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Biological assessment (antiviral and antioxidant) and acute toxicity of essential oils from *Drimys angustifolia* and *D. brasiliensis*

Madson Ralide Fonseca Gomes,*[*,1] Roselena Silvestri Schuh,[1] Ana Laura Bemvenuti Jacques,[1] Gilcéia G. Dorneles,[1] Jarbas Montanha,[2] Paulo Michel Roehe,[2] Sérgio Bordignon,[3] Eliane Dallegrave,[4] Mirna B. Leal,[5] Renata Pereira Limberger[1]

[1]Laboratório de Toxicologia, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Brazil,
[2]Laboratório de Virologia, Instituto de Ciências Básicas da Saúde, Departamento de Microbiologia, Universidade Federal do Rio Grande do Sul, Brazil,
[3]Laboratório de Botânica, Departamento de Morfologia Vegetal, Centro Universitário La Salle, Brazil,
[4]Centro de Informação Toxicológica do Rio Grande do Sul, , Fundação Estadual de Produção e Pesquisa em Saúde, Brazil.
[5]Laboratório de Farmacologia, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande do Sul, Brazil.

Article

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Abstract: The genus *Drimys* presents the widest geographical distribution of the Winteraceae family, which comprises seven genera and about 120 species. In Brazil, the genus is found from Bahia to Rio Grande do Sul and occur in two species, *Drimys angustifolia* Miers, and *D. brasiliensis* Miers, Winteraceae, popularly known as "casca-de-anta", characterized by the presence of flavonoids and essential oils. It is used in folk medicine as an antiscorbutic, stimulant, antispasmodic, anti-diarrheal, antipyretic, antibacterial, and against asthma and bronchitis, besides having insecticidal properties. In addition to the known biological activities, it is very important to explore new applications in the treatment of physiological disorders or diseases caused by parasites. Based on this information, in this study we propose to evaluate volatile oils of the species *D. brasiliensis* and *D. angustifolia*, as an antioxidant, using the model of the DPPH radical as an antiviral against human herpes virus type 1 (HSV-1) and acute toxicity *in vivo*. The two species were not able to reduce the DPPH radical and showed interesting antiviral activity, significantly reducing the virus titers *in vitro* assays. Regarding the *in vivo* toxicity in female Wistar rats, treatment with the two species showed interesting signs in animals such as salivation, ptosis, tremor, decreased motor activity. In addition the oils of *D. brasiliensis* to other signs, some animals showed increased urination and diarrhea.

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Introduction

The genus *Drimys* presents the widest geographical distribution of the Winteraceae family comprising seven genera and about 120 species. This genus comprises species *D. winteri* J.R. Forst. & G. Forst., *D. granadensis* L. f., *D. brasiliensis* Miers, and *D. angustifolia* Miers, which are classified by their morphological, anatomical and karyotypic characteristics (Ehrendorfer 1979; Lorenzi & Abreu Matos, 2002).

On the American continent, the area of occurrence of the genus extends from the southern tip of Argentina and Chile to Mexico, belonging to the genus *D. wintera* (Marchiori, 1997; Malheiros, 2005). In Brazil, the genus is found from Bahia to Rio Grande do Sul (Backes & Nardino, 1999) and occurs in two species, *D. angustifolia* and *D. brasiliensis*, popularly known as "casca-de-anta". Brazilians use it in folk medicine as an antiscorbutic, stimulant, antispasmodic, anti-diarrheal, antipyretic and antibacterial (Almeida, 1993; Winston, 1999), to treat asthma and bronchitis and it has insecticidal properties (Da Cunha et al., 2001). It is characterized by the presence of flavonoids and terpenoids (Witaicenis et al., 2007). Many plants containing essential oils present various biological

activities ranging from antiviral and antioxidant (Yunes & Filho, 2007).

Many authors have reviewed the benefits of using species of plants with antioxidant activity (Speroni & Scartezzini, 2000; Matkowski, 2008). Under stress, our body produces higher amounts of reactive oxygen species and this imbalance causes cell damage (Peuchant et al., 2004). Free radicals facilitate the development of cardiovascular, neurodegenerative and inflammatory diseases, and cancer. The antioxidant compounds contained in natural sources such as plants may therefore prevent certain diseases (Shahidi et al., 1992, Knekt et al., 1996).

