SUPPLEMENTARY MATERIAL

***In vitro* evaluation of cytotoxic activity of the ethanol extract and isolated compounds from the corms of *Liatris spicata* (L.) willd on HepG2**

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Abstract:

Investigation of the ethanol extract of the corms of *Liatris spicata* (L.) willd led to the isolation of two sterols: stigmasterol and its 3-*O*-glucoside, a triterpene: obtusifoliyl acetate, two benzofurans: euparin and 6-hydroxy-3-methoxytremetone, three phenolic acids: protocatechuic, vanillic and ferulic acid and a sesquiterpene lactone igalan. The structures of the isolated compounds were established on the basis of physicochemical properties and spectral analysis (IR, EI/MS, ¹H-NMR and ¹³C-NMR). The ethanol extract and its isolated compounds evidenced cytotoxic activities against human liver cancer cell line (HepG2), where igalan showed the highest potency (3.83 ± 0.043) $\mu\text{g/ml}$, its effect was comparable to that of the standard drug doxorubicin[®] (3.73 ± 0.036) $\mu\text{g/ml}$.

Keywords:

Liatris spicata, Cytotoxic activities, Igalan.

1. Experimental

1.1. General

Silica gel H (Merck, Darmstadt, Germany) for vacuum liquid chromatography (VLC), silica gel 60 (70 - 230 mesh ASTM; Fluka, Steinheim, Germany), Diaion HP-20 AG for column chromatography (75-150 μm , Mitsubishi Chemical Industries Co. Ltd) and Sephadex LH-20 (Pharmacia, Stockholm, Sweden) were used for column chromatography (CC). Thin-layer chromatography (TLC) was performed on silica gel GF₂₅₄ precoated plates (Fluka) using the following solvent systems: S₁, *n*-hexane/ethyl acetate (9.5:0.5, v/v); S₂, *n*-hexane/ethyl acetate (90:10 v/v); S₃, *n*-hexane/ethyl acetate (80:20 v/v); S₄, chloroform/methanol (95:5 v/v); S₅, chloroform/methanol (90:10 v/v) and S₆, chloroform/methanol (80:20 v/v). The chromatograms were visualized under UV light (at 254 and 366 nm) before and after exposure to ammonia vapor and spraying with natural products/polyethylene glycol reagent (diphenylboryloxyethyl-amin, NP/PEG), as well as after spraying with anisaldehyde/sulfuric acid spray reagent. Melting points (uncorrected) were determined on a D. Electrothermal 9100 instrument (Labequip, Markham, Ontario, Canada). UV spectra were recorded using a Shimadzu UV 240 (P/N 204 – 58000) spectrophotometer (Kyoto, Japan). Bruker NMR was used for ¹H NMR (400 MHz) and ¹³C NMR (125 MHz) measurements. IR spectra were measured on an infrared spectrophotometer FT/IR-460, Plus (Jasco, Oklahoma, Japan). The NMR spectra were recorded in CDCl₃ and CD₃OD and chemical shifts are given in δ (ppm) relative to TMS as internal standard.

1.2. Plant material

The corms of *Liatris spicata* (L.) willd were collected in January 2010 (flowering stage) from the Experimental Station of Medicinal Plants, Faculty of Pharmacy, Cairo University, Giza, Egypt. The plant was authenticated by Dr. M. Gibali (Senior Botanist). A voucher specimen was deposited at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt (30-5-2013-2).

1.3. Extraction, fractionation and isolation

The air-dried corms of *Liatris spicata* (L.) willd (1 kg) were powdered, then extracted with ethanol 95% by cold maceration till exhaustion. The ethanol extract was evaporated under reduced pressure to yield 160 g dried extract. The dried residue were suspended in water (250 ml) and partitioned successively between petroleum ether (6 x 500 ml), chloroform (6 x 500 ml),

ethyl acetate (6 x 500 ml) and *n*-butanol saturated with water (4 x 500 ml), the petroleum ether, chloroform, ethyl acetate and *n*-butanol fractions were evaporated to yield 10.6 g, 4.4 g, 4.3 g and 23.9 g respectively.

