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LC-DAD-UV and LC-ESI-MS-based Analyses, Antioxidant Capacity, and Antimicrobial Activity of a Polar Fraction from *Iryanthera ulei* Leaves

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Abstract: LC-DAD-UV and LC-ESI-MS-based analyses were performed in order to chemically characterize a phenol-enriched fraction obtained from *Iryanthera ulei* leaves-derived ethanol extract. Eight glycosylated flavonoids, two free-flavonoids and two neolignans were detected to be part of its isopropyl acetate-soluble (iPS) fraction. Presence of afzelin **1** was confirmed by isolation. Total Phenolic (TPC) and Total Flavonoid Contents (TFC), Antioxidant Capacity (DPPH, $ABTS^{\bullet+}$, and FRAP methods), as well as antimicrobial activity against five strains were determined.

Keywords: *Myristicaceae; Iryanthera ulei*; LC-UV-DAD; LC-ESI-MS; Flavonoids; Antioxidant; Antimicrobial.

1. Plant Source

Iryanthera ulei Warb. is a tree belongs to the Myristicaceae family, which comprises flowering plants distributed along pantropical regions at the world [1-2]. Whole leaves of *I. ulei* were collected in 2006 on Florencia, Caquetá (Department of Colombia). A voucher specimen was deposited at Herbario Nacional Colombiano under code COL519611.

2. Previous Studies

Flavonols, dihydrochalcones, lignans, diarylpropanes, γ-lactones, and iryantherins have been isolated from *I. ulei* which have been illustrated in four separated studies [3-6].

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3. Present Study

Leaves of *I. ulei* were dried at room temperature, powdered and extracted with 96% ethanol. Mixture was concentrated at reduced pressure, and then was extracted in a Soxhlet apparatus sequentially with solvents of increasing polarity (petroleum ether, chloroform, and isopropyl acetate). Resulting isopropyl acetate-soluble (iPS) fraction was submitted to different procedures in order to get single, pure compounds, including: 1) separation by column chromatography (adsorption chromatography, partition chromatography, and exclusion chromatography); and 2) preparative RP-HPLC separation (For further experimental details see Supporting Information). After performing these procedures, a single compound (**1**) was only favorable to be purified, whose structure was elucidated by spectroscopic methods (1D and 2D NMR and HRMS) to be afzelin in comparison with literature data [7-8].

Separation of iPS fraction employing a HPLC with a UV-DAD detector was accomplished after optimization conditions (data not shown). The optimized separation of this fraction is shown in Figure-1. Chemical nature of major components was determined as flavonoids, based on detailed UV spectra analyses. They were furthermore considered to be glycosylated because of their polarity.

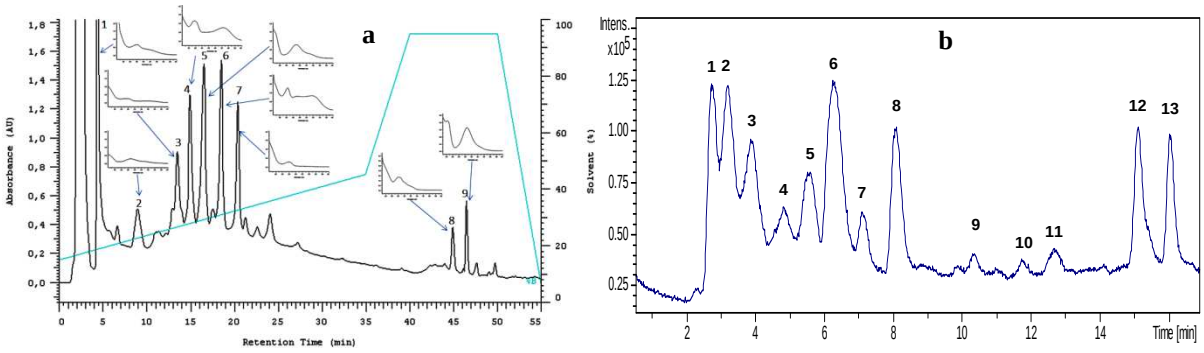

Figure 1. Chromatographic profile for iPS fraction. a) HPLC-UV-DAD system. Phenomenex Ultracarb C30 column (150 x 4.6 mm, 5 µm), gradient elution using acetonitrile-water mixture (blue line). b) HPLC-UV-HRMS system. Phenomenex Luna C18 column (100 x 2.0 mm, 2.5 µm); gradient elution using methanol/0.05% TFA-acidulated water mixture.

Analyzed flavonoids could be classified on different types according to saturation degree and oxidation degree on tricarbon segment (C ring) [9-10]. Position and relative intensities of the two bands on UV spectra of flavonoids provide important information about flavonoid nature and their oxygenation pattern [9,11]. UV spectra extracted from peaks on the HPLC-UV-DAD profile were submitted to comprehensive analysis in order to get information summarized in Table-1.

