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Anti-*Candida* activity of terpenes from *Salvia ovalifolia*, *S. procurrens* and *S. uliginosa*, native to South Brazil

Cristiano da Silva Sousa[a], Letícia Mezzomo[a], Juliana Butzge[a], Gabriela de Carvalho Meirelles[a], Sérgio Augusto de Loreto Bordignon[b], Gilsane Lino von Poser[a], Miriam Anders Apel[a,*]

[a]*Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil;* [b]*Postgraduate Program of Environmental Impact Assessment. UNILASALLE University, Canoas, RS, Brazil*
**Corresponding author: miriam.apel@ufrgs.br*

The essential oils of *Salvia ovalifolia*, *S. procurrens* and *S. uliginosa* were obtained by hydrodistillation and analyzed by gas chromatography-mass spectrometry. The three species displayed very low amounts of essential oils, consisting of a few sesquiterpenes, and aliphatic compounds such as aldehydes and long-chain fatty acids. From the leaves of *S. uliginosa*, an exudate was obtained which presented the diterpene icetexone as the major component. The exudate and icetexone were evaluated for the activity against *Candida* species, both showing inhibition of fungal growth.

Keywords: *Salvia*, Lamiaceae, terpenes, HPLC analysis, *Candida*.

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Introduction

The Lamiaceae family contains 236 genera and about 7,173 species, widespread all over the world, not found only in the coldest regions of high latitude (1). The largest genus of the family is *Salvia* L., comprising ca. 900 species of cosmopolitan distribution (2), with major centers of diversity in East and West Asia and Central and South America. The genus thrived specially in America where ca. 500 species belonging to the subgenus *Calosphace* (Benth.) Epling have been described. This subgenus is exclusively American and groups the majority of the species of the genus (1). In Brazil, 68 species were found being 47 endemics to the country (3).
The phytochemical investigation of the genus *Salvia* indicates the presence of diterpenes (4-6) and essential oils (7) as the main constituents. Due to the presence of these important groups of natural compounds, *Salvia* species are used in the traditional medicine, as well as and in the manufacture of perfumes, cosmetic and toiletry products.
Previous studies have demonstrated the antiprotozoal activity of the exudate of *Salvia uliginosa* and its main diterpene, icetexone (8). In this context, the purpose of this study was to characterize the essential oils obtained by hydrodistillation from *Salvia* species native to southern Brazil (*S. ovalifolia*, *S. procurrens* and *S. uliginosa*), using gas chromatography coupled to mass spectrometry. Furthermore, the essential oils, the exudate and an isolated diterpene, icetexone, were evaluated concerning the antifungal effect against *Candida* spp.

Materials and Methods

Plant Material

The plants (*S. ovalifolia*, *S. procurrens* and *S. uliginosa*) were collected in the Rio Grande do Sul state, South Brazil (SisGen-Brazil access registration AAEF4), and voucher specimens were deposited in the herbarium of the Universidade Federal do Rio Grande do Sul (ICN).

Essential oils extraction

One hundred grams of fresh aerial parts of S. *ovalifolia*, *S. procurrens* and *S. uliginosa* were submitted to hydrodistillation in a Clevenger-type apparatus, for 4 h. The oils were collected, dried over anhydrous sodium sulphate and stored in the dark, at -20 °C, until the GC-MS analyses. The yields were expressed as volume (mL) of essential oil per 100 g of fresh plant material.

GC-MS analyses

For chemical analysis, the essential were diluted in ethyl ether at a ratio 2:100 (v/v). The chemical composition was determined by gas chromatography coupled with mass spectrometry (GC-MS) (Shimadzu QP5000) equipped with a capillary column of fused silica Durabond-DB5 (30 m × 0.25 mm × 0.25 µm). The injector and detector temperatures were set at 220 °C and 250 °C, respectively, using a column temperature programming of 60 °C to 300 °C at 3 °C.min^{-1}, with helium as the carrier gas at a flow rate of 1 mL.min^{-1}. Ionization of the sample components was performed in the EI mode (70 eV). The injected volume was 1 µL. The identification of compounds was based on the comparison of retention indices calculated by linear interpolation

relative to retention times of a series of n-alkanes (C8–C23), and their mass spectra with authentic samples and with data collected from the literature (9) and also by comparison with mass spectra recorded in the database of NIST 62 and NIST 12 (National Institute of Technology and Standards). The relative amounts of the components were calculated based on GC peak areas by normalization.

