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Article

Chemical Characterization of Essential Oils from *Drimys angustifolia* Miers (Winteraceae) and Antibacterial Activity of their Major Compounds

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Nos últimos anos, os óleos essenciais vêm sendo intensamente estudados como fonte natural de novos agentes antimicrobianos. Neste trabalho, os óleos essenciais das folhas e galhos de *Drimys angustifolia* do sul do Brasil foram obtidos por hidrodestilação e analisados por cromatografias gasosas com detector de ionização de chama (GC-FID) e com espectrômetro de massa (GC-MS). Os constituintes drimenol e biciclogermacreno foram isolados por cromatografia em coluna do óleo essencial dos galhos e folhas, respectivamente. Os óleos, os constituintes isolados e combinações destes foram testados contra bactérias Gram-(+) e Gram-(–). Os óleos essenciais foram mais ativos contra *Bacillus cereus*, com MIC (concentração inibitória mínima) de 125 e 250 μg mL^{-1} para os galhos e folhas, respectivamente, inibindo fortemente o crescimento bacteriano. Biciclogermacreno foi mais ativo que drimenol, fornecendo um valor de MIC de 167 μg mL^{-1} contra *B. cereus*. Não foi observado qualquer efeito sinérgico nas combinações testadas.

Essential oils have been extensively studied in recent years as a natural source of new antimicrobial agents. In this work, essential oils of leaf and branch from *Drimys angustifolia* growing in Southern Brazil were obtained by hydrodistillation and analyzed by gas chromatographies with flame ionization detector (GC-FID) and with mass spectrometer (GC-MS). Drimenol and bicyclogermacrene were isolated by column chromatography from branch and leaf essential oils, respectively. Oils, isolated compounds and combinations of them were assayed against Gram-(+) and Gram-(–) bacteria. The oils showed to be more active against *Bacillus cereus*, with minimum inhibitory concentration (MIC) 125 and 250 μg mL^{-1} for branch and leaf oils, respectively, strongly inhibiting bacterial growth. Bicyclogermacrene was more active then drimenol, providing a MIC value of 167 μg mL^{-1} against *B. cereus*. Synergism was not observed in any of the combinations tested.

Keywords: *Drimys angustifolia*, essential oils, drimenol, bicyclogermacrene, antibacterial activity

Introduction

The importance of essential oils and essential oil components relies mainly on their antimicrobial and antioxidant activities, besides their sensorial properties. Therefore, they have been used in food and feed additives, as flavoring and cosmetic ingredients and also as biocide. More recently, essential oils are gaining even more importance in animal feeding to substitute in-feed antibiotics.[1,2]

Drimys is a genus that contains about seven species distributed from the Strait of Magellan to Southern Mexico.[3]

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Three species grow wild in Brazil, *Drimys angustifolia* Miers, *D. brasiliensis* Miers and *D. roraimensis (A. C. Sm.) Ehrend. & Gottsb*. The first two occur in Southern Brazil and the last one in the Amazonia forest.[4] *D. angustifolia*, known as "casca d'anta", "casca-de-anta" and "cataia", occurs more restrictively at high altitudes.[3,5] In folk medicine, the genus *Drimys* is used as a stimulant and tonic, also as analgesic and anti-inflammatory, and to treat ulcer, diarrhea, cancer, affection of respiratory tract and as substitute for quinine in treating malaria and other feverish conditions.[5,6]

Some attention has been given to the chemical characterization and properties of essential oils and volatile constituents of this genus. Muñoz-Concha *et al.*[7] identified the sesquiterpene drimenol in leaf and stem bark of *D. winteri* and poligodial in leaf of *D. andina* in different populations from Chile. *D. angustifolia* from Rio Grande do Sul State, Southern Brazil, provided essential oil from leaf containing α-pinene (5.9%), sabinene (11.4%), myrcene (8.2%), terpinen-4-ol (7.5%), bicyclogermacrene (20.0%) and safrole (5.4%) as the major compounds. On the other hand, stem bark essential oil showed very low concentration of monoterpenes, with the sesquiterpenes bicyclogermacrene (25.4%), spathulenol (10.0%), drimenal (13.4%) and drimenol (26.2%) as the main constituents.[5] The essential oils from leaf, stem bark and fruit of *D. brasiliensis* were characterized by the presence of cyclocolorenone and bicyclogermacrene, being observed high concentration of monoterpenes in the former oil.[5]

