Chemical investigation, antifungal activity and anatomical aspects of *Protium puncticulatum* J.F Macbr. and Protium tenuifolium (Engl.) Engl

Henrique Cativo dos Santos[1], Samirimi Januario Silva[1], Claudete Catanhede do Nascimento[2], Jorge Alves de Freitas[2], João Vicente Braga de Souza[3], Ana Claúdia Alves Cortez[3], Antonio Gilberto Ferreira[4], Sérgio Scherrer Thomasi[4], Marcia Ortiz Mayo Marques[5], Roselaine Facanali[5], Maria da Paz Lima[1*]

[1]Laboratório de Química de Produtos Naturais, Instituto Nacional de Pesquisas da Amazônia, 69011-970, Manaus-Amazonas, Brazil

[2]Laboratório de Tecnologia da Madeira, Instituto Nacional de Pesquisas da Amazônia, 69011-970, Manaus-Amazonas, Brazil

[3]Laboratório de Micologia Médica, Instituto Nacional de Pesquisas da Amazônia, 69011-970, Manaus-Amazonas, Brazil

[4]Laboratório de Ressonância Magnética Nuclear, Universidade Federal de São Carlos, 13565-905, São Carlos-São Paulo, Brazil

[5]Centro de Pesquisa de Recursos Genéticos Vegetais, Instituto Agronômico, 13020-902, Campinas-São Paulo, Brazil

Corresponding author: mdapaz@inpa.gov.br

Abstract

*Protium Burm. f. (Burseraceae) is well known in the Brazilian Amazon for its diversity of species, though some of them are difficult to identify based on only morphological characteristics. We investigated the species Protium puncticulatum J.F. Macbr. and Protium tenuifolium (Engl.) Engl. in relation to their chemical constituents and some biological aspects. The phytochemical study of the hexane extract from the trunk of P. puncticulatum led to the identification of a mixture of triterpenes: α, β-amyrin (**1** and **2**), and lupeol (**3**); the methanolic extract gave the lignans 7-oxo-parabenzolactone (**4**) and 7'-hydroxy-9α-methylcubebin (**5**); this last lignan showed a MIC of 320 µg/mL for Candida albicans and 160 µg/mL for Cryptococcus neoformans and C. gattii. The hexane extract from the branches of P. tenuifolium also provided a mixture of α and β-amyrin (**1**, **2**); the methanolic extract gave dimeric alkylresorcinols named integracin B (**6**) and integracin A (**7**). Analyses of anatomical characteristics confirmed the identity of the species Protium tenuifolium (Engl.) Engl. Essential oils obtained via hydrodistillation from the fresh bark of the trunk of P. tenuifolium showed a predominance of the monoterpenes limonene (56.17%), α-phellandrene (16.22%) and ρ-cymene (10.52%). This study is important since it increases knowledge on the volatile and non-volatile chemical constituents of the woody parts of two species of Protium from the Amazon.*

Keywords: *triterpenes; lignans; alkylresorcinols derivatives; essential oil; monoterpenes.*

1. Introduction

Protium Burm. f. (Burseraceae), commonly known as "breu" in the Brazilian Amazon, is well known for its diversity of regional species. There are approximately 73 species, 42 of which are endemic to the region (Daly, 1992). However, the genus has species that are difficult to identify based on only morphological characteristics (Daly, 1989). Studies related to the non-volatile chemical constituents of Amazonian species demonstrate the predominance of pentacyclic triterpenes with ursane and oleanane skeletons in leaves (Guimarães and Siani, 2007), stem bark (Zoghbi et al., 1993; Costa et al., 2012) and resins (Almeida et al., 2015; Susunaga et al., 2001); and lignans of dibenzylbutitolactone and arylnaphthalene types have been found in the trunk wood (Siqueira et al., 1995; Siani et al., 1998). As these are resinous species, there is considerable interest in studies of their essential oils, since these have high yields and their volatile constituents are predominantly monoterpenes (Lima et al., 2014; 2016; Zoghbi et al., 2005; Pinto et al., 2013) and sesquiterpenes (Oliveira et al., 2018; Carvalho et al., 2010).

The species *Protium puncticulatum* J.F. Macbr. [syn. *Protium juruense* Swart] has confirmed occurrence in northern Brazil (Acre, Amazonas, Rondônia), *Protium tenuifolium* (Engl.) Engl. [syn. *Protium neglectum* Swart; *Icicopsis tenuifolia* Engl.] is distributed in the Amazon (Flora do Brasil, 2021). Both species, known as "breu" and breu-vermelho", lack studies related to their volatile and non-volatile chemical constituents.

2. Materials and Methods

2.1 General experimental procedures

NMR spectra of compounds **1-5** were measured using a Bruker Fourier-300 apparatus. Analyses of compounds **6** and **7** by LC-DAD SPE/NMR were performed on a chromatograph (1200 series; Agilent GmbH) equipped with a quaternary pump (G1311A) and a degasser (G1322A), a variable wavelength diode array detector (G1315D), and an autosampler (Bruker Biospin GmbH). The LC system was controlled by HyStar 2.3 software (Bruker). A Knauer (K120 Knauer Smartline Pump Control 100, Bruker Daltonics GmbH©, V01.11) make-up pump diluted the post-column flow with water before the peaks were trapped using a Prospekt 2 SPE unit. The 1D and 2D NMR spectra of the isolated compounds were acquired at 299 K with the use of a Bruker Avance III instrument (14.1 Tesla/600 MHz) equipped with an automatic sample changer and a 5 mm inverse triple resonance cryoprobe (1H/13C/15N) and with a z-field gradient. LC-HRMS measurements were obtained using a MicroTOF-QII (Bruker Daltonics) mass spectrometer connected to an LC (Prominence UFLC, Shimadzu). Mass spectra were acquired using an ion trap spectrometer (LCQ Fleet^{TM}, Thermo Scientific) equipped with an electrospray source, operating in positive mode. Analyses of volatile constituents were performed on a Shimadzu QP5000 instrument equipped with a DB-5 fused silica capillary column.