Exudate preparation

Aerial parts of *S. uliginosa* (50 g) were immersed in dichloromethane in an amount sufficient to cover the plant material. After 30 s, the extract was filtered and the solvent was evaporated under reduced pressure. The crude exudate was partitioned by column chromatography over silica gel using mixtures of n-hexane and dichloromethane of increasing polarity as mobile phase. A main compound was obtained and identified as the diterpene icetexone by comparison with an authentic sample.

Exudate analysis

The qualitative analysis of exudate was performed using a HPLC method previously validated (not published data). The analyses were achieved using on a Waters 2695 instrument with a 2998 PDA detector. The separations were fulfilled with a system composed by water (A) and acetonitrile (B), both containing 0.1% of formic acid. A gradient system of 10→100% (B) in 30 minutes was applied, followed by 70→10% (B) for 20 minutes. A Waters Nova-Pack C18 column (4 µm, 3.9 mm x 150 mm) attached to a guard column Waters Nova-Pack C18 60 Å (3.9 mm x 20 mm). The column flow rate was 1 mL/min and the injection volume was 10 µL. The detector sensibility was 1 AUFS and the analysis was performed in λ= 330 nm at 25 ºC.

Evaluation of the antifungal activity

Microorganisms

The microorganisms selected for this study were provided by the Biomicolab Laboratory (Mycology Laboratory of the Federal University of Rio Grande do Sul). The fungi used were five species of the genus *Candida*: *C. albicans* (ATCC 24433), *C. glabrata* (ATCC 2001), *C. krusei* (ATCC 6258) *C. tropicalis* (ATCC 2551) and *C. parapsilosis*. All strains were stored in Tryptone Soya Broth (TSB; Himedia®) plus 10% glycerol and frozen at -18 °C. At the time of use, the strains were reactivated by inoculating 10 µL of the stock culture in tubes with Sabouraud dextrose agar with chloramphenicol (Kasvi®), and incubated at a temperature of around 30 °C for 48 h.

Disk diffusion test

The disk diffusion test to evaluate antifungal activity was performed as a screening, determining the size of the zones of inhibition (mm) produced by the samples against the *Candida* strains. The fungal suspension of each test strain was prepared and using the Bio-Spectrophotometer SP-220, the suspension was adjusted to $1x10^6$ CFU (Colony Forming Units), turbidity corresponding to 0.5 on the McFarland scale. This suspension was spread on sterile Petri dishes containing Sabouraud dextrose agar culture medium with chloramphenicol (Kasvi®). The test was performed following the protocol described in document M44 of the Clinical and Laboratory Standards Institute, CLSI (10). Filter paper discs (diameter of 10 mm) were impregnated with 15 µL of each oil sample and placed on the surface of the previously inoculated medium. The plates were incubated at 30 °C and the inhibition zones were read after 48 h. The antifungal used as positive control was itraconazole, 10 µg discs (Cecon / Brazil) and the breakpoints used were that recommended by document M44 of CLSI [10], in which ≥20 mm halo means sensitive, 15 mm-19 mm halo means dose dependent/sensitive intermediate and ≤14 mm halo means resistant. The active samples were selected for further study for determination of the Minimum Inhibitory Concentration (MIC).

Minimum Inhibitory Concentration (MIC)

The determination of the Minimum Inhibitory Concentration (MIC) was carried out by the broth microdilution method, as recommended by the CLSI (11). The samples were dissolved in dimethyl sulfoxide - DMSO (Nuclear®). The concentration range used for samples was 2000 $\mu g.mL^{-1}$ to 3.9 $\mu g.mL^{-1}$. The fungal suspensions were prepared in the same way as in the disk diffusion test, resulting in $1x10^6$ $CFU.mL^{-1}$ (Colony Forming Units), turbidity corresponding to 0.5 on the McFarland scale. Dilutions were subsequently made, 1:50 in 0.85% sterile saline and then again diluted 1:20 with RPMI 1640 (Sigma Aldrich®), resulting in a final suspension of $1x10^3$ $CFU.mL^{-1}$. The microplates were incubated at 30 °C and inspected after 24 h for visual verification of growth (10-12). The experiment was carried out in duplicate. MIC was defined as the lowest concentration of essential oil in which it was not possible to see any growth when compared to controls.

Results and Discussion

The *Salvia* species exhibited very low amounts of essential oils, confirming previous preliminary studies by our research group (13). Chemical analysis of the essential oils demonstrated that all species presented only sesquiterpenes, and aliphatic compounds such as aldehydes and long chain fatty acids (Figure 1). Among the latter, nonadecanal was found in all species, being abundant in *S. procurrens* and *S. ovalifolia* (19.9% and 11.3%, respectively). Regarding the composition of the sesquiterpenes, it was observed the occurrence of α-bisabolol in relatively large amounts (17.3%) in *S. ovalifolia* oil. This species also showed the presence of sesquiterpene α-guaiene in quantity inferior to 0.1%.

