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Chemical Constituents from the Leaves of *Morus nigra* L. (Moraceae) Collected in Casa Nova, Bahia, Brazil

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Constituintes Químicos Isolados das Folhas de *Morus nigra* L. (Moraceae) Coletadas em Casa Nova, Bahia, Brasil

Resumo: *Morus nigra* L. pertence à família Moraceae, é utilizada popularmente para o tratamento de diabetes, dislipidemias, doenças cardiovasculares e gota. O presente trabalho descreve o isolamento e identificação de compostos a partir das folhas de *M. nigra*. A investigação fitoquímica da fase hexânica das folhas resultou na identificação do esteroide β -sitosterol (**1**), e na fase acetato de etila foram identificados dois flavonoides glicosilados, kaempferol-3-*O*-glicosídeo (**2**) e quercetina-3-*O*-glicosídeo (**3**). Os compostos foram identificados através da análise de ressonância magnética nuclear 1D (RMN de ^1H e ^{13}C) e 2D (^1H - ^1H COSY, ^1H - ^{13}C HSQC, ^1H - ^{13}C HMBC), assim como por comparação com dados da literatura. Nossos resultados mostraram que esta espécie é uma fonte promissora de flavonoides e tem alta relação com outras espécies de *Morus*, além de ser uma candidata em potencial para a investigação de suas propriedades farmacológicas.

Palavras-chave: Moraceae; *Morus nigra* L.; flavonoides.

Abstract

Morus nigra L. belongs to the family Moraceae, and is popularly used for the treatment of diabetes, dyslipidemias, cardiovascular diseases and gout. The present work describes the isolation and identification of compounds isolated from the leaves of *M. nigra*. The phytochemical investigation of hexane phase from the leaves of this species allowed the identification of the steroid β -sitosterol (**1**), while the ethyl acetate phase led to the characterization of two glycosylated flavonoids, kaempferol-3-*O*-glycoside (**2**) and quercetin-3-*O*-glycoside (**3**). The compounds were identified by Nuclear Magnetic Resonance analysis 1D (^1H and ^{13}C NMR data) and 2D (^1H - ^1H COSY, ^1H - ^{13}C HSQC, ^1H - ^{13}C HMBC), as well as through the comparison with literature data. Our findings showed that this species is a promising source of flavonoids and have high relationship with other *Morus* species, besides being a potential candidate for the investigation of pharmacological properties.

Keywords: Moraceae; *Morus nigra* L.; flavonoids.

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Chemical Constituents from the Leaves of *Morus nigra* L. (Moraceae) Collected in Casa Nova, Bahia, Brazil

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1. Introduction

The family Moraceae is composed of 70 genera and around 1500 species,¹ distributed mainly in tropical regions, although there are representatives in a temperate climate. In Brazil, species from this family are traditionally cultivated by indigenous, and popularly known as "blackberry", "breadfruit", "jaca" and "fig".² Regarding the species of Moraceae, *Morus* L. has approximately 24 species and a subspecies, with at least 100 known varieties,³ which are widely used in traditional Chinese medicine as

antiphlogistic, hepatoprotective, hypotensive, antipyretic, analgesic, diuretic, expectorant and antidiabetic. In addition, the species of this genus are recognized to exhibit a variety of phenolic compounds, mainly isoprenylated.^{4,5}

Morus nigra L. is a tree popularly known in the San Francisco Valley region as "amora-miúra". This species is commonly employed in the treatment of diabetes, dyslipidemias, cardiovascular diseases, obesity and gout.³ Previous pharmacological investigations on this species described antidiabetic, antioxidant, anti-inflammatory and antihyperlipidemic activities, that are

frequently associated with the presence of phenolic compounds, including flavonoids, anthocyanins and carotenoids.⁶

Phytochemical studies have previously been reported for this species. Chemical analysis of the stem bark resulted in the isolation of two new prenylated flavonoids, mornigrol E and mornigrol F,⁷ while from the leaves were identified betulinic acid, β -sitosterol and germanicol.⁶ Studies performed from the twigs of *Morus nigra* showed the identification of 11 compounds: a novel dihydroflavonol, four new flavanones and six known isoprenylated flavonoids.⁸

In the present investigation, we report the isolation and characterization of three chemical constituents from the hexane and ethyl acetate fractions obtained by partition of the crude ethanolic extract of the leaves of *M. nigra*.

