Therapeutic switching: from antidermatophytic essential oils to new leishmanicidal products

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This study examined whether the antidermatophytic activity of essential oils (EOs) can be used as an indicator for the discovery of active natural products against Leishmania amazonensis. The aerial parts of seven plants were hydro-distilled. Using broth microdilution techniques, the obtained EOs were tested against three strains of dermatophytes (Trichophyton mentagrophytes, Microsporum gypseum and Microsporum canis). To compare the EOs antifungal and antiparasitic effects, the EOs activities against axenic amastigotes of L. amazonensis were concurrently evaluated. For the most promising EOs, their antileishmanial activities against parasites infecting peritoneal macrophages of BALB/c mice were measured. The most interesting antifungal candidates were the EOs from Cymbopogon citratus, Ocota nthus azureus and Protium heptaphyllum, whereas O. azureus, Piper hispidum and P. heptaphyllum EOs exhibited the lowest 50% inhibitory concentration (IC50) values against axenic amastigotes, thus revealing a certain correspondence between both activities. The P. hispidum EO was identified as the most promising product in the results from the infected macrophages model (IC50 4.7 µg/mL, safety index: 8). The most abundant compounds found in this EO were sesquiterpenes, notably curzerene and furanodiene. Eventually, the evaluation of the antidermatophytic activity of EOs appears to be an efficient method for identifying new potential drugs for the treatment of L. amazonensis.

Key words: therapeutic switching - antifungal agents - antiparasitic agents - Leishmania - peritoneal macrophages - sesquiterpenes

A promising, current strategy for the discovery of bioactive natural products is based on bioinspiration. The aim is to understand the functional role of secondary metabolites in living organisms and transpose the desirable properties to a corresponding research field. Gaining inspiration from the abilities of plants or microorganisms to produce adapted bioactive molecules under environmental pressure has led to some promising results, for example, in the search for antibiotic or antiviral agents (Pan et al. 2010) or natural antifungal products (Basset et al. 2012). Essential oils (EOs) are composed of volatile odoriferous compounds which play a major role in the complex interactions taking place between plants and pollinators, herbivorous insects, larger herbivores or microorganisms. In particular, they are among the most efficient antimicrobial compounds of plants’ chemical defense systems (Unsicker et al. 2009). This antimicrobial activity points to the use of a bioinspired strategy for the search for antifungal compounds within EOs. In the context of the growing interest in the uses of medicinal plants and, especially, EOs as new antifungal agents (Rios & Recio 2005), we examined seven EOs obtained from particularly fragrant plant species from French Guiana, presenting a distinctive richness and complexity of volatile compounds that potentially exhibit antimicrobial activity. In addition, the extensive search for new drugs to treat leishmaniasis is definitely necessary because the limited number of currently available products present noticeable side effects and the resistance to these products is increasing (Rocha et al. 2005). Known antifungal drugs such as amphotericin B, miltefosine and azoles have also demonstrated activity against Leishmania parasites (Moskowitz & Kurban 1999, Tong et al. 2007, Shakya et al. 2011b). These successful results led to the development of the “therapeutic switching” or “alternative drug use” strategy (Shakya et al. 2011a). In accord with this perspective, we evaluated the antileishmanial properties of selected antidermatophytic EOs. To our knowledge, the correspondence between these two activities has never been investigated for these particular natural products.

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MATERIALS AND METHODS

General remarks - Plant material and sample preparation - Seven EOs were obtained: Achetaria guianensis Pennell (Scrophulariaceae, leaves and stems), Cymbopogon citratus (DC.) Stapf (Poaceae, leaves), Mikania micrantha Kunth (Asteraceae, aerial parts), Ocatacanthus azureus (Linden) Ronse (Plantaginaceae, aerial parts), Piper hispidum Sw. (Piperaceae, leaves), Protium heptaphyllum (Aubl.) Marchand (Burseraceae, fresh green fruits), Vouacapoua americana Aubl. (Fabaceae, wood). Herbarium vouchers (respectively Silland 8, 40, 31, 30, 23, 20 and Rodrigues 6) were deposited in the French Guiana herbarium (CAY), where specialists (S Gonzalez, MF Prevost, F Crozier) and members of our laboratory (E Houël, A Rodrigues) confirmed identification. Plants were collected in French Guiana near Matoury and Cayenne, mainly during the rainy season (April-July) except for A. guianensis which was collected during the dry season (November). The fresh parts collected from each plant were hydrodistilled and the EOs were stored at -18°C until the subsequent analyses were performed. The material under study is endozone free.