With regard to viral infections, the human herpes virus type 1 (HSV-1) causes cold sores and is contracted in childhood and adolescence by direct oral contact and, if symptomatic, is characterized by orolabial or facial lesions. However, in recent studies, HSV-1 has emerged as one of the main agents causing genital herpes in some developed countries. It is one of the most prevalent infections in Brazil and worldwide (Clemens & Farhat, 2010). Other studies of plants containing volatile oils have been successful against HSV-1 (Hayashi et al., 1995; Reichling et al., 2005; Duschatzky et al., 2005). In Brazil, according to the National Therapeutic Form 2010, it only includes acyclovir as the main drug in the prophylaxis and treatment of HSV. There are few options for treatment, and therefore studies and investment in research and the discovery of new antiviral agents are of paramount importance (Fonseca, 1999).

As for medicinal use, both extracts and pure substances besides being pharmacologically active need to be safe and it is therefore essential to evaluate possible toxic effects. Pure substances and extracts should be evaluated pre-clinically for their potential toxicity as a predictor of possible acute and chronic adverse effects on reproduction or on neurological development, long before the initiation of more advanced phases which include the screening clinical trials. Regulatory agencies such as Anvisa (National Agency for Sanitary Vigilance), FDA (Food and Drug Administration), EPA (Environmental Protection Agency) require as preliminary tests of toxicity *in vivo*, among others, acute toxicity, subchronic and reproduction tests. Currently, the OECD (Organization for Economic Co-operation and Development), advocates internationally accepted protocols for such biological assays (Barros & Davinos 2003).

Thus, the chemical study of volatile oils of the species *D. angustifoli*a and *D. brasiliensis*, research on new biological activities and evaluation of their toxicity allow us to learn more about their therapeutic potential, as well as their possible adverse effects, thus increasing the safety of their use by the population. For this purpose, in this study, we evaluated the volatile oils of the two species, as an antioxidant using the DPPH assay, as compared to the antiviral HSV-1 and acute toxicity using Wistar rats.

Materials and Methods

Plant material

Collection of the DA and DB leaves

Drimys angustifolia Miers, Winteraceae (DA) was collected at the Center for Research and Nature Conservation Pró-Mata, São Francisco de Paula-RS, Brazil and *D. brasiliensis* Miers, Winteraceae (DB), was collected in São Jerônimo-RS, 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 DA and DB 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 and another one with carbowax 20 M. Injector and detector temperatures were set at 220 and 250 °C, respectively; the oven temperature was programmed from 60-300 °C to DB-5 column and 60-230 °C to carbowax one at 3 °C/min. 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 (Barrero et al., 2000; Adams, 2001; Limberger et al., 2007).

Assays

Evaluation of antioxidant activity

The antioxidant activity of essential oils was

evaluated quantitatively against the stable radical DPPH (2,2-diphenyl-1-picrylhydrazyl). Spectrophotometric measurements of the consumption of this radical were performed in the presence of oils. Dilutions (10, 25, 50, 100, 200, 300, 400 and 500 µg/mL) were made for both of them, added to cuvettes with ethanol solution of DPPH (molar absorption coefficient 517 nm: 11500M-1 cm-1). Absorbance was measured immediately after mixing in a UV-visible spectrophotometer at λ=517 nm, with measurements every 1 s for 600 s. The experiments were performed in triplicate using quercetin (Merck®) as the reference antioxidant substance.

Evaluation of antiviral activity

Cells and viruses

African green monkey kidney cells (Vero cell line CCL-81-ATCC) were grown in Eagle's minimum essential medium (MEM) supplemented with 10% newborn calf serum, 2 µg/mL of Amphotericin B, 100 UI/ml of penicillin G and 100 µg/mL of streptomycin. A virus stock of herpes simplex virus type I, VR733 (ATCC) was prepared on Vero cells infected at a low multiplicity of infection (0,01), incubated for 1-2 days, frozen/thawed, before clearing the preparation by centrifugation at low speed to remove the cell debris. Virus stocks were maintained at -70 °C until use. Virus titration was performed by the Kärber method using 96-well microtiter plates (Payment & Trudel, 1989). The virus titer was estimated from cytopathogenicity and expressed as 50% tissue culture infectious doses (TCID50/50 µL). It was 105.25 TCID50/50 µL for strain VR733 (ATCC).

