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Artigo

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Metabólitos Especiais Isolados de *Ouratea cuspidata* Engl. (Ochnaceae)

Abstract: Chromatographic fraction of extracts from the leaves and branches of *Ouratea cuspidate* (Ochnaceae) allowed the isolation and identificationof the mistures: of sitosterol and stigmasterol; two news maslinic acid ester derivative: 3-β-O-stearoyl/palmitoyl-maslinic acid and 2-α-O-stearoyl/palmitoyl-maslinic acid, and lupeol, α- and β-amyrin, besides the pure compounds: maslinic acid, amentoflavone and putraflavone, flavonoids 4',5,7-trihydroxy-3',5'-dimethoxy-flavone, 3,5,7,4'-tetrahydroxy-3'-methoxy-flavone, 5,7,4'-trihydroxy-3'-methoxy-3-O-β-D-galactopyranosyl-flavone as well as the carbohydrate methyl-β-D-glucopyranoside. The structures of compounds were deduced by spectral data analysis of NMR ^{1}H, ^{13}C (HSQC, HMBC, COSY) besides ESI-MS/MS, GC/MS and comparison with literature data.

Keywords: Steroids; triterpenes; biflavonoids; maslinic acid; flavonoid glycosides.

Resumo

Fracionamento cromatográfico dos extratos de folhas e de galhos de *Ouratea cuspidata* (Ochnaceae) permitiu o isolamento das misturas: sitosterol e estigmasterol; dois novos ésteres derivados do ácido maslinico, 3-β-O-estearoil/palmitoil-maslínico e ácido 2-α-O-estearoil/palmitoil-maslínico; lupeol, α- e β-amirina. Além dos compostos puros: ácido maslínico; ácido maslinico, amentoflavona e putraflavona; 4',5,7-trihidroxi-3',5'-dimetoxi-flavona; 3,5,7,4'-tetrahidroxi-3'-metoxi-flavona, 5,7,4'-trihidroxi-3'-metoxi-3-O-β-D-galactopiranosil-flavona, assim como do carboidrato metil-β-D-glicopiranosil As estruturas dos compostos foram deduzidas por análises dos dados espectrais de RMN ^{1}H, ^{13}C (HSQC, HMBC, COSY), além de ESI-EM/EM, CG/EM e comparação com dados da literatura.

Palavras-chave: Esteroides; triterpenos; biflavonoides; ácido maslinico; flavonoides glicosilados.

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Special Metabolites Isolated from *Ouratea cuspidata* Engl. (Ochnaceae)

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1. Introduction

In the course of the phytochemical investigation of the genus *Ouratea* (Ochnaceae) it has been reported the presence of terpenoids, isoflavonoids, flavonoid glycosides and biflavones,[1-3], beside the some spectral data analysis of biflavonoids and their derivatives have been discussed.[4,5] In those studies, it was also reported some reviews in which was proposed a rational nomenclature and was described the pharmacological potential of the genus.[6] The frequency of amentoflavone, agathisflavone and its derivatives, has allowed to consider these biflavonoids as chemotaxonomic markers for this genera.

In farmacological study it has been reported the DNA topoisomerase inhibition, cytotoxica and antitumor activities.[7-9]

As part of our phytochemical study, on *Ouratea* species, we describe the first phytochemical study of *Ouratea cuspidata* St. Hil, reporting the identification of two new acyl-maslinic acid derivatives, besides eleven known compounds including the two chemotaxonomic markers for this genus.

In Brazilian traditional medicine, *Ouratea* species have been indicated for the healing of palsy, erysipelas and wounds in the

uterus[10]. Leaves of *O. spectabilis* are used as stomachic and vermifuge, as well as gastric distress[11]. Leaf infusions of *O. parviflora* have long been prescribed for the treatment of inflammation-related diseases such as rheumatism, sprains and arthritic disorders[12]. The identification of maslinic acid and derivative in the non-polar fractions, reveal compounds that justify the use of *Ouratea* species. Different properties such as antitumoral, cardioprotective, anti-inflammatory activity, and antioxidant protection of maslinic acid have been described.[13,14]