Nuclear magnetic resonance (NMR) spectroscopy - The 1H NMR spectra and 13C NMR spectra were recorded at 400 MHz and 100.6 MHz, respectively, using a Varian 400 MR spectrometer equipped with a 5 mm inverse probe (Auto X PGF 1H/15N-13C). The EOs were dissolved in deuterated chloroform (CDCl3) in 5 mm tubes.

Gas chromatography-mass spectrometry (GC-MS) analysis - A Varian 450-GC fitted with a MS240 ion-trap MS and a Combipal autosampler was used for the GC-MS analysis. The GC was run with a non-polar Varian FactorFour VF-5ms column (30 m × 0.25 mm ID, 0.25 μm film) commonly used for the analysis of VOCs. The injection volume (EO dissolved in chromatography-grade hexane) was 1 μL. Helium was used as the carrier gas at a constant flow of 1 mL/min. The column temperature increased from 50-150°C at 4°C/min, then from 150-175°C at 1.5°C/min and from 175-300°C at 20°C/min for a total analysis time of 58.42 min. The injector temperature was set to 250°C and the injection was made with a split ratio of 1/50 during the whole run. The MS was operated in the electron impact mode at 70 eV, with a scan range of 40-400 m/z. The temperatures were set to 200°C for the ion trap, 50°C for the manifold and 305°C for the transfer line. The relative proportions of constituents of the EOs were expressed as the percentages obtained by peak area normalisation.

Component identification - The identification of the components of the EOs was based on the following: (i) GC retention indices (RI) on a non-polar column, (ii) computer matching with commercial mass spectral libraries (NIST 98 MS, ADAMS) (Adams 2007), (iii) comparisons of RI and spectra with those from previous work (Courtois et al. 2009, Houël et al. 2014) and from an in-house library of analyses of commercial EOs of known composition (Aromazone) and (iv) NMR spectroscopy.

Fungal strains - One clinical isolate of a Trichophyton species (Trichophyton mentagrophytes LMGO 1931) and two clinical isolates of Microsporum species (Microsporum gypseum LMGO 10 and Microsporum canis LMGO 22) were kindly provided by Dr Maria do Rosario Silva (University Hospital, Federal University of Goiás, Brazil). The cultures were maintained on potato dextrose agar and were cultured onto a new agar plate at 28°C for five days prior to antimicrobial tests.

Parasites and cultures - A cloned line of Leishmania amazonensis (strain MHOM/BR/76/LTB-012) was used in all of the experiments. An axenically grown amastigote form of L. amazonensis was maintained by weekly subculturing in MAA20 medium at 32±0.5°C in 25 cm² tissue culture flasks with 5% CO₂ and supplemented with 20% heat-inactivated foetal bovine serum (FBS), as previously described (Estevez et al. 2007).

Minimal inhibitory concentration (MIC) - The standard microdilution test was used to determine the MIC of the EOs. The experimental details were similar to those described previously (Houël et al. 2014). All assays were conducted in triplicate.

Cytotoxicity assay using VERO cells - VERO cells (African Green Monkey kidney epithelial cells) were seeded (5 × 10⁴ cells mL⁻¹, 100 μL per well) in 96-well flat-bottom plates at 37°C with 5% CO₂. RPMI-1640 medium without phenol red and supplemented with 10% heat-inactivated FBS was used. After the EOs were added, the cells were cultured for 48 h. The effects of the treatments were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay. Four hours after the addition of MTT, 100 μL of lysis buffer [50% isopropanol, 10% sodium dodecyl sulfate (SDS)] was added and the cells were shaken for 30 min at room temperature (RT). The optical density (OD) was read at 595 nm using a 96-well plate reader (Chameleon, Hidex; Finland). All experiments were performed in triplicate. The median toxic dose (TD₅₀) values were determined using linear regression analysis. The TD₅₀ was defined as the concentration of the test sample that resulted in a 50% reduction of absorbance compared to controls.

