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NOVEL FUROCARBAZOLE A LKALOIDS AND ANTIBACTERIAL ACTIVITY OF ETHANOL EXTRACT FROM *Zanthoxylum fagara* (L.) Sargent

NUEVOS ALCALOIDES FUROCARBAZÓLICOS Y ACTIVIDAD ANTIBACTERIANA DEL EXTRACTO ETANÓLICO DE *Zanthoxylum fagara* (L.) Sargent

NOVOS ALCALÓIDES FUROCARBAZÓLICOS E ATIVIDADE ANTIBACTERIANA DO EXTRATO ETANÓLICO DE *Zanthoxylum fagara* (L.) Sargent

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ABSTRACT

From ethanol-soluble extract of the bark from *Zanthoxylum fagara* (L.) Sargent. were isolated two novel furocarbazole alkaloids, 4-methoxy-10*H*-furo[3,2-a]carbazole (**1**) and 10*H*-furo[3,2-a]carbazole (**2**), whose structures were elucidated on the basis of IR, MS and NMR (including 1D and 2D) techniques. In addition, the antibacterial effect of the ethanol extract of bark was evaluated against Gram-negative bacteria *Escherichia coli, Salmonella typhi, Shigella boydii, Vibrio cholerae* El Tor, and *Vibrio cholerae* clinical lysate; and Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus epidermidis*, using the Agar-well diffusion method. In above-mentioned assay was found that the ethanol extract of bark exhibited inhibition against strains *B. subtilis* (17mm), *V. cholerae* El Tor (11mm), *V. cholerae* clinical lysate (10mm), and *S. epidermidis* (9mm).

Key words: *Zanthoxylum fagara*, Rutaceae, antibacterial, furocarbazole alkaloids.

RESUMEN

Del extracto etanólico de corteza de *Zanthoxylum fagara* (L.) Sargent se aislaron dos nuevos alcaloides de núcleo furocar-

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bazólicos, 4-metoxi-10*H*-furo[3,2-a]carbazol (**1**) y 10*H*-furo[3,2-a]carbazol (**2**), cuyas estructuras fueron elucidadas utilizando técnicas de IR, EM y RMN (en una y dos dimensiones). Con el extracto etanólico de corteza se realizaron ensayos de actividad biológica antibacteriana con las cepas gram-negativas *Escherichia coli, Salmonella typhi, Shigella boydii, Vibrio cholerae* El Tor, *Vibrio cholerae* caso clínico; y gram-positivas *Bacillus subtilis*, y *Staphylococcus epidermidis*, utilizando el método de difusión en agar. En este ensayo se encontró que el extracto etanólico de corteza presentó inhibición para las cepas de *B. subtilis* (17 mm), *V. cholerae* El Tor (11 mm), *V. cholerae* caso clínico (10 mm) y *S. epidermidis* (9 mm).

Palabras clave: *Zanthoxylum fagara*, Rutaceae, antibacteriana, alcaloides furocarbazólicos.

RESUMO

O extrato etanólico da casca de *Zanthoxylum fagara* (L.) Sargent. foram isolados dois novos alcalóides furocarbazolicos, 4-metoxi-10*H*-furo[3,2-a]carbazol (**1**) e 10*H*-furo [3,2-a]carbazol (**2**), cujas estruturas foram elucidados usando técnicas de IR, EM e RMN (em uma e duas dimensões). Com o extrato etanólico da casca foi testada atividade biológica antibacteriana com Gram-negativas estirpes *Escherichia coli*, *Salmonella typhi*, *Shigella boydii*, *Vibrio cholerae* El Tor, *Vibrio cholerae* caso clínico; e Gram-positivas *Bacillus subtilis e Staphylococcus epidermidis*, utilizando o método de difusão em ágar. Neste ensaio foi encontrado que o extrato etanólico da casca apresentou inibição para as estirpes de *B. subtilis* (17mm), *V. cholerae* El Tor (11mm), *V. cholerae* caso clínico (10mm) e *S. epidermidis* (9mm).

Palabvras-chave: *Zanthoxylum fagara*, Rutaceae, antibacteriana, alcalóides furocarbazólicos.

INTRODUCTION

According to the Herbario Nacional Colombiano (HNC), there are reported 150 genera and 900 species belonging to the family Rutaceae in Colombia (1), many of which have been used not only in traditional medicine as digestive healing, stomathic tonics, diuretics, sedatives, among others (2-5), but also in determining its biological activity for antiplasmodial and cytotoxic properties (5).