2.2 Obtaining samples and extractions

Samples from *Protium puncticulatum* (trunk) and *Protium tenuifolium* (branches and stem bark) were provided by the Wood Technology Laboratory at the Instituto Nacional de Pesquisas da Amazônia (INPA), and originated from the leftovers of studies of the wood species that were collected in the Estação

Experimental Silvicultura Tropical (53 Km north of the city of Manaus, Amazonas state). The samples from the trunk and branches were submitted to drying and then pulverized in a knife mill. Subsequently, extracts were obtained through successive macerations with hexane and methanol for a period of 7 days for each solvent at room temperature. Samples of fresh trunk bark of *P. tenuifolium* were submitted to hydrodistillation (triplicate) for 4 hours using a Clevenger-type apparatus.

2.3 Chromatographic fractionation of extracts from Protium puncticulatum

The hexane extract from the trunk (215 mg) was fractionated in a silica gel column (70-230 mesh; h X Φ = 39.4 X 2.3 cm) using hex:EtOAc (9:1) as the gradient to provide a mixture (9.2 mg) of triterpenes α,β-amyrin (**1**, **2**) and lupeol (**3**), as well as a mixture (22.1 mg) of the known steroids β-sitosterol and stigmasterol. The methanolic extract (7.35 g) was subjected to a fractionating column with Sephadex LH-20 eluted in methanol that generated 20 fractions, fractionation of fr. 4 (2.3 g) in a silica gel column (70-230 mesh; h X Φ = 54.8 X 3.2 cm) eluted with CH_2Cl_2:MeOH (98:2 → 9:1) generated 14 subfractions, for which the subfr grouped 8-9 by the process of recrystallization with methanol to give compound **4** (124.4 mg); fractionation of the subfr 10-12 in Sephadex LH-20 column eluted with MeOH followed by silica gel column (230-400 mesh) eluted with CH_2Cl_2: EtOAc (98:2 → 9:1) gave compound **5** (10 mg).

2.4 Chromatographic fractionation of extracts from Protium tenuifolium

The hexane extract of samples from the branches was fractionated over a chromatographic column of silica gel (70-230 mesh; h X Φ = 33.0 X 2.8 cm), using hexane, hex: EtOAc (9:1→8:2) as the gradient to give a mixture of α, β-amirina (**1** and **2**; 36 mg), and also a mixture of β-sitosterol and estigmasterol (115 mg). The methanolic extract (10.9 g) was successively partitioned with dichloromethane, ethyl acetate, and methanol. The dichloromethane phase (1.6 g) was fractionated in a sílica gel chromatography column (70-230 mesh; h X Φ = 27.0 X 3.5 cm) eluted with hexane, hex:AcOEt (9:1→7:3). Fractions 14-15 (12 mg) were rechromatographed in a Sephadex LH-20 column eluted with MeOH and then subfractions 11-16 (5 mg) were analyzed using LC-DAD SPE/NMR.

2.4.1 Isolation of compounds 6 and 7 from subfractions 11–16

A 5 mg portion of the subfr 11-16 was dissolved with 1.5 mL of acetone, and the solution was filtered through a PVDF membrane syringe filter (25 mm, 0.45 μm; Tedia, Brazil) prior to the LC analysis. The chromatographic separations were carried out using an analytical Eurobond Prontosil C18 column (125 × 4.0 mm, 5 μm) with a 20 μL injection volume. Gradient elution was performed using a combination of CH_3CN/Milli-Q H_2O with a linear gradient varying from 0-100% of CH_3CN, with a flow rate of 0.8 mL/min for the separation of the two chromatographic peaks of interest. The two compounds were adsorbed on solid-phase extraction cartridges (HySphere Resin GP, 10 mm × 2 mm, 10 μm spherical polydivinylbenzene stationary phase) using an automatic cartridge exchanger (Bruker Biospin GmbH,). After the adsorption process, the cartridges were dried with nitrogen for 30 min to remove residual solvent. CD_3OD (99.8% D) was used to elute compounds **6** and **7** from the SPE cartridges directly into NMR tubes (Bruker, 3 mm o.d.) for analysis.

2.5 Essential oil analysis

Quantitative analysis of the volatile constituents were performed on a GC-MS (QP5000, Shimadzu), operating by electron impact (70 eV), which used a DB-5 (30 m × 0.25 mm × 0.25 μm) capillary column in the GC experiment. The operating conditions were as follows: carrier gas was helium (flow 10 $mL.min^{1}$); temperature program at 60-240 ºC (3 $ºC.min^{-1}$); injection size of 1.0 μL; sample injection temperature at 250 ºC; detector temperature 290 ºC; split 1:20. The compounds were identified by comparing their mass spectrum to those of the database of the GC-MS (NIST 62.lib), literature (McLafferty and Stauffer, 1989) and retention indices (Adams, 2007). Quantitative analysis was performed using a GC 2010 GC-FID (Shimadzu) using the same conditions as the GC-MS method.