Activity on axenic amastigotes - All experiments were performed in triplicate. The in vitro leishmanicidal activities of the EOs were determined in axenic cultures of the amastigote form of L. amazonensis. To estimate the 50% inhibitory concentration (IC₅₀) of the extracts, the MTT was used as previously described (Estevez et al. 2007). Results were expressed as the percentage reduction of parasite burden compared to the level in untreated control wells and the IC₅₀ was determined from the concentration response curves (Excel software). Briefly, axenically grown amastigotes during the late log phase of growth were seeded in 96-well flat bottom microtitre plates. EOs, dissolved in dimethyl sulfoxide (DMSO), were added at final concentrations ranging from 100-10 μg/mL. The final DMSO concentration was never > 0.1%. After 72 h of incubation, 10 μL of MTT (10⁻³ μg/μL) was added to each well and the plates were further incubated for 4 h. After these 4 h, the enzymatic reaction was stopped with 100 μL of a 50% isopropanol and
In vitro antifungal activity of EOs - The in vitro antifungal activities of the seven EOs are presented in Table I. To improve the clarity of the results, a score representing the global antifungal activity was attributed to each EO. A MIC greater than 500 µg/mL received a 0, a MIC of 500 µg/mL received a 1, a MIC of 250 µg/mL received a 2 and each subsequent reduction in MIC by a factor of 500 µg/mL received an increment of 1. According to these scores, the most active antifungal EOs are those of C. citratus, with a score of 17 (representing MICs of 16, 8 and 62 µg/mL against M. gypseum, T. mentagrophytes and only twice that obtained for fluconazole). The EOs of M. micrantha (5) and A. guianensis (0) exhibited weak to non-existent activity against the selected dermatophytic filamentous fungi (MIC values from 125 to > 500 µg/mL). Among the remarkably active oils, the MICs were as low as 8 µg/mL against T. mentagrophytes and 16 µg/mL against M. canis and 62 µg/mL against M. gypseum.

Effects of EOs activities on the growth of axenic amastigotes and cytotoxic effects on BALB/c mouse peritoneal macrophages - The seven EOs were concurrently tested against axenic amastigotes of L. amazonensis. The
results are presented in Table I. While the EOs from *C. citratus*, *O. azureus* and *P. heptaphyllum* were the most interesting antifungal candidates, the EOs of *O. azureus*, *P. hispidum* and *P. heptaphyllum* exhibited the lowest IC$_{50}$ against axenic amastigotes, thus revealing a certain level of correspondence between both activities. A very high in vitro activity (IC$_{50}$ of 0.7 µg/mL) was measured for the *O. azureus* EO. This value is in the same range as the one obtained for the reference compound amphotericin B (0.3 µg/mL). The *P. heptaphyllum* and *P. hispidum* EOs were also remarkably active against the parasite (IC$_{50}$ values of 3.7 and 3.4 µg/mL, respectively). Overall, IC$_{50}$ values < 10 µg/mL were recorded for all seven EOs.

We also evaluated the selectivity index (SI) based on the toxicity measured on healthy macrophages. The most interesting oil in this respect was *O. azureus*, which had an SI value of 51. Among the other oils identified as the most active against *L. amazonensis*, the EOs of *P. heptaphyllum* and *P. hispidum* exhibited reasonably high selectivity indices of 19 and 11, respectively, which were comparable to the value of 12 obtained for amphotericin B. In contrast, even though the *C. citratus* EO was identified as the most potent antidermatophytic product and also exhibited high antileishmanial activity, this EO was shown to have a low SI of only 5 and thus is not as good of a candidate as the other three EOs with relatively high SIs.

Based on these results, the EOs of *O. azureus*, *P. heptaphyllum* and *P. hispidum* were selected to be further evaluated for their antileishmanial activity against parasites infecting BALB/c mice peritoneal macrophages.

**Cytotoxicity assay on VERO cells** - The toxicities of the EOs towards VERO cells are presented in Table I. Interestingly, the three most antileishmanial EOs (*O. azureus*, *P. heptaphyllum* and *P. hispidum*) exhibited no cytotoxicity against VERO cells (TD$_{50}$ > 100 µg/mL). The *M. micrantha* EO was also not cytotoxic. However, the EOs of *C. citratus*, *A. guianensis* and *V. americana* were all cytotoxic towards VERO cells at concentrations between 10-35 µg/mL. These results confirmed the selection of *O. azureus*, *P. heptaphyllum* and *P. hispidum* for further evaluation.

**Leishmanicidal activity in *L. amazonensis*-infected BALB/c mice peritoneal macrophages** - To evaluate the potential of the three selected EOs as clinical antileishmanial agents, they were added to a culture media containing *L. amazonensis*-infected BALB/c mice peritoneal macrophages (Table II). Notably, the *P. hispidum* EO exerted the highest leishmanicidal effect, with an IC$_{50}$ of 4.7 µg/mL. While this value is superior to the one recorded for amphotericin B (0.6 µg/mL), the safety indices are very similar.

The infection reduction indices were also calculated. In this respect, the *P. hispidum* EO was the most active causing a 97.5% reduction of the infection at a dose of 20 µg/mL. The same activity was obtained at 2 µg/mL for amphotericin B.