2. Results and Discussion

The phytochemical study of *O. cuspidata* allowed to isolate and identify two new triterpenoid ester derivatives, 3-β-O-stearoil/palmitoyl-maslinic acid and 2-α-O-stearoil/palmitoyl-maslinic acid (**1 + 2**), and the known compounds (**Figure-1**): four triterpenes, maslinic acid (**3**),[15] lupeol, α- and β-amyrin (**4 + 5 + 6**),[3,16,17] two steroids sitosterol and stigmasterol (**7 + 8**),[18] two biflavonoids, amentoflavone (9)[1] and putraflavone (**10**),[19] the flavones 5,7,4'-trihydroxy-3',5'-dimethoxy-flavone (**11**),[20] 3,5,7,4'-tetrahydroxy-3'-methoxy-flavone (**12**),[21] that were isolated from the extract of leaves. From the branch extracts were isolated the glycoside 5,7,4'-tri-hydroxy-3'-methoxy-3-O-β-D-galactopyranosyl-flavone (**13**)[21] and methyl-β-D-glucopyranoside (**14**).[22]

The structures of compounds were deduced by ^{1}H and ^{13}C NMR, and mass spectra analysis and comparison with literature data. The methyl and acetyl derivative of **9** were prepared analyzed by NMR data and confirmed the proposed structure.

The structures of triterpene acids esters were identified in the mixture (**1 + 2**) by analysis of ^{1}H and ^{13}C NMR spectra, COSY, HSQC, HMBC, comparison with literature data of maslinic acid,[15] besides the analysis of ESI-MS/MS and CG-MS of fraction containing bath compounds.

The ^{1}H NMR spectrum showed signals of seven methyl groups, signal of olefinic proton (δ_H 5.29) and four signals of HC-O similar to those of ring A of maslinic acid supporting acyl unities, that were confirmed by the $^{1}Hx^{1}H$-COSY analysis, δ_H 4.97 (ddd, J=4.1, 10.7, 10.4 Hz, H-2) coupling with δ_H 3.23 (d, J=10.1 Hz, H-3) and 4.53 (d, J=10.1 Hz, H-3) coupling with 3.81 (ddd, J=4.7, 11.0, 10,1 Hz, H-2).

The analysis of ^{13}C NMR (DEPTQ experiment) and comparison with the spectrum of maslinic acid (**3**) allowed to identify the signals of CH_3, CH_2, CH and quaternary carbons of the triterpene (**3**), four signals of oximethine carbons besides signals of the acyl unity [δ_C: 175.3/174.5 (C-1'), signals of (CH_2) CH_3]. These values are in accordance with each structure containing an acyl and a hydroxyl group, δ_{CH} 67.8 (C-2) and 84.8 (C-3) for **1**, and δ_{CH} 73.0 (C-2) and 80.9 (C-3) for **2**. The signals detected in HMQC spectrum confirmed this proposition with δ_H/δ_{CH}: 4.97/84.8, 4.53/73.0, 3.81/80.9, and 3.23/67.8. The mass spectrum (ESI-MS) in negative mode of fraction containing **1** + **2** showed two $[M-H]^{-\cdot}$ ion peak at *m/z* 709.5777 (of $C_{46}H_{78}O_5$, calc 709.5771) and 737.6074 (of $C_{48}H_{82}O_5$, calc 737.6984), and the ESI-MS/MS of *m/z*: 709.5777 furnished *m/z* at 709.5758 (60%), 453.3370 (30%, of triterpene acid fragment, $C_{30}H_{45}O_3$, calc. 453.3368), 255.2326 (100%, of acyl unity, $C_{16}H_{31}O_2$, calc. 255.2324). These data and the difference between the value of bath [M-H] allowed to include two acyl unities, hexadecanoyl/octadecanoyl, linked at C-3 in **1**, and at C-2 in **2**.

The HMQC experiments showed $^{2,3}J_{CH}$ long-range correlations between the H-12 (δ_H 5.29) and carbons C-14 (δ_C 41.6), C-9 (δ_C 47.5), and C-27 (δ_C 25.9); between H-3 (δ_H 4.97) and C-1' (δ_C 175.5), C-2 (δ_C 67.8), C-4 (δ_C 39.3), C-23 (δ_C 28.5) and C-24 (δ_C 17.1); between H-18 (δ_H 2.85) and C-12 (δ_C 122.6), C-13 (δ_C 143.8), C-14 (δ_C 41.6), C-16 (δ_C 22.8) and C-28 (δ_C 184.2); between H-1 (δ_H 2.07) and C-2 (δ_C 67.8), C-3 (δ_C 84.8), C-10 (δ_C 38.2) and C-25 (δ_C 16.5), between H-2'(δ_H 2.40) and

