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Short communication

Seasonal phytochemical study and antimicrobial potential of *Vetiveria zizanioides* roots

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This paper describes the seasonal phytochemical variation and the antimicrobial potential of *V. zizanioides* roots collected in Brazil. Considering the high levels of chemical constituents and their biological activity in dichloromethane fraction, the plants were grown in different seasons and the respective dichloromethane fractions were analyzed by gas chromatography-mass spectrometry. The antimicrobial activity was evaluated against several pathogenic microorganisms by determining the minimum inhibitory concentration (*MIC*) using the agar dilution method. Yields of dichloromethane fractions from plants collected in the autumn and spring occurred in a higher proportion than in other seasons. Khusimol (**2**) was isolated by column chromatography and identified by NMR and CG-MS, along with other sesquiterpenes, including β-vetivenene (**1**), vetiselinenol (**3**), isovalencenol (**4**), vetivenic acid (**5**), α-vetivone (**6**) and β-vetivone (**7**). Some extracts showed promising antimicrobial effects, with *MIC*s ranging from 31.25 to 500 μg mL^{-1}. Kushimol was slightly active against the tested microorganisms.

Keywords: Vetiveria zizanioides (Poaceae), seasonality, sesquiterpenes, khusimol, antimicrobial activity

Medicinal plants have emerged as an important source of medicinal agents to treat a variety of diseases, including human infections caused by pathogenic and resistant microorganisms. The search for new medicinal agents against the genera *Microsporum* and *Trichophyton* is very important because they are the main agents responsible for dermatophytoses, which are among the most common infectious diseases worldwide and require appropriate treatment (1, 2). *Vetiveria zizanioides* (Linn.) Nash (*Poaceae*), known as »vetiveria« or »falso-patcholi« in Brazil, is an aromatic and ornamental plant, popularly used for bathing and perfumery. There are several studies indicating its use for therapeutic purpose, and experimental studies have confirmed some of its biological effects (3, 4). Several authors have demonstrated its chemical composition, sesquiterpenes being the main com-

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ponents in the roots and phenolic compounds in the leaves (5, 6). However, little is found in the literature about the species adapted in Brazil. Preliminary studies in our laboratory indicate that crude extracts of this plant act against some pathogenic microorganisms. The present study therefore evaluates the seasonal phytochemical variation, using gas chromatography and the antimicrobial potential of the roots of *V. zizanioides*.

EXPERIMENTAL

General procedures

Thin layer chromatography (TLC) was performed on 0.25-mm thick silica gel Merck 60 F_{254} (Germany) and column chromatography on silica gel 60 (060–0.20 mm, VETEC, Brazil). Spots were visualized under UV light or with sulfuric anisaldehyde, indicative of steroids and terpenes. ^{1}H NMR spectra were recorded at 300 MHz and ^{13}C NMR spectra at 75 MHz using deuterated chloroform as a solvent and tetramethylsilane (TMS) as the internal reference on a Bruker DPX300 apparatus (Bruker, Germany). Chemical shift values are expressed in ppm and coupling constants (*J*) in Hz. GC-MS analyses were carried out on a Shimadzu GC-MS-QP2010S apparatus (Shimadzu, Japan).

Plant material

V. zizanioides (*Poaceae*) roots were collected in the gardens of UNIVALI (Itajaí-SC, Brazil) in December of 2010, April, July and September of 2011. The plant material was authenticated by Prof. Oscar Iza (UNIVALI, Itajaí-Brazil) and a voucher specimen is deposited at the Barbosa Rodrigues Herbarium (Itajaí, Brazil).

Preparation of extracts and fractions

A hundred grams of roots collected in different months was macerated separately with methanol at room temperature for about two weeks. After solvent removal under reduced pressure using a rotating evaporator, a crude methanolic extract was obtained, which was later used for biological assays and phytochemical analyses. Crude extracts were extracted with dichloromethane. The yields are given in Table I.

Isolation of khusimol

The methanolic extract of *V. zizanioides* roots collected in autumn (3 g) was dissolved in dichloromethane giving 2.5 g of this fraction. The extract was successively chromatographed on a silica gel column eluted with dichloromethane/methanol (98:2, *V*/*V*), resulting in 60 mg of khusimol (**2**), which was identified by spectroscopic analysis (^{1}H- and ^{13}C NMR) in comparison with the literature data (7).