The essential oil from a mixture of leaf and stem bark of *D. brasiliensis* with cyclocolorenone and bicyclogermacrene at concentrations of 30.4 and 11.8%, respectively, was tested against larvae of cattle tick *Rhipicephalus microplus* and dog *R. sanguineus*, giving promising results.[8]

D. brasiliensis collected in Mogi Guaçu City, São Paulo State, Southeastern Brazil, showed the following main constituents from leaf: sabinene, 9.5%; myrcene, 10.5%; limonene, 10.6% and cyclocolorenone, 28.3% and from stem bark: spathulenol, 22.9%; globulol, 6.3%; cyclocolorenone, 28.3% and the arylpropanoid myristicin, 5.2%.[9] In the search for natural fungicides to the protection of wheat crops, essential oil from bark of *D. winteri* was assayed by contact and as volatile against the soil fungus *Gaeumannomyces graminis*. Both procedures presented an elevated effect, especially the latter, which inhibited the growth of the fungus by 50% at 30.37 mg L^{-1}.[10]

Lago *et al.*[11] studied the chemical composition and anti-inflammatory property of the essential oils of *D. brasiliensis* from Campos do Jordão City, São Paulo State, Brazil. Although polygodial has been described as antinociceptive and anti-inflammatory agent from *D. winteri*,[12] the oil from stem bark showed superior activity than this dial sesquiterpene. The oil was characterized by the presence of monoterpenes (ca. 90%), with α-pinene as the major metabolite (39.5%). A possible chemomarker for this specie,[4] the sesquiterpene cyclocolorenone was not detected in the essential oils analyzed. Finally, *Portulaca oleraceae* L. and *Amaranthus hybridus* L. are annual weeds of tropical and subtropical crops, which have become cosmopolitan weeds distributed in a variety of soils and climates. The herbicide activity of the essential oil from leaf of *D. winteri* was evaluated against *P. oleraceae*, effectively reducing germination and showing phytotoxic effects on seedling growth; however being inactive against *A. hybridus*. Oxygenated sesquiterpenes constituted the predominant chemical group in *D. winteri* leaf essential oil, counting for about 58% of the total oil composition, with γ-eudesmol as the major representative ($21.65 \pm 0.41\%$).[13]

This work describes the chemical composition of the essential oils from dry leaf and branch of *D. angustifolia* collected in Santa Catarina State, Southern Brazil, and the isolation of two main sesquiterpenes. The antibacterial activity of the oils, of the pure isolated compounds and of binary mixtures of them was investigated.

Experimental

Plant material and essential oil extraction

Leaf and branch of *D. angustifolia* were collected in June 2009 and February 2011 at Papagaio Farm in Nova Veneza City (S28º57'08"; W49º40'51", 1228 m altitude), Santa Catarina State, Southern Brazil. The species was identified by the plant taxonomist André L. de Gasper by comparison with a voucher specimen deposited in the Herbarium Dr. Roberto Miguel Klein of the Universidade Regional de Blumenau (FURB) under the number 12287.

The plant material was dried under shadow at room temperature until constant mass. The branch was subsequently crushed in mill Tecnal-TE 648. The oils were obtained by hydrodistillation for 4 h in a modified Clevenger-type apparatus under nitrogen atmosphere. After extraction, the leaf oil was collected in a measuring cylinder dried with anhydrous magnesium sulfate, transferred to a 5 mL vial and stored in a refrigerator. The branch oil was solubilised with dichloromethane, dried with anhydrous magnesium sulfate, filtered, concentrated in rotary evaporator, transferred to a 5 mL vial and stored in a refrigerator.

GC-FID and GC-MS analyses

The analyses were performed using a Shimadzu 14-B gas chromatograph with a capillary column (Simplicity-1, Supelco, 30 m × 0.25 mm i.d. × 0.25 μm film thickness), and a Varian CP-3800 gas chromatograph with a capillary column (CP-Sil 8 CB, 30 m × 0.25 mm i.d. × 0.25 μm film thickness) equipped with a Saturn 2000 mass selective detector set at 70 eV. Gas chromatography with flame ionization detector (GC-FID) and gas chromatography-mass spectrometer (GC-MS) analyses were performed using the following analytical conditions: sample injection (0.5 μL); carrier gas helium, flow rate 1 mL min^{-1}; split mode; injector temperature 220 ºC and FID 240 ºC when appropriate. The oven temperature was programmed from 60 (0 min) to 195 ºC at 3 ºC min^{-1} gradient, then increasing at a rate of 20 ºC min^{-1} from 195 to 235 ºC, which was held for 30 min.

