

CHEMICAL CONSTITUENTS OF THE WOOD FROM *Zanthoxylum quinduense* Tul. (Rutaceae)

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Phytochemical investigation of the wood from *Zanthoxylum quinduense* Tul. allowed the isolation and identification of norchelerythrine, decarine, 6-acetyldihydrochelerythrine, syringaresinol, evofilin C, *p*-hydroxybenzaldehyde, vanillic acid, a mixture of  $\beta$ -sitosterol, stigmasterol and campesterol and a mixture of saturated and unsaturated fatty acids, and their esters derivatives. The structures of the isolated compounds were elucidated by spectroscopic techniques and comparison with literature data and the mixture of sterols and fatty acids were identified by GC/MS. The antifungal activity of the ethanolic extract, fractions and pure compounds against *Fusarium oxysporum* f. sp. *lycopersici* was determined by bioautography. Evofilin C and norchelerythrine were the only substances that present antifungal activity.

Keywords: *Zanthoxylum quinduense*; benzophenanthridine alkaloids; antifungal activity.

## INTRODUCTION

The genus *Zanthoxylum* (Rutaceae) comprises some 549 species worldwide distributed.<sup>1</sup> Economically, it has appreciable importance as a source of edible fruits, raw material for cosmetics and perfume industries, and by their culinary applications.<sup>2-4</sup> Many species of *Zanthoxylum* have applications in traditional medicine especially in America, Africa and Asia.<sup>2-7</sup> The leaves, bark and root bark of *Z. ailanthoides* Sieb. & Zucc. have used as folk medicines in Taiwan and China to treat the common cold, stasis, contusions, snakebites and to stimulate blood circulation.<sup>5</sup> *Z. rhoifolium* Lam has been popularly used in South America for inflammatory, microbial, cancerous and malaria processes.<sup>4,6</sup>

Chemical studies carried out on *Zanthoxylum* species have revealed the occurrence of alkaloids, lignans, amides and coumarins, of which some show fungitoxic potencial.<sup>3-9</sup> Extracts of some *Zanthoxylum* species shown significant antifungal activity. The bark methanol extract of *Z. monophyllum* showed significant activity against seven human pathogen fungi.<sup>10</sup> The ethanolic extracts of leaves, fruits, steam bark and root of *Z. americanum* demonstrated a broad spectrum of antifungal and antibacterial activity against *Candida albicans*, *Aspergillus fumigates*, *Cryptococcus neoformans* and *Fusarium oxysporum*.<sup>11</sup>

*Z. quinduense* Tul. [syn. *Fagara quinduense* (Tul.) Engl., *F. macrosperma* (Tul.) Engl., *Z. macrospermum* (Tul.)] is a tree that grows in South America (Colombia, Venezuela, Ecuador and Peru).<sup>12</sup> In previous publication we reported the presence of four benzophenanthridine alkaloids and one triterpene in its bark.<sup>13</sup> The chemical constituents and biological activities of its wood have never been studied. As a continuation of our studies on this species, the present communication report the isolation and identification of the benzophenanthridine alkaloids, lignans, phenylpropens, benzenoids, sterols and fatty acids from wood of *Z. quinduense*; as well as the results of antifungal activity evaluated for the crude ethanolic extract, fractions and of the pure compounds.

## RESULTS AND DISCUSSION

Chromatographic purification of the petroleum ether, dichloromethane and ethyl acetate fractions of the wood of *Z. quinduense* Tul. on silica gel column and preparative thin-layer chromatography (TLC)

afforded known compounds: norchelerythrine (**1**), 6-acetyldihydrochelerythrine (**2**), *p*-hydroxybenzaldehyde (**3**), vanillic acid (**4**), evofilin C (**5**), syringaresinol (**6**), decarine (**7**), mixture of sterols **M1** [ $\beta$ -sitosterol (**8**), stigmasterol (**9**) and campesterol (**10**)] and a mixture of saturated and unsaturated fatty acids, and their derived methyl esters **M2**. The compounds **1-7** were elucidated by spectroscopic techniques and comparison with the data described in the literature, and by comparison with authentic samples (TLC). The mixture of sterols **M1** and fatty acids **M2** were analyzed by GC/MS.