Salvia uliginosa essential oil was characterized by nonadecanal (40.2%) and heptadecanone (25.6%). In this species, hexadecanoic acid was observed only in traces.
Among the three species investigated, only *S. uliginosa* was previously accessed in more details regarding the essential oils (2). Interestingly, this plant, cultivated in Europe, exhibited a different composition. The extraction yield was also very low (0.02%) but other compounds were found. Sesquiterpenes represent the most abundant terpene fraction (89.56%). The main compound was bicyclogermacrene (16.3%) followed by germacrene D (14.8%), β-caryophyllene (8.6%) and δ-cadinene (8.5%), among the hydrocarbons. Spathulenol (12.7%) dominated the oxygenated sesquiterpene fraction.
Salvia uliginosa was also previously investigated by our research group regarding the diterpene composition. Cezarotto et al. (8) reported the occurrence of icetexane-type diterpenes, present in glandular trichomes found in the aerial parts of the plant. Of these diterpenes, a major component was identified as icetexone (Figure 1).

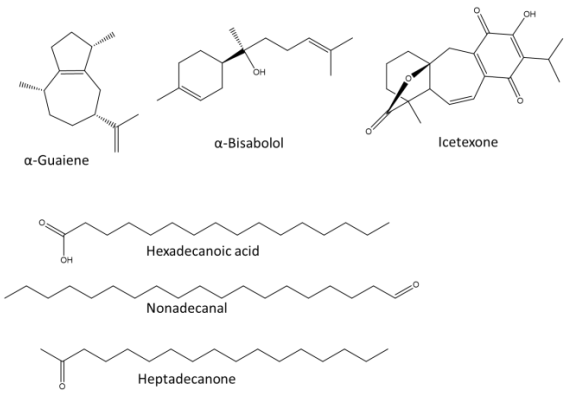

Figure 1. Chemical structure of the main compounds.

The exudate obtained in this study, analyzed by HPLC, showed the presence of several components, some of them with an ultraviolet profile of the icetexane diterpenes. By the chromatogram, it was observed that the main compound exhibits the same retention time and UV profile of icetexone.
In order to verify the susceptibility of strains of *Candida albicans*, *C. glabrata*, *C. krusei*, *C. tropicalis* and *C. parapsilosis* against the *S. ovalifolia*, *S. procurrens*, *S. uliginosa* essential oils, *Salvia uliginosa* exudate and icetexone, tests were performed to evaluate the antifungal activity of the samples. The anti-*Candida* activity was assessed by agar disk diffusion method and further by Minimum Inhibitory Concentration (MIC).
The results of the diffusion disk test, used as a screening, are shown in Table 1, in which the sizes of the inhibition halos are presented, in mm, and were obtained with the tested samples and with itraconazole, the antifungal used in this assay as positive control. None of essential oils demonstrated activity against the *Candida* species, since no inhibition zones were observed. Concerning itraconazole, *C. albicans* showed no inhibition zone, being, therefore, resistant to the antifungal substance control. The strain of *C. tropicalis* showed a 20 mm inhibition zone with the itraconazole disk, thus being sensitive to this antifungal. Considering *C. glabrata*, *C. krusei* and *C. parapsilosis*, the positive control demonstrated intermediate action with a 15 mm halo inhibition.
Differently from the essential oils, *S. uliginosa* exudate and icetexone were active against *C. albicans*, displaying inhibition zones of 30 mm and 25 mm, respectively (Figure 2). It must be considered that icetexone was isolated from *S. uliginosa*, where this diterpene is the major compound in the dichloromethane exudate and seems to be, at least, one of the active substances responsible for the observed effect.

Table 1. Size of inhibition halos (mm) of itraconazole (ITRA) and tested samples against *Candida albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*.

***Candida* sp.**	**ITRA**	***Salvia uliginosa* exudate**	**Icetexone**
C. albicans	10	30	25
C. glabrata	15	NI	NI
C. krusei	15	NI	NI
C. parapsilosis	15	NI	NI
C. tropicalis	20	NI	NI

[1]NI: no inhibition halo

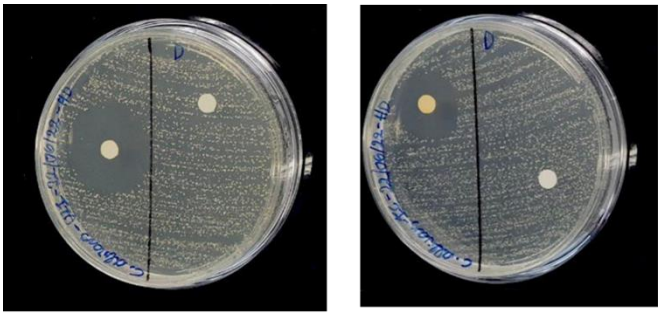

Figure 2. Inhibition zone by disc-diffusion test with Salvia uliginosa exudate (left) and icetexone (right). The disks in the right part of the plates contain the solvent used to dilute the samples (dichloromethane) which showed no effect against the fungi.

