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Phytochemical Characterization, Antifungal Activity and Docking Investigation of The Isolated Compounds from *Lippia callicarpifolia*

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Abstract: Bioassay-guided fractionation of extracts of aerial parts from *Lippia callicarpifolia* via chromatographic techniques led to the isolation of two *D*:*C*-friedours-7-ene compounds, namely *D:C*-3-oxo-friedours-7-en-28-oic acid (**1**) and a new callicarpifolic acid (**2**). Furthermore, additional known compounds, *β*-sitosterol glucoside (**3**) and genkwanin (**4**) were also identified. The structures of the natural products were established by 1D and 2D NMR, IR, and spectrometric analyses. In the antifungal assays, *Fusarium sporotrichioides* NRLL was sensitive to compounds **1** and **4** in the concentration range of 0.25–1 mg/mL. The docking investigation revealed high inhibition of the trichodiene synthase enzyme by compound **4**.

Keywords: *Lippia callicarpifolia*; *Fusarium sporotrichioides*; genkwanin; callicarpifolic acid

1. Plant Source

Lippia callicarpifolia Kunth was collected during the flowering stage (December 2023) in San José de los Amates, Tonatico, Estado de México. A voucher specimen (IZTA 3505) was deposited at the herbarium of Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlalnepantla, Estado de México, where Ma. Edith López-Villafranco identified the plant material.

2. Previous Studies

The genus *Lippia* (Verbenaceae) encompasses plant species used globally in traditional culinary practices and medicinal applications [1]. In Mexico, several species of the genus *Lippia* are integrated into traditional Mexican medicinal practices, including *L. dulcis*, *L. myriocephala*, *L. queretarensis*, *L. alba*, *L. stoechadifolia*, and *L. graveolens* [2]. Chemical investigations of *Lippia* species have frequently focused on essential oils. Furthermore, their extracts were also examined. The predominant

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nonvolatile metabolites identified within the genus include triterpenes, flavonoids, naphthoquinones, and iridoids [1, 3]. *L. callicarpifolia* is a perennial plant used in Mexico for diverse medicinal purposes [4-5]. Previous investigations have been conducted on the pharmacological properties of essential oils derived from this plant, including chemical analysis of the essential oils extracted from its aerial parts. Therefore, inspired by previous investigations and the established *in vitro* antifungal properties of *Lippia* species [1, 3, 6], this study aimed to explore the assay-guided extraction of antifungal compounds from *L. callicarpifolia.*

3. Present Study

In this investigation, we conducted bioassay-guided isolation of extracts of the aerial parts of *L. callicarpifolia* to evaluate their effects against human fungal pathogens and fungi of environmental importance (Supplementary material). The *n*-hexane and methanol extracts from the leaves/stems and flowers of *L. callicarpifolia* did not exhibit antifungal activity during screening. The EtOAc extracts of leaves/stems and flowers inhibited the growth of *Fusarium sporotrichioides*, with percentages of inhibition of 58% ± 3.8 and 87% ± 0.0 at a concentration of 4 mg/mL, respectively. Consequently, the findings from this initial screening process served as a foundation for investigating the antifungal potential of the compounds of the EtOAc extracts against this microorganism. After fractionating the EtOAc extract from the leaves/stems, fractions F-4, F-6, and F-39 exhibited antifungal activity. F-4 promoted antifungal bioactivity attributed to triterpene **1** at a concentration of 0.25 mg and had inhibitory effect of 37.8% ± 3.8, whereas concentrations of 0.5 and 1 mg/mL had inhibitory effects of 40% ± 0.0 and 48.9% ± 3.8, respectively, on the growth. Notably, F-6 inhibited mycelial growth, whereas triterpene **2**, isolated from this fraction, did not exhibit antifungal effect. Furthermore, the inactive *β*-sitosterol glucoside (**3**) was successfully identified in F-39 [7]. The fractionation of the EtOAc extract from the flowers by column chromatography facilitated the identification of antifungal activity, which was predominantly concentrated in the F-17 fraction. Consequently, the F-17 partition was selected for the subsequent isolation of the bioactive compounds. Compound **4**, identified as genkwanin [8], was isolated from the active mixture and evaluated for antifungal activity, with inhibition percentages of 31% ± 3.8, 48% ± 0.0, and 62% ± 3.8 against *F. sporotrichioides* at concentrations of 0.25, 0.5, and 1 mg/mL, respectively. Notably, the EtOAc extracts did not exhibit antifungal activity against *Candida* species or other tested molds. The framework detailing the fractionation strategy, including the yields of the compounds, is provided in the supplementary material.