2. Experimental

2.1. General experimental procedures

One-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) experiments were acquired in CDCl_3 or DMSO-d_6 at 293K on a ASCEND III 400 NMR spectrometer (Bruker, Karlsruhe, Germany), operating at 9.4 Tesla, observing the ^1H and ^{13}C nuclei at 400.13 and 100.13 MHz, respectively. The spectrometer was equipped with either a 5-mm multinuclear direct detection probe with a z-gradient. One-bond and long-range ^1H - ^{13}C correlation from HSQC and HMBC NMR experiments were optimized for an average coupling constant $^1J_{(\text{C,H})}$ and $^{\text{LR}}J_{(\text{C,H})}$ of 140 and 8 Hz, respectively. All ^1H and ^{13}C NMR chemical shifts (δ) are given in ppm related to the TMS signal at 0.00 ppm as an internal reference, and the coupling constants (J) in Hz. Silica gel 60 (70-230 mesh) was used for column chromatography (CC) with 0.063-0.200 mm particles (Merck), while silica gel 60 F_{254} was used for thin-layer chromatography (TLC). Fractions were monitored by analytical

thin-layer chromatography (TLC - Aluminum F_{254}), determining the purity of the sample when a single spot was observed after exposure under UV irradiation chamber (254 and 365 nm), eluted with at least three separate solvent systems.

2.2. Plant material

Leaves of *M. nigra* were collected in the city of Casa Nova, State of Bahia, Brazil, in February 2010. The identity of the plant was confirmed by the botanist José Alves de Siqueira Filho and a voucher specimen (#1764) has been deposited in the Herbário Vale do São Francisco (HVASF) at the Federal University of San Francisco Valley (UNIVASF). All procedures for access to genetic patrimony and associated traditional knowledge were carried out and the project was registered in SisGen (Register #AC34CFC).

2.3. Extraction and isolation

Leaves of *M. nigra* were dried in an oven with circulating air at an average temperature of 40 °C for 72 h, obtaining the dried and pulverized plant material (1196 g). The dried and powdered plant material was submitted to maceration with ethanol 95 %. The extractive solution obtained was concentrated in rotatory evaporator at reduced pressure (50 °C) affording the crude ethanolic extract (CEE), which weighed 64 g (yield of 5.5 %). Amounts of CEE (4.0 g) were separated to biological tests and to assess preliminary phytochemical screening. The remainder (60.0 g) was partitioned to isolate the chemical constituents.

CEE was solubilized in a mixture of $\text{MeOH:H}_2\text{O}$ (3:7) and then fractionated with hexane, chloroform and ethyl acetate solvents in ascending order of polarity, yielding three fractions: hexane (FH), chloroform (FC), ethyl acetate (FA).

FH fraction (9.0 g) was initially submitted to column chromatography using silica gel 60 as

stationary phase (CC) eluted with a gradient system, comprising of increasing concentrations of CH₂Cl₂ in *n*-hexane (100:0 to 0:100, v/v), followed by EtOAc in CH₂Cl₂ (100:0 to 0:100, v/v), and MeOH in EtOAc (100:0 to 0:100, v/v), resulting in 141 fractions (10 ml each). The eluted fractions were evaluated and pooled according to TLC analysis, to provide 29 groups (GF1-14 to GF141). GF47 was submitted to preparative thin-layer chromatography (CCDP), and resulted in the isolation of the compound in the form of crystal needle, identified as compound **1** (60.0 mg).