In the disk diffusion test the best results were obtained with the exudate of *S. uliginosa* and with icetexone against *C. albicans*. Thus, these samples were selected to determine the minimum inhibitory concentration (MIC). The MIC values obtained with the S. *uliginosa* exudate and icetexone, against *C. albicans* were 250 µg.mL^{-1} and 500 µg.mL^{-1}, respectively. It is interesting to note that the exudate of *S. uliginosa* showed better MIC compared to icetexone, confirming that the diterpene alone, although it is the major component of the exudate, is only one of those responsible for the effect observed against *C. albicans*.
Although no reports on the antifungal property of *S. uliginosa* exudate were found in the literature, other biological activities were reported. Cezarotto et al. (8) identified potent antichemotactic and leishmanicidal effects in diterpenes isolated from *S. uliginosa* exudate.

The diterpenes isolated were isoicetexone (IsoICT), icetexone diterpenes (ICT) and 7-acetoxy-6,7-dihydroicetexone, of which the first two showed high leishmanicidal activity against *Leishmania amazonensis*.

Conclusions

The results reported here highlight the biological properties of *S. uliginosa* exudate and corroborate its potential as a new antifungal therapeutic alternative.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

References

1. Jiahui S, Peipei S, Chuanzhi K, Lanyue Z, Lanping G. Chemical composition and biological activities of essential oils from six Lamiaceae folk medicinal plants. Front Plant Sci. 2022; 131:919294.
2. O'Leary N, Moroni P. Tipificaciones en *Salvia* (Lamiaceae). Novon: A J Bot Nomencl. 2016, 24(4): 373-379.
3. *Salvia* in Flora do Brasil 2020. Jardim Botânico do Rio de Janeiro. Available in: <http://floradobrasil.jbrj.gov.br/reflora/floradobrasil/FB8296>. Accessed in october 2022.
4. Kabouche A, Kabouche Z. Bioactive diterpenoids of *Salvia* species. Studies in Natural Products Chemistry, v. 35, 2008.
5. Wu YB, Ni ZY, Shi QW, Dong M, Kiyota H, Gu YC, Cong B. Constituents from *Salvia* and their biological activities. Chem Rev. 2012; 112:5967-6026.
6. Karalija E, Dahija S, Tarkowski P, Zeljković S. Influence of Climate-Related Environmental Stresses on Economically Important Essential Oils of Mediterranean *Salvia* sp. Front Plant Sci. 2022; 13: 864807.
7. Cezarotto CS, Dorneles A, Baldissera FG, da Silva MB, Markoski MM, Júnior LCR, Peres A, Fazolo T, Bordignon SAL, Apel MA, Romão PRT, von Poser GL. Leishmanicidal and antichemotactic activities of icetexanes from *Salvia uliginosa* Benth. Phytomedicine. 2019; 58:152748.
8. Adams RP. Editor. Identification of Essential Oil Components by Gas Chromatography/Mass Spectroscopy. Carol Stream (IL): Allured Publishing, 2017. E-book.
9. Clinical and Laboratory Standards Institute (CLSI), Performance Standards for Antifungal Susceptibility Testing of Yeasts. M44, 2018.
10. CLSI, Performance Standards for Antifungal Susceptibility Testing of Yeasts, M60. 2017;1-12.
11. Clinical and Laboratory Standards Institute (CLSI), Reference method for broth dilution, M27-A3 Ref. Method Broth Dilution Antifung. Susceptibility Test. Yeasts; Approv. Stand. - Third Ed. 2008; 28(14).
12. Pinto PS. Terpenoides em espécies do gênero *Salvia* (Lamiaceae) [dissertation]. Porto Alegre: Programa de Pós-graduação em Ciências Farmacêuticas. Universidade Federal do Rio Grande do Sul, 2012.
13. Giuliani C, Ascrizzi R, Tani C, Botton M, Bini LM, Flamini G, Fico G. *Salvia uliginosa* Benth.: Glandular trichomes as bio-factories of volatiles and essential oil. Flora. 2017;, 233:12-21.