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The Importance of Monoterpenes in the Antibacterial Activity of *Osteophloeum platyspermum* Essential Oils

Sinária R. N. de Sousa [a], Jefferson de S. Silva [a,b], Mateus L. B. Paciencia [a], Sergio A. Frana [a,b], Ingrit E. C. Díaz [c] and Ivana B. Suffredini [a,b*]

[a] Center for Research in Biodiversity, Universidade Paulista, São Paulo, Brazil.
[b] Graduate Program in Environmental and Experimental Pathology, Universidade Paulista, São Paulo, Brazil.
[c] Faculdad De Ingeniería Química y Textil/Faculdad de Ciencias, Universidad Nacional De Ingeniería, Lima, Peru, Brazil.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate antibacterial and cytotoxic activities of essential oils (EO's) obtained from *Osteophloeum platyspermum (ucuuba-chico-de-assis*; Myristicaceae) fresh leaves against *Staphylococcus aureus*, MCF-7 breast, and PC-3 prostate cancer cell lines.
Study Design: Thirteen EO's were submitted to antibacterial and cytotoxicity assays that included 18 compounds commonly present in the EO's. The relationship between the EO's biological activities and the dry (DS) or rainy (RS) seasonal variation was accessed using the relative percentage of the terpenes.
Place and Duration of the Study: The study was conducted at the Center for Research in Biodiversity (Microbiology Laboratory and Cell Culture Laboratory), Paulista University. Biological activity assessment was conducted between October/2016 and November 2019).
Methodology: Thirteen essential oils were obtained from the leaves of *O. platyspermum* and were

**Corresponding author: E-mail: ibsuffredini@yahoo.com.br, ivana.suffredini@docente.unip.br;*

previously chemically characterized. From the analyses, 18 terpenes were determined to commonly occur in the 13 EO's. Microdilution broth and cytotoxicity assays were performed to obtain minimal bactericidal concentrations (MBCs) and 50% (EC50) effective concentrations for the cytotoxicity assays. Data from the 18 terpenes were submitted to two-way ANOVA, cluster (CA), principal component (PCA), canonical correspondence analyses (CCA), and one-sample t-test. The relationship between the biological activities of EO's and seasonal variability (dry season, DS, or rainy season, RS) was assessed. One sample t-test was performed to verify the potency of the cytotoxic effects of EO's.

Results: Previously, 18 terpenes were identified in all the 13 EO's. From those, α-terpineol, limonene, myrcene, linalool, and terpinen-4-ol were relevant to the ordination of the DS EO set, while spathulenol, α-pinene, β-pinene, isospathulenol, α-cadinene, ledol, cubenol-1-epi, neo-intermedeol, elemol, β-elemene, γ-elemene and viridiflorol were relevant to the ordination of the RS EO set. The biological activities were more related to the EO's collected in the RS than those in the DS. Also, biological activities were shown to be related to the occurrence of limonene, myrcene, elemol, β-pinene, α-pinene, and terpinen-4-ol.

Conclusions: Although the presence of the 18 terpenes is necessary to maintain the species adaptability to the environment, the occurrence of limonene, myrcene, elemol, β-pinene, α-pinene, and terpinen-4-ol had a significant role in the effective expression of the biological activity of EO's from *O. platyspermum*, particularly those that occur during the rainy season, in the Amazon rain forest.

Keywords: Myristicaceae; terpenes; biological activity; breast cancer; prostate cancer; staphylococcus aureus.

ABBREVIATION

CCA-canonical correspondence analysis; CFU-colony forming units; DMSO-dimethylsulfoxide; DOXO-doxorubicin; DS-dry season; EO-essential oils; IBAMA- Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (Environmental Agency); MMA-Ministério do Meio Ambiente (Environmental Ministry); PCA-principal component analysis; RS-rainy season, Sau-*Staphylococcus aureus*; SRB-sulforhodamine B; TCA-trichloroacetic acid;

1. INTRODUCTION

Osteophloeum platyspermum (Spruce ex A. DC.) Warb. (Myristicaceae), popularly known as *ucuuba-chico-de-assis*, occurs in the Amazon Rain forest as a single species of the genus [1-3]. Reports on its hallucinogenic properties [4] and description of compounds as (-)-kaur-16-en-19-oic, acid, sitosterol, stigmasterol, (±)-3-demethylhomopterocarpin, and (±)-maackiain [5], eperu-8(20),13-dien-3α, 15-diol, glyceryl laurodimyriaste, glyceryl 1,3-lauromyristate, dihydroguaiaretic acid, hydroxyotobain, hydroxyoxootobain, guaiacin, and otobaphenol [6] have been done, as was the leaves and pericarp essential oils (EO's) evaluation [7].