2.6 Antifungal assay

Cryptococcus neoformans (ATCC 90112), *Cryptococcus gattii* (ATCC 32269) and *Candida albicans* (ATCC 60193), from the culture collection at the Instituto Nacional de Pesquisas da Amazônia (INPA) in Manaus, Amazonas state, Brazil, were used as references. Minimum inhibitory concentration (MIC) assays were performed using the broth microdilution method, as described by the Clinical and Laboratory Standards Institute in documents M27-A3 (CLSI, 2008). Amphotericin B was used as the antifungal standard.

2.7 Macroscopic analysis of Protium tenuifolium

The woody species was identified via macroscopic analysis and comparison with samples available in the xylotheque of the Instituto Nacional de Pesquisas da Amazônia (INPA).

2.8 Spectroscopic data of compounds

α and β-amyrin(**1** and **2**) and lupeol (**3**). ^{1}H NMR (300 MHz, TMS, $CDCl_3$, δ, ppm, J/Hz): Text. ^{13}C NMR and DEPT (75 MHz, $CDCl_3$, δ, ppm): Text.

7-*oxo*-Parabenzolactone (**4**). HRMS m/z 369.0975 $[M+H]^{+}$. ^{1}H NMR (300 MHz, TMS, $CDCl_3$, δ, ppm, J/Hz): δ 7.28 (1H, dd, J = 8.0 and 1.7, H-6), 7.19 (1H, d, J = 1.6, H-2), 6.81 (1H, d, J = 8.0, H-5), 6.59 (1H, sl, H-2'), 6.62 (1H, d, J = 8.0, H-5'), 6.54 (1H, dd, J = 8.0 and 1.7, H-6'), 6.06 and 5.88 (4H, sl, OCH_2O), 4.42 (1H, t, J = 8.5, H-9a), 4.16 (1H, t, J = 8.4, H-9b), 4.05 (1H, m, H-8), 3.49 (1H, m, H-8'), 3.08 (1H, dd, J = 14.1 and 6.5, H-7a'), 2.94 (1H, dd, J = 14.1 and 6.5, H-7b'). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 194.5 (C-7), 177.0 (C-9'), 152.6 (C-3), 148.5 (C-4), 147.9 (C-3'), 146.5 (C-4'), 130.6 (C-1'), 130.3 (C-1), 124.8 (C-6), 122.5 (C-6'), 109.6 (C-5'), 108.0 (C-2'), 108.2 (C-2), δ 107,9 (C-5), 102.1 ($O\underline{C}H_2O$), 101.0 ('$O\underline{C}H_2O$'), 68.1 (C-9), 46.6 (C-8), 44.8 (C-8') and 34.6 (C-7'). HSQC (300/75 MHz, $CDCl_3$): Text. HMBC (300/75 MHz, $CDCl_3$): 7.28 → 108.29, 148.52, 194.54; 7.19 → 148.52, 124.84, 152.65, 194.54; 6.81 → 130.30, 152.65, 148.52; 6.62 → 34.63, 122.50, 130.68, 146.58, 6.59 →122.50, 130.68, 194.54; 6.54 → 34.63, 107.92, 109.66, 146,58; 6.06 → 148.52, 152.65; 5.89 and 5.88 → 146.58, 147.90, 4.05 → 34.63, 44.82, 68.18, 177.05, 194.54; 4.42 → 44.82; 4.16 → 44.82; 3.49 → 34.63, 46.69, 130.68, 177.05, 194.54; 3.08 → 44.82, 46.69, 109.66, 122.50, 130.68, 177.05.

7'-Hydroxy-9α-methylcubebin (**5**). HRMS *m/z* 387. 3481[M+H]$^+$. ^{1}H NMR(300 MHz, TMS, $CDCl_3$, δ, ppm, J/Hz,): Table 1. ^{13}C NMR (75 MHz, $CDCl_3$): Table 1. HSQC (300/75 MHz, $CDCl_3$): Text. HMBC (300/75 MHz, $CDCl_3$): Table 1.

Integracin B (**6**). ^{1}H NMR (600 MHz, TMS, MeOD, δ, ppm, J/Hz): Table 2. ^{13}C NMR (150 MHz, MeOD,): Table 2. HSQC and HMBC: Text.

Integracin A. (**7**). ^{1}H NMR (600 MHz, TMS, MeOD, δ, ppm, J/Hz): Table 2. ^{13}C NMR (150 MHz, MeOD,): Table 2. HSQC and HMBC: Text.

3. Results and Discussion

3.1 Protium puncticulatum - Identification of compounds and antifungal activity of lignans 4 and 5

A mixture of the triterpenes α- and β-amyrin (**1** and **2**), and lupeol (**3**) from the hexane extract was identified using NMR. The ^{1}H NMR spectrum showed signals of methyl hydrogens between δ 0.79 – 1.61, olefinic hydrogens at δ 5.13 and δ 5.19 (t, J = 3.6 Hz) from H-12 from the mixture of α- and β-amyrin (**1** and **2**), double-terminal vinyl hydrogens (H-29) at δ 4.57 and 4.69 from the lupeol (**3**). The analysis of spectrum ^{13}C NMR and DEPT-135º confirms the occurrence of the three triterpenes, the typical signals of olefinic carbons were verified at δ 124.40 (C-12) and 139.55 (C-13) from α-amyrin (**1**), δ 121.71 (C-12) and 145.19 (C-13) from β-amyrin (**2**), δ 150.95 (C-20) and 109.32 (C-29) of lupeol (**3**). Carbinolic carbons were found at δ 79.05, 79.04 and 78.84, respectively. Based on the intensities of the ^{1}H and ^{13}C NMR signals, it appears that in the triterpene mixture α-amyrin is the most abundant, followed by β-amyrin and lupeol. The mixture of α-amyrin (ursane skeleton) and β-amyrin (oleanane skeleton) has been widely reported in *Protium* species from the Amazon region, however this is the first report of it appearing in *P. puncticulatum.*