Due to the unfeasibility of total recognition of the chemical structures for those components into iPS fraction, an analysis by HPLC coupled to HRMS via electrospray ionization (ESI) interface was carried out. Taking into account the results of HPLC-UV-DAD analysis, as well as molecular ion peaks, the main components of the fraction were identified as shown in Table-2. Main components were determined to be dihydroflavonol glycosides, which is in agreement to that of previously predicted by LC-UV-DAD information.

Mass spectra let us to confirm the nature of the compounds because of the presence of ion peaks such as $[M+H]^+$, $[M+Na]^+$, and $[Aglycone(A)+H]^+$. Above information is illustrated by compound at retention time 8.1 min (Table-2) identified as afzelin because of the presence of peak at m/z 455.0952 which is suggested to be the *pseudo*-molecular ion $[M+Na]^+$. Moreover, this structure was confirmed by peak at *m/z* 433.1140 ($[M+H]^+$) and the respective aglycone (kaempferol) at *m/z* 287.0552 $[A+H]^+$ (Figure-2a).

Table 1. Identification of flavonoid types from iPS fraction of *I. ulei* by HPLC-UV-DAD.

Peak	R_t (min)	λ_{max} (nm)		Flavonoid Type
		Band II	Band I	
1	4.40	281	324 (s)	Flavanone or dihydroflavonol
2	9.03	288	331 (s)	Flavanone or dihydroflavonol
3	13.55	281	340 (s)	Flavanone or dihydroflavonol
4	15.00	258	344	Flavone
5	16.55	289	336 (s)	Flavanone or dihydroflavonol
6	18.51	264	339	Flavonol
7	20.40	280	-	-
8	44.96	264	304 (s)	Isoflavone
9	46.55	287	-	Dihydrochalcone

Table 2. Flavonoids identified in iPS fraction of *I. ulei* by LC-HRESIMS.

Peak	R_t (min)	Mass fragments (*m/z*)	Compound
1	2.8	365.1042 $[M+H]^+$ 203.0537 $[A+H]^+$	shikimate ethyl ester 3-*O*-glucoside
2	3.2	475.0977 $[M+Na]^+$ 377.0839 291.0859 $[A+H]^+$	sieboldin
3	3.9	459.0892 $[M+Na]^+$ 305.0652 $[M+H-Rha]^+$	taxifolin 3-*O*-pentoside
4	4.8	473.1047 $[M+Na]^+$ 305.0595 $[M+H-Rha]^+$	taxifolin 3-*O*-rhamnoside (astilbin, neoastilbin, isoastilbin or neoisoastilbin)
5	5.5	475.0992 $[M+Na]^+$ 343.0812 [A+Na]	5,7,3',4',5'-pentahydroxy-dihydroflavonol 3-*O*-pentoside
6a	6.1	457.1096 $[M+Na]^+$ 289.0705 $[A+H]^+$	eriodictyol 7-*O*-rhamnoside
6b	6.4	471.0897 $[M+Na]^+$ 303.0494 $[A+H]^+$	quercitrin
7	7.1	457.1098 $[M+Na]^+$ 289.0712 $[A+H]^+$	dihydrokaempferol 3-*O*-rhamnoside
8	8.1	455.0952 $[M+Na]^+$ 287.0552 $[A+H]^+$	afzelin
9	10.3	471.0670 $[M+Na]^+$ 449.0859 $[M+H]^+$ 275.1615 $[C_{17}H_{23}O_3]$	mururin A
10	11.7	465.1764 $[M+Na]^+$ 443.1916 $[M+H]^+$	4,5,7-Trihydroxy-6,8-dimethylflavan 5,7-di-*O*-glucoside
11	12.6	427.1720 $[M+Na]^+$ 405.1744$[M+H]^+$ 301.1065	glechonin A
12	15.1	367.1510 $[M+Na]^+$ 327.1580 $[M-H_2O+H]^+$	machilin C
13	16.0	325.1046 $[M+Na]^+$ 303.1219 $[M+H]^+$	3',7-dihydroxy-2',4'-dimethoxyisoflavan

Similarly, peak at retention time 3.9 min showed mass spectra with peaks at *m/z* 459.0892 and 305.0652 which can be assigned to $[M+Na]^+$ and $[A+H]^+$, respectively (Figure-2b). Above-mentioned *m/z* signals can be attributable to a pentoside dihydroquercetin (a dihydroflavonol). However, due to the known MS limitations, a reliable pentose attached to flavonol on the glycoside was not achieved. Presence of afzelin was confirmed by isolation and structural elucidation of compound **1** (Spectroscopic data provided in Supporting Information)**.**