D:C-3-oxo-friedours-7-en-28-oic acid ***(1)***: White solid; IR C-H (2971, 2957 cm^{-1}) C=O (1706 cm^{-1}) carboxylic acid C=O (1681 cm^{-1}); EIMS *m/z* 439 $[M\text{-}CH_3]^+$; ^1H NMR (300 MHz, $CDCl_3$) 1.98 (m, H-1a), 1.46 (m, H-1b), 2.76 (ddd, *J*= 14.6, 14.6, 5.7 Hz, H-2a), 2.26 (m, H-2b), 1.72 (t, *J*= 8.9 Hz, H-5), 2.33 (m, H-6a), 2.12 (m, H-6b), 5.47 (m, H-7), 2.33 (m, H-9), 1.64 (m, H-11 and H-12), 1.58 (m, H-15), 1.93 (m, H-16a), 1.48 (m, H-16b), 2.37 (brs, H-18), 1.15 (m, H-19), 1.12 (m, H-20), 1.13 (m, H-21), 2.27 (m, H-22a), 1.68 (m, H-22b), 1.05 (s, CH_3-23), 1.11 (s, CH_3-24), 1.01 (s, CH_3-25), 1.11 (s, CH_3-26), 1.04 (s, CH_3-27), 1.06 (d, *J*= 6.9 Hz, CH_3-29), 0.87 (d, *J*= 3.6 Hz, CH_3-30). ^{13}C NMR (75.4 MHz, $CDCl_3$) 38.2 (C-1), 34.9 (C-2), 216.9 (C-3), 47.8 (C-4), 52.1 (C-5), 24.5 (C-6), 116.9 (C-7), 144.7 (C-8), 47.9 (C-9), 35.3 (C-10), 16.5 (C-11), 32.5 (C-12), 37.1 (C-13), 41.1 (C-14), 28.3 (C-15), 32.5 (C-16), 44.7 (C-17), 47.5 (C-18), 36.7 (C-19), 32.3 (C-20), 28.9 (C-21), 26.0 (C-22), 24.4 (C-23), 21.5 (C-24), 12.6 (C-25), 23.6 (C-26), 21.9 (C-27), 186.3 (C-28), 23.4 (C-29), 21.3 (C-30).

Callicarpifolic acid (2): White solid; IR OH (3407, 3227 cm^{-1}), C-H (2946, 2925 cm^{-1}), C=O (1691 cm^{-1}), and C-O (1042 cm^{-1}). HRESIMS *m/z* 495.3443 $[M + Na]^+$ [calcd as 495.3450 for: $C_{30}H_{48}O_4Na^+$]; ^1H NMR (300 MHz, $CDCl_3$) 1.84 (dd, *J*= 10.4, 2.3 Hz, H-1a), 1.19 (m, H-1b), 3.87 (m, H-2), 2.99 (d, *J*= 2.1 Hz, H-3), 1.32 (m, H-5), 2.08 (m, H-6a), 1.98 (m, H-6b), 5.41 (m, H-7), 2.17 (m, H-9), 1.53 (m, H-11), 1.55 (m, H-12a), 1.26 (m, H-12b), 1.28 (m, H-15), 1.74 (td, *J*= 9.8, 4.0 Hz, H-16a), 1.51 (m, H-16b), 2.31 (brs, H-18), 1.07 (m, H-19), 1.07 (m, H-20), 1.52 (m, H-21a), 1.46 (m, H-21b), 2.19 (m, H-22a), 1.60 (m, H-22b), 0.86 (s, CH_3-23), 0.98 (s, CH_3-24), 0.92 (s, CH_3-25), 1.01 (s,

CH_3-26), 0.98 (s, CH_3-27), 1.00 (d, *J*= 6.0 Hz, CH_3-29), 0.82 (d, *J*= 2.7 Hz, CH_3-30). ^{13}C NMR (75.4 MHz, $CDCl_3$) 42.5 (C-1), 70.1 (C-2), 77.3 (C-3), 37.8 (C-4), 50.0 (C-5), 23.8 (C-6), 116.8 (C-7), 144.0 (C-8), 48.2 (C-9), 34.2 (C-10), 15.9 (C-11), 32.0 (C-12), 36.6 (C-13), 40.6 (C-14), 28.9 (C-15), 32.2 (C-16), 43.8 (C-17), 47.6 (C-18), 36.2 (C-19), 31.6 (C-20), 27.8 (C-21), 25.6 (C-2), 29.2 (C-23), 16.7 (C-24), 14.1 (C-25), 23.3 (C-26), 21.4 (C-27), 181.6 (C-28), 23.2 (C-29), 21.2 (C-30).

