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Artigo

VOLATILE AND NON-VOLATILE COMPOUNDS AND ANTIMICROBIAL ACTIVITY OF *Mansoa difficilis* (Cham.) Bureau & K. Schum. (Bignoniaceae)[#]

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Essential oil from the leaves of *Mansoa difficilis* was analyzed by GC/MS. Oct-1-en-3-ol (49.65%) was the major compound, but diallyl di- and trisulfide were also present (0.85 and 0.37%, respectively), justifying the garlic-like odor of the crushed leaves. The hexane and methanol extracts of the leaves and stems afforded as main constituents a mixture of linear hydrocarbons, spinasterol, stigmasterol, ursolic and oleanolic acids, two apigenin derivatives and verbascoside. The hexane and methanol extracts of leaves were tested for antimicrobial activity against ten microorganisms. The hexane extract was active against both *Psedomonas aeruginosa* and *Staphylococcus aureus*.

Keywords: *Mansoa difficilis*; chemical composition; antimicrobial inhibition.

INTRODUCTION

Bignoniaceae is predominantly a neotropical family and comprises almost 800 species and 104 genera.[1] Around 316 species, grouped into 55 genera, are known in Brazil.[2] Among the genus *Mansoa*, *M. alliacea* (Lam.) A. H. Gentry, *M. angustidens* (DC.) Bureau & K. Schum., *M. difficilis* (Cham.) Bureau & K. Schum. and *M. standleyi* (Steyerm.) A. H. Gentry are common in the North of Brazil.

Mansoa species are known for their pungent garlic-like smell when their vegetative and reproductive organs are crushed, particularly *M. alliacea. Mansoa difficilis*, locally known as cipó-sino, cipó-de-alho-do-mato and cipó-una, also has a garlic-like odor, but not as strong as that of *M. alliacea* and *M. standleyi*, whose essential oils are rich in organosulfur compounds.[3,4]

Antimicrobial activities have been observed in many species of Bignoniaceae: extracts, and essential oils of *Mansoa alliacea* were found to be active against *Alterniaria brassicae*,[5] *Drechslera oryzae*,[6] *Colletotrichum capsici*, *Curvularia lunata*, *Alternaria alternata*, *A. brassicae*, *A. brassicola*, *A. carthami*, *Fusarium oxysporum* and *F. udum*.[7] Crude extracts of *M. hirsuta* DC. inhibited the growth of standardized cultures of *Aspergillus niger* and *Fusarium oxysporum*.[8] Extracts of *M. hymenaea* (DC.) A. H. Gentry have shown high antifungal activity, especially against *Trichophyton mentagrophytes*, *Microsporum gypseum*,[9,10] *Trichophyton rubrum*, and dermatophyte fungi.[8]

A survey of the literature revealed that no studies on the volatile and non-volatile compounds or antimicrobial activity of *M. difficilis* have been published to date. This species has been used in handicraft as a raw material for baskets, fishing and planting equipment.[11] The aim of this study was to characterize the chemical composition of volatile and non-volatile compounds of *M. difficilis* and to test the hexane and methanol extracts of the leaves against a diverse range of organisms comprising Gram-positive and Gram-negative bacteria and yeasts.

EXPERIMENTAL

Botanical material

The samples of *M. difficilis* were collected in the municipality of Santa Luzia do Pará, in the Northeast of the State of Pará (Brazil), at their full flowering stage, in June 2008. A voucher specimen was deposited in the Herbarium of the Museu Paraense Emílio Goeldi (MG190,032).

Extractions of volatile and non-volatile compounds

Samples of fresh leaves (300.7 g) were hydrodistilled for 3 h using a Clevenger-type apparatus with maintenance of the cooling water at 12 °C. The oils obtained were centrifuged for 5 min (3,000 rpm), dried over Na_2SO_4, centrifuged again, and immediately submitted to GC/MS analysis. A solution containing 2 µL of the oil in 1 mL of hexane was immediately prepared for gas chromatography analysis. The total oil yield was expressed in percentage (volume/mass) on the basis of free water material. The amount of water was measured using infrared light on a Mater 50 device.

