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Chemical diversity and leishmanicidal activity of *Manekia obtusa* Miq (Piperaceae)

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Piperaceae species have long been used for therapeutic purposes, a reason for the great interest in their use as a source of raw material in phytochemical studies. The aim of this work is to investigate the chemical composition and biological potential of *Manekia obtusa* Miq, a native rare Piperaceae species in Brazil. Phytochemical studies of nonpolar extract from leaves of *M. obtusa* led to the isolation and characterization of the flavone 7,4'-dimethylapigenine and 2', 6'-dihydroxy-4'-methoxychalcone from the hexane extract. This flavonoid rich fraction was able to inhibit both growth and viability of *Leishmania amazonensis* in a dose dependent way. Minimum inhibitory concentration (MIC) and inhibitory concentration of 50% (IC_{50}) were calculated at 49.25 and 26.03 µg/ml, respectively. The leaves and stems of *M. obtusa* had their essential oils extracted by hydrodistillation and analyzed by gas chromatography-mass spectrometry (GC-MS). The constituents δ-3-carene (55.3%), α-pinene (5.9%), δ–cadinene (2.9%), β-pinene (2.7%) and α-cadinol (2.5%) were identified as the major components of the leaf essential oil, while in the stems, δ-3-carene (46.2%) was the major constituent followed by safrole (9.3%), α-cadinol (3.5%), β-pinene (3.2%) and α-pinene (3.1%). These results contributed to improving the knowledge of this native species rarely studied in Brazil. All compounds have not been previously been described with respect to this species.

Key words: *Manekia obtusa*, Piperaceae, *Manekia*, essential oil, flavonoids.

INTRODUCTION

Piperaceae species are widely distributed in tropical and sub-tropical regions reaching around 2000 species throughout the world. The Piperaceae family includes 4 genera: *Piper, Peperomia, Manekia* and *Zipelia* (Guimarães and Silva, 2009). In Brazil, Piperaceous species are present in many different biomes from the North to the South of the country, being found in a variety of altitudes (Monteiro and Guimarães, 2009). Several species have been used historically in religious and in traditional medicine within ethnic groups in Brazil and South East Asia (Cunico et al., 2005; Chaveerach et al., 2006). In the North of Brazil, some Piper species are

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reknowned due to their anesthetic properties, being popularly known as “anesthesia”. Traditional communities in the Brazilian Amazonia use its root’s aerial parts for the treatment of odontalgia by chewing or mouth washing (Cunico et al., 2005). Phytochemical studies on members of Piperaceae species have shown a variety of bioactive metabolites including alkaloids, chromenes, amides, flavonoids and terpenoids which are of economic and medicinal importance (Parmar et al., 1997; Raimundo et al., 2009; Marques et al., 2010a, 2013b; Silva et al., 2010; Moraes et al., 2011; Rebelo et al., 2012; Lara Jr et al., 2012).

Due to the high content of essential oil, many aromatic species of Piperaceae have been the object of studies, showing a wide variety of constituents, mainly terpenoids and arylpropanoids (Santos et al., 2001; Andrade et al., 2009; Trindade et al., 2010). However, the genus *Sarcorhachis* Trel. synonymous with *Manekia* Trel. (Monteiro and Guimarães, 2009; Schubert et al., 2012), had undergone only a few phytochemical studies in specialized literature. Despite the existence of numerous botanical studies of the family, phytochemical investigations are rarely described in literature regarding the genus *Manekia*. This investigation of *Manekia obtusa* was carried out in order to explore native Brazilian Flora biodiversity and to increase the knowledge of secondary metabolites of species from native Piperaceae species not yet studied in Brazil as well as their potential biological properties.