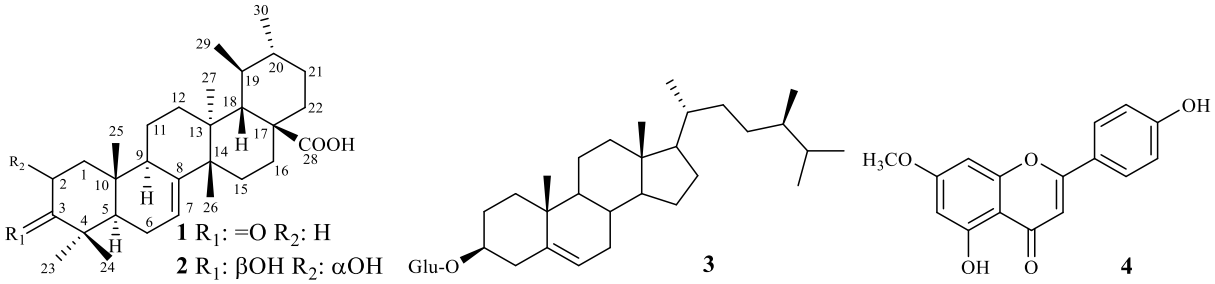

Figure 1. Chemical structures of natural compounds isolated from *L. callicarpifolia*

Compound **1** was isolated as a white solid. Analysis of the ^{1}H NMR spectrum (300 MHz, $CDCl_3$) revealed the presence of vinylic protons at δ_H 5.47 (1H, m, H-7) attributed to the trisubstituted double bond. One-proton resonance at δ_H 2.76 indicated that H-2 is a ddd, *J*= 14.6, 14.6, 5.7 Hz, characteristic of an axial proton adjacent to a carbonyl moiety. Five tertiary methyl protons were observed at δ_H 1.11 (3H, s, CH_3-24), 1.11 (3H, s, CH_3-26), 1.05 (3H, s, CH_3-23), 1.04 (3H, s, CH_3-27), and δ_H 1.01 (3H, s, CH_3-25), and two protons belong to secondary methyl groups at δ_H 1.06 (3H, d, *J*= 6.9 Hz CH_3-29) and δ_H 0.87 (3H, d, *J*= 3.9 Hz, CH_3-30), suggesting an ursan triterpene framework. Triterpene **1** was previously found in *Davidsonia pruriens* [9]; however, an inspection of the reported NMR data revealed that its ^{13}C NMR assignments have not been described. Analysis of the DEPT and 2D NMR HSQC and HMBC spectra revealed full ^{13}C NMR chemical shift assignments of **1** (Table S1, Supporting Information). The ^{13}C NMR spectrum (75.4 MHz, $CDCl_3$) exhibited 29 carbon signals, of which ketone C-3 appeared at δ_C 216.9, carboxylic acid C-28 at δ_C 186.3, vinylic carbons C-8 at δ_C 144.7, and C-7 at δ_C 116.9. In addition, the experiment exhibited five quaternary, seven tertiary, nine secondary, and five primary carbons at δ_C 52.1 to 12.6, which was corroborated by the DEPT experiment. The HMBC correlations (Figure 2) established the chemical shift assignments. For example, in the HMBC spectrum, the signals of CH_3-23 and CH_3-24 were correlated with carbonyl ketone C-3, whereas H-5, CH_3-23, and CH_3-24 were correlated with C-4. The signals of CH_3-23, CH_3-24, and CH_3-25 were correlated with C-5, whereas the signal of CH_3-26 was correlated with the vinyl carbon C-8. In addition, CH_3-24 and CH_3-26 exhibited the same chemical shift at δ_H = 1.11. The HMBC correlations allowed us to distinguish the methyl protons by correlating CH_3-23 with C-24. The signals corresponding to the C, D, and E rings can be assigned by correlating H-18 with the quaternary C-17 carbon. The methine protons H-29 and H-30 were correlated with C-19 and C-20, respectively. The full ^{13}C signals were assigned by comparison with the reported data of an analogous molecular structure (Table S1, Supporting Information) [9-11]. The EIMS spectral fragmentation pattern of **1** revealed the presence of a molecular ion $[M\text{-}CH_3]^+$ at *m/z* 439, indicating the loss of an allylic methyl group, and characteristic product ions at *m/z* 235 and 245, which were similar to those reported for myrtifonic acid and bauerenone indicated that this compound has a bauer skeleton. The ^{1}H NMR and IR data (Supporting Information) agreed with the data reported for *D:C*-3-oxo-friedours-7-en-28-oic acid (3-oxo-bauer-7-en-28-oic acid, **2**) [9-10].