GC-MS analysis

Samples were analyzed according to the following methodology. Dichloromethane fractions were dissolved in chloroform and a 1-μL aliquot of the solution of each fraction

was injected into the GC-MS system. GC-MS analysis was performed using an Agilent 7890A (Agilent, USA) series apparatus interfaced with a 5975C mass detector. A HP-HP-5 (30 m × 0.25 mm, 0.10 μm) dimethylpolysiloxane analytical column was used for the separation. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1}. The injector (splitless mode, 1:100 split ratio) was maintained at 300 °C. The initial column temperature was set at 50 °C and kept for 5 min. It was then ramped by 20 °C min^{-1} up to 200 °C, then by 20 °C min^{-1} up to 290 °C and, finally, by 20 °C min^{-1} up to 310 °C, where it was kept for 15 min. MS detector in a scan (m/z = 30 to 450 Da) and positive electron impact ionization (EI) modes were used and data were collected using single ion monitoring (SIM).

Biological assay

For antimicrobial evaluation, strains from the American Type Culture Collection (ATCC, USA), CEREMIC (C), Centro de Referencia Micológica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Argentina and Control Lab (CL), Brazil, were used. The bacteria used were *Bacillus subtilis* (ATCC 14579), *Escherichia coli* (ATCC 11775), *Staphylococcus aureus* (ATCC 6538P), and *Staphylococcus saprophyticus* (ATCC 35552. The fungi used were the yeasts *Aspergillus fumigatus* (ATCC 26934) and *Rhizopus sp.* (CL 35) and the dermatophytes were *Microsporum canis* (C 112), *Microsporum gypseum* (C 115), *Trichophyton metagrophytes* (ATCC 9972), *Trichophyton rubrum* (C 137) and *Candida albicans* (ATCC 10231).

Media and inocula

The bacteria used were cultivated on Müller-Hinton agar (MHA, Difco, France) at 35 °C for 24 h. Cell suspension in saline (0.86 %) was adjusted to give a final concentration of 1.5×10^8 cell mL^{-1}, standardized with 0.5 on the McFarland scale (λ = 530 nm). The fungi were cultivated on Sabouraud dextrose agar (SDA, Difco). For filamentous fungi, suspensions were obtained according to the reported procedures and were adjusted to the range 1.0×10^6 to 5.0×10^6 spores mL^{-1} by microscopic enumeration using a hemocytometer. The yeasts were prepared by adjusting the suspension so as to give a final concentration of between 1.0×10^6 and 5.0×10^6 cell mL^{-1}, also standardized with 0.5 on the McFarland scale (λ = 530 nm).

Antimicrobial evaluation

The minimum inhibitory concentration (*MIC*) was determined for the microorganisms by the agar dilution method, which was carried out on slants (1 mL). Stock solution of each fraction or khusimol in dimethylsulfoxide (DMSO) was diluted to give serial two-fold dilutions, which were added to each medium (MHA for bacteria and SDA for fungi), resulting in ten different concentrations ranging from 10 to 1000 μg mL^{-1}. Afterwards, a volume of 1 μL of previously prepared inoculum suspension was inoculated with a sterile loop to each slant, with the exception of the sterile control. The antibacterial and antifungal agents, cefoxitin and ketoconazole (both from Sigma, USA) were included in the assay as positive control. The final concentration of DMSO in the assay did not exceed 2 %. A drug-free saline solution (0.86 %) was used as a blank control. The slants were incubated at 35 °C for the bacteria and yeasts and at 25 °C for the dermatophyte strains. *MIC*s were visually recorded

after 24 h for bacteria and after 48 h for yeasts, and at a time according to the control fungus growth for the fungi. The experiments were performed in triplicate.

RESULTS AND DISCUSSION

Table I shows that the yield of methanolic extracts differed between seasons, especially when collected in winter and spring, in which period they were 2 to 4 times higher than in summer and autumn.

Table I. Yield of methanol extract and dichloromethane fraction of V. zizanioides *collected in different seasons*

Season	Yield of methanolic extract[a] (%)	Yield of dichloromethane fraction[b] (g)	Yield of dichloromethane fraction[b] (%)
Summer	4.4	2.6	59.1
Autumn	3.1	2.6	83.9
Winter	10.3	8.2	79.6
Spring	14.2	11.2	78.9

[a] From 100 g dried roots.
[b] From methanolic extract.