The composition of the essential oil was previously determined [7]. Briefly, the leaves of one individual tree of *O. platyspermum* [*M.B. Paciencia*, 846 (UNIP Herbarium)] were collected 13 times in a period from November/2009 to October/2011, under license IBAMA/MMA/Brazil #12A/08. Plant was accessed in a *terra firme* forest near Manaus, AM, Brazil. A voucher of the species was deposited at UNIP Herbarium #UNIP5720. The leaves were collected during dry season, characterized by precipitation diminish, and rainy season, indicated by rainfall increase. The dry season is commonly known as "summer" and the rainy season "winter", in the Amazon Forest. Each collection was subsequentially numbered, from collection # 2 to collection # 14.

At each collection, leaves of the same specimen were taken and subjected to steam distillation in Clevenger apparatus for four h. Essential oils were collected using pentane, the pentane was then removed by air evaporation, and sodium sulfate anhydrous was added to remove the remaining water. Then, the essential oils were kept in amber flasks under -10^{o}C until use. Essential oils (EO's) were diluted to concentrations of 10%, 5%, 2.5%, and 1.25% in dimethylsulfoxide (DMSO, Synth) before being tested in biological assays; GC-MS analyzed

each essential oil according to the procedures described elsewhere [7].

According to the previous work [7], the yields of the essential oils were 408OE2 (0.236%), 408OE3 (0.7343%), 408OE4 (0.4202%), 408OE5 (0.5958%), 408OE6 (0.3025%), 408OE7 (0.3587%), 408OE8 (0.3587%), 408OE9 (0.6873%), 408OE10 (0.2916%), 408OE11 (0.5706%), 408OE12 (0.2276%), 408OE13 (0.2432%) and 408OE14 (0.2904%). Of the oils, 50 terpenes were previously identified in the species, and 18 terpenes that occurred in all 13 essential oils were used in the statistical analyses, in the present work: are α-pinene (9.8 %), β-pinene (34.6 %), myrcene (7.4 %), limonene (21.0 %), linalool (1.3 %), terpinen-4-ol (1.0 %), α-terpineol (5.5 %), β-elemene (0.8 %), γ-elemene (0.7 %), δ-amorphene (0.5 %), elemol (0.3 %), spathulenol (2.8 %), viridiflorol (1.2 %), ledol (0.7 %), cubenol-1-epi (0.7 %), isospathulenol (1.3 %), α-cadinol (1.8 %), neo-intermedeol (0.6 %).

Some information regarding *O. platyspermum* has been reported, although information about the biological activity of EO is still unfulfilled. This report aims to fulfill that gap by considering the biological activities of 13 essential oils obtained from leaves collected from the wild plant against *Staphylococcus aureus* and human breast and prostate cancer cell lines based on the light of multivariate analysis.

Highlights

- The essential oils of *O. platyspermum* showed anti-*Staphylococcus aureus* activity
- The essential oils of *O. platyspermum* showed cytotoxicity against cancer cell lines
- Limonene, myrcene, elemol, β- and α-pinene, and terpinen-4-ol highlight biological activity
- The active terpenes are representative of the rainy season in the Amazon

2. MATERIALS AND METHODS

2.1 Microdilution Broth Assay and Determination of Minimal Bactericidal Concentrations

EO's were tested using the microdilution broth assay (MBA), in sterile conditions [8,9]. Briefly, 190-µL aliquot of the bacterial suspension of *Staphylococcus aureus* (ATCC 29213; Thermo Oxoid, USA) adjusted to 1.5×10^8 colony-forming units per mL (CFU/mL) in Müller-Hinton broth was dispensed into 96-well microplates. A 10µL aliquot of each EO at the adequate concentration was added to the correspondent wells. Microplates were incubated at 36°C for 24 h. The inhibition of bacterial growth was visually assessed, and bacterial suspensions from all test wells were sub-cultured in sterile Müller-Hinton agar to evaluate the treatment effectiveness, as first was described for *Enterococcus faecalis* [8] and *Streptococcus mutans* [9]. Minimal bactericidal concentrations (MBCs) against *S. aureus* were obtained for the EO's, using the pre-determined EO concentrations previously described. EO's final test concentrations (20 times fold dilution) for the antibacterial assay were achieved to end up with 0.5%, 0.25%, 0.125%, and 0.0625%, and a 400 times fold dilution was performed to each of the oils used in the cytotoxic assay, resulting in a final concentration of 0.025%. Percentages are given in v/v.