The petroleum ether (10 g) was chromatographed over a VLC column (6 x 20 cm, silica gel H, 250 g). Gradient elution was carried out using *n*-hexane/chloroform mixtures and chloroform/ethyl acetate mixtures. Fractions of 200 ml each were collected and monitored by TLC to yield four main fractions (A – D). Fraction A (45-50% chloroform/*n*-hexane) was washed with acetone to give compound **1** (34 mg, white needle crystals, $R_f = 0.89$ in S_1). Fraction B (60-70 % chloroform/*n*-hexane) was rechromatographed over a silica gel 60 column, using *n*-hexane/ethyl acetate (9.9: 0.1 v/v) as eluent, to give compound **2** (690 mg, yellow needle-shaped crystals, $R_f = 0.63$ in S_2). Fraction C (85-90 % chloroform/*n*-hexane) was purified in the same way using *n*-hexane/ethyl acetate (9.9:0.1 v/v) as an eluent to give compound **3** (23 mg, white needle-shaped crystals, $R_f = 0.61$ in S_3). Fraction D (20% ethyl acetate/chloroform) was similarly purified using *n*-hexane/ethyl acetate (9.8:0.2 v/v) as eluent to give compound **4** (63 mg, white needle crystals, $R_f = 0.57$ in S_3).

Chloroform fraction (4 g) was chromatographed on 20 g silica gel in a vacuum liquid chromatography column (VLC) (5x15 cm). Gradient elution was carried out with chloroform and increasing the polarity by 5% stepwise addition of ethyl acetate till 100% ethyl acetate, followed by increasing the polarity with methanol in 5% increment till 50% methanol in ethyl acetate. Fractions of 100 ml each were collected and monitored by TLC to yield two main fractions. First fraction (35-40% ethyl acetate/chloroform) was rechromatographed over a silica gel column and eluted with *n*-hexane /ethyl acetate (9.8:0.2 v/v) as eluent to give compound **5** (38mg, yellow oil , $R_f = 0.45$ in S_3). The second fraction (60-70 %) ethyl acetate/chloroform was purified in the same way using chloroform/methanol (9.8:0.2 v/v) as eluent to give compound **6** (24 mg, white microcrystalline powder, $R_f = 0.62$ in S_3).

Ethyl acetate fraction (4.3 g) was fractionated over a Sephadex LH-20 column using water/ methanol gradient elution (3 x 25 cm). Fractions were collected and purified using silica gel 60 columns, using chloroform/methanol (9.9:0.1) to yield two compounds, **7** (28 mg, $R_f = 0.43$ in S_6) and **8** (123 mg, $R_f = 0.58$ in S_6).

Butanol fraction, previously prepared (10 g) was loaded to a diaion HP-20 AG (250 g, 5 x 120 cm) packed in water. Elution was carried out with water, followed by methanol/water (50%) and methanol (100%). Fraction eluted with 50% methanol in water showed one major spot ($R_f = 0.72$, S_6) gave after evaporation 85 mg of yellow residue; this fraction was purified on sephadex LH - 20 column and eluted with methanol/water (1: 1 v/v) to give compound **9** (12 mg, $R_f = 0.72$, S_6).

Obtusifoliyl acetate **1**: White needle crystals, m.p. 111- 113 °C, m/z at 468 $[M]^+$. 1H -NMR: δ 2.05 (3H,s, CH_3O), Two methylene protons at 4.67 and 4.71. Seven methyl groups at 0.71 (3H,s, CH_3 -18), 0.86 (3H, d, $J = 6.6$, CH_3 -30), 0.89 (3H,s, CH_3 -29), 0.98(3H,s, CH_3 -19), 0.93 (3H,d, $J = 6.6$, CH_3 -21), 1.02 (3H, d, $J = 6.6$, CH_3 -26), 1.03 (3H, d, $J = 7.1$, CH_3 -27). ^{13}C NMR: δ 171 (C=O), 156 (C-24), 134.5 (C-9), 134.2 (C-8), 105 (C-30), 80.9 (C-3), 50.5 (C-17), 50.3 (C-14), 49.8 (C-5), 44.4 (C-13), 37.8 (C-4), 36.8 (C-20), 36.4 (C-10), 35.2 (C-22), 34.9 (C-1), 33.8 (C-25), 31.2 (C-15), 30.9 (C-2), 30.8 (C-16), 28.2 (C-23), 27.9 (C-7), 26.3 (C-12), 24.2 (C-28), 24.1 (C-27), 21.9 (C-11), 21.8 (C-26), 21.3 (CH_3O), 21 (C-6), 18.7 (C-19), 18 (C-21), 15.8 (C-18), 14.3 (C-29). IR ν_{max} : 1735, 1630, 1042 and 880 cm^{-1} .