Leaves and stems (1,000 and 500 g, respectively), dried for 7 days in an air-conditioned room (at low humidity) and ground, were successively extracted with hexane and methanol at room temperature yielding, after vacuum concentration, the hexane and methanol extracts of the leaves and stems. The hexane extracts (MdLH:22.0 g and MdSH:2.5 g) and the methanol extracts (MdLM:185.0 g and MdSM:31.2 g) of leaves and stems, respectively, were further fractionated. Parts of the MdLM (40.0 g) and MdSM (32.0 g) were partitioned with dichloromethane (D), ethyl acetate (Et) and *n*-butanol (B) yielding, after vacuum concentration, the respective phase residues from the leaves (MdLMD:7.6 g; MdLMEt:15.0 g

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and MdLMB:6.0 g) and stems (MdSMD:5.8 g, MdSMEt:5.3 g and MdSMB:3.3 g).

Analysis of volatile compounds

The oil was analyzed using a Shimadzu GC/MS Model QP 2010 Plus, equipped with a Rtx-5MS (30 m x 0.25 mm; 0.25 μm film thickness) fused silica capillary column. Helium was used as the carrier gas adjusted to 1.2 mL min^{-1}; with splitless injection of 1 μL of a hexane solution; injector and interface temperature were 250 °C; oven temperature programmed was 60-240 °C at 3 °C min^{-1}. EIMS: electron energy, 70 eV; ion source temperature was 200 °C. Identification of the compounds were made by comparing their GC mass and retention data with those held in the NIST-05 library and cited in the literature data.[12,13] Retention indices were calculated using *n*-alkane standard solutions (C8-C26) available from Fluka S. A., under the same chromatographic conditions. Quantitative data were obtained from the electronic integration of the total ion chromatogram (TIC) peak areas.

Classic chromatographic procedures

The MdLH (15.0 g), MdSH (1.7 g), MdLMD (3.0 g), MdSMD (3.5 g), MdLMEt (12.0 g) and MdSMEt (3.5 g) were fractionated by column chromatography (CC) on silica (70-230 mesh) using mixtures of hexane, EtOAc and methanol with increasing order of polarity as mobile phase.

Column chromatography of MdLH (40 g) on silica afforded eight fractions (A-H) that were eluted with hexane (A) and hexane-EtOAc 2.5% (B-D), hexane-EtOAc 4% (E), hexane-EtOAc 10.0% (F), hexane-EtOAc 15.0% (G) and hexane-EtOAc 50% (H). Compounds **1** (400 mg) and **2** (440 mg) were the main constituents of fractions A and B, respectively. Fractions C-G were further fractionated by CC on silica using mixtures of hexane and EtOAc as eluents: fraction C (hexane--EtOAc 1.5%) afforded compounds **3** (13 mg) and **4** (30 mg); fraction D (hexane-EtOAc 1.25%) yielded compound **5** (8 mg); fraction E (hexane-EtOAc 10%) yielded **6** (45 mg); fraction F (hexane-EtOAc 3%) led to a mixture of **7** and **8** (357 mg) purified by crystallization with hexane-EtOAc; fraction G (hexane-EtOAc 12-14%) afforded a mixture of **9** and **10** (95 mg). CC on silica of MdLMD (3 g) led to isolation of **11** (8 mg) when using hexane-EtOAc 9% as the eluent and to additional quantities of the mixture of **9** and **10** (1,100 mg). CC fractioning of MdLMEt (12 g) on silica with further purification on Sephadex LH-20 led to compounds **12** (198 mg) and a mixture of **13** and **14** (45 mg).

Similar CC procedures of MdSH (1.7 g) led to the isolation of **1** (30 mg), **6** (145 mg), **7-8** and **15** (175 mg) and **9**-**10**. CC of MdSMD (3.5 g) on silica using EtOAc-MeOH 5% as the eluent afforded the mixture of **16**-**17** (13 mg). CC of MdSMEt (3.5 g) led to additional quantities of **13**-**14** (423 mg).