2.2 Cytotoxicity Assay

Breast adenocarcinoma (MCF-7) and prostate carcinoma (PC-3) cancer cell lines were obtained from the National Cancer Institute (NCI/NIH/USA), and were kept cryopreserved up the use. The experiment was performed as previously described [10]. They were weekly cultivated in cell culture flasks with RMPI-1640 supplemented with 5% fetal bovine serum, 1% glutamine, and 1% gentamycin. Flasks were kept in an incubator at 37° C, with 5% CO_2 and 100% relative humidity. Similar conditions were maintained during the assay. Cells were seeded in 96 microplates at densities of 7,500 PC-3 cells/well [11] and 10,000 MCF-7 cells/well [12]. Cultures were incubated for 24h before the essential oils were added. The oils remained in contact with the cells for 48h in the microculture assay. After that, percentages of cell growth were obtained by the sulforhodamine B (SRB, Sigma, USA) assay, as described for lung, colon, and central nervous system cell lines [13]. Doxorubicin (DOXO, Synth, Brazil) was used as a reference drug.

2.3 Sulforhodamine B (SRB) Assay

SRB is a tetrazolium derivative dye that binds to mitochondrial proteins of viable cells, was used in the assay. Viable cells were fixed to the

microplates' wells by adding 50 µL of cold 50% trichloroacetic acid (TCA; Synth, Brazil) for one h under refrigeration. Microplates were washed with water for five times until complete removal of non-viable cells. Plates were left to air-dry for 24 h. An amount of 100 µL of SRB 0.4% in acetic acid was added to each well and kept in contact for 10 min in a plate shaker. After that, unbound SRB was removed from the plate by washing the wells four times with 0.1% acetic acid with a plate washer. The remaining dye was then resuspended with the addition of 100 µL of Trizma Buffer (Merck, Germany) and shaken for 10 min. The total of viable cells was measured by the optical densities of the wells in a microplate spectrophotometer reader at 515 nm. The percentage of cell growth was obtained by the formula [(T-T0)/(C-T0)] * 100 = %CG, where T corresponds to treated cells, C corresponds to control or untreated cells, and T0 corresponds to cell growth in the first 24h of the assay, before treatment being added to the corresponding wells [10]. If the results are negative, cell lethality (CL) has occurred, meaning that the EO reduced the number of cells to a number lower than that registered in T0, and the expression of the results is given in %CL.

2.4 Statistical Analyses

To analyze the relationship among terpenes (variable), biological activities of the 13 essential oils, and seasonal variation (DS and RS), cluster, principal component (PCA), and canonical correspondence analyses (CCA; MVSP statistical package) were performed based on the relative percentage of the 18 terpenes commonly occurring in the 13 essential oils. Seasonal information (dry season, DS, or rain season, RS) and antibacterial/cytotoxicity results expressed as MBCs and %CLs were used in the analyses as factors to ordinate and to perform CCA [14]. Data on the percentage of cell lethality from the cytotoxicity were analyzed by Shapiro-Wilk normality test and by one-sample t-test (GraphPad Prism 7.0 package) considering the percentage of growth inhibition obtained for doxorubicin, the standard drug, as the hypothetical value of -0.42 and -12.24 for breast and prostate cancer cell lines, respectively.

3. RESULTS AND DISCUSSION

Table 1 shows the biological activity observed for the EO's from leaves of *O. platyspermum*. The 13 EO's were significantly active against *S. aureus* and cytotoxic to MCF-7 and PC-3 cancer cells.

Table 1. Results obtained from the cytotoxic analysis against MCF-7 breast and PC-3 prostate cancer cell lines and minimal bactericidal concentrations obtained from antibacterial analysis against *Staphylococcus aureus* (Sau) of the 13 essential oils obtained from the leaves of *Osteophloeum platyspermum*, Myristicaceae, rated by the SRB and microdilution broth assays, respectively. Minimal bactericidal concentrations, MBCs, are expressed in µg/mL and % of growth inhibition, %CL's are expressed in percentage, concerning time zero growth. NT=not tested

	MBC Sau	MCF-7 %CL	PC-3 %CL
408OE2 (DS)	0.25	-7.67	-32.56
408OE3 (RS)	0.25	-14.61	-37.71
408OE4 (RS)	0.5	-25.42	-57.08
408OE5 (RS)	0.06	-3.15	5.39
408OE6 (RS)	0.5	-30.37	-55.78
408OE7(DS)	0.5	-15.38	27.36
408OE8 (DS)	0.13	13.94	26.26
408OE9 (RS)	0.06	7.08	22.87
408OE10 (RS)	0.5	-15.79	-21.82
408OE11 (RS)	0.06	-32.34	-39.78
408OE12 (DS)	0.25	-7.11	-13.08
408OE13 (DS)	0.25	-20.66	-42.23
408OE14 (DS)	0.5	-13.88	-50.75
Doxorubicin	NT	-0.42	-12.24

DS=dry season; RS=rainy season; NT=not tested; CL=cell lethality compared to a T0 cell growth at 24 h.