Euparin **2**: Yellow needle crystals, m.p. 97-100 °C, m/z at 216 $[M]^+$. 1H -NMR: δ 12.48 (1H, s, OH), 7.86 (1H, s, H-4), 6.42 (1H, s, H-7), Two Methyl groups at 2.6 (3H, s), 2.08 (3H, s). Two well separated methylene protons at 5.72 (1H, s, H-11a) and 5.16 (1H, s, H-11b). The furanoid proton (H-3) at 6.95. ^{13}C NMR: δ 156.12 (C-2), 98.22 (C-3), 120.36 (C-4), 121.24 (C-5), 159.12 (C-6), 102.17 (C-7), 161.12 (C-8), 115.38 (C-9), 131.48 (C-10), 111.26 (C-11), 19.68 (C-12), 202.75 (C-13), 28.45 (C-14). IR ν_{max} : 1635, 1475, 1360, 1317, 1127, 1032 and 902 cm^{-1} .

Igalan **3**: White needle crystals, m.p. 79–79.5°C, m/z at 232 $[M]^+$. 1H -NMR: δ 6.35 and 5.56 (2H,d, $J = 4.5$, H-9), 5.73 (1H, m, H-10), 4.96, 4.92, 4.88, and 4.58 (4H,s, , H-11, 13), 4.82 (1H, m, H-3), 3.2 (1H, m, H-4), 1.73 (3H,s, H-14) and 1.06 (3H, s, H-15). ^{13}C NMR: δ 40 (C-1), 45.9 (C-2), 75.8 (C-3), 38.8 (C-4), 26.6 (C-5), 42.7 (C-6), 137.2 (C-7), 170.5 (C-8), 120.4 (C-9), 147.3 (C-10), 111 (C-11), 145.7 (C-12), 113 (C-13), 18.3 (C-14), 22.7 (C-15). IR ν_{max} : 1770, 1660, 1640, 1410, 1110, 909 and 892.

Stigmasterol **4**: White needle crystals, m.p. 169-170°C, m/z at 412 $[M]^+$. 1H -NMR: δ 0.71 (3H, d, $J = 5.4$ Hz, Me-21), 0.85 (3H, t, $J = 6.3$, Me-29), 0.90 (3H, d, $J = 6.4$ Hz, Me-26), 1.043 (3H,

d, $J = 6.3$ Hz, Me-27), 1.27 (3H, s, Me-18), 1.66 (3H, s, Me-19), 3.51 (1H, m, H-3), 5.15 (dd, 1H, $J = 8.3$, 15.4, H-22), 5.21 (1H, dd, $J = 8$, 15.2, H-23) and 5.4 (1H, br.s., H-6). ^{13}C NMR: δ 140.76 (C-5), 138.31 (C-22), 129.28 (C-23), 121.69 (C-6), 71.78 (C-3), 21.23 (C-21), 21.09 (C-27), 19.4 (C-19), 19.0 (C-26), 12.25 (C-29), 12.06 (C-18). IR ν_{max} : 3476, 2944, 1646, 1556, 1370, 1214, 1168, 1114, 1062 and 894 cm^{-1} .

6-Hydroxy-3-methoxytremetone **5**: Yellow oil, m/z : 248 $[\text{M}]^+$. ^1H -NMR: δ 1.82 (s, CH_3 -12), 2.57 (s, CH_3 -14), 3.5 (s, 6-OMe), 12.48 (6-OH), 5.08 (1H, s, H-11a), 4.93 (1H, s, H-11b), 5.12 (m, H-3), 5.25 (m, H-2), 6.43 (s, H-7), 7.62 (s, H-4). ^{13}C NMR: δ 55.3 (OMe), 83.3 (C-2), 92.2 (C-3), 129.2 (C-4), 117.8 (C-5), 167.1 (C-6, C-8), 92.9 (C-7), 114.4 (C-9), 141.1 (C-10), 113.2 (C-11), 17.5 (C-12), 202.2 (C-13), 29.7 (C-14). IR ν_{max} : 2920, 1634, 1484, 1400, 1370 and 1262 cm^{-1} .