**Determination of the composition of the *P. hispidum* EO by GC-MS and NMR analyses** - As the *P. hispidum* EO was identified as the most promising product in the infected macrophages model it was submitted to detailed chemical analysis. There were 64 compounds identified in the *P. hispidum* EO, accounting for 90.5% of the composition of the oil. The details of the identifications and relative concentrations of the compounds found in the hydrosdistilled oil of *P. hispidum* are reported in Supplementary Table. The compounds representing more than 1% of the EO are described in Table III. The chemical composition of the *P. hispidum* EO obtained in this study revealed that sesquiterpenes are the most abundant compounds; the five most abundant compounds identified by the GC/MS analysis were curzerene (15.7%), germacrene B (10.9%), α and β-selinene (10.5 and 7.6%, respectively) and β-caryophyllene (4.7%) (Figure). It is known that curzerene can be produced from furanodione through a thermal Cope rearrangement, with 1,4-diienes being involved in this [3,3]-sigmatropic reaction due to the high temperatures that occur during the injection of the sample into the GS (Baldovini et al. 2001). The comparison of the $^{13}$C NMR spectra of the crude oil with the data in the literature allowed us to confirm the presence of curzerene, but also revealed the presence of the heat-sensitive compound furanodione in the crude EO.

| Leishmania amazonensis | IC$_{50}$ BALB/c mice infected peritoneal macrophages | Safety index on macrophages | Infection reduction index (%) (maximum concentration, µg/mL) |
|------------------------|------------------------------------------------------|----------------------------|----------------------------------------------------------|
| *Otacanthus azureus*   | 16.1                                                 | 2                          | 64.7 (20)                                                |
| *Piper hispidum*       | 4.7                                                  | 8                          | 97.5 (20)                                                |
| *Protium heptaphyllum* | 34.9                                                 | 2                          | 59.6 (40)                                                |
| Amphotericin B         | 0.6                                                  | 6                          | (2)                                                      |

**TABLE II**
Antileishmanial activity [50% inhibitory concentration (IC$_{50}$) (µg/mL)] against infected BALB/c mice peritoneal macrophages, safety index for BALB/c mice peritoneal macrophages and infection reduction index at the maximum concentration measured for the three most promising essential oils (EOs) and the reference antileishmanial drug (amphotericin B)
even if the relative proportions could not be evaluated (Baldovini et al. 2001). Hence, the curzerene identified in the GC/MS analysis in fact originates from curzerene already present in the EO and from its precursor, furanodiene; thus, the quantitative data are affected by the contribution from the Cope rearrangement.

### TABLE III

Main components (> 1 %) of the *Piper hispidum* essential oil identified by the gas chromatography-mass spectrometry analysis

| RI  | Composition (%) | Compound          | Courtois et al. (2009) | Adams (2007) | Houël et al. (2014) |
|-----|----------------|-------------------|------------------------|--------------|---------------------|
| 935 | 1              | α-pinene          | 940                    | 932          | 936                 |
| 980 | 1.4            | β-pinene          | 985                    | 974          | 981                 |
| 1379| 1.2            | α-copaene         | 1381                   | 1374         | 1380                |
| 1391| 2.6            | β-elemene         | 1385                   | 1389         | 1391                |
| 1423| 4.7            | β-caryophyllene   | 1427                   | 1417         | 1424                |
| 1432| 1.5            | γ-elemene         | 1432                   | 1434         | -                   |
| 1434| 1.2            | β-copaene         | -                      | 1430         | -                   |
| 1460| 2.2            | α-humulene        | 1462                   | 1452         | 1461                |
| 1476| 1.1            | selina-4,11-diene | 1482                   | -            | -                   |
| 1493| 7.7            | β-selinene        | 1496                   | 1489         | -                   |
| 1497| 15.7           | curzerene<sup>b</sup> | -                    | 1499         | -                   |
| 1499| 10.5           | α-selinene        | 1496                   | 1498         | 1499                |
| 1515| 1.1            | γ-cadinene        | 1518                   | 1513         | -                   |
| 1519| 3.4            | δ-cadinene        | 1521                   | 1522         | -                   |
| 1524| 1.4            | calamenene (UI)   | -                      | 1521/1528    | 1524                |
| 1561| 10.9           | germacrene B      | 1567                   | 1559         | -                   |
| 1597| 1.4            | viridiflorol      | -                      | 1592         | 1599                |
| 1620| 1.3            | 1,10-di-epi-cubene| -                      | 1613<sup>c</sup> | -                |
| 1657| 3.9            | 7-epi-α-eudesmol  | -                      | 1662         | -                   |
| 1660| 4.6            | junicedranone     | -                      | 1664         | -                   |
| Total| 78.9         | -                 | -                      | -            | -                   |

<sup>a</sup>: the identified constituents are listed in their order of elution from a non-polar column (Varian FactorFour VF-5ms); <sup>b</sup>: from curzerene and furanodiene. Quantitative data are affected by thermal rearrangement; <sup>c</sup>: Cicció and Chaverri (2008); RI: retention indices; UI: undetermined isomer.