Within this family there is the genus *Zanthoxylum* (6), whose use in folk-medicine is also common including treatments for coughs, enteritis, diarrhea, colds, rheumatism and ulcers (7-11). Furthermore, biological activity assays on *Zanthoxylum* have shown to be effective as antimicrobial (7-9), cytotoxic, (12-13), platelet aggregation inhibitor (14-15), and antitumor agent (16).

Wide-range of ethnobotanical and pharmacological applications makes extracts (or isolated compounds), from species belonging to genus *Zanthoxylum*, a raw material for the search for new biologically active chemical entities being closely associated with the wealth of secondary metabolites such as alkaloids, lignans, terpenes, flavonoids, coumarins, among others, becoming genus *Zanthoxylum* a target for phytochemical and biological purposes.

A contribution to the chemistry of genus *Zanthoxylum* is made in the present work through phytochemical exploration

of ethanol extract from bark of *Zanthoxylum fagara* (commonly known as *wild lime*), allowing isolating two novel furocarbazole alkaloids **1** and **2**. Additionally, in order to determine the biological potential of *Z. fagara* (in accordance to that reported for the genus), antibacterial activity was evaluated for bark ethanol extract.

MATERIALS AND METHODS

General

Infrared spectra (IR) were recorded (KBr window) on a Perkin Elmer 1000 series FT-IR Panagon 500. ^{1}H and ^{13}C NMR spectra, DEPT, HMQC, HMBC, and COSY experiments were performed on a Bruker Avance 400 ($CDCl_3$, CD_3OD) using TMS as internal standard. High Resolution Mass Spectra (HRMS) were determined on a Shimadzu LCMS-IT-TOF mass spectrometer system with electrospray ionization (ESI) in a positive ion mode. Column chromatography (CC) was performed using silica gel (Merck, 40-63 mm) and thin layer chromatography (TLC) using silica gel chromatoplates HF_{254} (Merck, 0.3 mm thickness). Solvents were purified before use such as benzene, isopropyl acetate (*i*PrOAc) toluene (Tol) and ethyl acetate (EtOAc).

Plant material

Plant material of *Zanthoxylum fagara* (L.) Sargent was collected in the mountain Los Montes de María, Department of Bolívar, Colombia (coordinates: 9°53'17''N 75°26'05''W; elevation: 453 m), on November 2004. Specimen was determined by botanist Eduino Carbonó de la Hoz and a voucher is kept at the Herbarium of the University of Magdalena UTM-12000 with the accession code: "*Zanthoxylum fagara* (L.) Sarg. Colombia. Bolívar. Montes de María. Marzo del 2007.Y.Montes y O. García No 04 (UTMC)".

Extraction and isolation

Air-dried, ground bark (700 g) of *Z. fagara* was subjected to extraction by percolation with ethanol 96% for a week. Resulting extract (28 g), after removing the solvent, was fractionated by CC on silica gel using mixtures of benzene:EtOAc by gradient elution to collect fifteen fractions (monitored by CCD). Fractions were dried, subsequently weighed and IR-controlled. Fraction 5 (1084.4 mg) was purified by CC on silica gel using mixtures of toluene:*i*PrOAc by gradient elution to afford eleven fractions. Because of chromatographic profile, fraction 5-6 (197.1 mg) were purified by preparative TLC using a mixture of toluene: *i*PrOAc 1:1 as eluent to obtain **1** (70.0 mg). On the other hand, an acid-base alkaloid extraction procedure was performed on 20 g of crude ethanol extract. Resulting chloroform-soluble fraction (4742.8 mg) was submitted to CC on silica gel using a mixture of toluene: *i*PrOAc 7:3 as eluent to collect fourteen fractions. Compound **2** (230.2 mg) was found to be a single component of fraction 9.