The identification of components was made by computer library search based on matching of MS spectra (NIST 98), comparison with literature data[14] and experimental retention indices (RI), which were calculated by using a homologous series of *n*-alkanes analyzed under the same GC-FID conditions previously described. The component quantification was based on their GC peak areas, without correcting for response factors. Pure isolated compounds were also analyzed by GC-MS.

NMR analysis

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of pure compounds were recorded on a Varian AS 400 spectrometer operating at 400 and 100 MHz, respectively. Deuterated chloroform was used as solvent and trimethylsilane as internal standard. Hydrogen and carbon chemical shifts (δ) are reported in parts *per* million (ppm). The NMR identification was made by comparison to published data.

Drimenol isolation and characterization

Hexane was added to the crystallized branch essential oil from *D. angustifolia* in a 1:1 ratio (v/v). Heating was used to give a homogeneous mixture, which was then cooled in a refrigerator for several hours. Drimenol was separated as colorless crystals. This procedure was repeated twice. Its purity was determined by GC-FID under the same conditions used for the essential oil analysis, and it was superior to 98%. Its melting point was obtained in a digital Kofler type apparatus-Microquimica APF-301 and is uncorrected. The specific rotation was determined in a QUIMIS polarimeter-Q760M at 22 ºC using a solution of the titled compound in benzene (2.41 mg mL^{-1}).

Bicyclogermacrene isolation and characterization

It was obtained by column chromatography using a 30:1 ratio of silica gel 60 (70-230 mesh):leaf essential oil. Bidistilled hexane was used as eluent and fractions with volume of 10 mL each were collected. After analysis by GC-FID, the fractions containing pure bicyclogermacrene were combined and concentrated in rotatory evaporator using a bath temperature of 50 ºC. Its purity was determined by GC-FID under the same conditions used for the essential oil analysis, and it was superior to 98%.

Microorganisms

The bacterial strains were acquired from The American Type Culture Collection (ATCC). Tests were carried out in duplicate with strains of Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923) and *Bacillus cereus* (ATCC 11778) and Gram-negative bacteria *Acinetobacter baumanii* (ATCC 17978), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). The identification of strains was confirmed by the use of biochemical profiles according to the recommendations of the Manual of Clinical Microbiology.[15]

Minimum inhibitory concentration (MIC)

The direct antibacterial effect was evaluated by the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute[16] for determination of MIC for the leaf and branch essential oils and isolated compounds *D. angustifolia*. The essential oil or single compound (16 mg mL^{-1}) was dissolved in an aqueous solution of DMSO (dimethylsulfoxide, 20%, v/v). This solution was transferred to 96-well plates (100 μL *per* well) and was also diluted with sterile Mueller-Hinton broth (100 μL *per* well). Then, serial dilutions were made resulting in concentrations of 8,000 to 62.5 μg mL^{-1}. The inoculum (5 μL) containing 1.5×10^{8} colony-forming unit *per* mL (CFU mL^{-1}) (0.5 McFarland scale) of each microorganism was added to each well. A number of wells was reserved on each plate for sterility control (no inoculum added), inocula viability (no essential oil added) and the positive control (gentamicin added in different concentrations).[17] Plates were aerobically incubated for 18-24 h at 35 ºC, and 10 μL of methanol solution (5 mg mL^{-1}) of 2,3,5-triphenyl-tetrazolium chloride (TTC, Vetec, São Paulo, Brazil) were added to each well

to detect the active bacterial metabolism. MIC was defined as the lowest concentration of essential oil that visibly inhibited growth of bacterial spots detected with TTC (indicator 2,3,5-triphenyltetrazolium).[18] The assays were performed in triplicate to ensure reproducible results.

Results and Discussion

Essential oil composition

The growing demand for medicinal plants by the pharmaceutical industry requires the use of dry material for large production. This has stimulated the study of different drying procedures applied to plant species.[19] Furthermore, the capability to preserve fresh material largely depends on the quantity collected and storage resources for transportation and extracting period, which takes a quite long time in laboratory scale. Therefore, to ensure a more reproducible and accurate results, the oils were obtained by hydrodistillation of dry leaf and branch from *D. angustifolia*, providing yield of 1.53 and 0.47%, respectively. A more complex mixture was observed in the leaf oil, where 18 compounds were identified by GC-FID and GC-MS, representing 95.7% of its composition. In the branch essential oil, 11 compounds were identified, accounting for 92.1% of the oil composition. The qualitative and quantitative analyses are shown in Table 1, which also contains the main chemical group distribution. The monoterpenic content of the leaf essential oil was high (69.7%) and the major components were sabinene (17.2%) and myrcene (16.0%). The sesquiterpenes were represented by bicyclogermacrene (14.9%).