#### DISCUSSION

The three most active antifungal EOs were those from *C. citratus*, *O. azureus* and *P. heptaphyllum*. The EO of *C. citratus* has largely been described as antifungal (Shin & Lim 2004, da Silva et al. 2008). In our study, the *C. citratus* EO was mainly composed of neral (31%) and geranial (56%), corroborating the already well-known antifungal activity of citral, known to act by forming a charge transfer complex with an electron donor of fungal cells and thus causing fungal death (da Silva et al. 2008). The antidermatophytic activity and chemical composition of the *O. azureus* EO has been further studied elsewhere (Houël et al. 2014). It was shown to be largely composed of sesquiterpenes, with the main component being β-copaen-4-α-ol (23%), alongside α-humulene (10.6%), α-copaene (8.8%), myrtenal (5.6%), viridiflorol (5.1%) and trans-pinocarveol (4.3%). Concerning the EO of *P. heptaphyllum*, we have demonstrated for the first time that the oil extracted from fresh green fruits is a highly potent antifungal agent against dermatophytic filamentous fungi. Moreover, this oil exhibited no cytotoxicity against VERO cells (TD<sub>50</sub> > 100 µg/mL). Further studies should be conducted on this EO to confirm the fact that it represents a promising prod-
uct for the treatment of human superficial dermatomyco-
ses. In our extract, the *P. heptaphyllum* EO was mainly
composed of limonene (82%), along with small propor-
tions of other monoterpenes (α-pinene 5.4%, β-pinene
2.5%, *p*-cyrene 1.5%, *trans*-carveol 0.9%, β-myrcene
0.7% and carvone 0.7%). This composition differs from the
one already published for immature fruits (Pontes et al.
2007), which indicated that the primary component was α-terpinene.
We tested the three main compounds for their antidermatophytic
activities, but all of them were inactive. Similarly to the *O. azureus* EO, the antifungal activity could thus be due to a synergistic effect
of multiple compounds, as that described for limonene and α-pinene on *S. cerevisiae* or to the activity of a minor component (Tserennadmid et al. 2011). In addition, the
EOs of *V. americana* and *P. hispidum* also exhibited sig-
ificant antifungal activity. Antidermatophytic as well as
antimicrobial activity have already been described in the litera-
ture for some *P. hispidum* EOs (Morales et al. 2013,
Tangariñe-Castaño et al. 2014).

The seven oils were concurrently tested against ax-
enic amastigotes of *L. amazonensis*. Infections with this
parasite result in a clinical spectrum of manifestations that
includes all three forms of leishmaniasis (cutaneous,
mucosal and visceral) (Rocha et al. 2005). Of the
three most antifungal EOs, two of them (*O. azureus* and
*P. heptaphyllum*) also exhibited remarkable antileish-
manial activities, especially *O. azureus* (IC₅₀ 0.7 µg/mL).
Concerning *O. azureus* EO, none of its main components
to our knowledge have been clearly identified as antileish-
manial. However, *P. heptaphyllum* EO was shown to be
mainly composed of limonene, recently demonstrated to
attack the plasma membrane of the parasite (Camargos
et al. 2014). A third oil, that of *P. hispidum* leaves, was
also identified as a potent antiamastigote agent with an
IC₅₀ of 3.4 µg/mL. We had previously observed that this
EO exhibited significant antifungal activity, demonstrat-
ed by a high score for activity (8) and MIC values rang-
ing from 62-500 µg/mL. Notably, the *C. citratus* EO was
identified as the most potent antidermatophytic product and
also demonstrated significant anti-amastigote ac-
tivity. This dual activity against both filamentous der-
matophytic fungi and *Leishmania* sp. amastigotes has
already been observed with miltefosine, amphotericin B
and azoles (Moskowitz & Kurban 1999, Tong et al. 2007,
Shakya et al. 2011b). In fact, amphotericin B and azoles,
which were initially developed as antifungals and are now
used (or have been successfully tested) against *Leishma-
nia* sp., are both involved in interactions with the sterols of
fungal membranes that lead to cell death. The former
cause death by inhibiting the demethylation of lanosterol
and the latter disrupts the synthesis of ergosterol (Ghan-
noum & Rice 1999). The antileishmanial activities of
these molecules is thus due to the relatively high content
of ergosterol in the membranes of *Leishmania* and the
result of similar mechanisms to those occurring in fungi
(Gebre-Hiwot & Frommel 1993). In addition, miltefos-
ine interferes with phospholipid metabolism (Tong et al.
2007). Targeting antifungal natural products potentially
having an effect on *Leishmania* cell membrane is thus
relevant (Bou et al. 2014). To our knowledge, this is the
first time that such a correspondence in activity has been
shown for EOs, even though the modes of actions should
be investigated further.