The compounds (**1**) and (**7**) gave positive to Dragendorff's reagent. Their <sup>1</sup>H NMR spectrum present the typical signals for benzophenanthridine alkaloids: two signals in the aromatic region enough displaced to low field due to the proximity of the nitrogen, one singlet characteristic of the proton attached to C-6 [ $\delta_{\text{H}}$  9.74 (1H, s) to (**1**) and  $\delta_{\text{H}}$  9.63 (1H, s) to (**7**)] and another typical of the proton of the position C-4 [ $\delta_{\text{H}}$  8.72 (1H, s) to (**1**) and  $\delta_{\text{H}}$  8.66 (1H, s) to (**7**)]; singlet for a methylenedioxy group located on the positions C-2 and C-3 [ $\delta_{\text{H}}$  6.13 (2H, s) to (**1**) and  $\delta_{\text{H}}$  6.19 (2H, s) to (**7**)]. Also, the aromatic region of the <sup>1</sup>H NMR spectrum exhibited two pairs of one-proton doublets [ $\delta_{\text{H}}$  8.35 (1H, d, *J* = 9.0 Hz, H-11), 8.34 (1H, d, *J* = 9.0 Hz, H-10), 7.84 (1H, d, *J* = 9.0 Hz, H-12) and 7.59 (1H, d, *J* = 9.0 Hz, H-9) to (**1**), and  $\delta_{\text{H}}$  8.49 (1H, d, *J* = 9.0 Hz, H-11), 8.45 (1H, d, *J* = 8.9 Hz, H-10), 7.95 (1H, d, *J* = 9.0 Hz, H-12) and 7.61 (1H, d, *J* = 8.9 Hz, H-9) to (**7**)]. The coupling pattern for the aromatic protons indicated the substituents groups to be at the C-7 and C-8 position, similar to the chelerythrine.<sup>14</sup> The similar <sup>1</sup>H and <sup>13</sup>C NMR data for rings A-D of (**1**) and (**7**) showed them to have the same substitution pattern. The only difference between (**1**) and (**7**) was the presence of an additional methoxyl group for (**1**) instead of hydroxyl group in (**7**). The position of the substituents and all non-hydrogenated carbons of (**1**) and (**7**) were achieved with the aid of the HMBC spectra. By comparison of the <sup>1</sup>H and <sup>13</sup>C NMR data of these compounds with the reported data in the literature, the identity of the compounds (**1**) and (**7**) were established as norchelerythrine and decarine, respectively.<sup>14,15</sup>

The compound (**2**) presents a similar structure to norchelerythrine (**1**) and decarine (**7**). In contrast to (**1**) and (**7**), the <sup>1</sup>H NMR spectrum showed typical aromatic signals for a benzophenanthridine alkaloid with the chelerythrine substitution pattern (except signal characteristic of the proton attached to C-6) confirming that the compound is a dihydrochelerythrine substituted at C-6. The presence of an acetyl group at C-6 was indicated by a singlet for a methyl group ( $\delta_{\text{H}}$  1.97) and the AMX system  $\delta_{\text{H}}$  2.25 (1H, dd, *J* = 15.0, 3.6 Hz), 2.61 (1H, dd,

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$J = 15.0, 11.2$  Hz) and  $5.06$  (1H, dd,  $J = 11.2, 3.6$  Hz, H-6) due to H-6 and the acetyl methyl protons in its  $^1\text{H}$  NMR spectrum and signals at  $\delta_{\text{C}}$  207.7 (C=O), 31.0 (COCH<sub>3</sub>) and 46.8 (-CH<sub>2</sub>CO) in the  $^{13}\text{C}$  NMR spectrum. On the basis of this evidence, their optical rotation value,  $[\alpha]_{\text{D}}^{25} -135^\circ$  ( $c$  0.13, CHCl<sub>3</sub>), and literature data the alkaloid was identified as (-)-6-acetyldihydrochelerithrine (**2**).<sup>16</sup>