Antibacterial assay

Antibacterial activity tests for the ethanol extract of bark was carried out using Gram-negative bacteria *Escherichia coli* (ATCC 25922), *Salmonella typhi* (ATCC 19430), *Shigella boydii* (ATCC 8700), *Vibrio cholerae* El Tor (ATCC 39541), and *Vibrio cholerae* clinical lysate; and

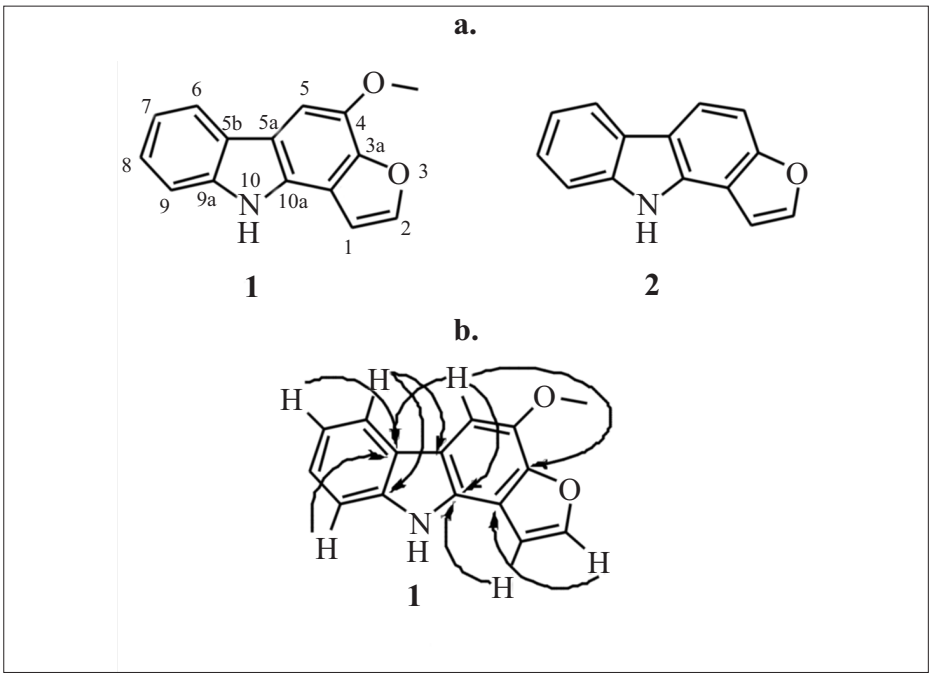

Figure 1. (a) Structures of novel furocarbazole alkaloids isolated from Z. fagara 1-2, (b) Selected HMBC correlations of alkaloid 1.

Gram-positive bacteria *Bacillus subtilis* (ATCC 6633), and *Staphylococcus epidermidis* (+) (ATCC 12228), following Agar-well diffusion method, previously reported by Vanden-Berghe *et al*. (17), using a concentration of 2 mg/mL extract, and 30 mg kanamycin discs (Bigaux E-03) as positive control.

RESULTS AND DISCUSSION

Compound **1** is a yellow oil (positive to Dragendorff test), whose molecular formula is $C_{15}H_{11}NO_2$ assigned by HRESIMS analysis ($[M+H]^+$ *m/z* 238.0852, calcd for $C_{15}H_{12}NO_2$, 238.0868). Its IR spectrum showed a signal at 3433 cm^{-1} due to the N-H tension for a free secondary amine, checked by N-H bending band at 1645 cm^{-1}, a signal at 3071 cm^{-1} due to =C-H stretching for aromatic compounds was confirmed with band at 1581 cm^{-1} of C=C stretching, and at *ca* 1050-1150 cm^{-1} range there were C-O stretching bands of ethers. ^{1}H NMR spectrum (Table 1) exhibited four signals at δ_H 8.65 (*d*, *J* = 8.2 Hz, 1H), 8.04 (*d*, *J* = 7.8 Hz, 1H), 7.67 (*dt*, *J* = 8.0, 1.0 Hz, 1H) and 7.50 (*dt*, *J* = 7.8, 1.0 Hz, 1H), corresponding to an ABCD-type aromatic system (18-20). This system is confirmed on observing the ^{1}H-^{1}H COSY experiment by correlations between hydrogens at δ_H 8.04 and 7.50, and similarly between hydrogens at δ_H 7.67 and 8.65, confirming hydrogens at δ_H 7.50 and 7.67 show an *ortho-meta*-correlated system. Furthermore, there is also a signal at δ_H 7.19 (s, 1H) corresponds to an aromatic