The differences between percentages of terpenes concerning seasonal occurrence are reported [Fig. 1]. Terpenes interfered with 97.4 % of the variance ($F_{17,198}$=863.8; *P*<.0001), the seasonality contributed with 0.0009 % of the variance ($F_{1,198}$=0.1286; *P*=.7203), and the interaction corresponded to 0.4731 % of the variance ($F_{17,198}$=4.196; *P*<.0001). It was observed that β-pinene occurred more in the RS than in the DS (*P*=.0003), while limonene (*P*=.0326) and α-terpinene (*P*<.0001) occurred in higher amounts during the DS.

In the multivariate analyses, the 18 common terpenes were considered the variables while the 13 essential oils were considered the cases. Fig. 2 shows the results obtained in cluster analysis (UPGMA, Bray-Curtis). In the present work, cluster analysis [Fig. 2] was performed to explore the possibility of ordinating the 13 essential oils according to the dry and rainy seasons, using the variation in percentage within the 18 variables. The ordination was observed for the RS essential oils, which in Fig. 2 are highlighted in green, and for the DS essential oils, highlighted in blue. Three oils did not cluster, 408OE4, 408OE7, and 408OE11.

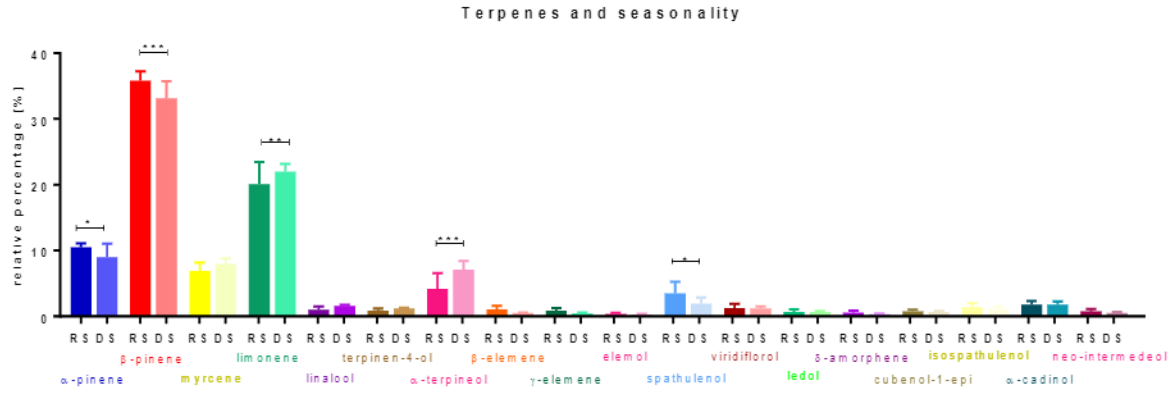

Fig.1. Differences in the terpene percentage in the essential oils obtained from the leaves of wild *Osteophloeum platyspermum* (Myristicaceae) concerning the seasonal occurrence. Two-way ANOVA evaluation followed by Sidak's multi-comparison post-test considering the seasonal factor to compare each terpene expression variation. **P*<.05; ****P*<.0001. Interaction $F_{17,198}$=4.196; *P*<.0001; Seasonality $F_{1,198}$=0.1286; *P*=.7203; Terpenes $F_{17,198}$=863.8; *P*<.0001