MATERIALS AND METHODS

Plant and essential oil extraction

The species *M. obtusa* was collected in August 2009 by Dr. Edemilson Pinto da Silva in the city of Itajubá, MG. The botanical material was identified by the botanist Dr. Elsie Franklin Guimarães and a sample was deposited in the RB Herbarium of Botanical Garden of Rio de Janeiro Research Institute, under the number RB480844. Fresh leaves and stems (100 g) from *M. obtusa* were separated, reduced to small pieces, and were submitted to hydrodistillation for 2 h in a modified Clevenger-type apparatus. The obtained essential oils (EO) were dried over anhydrous sodium sulphate and were immediately stored in closed dark vials at 4°C until further analysis.

Gas chromatography-mass spectrometry (GC-MS) analysis

Gas chromatography flame ionization detector (GC-FID) analysis

The gas chromatography analyses were carried out on a GC 2010 Shimadzu with a ZB-1MS fused silica capillary column (30 m × 0.25 mm × 0.25 µm film thickness). The operating temperatures used were as follows: injector 260°C, detector 290°C and column oven 60°C up to 290°C (10°C min^{-1}). Hydrogen at 1.0 ml min^{-1} was used as a carrier gas. The pure isolated compounds were analysed. The percentages of the flavonoids as well as the volatile compounds in the crude EO were obtained by GC-FID analysis. The EO data are the means of three experiments performed in triplicate. The results are presented as average of three analyses ± standard deviation (SD).

GC-MS analysis

Qualitative analyses were carried out on a GCMS-QP2010 PLUS Shimadzu with a ZB-5MS fused silica capillary column (30 m × 0.25 mm × 0.25 µm film thickness) under the experimental conditions reported for GC-FID analysis. The flavonoids and the essential oil components were identified by comparing their retention indices and mass spectra to the published data and computer matching with WILEY 275 and the National Institute of Standards and Technology (NIST 3.0) libraries provided by a computer-controlled GC-MS system (Adams, 2001). The retention indices were calculated for all the volatile constituents using the retention data record of a linear n-alkanes C_8–C_{24} standard curve.

Nuclear magnetic resonance (NMR) spectroscopy

Carbon and hydrogen nuclear magnetic resonance spectra (NMR, ^{1}H and ^{13}C), were obtained using a VNMRS 500 Spectrometer Varian-Gemini operating at 400 MHz/100 MHz, using deuterated solvents, $CDCl_3$, DMSO-D_6. Special uni- and bidimensional techniques such as: COSY, HMBC, and HSQC were also performed. The chemical shift values (δ) were referred to an internal standard (TMS), represented in parts per million (ppm) of the frequency applied for each experiment and the coupling constants were measured in Hertz. The obtained data were compared with literature data (McLafferty and Stanffer, 1989; Torres-Santos et al., 1999; Baldoqui et al., 2009).

Preparation of extracts

The plant material was separated according to its different organs: leaves, stems and roots, which were reduced to small fragments using a pruning scissors and immediately submitted to extraction with organic solvents. The dry and crushed leaves of *M. obtusa* (454.0 g) were extracted by static maceration, successively with hexane, replaced daily, for thirteen days, producing 25.3 g of hexane extract, and with methanol replaced daily for ten days, resulting in 50.5 g of methanol extract.

Isolation of pure compounds

The hexane crude extract (10 g) from leaves of *M. obtusa* was subjected to a liquid silica gel chromatographic column, eluted with solvent systems prepared with binary mixtures using hexane, ethyl acetate and methanol. In total, 200 fractions were obtained from this column.

Isolation of 7, 4’-dimethylapigenine

From the hexane leaf extract chromatographic column, the fraction 50 to 59 (67 mg) eluted with Hex/AcOEt 5%, obtained as yellowish crystalline solid, with a profile of a single yellow spot on thin layer chromatography (TLC). The sample was initially subjected to a GC-FID analysis, which showed the presence of a single major signal on the chromatogram (Figure 1). The sample was also subjected to gas chromatography coupled with mass spectrometry and NMR analysis.