Table 1. ^{1}H and ^{13}C NMR spectroscopic data of compounds 1 and 2

	1					2				
C	δ_C	δ_H **(mult., *J* (Hz), ∫)**	**DEPT**	**HMBC**		δ_C	δ_H **(mult., J (Hz), ∫)**	**DEPT**	**HMBC**	
				^{2}J	**^{3}J**				**^{2}J**	**^{3}J**
1	113.8	7.79 (d, 5.1, 1H)	CH	129.4	127.5[a]	116.3	7.98 (d, 5.1, 1H)	CH	145.6	127.3[a]
2	145.7	8.72 (d, 5.1, 1H)	CH	113.8	129.4-136.7[a]	145.6	8.83 (d, 5.1, 1H)	CH	116.3	130.0
3a	155.3	-	C	-	-	159.5	-	C		
4	154.5	-	C	-	-	119.3	8.07 (d, 9.8, 1H)	CH	159.5	-
5	109.7	7.19 (s, 1H)	CH	127.5	155.3-136.7	129.0	7.00 (d, 9.8, 1H)	CH	127.3	138.2
5a	127.5	-	C	-	-	127.3	-	C	-	-
5b	125.0	-	C	-	-	126.1	-	C	-	-
6	122.5	8.04 (d, 7.8, 1H)	CH	-	139.0-130.5-127.5	117.4	8.67 (d, 8.2, 1H)	CH	125.0	131.1
7	125.8	7.50 (dt, 7.7, 0.9, 1H)	CH	-	125.0-117.5	125.0	7.53 (dt, 7.6, 0.9, 1H)	CH	-	126.0-117.4
8	130.5	7.67 (dt, 7.7, 0.9, 1H)	CH	-	139.0-122.5	131.1	7.71 (dt, 7.8, 1.0, 1H)	CH	122.8	139.0
9	117.5	8.65 (d, 8.2, 1H)	CH	139.0	125.0	122.8	8.11 (d, 7.8, 1H)	CH	139.0	-
9a	139.0	-	C	-	-	139.0	-	C	-	-
10a	136.7	-	C	-	-	138.2	-	C	-	-
10b	129.4	-	C	-	-	130.0	-	C	-	-
OCH_3	57.0	4.04 (s, 3H)	CH_3	-	154.5	-	-	-	-	-

[a] Four-bond heteronuclear correlation.

proton (20-22) deduced by its chemical shift and multiplicity.

^{1}H NMR spectrum displays other signals at δ_H 8.72 (*d*, J = 5.1, 1H) and 7.79 (*d*, J = 5.1, 1H) are consistent with two hydrogens on a furan ring defined by their chemical shift, coupling constants and multiplicity (24). ^{1}H-^{1}H COSY spectrum shows the correlation between these two hydrogens, confirming its neighborhood with no other nearby hydrogen. Finally, ^{1}H NMR spectrum exhibited a signal at δ_H 4.04 (*s*, 3H) corresponding to an aromatic *O*-methyl group. ^{13}C NMR spectrum evidenced the presence of fifteen carbons discriminated to be three carbons attached to oxygen (δ_C 155.3, 154.5, and

145.7) (23-24), and two carbons attached to nitrogen (δ_C 139.0 and 136.8) according to their chemical shifts (18-23), led to establish *N*-linked aromatic rings. ^{13}C NMR spectrum similarly showed a signal at δ_C 56.9 (18-19) concerning to an aromatic *O*-methyl group, and seven carbons shifted between δ_C 130.5 and 109.7 pertaining to sp^2-hybridized methine carbons as confirmed by DEPT experiments. Adequate assignments were established by determination of C-H connectivities using the two-dimensional heteronuclear HMQC experiment in conjunction to the heteronuclear HMBC experiment, which allowed establishing long-range correlations between some hydrogens and carbons (Table 1). *N*-attached quaternary carbons on carbazole ring (δ_C 139.0 and 136.7) were positioned by means of HMBC correlation with hydrogens at δ_H 8.04 and 7.19, respectively. Quaternary carbons at δ_C 125.0 and 127.5 (bearing carbazole ring) were defined by HMBC correlation with hydrogens at δ_H 8.04 and 8.65, respectively, as well as quaternary carbons on furan ring (δ_C 129.4 and 155.3) were assigned by the correlation with hydrogens at δ_H 8.72 and 7.19. Selected HMBC correlations of compound **1** are shown in Figure 1b. Above-mentioned spectroscopic information led to determine the structure of **1** as 4-methoxy-10*H*-furo[3,2-a]carbazole (Figure 1a).