Structural determination of non-volatile constituents

Structures of the isolated compounds were proposed based on the analysis of ^{1}H- and ^{13}C-NMR spectral data and GC-MS and by comparison with literature data. NMR spectra were recorded on a Mercury 300 – Varian instrument using $CDCl_3$, CD_3OD, C_5D_5N or DMSO-d_6 as solvents.

Microbial strains

The following bacteria and yeasts were used for the experiments: *Escherichia coli* CCMB 261 (sensitive to trimethoprim and resistant to sulphonamide), *Pseudomonas aeruginosa* CCMB 268, *Salmonella* sp. CCMB 281, *Staphylococcus aureus* CCMB 262 (resistant to streptomycin and dihydrostreptomycin), *Staphylococcus aureus* CCMB 263, *Staphylococcus aureus* CCMB 285, *Bacillus cereus* CCMB 282, *Candida albicans* CCMB 286, *Candida albicans* CCMB 266 and *Candida parapsilosis* CCMB 288 (resistant to amphotericin-B). All microorganisms were cultured on Müeller-Hinton agar (MHA). The bacterial strains were cultured at 37 °C for 24 h and yeasts at 28 °C for 48 h. All the microbial tests were performed in triplicate.

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the hexane and methanol extracts from the leaves of *M. dificillis* was determined based on a microdilution method in 96 multi-well microtiter plates.[14] All microbial tests were performed in MHA. The extracts were dissolved in a DMSO-water solution (1:1) and sterilized by filtration through a cellulose acetate membrane (0.22 mm). Serial dilutions from 10 to 0.078 mg mL^{-1} of the extracts were prepared. Each well received 10 μL of suspension of each micro-test. The purity of the suspension of the inoculums was verified in a simultaneous incubation. After the period of incubation, 50 μL of triphenyl tetrazolium chloride 2-3-5 (TTC) was added to a final concentration of 0.40 mg mL^{-1} (final concentration; assays with yeasts) and 30 μL of rezasurine (RZ, assays with bacteria) to a final concentration of 0.01% for qualitative analysis of microbial growth in the wells in order to determine the antimicrobial activity of each dilution of the samples. Nystatin (20 mg mL^{-1}) and chloramphenicol (10 mg mL^{-1}) were used as positive controls. Controls were performed to test the viability of microorganisms and the sterility of the culture medium. The MIC was considered the lowest extract concentration where there was no visible microbial growth after the color indicator (TTC and RZ) step.

Minimal microbicidal concentration (MMC)

Petri dishes containing MHA were used for this assay. Volumes of 5 μL from each MIC well were transferred to MHA and cultured at 28 °C for 48 h (yeasts) and at 37 °C for 24 h (bacteria). The MMC was considered the lowest extract concentration where there was no cellular growth.

RESULTS AND DISCUSSION

Volatile compounds

The chemical composition of the oil, and retention indices of the constituents, is given in Table 1. Oil yield was 0.04% in a sample free of water (percentage of water was 55.89%). GC/MS data on the volatile components of *M. difficilis* leaves was characterized by a mixture of aliphatic alcohols, ketones, esters, terpenes, allyl sulfides and the phenylpropanoid known as safrole. Twenty-seven compounds were identified, among them oct-1-en-3-ol (49.65%), linalool (14.93%) and *cis*-phytol (12.83 %) were the most prominent. (E)-α-Ionone, (E)-β-ionone, β-damascenone and dehydro-*ar*-ionene (1,16-trimethyl-1,2-dihydronaphthalene) were previously reported to occur in other species of Bignoniaceae (*Macfadyena unguis-cati* L.).[15] It is noteworthy that (E)-α-ionone, (E)-β-ionone and β-damascenone were only encountered in the essential oil from leaves of *M. standleyi* when dried for 12 h under sunlight, however, when the leaves of *M. standleyi* were dried at room temperature, in an air-conditioned room or in a freezer these compounds were not observed.[16] Although the garlic like-odor of the crushed leaves was easily detected, the amounts of diallyl disulfide (0.85%) and diallyl trisulfide (0.37%)

were lower than those of the oils of *M. alliacea*[3] and *M. standleyi*.[4] According to Campbell and coworkers,[17] cyclic polysulfides, such as 5-methyl-1,2,3,4-tetrathiane, are formed during gas chromatographic procedures when the temperature injection is over 170 °C; therefore, this constituent was probably formed during the analysis and is not actually present in the oil of *M. difficilis*.