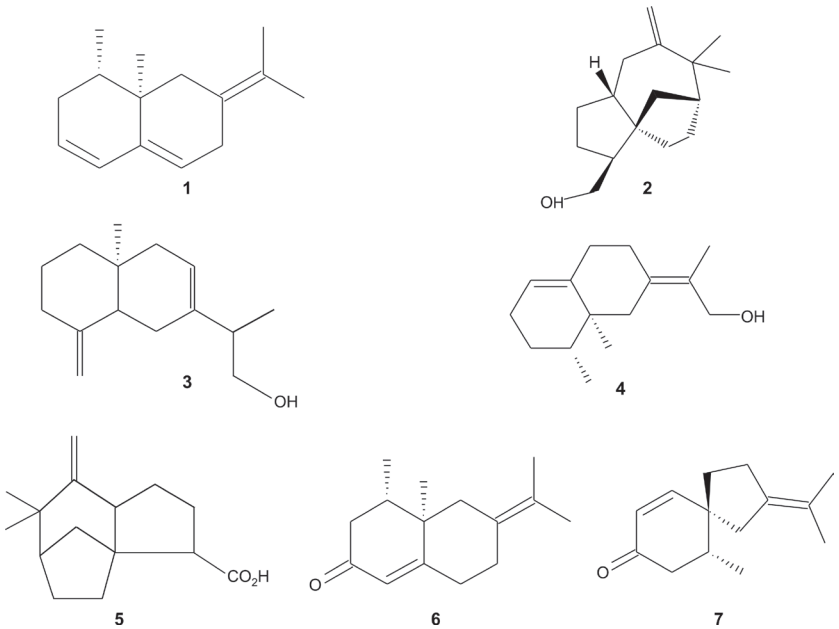

Fig. 1. Molecular structures of substances identified in roots of *V. zizanioides:* β-vetivenene (**1**), khusimol (**2**), vetiselinenol (**3**), isovalencenol (**4**), vetivenic acid (**5**), α-vetivone (**6**) and β-vetivone (**7**).

Evaluation by GC-MS evidenced the presence of several components, seven of them identified as β-vetivenene (**1**), khusimol (**2**), vetiselinenol (**3**), isovalencenol (**4**), vetivenic acid (**5**), α-vetivone (**6**) and β-vetivone (**7**) in CH_2Cl_2 fractions, with compounds **2**, **4** and **5** being the major compounds (Fig. 1). It is interesting to note that a similar profile of the extracts collected in different seasons was seen. However, plants collected in winter showed significant differences in chemical constitution, both qualitative and quantitative (Fig. 2). For example, the sesquiterpene vetiselinenol (**3**) appeared only during winter, as well as another compound not yet identified with t_R of 9.55 min (Fig. 2). The components of the fraction collected in winter were quantified furnishing the following percent yields: **1** (4.1), **2** (8.2), **3** (7.2), 4 (16.7), **5** (17.5), **6** (6.1) and **7** (5.1).