Compound **2** is a yellow-green, oily-appearance liquid (positive Dragendorff test) whose molecular formula was assigned $C_{14}H_9NO$ as deduced by HRESIMS analysis ($[M+H]^+$ *m/z* 207.0677, calcd for $C_{14}H_{10}NO$, 207.0684). ^1H NMR spectrum (Table 1) shows characteristic signals for an aromatic compound, evidencing the presence of a furan ring because of signals at δ_H 8.83 (*d*, J = 5.1 Hz, 1H) and 7.98 (*d*, J = 5.1 Hz, 1H), without correlation with other hydrogen according to the ^1H-^1H COSY spectrum (23). Analysis of the signals at δ_H 8.67 (*d*, J = 8.2 Hz, 1H), 8.11 (d, J = 7.8 Hz, 1H), 7.71 (*dt*, J = 7.8 Hz, 1H), and 7.53 (*dt*, J = 7.6 Hz, 1H) on the ^1H NMR and ^1H-^1H COSY spectra, indicated that an ABCD-type system was also present (18- 20), similarly to that of **1**. Signals at δ_H 7.00 (*d*, J = 9.8 Hz, 1H) and 8.07 (*d*, J = 9.8 Hz, 1H) correspond to two hydrogens attached to an aromatic ring according to its chemical shift, multiplicity, and coupling constant (19-20). Structure of **2** was found to be very similar to that of **1**, except for the absence of methoxy group. From these facts, the structure of **1** was determined as 10*H*-furo[3,2-a]carbazole (Figure 1a).

Although genus *Zanthoxylum* had been usually equated with *Fagara* (a genus taken from Arabic and firstly used and latinized by Linnaeus), and according to Austin and Felger, taxonomic disposition of *Fagara* has varied, and it has been considered a separate genus or used as a synonym or infrageneric taxon (25). However, *Z. fagara* might be excluded from this etymologycal discussion, since the term *fagara* is referred to the sharp spines on the trunk of this plant to establish the name given by Linnaeus (formerly *Schinus fagara*) (25). In addition, although carbazole alkaloids are frequently presented in *Murraya* species (19-23), finding of these two non-common alkaloids from a *Zanthoxylum* specimen could be considered as an interesting indication of a chemotaxonomic variation possibly due to either evolutive processes or ecological

Table 2. Results of antibacterial activity of ethanol extract from *Z. fagara*

Bacterial strains	Inhibition zone[a] (mm) of EtOH extract	Inhibition zone[a] (mm) of kanamicin
Vibrio cholerae El Tor	11	18
Vibrio cholerae clinical lysate	10	-
Staphylococcus epidermidis	9	20
Bacillus subtilis	17	22

[a] concentrations at 2 mg/mL.

conditions, since *Zanthoxylum* alkaloids are mostly related to benzylisoquinoline-type (26).

Ocurrence of compounds **1**-**2** is supported by the corresponding biosynthetic pathway, whose initiator should be anthranilic acid, and after multiple sequentially chemical transformations (such as phosphorylation, protonation, ring-opening, dehydration, intermolecular C-C coupling, decarboxylation, prenylation, elimination, and oxidation) compound **2** is produced (25-26). Subsequent hydro- xylation preferentially occurs at para-position whether it is unsubstituted, or at ortho-position if it is substituted. In the latter case, proposed hydroxylation occurs on adjacent position to oxygenated carbon at furan ring. After this hydroxylation, a methylation by SAMe should be produced to generate **1** (27-28).

Antibacterial effects for ethanol extract of bark from Z. *fagara* were evaluated against strains *B. subtilis*, *S. epidermidis*, *V. cholerae* El Tor and *V. cholerae* clinical lysate (Table 2). Neither strain was inhibited by the extract at higher inhibition zone values to those of positive control. Nevertheless, some trends were clearly observed. Both Gram(+) bacteria were susceptible to bark ethanol extract treatment (being more resistant the *S. epidermidis* strain than *B. subtilis*). Additionally, although most of the Gram(-) bacteria were not susceptible to the same treatment, the two *V. cholerae* strains (El Tor and clinical lysate biotypes) were inhibited by the bark ethanol extract involving a very small difference between them. These results indicate a selective antibacterial action of this extract, but further studies are required to draw unambiguous conclusions. However, these results indicate that bark extract from *Z. fagara* could serve either as a source of antibacterial compounds or potential treatment for some infectious problems.

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