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Phenolic Constituents from *Alchornea castaneifolia*

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Abstract: *Alchornea castaneifolia* is a tree growing commonly in several South American countries. It is best known under its Peruvian name Iporuru. The leaves are being used as a folk remedy against numerous symptoms associated with pain and inflammation. It has a reputation for being a tonic and booster of male libido. In this study, using a combination of chromatographic techniques, we isolated phenolic constituents present in leaves, and elucidated their structures using MS and NMR techniques. The isolated and characterized compounds were myricetin glucoside, myricetin galactoside, proanthocyanidin A1 and A2, epicatechin, gallic acid, shikimic acid, putranjivain A, elaeocarpusin and never before isolated methyl ester of repandusinic acid A.

Keywords: *Alchornea castaneifolia*; phenolics; ellagitannins; spectroscopic techniques; methyl ester of repandusinic acid A.

1. Introduction

The *Euphorbiaceae* is a large family including 300 genera and about 5000 species, out of which, the genus *Alchornea* comprises about 60 species [1]. *Alchornea castaneifolia* A. Juss. belongs to the Euphorbiaceae family and is best known as "iporuru" or "sarã". It is a medium size tree, commonly growing alongside riverbanks, both in sandy and clay soils. Some of *Alchornea* species have been investigated phytochemically and the presence of several classes of secondary metabolites, such as triterpenoids from *A. latifolia* and *A. sidifolia*, phenolics from *A. cordifolia*, *A. castaneifolia*, *A. laxiflora* and *A. triplinerva*, alkaloids from *A. floribunda* were reported [1-6]. Isolation of quercetin-3-galactopyranoside, quercetin-3-arabinopyranoside, myricetin-3-arabinopyranoside, quercetin,

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amentoflavone, gallic acid, and gallic acid methyl ester from *A. castaneifolia* has been described [5]. In the Brazilian "savannah" two species of *Alchornea* are used by the local population: *A. glandulosa* (Poepp. & Endl.) and *A. castaneifolia* (A. Juss.) [7].

Leaves and bark of this plant are commonly used in the treatment of rheumatism, arthritis, ulcer, gastritis and muscular pains [5,6]. The methanol extract obtained from stem bark of this species exhibits moderate anti-inflammatory activity *in vitro* and *in vivo* [8]. Hiruma-Lima *et al*. found that the hydroethanolic extract of the leaves and bark obtained from *A. castaneifolia* shows antiulcerogenic activity. Similar effects of ethyl acetate fraction from *A. triplinerva* on gastric ulcer in rats was observed [9]. Also anti-inflammatory activity of an ethyl acetate fraction obtained from the methanol extract of the leaves of *A. glandulosa* was reported [10]. Despite the popular use of "iporuru" in folk medicine, there are limited data available about its chemistry and pharmacology. In this paper we describe isolation of ten polyphenols present in the leaves of *A. castaneifolia,* including two known ellagitannis and one new - methyl ester of repandusinic acid A. The structures of the isolated compounds were elucidated by a combination of spectroscopic methods (HRESIMS, ^{1}H and ^{13}C-NMR) and comparisons with the literature data.

2. Materials and Methods

2.1. General Experimental Procedures

^{1}H, ^{13}C and 2D NMR spectra were obtained on a Bruker Avance III 600 NMR spectrometer, operating at 600.13 MHz and 150.92 MHz, respectively, using standard pulse programs. Spectra were recorded in methanol-d4 at 295 K and referenced to solvent signals at 3.31 (δ_H) and 49.15 ppm (δ_C).

The HRMS measurements were conducted on an Apex-Qe Ultra 7T instrument (Bruker Daltonics, Bremen, Germany) equipped with a dual ESI source and a heated hollow cathode dispenser. The instrument was calibrated with the Tunemix TM mixture (Bruker Daltonics). Analysis of the obtained mass spectra was carried out using a Data Analysis (Bruker Daltonics) software. The instrumental parameters were as follows: scan range, 100–1600 *m/z*; dry gas, nitrogen; temperature, $200^{O}C$; potential between the spray needle and the orifice, 4.2 kV. Compounds dissolved in methanol containing 10^{-2} mole of NH_4HCO_3 were analyzed in negative ion mode as $[M-H]^-$ and $[M-2H]^{2-}$ ions.

2.2. Plant Material

Leaves of *Alchornea castaneifolia* A. Juss. were purchased from Universal Herbs, Inc©, Union City, CA, USA. A voucher specimen (PA-2010/VIII-128) of the leaves of *Alchornea castaneifolia* has been deposited at Planta Analytica LLC.