Evaluation of cytotoxicity

The solutions to be tested in the antiviral experiments were prepared by dissolving the extracts in PBS and when necessary, DMSO at sub toxic concentration (maximum of 0.019% was added).To assess the effect of oils on uninfected Vero cells, dilutions ranging from 5 mg/mL to 0.019 mg/mL in the maintenance medium, were added to Vero monolayers (using a 96-well microplate with 3.0×104 cells per well). After 72 h of incubation at 37 °C, cytotoxicity was determined by microscopic examination of the cell morphology in treated and untreated cultures. The maximum concentration at which no effect on the growth of host cell was observed (compared to controls) was considered as the maximum tolerated concentration (MTC) (Fritz et al., 2007). The MTC was determined for two volatile oils before proceeding to the antiviral activity assays. All assays were carried out in triplicate.

Antiviral activity

Dilutions of the extracts and compounds were prepared starting from the previously determined MTC. The samples from MTC, MTC/2, MTC/4, MTC/8 and MTC/16 were added on confluent 24 h old monolayer of Vero cells grown in microtiter tissue culture plates just before virus inoculation. One hundred tissue culture infection doses per 50 µL (TCID50) of the HSV-1 ATCC-VR733 strains were added to each of the wells. Toxicity controls, cell and virus controls titration were run simultaneously. Plates were incubated for 72 h at 37 °C, and then examined for the presence of cytopathic effects (CPE). Toxicity controls, cell and virus controls titration were run simultaneously. Plates were incubated for 72 h at 37 °C, and then examined for the presence of cytopathic effects (CPE). Acyclovir, Sigma, at 0.01 mg/mL was used as positive control for HSV-1 inhibition. In order to quantify the antiviral activity, the contents of the four identical wells were harvested, mixed, and clarified by low-speed centrifugation, and virus titration were performed on the supernatant fluid by Kärber method (Payment & Trudel, 1989), using a 96-well microtiter plates. The antiviral of each extract was determined as the viral titer reduction factor (log 10) by comparison with untreated controls (Fritz et al., 2007).

Acute toxicity

The acute toxicity tests were approved by the Ethics and Research Committee of the State Foundation of Production and Research in Health of Rio Grande do Sul, Brazil, with protocol number 003/2009.

They were performed according to the protocol of the OECD, which advocates the use of the Up or Down test (OECD 425) to estimate the median lethal dose (LD50), in addition to providing evidence of acute toxicity and possible target organs of toxicity. The following signs of toxicity were observed: skin changes, hair (piloerection), mucous membranes, eyes, circulatory and respiratory pattern, abnormal locomotion, reaction to stimuli, diarrhea, drooling, tremor, ptosis, changes in muscle tone, hypnosis, seizures and writhing. Mortality was observed during the first 24 h and daily for fourteen days after administration. The variation in body mass was also observed daily.

Animals

Adult, female Wistar rats (90 days old) were used, kept under standard vivarium conditions and previously adapted to them. Groups of up to five females, fasting, abstaining from solids for 8 h and from liquids for 2 h, were treated orally (gavage) (maximum of 10 mL/kg)

with a single dose of volatile oil from leaves of each species, starting with the dose of 175 mg/kg, then using doses of 550 and 1000 mg/kg, as needed. Each animal was observed for one min at 0, 5, 10, 15, 30, 60, 120, 180, 240, 300 and 360 min periods and after this, every 24 h of administration of treatments, until a period of fourteen days. At the end of the observation period, all survivors were euthanized and necropsied. If macroscopic alterations were observed at necropsy, histopathological studies of organs affected would be performed. Euthanasia was done under anesthesia with sodium thiopental at a dose of 40 mg/kg intraperitoneally, followed by opening the abdominal cavity and sequential sectioning of the diaphragm.

Statistical analysis

Statistical analysis for body weight gain was evaluated by analysis of variance (ANOVA) of repeated measures and relative organs weight was done by one-way ANOVA. Bonferroni's post hoc test for multiple comparison was applied.

Results and Discussion

Yield and chemical composition of oils extracted from leaves of D. angustifolia *and* D. brasiliensis

Volatile oils are very complex natural mixtures which can contain about 20-60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20-70%) compared to others components present in trace amounts (Croteau et al., 2000; Betts, 2001; Bowles, 2003; Pichersky et al., 2006).

The oil yield of *Drimys angustifolia* Miers, was 0.5% and of *D. brasiliensis* Miers, Winteraceae, 0.3%. The chemical analysis of the plant species in this study showed that 91.6% of the compounds of *D. angustifolia* were identified, the major ones being bicyclogermacrene with 19.7%, sabinene with 9.7% and terpinen-4-ol with 6.4%. These values agree with previous studies of our research group in which extraction was performed from fresh leaves (Limberger et al., 2007).