The ^{1}H NMR spectra of compounds **4** and **5** exhibited six hydrogens that are characteristic of the aromatic system between δ 7.28-6.36, with a substitution pattern that indicates trisubstituted aryl groups. The signals at δ 6.06 and 5.88 (**4**), 5.94 and 5.91 (**5**) integrated by two hydrogens each, are indicative of a substitution of the methylenedioxy group of the aromatic rings. The butyrolactone system was verified for compound **4** due to the signals of methine hydrogens at δ 4.05 (H-8) and 3.49 (H-8') and methylene hydrogens at δ 4.42 (H-9a) and 4.16 (H-9b). For compound **5**, there were signals of methine hydrogens at δ 2.40 (H-8) and 2.37 (H-8'), oxymethine at δ 4.70 (H-9) and 4.59 (H-7'), oxymethylene at δ 4.18 and 3.92 (H-9') and oxymethylic at δ 3.36.The correlations observed in the HMBC experiment of compound **5** justify the substitution for the presence of methoxy at C-9 and hydroxyl at C-7' (table 1). The orientation of the α-methoxy group at C-9 was based on the coupling constants and comparison with the literature (Marco et al., 1996). Thus, based on the ^{13}C NMR data analyzed together with DEPT, HSQC and HMBC, lignan **4** was identified asv7-oxo-parabenzolactone, and **5** as 7'-hydroxy-9α-hydroxy-9α-methylcubebin. 7-oxo-parabenzolactone lignan was previously isolated from *Protium tenuifollum* trunk wood (Siqueira et al., 1995), 7'- hydroxy-9α-methylcubebin is unprecedented in the literature.

Antifungal tests showed that 7-oxo-parabenzolactone (**4**) in the concentration of 320 µg/ml did not show any growth reduction in strains of three fungal species. Lignan **5** (7'- hydroxy-9α-methylcubebin) showed a MIC of 320 µg/mL for *Candida albicans* and 160 µg/mL for *Cryptococcus neoformans* and *C. gattii*. The greatest activity of **5** is probably due to the presence of hydroxyl and methoxy that influenced it in some way and produced toxicity to fungi.

Table 1. NMR data for compound **5** ($CDCl_3$)

Position	^{1}H NMR	^{13}C NMR	HMBC
1		132.58	
2	6.36 d (J = 1.5 Hz)	109.01	C-3, 4, 6, 7
3		147.46	
4		145.88	
5	6.62 d (J = 7.8 Hz)	107.75	C-1, 3
6	6.41 dd (J = 7.8, 1.5 Hz)	121.77	C-2, 4, 7
7	2.30 dd (J =12.2, 7.5 Hz; H-7a)	38.89	C-1, 2, 6, 8, 9
	2.67 dd (J =12.2, 7.5 Hz; H-7b)		
8	2.40 m	49.72	C-1, 7, 7', 9
9	4.70 sl	108.12	C-7, 8, 8', 9', O**C**H_3
1'		137.80	
2'	6.62 d (J =1.5 Hz)	106.01	C-3', 4', 6', 7'
3'		146.32	
4'		147.50	
5'	6.64 d (J = 7.8 Hz)	107.84	C-2', 3', 4', 6'
6'	6.62 dd (J = 7.8, 1.5 Hz)	118.59	C-2', 7'
7'	4.59 d (J = 4.4 Hz)	73.60	C-8, 2' 8', 9'
8'	2.37 m	47.80	C-1, 7, 8, 9, 7',
9'	4.18 t (J = 8.9 Hz; H-9a)	69.02	C-8', 8, 9, 7'
	3.92 dd (J = 8.9, 6.2 Hz; H-9b)		C-8, 7'
OCH_3	3.36 sl	54.00	C-9
CH_2O_2 (3, 4)	5.94 d (J =1.5 Hz)	100.94	C-3, 4
CH_2O_2 (3', 4')	5.91 d (J =1.5 Hz)	100.88	C-3', 4'