2.3. Extraction and Isolation

Alchornea castaneifolia extract (202 g) was obtained by extracting 3.6 kg of the plant material with 16 L of methanol. The extract was purified by open column chromatography on a silica gel (Purosil; 230-400 mesh). The column size was 8 x 88 cm and 1.3 kg of silica gel was taken for chromatography. Before sample application the column was conditioned with ethyl acetate : methanol : water (10 : 1 : 1 v/v/v). Afterwards the sample was loaded at the top of the column and the solvent system was changed gradually from (10 : 1 : 1 v/v/v) to (5: 4 : 1 v/v/v) during chromatographic mode. The last two fractions were eluted with methanol : water (5 : 5 v/v). Approximately one liter fractions were collected and analyzed by TLC and HPLC.

After this step of purification 20 fractions were collected (I to XX). Solvent evaporation yielded the corresponding fractions: I - 2.5 g; II - 14.1 g; III - 8.0 g; IV - 17.0 g; V - 17.3 g; VI - 7.6 g;

VII - 10.2 g; VIII - 6.1 g; IX - 12.8 g; X - 5.0 g; XI - 3.7 g; XII - 5.0 g; XIII - 4.4 g; XIV - 3.1 g; XV - 6.3 g; XVI - 2.7 g; XVII - 6.4 g; XVIII - 6.2 g; XIX - 6.6 g; XX - 4.0 g.

Fraction III (8.0 g) was further purified by countercurrent chromatography using Kromaton FCPC with a 1 L rotor (Kromaton Technologies, France) in the following solvent system: hexane : ethyl acetate : methanol : water (4 : 12 : 3 : 5 v/v/v). The rotor was filled with the lower phase acting as a stationary phase. Eight grams of the sample was dissolved in a mixture of both phases, each 50 mL, and pumped into the rotor using Waters HPLC pump model 590. The rotational speed was set at 800 rpm, and the upper phase of the solvent system was pumped at 8 mL/min. Eluent was collected into 25 mL test tubes and analyzed by TLC or HPLC and combined according to similar profiles. Subfraction III-H (300 mg) that produced one main spot by TLC and two main peaks by HPLC was finally purified by preparative HPLC. Preparative HPLC was performed on a Waters system consisting of a controller and pump model 600 and UV detector model 600 at 230 nm. A chromatographic column YMC ODS 150 x 30 mm, 120 nm, 5 μm in isocratic mode (15% AcCN/water) was used. As a result of preparative HPLC compound **4** (83 mg) has been obtained. Similarly subfraction III-G (450 mg) was rechromatographed by preparative HPLC using the same conditions. As a result the following compounds with high purity were obtained: **6** (12 mg) and **5** (132 mg) as a most abundant compound in this fraction. Changing slightly solvent system in preparative HPLC to 5% of AcCN in water (isocratic mode) 42 mg of compound **10** was obtained from subfraction III-E (290 mg).

Fraction VIII (6.1 g) was purified by FCPC in the following solvent system; hexane : ethyl acetate : methanol : water (1:12:2:5 v/v/v/v) in ascending mode giving three main subfractions VIII-A (520 mg), VIII-B (510 mg) and VIII-C (1.6 g). Following preparative HPLC with 32% MeOH as a mobile phase (isocratic mode) from subfraction VIII-A after five runs compounds **7** and **8** were obtained as a mixture (110 mg). Subfraction VIII-C (1.6 g) has been partitioned between n-butanol and water (1:1 v/v). The butanol layer after evaporation gave 0.5 g of VIII-C1, whereas the water layer 1.1 g of VIII-C2. Sample VIII-C2 was further purified by preparative HPLC (gradient mode starting from 1% MeOH to 34% MeOH) giving respectively compound **9** (127 mg) and **1** (11 mg).

Fraction X (5.0 g) affording after several preparative HPLC runs with 10% MeCN as a solvent system (isocratic mode) a pure compound **2** (15 mg).

FCPC purification of fraction XII (5.0 g) with solvent system: ethyl acetate : butanol : water (3:2:5 v/v/v) was performed in ascending mode. Final purification of subfractions having similar HPLC profile XII-B (126 mg) and XII-C (120 mg) was achieved by preparative HPLC (gradient mode starting from 20% MeOH to 30% MeOH) giving compound **3** (9 mg).