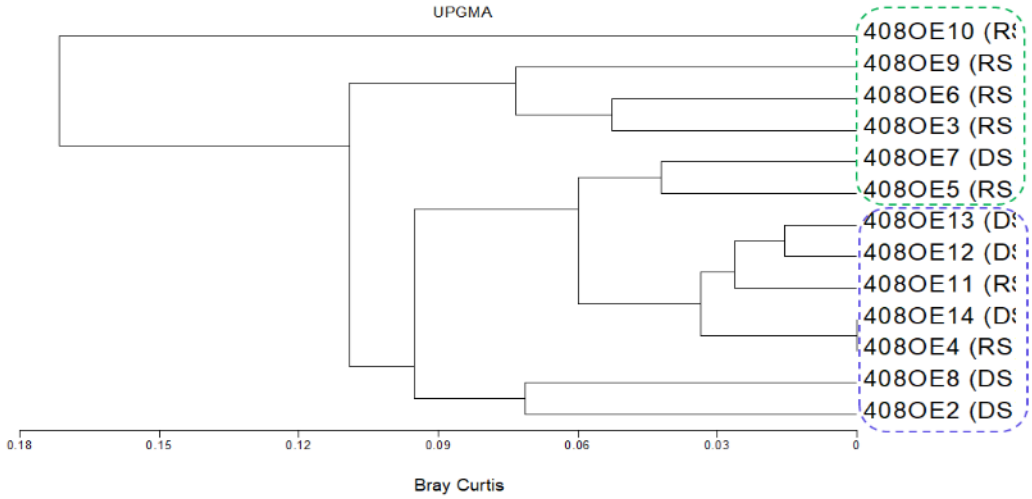

Fig. 2. Cluster analysis results considering 18 terpenes occurring concomitantly in the 13 essential oils obtained from the leaves of *Osteophloeum platyspermum* (Myristicaceae). Blue dots = dry season; green dots = rainy season

Fig. 3 shows the principal component analysis, as seasonally ordinated. The cumulative percentage in the first axis is 53.945, in the second axis is 84.140 and on the third axis is 95.113. The analysis resulted in the EO's ordination according to the seasonal variation related to the dry and rainy seasons. The EO's that composed the DS group were 408OE2, 408OE7, 408OE8, 408OE12, 408OE13, and 408OE14, which were influenced by the presence of α-terpineol, limonene, myrcene, linalool, and terpinen-4-ol. In contrast, the RS group, composed of EO's 408OE3, 408OE5, 408OE6, 408OE9 and 408OE10, were influenced by the presence of the other 13 terpenes, such as spathulenol, α-pinene, β-pinene, isospathulenol, α-cadinol, ledol, neo-intermedeol, δ-amorphene, cubenol-1-epi, elemol, β-elemene, γ-elemene, and viridiflorol. In both cluster analysis and PCA, EO's 408OE4 and 408OE11 did not group in the respective RS set, although 408OE7 was ordinated in its respective DS set in the PCA.

The essential oils of *O. platyspermum* were obtained from the leaves collected from a single tree from November 2009 to October 2011. They were tested against Sau and against breast and prostate human cancer cell lines to evaluate possible correlations of the biological activity to both dry and rain seasons (Table 1). Multivariate analyses are being regularly used to cope with the interpretation of data obtained from essential oil compounds and their relationship with factors as biological activities [15], seasonal expression, and analysis of the influence of climatic factors [14] on the essential oil production [16]. Cluster analysis and PCA were adopted as a tool to discriminate three Myrtaceae essential oils [17], while PCA was used to discriminate clones of *Juniperus communis* and wild *Juniperus* sp. [18]. Variation in the essential oils of five wild populations of *Dorema aucheri* using cluster analysis, PCA and CCA was used to study the relationship of environmental parameters on the amount of some components as α-caryophyllene, thymol, cuparene, and caryophyllene oxide, and β-gurjunene [19].