At this stage of the study, the EOs found to exhibit both
the best antifungal activity and the lowest IC₅₀ against ax-
enic amastigotes were those of *O. azureus*, *C. citratus*,
*P. heptaphyllum* and *P. hispidum*. The toxicities of these
eOSs towards BALB/c mice peritoneal macrophages were
then also evaluated. The best selectivity indices regard-
ing antiparasitic activity were obtained for the *O. azureus*
(71), *P. heptaphyllum* (19) and *P. hispidum* (11) EOs. These
three EOs were also non-toxic to VERO cells, whereas
the *C. citratus* EO had a TD₅₀ of 30.7 µg/mL. It should
be noted that the *O. azureus* and *P. heptaphyllum* EOs
or extracts have never been described as antileishmanial
agents. Though *P. hispidum* extracts are already known
for their antileishmanial activity against *L. amazonensis*
(Estevez et al. 2007, Ruiz et al. 2011), this is the first time
that these properties are described for the EO.

To confirm the potential use of these EOs as antileish-
manial agents and corroborate the results indicating that
the examination of alternative uses of natural antifungal
products could lead to the discovery of promising anti-
leishmanial drugs, we evaluated the activity of these last
three oils on *L. amazonensis*-infected BALB/c mice peri-
toneal macrophages, excluding the *C. citratus* EO be-
cause of its relative toxicity. As *Leishmania* parasites survive
and multiply within mammalian macrophages, this model
produces results more closely related to in vivo results and
a therapeutic drug can only demonstrate activity if it can
cross the host cell membrane and act on the intracellular
amastigotes (Kyriazis et al. 2013, Rodrigues et al. 2013).
The EO of *P. hispidum* was clearly the most potent and
promising oil, with an IC₅₀ of 4.7 µg/mL and a safety index
of 8, a value superior to the one calculated for the refer-
ence drug amphotericin B. This EO reduced the infection
by 97.5% at 20 µg/mL. The present results confirm the
interest of natural compounds study, including crude ex-
tracts or fractions, for the discovery of potent antileish-
manial compounds, as underlined by Rabito et al. (2014).

The compositions of some *P. hispidum* EOs have al-
ready been described in the literature (Pino et al. 2004,
Benitez et al. 2009, Cruz et al. 2012, Assis et al. 2013,
Morales et al. 2013). Our findings are consistent with
previous results; the *P. hispidum* EO extracted in this
study was mainly composed of sesquiterpenes, though
the proportions of oxygenated sesquiterpenes and ses-
quiterpene hydrocarbons are highly variable. This re-
sult possibly being due to seasonal or environmental
variations (Figueiredo et al. 2008, Duarte et al. 2009),
repeating this study on new *P. hispidum* collections and
extractions could therefore help to assure the correla-
tion between the EO composition and biological activity.
Curzerene has been previously identified in some oils,
but never as the major component (Benitez et al. 2009).
In our hands, the *P. hispidum* EO was shown to contain
both curzerene and its precursor furanodiene and the
relative proportion of curzerene calculated by GC analy-
sis was thus overestimated. Other furanosesquiterpenes
were detected by GC/MS and NMR analysis but could
not be identified. Curzerene has already been found in
other antileishmanial EOs (Rodrigues et al. 2013) and, considering our findings, furanosesquiterpenes could contribute to the antileishmanial activity of the *P. hispidum* EO. Moreover, β-caryophyllene, which accounts for 4.7% of this EO, is known to be an antileishmanial compound, possibly having an antileishmanial activity associated with the inhibition of the biosynthesis of cellular isoprenoids (Santos et al. 2008). According to these data and those concerning the other active EOs, correlating the chemical composition of the EOs and their biological activity, for example through a metabolomic approach, could lead to valuable information. Indeed, β-caryophyllene representing in particular only 0.52% of *O. azures* EO (Houël et al. 2014) and not having been identified in *P. heptaphyllum* EO, other potent antileishmanial molecules could thus be revealed.

In conclusion, the bioinspired selection of fragrant species successfully led to the identification of strongly antifungal compositions. This study also demonstrated the significant antileishmanial potential of the EO of *P. hispidum* against *L. amazonensis*, pending confirmation with in vivo assays. It would also be an interesting perspective to perform synergy studies between the most abundant compounds and antileishmanial chemotherapeutics as amphotericin B, as well as further investigate the role of synergy concerning biological activity and selectivity of the crude oil itself. Eventually, the evaluation of the antidermatophytic activities of EOs appears to be a promising strategy for the discovery of new natural antileishmanial products, a significant achievement within the context of the alternative drug use, especially considering factors such as the low cost, high accessibility, high availability and reduced cytotoxicity of these products.