On the other hand in *D. brasiliensis*, we observed that 96.6% of compounds were identified, the major ones being cyclocolorenone with 18.3%, terpinen-4-ol with 8.4% and myristicin with 6.6%. These data corroborate previous data from our group where cyclocolorenone appears as major constituent and characteristic of oil of *D. brasiliensis* with a content of 32.3%, depending on the season and the collection of plant material and the part of the plant used (Limberger et al., 2007). Thus, as to the chemical composition, the species differ in the major component, to which to the biological activity is usually attributed (Bakkali et al., 2008).

Evaluation of antioxidant activity

The volatile oils of the plants were not able to consume the stable DPPH radical, and thus, in this model studying antioxidant activity the oils were inactive.

Evaluation of antiviral activity

The maximum non-toxic concentrations for the volatile oils of species *D. angustifolia* and *D. brasiliensis* were respectively, 156.3 µg/mL and 625 µg/mL (Table 1). Based on these concentrations antiviral tests were performed against HSV-1. With these values, the oil of D. angustifolia is four times more toxic than *D. brasiliensis* for the Vero Cells.

In both samples reduced the viral titer of DNA virus tested HSV-1 strain VR733 (ATCC). This effect on HSV-1 replication was quantified by infectious titer reduction after several round of multiplication, the culture being inoculated at 100 infectious doses. We can consider a reduction titer of 0.5 to 0.9 log10 moderate activity (Sidwell & Huffman, 1971) (Table 1). It should be mentioned that this is the first antiviral study performed with both species. Disruption of the HSV viral envelope by essential oils could also be observed by electron microscopy preventing the host cells from infection (Schnitzler et al., 2007).

Table 1. The antiviral activity of volatile oils (MTC) of *Drimys angustifolia* e *D. brasiliensis* determined as the viral titer reduction factor (log10) by comparison with untreated controls.

Sample	MTC (µg/mL)	Yields reduction (log10)[a]
		VR733 (ATCC) strain
D. angustifolia	156.3	0.75
D. brasiliensis	625.0	1.00

The data represent the mean±SD for four replicate samples of three separated experiments. a: When compared with controls. HSV-1 titers: 105,25 TCID50.µL

Acute toxicity

It is the first time that the acute toxicity study is performed only with the volatile oils of two species. There exist several variations of the up-and-down experimental design for estimating an LD50 and (OECD, 2008). Normally female rats are used (Lipnick et al., 1995). This is because literature surveys of conventional LD50 tests show that usually there is little difference in sensitivity between sexes, but in those cases where differences are observed, females are generally slightly more sensitive (OECD, 2000).

The acute effects of *D. angustifolia* and *D.*

brasiliensis essential oils after oral administration to rats are described in Table 2. The signs of ptosis, ataxia (cerebellar action) in addition to reduced motor activity are characterized as signs of central nervous system depressors that can be explained by lipid solubility and the size of the molecules contained in the oils, easily reaching the nervous system and leading to the onset of the signs observed (Bakkali et al., 2008). Another sign observed is salivation that can be explained by the action of oil components in muscarinic receptors (M3) of acetylcholine causing excessive oral secretions (De Almeida, 2006).

After statistical analysis, no significant differences were observed in body weight and organ mass (Figure 1).

The weight reduction as well as the decreased intake of food and water, suggest systemic toxicity (Mello et al., 1997; Mello, 2001). Thus, these signs were not observed in animals treated by the oils of the two species.

Finally, the experiment was carried out until a dose of 1000 mg/kg in order to reduce the amount of animals, since neither deaths nor signs were observed in animals treated that would justify increasing doses with the volatile oils to this dose (Cazarin et al., 2004; OECD, 2008). In another study, extracts of leaves and stems barks of *D. angustifolia* showed that deaths occurred only at doses above 3500 mg/kg (one male and one female in a group of five animals each) and at a dose of 5250 mg/kg (one male and three females in a group of five animals each). They presented exophthalmia at all doses tested (Witaicenis, 2007).

The test procedure described of value in minimizing the number of animals required to estimate the acute oral toxicity of a chemical. In addition to the estimation of LD50 and confidence intervals, the test allows the observation of signs of toxicity. This information is useful to determine the relevance of the test for the protection of human health (OECD, 2008). The estimated LD50 cannot be calculated because no deaths were observed during the experiment.