Compound **2** was isolated as a white solid. The FTIR spectrum exhibited absorption bands of OH (3407 cm^{-1}), carboxylic acid C=O (1691 cm^{-1}), and C-O (1042 cm^{-1}). The HRESI-MS spectrum exhibited an $[M + Na]^+$ ion at *m/z* 495.3443 (calcd as 495.3450 for $C_{30}H_{48}O_4Na^+$). The ^{1}H NMR data (300 MHz, DMSO-d_6) showed the characteristic signals for *D*:*C*-friedours-7-ene compounds with the presence of two oxymethine protons at δ_H 3.87 (1H, m, H-2), and δ_H 2.99 (1H, d, *J*= 2.4 Hz, H-3). The ^{13}C NMR analysis (75.4 MHz, DMSO-d_6) exhibited that the structure was composed of 30 carbons, including the presence of carboxylic acid C-28 at δ_C 181.6, vinylic carbons C-8 and C-7 at δ_C 144.0

and 116.8, respectively, and two oxymethine carbons resonate at δ_C 77.3 (C-3) and δ_C 70.1 (C-2). Herein, the NMR data were also recorded at 700 MHz (Supporting Information). These analyses were useful for establishing the molecular connectivity and stereochemistry of the molecule.

In the COSY spectrum of **2**, the oxymethine proton H-2 was correlated with H-3 and H-1, whereas H-3 was correlated with H-2, confirming its vicinity. The vinylic proton H-7 showed a correlation with H-6, whereas H-6 exhibited a cross peak with H-5 (Figure 2). The analysis of the 2D NMR spectra COSY and HSQC allowed full ^{13}C NMR chemical shift assignments.

The assignment of the signals of rings A and B was confirmed by HMBC correlations (Figure 2). The hydroxyl positions were assigned to C-3 due to the correlation with CH_3-23, CH_3-24, and H-1; the second hydroxyl group was positioned at C-2 since H-1 was correlated with C-2. The vinylic positions were attached to nonprotonated C-8 connected to bonded vinylic C-7 due to the correlation observed between CH_3-26 and C-8, which was confirmed by the correlation between H-6 and C-7. In addition, the HMBC experiment confirmed the positional assignment of the oxymethine carbon C-5 because H-1, CH_3-23, CH_3-24, and CH_3-25 were correlated with C-5, whereas H-1 and CH_3-25 exhibited correlations with C-9. The signals of the ^{13}C corresponding to rings C, D, and E can be assigned by the correlation between the methine proton H-18 and the methylene protons H-16 and H-22 with C-28, confirming the presence of the carboxylic acid at C-28. Furthermore, C-13 and C-14 were differentiated on the basis of their HMBC correlations. Although both the methyl protons CH_3-26 and CH_3-27 showed correlations with the quaternary carbons, the distinction was possible because the signal of the methine proton H-18 correlated with C-13. Given that each carbon signal exhibited the expected pattern of correlations in HMBC for *D*:*C*-friedours-7-ene compounds [14], the assignments of methylene C-11, C-12, C-15, and C-22, methine C-19 and C-20, and quaternary and C-10 were unambiguously assigned (Figure 2).

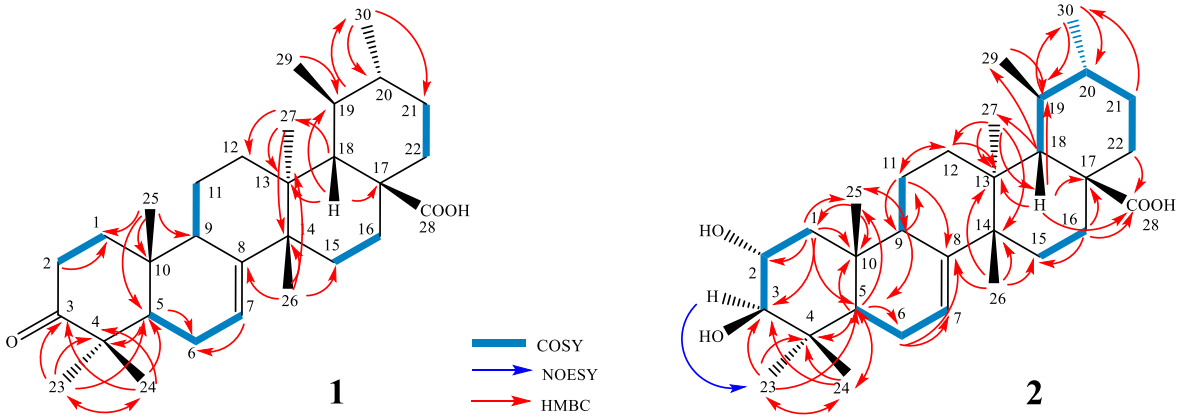

Figure 2. Key COSY, NOESY, and HMBC correlations of natural compounds **1** and **2**