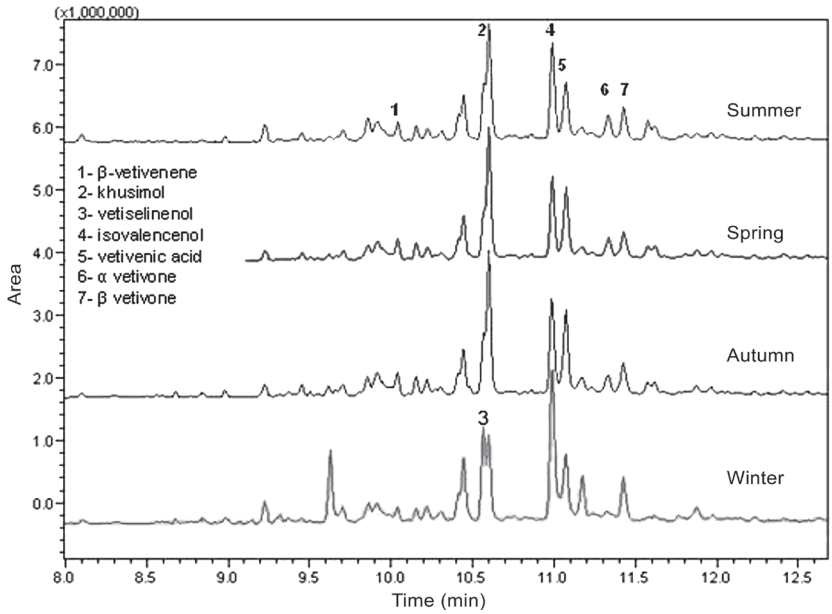

Fig. 2. GC chromatographic profile of dichloromethane fractions collected in different seasons: β-vetivenene (**1**), khusimol (**2**), vetiselinenol (**3**), isovalencenol (**4**), vetivenic acid (**5**), α-vetivone (**6**) and β-vetivone (**7**).

Table II shows the antifungal effect of methanolic extracts of roots of *V. zizanioides* collected in different seasons and of the isolated compound khusimol. According the Rios and Recio (8), who suggested good antimicrobial properties for this extract with *MIC* less than 100 μg mL^{-1}, all the evaluated extracts were promising against *Microsporum canis, Microsporum gypseum, Trichophyton mentagrophytes* and *Trichophyton rubrum* with *MIC* values between 62.5 and 31.25 μg mL^{-1}. Khusimol, one of the major compounds in all extracts, was weakly active against these fungi, suggesting that the antifungal potential was related to the presence of other substances or the occurrence of synergic effects.

Table III indicates that, in general, the extract of *V. zizanioides* collected in winter exerted the most promising antimicrobial effects. This fact may be related to the presence of

Table II. Minimal inhibitory concentration of methanolic extracts of V. zizanioides *collected in different seasons, khusimol and ketoconazole against pathogenic microorganisms*

Sample	*MIC* (μg mL^{-1})						
	M. canis	*M. gypseum*	*T. metagrophytes*	*T. rubrum*	*A. fumigatus*	*Rhizopus sp*	*C. albicans*
Summer	62.5	62.5	62.5	31.25	500	>1000	500
Autumn	62.5	31.25	31.25	31.25	1000	>1000	500
Winter	62.5	31.25	31.25	31.25	>1000	>1000	1000
Spring	62.5	62.5	62.5	31.25	>1000	>1000	250
Khusimol	500	250	> 1000	>1000	> 1000	500	500
Ketoconazole	8	6	8	3	7	15	0.3

DMSO – negative control, inactive.

Table III. Minimal inhibitory concentration (μg mL^{-1}) of dichloromethane fractions of V. zizanioides *collected in different seasons and khusimol against pathogenic bacteria*

Sample	*MIC* (μg mL^{-1})			
	S. aureus	*S. saprophyticus*	*B. subtilis*	*E. coli*
Summer	500	250	250	500
Autumn	500–250	250	250	1000
Winter	250	125	500	500
Spring	500–250	125	500	500
Khusimol	500	250	> 1000	>1000
Cefoxitin	2	4	1	4

DMSO – negative control, inactive.

vetiselinenol (**3**) and other compounds, such as that with t_R of 9.55 min, or to the additive and/or synergistic effects (9).

Considering that sesquiterpenes are the major compounds of these extracts, as evidenced by chromatographic analyses and previous investigations of this plant (10). As can be seen, all the fractions exhibited only moderate or weak effects, with *E. coli* being the most resistant microorganism tested. Barros and co-workers (10) studied the antibacterial effects of essential oil of *V. zizanioides* collected in winter in another region of Brazil and demonstrated it to be a little more active than our dichloromethane fraction against *S. aureus*, with *MIC* of 150 μg mL^{-1}. They also found bioactivity against other microorganisms, including *Bacillus cereus* and *Micrococcus roseus* (*MIC* 190 μg mL^{-1}). On the other hand, they evidenced that the crude ethanolic extract or dichloromethane fractions were much less active than the essential oil. However, dichloromethane fractions from our study showed higher activity, probably due to the significant difference in the chemical composition of extracts.

CONCLUSIONS

In conclusion, our results show that *V. zizanioides* exhibits a promising antimicrobial potential, especially against dermatophytes and *C. albicans*, a fungus that is very resistant to the action of extracts of vegetable origin. Kushimol, one of the main identified compounds, was slightly active against the tested microorganisms, suggesting other bioactive compounds or additive/synergistic effects.

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