The  $^1\text{H}$  NMR spectra of (**5**) show the characteristic pattern of 1,4-disubstituted benzenes with two first order doublets at  $\delta_{\text{H}}$  7.31 (2H, d,  $J = 8.7$  Hz, H-2, H-6) and 6.86 (2H, d,  $J = 8.7$  Hz, H-3, H-5). The chemical shifts of the aromatic protons indicate an oxy-substituent in the *para*-position to an alkyl group. The substituent can be identified as 3',3'-dimethylallyloxy group by the singlets for two methyl groups [ $\delta_{\text{H}}$  1.74 (3H, s, H-4') and 1.80 (3H, s, H-5')] and an olefinic group [ $\delta_{\text{H}}$  5.49 (1H, m, H-2'')], and the doublet for a methylene group [ $\delta_{\text{H}}$  4.50 (2H, d,  $J = 6.8$  Hz, H-1')] in its  $^1\text{H}$  NMR spectrum and signals at  $\delta_{\text{C}}$  18.2 (CH<sub>3</sub>), 25.8 (CH<sub>3</sub>), 64.8 (OCH<sub>2</sub>), 119.6 (CH) and 138.3 (C) in the  $^{13}\text{C}$  NMR. The second substituent is a 3-hydroxy-1-propenyl moiety, with the typical pattern of an ABC system with resonances at  $\delta_{\text{H}}$  6.55 (1H, br d,  $J = 15.9$  Hz, H-7), 6.23 (1H, dt,  $J = 15.9, 6.0$  Hz, H-8), and 4.29 (2H, dd,  $J = 6.0, 1.0$  Hz, H-9), for -CH=CH-CH<sub>2</sub>OH. The large olefinic coupling constant is characteristic of a *trans* configured double bond. Compound (**5**) therefore, was identified as the already known evofilin-C.<sup>17</sup>

In the same way, the substances (**3**) and (**4**) were identified as derived of the benzoic acid, *p*-hydroxybenzaldehyde and vanillic acid, respectively. These compounds have been previously isolated in *Z. zanthoxyloides*<sup>18</sup> and *Z. schinifolium*.<sup>19</sup>

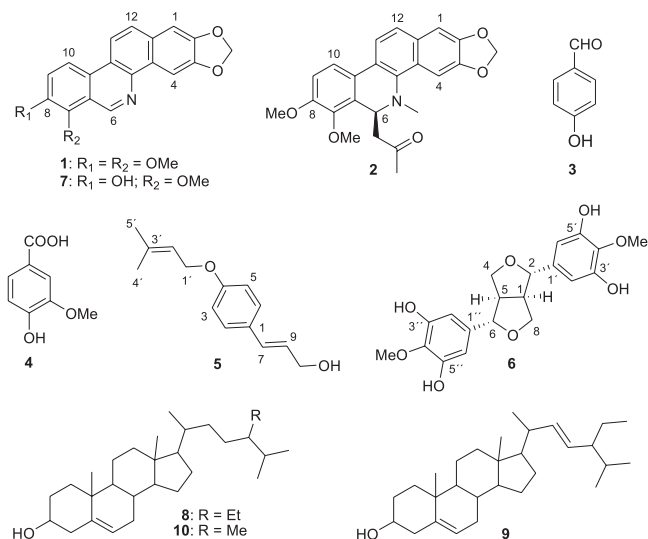
(+)-Syringaresinol (**6**) was isolated as colorless oil from dichloromethane fraction. The  $^1\text{H}$  NMR spectrum of (**6**) present the typical signals for bicyclooctane lignans: signals for the 3,3-*O*-bicyclooctane moiety with eight aliphatic protons [ $\delta_{\text{H}}$  3.09 (2H, m, H-1, H-5), 3.91 (2H, m, H-4<sub>ax</sub>, H-8<sub>ax</sub>), 4.27 (2H, dd,  $J = 9.0, 6.4$  Hz, H-4<sub>eq</sub>, H-8<sub>eq</sub>) and 4.72 (2H, d,  $J = 4.5$  Hz, H-2, H-6)]. The coupling constants of H-2 and H-6 were 4.2 Hz and showed the relative configuration of H-1 and H-2, H-5 and H-6 to be *trans*-form.<sup>20</sup> Also, the  $^1\text{H}$  NMR spectrum exhibited a signals for tetrasubstituted benzene moieties each one with two methoxy groups [ $\delta_{\text{H}}$  3.88 (6H, s)] and one hydroxyl group at  $\delta_{\text{H}}$  5.57. The simplicity of the  $^1\text{H}$  NMR spectrum revealed (**6**) to be a symmetrical lignan. With the dextrorotatory optical rotation,  $[\alpha]_{\text{D}}^{25} +41.6^\circ$  ( $c$  0.15, CHCl<sub>3</sub>), the structure of (**6**) was elucidated as (+)-syringaresinol, which was further confirmed by  $^{13}\text{C}$  NMR, DEPT, HMQC and HMBC spectra and by comparison of literature data.<sup>21</sup>

$^1\text{H}$  and  $^{13}\text{C}$  NMR analysis for mixture **M1** and the comparison with literature data showed the presence of  $\beta$ -sitosterol (**8**) and stigmasterol (**9**) in proportion of 10:1. However, the chromatogram of total ions of the analysis for it GC/MS showed three picks with retention times 49.7, 50.4 and 52.1 min and integrated area of 16.44, 4.55 and 79.02%, respectively. The comparison of the mass spectra corresponding to each pick, with the reported in the literature suggested that they correspond to stigmasterol (**9**), campesterol (**10**) and  $\beta$ -sitosterol (**8**), respectively.<sup>22</sup>