The stereochemistry of compound **2** was elucidated by vicinal coupling constant data H-3-H-2 (J= 2.1 Hz), complemented by the NOESY correlations between H-3 and CH_3-23, indicating that the hydroxyl groups were at the 2α and 3β positions. Furthermore, the relative configuration of the H-18 proton was found to be 18β configuration due to its correlation with the double signal assigned to CH_3-29. The structural configuration was determined to be callicarpifolic acid (**2**) (*D:C*-2α,3β-dihydroxy-friedours-7-en-28-oic-acid or 2α,3β-dihydroxy-bauer-7-en-28-oic-acid) on the basis of the results obtained from 1D and 2D correlations and the comparison of the NMR data with those of analogous structures [7, 9-11], Supporting Information. To our knowledge, this compound has not been characterized previously. This triterpenoid skeleton is derived from ursanyl cation through a series of rearrangements, resulting in the formation of the *D*:*C*-friedours-7-ene compound [11].

The NMR data for compound **3** were consistent with those of β-sitosterol glucoside [7]. Compound **4** was isolated as a yellow solid. The spectroscopic and spectrometric data were in accordance with those of genkwanin (7-OMe-epigenin, **4**) [8]. Pharmacological investigations have demonstrated that **4** has a various effects such as antiinflammatory, anticancer, antiplasmodial, and

antibacterial properties [8]. In the present study, compounds **1** and **4** showed antifungal activity against *F. sporotrichioides*, a phytopathogen that synthesizes a variety of mycotoxins, including trichothecenes type A mycotoxins, such as T2 and HT-2, and presented significant risks to agricultural products and animal health [12-13]. Genkwanin had the highest inhibitory effect on the growth of *F. sporotrichioides*; therefore, virtual screening of compound **4** with the protein structure 1JFA associated with the biosynthetic pathway of these mycotoxins in *F. sporotrichioides* was performed [12]. The virtual screening results revealed that genkwanin (**4**) had a high affinity energy of −7.3 kcal/mol. Figure 3 shows the residues that interact with **4**. Two hydrogen bonds are formed with LYS-198 and PHE-114. The hydrophobic interactions are summarized in Table S2 (Supplementary Material).

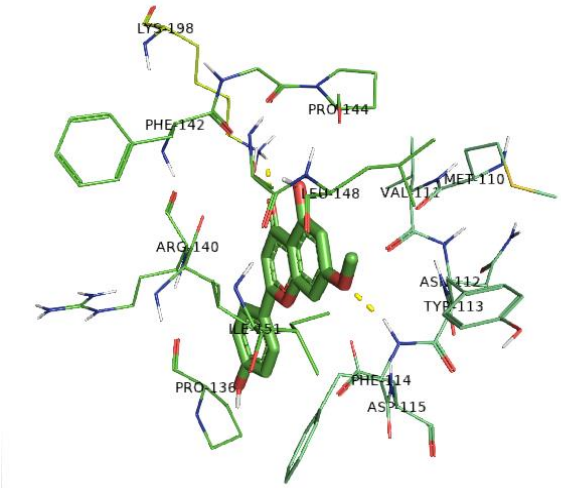

Figure 3. Bonding interactions of **4** with the residues of trichodiene synthase

This enzymatic route is vital for the generation of hazardous compounds such as T-2 and HT-2 toxins, which are categorized as type A trichothecenes. Understanding the genetic and biochemical pathways involved in mycotoxin production, as well as the morphological responses to environmental stressors, is crucial for developing effective control measures against this pathogen [13]. In summary, the function of 1JFA in *F. sporotrichioides* is integral to the biosynthesis of trichothecenes, which are key factors in the pathogenicity of this fungus. In conclusion, a new compound, callicarpifolic acid (**2**), along with known compounds **1**, **3** and **4** were extracted from the aerial parts of *L. callicarpifolia*. Among these compounds, **1** and **4** inhibited the growth of *F. sporotrichioides* NRLL. Furthermore, the docking investigation revealed high inhibition of the trichodiene synthase enzyme by compound **4**.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

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