Isolation of 2’,6’-dihydroxy-4’-methoxychalcone

Column purification on silica gel was carried out with fraction 123 to

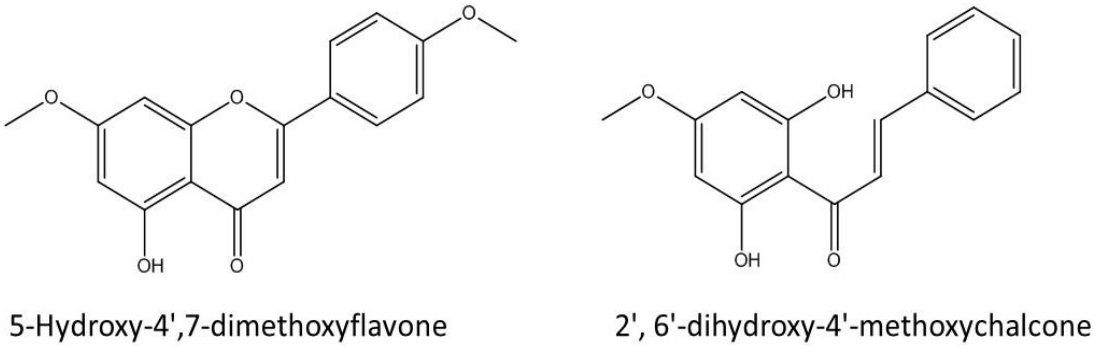

Figure 1. Isolated flavonoids from the hexane extract of *Manekia obtusa*.

140 (180 mg) obtained from the hexane leaf extract fractionation of *M. obtusa*. Solvent systems prepared with binary mixtures of hexane, ethyl acetate and methanol in increasing polarity were used, resulting in a further 62 fractions. The fraction eluted with AcOEt/MeOH 10% yielded a greenish solid that was encoded as AM56 (6 mg) and that was subjected to CG-FID, CG-MS and NMR analysis.

Antileishmanial activity

Minimum inhibitory concentration (MIC)

Protozoa in exponential growth phase (10^6 parasites/ml) were plated in the presence of micro serial dilutions of extracts, essential oil and purified compounds, starting from a concentration of 0.5 mg/ml to 0.97 µg/ml. After incubation at 26°C for 72 h, cell viability was measured by the colorimetric method reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) salt (Dutta et al., 2005). MIC and inhibitory concentration of 50% (IC_{50}) of extracts, compounds and essential oil on *Leishmania amazonensis* and *Leishmania chagasi* were calculated by Microsoft Excel® linear regression.

RESULTS AND DISCUSSION

Identification of the essential oil

The identification of the EO components from the leaves and stems of *M. obtusa* (Table 1) was carried out by chromatographic profile analysis of the aromatic oils obtained by hydrodistillation. From the leaf EO chromatographic profile analysis, it was possible to identify chemical components responsible for more than 88.3% of the oil. The constituents δ-3-carene (57.0 ± 2.9%), α-pinene (6.3 ± 1.7%), safrole (4.0 ± 2.1), α-cadinol (2.7 ± 0.4%), β–elemene (2.3 ± 0.4%) and β-pinene (2.0 ± 1.2%) and were identified as the major components of this essential oil. In the essential oil obtained from the stems, the major compound identified was δ-3-carene (41.0 ± 3.7%), followed by safrole (8.3 ± 3.5%), β–elemene (7.2 ± 1.3%), β-pinene (3.9 ± 0.7%), α-pinene (3.6 ± 0.5%) and α-cadinol (3.3 ± 1.2%), resulting in the identification of 93.2% of the volatile components. The chemical structures of the major volatile components from *M. obtusa* essential oils are presented in the Figure 2.