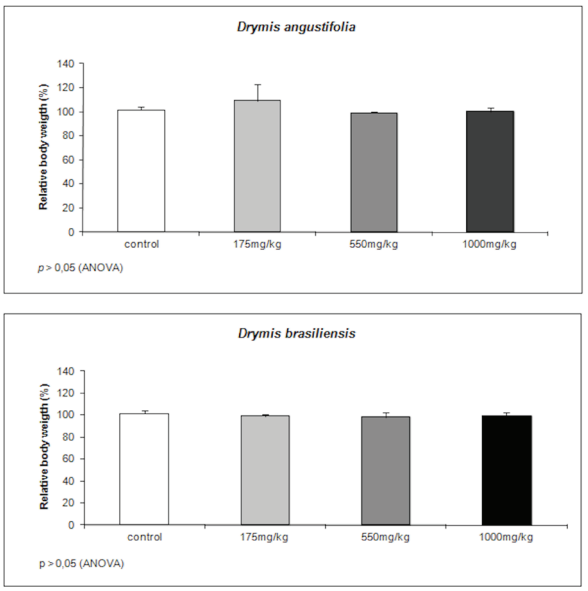

Figure 1. Relative body weight for essential oils of *Drimys angustifolia* and *D. brasiliensis.*

Table 2. Acute effects of *Drimys angustifolia* and *D. brasiliensis* essential oils after oral administration to rats (n=5 female per group).

Toxic signs	*D. angustifolia* (%)				*D. brasiliensis* (%)			
	Control	175 mg/kg	550 mg/kg	1000 mg/kg	Control	175 mg/kg	550 mg/kg	1000 mg/kg
Reduction of locomotor activity	0	60	100	100	0	100	100	100
Occurrence interval (h)	--	0-6	0-4	0-6	--	0-6	0-6	0-6
Ptosis	0	60	40	40	0	60	20	60
Occurrence interval (h)	--	0-1	1-3	0-2	--	0-3	0-1	0-3
Exophthalmia	0	0	40	60	0	20	20	40
Occurrence interval (h)	--	--	0-1	0-3	--	0-3	1-2	0-1
Urination	0	0	0	0	0	60	40	0
Occurrence interval (h)	--	--	--	--	--	0-1	0-1	--
Diarrhea	0	0	0	0	0	0	0	20
Occurrence interval (h)	--	--	--	--	--	--	--	0-1
Salivation	0	40	20	20	0	20	40	40
Occurrence interval (h)	--	0-1	0-1	0-1	--	0-1	0-1	0-4
Tremors	0	40	20	0	0	20	20	40
Occurrence interval (h)	--	0-1	0-1	--	--	0-1	0-1	0-4
Increased respiration rate	0	20	0	20	0	60	20	20
Occurrence interval (h)	--	2-3	--	0-1	--	0-1	0-1	0-1
Writhing	0	60	40	40	0	80	20	40
Occurrence interval (h)	--	0-1	1-2	0-1	--	0-1	1-2	0-1

%=The percentage refers to the proportion of animals in the group that expressed the respective signals at some point during the observational period (up to 6h).

Conclusions

The species showed that the chemical composition and major components of different. *Drimys angustifolia* Miers, showed higher antiviral activity than the species *Drimys brasiliensis* Miers, Winteraceae. However, from the toxicological standpoint, it presented higher toxicity in the cytotoxicity assays and showed no antioxidant activity using the DPPH model.

In acute toxicity the animals showed significant signs of toxicity as: reduction of locomotor activity, ptosis, exophthalmia, urination, diarrhea, salivation, tremors, increased respiration rate, writhing.

Thus, studies evaluating the toxicity are of paramount importance to ensure the use of medicinal plants by the population, because few studies are directed toward this area, even though the regulatory agencies require them to register drug products based on these plants.

Importantly, the investigation of the antioxidant, antiviral and acute toxicity of the volatile oils of two species were first performed, highlighting the importance and originality of the work, which will assist in understanding and rational use of these medicinal plants.

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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, GGD, JM, PMR, ED, MBL contributed to biological studies. SB and RPL contributed in plant identification and herbarium confection. MRFG, GGD and RPL contributed to chromatographic analysis. JM, ED, MBL and RPL contributed to critical reading of the manuscript. SB, GGD and RPL contributed to plant collection. JM, PMR, ED, MBL and RPL designed the study, supervised the laboratory work and contributed to critical reading of the manuscript.

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***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. +55 51 8102 3822, 49 99434 368, 51 3059 6141, 51 3308 5297
Fax: +55 51 3308 5243