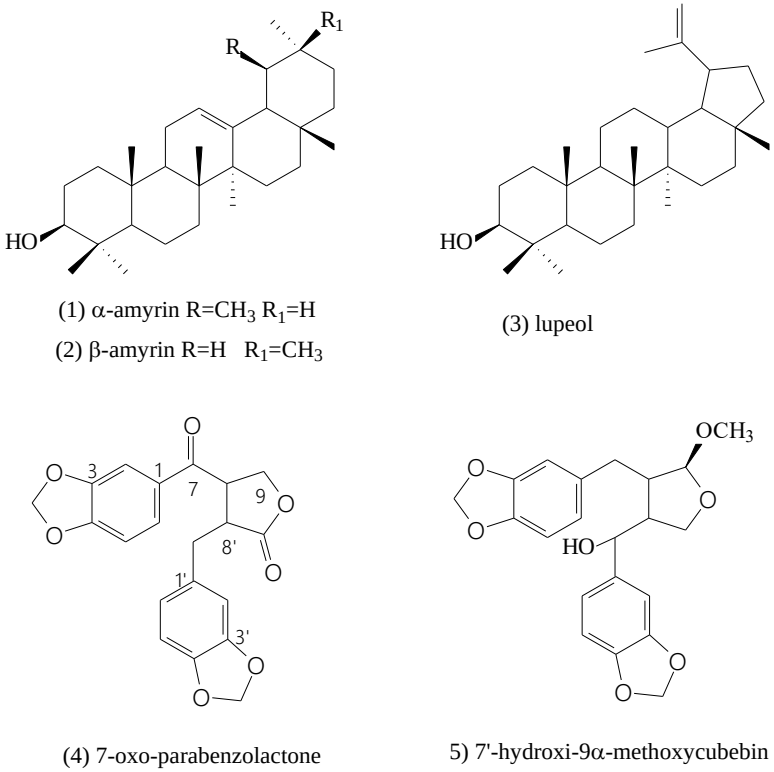


Figure 1. Compounds from *Protium puncticulatum* trunk wood

3.2 Protium tenuifolium - Identification of compounds and wood anatomy

A mixture of the triterpenes α- and β-amyrin (**1**, **2**) was also identified from the hexane extract of *P. tenuifolium* branches. The ^{1}H and ^{13}C NMR spectrum showed the characteristic signals of these triterpenes as methylic, carbinolic and olefinic groups, whose signal intensity indicated that α-amyrin is present with a high concentration in the mixture.

The ^{1}H NMR spectra (Table 2) of compounds **6** and **7** showed methyl triplets at δ 0.94 and 0.90 Hz of compound **6**, δ 0.96 and 0.92 of compound **7**; oxymethine hydrogens as multiple's at δ 5.24 and 3.50 (**6**); δ 5.27 and 4.88 m (**7**). Two meta coupled aromatic hydrogens located on the tetrasubstituted phenyl ring, and three aromatic hydrogens on the 1,3,5-trisubstituted phenyl ring were observed for **6** and **7**. Methylene hydrogens were observed at δ 2.80-1.31. The ^{1}H NMR spectrum of **7** displayed an acetyl methyl singlet at δ 1.99. The ^{13}C NMR (Table 2) and HSQC spectra of **6** exhibited signals of aromatic carbons for two substituted aromatic rings, two oxymethines, one ester carbonyl carbon, two methyl groups and aliphatic methylene groups, which accounted for 18 carbons. Via analysis of these spectral data associated with the correlations established by HMBC experiments and comparison with literature, these two dimeric alkylresorcinols were identified as integracin B (**6**) and integracin A (**7**) (Singh et al., 2002). Phenolic lipids or long-chain phenols called 5-alkylresorcinols have been reported in some families including Anacardiaceae (Dang et al., 2019), which are taxonomically close to Burseraceae (APG, 2021).

Table 2. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) in MeOD of **6** and **7**

Position	^{1}H NMR		^{13}C NMR	
	6	**7**	**6**	**7**
1			159.3	159.3
2	6.06 d (2.2 Hz)	6.07 d (2.2 Hz)	101.0	101.0
3			159.3	159.3
4	6.10 d (2.2 Hz)	6.11 d (2.2 Hz)	107.9	107.9
5			146.2	146.3
6	6.10 d (2.2 Hz)	6.11 d (2.2 Hz)	107.9	107.9
7	2.41 t (7.9 Hz)	2.43 t (7.5 Hz)	37.0	37.0
8	1.50 m	1.50-1.56 m	32.4	32.4
9	1.31-1.41 m	1.27-1.45 m	31.2	30.3
10	1.31-1.41 m	1.27-1.45 m	30.3	30.4
11	1.31-1.41 m	1.27-1.45 m	30.4	30.7
12	1.31-1.41 m	1.27-1.45 m	26.8	26.7
13	1.65 m	1.65 m	35.5	35.5
14	5.24 m	5.27 m	76.6	76.6
15	1.65 m	1.65 m	37.8	37.7
16	1.31-1.41 m	1.39 m	19.9	19.9
17	0.94 t (7.4 Hz)	0.96 t (7.2 Hz)	14.3	14.3
1’			166.1	166.0
2’	6.15 d (2.5 Hz)	6.16 d (2.5 Hz)	102.0	101.9
3’			163.8	163.7
4’	6.19 d (2.5 Hz)	6.20 d (2.5 Hz)	112.0	111.9
5’			149.0	149.0
6’			105.7	105.7
7’			172.8	172.7
8’	2.80 m	2.83 m	37.8	37.7
9’	1.50 m	1.52-1.54 m	33.6	33.6
10’	1.31-1.41 m	1.27-1.45 m	31.1	31.0
11’	1.31-1.41 m	1.31 m	30.5	30.6
12’	1.31-1.41 m	1.31 m	30.9	30.6
13’	1.31-1.41 m	1.37-1.32 m	26.6	26.4
14’	1.31-1.41 m	1.50 m	38.4	35.5
15’	3.50 m	4.88 m	72.2	75.4
16’	1.31-1.41 m	1,67 m	40.7	37.5
17’	1.31-1.41 m	1.31 m	19.9	19.6
18’	0.90 t (7.2 Hz)	0.92 t (7.2 Hz)	14.5	14.3
1’’				172.9
2’’		1.99 s		21.1