The essential oil yields obtained by hydrodistillation of fresh plant material was 1.4 and 1.2% (w/w) for leaves and stems, respectively. The monoterpene δ-3-carene was the major volatile compound found in both organs of *M. obtusa*. The essential oil of *M. obtusa* collected in Paraná State, South of Brazil, was previously described by Fernandes (2006). In this study, 12 volatile compounds were identified in the leaf essential oil. The major volatile components present in the leaf oil were β-phellandrene (17.4%), α-pinene (15.1%), caryophyllene (9.4%) and biciclogermacrene (7.9%). In the characterization of the essential oil of the stems, 16 major components were observed as follows: β-phellandrene (17.2%), in a similar concentration found in the leaves, followed by caryophyllene (15.6%), α-pinene (9.7%) and α-humulene (8.7%). Curiously, the δ-3-carene and safrole were not observed in any of the oil studied from the Southern Brazil specimen. The characterization of δ-3-carene as a chemical marker of the essential oil for the species collected in Minas Gerais Stade as well as the high mono- and sesquiterpene variability in the southern species suggest the possibility of a new distinct chemotype of this species, now rich in δ-3-carene instead of β-phellandrene. For this study, three different samples of *M. obtusa* from the same area were collected in Minas Gerais State. Unfortunately, it was not possible to compare the volatile chemical profile of different location collections, as this species is not usually found in Brazil. Because of this, studies have rarely been carried out with *M. obtusa*.

In Costa Rica, Chaverri et al. (2011) studied the leaf essential oil of *Manekia naranjoana* resulting in the characterization of the chemical constituents β–pinene (30.6%), α-pinene (18.8%), limonene (13.7%) and β-caryophyllene (6.1%) as the major components present in the essential oil of the aerial parts of this plant. The monoterpenes α-pinene and β–pinene were also found in *M. obtuse* from Minas Gerais, but as minor compounds of the volatile mixture, while β-caryophyllene was present just in the specimen of *M. obtusa* from Parana.

Table 1. Components of the essential oils of leaves and stems of *M. obtusa* obtained by hydrodistillation.

S/N	Compound	IR^{Cal}	IR^{Lit}	Leave (%)	Stem (%)
1	α-pinene	938	939	6.3 ± 1.7	3.6 ± 0.5
2	sabinene	976	976	0.3 ± 0.4	0.2 ± 0.5
3	β-pinene	982	980	2.0 ± 1.2	3.9 ± 0.7
4	β-myrcene	990	991	1.5 ± 0.6	3.0 ± 3.2
5	δ-3-carene	1014	1011	57.0 ± 2.9	41.0 ± 3.7
6	limonene	1033	1029	1.0 ± 0.3	1.3 ± 0.3
7	β-phellandrene	1035	1031	0.2 ± 0.7	0.9 ± 0.5
8	γ-terpinene	1057	1062	0.4 ± 0.8	0.5 ± 01
9	isoterpinolene	1083	1086	0.2 ± 0.1	0.4 ± 0.2
10	terpinolene	1087	1088	1.6 ± 0.0	0.5 ± 0.6
11	safrole	1293	1287	4.0 ± 2.1	8.3 ± 3.5
12	copaene	1375	1376	0.6 ± 0.5	1.0 ± 0.1
13	β-elemene	1388	1391	2.3 ± 0.4	7.2 ± 1.3
14	β-cedrene	1418	1418	0.3 ± 0.8	1.0 ± 0.0
15	α-caryophyllene	1454	1454	0.8 ± 1.0	0.3 ± 1.4
16	γ-muurolene	1475	1477	0.5 ± 0.7	2.6 ± 2.0
17	D-germacrene	1480	1480	1.0 ± 0.5	0.8 ± 0.7
18	epinazorene	1496	1497	0.3 ± 0.0	0.1 ± 0.2
19	α-farnesene	1505	1508	1.2 ± 0.4	1.3 ± 1.0
20	cubebol	1514	1514	0.2 ± 0.6	1.2 ± 0.5
21	δ-cadinene	1517	1524	1.8 ± 1.2	2.6 ± 0.0
22	τ-cadinol	1642	1640	0.9 ± 0.0	0.3 ± 0.9
23	muurolol	1643	1641	1.1 ± 0.2	1.5 ± 0.1
24	α-muurolol	1646	1645	0.1 ± 0.6	0.4 ± 0.8
25	α-cadinol	1656	1653	2.7 ± 0.4	3.3 ± 1.2
Percentage of identified compounds				88.3	93.2