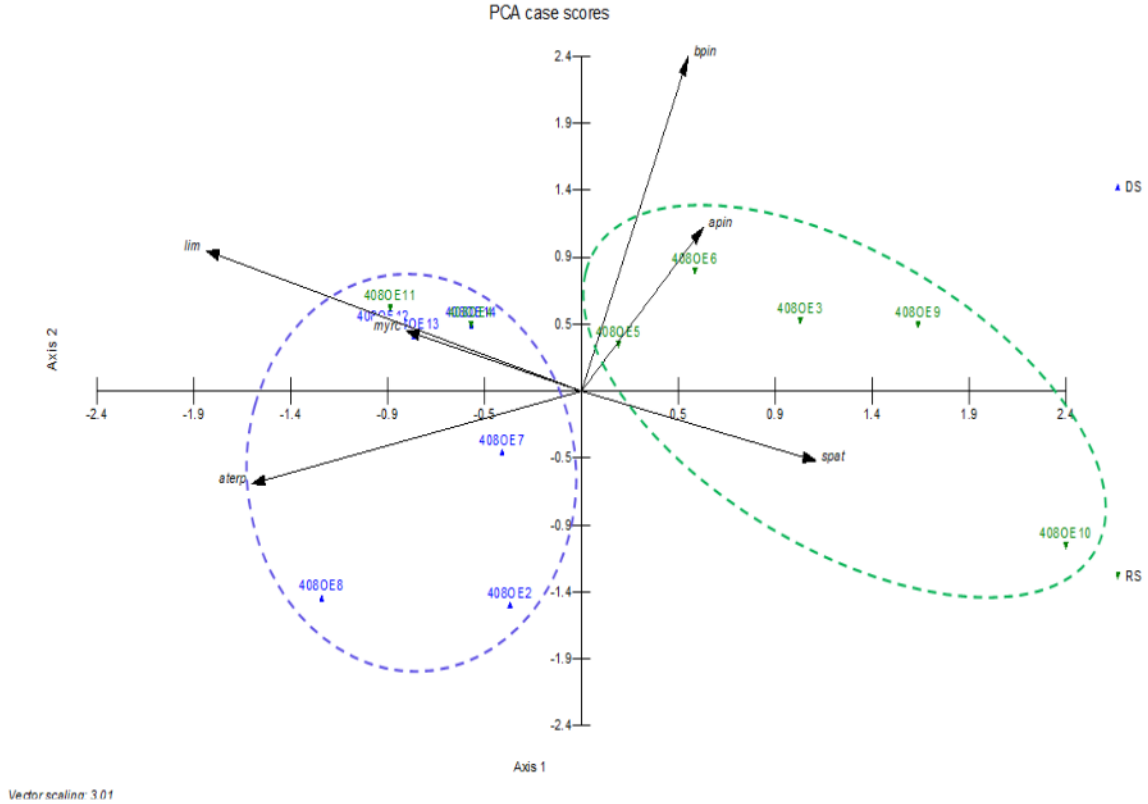

Fig. 3. Results of principal component analysis (PCA) considering 18 terpenes (variables) which concomitantly occur in 13 essential oils (cases). The essential oils were obtained from leaves of *Osteophloeum platyspermum* (Myristicaceae). Significance was considered as α <.05

Fig. 4 shows the CCA results obtained by considering the relative percentage of the 18 terpenes (variables) in each of the 13 EO's (cases), compared to the MBCs and %CL's. The cumulative percentage in the first axis is 73.859 and in the second axis is 91.898. The best essential oils' antibacterial and cytotoxic activities are represented by low MBC's and %CL's values [14]. In Fig. 4A, vectors representing biological activities oppose the active EO's location. It is possible to identify the EO set that is more active against Sau (408OE5, 408OE8, 408OE9, and 408OE11). The same is observed for the more active ones against MCF-7 (408OE4, 408OE6, 408OE11, and 408OE13) and those that were more active against PC-3 (408OE2, 408OE3, 408OE4, 408OE6, 408OE10, 408OE11, 408OE13, and 408OE14). It was observed that the prostate cancer cell line was more sensitive to the activity of the essential oils. Moreover, Fig. 4B shows that limonene, myrcene, and elemol strongly influence the ordination of 408OE4, 408OE11, 408OE12, 408OE13, and 408OE14, and consequently the biological activity. Limonene was found to be present in high amounts, in the Amazon species *Pectis elonglata* [20]. The presence of β-pinene, α-pinene, and terpinen-4-ol is also relevant for the biological activity of the *O. platyspermum* EO's.