The ethyl acetate fraction (FA) was submitted to a Sephadex® molecular exclusion chromatography column due to the suspected presence of glycosylated flavonoids, and resulting in 86 fractions, that were evaluated and pooled according to TLC analysis, to provide 9 groups (GF1-7 to GF65-86). GF45 resulting from this procedure was washed with chloroform and resulted in the mixture of two compounds **2** and **3** (42.0 mg) as yellow amorphous powder.

***β*-sitosterol (1):** Crystal needle; ¹H-NMR (CDCl₃, 400 MHz) δ: 0.68 (3H, s, H-18), 0.81 (3H, d, J = 6.9 Hz, H-27), 0.83 (3H, d, J = 6.9 Hz, H-26), 0.84 (3H, t, J = 7.7 Hz, H-29), 0.92 (3H, d, J = 6.7 Hz, H-21), 1.00 (3H, s, H-19), 2.26 (2H, m, H-4), 3.52 (1H, m, H-3), 5.35 (1H, d, J = 5.2 Hz, H-6); ¹³C-NMR (CDCl₃, 100 MHz) δ: 37.4 (C-1), 31.9 (C-2), 72.0 (C-3), 42.4 (C-4), 140.9 (C-5), 121.9 (C-6), 31.8 (C-7), 32.0 (C-8), 50.3 (C-9), 36.7 (C-10), 21.2 (C-11), 39.9 (C-12), 42.5 (C-13), 56.9 (C-14), 24.5 (C-15), 28.4 (C-16), 56.2 (C-17), 12.1 (C-18), 19.4 (C-19), 36.3 (C-20), 18.9 (C-21), 34.1 (C-22), 26.2 (C-23), 46.0 (C-24), 29.3 (C-25), 20.1 (C-26), 19.6 (C-27), 23.2 (C-28), 12.2 (C-29); identified by comparison with literature data (¹³C-NMR).⁹

Kaempferol 3-O-glycoside (2): Yellow amorphous powder; ¹H-NMR (DMSO-d₆, 400 MHz) δ: 12.65 (1H, s, OH-5), 6.22 (1H, d, J = 2.0 Hz, H-6), 6.44 (1H, d, J = 2.0 Hz, H-8), 8.04 (2H, d, J = 8.8 Hz, H-2'/H-6'), 6.89 (2H, d, J = 8.8 Hz, H-3'/H-5'), 5.47 (1H, d, J = 7.3 Hz, H-1''), 3.18

(1H, m, H-2''), 3.08 (1H, m, H-3''), 3.08 (1H, m, H-4''), 3.21 (1H, m, H-5''), 3.32 and 3.57 (2H, m, H-6''); ¹³C-NMR (DMSO-d₆, 100 MHz) δ: 156.5 (C-2), 133.3 (C-3), 177.5 (C-4), 161.2 (C-5), 98.7 (C-6), 164.2 (C-7), 93.7 (C-8), 156.2 (C-9), 104.0 (C-10), 120.9 (C-1'), 130.9 (C-2'/C-6'), 115.2 (C-3'/C-5'), 160.0 (C-4'), 100.8 (C-1''), 74.3 (C-2''), 77.5 (C-3''), 69.9 (C-4''), 76.4 (C-5''), 60.9 (C-6''); identified by comparison with literature data (¹H and ¹³C-NMR).¹⁰

Quercetin 3-O-glycoside (3): Yellow amorphous powder; ¹H-NMR (DMSO-d₆, 400 MHz) δ: 12.63 (1H, s, OH-5), 6.20 (1H, d, J = 2.1 Hz, H-6), 6.42 (1H, d, J = 2.1 Hz, H-8), 7.58 (1H, d, J = 2.1 Hz, H-2'), 6.85 (1H, d, J = 9.0 Hz, H-5'), 7.59 (1H, dd, J = 9.0 and 2.1 Hz, H-6'), 5.48 (1H, d, J = 7.7 Hz, H-1''), 3.18 (1H, m, H-2''), 3.07 (1H, m, H-3''), 3.07 (1H, m, H-4''), 3.21 (1H, m, H-5''), 3.32 and 3.57 (2H, m, H-6''); ¹³C-NMR (DMSO-d₆, 100 MHz) δ: 156.4 (C-2), 133.1 (C-3), 177.4 (C-4), 161.2 (C-5), 98.6 (C-6), 164.1 (C-7), 93.5 (C-8), 156.3 (C-9), 103.9 (C-10), 121.2 (C-1'), 116.2 (C-2'), 144.8 (C-3'), 148.5 (C-4'), 115.0 (C-5'), 121.7 (C-6'), 100.8 (C-1''), 74.1 (C-2''), 77.6 (C-3''), 69.8 (C-4''), 76.5 (C-5''), 60.8 (C-6''); identified by comparison with literature data (¹H and ¹³C-NMR).¹¹