IR^{cal}: calculated retention index; IR^{Lit}: literature retention index; Data are the means of three experiments performed in triplicate. The results are presented as average of three analyses ± standard deviation (SD).

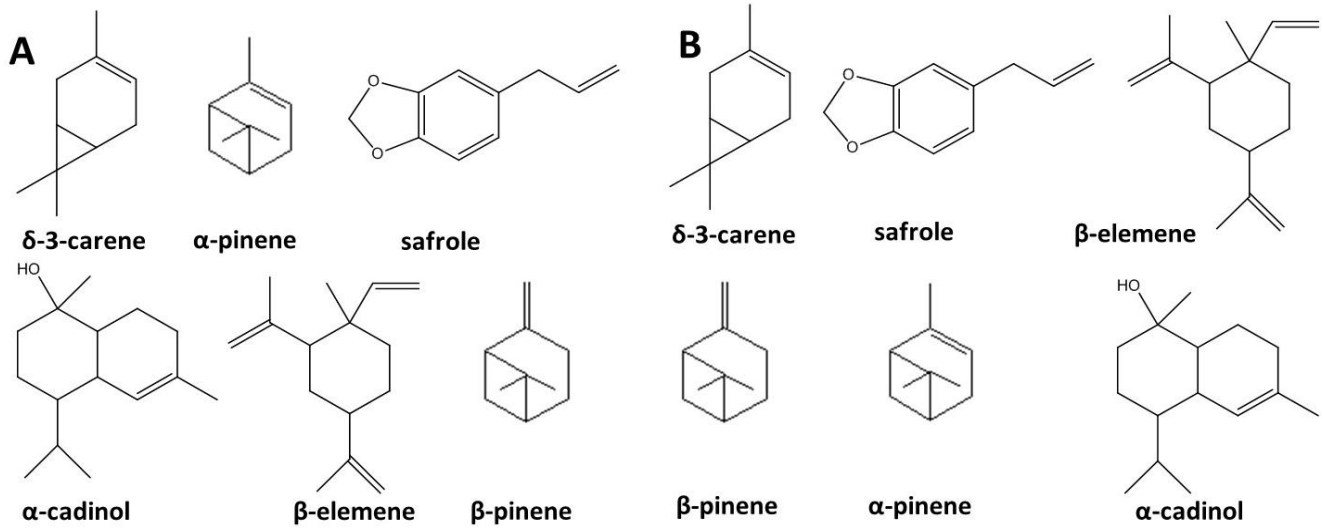

Figure 2. Chemical structures of some components present in stem (A) and leaf (B) essential oils of *Manekia obtusa*.

