are involved in the antiproliferative effect of quercetin in human bladder cancer T24 cells. *Urol Oncol-Semin O I*: doi.org/10.1016/j.urolonc.2011.10.009.
- Sacchetti G, Maietti S, Muzzoli M, Scaglianti M, Manfredini S, Radice M, Bruni R 2005. Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in food. *Food Chem* 91: 621-632.
- Sakulku U, Nuchuchua O, Uawongyart N, Puttipatkhachorn S, Soottitantawat A, Ruktanonchai U 2009. Characterization and mosquito repellent activity of citronella oil nanoemulsion. *Int J Pharm* 372: 105-111.
- Shah P, Bhalodia D, Shelat P 2010. Nanoemulsion: A pharmaceutical review. *Sys Rev Pharm* 1: 24-32.
- Silva J, Abebe W, Souza SM, Duarte VG, Machado MIL, Matos FJA 2003. Analgesic and anti-inflammatory effects of essential oils of *Eucalyptus*. *J Ethnopharmacol* 89: 277-283.
- Silva MT, do Vale A, dos Santos NM 2008. Secondary necrosis in multicellular animals: an outcome of apoptosis with pathogenic implications. *Apoptosis* 13: 463-482.
- Stylli SS, Kaye AH 2006. Photodynamic therapy of cerebral glioma-A review Part II-Clinical studies. *J Clin Neurosci* 13: 709-717.
- Tiwari SB, Amiji MM 2006. Nanoemulsions formulations for tumor-targeted delivery, nanotechnology for cancer therapy. United States: Mansoor M. Amiji, Taylor and Francis Group.
- Turina AV, Nolan MV, Zygadlo JA, Perillo MA 2006. Natural terpenes: self-assembly and membrane partitioning. *Biophys Chem* 122: 101-113.
- Yasuhara S, Zhu Y, Matsui T, Tipirneni N, Yasuhara Y, Kanemi M, Rosenzweig A, Martyn JA 2003. Comparison of comet assay, electron microscopy, and flow cytometry for detection of apoptosis. *J Histochem Cytochem* 51: 873-885.
- Yunes RA, Filho VC 2007. Química de produtos naturais, novos fármacos e a moderna farmacognosia. Santa Catarina: Univali Editora.

***Correspondence**

Madson Ralide Fonseca Gomes
 PPGCF, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul
 Av. Ipiranga, 2752, 6º andar, sala 605, 90610-000 Porto Alegre-RS, Brazil
 00140101@ufrgs.br
 Tel: 51 81023822, 49 99434368, 51 30596141, 51 33085297
 Fax: 51 33085243