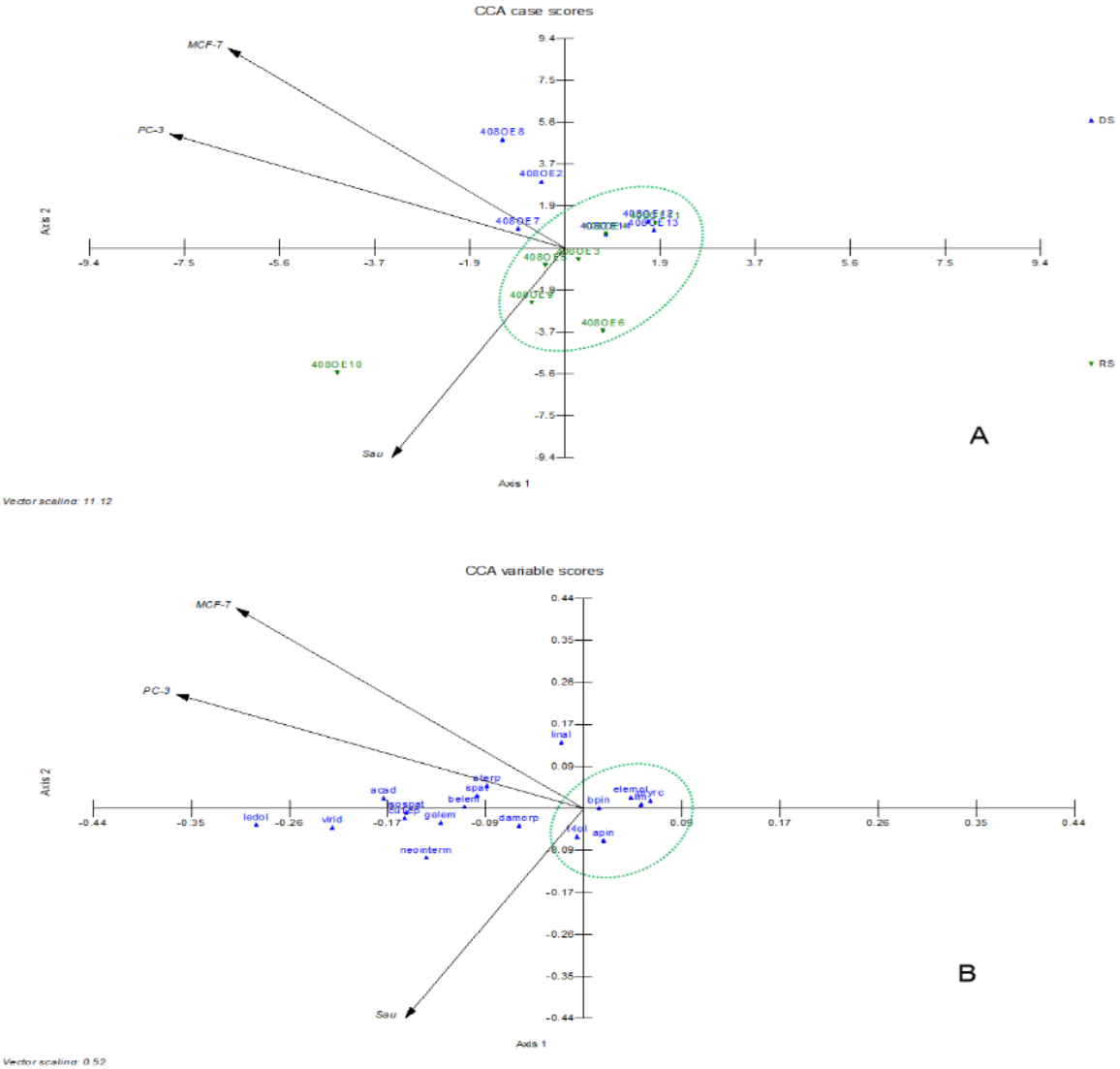

Fig. 4. Canonical correspondence analysis considering 18 terpenes, expressed as the variables in the statistical analyses and that concomitantly occur in the 13 essential oils, defined as the cases, obtained from the leaves of *Osteophloeum platyspermum* (Myristicaceae) and the results obtained from antibacterial and cytotoxic assays. A. Case scores B. Variable scores. Significance was considered as α<.05.

Fig. 5 reports the results of the one-sample t-test performed with data obtained from the breast and prostate cancer cell line cytotoxicity assays. The Shapiro-Wilk normality test was adopted to evaluate the normality of %CL values obtained from breast and prostate cancer cytotoxic assay (*P*=.7665 and *P*=.0716, respectively). One-sample t-test was performed to access the EO's cytotoxic activity compared to doxorubicin (DOXO), the reference drug used in the assay (reference %CL -0.42; actual mean=-12.72, discrepancy of -12.3, where t=3.283; df=12 and *P*=.0065). Also, one-sample t-test made with prostate cancer cells did not show significance (t=0.9683; df=12 and *P*=.3520; theoretical mean = -12.24, actual mean=-20.69, discrepancy of -8.445).

Although the essential oils 408OE4 and 408OE11 were made from the leaves collected in RS, they did not cluster or gather in their respective set. Nonetheless, they showed to be the most active EO's among the 13 that were tested, once 408OE4 was significantly cytotoxic against both breast and cancer cells, and 408OE11 was active against both the bacteria and cytotoxic against the breast cancer cell.

Table 1 shows results from minimal bactericidal concentrations (MBCs) obtained against *Staphylococcus aureus* ATCC 29213 (Sau), which were tested in the microdilution broth assay and expressed as percentage (v/v). Table 1 also shows results obtained from the cytotoxicity analyses performed with EO's against human breast (MCF-7) and prostate (PC-3) cancer cell lines, expressed as %CL. According to our findings, EO's 408OE5, 408OE9, and 408OE11, in which MBCs were 1.25 µg/mL, showed the highest activity against Sau, while 408OE4, 408OE6, 408OE7, 408OE10, and 408OE14 were less active against the bacteria. EO's 408OE4, 408OE6, and 408OE11 were significantly cytotoxic against the breast cancer cell, while 408OE4, 408OE6, and 408OE14 were more active against the prostate cancer cell line. Essential oils obtained from leaves collected in the rainy season showed that they are more active against Sau (408OE5, 408OE9, and 408OE11), in addition to being more cytotoxic against the cancer cells (408OE4, 408OE6, 408OE11). Oils 408OE4 and 408OE6 were cytotoxic to both cell lines while less active against the bacteria.