Table 1. Constituents (%) identified in the essential oil of the leaves of *Mansoa difficilis*

Constituents	RI*	%
(3*Z*)-hexenol	867	3.49
oct-1-en-3-ol	979	49.65
octan-3-ol	997	1.65
diallyl disulfide	1082	0.85
linalool	1102	14.93
3-hexenyl butanoate	1188	0.88
α-terpineol	1194	1.19
methyl salicylate	1198	0.29
nerol	1230	0.43
(3*Z*)-hexenyl 2-methyl butanoate	1234	0.65
geraniol	1256	1.25
safrole	1292	0.99
diallyl trisulfide	1303	0.37
1,2-dihydro-1,1,6-trimethylnaphthalene	1357	0.20
5-methyl-1,2,3,4-tetrathiane	1367	0.26
(3*Z*)-hexenyl hexanoate	1383	1.16
(*Z*)-β-damascenone	1388	1.82
(2*E*)-hexenyl caproate	1391	0.27
(*E*)-β-damascenone	1419	0.43
β-caryophyllene	1424	0.56
(*E*)-α-ionone	1432	0.20
(*E*)-β-ionone	1491	0.42
(*E*)-nerolidol	1563	0.11
(3*Z*)-hexenyl benzoate	1571	0.42
pentadecanal	1717	0.59
6,10,14-trimethyl-2-pentadecanone	1848	0.30
cis-phytol	2117	12.83
butyl palmitate	2190	0.29
Total		96.48

*RI on Rtx-5MS

Non-volatile compounds

Classic chromatographic procedures of the hexane and methanol extracts of leaves and stems led to the identification of known compounds. Linear hydrocarbons that varied from C_9 to C_{22} (**1**)[12] and squalene (**2**)[18] were identified, together with mixtures of fatty alcohols (**3**), fatty acid methyl esters (**4**), phytol fatty esters (**5**) and fatty acids (**6**)[19] that were not analyzed further. The steroids spinasterol (**7**), stigmasterol (**8**), sitosterol (**15**), 3-*O*-β-D-glucopiranosyl-stigmasterol (**16**) and 3-*O*-β-D-glucopiranosyl-sitosterol (**17**) were identified,[20] as well as the two triterpenoids ursolic (**9**) and oleanolic acids (**10**)[21] and the diterpenoid phytol (**11**).[22] A mixture of two flavones, 7-*O*-β-glucopiranosyl-apigenin (**13**) and 7-*O*-β-glucopiranosyl-6-hydroxyapigenin (**14**),[23] and the glycoside verbascoside (**12**)[24] were identified.

The chemical composition of the non-volatile compounds from the stems of *M. difficilis* closely resembled that found for the leaves, except for verbascoside (**12**) which was only identified in the leaves. Ursolic acid was also previously isolated from some Bignoniaceae species, such as from *Clytostoma ramentaceum* (Mart. ex DC.) Bureau & K. Schum., *Arrabidaea triplinervia* (Mart. ex DC.) Baill. ex Bureau and *Arrabidaea samydoides* Sandw.[8,25] The occurrence of verbascoside was previously reported in some Bignoniaceae genera, such as *Arrabidaea*,[26] *Deplanchea*, *Jacaranda, Mussatia*, *Tecoma*, *Newboldia*,[27] and also the species *Barnettia kerri* (Barnett & Sandwith) Santisuk and *Markhamia stipulata* (Wall.) Seem.,[28] but this is the first report of the compound in *Mansoa*. The structures of some of the identified compounds from *M. difficilis* are shown in Figure 1.