C-1' (δ_C 175.5 $^2J_{CH}$) for compound **1**. For compound **2** was observed correlations between the H-3 (δ_H 3.81) and C-2 (δ_C 73.0), C-4 (δ_C 39.3) and C-24 (δ_C 17.1); between H-2 (δ_H 4.53) and C-3 (δ_C 80.9) and C-1' (δ_C 174.5); between H-1 (δ_H 2.07) and C-2 (δ_C 73.0), C-3 (δ_C 80.9), C-10 (δ_C 38.2) and C-5 (δ_C 55.1); between H-2'(δ_H 2.31) and C-1' (δ_C 174.5 $^2J_{CH}$). The correlation of $^3J_{CH}$ between H-3 (δ_H 4.97) and C-1' (δ_C 175.5 $^2J_{CH}$) and between H-2 (δ_H 4.53) and C-1' (δ_C 174.5 $^3J_{CH}$) were used to confirm the acyl group in the compounds **1** and **2**, respectively.

The GC-MS analysis of the methyl esters prepared by transesterification of the fraction containing both compounds by acid hydrolysis (methanol and HCl) confirmed the proposed acyl unities. The mass spectrum of both methyl ester presented, besides other values, the *m/z* of molecular ion at 270 ($M^{+\cdot}$, 5%) for **1**, and 298 ($M^{+\cdot}$, 5%) for **2**, respectively, compatible with molecular formula $C_{17}H_{34}O_2$ and $C_{19}H_{38}O_2$ of bath methyl esters. The comparison of these spectra with NIST library were used to confirm the bath proposed structure of methyl esters derivatives and defined the structures of compounds **1** and **2**.