3. Results and Discussion

Phytochemical analysis of the leaves of *M. nigra* resulted in the isolation of three compounds, the steroid *β*-sitosterol (**1**) and two glycosylated flavonoids, namely, kaempferol 3-*O*-glycoside (**2**) and quercetin 3-*O*-glycoside (**3**). The chemical structures are presented in Figure 1. All compounds were identified by NMR 1D and 2D (COSY, HSQC and HMBC), as well as by comparison with data reported in the literature.

Compound **1** was isolated in the form of crystal needle soluble in CHCl₃. The ¹H-NMR spectrum of **1** showed the presence of various signals in the region between δ 0.68 to δ 2.27 ppm, as well as absence of signals in the region of aromatic hydrogens (δ 6.00 to δ 9.0

ppm), evidencing its aliphatic nature. The singlets at δ 0.68 and 1.00 confirming the presence of two methyl groups attached to quaternary carbons, while the presence of three doublets at δ 0.81, 0.83 and 0.92 showed the methyl groups attached to methinic carbons. Already the triplet at δ 0.84 revealed the existence of a methyl group attached to methylene carbon. The $^{13}\text{C}\{^1\text{H}\}$ and DEPT-135 NMR spectra, as well as one-bond and long-range ^1H - ^{13}C correlation maps from HSQC and HMBC NMR experiments indicated a total of 29 carbons. These carbons comprised six methyl, eleven methylene, nine methine and three quaternary carbons. The hydrogen at δ 3.52 showed one-bond ^1H - ^{13}C HSQC correlation with the carbon at δ 72.0 (C-3), supporting the presence of a OH group at C-3 in the structure of **1** (Fig. 1). The doublet at δ 5.35 showed one-bond ^1H - ^{13}C HSQC correlation with the carbon at δ 121.9 (C-6) and long-range ^1H - ^{13}C HMBC correlation with the carbons at δ 42.4 (C-4), 31.7 (C-7) and δ 32.0 (C-8), indicated that the presence of one olefinic hydrogen in C-6. In addition, the complex multiplets at δ 2.29 revealed that the two CH_2 adjacent to carbon attached to OH group. This hydrogen showed long-range ^1H - ^{13}C HMBC correlation with the carbons at δ 31.9 (C-2), 72.0 (C-3), 140.9 (C-5), 121.9 (C-6) and δ 36.7 (C-10). The observed chemical shift values in NMR spectra are very close to values reported in the literature for β -sitosterol.⁹ Based on the ^1H and ^{13}C NMR 1D/2D data, and comparison with literature data,⁹ compound **1** was identified as β -sitosterol (Fig. 1).

On the other hand, compounds **2** and **3** were obtained in mixture, once its NMR data indicated the presence of two set of signals with different ratios. The compounds **2** and **3** were obtained as a yellow amorphous powder, soluble in CH_3OH . The analysis by TLC revealed with the reagent of amino-2-ethyl diphenyl borinate (NEU-PEG) yellow coloration, indicating a positive reaction for flavonoids. The first set of signals in the ^1H NMR spectrum showed two doublets in δ 6.22 (1H, d , J = 2.0 Hz) and 6.44 (1H, d , J = 2.0 Hz),