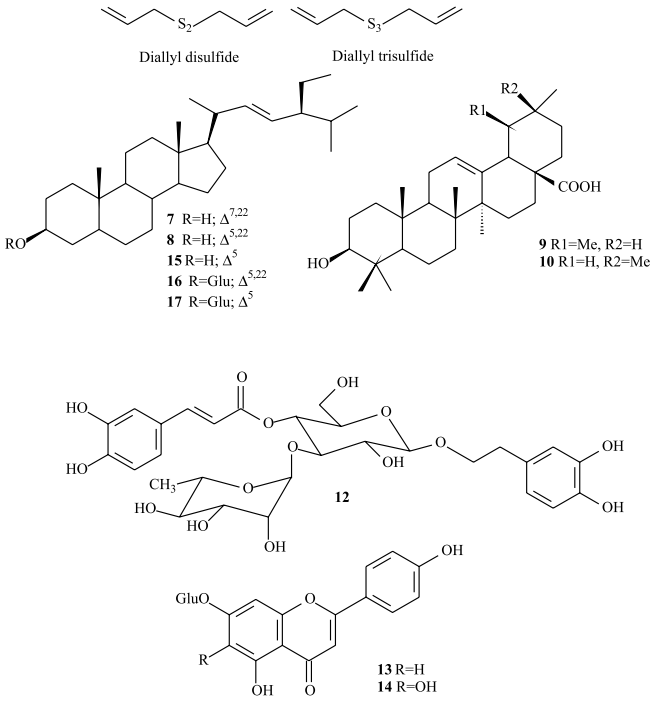

Figure 1. *Structures of some compounds of Mansoa difficilis*

Antimicrobial activity

The MIC and MCC data on the hexane and methanol extracts obtained from the leaves of *M. difficilis* are shown in Table 2. Both extracts showed inhibition of the tested microorganisms. The hexane extract showed higher activity than the methanol extract, inhibiting *Salmonella* sp. CCMB 281 (MIC = 0.16 mg mL^{-1}), *P. aeruginosa* CCMB 268 (MIC = 0.08 mg mL^{-1}), *S. aureus* CCMB 262 (MIC = 0.08 mg mL^{-1}). The best results were observed against *P. aeruginosa* 268 CCMB and *S. aureus* CCMB 262, where the values of MIC were lower than the control (chloramphenicol) (Table 2). The methanol extract showed higher inhibition against yeasts than the bacteria tested and the best result was observed against *C. albicans* CCMB 266 (MIC = 1.25 mg mL^{-1} and MMC 5.00 mg mL^{-1}).

According to Fontanay and coworkers,[29] MIC values below 10 μg mL^{-1} are considered good and those around 50 μg mL^{-1} moderate, for antibacterial activity. MIC values that equal hundreds of μg mL^{-1} indicate that the compound has no activity. Thus, it can be considered that the hexane extract of *M. dificillis* has moderate activity against *P. aeruginosa* CCMB268 and *S. aureus* CCMB 262.

Linear hydrocarbons (C_9 to C_{22}) (**1**), sterols (**7**, **8**, **15**), ursolic (**9**)

Table 2. Minimum inhibitory concentration (MIC) and minimum microbicide concentration (CMM), in mg mL^{-1}, of the methanol and hexane extracts of *Mansoa difficilis*

Microorganisms	MeOH extract		Hexane extract		Controls	
	MIC	MMC	MIC	MMC	Nist/Chlorf	DMSO
E. coli CCMB 261	2.50	10.00	5.00	5.00	R	5.00
P. aeruginosa CCMB 268	2.50	2.50	0.08	10.0	0.31	5.00
Salmonella sp. CCMB 281	5.00	10.00	0.16	10.0	0.16	5.00
S. aureus CCMB 262	2.50	5.00	0.08	10.0	0.31	5.00
S. aureus CCMB 263	2.50	5.00	2.50	5.00	0.31	10.00
S. aureus CCMB 285	2.50	2.50	10.00	(-)	R	10.00
B. cereus CCMB 282	2.50	5.00	5.00	10.00	0.16	5.00
C. albicans CCMB 286	1.25	10.00	2.50	5.00	0.63	10.00
C. albicans CCMB 266	1.25	5.00	2.50	5.00	0.08	10.00
C. parapsilosis CCMB 288	1.25	10.00	2.50	(-)	R	10.00