2.4. TLC and analytical HPLC analysis

TLC was performed on silica gel 60 F_{254} plates (Merck), developed at room temperature with a solvent system consisting of ethyl acetate : methanol : water (10 : 2 : 1 v/v/v) and compounds were visualized with universal spray reagent ($Cr^{+3}/Mo^{+6}/H_2SO_4$) after heating. HPLC chromatographic separation was achieved using a Hewlett Packard HP Series 1050 system equipped with an autosampler system and HP 1050 photodiode array detector. The column YMC PackPro C18 (3 μm, 150 mm x 4.6 mm i.d.) was used during all analysis and optimized gradient of MeCN in 0.1%TFA was taken as a solvent system. The system was run with the following gradient program: 0.0 - 15.0 min, 10% MeCN to 35% ; 15.0 - 15.3 min, 35% MeCN to 90%; 15.3 - 20.3 min 90% MeCN (isocratic); 20.3-25 min, 90% MeCN to 10%. Peaks were monitored at 210, 230 and 275 nm, at a flow rate of 0.8 mL/min.. HP Chemstation was used to control the operation of the system and performed data analysis.

3. Results and Discussion

The ^{13}C NMR spectrum of (**1**) displays 40 distinct signals, of which 7 belong to carbonyl ester carbon atoms, 24 to aromatic/double bonded carbons, 8 to oxygen-bearing aliphatic carbons and one to aliphatic CH group. The ^{1}H NMR spectrum is in comparison much less crowded, showing only 14 distinct multiplets. The analysis of ^{1}H and ^{13}C NMR (Table 1) signals suggest that the studied compound possesses four highly oxygenated aromatic rings, found in ellagitannin-like substances. Indeed, six of the oxygen-bearing carbons are assignable to a glucose moiety, esterified at four oxygen atoms, of which one belongs to galloyl moiety and one to hexahydrodiphenyl (HHDP) moiety (substituted at atoms 3 and 6). The remaining moiety is substituted at the O4 atom. The detailed analysis of HMBC correlations (Figure 1) shows that (**1**) has very similar chemical shifts to repandusinic acid A[11], with only one additional ^{1}H singlet at 3.69 ppm (corresponding to three protons) and one ^{13}C signal at 53.84 ppm, both belonging to oxygenated methyl group. The proton singlet correlates with carbonyl carbon signal at 171.50 ppm. Therefore (**1**) was concluded to be 1-O-galloyl-3,6-(R)-HHDP-4-O-(1'-methoxy-4'-dehydrochebuloyl)-β-D-glucose.

High resolution mass spectrum measured for compound (**1**) in negative ion mode revealed two peaks: $[M-H]^-$ at *m/z* 983.1024 indicating the molecular formula of (**1**) as $C_{42}H_{31}O_{28}$ (calc. 983.1007) and $[M-2H]^{2-}$ at *m/z* 491.0471 (calc. 491.0467). The mass accuracy was within 3 ppm. Both formulas generated for -1 and -2 ions corresponds to $C_{42}H_{32}O_{28}$ and are consistent with the molecular formula of the methyl ester of repandusinic acid A.

To make sure that (**1**) is not an artifact resulting from extraction with MeOH, a small amount of the leaves was extracted with aqueous acetone and positively compared against the solution of (**1**) by HPLC analysis.

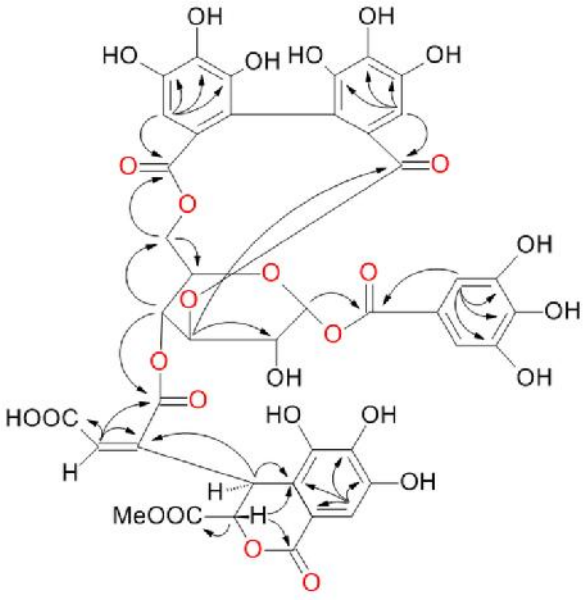

Figure 1. Selected HMBC correlations for methyl ester of repandusinic acid A (**1**).