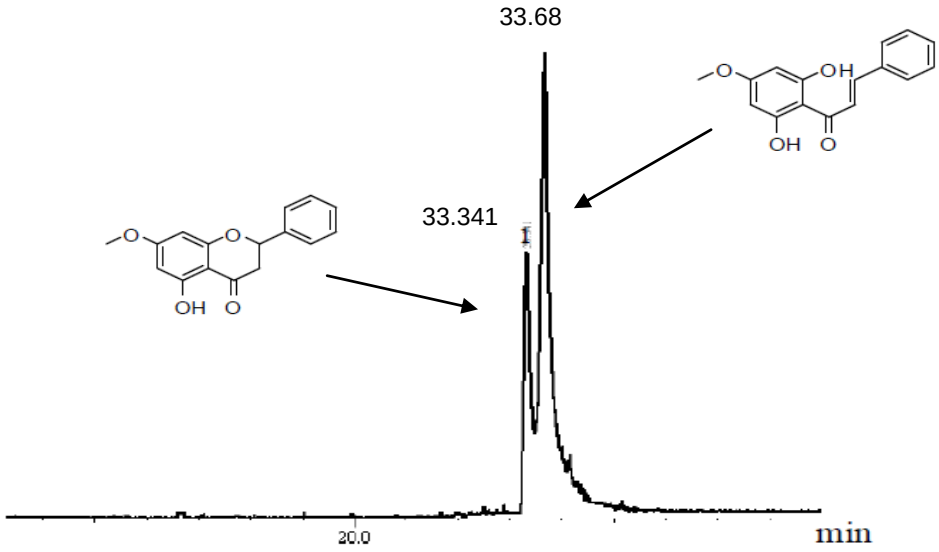

Figure 3. GC-FID chromatogram shows the presence of the 2',6'-dihydroxy-4'-methoxychalcone and its isomer, the 5-hydroxy-7-methoxyflavanone.

Flavonoids of *M. obtusa*

Two free flavonoids were isolated from the hexane extract of *M. obtusa*. The flavonoid rich fractions were obtained from chromatographic fractionation on silica gel column. TLC analyses using NP/PEG reagent indicated the presence of free flavonoids. The fraction 50 to 59 (67 mg), eluted with Hex/AcOEt 5%, was obtained as a pale yellowish crystalline solid, with a profile of a single yellow spot on TLC. The pure compound initially was analyzed by GC-FID and GC-MS that revealed a signal for a molecular ion at m/z 298. Fragmentation pattern analyses of the substance supports the proposition of its molecular formula as $C_{17}H_{14}O_5$. The fragmentation pattern observed in the spectrum was related to flavone, which was compared with literature data (McLafferty and Stanffer, 1989). The subsequent analyses of ^{12}H-NMR, ^{13}C-NMR, as well as bidimensional NMR techniques were compared to literature data and the substance was determined to be the flavone 7,4'-dimethylapigenine (5-Hydroxy-4',7-dimethoxyflavone) (Figure 2).

The second flavonoid came from the fraction 123 to 140 obtained from the hexane leaf extract fractionation of *M. obtusa*. After chromatographic procedures, it was possible to obtain a yellowish solid that was encoded as AM56 (6 mg) and that was subjected to CG-FID, CG-MS and NMR analysis.

The chromatogram profile showed the presence of two mixed substances with predominance of a single component. A sample analyzed through GC-MS confirmed the presence of a metabolite identified as 2', 6'-dihydroxy-4'-methoxychalcone in equilibrium with a fraction of its isomer 5-hydroxy-7-methoxyflavanone (Figure 3).

The 2', 6'-dihydroxy-4'-methoxychalcone is frequently accompanied by its isomer (5-hydroxy-7-methoxyflavanone) when analyzed by GC-FID and GC-MS. However, results of the NMR analyses indicated the presence of just one metabolite in the sample. All the ^{1}H-NMR and ^{13}C-NMR spectra signs were checked in agreement to the isolated chalcone structure, which was confirmed by comparison to literature data.

Leishmanicidal activity

The crude methanol leaf extract, the hexane leaf extract, as well as leaf essential oil and pure isolated flavonoids were tested for antileishmanial activity on *L. amazonensis* and *L. chagasi* strains. The results are summarized in Table 2. The results revealed that methanol leaf extract is not active against *L. amazonensis*, being just moderately active against *L. chagasi*. The hexane leaf extract is able to inhibit both growth and viability of *L. amazonensis* in a dose dependent way (Table 3 and Figure 4). Against *L. chagasi*, this nonpolar extract was less effective. MIC and IC_{50} were calculated as 49.25 and 26.03 µg/ml, for *L. amazonensis* and 139 and 88 µg/ml for *L. chagasi*.