In the branch essential oil monoterpenes accounted for only 10.6% of its composition, being represented by α-pinene, isoterpinolene and linalool. The arylpropanoid safrole was detected in low concentration in both oils. The most abundant compounds in the branch oil of *D. angustifolia* were sesquiterpenes, representing 80.2% of its composition with drimenol, an oxygenated sesquiterpene, as the major constituent (50.0%).

Besides *D. winteri*[7,20] and *D. angustifolia*,[4] this sesquiterpene has been found in many other natural sources, as mentioned by Aricu.[21] Drimenol has been used in the synthesis of a series of natural biologically active drimanes and nordrimanes.[21,22] Drimenol can also be used as starting compound for (–)-ambrox, the most important equivalent of Ambergris, widely used in perfumery.[23,24] Additionally, it has been the starting material for nitrogenated drimane with guanidine moiety possessing antifungal activity[25] and the cytotoxic *ent*-cyclozonarone especially towards MS-1, mice endothelial cells.[26] These selected examples illustrate the importance of this secondary metabolite and moreover the necessity to identify new natural sources for this compound due to the restricted occurrence of *D. winteri*.[20,27]

Table 1. Percentage composition of leaf and branch oils from *Drimys angustifolia* collected in June 2009, Nova Veneza City, Santa Catarina State, Brazil

Compound	Leaves / %	Branch / %	RI_{obs}	RI_{lit}
α-Pinene	2.8	1.5	928	932
Sabinene	17.2	–	968	969
β-Pinene	6.0	–	972	974
Myrcene	16.0	–	985	988
α-Terpinene	2.6	–	1012	1014
Limonene	3.4	–	1024	1024
β-Phellandrene	7.0	–	1025	1025
Z-β-Ocimene	3.4	–	1031	1032
Isoterpinolene	1.5	4.5	1083	1085
Linalool	3.4	4.6	1096	1095
Terpinen-4-ol	6.4	–	1172	1174
Safrole	1.5	1.3	1283	1285
Bicyclogermacrene	14.9	19.0	1492	1500
Spathulenol	1.6	3.9	1573	1577
Globulol	2.5	2.3	1580	1590
Viridiflorol	1.3	1.3	1587	1592
Drimenol	1.6	50.0	1758	1766
Rimuene	–	1.1	1886	1896
Kaurene	2.6	2.6	2041	2042
Monoterpenes	69.7	10.6	–	–
Sesquiterpenes	24.5	80.2	–	–
Total	95.7	92.1	–	–

RI_{obs}: Observed Retention Indices; RI_{lit}: Literature Retention Indices.

The main advantage of using branch of *D. angustifolia* as a source of drimenol relies on the possibility of regular pruning to access large amounts of plant material without compromising the plant integrity.

Since the chemical composition of a plant is affected by several different factors such as genetic, ontogenic and climate,[28] it is currently under investigation in our group the prospection of *D. angustifolia* from Santa Catarina State, Southern Brazil, with high content of drimenol. The results will be published in due course.

Antibacterial assays

The results of the antibacterial activity of the leaf and branch essential oils from *D. angustifolia* are shown in

Table 2. Minimum inhibitory concentration (μg mL^{-1}) of oils, binary mixture of the oils, drimenol (DR), bicyclogermacrene (BC) and binary mixtures of these isolated compounds

	S. aureus	*B. cereus*	*A. baumanii*	*E. coli*	*P. aeruginosa*
LO[a]	2,000	250	1,000	2,000	2,000
BO[b]	500	125	500	2,000	1,000
LO:BO[c]	1,000	250	1,000	2,000	1,000
Drimenol[d]	667	667	583	1,333	667
Bicyclogermacrene[d]	292	167	500	500	417
DR:BC (4:1)	GR[b]	500	1,000	GR	250
DR:BC (3:2)	GR	500	1,000	GR	500
DR:BC (1:1)	GR	500	1,000	GR	500
DR:BC (2:3)	GR	250	GR	GR	GR
DR:BC (1:4)	1,000	250	GR	GR	GR
Control[e]	0.5	0.6	0.6	1.0	1.0

[a]LO: leaf oil; [b]BO: branch oil; [c]LO:BO: 1:1 mixture (m/m); [d]MICs for drimenol and bicyclogermacrene are medium values from 3 independent experiments, each of them carried out in duplicate; GR: bacterial growth; [e]gentamicin.