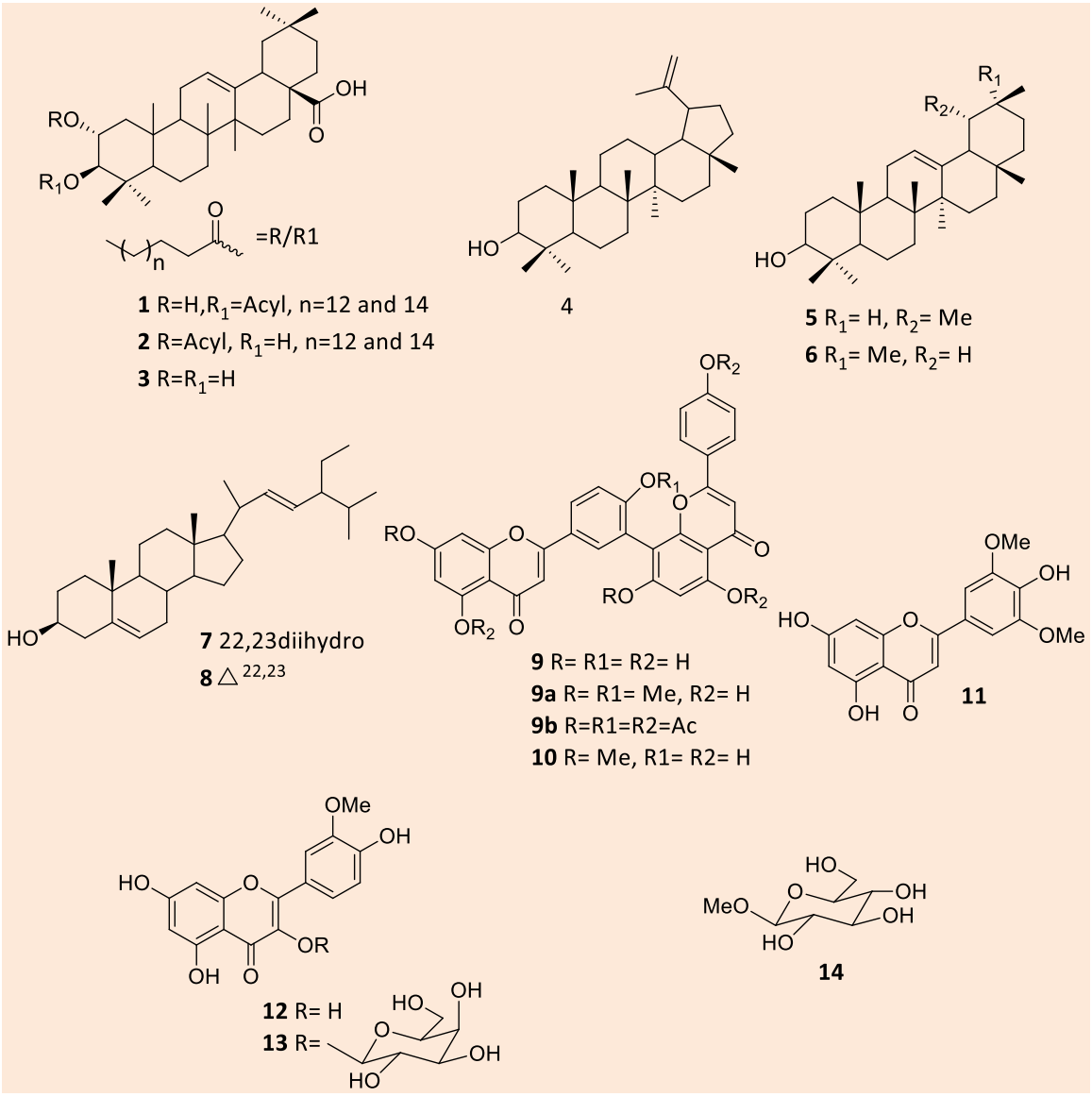

Figure 1. Structure of the compounds

3. Material and Methods

3.1. Equipment and chemicals

^{1}H and ^{13}C NMR spectra were obtained on a Bruker AC-400 (400 and 100 MHz) and AC-500 (500 and 125 MHz) spectrometer using DMSO-d_6, $MeOD_4$ or $CDCl_3$ as solvents with TMS as the internal reference.

Electrospray ionization–high resolution spectra were measured on a quadrupole-time of flight instrument (micrOTOF II and UltrOTOFQ, Bruker Daltonics, Billerica, MA).

The methyl esters analysis was made by gas chromatograph coupled to a mass spectrometer (GC/MS – Shimadzu QP-2010 Plus) equipped with a Factor Four/Vf-5ms fused-silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness), using helium as carrier gas at 1 mL/min. The initial oven temperature was 60ºC, which after being held constant for 1 min. was increased at a rate of 10ºC min^{-1} to 250ºC. The sample injection volume was 1 µL in $CHCL_3$. The injector and detector temperatures were both 250ºC. The mass spectra were obtained in a range of *m/z* 10 – 300, by the electron impact technique at 70 eV.

Column chromatography was carried out with silica gel (Vetec and Aldrich 0.05-0.20 mm) and Sephadex LH-20 (Sigma, USA); silica gel F254 G (Vetec) was used form preparative TLC; aluminum backed (Sorbent silica gel plats W/UV254 were used form analytical TLC, with visualization under UV (254 and 366 nm), with $AlCl_3$-EtOH (1%), vanillin and iodine vapour.

3.2. Plant material

The leaves and branches of *O. cuspidata* were collected in the beach ridge of Barra de Maricá, municipality of Rio de Janeiro-RJ, Brazil, by Doctor L. de S. Valle. Voucher specimen (N° 206313) is deposited at the Herbarium of Museu Nacional do Rio de Janeiro.

3.3. Extraction and Isolation of the compounds

The dried and powdered leaves (OCL, 580 g) and branches (OCB, 650 g) were extracted exhaustively in sequence with CH_2Cl_2, ethyl acetate and methanol at room temperature. The solvents were removed under vacuum furnishing the residues OCLD (3.30 g), OCLAc (11.4 g), OCLM (130 g), OCBC (8.0g), OCBAc (20.0 g) and OCBM (65.0 g), respectively. Each extract was filtered on silica gel column using adequate eluent and the fractions were analyzed by TLC plate and reunited in groups. The fractions were crystallized or re-fractionated on silica gel and/or Sephadex LH-20 column eluting by each adequate eluent.

The residue OCLD was fractionated on a silica gel column eluted with hexane and mixture with ethyl acetate and methanol in increasing polarity and the fractions were analyzed by TLC plate. This residue yielded a mixture of steroids, sitosterol and stigmasterol (**7 + 8**), and a mixture of triterpenes, lupeol, α-amyrin and β-amyrin (**4 + 5 + 6**, respectively).