Table 1. NMR Spectroscopic Data (600 MHz, MeOD, T=295K) for methyl ester of repandusinic acid A (**1**).

Position	**δ_C, type**	**δ_H (*J* in Hz)**	**HMBC**
Glucose			
G1	95.72, CH	6.18, d (2.75)	G5, A7
G2	70.12, CH	4.05, bs	G3
G3	71.73, CH	4.82, bs	G2, G4, B7
G4	65.80, CH	5.59, d (2.80)	G3, G6, 7’
G5	74.47, CH	4.49, t (8.29)	G6, G1
G6	65.44, CH_2	4.23, dd (10.99; 7.94)	
		4.85 (overlapped)	G5, C7
Galloyl (ring A)			
A1	120.99, C	-	A2/6, G1
A2	111.29, CH	7.06, s	A1, A3/5, A6, A7
A3	146.92, C	-	A2/6
A4	141.23, C	-	A2/6
A5	146.92, C	-	A2/6
A6	111.29, CH	7.06, s	A1, A3/5, A7
A7	167.06, C	-	A2/6, G1
HHDP (rings B/C)			
B1/C1	116.84/117.69, C	-	B3/C3
B2/C2	125.55/125.83, C	-	
B3	110.60, CH	6.713, s	B1/C1, B4/C4, B5/C5, B7
C3	109.26, CH	6.711, s	B1/C1, B4/C4, B5/C5, C7
B4/C4	146.11/146.51, C	-	B3/C3
B5/C5	138.18/138.75, C	-	B3/C3
B6/C6	145.67/145.76, C	-	
B7	168.65, C	-	B3
C7	170.50, C	-	C3
4-Acyl (rings D/E)			
D1	117.41, C	-	D3, 2’, 3’
D2	117.73, C	-	D3
D3	109.81, CH	7.07, s	D1, D2, D4, D5
D4	147.13, C	-	D3
D5	140.93, C	-	D3
D6	144.58, C	-	1’
1’	171.50, C	-	2’, Me-1’
Me-C1’	53.84, CH_3	3.69, s	1’
2’	80.15, CH	5.46, d (2.89)	1’, 3’, 4’, D1, COO
3’	36.62, CH	5.40, bd (2.62)	2’, 4’, 5’, D1, D2
4’	140.53, C	-	2’, 3’, 5’
5’	134.35, CH	7.13, s	3’, 4’, 6’, 7’
6’	166.44, C	-	5’
7’	166.42, C	-	5’, G4
COO (ring E)	167.38, C	-	2’

Along with (**1**), nine known compounds have been isolated and their structures were determined on the basis of MS and NMR spectroscopies. The isolated compounds are ellagitannins: putranjivain A (**2**) and elaeocarpusin (**3**) [12,13], proanthocyanidins A_1 (**4**) and A_2 (**5**) [14], epicatechin (**6**), flavonoids; myricetin 3-O-glucoside (**7**) and myricetin-3-O-galactoside (**8**) [15,16], shikimic acid (**9**) [17] and gallic acid (**10**) [18]. Earlier NMR data for compound (**3**), published in 1995 [13], were incomplete. The detailed analysis with 2D NMR allowed us to assign all of the resonances and the assignments are gathered in Table 2.

Table 2. NMR Spectroscopic Data (600 MHz, MeOD, T=295K) for elaeocarpusin (**3**).