Williams et al. (2003) registered the isolation of the amide aegeline and its leishmanicidal activity. This compound was described as one of the chemical markers for *M. obtusa* and *M. naranjoana* (Williams et al., 2003; Jaramillo et al., 2001; Fernades et al., 2006). However, this amide was not found in the studied fractions of this investigation.

The hexane extract of *M. obtusa* leaves presents as major components chalcones, flavanones and flavones. Chalcones and terpenes isolated from *Piper aduncum* and *Piper claussenianium* have been shown to exhibit significant leishmanicidal activity against *L. amazonensis* (Torres-Santos et al., 1999; Marques et al., 2010;

Table 2. Leishmanicidal activity of *Manekia obtusa* samples.

Sample	*L. amazonensis*		*L. chagasi*	
	MIC (µg/ml)	IC_{50} (µg/ml)	MIC (µg/ml)	IC_{50} (µg/ml)
Leaf essential oil	432	173.5	> 500	-
Methanol leaf extract	> 500	-	276.5	160.4
Hexane leaf extract	49.25	26.03	139	88
Chalcone	> 500	-	> 500	-
Flavone	> 500	-	129.5	69.3

Table 3. Absolute values of the antileishmanial activity of Hexane extract. Dashes indicate that no cells were detected at the counting.

Hexane extract (µg/ml)	viable cells ($\times 10^5$ cells/ml)	unviable cells ($\times 10^5$ cells/ml)	Viability (%)
0	167	-	100
12.5	155	-	100
25	60	9	86.9
50	-	3	0
100	-	-	0
200	-	-	0

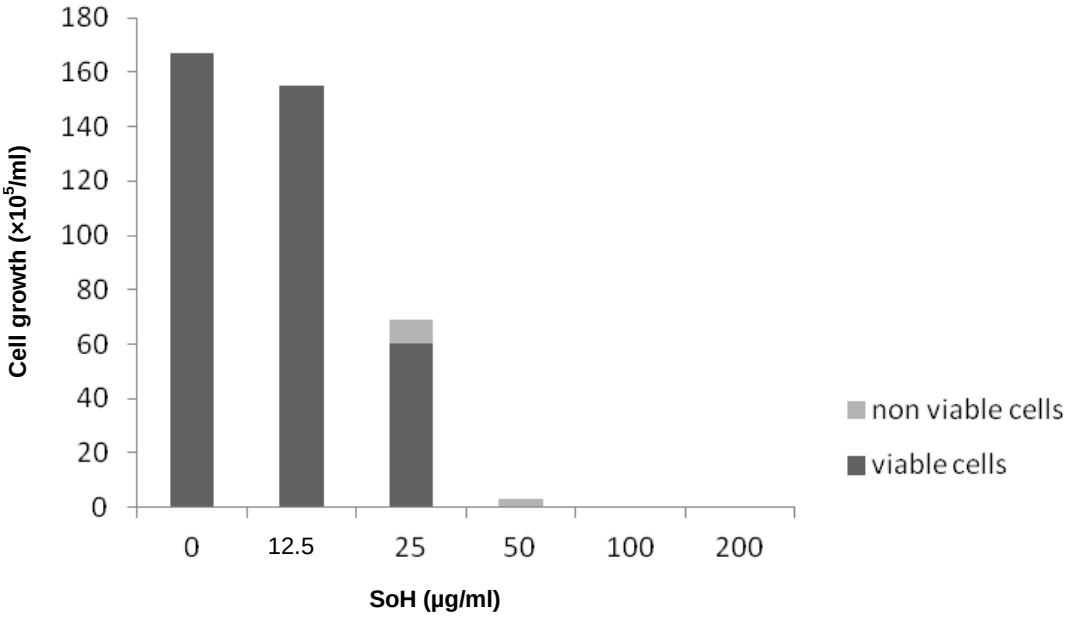

Figure 4. Antileishmanial activity of Hexane extract (SoH) against growth and viability of *L. amazonensis* promastigotes after a 72h incubation in Schneider's insect medium at 26°C. Light grey bars indicate portion of deceased cells, dark grey bars indicate viable cells, while whole bar indicates the total growth rate.