(-): no inhibition; Nist: nystatin; Chlorf: chloramphenicol; R: resistent.

and oleanolic (**10**) acids isolated from the hexane extract of *M. difficilis*, as well as verbascoside (**12**), found in the methanol extract of *M. difficilis* probably contribute to the antimicrobial activity of the extracts. According to Sharma[30], homologous series of *n*-alkanes exhibited relatively strong antibacterial action against *S. aureus* and *Klebsiella* and moderate action against *Staphylococcus albus*, *Steptococcus viridans*, *E. coli* and *Pseudomonas pyocyanea*; it also was postulated that the antibacterial activity did not vary with the molecular weights of the tested mixtures of *n*-alkanes. In the same study, Sharma showed that the common phytosterols stigmasterol and sitosterol showed strong action against Gram-positive bacteria and a moderate response against Gram-negative bacteria. Spinasterol showed activity against multiple antibiotic-resistant *Helicobacter pylory*[31] and broad activity against opportunistic *Candida* species, *Cryptococcus gattii and Sporothrix schenckii*.[32] Antimicrobial action of oleanolic acid against 21 microorganisms (6 Gram-positive and 12 Gram-negative bacteria and 3 *Candida* species) has been reported.[33] Ursolic acid has been shown to have antimicrobial activity against *S. aureus*, Gram-negative bacteria and *Microsporium lenosum.*[34] Ursolic acid was identified as one of the active compounds in rosemary that inhibits the growth of *S. aureus, E. coli, Lactobacillus brevis, Psedomonas fluorescens, Rhodotorula glutinis* and *Kluyveromyces bulgaricus* at a concentration of 150 μg mL^{-1}.[35] In a recent review, Krystyna and coworkers[36] revealed the appreciable antibacterial activity of ursolic and oleanolic acids. Verbascoside is known for its antibacterial activity against *Proteus mirabilis* and *S. aureus* including one methicillin-resistant strain.[37] A mixture of the isomeric compounds verbascoside and isoverbascoside was active against 5 Gram-positive bacteria (*S. aureus, Micrococcus luteus, Bacillus subtilis, Bacillus mycoides, Enterococcus faecalis*), 2 Gram-negative bacteria (*E. coli* and *Serratia marcensis*), and one yeast (*C. albicans*), whereas *P. aeruginosa* and *Mycobacterium smegmatis* were found to be resistant.[26]

CONCLUSION

This is the first report on the volatile and non-volatile compounds of *M. difficillis* including antimicrobial activity evaluation. This study highlighted another source of organosulfur and other compounds from the *Mansoa* genus. The results of the present investigation demonstrate that *M. difficilis* shows growth inhibition against several bacteria and yeasts. The hexane extract proved especially active against *P. aeruginosa* and *S. aureus*, including streptomycin- and dihydrostreptomycin-resistant strains.