Position	**δ_C, type**	**δ_H (*J* in Hz)**	**HMBC**
Glucose			
G1	93.21, CH	6.45 d (3.68)	G5, A7
G2	73.96, CH	5.34 d (3.44)	G1, G3, G4
G3	64.00, CH	5.90 d (3.44)	G2, G4, G5, B7
G4	69.43, CH	4.83 d (3.92)	G2, G3
G5	75.22, CH	4.62	G1, G4, G6
G6	64.90, CH_2	4.20 dd (11.97; 5.78)	G5, C7
		4.80 dd (12.03; 8.11)	G5, C7
Galloyl (ring A)			
A1	120.21, C	-	
A2	110.71, CH	7.10 s	A1, A3/5, A4, A6, A7
A3	146.76, C	-	
A4	140.87, C	-	
A5	146.76, C	-	
A6	110.71, CH	7.10 s	A1, A2, A3/5, A4, A7
A7	166.32, C	-	
HHDP (rings B/C)			
B1/C1	117.83/117.72, C	-	
B2/C2	124.95/122.67, C	-	
B3	109.62, CH	6.907 s	B1/C1, B2/C2, B4/C4, B5/C5, B7
C3	110.52, CH	6.902 s	B1/C1, B2/C2, B4/C4, B5/C5, C7
B4/C4	145.67/145.92, C	-	
B5/C5	138.87/138.46 , C	-	
B6/C6	145.44/145.52, C	-	
B7	167.83, C	-	
C7	169.39, C	-	
2,4-Acyl (rings D/E)			
D1	116.59, C	-	
D2	118.63, C	-	
D3	114.62, CH	7.23 s	D1, D2, D4, D5, D7
D4	148.31, C	-	
D5	137.06, C	-	
D6	148.94, C	-	
D7	166.30, C	-	
E1	52.75, CH	5.67 s	E2, E3, F2, E6, D1, D6
E2	50.36, CH	-	
E3	38.32, CH_2	2.16	E2, F2
E4	199.25, C	-	
E5	97.06, C	-	
E6	108.26, C	-	
E7	168.93, C	-	
F1	172.26, C	-	
F2	81.38, C	-	
F3	109.46, C	-	
F4	75.10, CH	4.56 t (5.29)	F1, F3, H1, H2
H1	90.64, CH	4.60 s	
H2	76.85, CH_2	4.07	H1, F3, F4

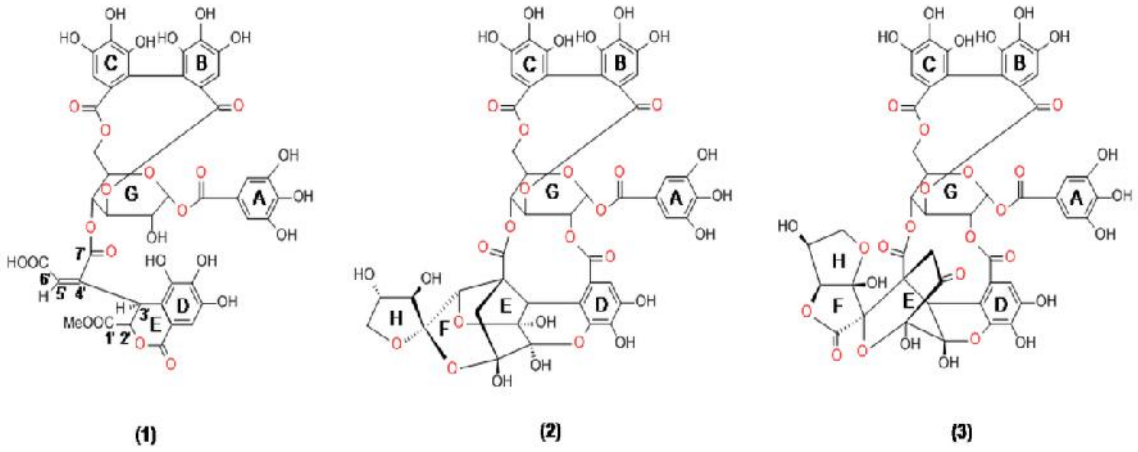

Figure 2. Structures of isolated ellagitannins: methyl ester of repandusinic acid A (**1**), putranjivain A (**2**), elaeocarpusin (**3**)

The high resolution spectra of compounds (**3**) and (**2**) measured in negative ion mode indicated presence of two signals: at *m/z* 1109.0935 (calculated for $C_{47}H_{33}O_{32}$ 1109.0960) and 1083.1181 (calculated for $C_{46}H_{35}O_{31}$ 1083.1168) corresponding to $[M\text{-}H]^-$ ions of elaeocarpusin and putranjivain respectively.

Myricetin 3-O-glucoside (**7**) and 3-O-galactoside (**8**) were isolated as a mixture. The ^1H NMR spectrum of (**7**) and (**8**) displays two set of resonances for sugar moieties, of different intensity with the ratio of ca. 1:4.9, thus making it identifiable and assignable. The ^1H and ^{13}C NMR chemical shifts of both compounds are in agreement with the literature data [15,16].

It is noteworthy that the presence of tannins seems to be the characteristic of Alchornea and may have chemotaxonomic significance. Gallic acid, methyl gallate, ellagic acid, and its derivatives have been found in several Alchornea species, such as *A. laxiflora*, *A. cordifolia* and *A. triplinervia* [1-5]. Thus our findings add ellagitannins as chemotaxonomic markers for the genus Alchornea and may contribute to future phytochemical investigation.

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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