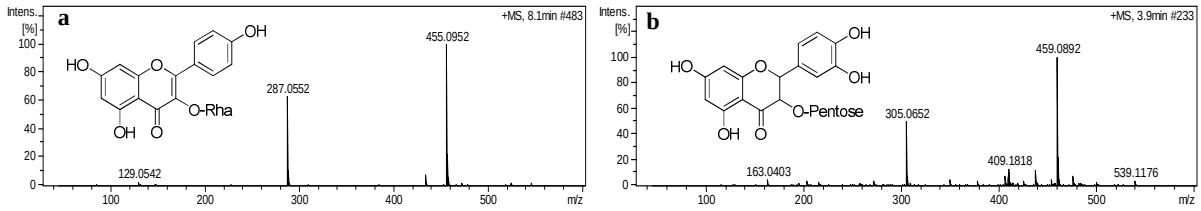

Figure 2. Positive ion mode ESI-MS of two components of iPS fraction: a) Peak at 8.1 min; b) peak at 3.9 min.

Detailed analysis of peak at 6.3 min let us recognize it as a combination of two peaks possessing different UV absorptions, concluding that peak was a mixture of two components with similar chromatographic behavior. Full analysis of mass spectra across the peak (at begin and at the end) was useful to determine the nature of both components as eriodictyol 7-*O*-rhamnoside (peak 6a) and quercitrin (peak 6b), respectively (table-2). (LC-MS data provided in Supporting Information).

Above-mentioned results are consistent with the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) values, which were determined by Folin-Ciocalteau [12] and aluminum chloride [13] colorimetric assays, respectively (detailed protocols provided in Supporting information). TPC value (see table-3) confirmed iPS fraction is a polar, phenol-enriched fraction from *I. ulei*, whose total content of free flavonoids was found to be *ca.* 6.6% to that of phenol-related compounds. From this fact, Antioxidant Capacity was assessed for iPS fraction employing DPPH, $ABTS^{\bullet+}$, and FRAP methods [12-14], whose results are shown in Table-3. On comparing DPPH and $ABTS^{\bullet+}$ results for iPS (exhibiting a dose-dependent behavior, see supporting information), $ABTS^{\bullet+}$ was found to be *ca.* 4.4 times more sensitive to the action of iPS, possibly due to the polar nature of this fraction [15], favoring a proton radical scavenging with comparable result to the positive control ($ABTS^{\bullet+}$ EC_{50}, 8.4 vs 6.1 μg/mL). The reducing power (FRAP) was also found to be consistent, with 80% of BHT potency.

Table 3. Total Phenolic, Total Flavonoid contents, and Antioxidant Capacity of iPS fraction[a]

Sample	TPC[b]	TFC[c]	Antioxidant Capacity		
			DPPH[d]	$ABTS^{\bullet+}$[d]	FRAP[e]
iPS fraction	779.0 ± 43.9	117.3 ± 1.1	36.8 ± 1.8	8.4 ± 0.8	0.671 ± 0.015
BHT[f]	-	-	9.3 ± 0.5	6.1 ± 0.6	0.541 ± 0.008

[a] Results are reported as mean ± standard deviation of three replicates (95% confidence); [b] mg galic acid equivalents/g iPS; [c] mg quercetin equivalents/g sample; [d] IC_{50} (μg/mL); [e] μM Trolox equivalents/mg sample; [f]positive control.

Finally, agar-well diffusion method [16] were used to test the antimicrobial activity for ethanol extract, iPS fraction, and compound **1** against four bacterial strains (*S. aureus, E. coli, P. aeruginosa, S. enterica*) and a fungal strain (*C. albicans*), using chloramphenicol and clotrimazole as positive control for bacteria and yeast, respectively (Table-4). Compound **1** exhibits no activity against tested microorganisms. However, EtOH extract and iPS fraction only exhibit activity against *S. aureus* by *ca.* 65% of the positive control potency. For the other microbes, EtOH extract and iPS fraction were inactive.

Table 4. Antimicrobial activity evaluation for fractions of *I. ulei*

Sample[a]	Inhibition zone (mm)[b]				
	S. aureus	*E. coli*	*P. aeruginosa*	*S. enterica*	*C. albicans*
EtOH extract	17	-	-	-	-
iPS fraction	16	-	-	-	-
Afzelin (**1**)	-	-	-	-	-
Positive control	26	26	16	24	37

[a] tested at 30 mg/mL for extract and fraction and 1.5 mg/mL for pure compound; Positive control: chloramphenicol 0.10 mg/mL (bacteria) and Clotrimazole 0.15 mg/mL (yeast); [b] results from triplicate measurements including hole diameter; - : No activity showed.

The presence of flavonoid glycosides as main components of a polar fraction from leaves of *I. ulei* was established in this study, employing different instrumental methods. Thus, further studies on this polar fraction of *I. ulei* leaves must be considered to be accomplished as an important source of botanical antioxidants

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Supporting Information

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

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