Fig. 5 represents the essential oils' breast and prostate cancer cytotoxicity statistics and corroborates findings for 408OE4, 408OE6, and 408OE11. According to our results, March, April, and May are relevant months in which the terpene composition in the essential oils from *O. platyspermum* supports better biological activities.

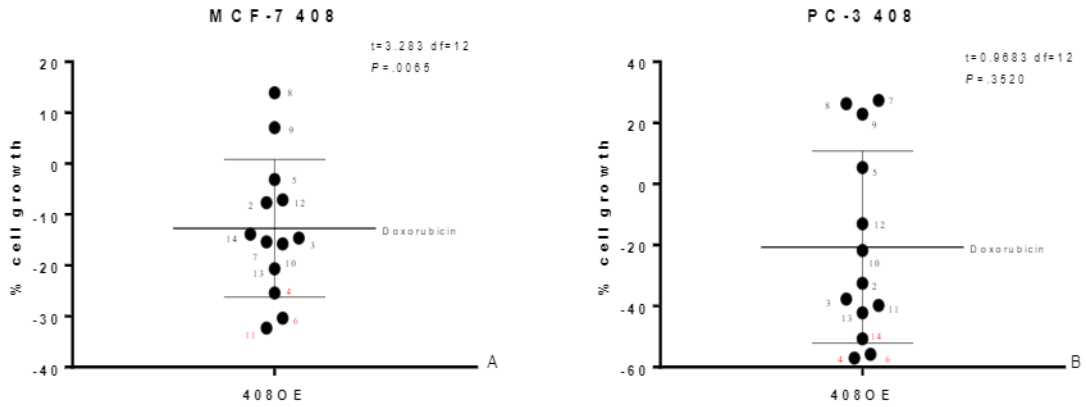

Fig. 5. A. One-sample t-test made with the percentage growth/lethality obtained in the cytotoxic assay using the human breast cancer cell line MCF-7. B. One sample t-test made with the percentage growth/lethality obtained in the cytotoxic assay using human prostate cancer cell line PC-3. Theoretical means were defined as the value corresponding to the percentage of growth/lethality obtained for doxorubicin, the standard drug, determined as -0.41 and -12.24, respectively. Significance was considered as α<.05

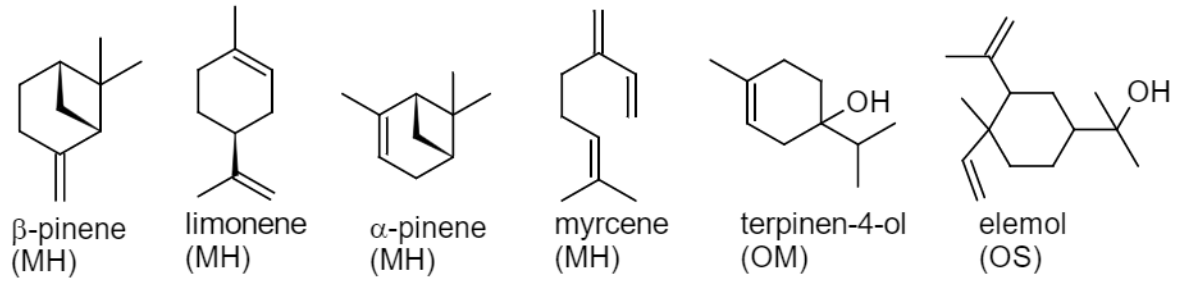

Fig. 6. Relevant terpenes involved in the biological activity of the essential oils obtained from the leaves of *Osteophloeum platyspermum*

4. CONCLUSIONS

The essential oils obtained from the leaves of *O. platyspermum* have been ordinated into two sets related to the Amazon rain forest seasonality. Essential oils also showed variability in antibacterial and cytotoxic activities. Although the 18 terpenes found in the EO's are relevant to the species, the occurrence of limonene, myrcene, elemol, β-pinene, α-pinene, and terpinen-4-ol [Fig. 6] played a significant role in the effectiveness of the biological activity, particularly considering the terpenes that are expressed during the rainy season, in the Amazon rain forest.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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