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REFERENCES

1. Fischer, E.; Theisen, I.; Lohmann, L. G. In *The families and genera of vascular plants*; Kadereit, J. W., ed.; Springer-Verlag: Heidelberg, 2004, vol. 7.
2. Gentry, A. H.; *Flora Neotropica* **1980**, *25*, 1.
3. Zoghbi, M. G. B.; Ramos, L. S.; Maia, J. G. S.; Silva, M. L.; Luz, A. I. R.; *J. Agric. Food Chem.* **1984**, *32*, 1009; Zoghbi, M. G. B.; Andrade, E. H. A.; Maia, J. G. S.; *Flav. Fragr. J*. **2002**, *17*, 133.
4. Zoghbi, M. G. B.; Pereira, R. A.; Lima, G. S. L.; Guilhon, G. M. S. P.; *J. Essent. Oil Res*. **2010**, *22*, 247.
5. Bhupendra, K. R.; Taneja, V.; Singh, U. P.; *Pharm. Biol.* **1999**, *37*, 13.
6. Chaturvedi, R.; Dikshit, A.; Dixit, S. N.; *Tropical Agriculture* **1987**, *64*, 318.
7. Rana, B. K.; Taneja, V.; Singh, U. P.; *Pharm. Biol.* **1999**, *37*, 13.
8. Rocha, A. D.; Oliveira, A. B.; Souza Filho, J. D.; Lombardi, J. A.; Braga, F. C.; *Phytother. Res.* **2004**, *18*, 463.
9. Freixa, B.; Vila, R.; Vargas, L.; Lozano, N.; Adzet, T.; Canigueral, S.; *Phytother. Res.* **1998**, *12*, 427.
10. Chirunthorn, R.; Supavita, T.; Intaraksa, N.; Kummee, S.; Junkong, N.; Chisorn, B.; Itharat, A.; *J. Sci. Technol.* **2005***, 27 (Suppl. 2),* 489.
11. Oliveira, J.; Zoghbi, M. G. B. In *Cipó-de-alho: aspectos botânicos, químicos e moléculas bioativas*; Souza Filho, A. P.; Nascimento, J. L. M., eds.; Embrapa: Brasília, 2012, cap. 4.
12. Adams, R. P.; *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, 4th ed.*,* Allured Pub. Corp.: Carol Stream, 2007.
13. Yayli, N.; Güleç, C.; Üçüncü, O.; Yasar, A.; Ülker, S.; Coşkunçelebi, K.; Terzioğlu, S.; *Turk. J. Chem.* **2006**, *30*, 71.
14. Sarker, S. D.; Nahar, L.; Kumarasamy, Y.; *Methods* **2007**, *42*, 321.
15. Aboutab, E. A.; Hashem, F. A.; Sleem, A. A.; Maamoun, A. A.; *Plant Product Res. J.* **2010**, *14*, 19.