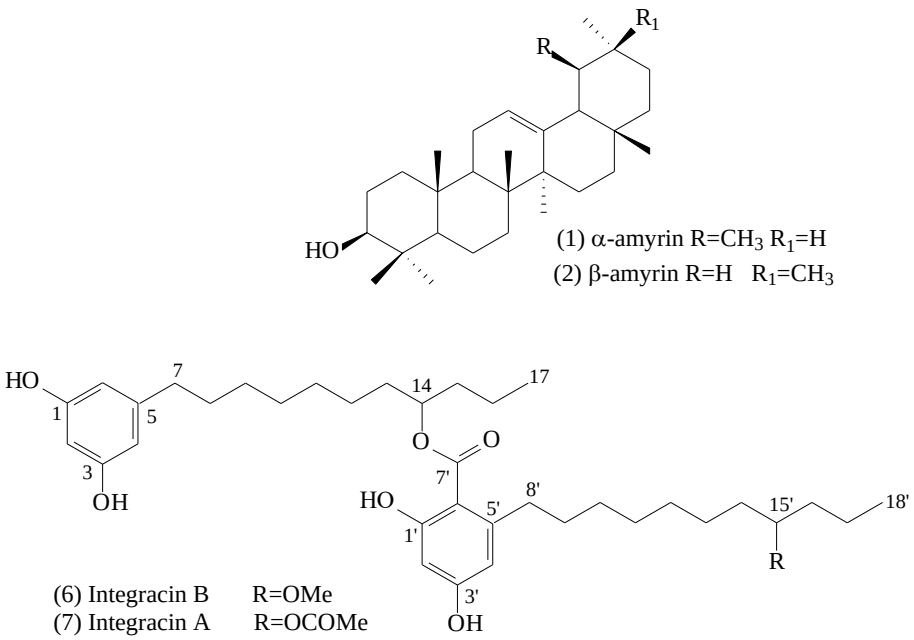
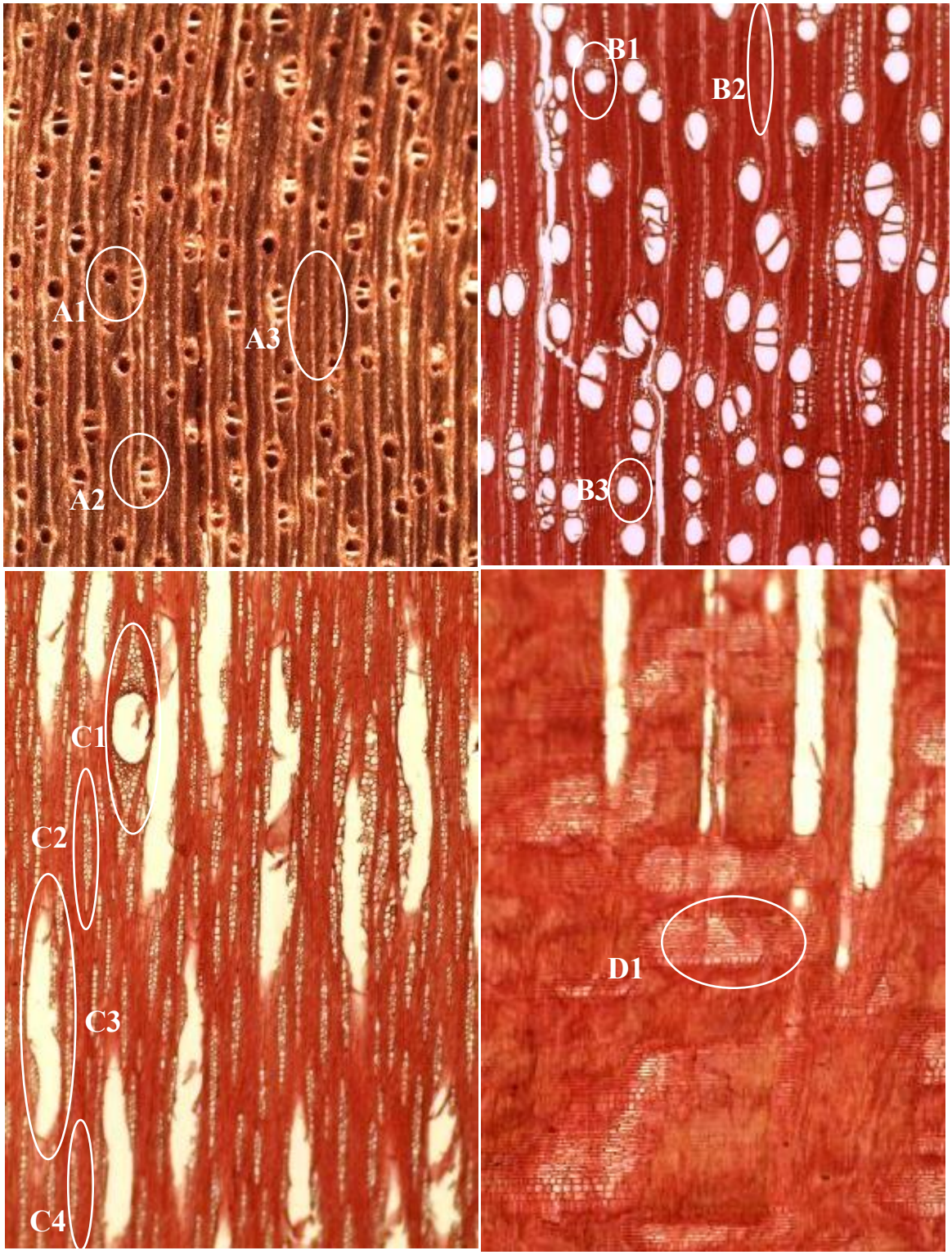


Figure 2. Compounds from *Protium tenuifolium* branches

Since the phenolic lipids identified in this research from *Protium tenuifolium* were not previously reported in Burseraceae species, it was decided to confirm the botanical identification of the wood using macroscopicanatomical analysis. This was performed with the aid of an X lens (Coradin and Muniz, 1992) and then divided into the following two groups: general or sensory characteristics and anatomical characteristics.