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**REFERENCES**

Adams RP 1995. Identification of essential oil components by gas chromatography/mass spectrometry, 3rd ed., Allured Publishing Corporation, Carol Stream, 466 pp.

Adams RP 2007. Identification of essential oil components by gas chromatography/mass spectrometry, 4th ed., Allured Publishing Corporation, Carol Stream, 401 pp.

Appel MA, Sobral M, Schapoval EES, Henriques T, Menut C, Besiere J-M 2004. Essential oil composition of *Eugenia florida* and *Eugenia mansonii*. *J Essent Oil Res* 16: 321-322.

Assis A, Brito V, Bittencourt M, Silva L, Oliveira F, Oliveira R 2013. Essential oils composition of four *Piper* species from Brazil. *J Essent Oil Res* 25: 203-209.

Baldovini N, Tomi F, Casanova J 2001. Identification and quantitative determination of furanodiene, a heat-sensitive compound, in essential oil by 13C-NMR. *Phytochem Anal* 12: 58-63.

Basset C, Rodrigues AMS, Eparvier V, Silva MRR, Lopes NP, Sabatier D, Fonty E, Espindola LS, Stien D 2012. Secondary metabolites from *Spirotropis longifolia* (DC) Baill and their antifungal activity against human pathogenic fungi. *Phytochemistry* 74: 166-172.

Benitez NP, Leon EMM, Stashenko EE 2009. Essential oil composition from two species of *Piperaceae* family grown in Colombia. *J Chromatogr Sci* 47: 804-807.

Bou DD, Tempone AG, Pinto EG, Lago JHG, Sartorelli P 2014. Antiparasitic activity and effect of casearins isolated from *Casearia sylvestris* on *Leishmania* and Trypanosoma cruzi plasma membrane. *Phytotherapy* 21: 676-681.

Camargos HS, Moreira RA, Mendanha SA, Fernandes KS, Dorta ML, Alonso A 2014. Terpenes increase the lipid dynamics in the *Leishmania* plasma membrane at concentrations similar to their IC50 values. *PLoS ONE* 9: e104429.

Cicció JF, Chaverri C 2008. Volatile constituents of the oils from *Pouteria quadriflora* (Lauraceae) from Alberto M Brenes Biological Reserve, Costa Rica. *Quim Nova* 31: 665-669.

Courtois EA, Paine T, Blandinieres P-A, Stien D, Bessiere J-M, Houel E, Baraloto C, Chave J 2009. Diversity of the volatile organic compounds emitted by 55 species of tropical trees: a survey in French Guiana. *J Chem Ecol* 35: 1949-1962.

Cruz SM, Cáceres A, Álvarez LE, Apel MA, Henriques AT 2012. Chemical diversity of essential oils of 15 *Piper* species from Guatemala. *Acta Hortic* 964: 39-46.

da Silva CB, Gutierrez SS, Weisheimer V, Schapoval EES 2008. Antifungal activity of the lemongrass oil and citral against *Candida* spp. *Braz J Infect Dis* 12: 63-66.

Duarte AR, Naves RR, Santos SC, Seraphin JC, Ferri PH 2009. Seasonal influence on the essential oil variability of *Eugenia dyssenterica*. *J Braz Chem Soc* 20: 967-974.

Estevez Y, Castillo D, Pisango MT, Arevalo J, Rojas R, Alban J, Deharo E, Bourdy G, Sauvain M 2007. Evaluation of the leishmanicidal activity of plants used by Peruvian Chayahuita ethnic group. *J Ethnopharmacol* 114: 254-259.

Figueiredo AC, Barroso JG, Pedro LG, Scheffer JC 2008. Factors affecting secondary metabolites production in plants: volatile components and essential oils. *Flavour Frags J* 23: 213-226.

Gebre-Hiwot A, Frommel D 1993. In vitro anti-leishmanial activity of inhibitors of ergosterol biosynthesis. *J Antimicrob Chemother* 32: 837-842.

Ghanounou MA, Rice LB 1999. Antifungal agents: mode of action, mechanisms of resistance and correlation of these mechanisms with bacterial resistance. *Clin Microbiol Rev* 12: 501-517.

Houel E, Rodrigues AMS, Jahn-Oyac A, Bessiere J-M, Eparvier V, Deharo E, Stien D 2014. In vitro antidermatophytic activity of *Ocotea azuarens* (Linden) Ronse essential oil alone and in combination with azoles. *J Appl Microbiol* 116: 288-294.