Marques et al., 2013). The nonpolar extract of *M. obtusa* demonstrated a strong activity on the growth and on the viability especially against *L. amazonensis*. It was thought that due to the presence of these compounds, which have already been reported with antiprotozoan activities (Torres-Santos et al., 1999; Kim et al., 2004), the hexane extract was biologically active against *Leishmania* strains. Torres-Santos et al. (2009) demonstrated that methoxy-chalcone is able to alter the membrane ergosterol biosynthesis pathway in *L. amazonensis* promastigotes. This same compound has been noticed to induce loss of mitochondrial function in parasites with consequent ultra-

structural modifications of this organelle. Chalcone derivatives have been shown to inhibit *Leishmania braziliensis* growth and several of them appear to have good selectivity indexes against parasite promastigotes when compared with Vero cells, what can be extremely relevant to prevent cytotoxic effects (Bello et al., 2011). Schinor et al. (2007) reported the isolation of several bioactive flavonoids such as the flavones apigenin, luteolin and crysoeriol as well as the flavonol kaempferol, against *L. amazonensis* amastigotes stages. It was found that the flavone apigenin in a concentration of 84 µg/ml was effective against cell viability of *L. amazonensis,* inhibiting 84% of amastigotes forms. In another study involving flavonoids, Torres-Santos et al. (1999) reported that 2',6'-Dihydroxy-4'-methoxychalcone purified from the dichloromethane extract of *P. aduncum* presented significant activity *in vitro* against promastigotes and intracellular amastigotes of *L. amazonensis*, with 50% effective doses of 0.5 and 24 µg/ml, respectively. Its inhibitory effect on amastigotes was indicated as an apparently direct effect on the parasites and is not due to activation of the nitrogen oxidative metabolism of macrophages, since the production of nitric oxide by both unstimulated and recombinant gamma interferon-stimulated macrophages was decreased rather than increased with the chalcone. Curiously, both isolated compounds from *M. obtusa* 7,4'-dimethylapigenine and 2', 6'-dihydroxy-4'-methoxychalcone were not effective against the tested *Leishmania* strains. The MIC for the chalcone was >500 µg/ml for both strains of *Leishmania*, while the flavone was >500 µg/ml for *L. amazonensis* and 129.5 µg/ml for *L. chagasi*.

Thus, the major flavonoids isolated from the hexane extract could not be related to the leishmanicidal activity. In order to investigate the possible constituents responsible for the biological activity found in the hexane extract, the leaf essential oil was also tested against the parasites. Once again, the MIC for the essential oil was quite similar to the isolated flavonoids, being very low to justify the activity of the hexane extract. In this way, the results suggest the hexane extract of *M. obtusa* as the most promising sample among all studied. The good level of inhibition encourages the continuous investigation of the compounds and/or synergism responsible for the leishmanicidal activity of this fraction. This study inspired us to continue performing the bioassay-guided isolation of bioactive principles that could provide new antiparasitic phytotherapeutic drugs or lead to compounds for future drug development.

Conclusion

This work is one of the few on the genus *Manekia* with respect to chemical and biological background and composition. This study resulted in the isolation and structural characterization of the flavone 7,4'-dimethylapigenine and 2',6'-dihydroxy-4'-methoxychalcone. The essential oil from the leaves was characterized displaying a different chemical profile that highlights δ-3-carene as the major metabolite. The absence of effective inhibition by the isolated compounds and the essential oil may suggest the presence of synergism involving the *Leishmania* strains inhibition mechanisms. Further investigations should be taken in order to clarify which metabolite and mechanisms could be responsible for the biological activity.

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