General/sensory characteristics: medium density wood; heartwood (light brown-pink) distinct from sapwood (light beige); regular grain; regular grain; medium texture; indistinct smell and taste.

Anatomical characteristics: axial parenchyma practically indistinct, even under the lens; pores seen with the naked eye, numerous, small to medium, predominant solitary, multiples of 2 to 4 (Figure 3A), empty, some clogged; vascular lines long and straight, sometimes interrupted. The rays at the top are thin and numerous, visible only with the aid of a lens; in the tangential side, they are low and irregularly distributed (Figure 3B); on the radial face, they are well contrasted (Figure 3C); growth layers demarcated by dark areas of fibrous tissue. Secretory channels observed in the tangential plane (Figure 3D).



3A:A1.solitary pores; A2.multiple pores; A3.rays (thin and light lines), fibers (wider and darker part). **3B:**B1 solitary pore; B2.rays; B3.axial parenchyma cells. **3C:**C1.secretory channel within the ray; C2.rays; C3.vase; C4.fiber; D1.rays

Figure 3. Microphotographs of transverse (A and B), tangential (C) and radial (D) planes

3.3 Protium tenuifolium - Essential oil composition of trunk bark

The essential oil from the bark of the trunk of *Protium tenuifolium* had a yield of 0.05%. The GC/MS analysis of the volatile components indicated the presence of 33 constituents, of which 24 were identified

(93.93%). The chemical profile (Table 3) revealed a high proportion of monoterpene hydrocarbons (84%). The major constituents were limonene (56.17%), α-phellandrene (16.22%) and ρ-cymene (10.52%). Studies on the essential oils found in the bark of *Protium* species are rare, and this is the first study of the chemical composition of the volatile compounds from *Protium tenuifolium.*

Table 3. Composition of the essential oil from *Protium tenuifolium*

Compounds	IR cal	%	Compounds	IR cal	%
α-pinene	930	0.58	α-zingiberene	1493	0.29
α-phellandrene	1003	**16.22**	α-muurolene	1497	0.26
α-terpinene	1014	0.27	δ-*amorphene*	1511	0.21
p-cymene	1021	**10.52**	7-*epi*-α-selinene	1520	1.21
limonene	1027	**56.17**	ni	1545	0.22
terpinolene	1085	0.24	ni	1573	0.39
linalool	1096	0.66	caryophyllene oxide	1580	0.26
trans-*p*-met-2,8-dien-1-ol	1118	0,23	viridiflorol	1589	0.26
trans-sabinol	1135	0.22	ni	1621	0.22
α-terpineol	1187	1.38	1-*epi*-cubenol	1624	0.87
ni	1192	0.22	ni	1628	0.30
piperitone	1251	1.14	ni	1638	1.89
α-cubebene	1348	0.73	α-muurolol	1642	0.58
α-copaene	1374	0.25	ni	1646	0.18
β-caryophyllene	1417	0.39	ni	1647	0.20
γ-gurjunene	1474	0.31	ni	1650	2.45
γ-muurolene	1478	0.68			
Hydrocarbon monoterpenes 84.0%			Hydrocarbon sesquiterpenes 4.33%		
Oxygenated monoterpenes 3.63%			Oxygenated sesquiterpenes 1.97%		
Not identified (ni) 6.07%			Total identified 93.93%		

4. Conclusion

This study is important since it increases the chemical knowledge of the woody parts of Amazonian species of *Protium*, principally as there are no previous reports of volatile and non-volatile chemical constituents in the vegetative parts of the two species evaluated. The presence of alkylresorcinol in the Burseraceae family is also an unprecedented finding.

5. Acknowledgements

The authors are grateful for the support from the Fundação de Amparo à Pesquisa Estado do Amazonas (FAPEAM), Grant No 062.00178/2019.

6. References

Adams, R.P. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*; Allured Publishing Corporation: Carol Stream, IL, USA, 2007.

Almeida, P. D., Boleti, A. P., Rüdiger, A. L., Lourenço, G. A., Veiga Junior, V. F. and Lima, E. S. (2015). Anti-inflammatory activity of triterpenes isolated from *Protium paniculatum* oil-resins. *Complementary and Alternative Medicine* **15**, 1-10. https://doi.org/10.1155/2015/293768

Angiosperm Phylogeny Group (APG). Available at http://www.mobot.org. Accessed March, 2021.

Carvalho, L. E., Pinto, D. S., Magalhães, L. A. M., Lima, M. P., Marques, M. O. M. and Facanali, R. (2010). Chemical constituents of essential oil of *Protium decandrum* (Burseraceae) from Western Amazon. *Journal of Essential Oil Bearing Plants* **13**, 181-184. https://doi.org/10.1080/0972060X.2010.10643809

CLSI (Clinical and Laboratory Standards Institute). *Reference method for broth dilution antifungal susceptibility testing of yeast*. Approved standard, M27-A3, 3rd ed. Wayne, PA, USA, 2008.