OCLAc and OCLM were filtered on a silica gel column and eluted initially with hexane:ethyl acetate (1:1) for OCLAc and ethyl acetate (100%) for OCLM increasing the polarity with ethyl acetate and methanol to 100% methanol. The impure fractions containing flavonoids, revealed by $AlCl_3$ in ethanol, were filtered on Sephadex LH20 using methanol as eluent. The fractions were analyzed with TLC plate and reunited in groups and the groups of fractions containing pure flavonoids were analyzed by ^{1}H and ^{13}C NMR spectra. From the OCLAc were isolated the biflavones amentoflavone **9** (100 mg) and putraflavone **10** (120 mg). The fractions OCLAc soluble in CH_2Cl_2 was filtered on a

silica gel column and eluted initially with hexane, CH_2Cl_2, ethyl acetate and methanol increasing the polarity. Two triterpenes, 3-β-O-stearoyl/palmitoyl-maslinic acid and 2-α-O-stearoyl/palmitoyl-maslinic acid (**1 + 2**) (38 mg) were identified, as mixture, and maslinic acid **3** (10 mg).

From OCLM were isolated amentoflavone **9** (30 mg), the flavones 4',5,7-trihydroxy-3',5'-dimethoxy-flavone **11** (20 mg), and mixtures of sugars and of tannins were identified in polar fractions. The same procedure was made with the residue from the branches (OCBC and OCBAc) and the mixtures of steroids (**7 + 8**) and triterpenes (**4 + 5 + 6**) were isolated from OCBC; from the OCBAc were isolated the flavonol 3,5,7,4'-tetrahydroxy-3'-methoxy-flavone **9** (10 mg), the glycosyl flavonoid **13** (15 mg), and the glucopyranoside, methyl-β-D-glucopyranoside **14** (80 mg). The analysis of OCBM by IR, ^1H and ^{13}C allowed to identify only mixtures of sugars and tannins. The biflavone **9** was treated with diazomethane to yield **9a** (gum, 12.0 mg) and with pyridine/acetic anhydride (1:1) yielding the peracetyl derivative **9b** (gum, 20.0 mg) using the same procedure described by Silva et al (2009).[23]

NMR data of the 3-β-O-Stearoyl/palmitoyl-maslinic acid and 2-α-O-Stearoyl/palmitoyl-maslinic acid (**1 + 2**):

^1H NMR (500 MHz, $CDCl_3$) δ (mult., *J* in Hz): 5.29 (sl, H-12), 4.53 (d, 10.1, H-3 of 3-β-acyl), 3.81 (tbr, H-2 of 3-β-acyl), 4.97 (tbr, H-2 of 2-α-acyl), 3.23 (d, 10.1, H-3 of 2-α-acyl), 2.07 (m, H-1), 2.85 (d, 11.0, H-18), 0.88 (s, Me-23), 0.90 (s, Me-24), 0.75 (s, Me-25), 0.92 (s, Me-26), 0.94 (s, Me-27), 1.00 (s, Me-29), 1.15 (s, Me-30), 2.40 (dd, 7.2, H-2'), 1.27-1.29 (m, H-4' – H-13'). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 47.8 (C-1), 67.8 (C-2), 84.8 (C-3), **for 1** and 47.6 (C-1), 73.0 (C-2), 80.9 (C-3) **for 2** 38.2 (C-4), 55.1 (C-5), 18.3 (C-6), 30.7 (C-7), 39.3 (C-8), 47.5 (C-9), 39.3 (C-10), 23.5 (C-11), 122.6 (C-12), 143.8 (C-13), 41.6 (C-14), 27.6 (C-15), 22.8 (C-16), 46.5 (C-17), 40.9 (C-18), 45.8 (C-19), 30.6 (C-20), 33.8 (C-21), 32.4 (C-22), 28.5 (C-23), 17.1 (C-24), 16.5 (C-25), 17.6 (C-26), 25.9 (C-27), 184.2 (C-28), 33.0 (C-29), 23.6 (C-30), 175,5/174.5 (C-1'), 34,7 (C-2'), 25,0 (C-3'), 29.2 – 29.7 (C-4'- C-13'/15'), 31.9 (C-14'/16'), 22.7 (C-15'/17'), 14.1 (C-16'/18'). ESI-MS *m/z*: 709.5766 [M-H, 1/2]$^-$, and 737.6078 [M-H, 1/2]$^-$, MS/MS: *m/z* 709.5758 (60%), 453.3370 (30%), 255.2326 (100%).

4. Conclusions

This first phytochemical investigation of *O. cuspidata* has further enriched the knowledge about the chemistry of *Ouratea* genus. The maslinic acid, and the new acyl-maslinic acids derivatives from leaves of *O. cuspidata* reveal the biodiversity of this plant and justify the use of *Ouratea* species in popular medicine in Brazil. The presence of biflavonoids, **9-10**, corroborates the use of these compounds as chemotaxonomic markers for the genus.

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