Stigmasterol -3-O- β -D-glucoside **6**: White microcrystalline powder, m.p. $290\text{ }^\circ\text{C}$, m/z : 575 $[\text{M} + \text{H}]^+$, 412 (M - sugar) $^+$. ^1H -NMR: δ 0.65 (3H, d, $J = 5.4$ Hz, Me-21), 0.74 (3H, t, $J = 6.3$, Me-29), 0.83 (3H, d, $J = 6.4$ Hz, Me-26), 0.89 (3H, d, $J = 6.3$ Hz, Me-27), 0.91 (3H, s, Me-18), 0.96 (3H, s, Me-19), 3.33 (1H, m, H-3), 4.21 (1H, d, $J = 7.5$, H-1'), 4.76 (m, 2H, H-22, 23) and 5.4 (1H, br.s., H-6). IR ν_{max} : 3402, 2940, 2870, 1635, 1458, 1370, 1258, 1165, 1072 and 1026 cm^{-1} .

Protocatechuic acid **7**: White needle crystals, m.p. $179\text{--}182\text{ }^\circ\text{C}$, m/z : 155 $[\text{M} + \text{H}]^+$. ^1H -NMR: δ 6.80 (d, 1H, $J = 8.1$ Hz, H-5), 7.30 (dd, 1H, $J = 2.0$, 8.1 Hz, H-6), 7.45 (d, 1H, $J = 2.0$ Hz, H-2). ^{13}C NMR: δ 170.3 (COOH), 151.5 (C-4), 146.0 (C-3), 123.8 (C-6), 123.2 (C-1), 117.7 (C-2), 115.7 (C-5). IR ν_{max} : 3381, 2945, 2836, 1681, 1456, 1055 and 1028 cm^{-1} .

Vanillic acid **8**: White needle crystals, m.p. $210\text{--}213\text{ }^\circ\text{C}$, m/z : 168 $[\text{M}]^+$. ^1H -NMR: δ 6.93 (d, 1H, $J = 8.7$ Hz, H-5), 7.65 (m, 2H, H-2 and H-6), 3.90 (3H, s, -OCH₃). ^{13}C NMR: δ 168.8 (C=O), 151.02 (C-4), 147.1 (C-3), 123.69 (C-6), 114.2 (C-2), 121.92 (C-1), 112.28 (C-5), 62.7 (-OCH₃). IR ν_{max} : 3376, 2945, 2835, 1655, 1457, 1114 and 683 cm^{-1} .

Ferulic acid **9**: White needle crystals, m.p. $168\text{--}171\text{ }^\circ\text{C}$, m/z : 194 $[\text{M}]^+$. ^1H -NMR: δ 3.58 (s, 3H, -OCH₃), 6.2 (d, $J = 15.6$ Hz, 1H, H-8), 6.76 (d, $J = 8.3$ Hz, 1H, H-5), 6.92 (dd, $J = 8.3$, 2.1 Hz, 1H, H-6), 7.01 (d, $J = 2.1$ Hz, 1H, H-2), 7.49 (d, $J = 15.6$ Hz, 1H, H-7). ^{13}C NMR: δ 64.33 (-OCH₃), 114.8 (C-8), 115.0 (C-2), 116.4 (C-5), 122.7 (C-6), 127.9 (C-1), 146.5 (C-4), 146.8 (C-7), 149.3 (C-3), 169.5 (-COOH). IR ν_{max} = 3450, 1690, 1605, 1510, 1275 and 940 cm^{-1} .

1.4. Biological activity

Assessment of cytotoxic activity

The cytotoxicities of ethanol extract of *L. spicata* and the compounds isolated were assessed using the sulforhodamine B (SRB) assay (Skehan *et al.*, 1990) against one human cancer cell line: liver (HepG2) adenocarcinoma and normal cell line melanocytes (HFB4). The assessment was performed at the National Cancer Institute in Egypt (NCI). The cells were plated in 96-multiwell plates (104 cells/ well) for 24 hours before treatment with the samples to allow attachment of the cells to the wall of the plate. Different concentrations of each of the tested samples (0, 5, 12.5, 25 and 50 µg/ml in DMSO) were added to the cell monolayer. Six wells were prepared for each individual dose. Monolayer cells were incubated with the extracts and compounds under test for 48 hours, at 37 °C and in atmosphere of 5% CO₂.

After 48 hours, the cells were fixed, washed and stained with Sulpho-Rhodamine-B stain. Excess stain was washed with acetic acid and the attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an ELISA reader and the relation between the surviving fraction and the sample concentration was plotted to get the survival curve of each tumor cell line after being treated with the specified sample. The IC₅₀ values (concentration which reduces the survival to 50%) were calculated together with the reference drug doxorubicin[®].

References

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