Coradin, V. T. R. and Muniz, G. I. B. *Normas de procedimentos em estudos de anatomia da madeira - I. Angiospermae II. Gimnospermae*. IBAMA: Brasilia, DF, 1991.

Costa, T. O. G., Almeida, R. A., Koolen, H. H. F., Silva, F. M. A. and Pinto, A. C. (2012). Constituintes químicos do caule de *Protium hebetatum* (Burseraceae). *Acta Amazonica* **42**, 557-540. https://doi.org/10.1590/S0044-59672012000400014

Daly, D. C. (1992). New taxa and combinations in *Protium* Burm. f. studies in neotropical Burseraceae VI. *Brittonia* **44**, 280-299. *ISSN*: 0007-196X

Daly, D.C. (1989). Studies in Neotropical Burseraceae II. Generic limits in Neotropical Protieae and Canarieae. *Brittonia* **41**, 17-27. *ISSN*: 0007-196X

Dang, P. H., Nguyen, L. T. T, Nguyen, H. T. T., Le, T. H., Do, T. N. V. and Nguyen, H. X. (2019). A new dimeric alkylresorcinol from the stem barks of *Swintonia floribunda* (Anacardiaceae). *Natural Product Research* **33**, 2883-2889. https://doi.org/10.1080/14786419.2018.1509329

Flora do Brasil. Available at http://servicos.jbrj.gov.br. Accessed March, 2021.

Guimarães, A. C. and Siani, A. C. (2007). Triterpenes from the leaves of *Protium strumosum*. *Revista Fitos* **3**, 67-76. ISSN: 1808-9569

Lima, T. A. A. C., Rocha, K. R. A., Melo, M. F. F., Marques, M. O. M, Facanali, R. and Lima, M. P. (2014). Aspectos morfológicos e químicos de *Protium spruceanum*: uma contribuição ao conhecimento de espécies aromáticas do Bosque da Ciência do INPA. *Scientia Amazonia* **3**, 6-10. ISSN 2238.1910

Lima, M. P., Lima, T. A. A. C., Ribeiro, J. E. L. S., Marques, M. O. M. and Facanali, R. (2016). Estimulo para produção de resina em *Protium hebetatum* Daly e avaliação dos constituintes químicos voláteis. *Scientia Amazonia* **5**, 21-24. ISSN 2238.1910

Oliveira, L. M., Queiroz, D. P. K., Melo, L. E..S., Marques, M. O. M., Facanali, R. and Lima, M. P. (2018). Constituintes voláteis dos galhos de quatro espécies de *Protium* ocorrentes na flora da Reserva Ducke. *Scientia Amazonia* **7**, 68-73. ISSN 2238.1910

Marco, J. A., Sanz-Cervera, J., Morante, M. D., Garcia-Lliso, V., Vallès-Xirau, J. and Jakupovic, J. (1996). Tricyclic sesquiterpenes from *Artemisia chamaemelifolia. Phytochemistry* **41**, 837-844. https://doi.org/10.1016/0031-9422(95)00702-4

McLafferty, F. W. and Stauffer, D.B. *Wiley/NBS Registry of Mass Spectral Data*. Wiley-Interscience: New York, USA, 1989.

Pinto, D. S., Carvalho, L. E., Lima, M. P., Marques, M. O. M., FacanalI, R. and Ribeiro, J. E. L. S. (2013). Volatiles of foliar rachis, branches and resin elicited by insects from *Protium hebetatum* grows wild in Amazon. *Journal Essential of the Oil Bearing Plants* **13**, 699-703. https://doi.org/10.1080/0972060X.2010.10643881

Siani, A. C., ZoghbI, M. G. B., Wolter, E. L. A. and Vencato, I. (1998). 5-Methoxyjusticidin A, a new arylnaphthalene lignan from *Protium unifoliolatum. Journal of Natural Product* **61**, 796-797. https://doi.org/10.1021/np9702291

Singh, S.B., Zink, D. L., Bills, G. F., Pelaez, F., Teran, A., Collado, J., Silverman, K. C., Lingham, R. B., Felock, P. and Hazuda, D. J. (2002). Discovery, structure and HIV-1integrase inhibitory activities of integracins, novel dimeric alkyl aromatics from *Cytonaema sp*. *Tetrahedron Letters* **43**, 1617-1620. https://doi.org/10.1016/S0040-4039(02)00083-7

Siqueira, J. B. G., Zoghbi, M. G. B., Cabral, J. A. and Wolter Filho, W. (1995). Lignans from *Protium tenuifolium. Journal of Natural* 5**8**, 730-732. https://doi.org/10.1021/np50119a011

Susunaga, G. S., Siani, A. C., Pizzolatti, M. G., Yunes, R. A. and Monache, F. D. (2001). Triterpenes from the resin of *Protium heptaphyllum. Fitoterapia* **72**, 709-711. https://doi.org/10.1016/S0367-326X(01)00289-1

Zoghbi, M. G. B., Siqueira, J. B. G., Wolter, E. L. A. and Pereira Junior, O. L. (1993). Constituintes químicos de *Protium paniculatum* (Burseraceae). *Acta Amazonica* **23**, 187-189. https://doi.org/10.1590/1809-43921993233189

Zoghbi, M. G. B., Andrade, E. H. A., Lima, M. P., Silva, T. M. D. and Daly, D. C. (2005). The essential oils of five species of *Protium* growing in the north of Brazil. *Journal of Essential Oil Bearing Plants* **8**, 312-317. https://doi.org/10.1080/0972060X.2005.10643458