16. Guilhon, G. M. S. P.; Bittencourt, R. M.; Lima, G. S. L.; Zoghbi, M. G. B. In *Cipó-de-alho: aspectos botânicos, químicos e moléculas bioativas*; Souza Filho, A. P.; Nascimento, J. L. M., eds.; Embrapa: Brasília, 2012, cap. 4; Zoghbi, M. G. B.; Pereira, R. A.; Oliveira, J. In ref. 11, cap. 5.
17. Campbell, C.; Gries, R.; Khaskin, G.; Gries, G.; *J. Appl. Entomol*. **2011**, *135*, 374.
18. Breitmaier, E.; Voelter, W.; *Carbon-13 NMR Spectroscopy*, 3rd ed., VCH: Weinheim, 1987.
19. Ragasa, C. Y.; Hofilena, J.; Rideout, J. A.; *Philippine J. Sci.* **2004**, *133*, 1; Knothe, G.; Kenar, J. A.; *Eur. J. Sci. Technol.* **2004**, *106*, 88; Ragasa, C. Y.; Lim, K.; *Philippine J. Sci.* **2005**, *134*, 63; Asahi Research Center; *Handbook of proton-NMR spectra and data*, Academic Press: Tokyo, 1985.
20. Forgo, P.; Kover, K. E.; *Steroids* **2004**, *69*, 43; Zhang, X.; Cambrai, A.; Miesh, M.; Roussi, S.; Raul, F.; Aoude-Werner, D.; Marchioni, E.; *J. Agric. Food Chem.* **2006**, *54*, 1196; Sultanova, N. A.; Abilov, Z. A.; Shul'ts, E. E.; Omurkamzinova, V. B.; *Chem. Nat. Compd.* **2004**, *40*, 192; Kojima, H.; Sato, N.; Hatano, A.; Ogura, H.; P*hytochemistry* **1990**, *29*, 2351; Zhang, L.; Yang, X-D.; Xu, L-Z.; Zou, Z-M.; Yang, S-L.; *J. Asian Natural Products Res.* **2005**, *7*, 649.
21. Ahmad, V. U.; Rahman, A.; *Handbook of natural products data: pentacyclic triterpenoids*, Elsevier: Amsterdam, 1994, vol. 2.
22. Rahman, A-U.; Ahmad, V. U.; *^{13}C-NMR of Natural Products*, Plenum Press: New York, 1994, vol. 2.
23. Oyama, K.-I.; Kondo, T.; *Tetrahedron* **2004**, *60*, 2025; Peng, Z. F.; Strack, D.; Baumert, A.; Subramaniam, R.; Goh, N. K.; Chia, T. F.; Tan, S. N.; Chia, L.S.; *Phytochemistry* **2003**, *62*, 219.
24. Krebs, H. C.; Habermehl, G. G.; *Magn. Reson. Chem.* **1992***, 30*, 56.
25. Leite, J. P. V.; Lombardi, J. A.; Chiari, E.; Oliveira, A. B.; *Rev. Bras. Farmacogn.* **2001**, *11*, 77; Pauletti, P. M.; Bolzani, V. S.; Young, M. C. M.; *Quim. Nova* **2003**, *26*, 641.
26. Lima, C. S. A.; Amorim, E. L. C.; Sena, K. X. F. R.; Chiappeta, A. A.; Nunes, X. P.; Agra, M. F.; Cunha, E. V. L.; Silva, M. S.; Barbosa Filho, J. M.; *Rev. Bras. Cien. Farm.* **2003**, *39*, 77.
27. Davioud, E.; Bailleul, F.; Delaveau, P.; Debray, M. M.; *Planta Med.* **1989**, *55*, 87; Jimenez, C.; Villaverde, M. C.; Riguera, R.; Castedo, L.; Stermitz, F. R.; *Phytochemistry* **1987**, *26*, 1805; Jimenez, C.; Villaverde, M. C.; Riguera, R.; Castedo, L.; Stermitz, F. R.; *Phytochemistry* **1988**, *27*, 2947; Jimenez, C.; Villaverde, M. C.; Riguera, R.; Castedo, L.; Stermitz, F. R.; *J. Nat. Prod*. **1989**, *52*, 408; Guiso, M.; Marra, C.; Piccioni, F.; Nicoletti, M.; *Phytochemistry* **1997**, *45*, 193; Gafner, S.; Wolfender, J. L.; Nianga, M.; Hostettmann, K.; *Phytochemistry* **1997**, *44*, 687.
28. Garcez, F. R.; Garcez, W. S.; Mahmoud T. S.; Figueiredo, P. O.; Resende, U. M.; *Quim. Nova* **2007**, *30*, 1887.
29. Fontanay, S.; Grare, M.; Mayer, J.; Finance, C.; Duval, R. M.; *J. Ethnopharmacol.* **2008**, *120*, 272.
30. Sharma, R. K.; *Bioorg. Chem.* **1993**, *21*, 49.
31. Wang, Y-C.; Li, W-Y.; Deng-Chyang Wu, D-C.; Wang, J-J.; Wu, C-H.; Liao, J-J.; Lin, C-K.; *Evidence-Based Complementary and Alternative Medicine* (2011), doi:10.1093/ecam/nep147.
32. Johann, S.; Mendes, B. G.; Missau, F. C.; Resende, M. A.; Pizzolatti, M. G.; *Braz. J. Microbiol.* **2011**, *42*, 1065.
33. Kuete, V.; Eyong, K. O.; Folefoc, G. N.; Beng, V. P.; Hussain, H.; Krohn, K.; Nkengfack, A. E.; *Pharmazie* **2007**, *62*, 552.
34. Liu, J.; *J. Ethnopharmacol.* **1995**, *49*, 57.
35. Collins, M. A.; Charles, H. P.; *Food Microbiol.* **1987**, *4*, 311.
36. Krystyna, I. W.; Grudniak, A. M.; Fiecek, B.; Kraczkiewicz-Dowjat, A.; Kurek, A.; *Cent. Eur. J. Biol*. **2010**, *5*, 543.
37. Didry, N.; Seidel, V.; Dubreuil, L.; Tillequin, F.; Bailleul, F.; *J. Ethnopharmacol.* **1999**, *67*, 197.