assigned to H-6 and H-8, respectively, of the flavonoid A-ring. The presence of a doublet in δ 6.89 (J = 8.8 Hz) performing an ortho coupling with another in δ 8.04 (J = 8.8 Hz) indicated the presence of an AA'BB' system in B-ring of flavonoid, assigned to the H-3'\H-5' and H-2'\H-6', respectively. Furthermore, the ^1H NMR spectrum showed a doublet at δ 5.47 (J = 7.3 Hz) typical of anomeric hydrogen unit glucose, one-bond ^1H - ^{13}C HSQC correlation with the carbon at δ 100.8 (C-1'') and long-range ^1H - ^{13}C HMBC correlation with the carbons at δ 74.3 (C-2''), 76.4 (C-5'') and δ 133.3 (C-3), supporting the presence of one glycosidic unit in C-3. Based on ^1H and ^{13}C NMR 1D/2D data and comparison with the literature,^{10,11} compound **2** was identified as kaempferol 3-O-glycoside (Fig. 1). This compound has already been identified in *Morus* species, among them *Morus nigra*¹² and *Morus atropurpurea*.¹³

The second NMR dataset in the ^1H NMR showed similar spins systems for compound **2**. However, in this case the ring B were replaced by signals that suggested the presence of the 3',4'-disubstituted type substitution pattern, which was confirmed on the basis of the long-range ^1H - ^{13}C correlation map from HMBC NMR experiment. The presence of two doublets at δ 6.85 (J = 9.0 Hz) and δ 7.58 (J = 2.1 Hz) for H-2' and H-5', respectively, as well as a double doublet at δ 7.59 (J = 9.0 and 2.1 Hz) for the H-6', compatible with the flavonoid B-ring. Furthermore, the ^1H NMR spectrum showed two doublets in δ 6.20 (1H, d , J = 2 Hz) and δ 6.42 (1H, d , J = 2 Hz) coupled in meta, concerning the hydrogens of A-ring, suggesting the existence of a 6,8-disubstituted ring. A glycosidic unit in the compound **3** was confirmed by the presence of doublet at δ 5.48 (J = 7.7 Hz) overlapping in the signal in C-1'' for compound **2**. Considering the ^1H and ^{13}C NMR 1D/2D data and comparison with the literature¹¹, Compound **3** was identified as quercetin 3-O-glycoside (Fig. 1). This compound has already been identified in some species of *Morus*, among them *Morus nigra*¹⁴, *Morus alba*¹⁵ and *Morus australis*¹⁶.

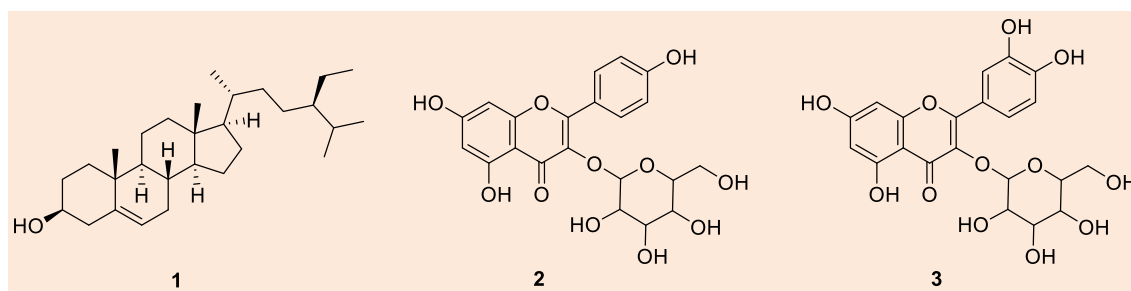


Figure 1. Chemical constituents isolated from the leaves of *M. nigra*

4. Conclusion

The phytochemical investigation of *Morus nigra* extracts led to the isolation and identification of three compounds, one steroid (β -sitosterol) and two glycosylated flavonoids (kaempferol 3-*O*-glycoside and quercetin 3-*O*-glycoside). These compounds described in this work had already been reported in this species, but are being described for the first time in the species collected in city of Casa Nova, at the region of the São Francisco Valley.

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