Table 2. Aligianis *et al.*[29] proposed a classification for the antimicrobial activity of plant extracts based on the MIC results as follows: strong inhibitors, MIC equal or below 500 μg mL^{-1}; moderate inhibitors, MIC between 500 and 1,500 μg mL^{-1}; weak inhibitors, MIC above 1,500 μg mL^{-1}.

Drimys angustifolia essential oils were active against all of the microorganisms tested. The leaf oil showed weak activity against *S. aureus*, *E. coli* and *P. aeruginosa*, moderate activity against *A. baumanii* and strong activity against *B. cereus*. The branch oil showed a MIC value of 2,000 μg mL^{-1} against *E. coli*. On the other hand, it showed strong activity against *S. aureus*, *B. cereus* and *A. baumanii* and moderate activity against *P. aeruginosa*. Our results indicate that *E. coli* was the most resistant bacteria while *B. cereus* was the most sensitive, presenting excellent MIC values, 125 and 250 μg mL^{-1} for branch and leaf oils, respectively.

B. cereus is a common food poisoning organism, but also systemic and local infections have been reported, especially when associated with immunologically compromised patients, neonates, drug addicts and patients with a history of traumatic or surgical wounds or catheters.[30]

Antimicrobial activities of essential oils are difficult to correlate to a specific compound due to their complexity and variability. The antimicrobial activities have been mainly explained through the presence of C_{10} and C_{15} terpenes with aromatic rings and phenolic hydroxyl groups able to form hydrogen bonds with active sites of the target enzymes, although other active terpenes, as well as alcohols, aldehydes and esters can contribute to the overall essential oil antimicrobial effect.[31]

Some drimanes are known to present antibacterial activity[22,32] as well as bicyclogermacrene.[33] This association may explain the strong activity related to branch essential oil from *D. angustifolia*.

In general, the antibacterial activity of *D. angustifolia* essential oil was more pronounced against Gram-positive than Gram-negative bacteria strains, a fact previously observed with essential oils from other plant species.[34,35] The reason for the different sensitivity between Gram-positive and Gram-negative bacteria could be ascribed to the morphological differences between these microorganisms. There is a possibility that Gram-negative organisms are less susceptible to the action of antibacterials since they posses an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering.[35,36] The Gram-positive bacteria should be more susceptible having only an outer peptidoglycan layer which is not an effective permeable barrier.[37]

The combination of essential oils is not a common practice although it might improve their properties.[35] In this case, the lowering of antibacterial activity was observed in comparison to the branch essential oil. It seems that the increase of monoterpene contents at the expenses of drimenol and bicyclogermacrene caused this unfavorable result. Since drimenol and bicyclogermacrene are the two main constituents, it was decided to investigate their properties more closely. Therefore, pure drimenol and bicyclogermacrene, as well as their binary mixtures, were also subjected to the same antimicrobial assays. The results are summarized in Table 2. As it can be noticed, bicyclogermacrene was the most active compound for

all tested bacteria, especially for Gram-positive one. The combination of these two sesquiterpenes did not improve the activity, indicating the absence of synergism at the proportions tested.

By taking bicyclogermacrene as leading compound in inhibiting the bacterial growth, it seems somewhat surprising the MIC observed for its pure form. One should consider that this hydrocarbon sesquiterpene accounts for only 20% of the total oil composition, therefore it would be expected a 5 fold activity increase as an isolate. The best result was obtained against *E. coli*, in which the MIC value changed from 2,000 to 500 μg mL^{-1}, but it was less expressive against Gram-positive bacteria, with very similar MIC values for bicyclogermacrene and total branch essential oil, 167 and 125 μg mL^{-1}, respectively. In this case, it is possible that bicyclogermacrene acts in synergism with some of the minor oil constituent, which could also explain the more pronounced activity of the branch oil over the leaf oil, although they have similar concentrations of this metabolite.

Conclusion

In conclusion, our results demonstrate that essential oils of *D. angustifolia* present significant *in vitro* antibacterial activity against *B. cereus*. Regardless to this activity, the branch oil was the most promising agent since it strongly inhibited both Gram-positive and Gram-negative bacteria. Bicyclogermacrene was more active than drimenol against the bacteria tested, however its activity as pure compound did not increase as expected, except for *E. coli*. No synergism was observed using different combinations of drimenol and bicyclogermacrene. Finally, the branch oil can be regarded as a sustainable source of (–)-drimenol.

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Supplementary Information

Supplementary data (Figures S1-S8) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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