Among the metabolites presented in the study, this is the first report on the occurrence of vanillic acid (**3**), *p*-hydroxybenzaldehyde (**4**), evofilin C (**5**), decarine (**7**) and fatty acids in *Z. quinduense*. Other secondary metabolites have been previously reported in its wood and bark.<sup>13,23</sup>

The mixture **M2** was analyzed by GC and GC/MS. A total of 8 different compounds accounting for 77.51% were identified as: methyl palmitate (2.51%), palmitic acid (C16:0; 32.81%), ethyl palmitate (8.74%), margaric acid (C17:0; 8.01%), methyl stearate (2.12%), oleic acid (C18:1,  $\Delta^9$ ; 6.84%), stearic acid (C18:0; 12.40%) and ethyl stearate (4.02%).

All the classes of compounds reported in this work have been found in other species of *Zanthoxylum* (including *Fagara*). The sterols, fatty acids and benzenoids found are common in many plant families. The



occurrence of evofilin C (**5**) and syringaresinol (**6**) has been described in a large number of rutaceous plants including *Zanthoxylum* genus. Benzophenanthridine alkaloids have great chemotaxonomic importance since they are widely distributed throughout the genus but not in the *Rutaceae* family.<sup>8,14</sup>

The antifungal activity was evaluated by means of direct bioautography in a TLC bioassay.<sup>24</sup> The ethanolic extract and its fractions (petroleum ether, dichloromethane, ethyl acetate and acetone) were inactive against *Fusarium oxysporum* f. sp. *lycopersici*. The inhibitory activity against the growth of the fungi was appreciable at 25  $\mu\text{g}$  for evofilin C (**5**) and at 50  $\mu\text{g}$  for norchelerythrine (**1**), while the other compounds were ineffective.

## EXPERIMENTAL

### General procedures

Optical rotations were measured on a Schmidt-Haensch polarimeter.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra as well as 2D spectra (COSY, HMQC, and HMBC) were recorded on a Bruker Avance 400 spectrometer operating at 400 MHz for  $^1\text{H}$  and 100 MHz for  $^{13}\text{C}$  using the solvent peaks as internal references. Silica gel (Merck, 230-400 mesh) was employed in the column chromatography separations, while silica gel 60 PF<sub>254</sub> (Merck) was used for analytical (0.25 mm) and preparative TLC (1.0 mm). The visualization of the compounds was carried out with iodine vapors, UV light or Dragendorff's reagent.

The GC/MS analyses were performed in EI mode on a Hewlett Packard-5890 GC system with a fused capillary column (50 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$ , HP-5MS, Crossbond 5% phenyl-95% dimethylpolysiloxane) directly coupled to on Hewlett Packard 5973 selective mass detector. Conditions of injection were the following: injector temperature 250  $^\circ\text{C}$ ; oven temperature program of 45 (2 min) – 150  $^\circ\text{C}$  (5 min) at a rate of 2  $^\circ\text{C}/\text{min}$ , 150  $^\circ\text{C}$  (2 min) – 280  $^\circ\text{C}$  (5 min) at a rate of 8  $^\circ\text{C}/\text{min}$ ; split 30:1 during 1.50 min, carrier gas He: 1 mL/min, constant flow; sample volume 1  $\mu\text{L}$ . The mass spectrometer was operated at 70 eV. The samples were prepared dissolving 1 mg of each sample in 1 mL of chloroform. The identification of the chemical constituents was based on comparison of their mass spectral pattern with those obtained from the Wiley 138.L and NBS 75K.L libraries.

### Plant material

The wood of *Z. quinduense* Tul. was collected from the town of Albán, Cundinamarca, Colombia, in August 2005. The plant sample

was identified by Adolfo Jara of the Herbario Nacional Colombiano of the Universidad Nacional de Colombia, and a voucher specimen of *Z. quinduense* Tul. (COL-511101) was deposited at the Herbario Nacional Colombiano of the Universidad Nacional de Colombia, Bogotá, Colombia.