Kyraizis JD, Aliagianis N, Polychronopolus P, Skaltsounis A-L, Dotsika E 2013. Leishmanicidal activity assessment of olive tree extracts. *Phytotherapy* 20: 275-281.

Moraes A, Rojas J, Moujir LM, Araujo L, Rondón M 2013. Chemical composition, antimicrobial and cytotoxic activities of *Piper hispidum* Sw. essential oil collected in Venezuela. *J Appl Pharm Sci* 6: 16-20.

Moskowitz PF, Kurban AK 1999. Treatment of cutaneous leishmaniasis: retrospectives and advances for the 21st century. *Clin Dermatol* 17: 305-315.

Pan S-Y, Pan S, Yu Z-L, Ma D-L, Chen S-B, Fong W-F, Han Y-F, Ko K-M 2010. New perspectives on innovative drug discovery: an overview. *J Pharm Pharmacuet Sci* 13: 450-471.

Pino JA, Marbot R, Bello A, Urquiola A 2004. Composition of the essential oil of *Piper hispidum* Sw. from Cuba. *J Essent Oil Res* 16: 459-460.
Rabito MF, Britta EA, Pelegrini BL, Scariot DB, Almeida MB, Nixdorf SL, Nakamura CV, Ferreira ICP 2014. In vitro and in vivo anti-Leishmania activity of sesquiterpene lactone-rich dichloromethane fraction obtained from Tanacetum parthenium (L.) Schultz-Bip. Exp Parasitol 143: 18-23.

Rios JL, Recio MC 2005. Medicinal plants and antimicrobial activity. J Ethnopharmacol 100: 80-84.

Rocha LG, Almeida JR, Macedo RO, Barbosa-Filho JM 2005. A review of natural products with antileishmanial activity. Phytotherapy 12: 514-535.

Rodrigues KADF, Amorim LV, Oliveira JMGD, Dias CN, Moraes DFC, Andrade EHDA, Maia JGß, Carneiro SMP, Carvalho FADA 2013. Eugenia uniflora L. essential oil as a potential anti-Leishmania agent: effects on Leishmania amazonensis and possible mechanisms of action. Evidence-Based Complementary and Alternative Medicine doi: 10.1155/2013/279726.

Ruiz C, Haddad M, Alban J, Bourdy G, Reategui R, Castillo D, Sauvain M, Deharo E, Estevez Y, Arevalo J, Rojas R 2011. Activity-guided isolation of antileishmanial compounds from Piper hispidum. Phytochem Lett 4: 363-366.

Santos AO, Ueda-Nakamura T, Dias Filho BP, Veiga Júnior VF, Pinto AC, Nakamura CV 2008. Effect of Brazilian copaiba oils on Leishmania amazonensis. J Ethnopharmacol 120: 204-208.

Shakya N, Bajpai P, Gupta S 2011a. Therapeutic switching in Leishmania chemotherapy: a distinct approach towards unsatisfied treatment needs. J Parasit Dis 35: 104-112.

Shakya N, Sane SA, Gupta S 2011b. Antileishmanial efficacy of fluconazole and miltefosine in combination with an immunomodulator - picroliv. Parasitol Res 108: 792-800.

Shin S, Lim S 2004. Antifungal effects of herbal essential oils alone and in combination with ketoconazole against Trichophyton spp. J Appl Microbiol 97: 1220-1226.

Tangarife-Castaño V, Correa-Royero JB, Roa-Linares VC, Pino-Benitez N, Betancur-Galvis LA, Durán DC, Stashenko EE, Mesa-Arango AC 2014. Anti-dermatophyte, anti-Fusarium and cytotoxic activity of essential oils and plant extracts of Piper genus. J Essential Oil Res 26: 221-227.

Tong Z, Widmer F, Sorrell TC, Guze Z, Jolliffe KA, Halliday C, O’k CI, Kong F, Wright LC, Chen SCA 2007. In vitro activities of miltefosine and two novel antifungal biscationic salts against a panel of 77 dermatophytes. Antimicrob Agents Chemother 51: 2219-2222.

Tserennadmid R, Tako M, Galgoczy L, Papp T, Pesti M, Vagvolgyi C, Almassy K, Krisch J 2011. Anti-yeast activities of some essential oils in growth medium, fruit juices and milk. Int J Food Microbiol 144: 480-486.

Unsicker SB, Kunert G, Gershenzon J 2009. Protective perfumes: the role of vegetative volatiles in plant defense against herbivores. Curr Opin Plant Biol 12: 479-485.