### Extraction and isolation

Dried and powdered wood (4550 g) of *Z. quinduense* was extracted by maceration successively with ethanol at room temperature. The resulting solutions were concentrated in vacuum to yield a crude extract (81.4 g) which was submitted to successive extraction by Soxhlet with petroleum ether (EP), dichloromethane (CH<sub>2</sub>Cl<sub>2</sub>), ethyl acetate (EtOAc), and acetone (Me<sub>2</sub>CO) solvents. Concentration of solvents yielded EP fraction (9.5 g), CH<sub>2</sub>Cl<sub>2</sub> fraction (6.5 g), EtOAc fraction (2.0 g) and Me<sub>2</sub>CO fraction (12.2 g).

Approximately, 7 g of the EP fraction was fractionated by column chromatography on silica gel, eluted with a mixture of EP:AcOEt at increasing polarity (9:1 to 5:5), to yield 16 fractions. In the fraction 1 (552.9 mg) denominated **M1**, analyzed by GC and GC/MS, was determined the occurrence of saturated and unsaturated fatty acids and their esters derivatives. Fraction 8 (1.7 g), by successive washing with acetone, yield a white solid (440.9 mg) that correspond to a mixture **M2** of sterols known as  $\beta$ -sitosterol (**8**), stigmasterol (**9**) and campesterol (**10**).

The supernatant from 8 was put together with fraction 9 and submitted to repeat column chromatography on silica gel, using C<sub>6</sub>H<sub>6</sub>:EtOAc (7:3) and C<sub>6</sub>H<sub>5</sub>CH<sub>3</sub>:EtOAc (7:3) as elution systems. Then, two benzophenanthridine alkaloids, norchelerythrine (**1**) (3.3 mg) and 6-acetyl-dihydrochelerythrine (**2**) (4.0 mg), were obtained by preparative TLC on silica gel, eluted with CH<sub>2</sub>Cl<sub>2</sub>:MeOH:HCOOH (95:5:0.5) and C<sub>6</sub>H<sub>5</sub>CH<sub>3</sub>:EtOAc:HCOOH (95:5:0.5), respectively.

The CH<sub>2</sub>Cl<sub>2</sub> and EtOAc fractions (7.5 g) were assembled and fractionated by column chromatography using a mixture of C<sub>6</sub>H<sub>5</sub>CH<sub>3</sub>:EtOAc at increasing polarity (8:2 to 3:7) to yield 14 fractions. Fraction 3 (102.7 mg) afforded norchelerythrine (**4**) (8.0 mg) by column chromatography eluting with mixture of C<sub>6</sub>H<sub>5</sub>CH<sub>3</sub>:EtOAc (8:2). Fraction 5 (357.3 mg) was submitted to column chromatography eluting with mixture of C<sub>6</sub>H<sub>5</sub>CH<sub>3</sub>:EtOAc (9:1), then by preparative TLC, eluting with mixture of CH<sub>2</sub>Cl<sub>2</sub>:MeOH (98:2), were isolated vanillic acid (**3**) (17.6 mg), *p*-hydroxybenzaldehyde (**4**) (24.3 mg) and mixture of evofilin *C* (**5**) and  $\beta$ -sitosterol (**8**) (7.3 mg; 10:7). Fraction 8 (570.4 mg) was submitted to column chromatography, eluting with mixture of CHCl<sub>3</sub>:MeOH (98:2) to yield syringaresinol (**6**) (62.7 mg) and decarine (**7**) (4.0 mg).

### Antifungal assay

The antifungal activity of the crude plant extract, fractions and pure compounds against *Fusarium oxysporum* f. sp. *lycopersici* was determined using the bioautographic technique. The microorganism used in the antifungal assay has been maintained at the Universidad Nacional de Colombia - Bogotá (Faculty of Science, Department of Chemistry, Laboratory of Vegetal Natural Products). Ten microliters of the solutions were prepared, in different concentrations, corresponding to 100, 50, 25, 10, 5, 2 and 1  $\mu$ g for pure compounds, and 300  $\mu$ g for the crude extract or fractions. The samples were applied to TLC plates, only extract and fractions were eluted with CHCl<sub>3</sub>-MeOH (95:5) or *n*-hexane-EtOAc (8:2) followed by complete removal of the solvent at room temperature.

The chromatograms were then sprayed with a spore suspension of fungi in glucose and salt solution and incubated for 72 h in the darkness in a moistened chamber at 25 °C. Exposure of TLC plates to UV light

(254 nm) and iodine vapours significantly enhanced contrast in order to detect inhibition zones, indicating the inhibitory activity against the growth of the fungi. Benomyl was used as positive control (1  $\mu$ g), and the solvents used to dissolved the samples were the negative controls.<sup>24</sup>

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