Chemical Constituents and Cytotoxic Effect of the Main Compounds of *Lythrum salicaria* L.

Azadeh Manayi[a], Soodabeh Saeidnia[b], Seyed Nasser Ostad[c], Abbas Hadjiakhoondi[a], Mohammad Reza Shams Ardekani[a,d], Mahdi Vazirian[a], Yasmin Akhtar[e], and Mahnaz Khanavi[a,d,*]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran. Fax: +98-21-66954706. E-mail: khanavim@tums.ac.ir
[b] Medicinal Plants Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
[c] Department of Toxicology and Pharmacology, Faculty of Pharmacy and Pharmaceutical Sciences Research Center, Tehran University of Medical Sciences, Tehran, Iran
[d] Persian Medicines and Pharmacy Research Center, Tehran University of Medical Sciences, Tehran, Iran
[e] Faculty of Land and Food Systems, University of British Columbia, Vancouver, BC, Canada

* Author for correspondence and reprint requests

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Lythrum salicaria L. (Lythraceae), a herbaceous plant growing widely in Iran, has been well known for many centuries for its astringent and styptic properties. A phytochemical investigation of this plant, based on spectroscopic analysis, identified fourteen compounds: 5-hydroxypyrrolidin-2-one (**1**), umbelliferone-6-carboxylic acid (**2**), 3,3',4'-tri-*O*-methylellagic acid-4-*O*-β-D-(2"-acetyl)-glucopyranoside (**3**), 3,3',4'-tri-*O*-methylellagic acid-4-*O*-β-D-glucopyranoside (**4**), daucosterol (**5**), phytol (**6**), dodecanoic acid (**7**), oleanolic acid (**8**), 3,3',4'-tri-*O*-methylellagic acid (**9**), corosolic acid (**10**), β-sitosterol (**11**), peucedanin (**12**), buntansin (**13**), and erythrodiol (**14**). All compounds, except for **8** and **11**, have been isolated from *L. salicaria* for the first time. Cytotoxic activities of the compounds were examined against three cancerous cell lines, colon carcinoma (HT-29), leukemia (K-562), and breast ductal carcinoma (T47D), and Swiss mouse embryo fibroblast (NIH-3T3) cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and methotrexate as positive control. Compounds **5**, **10**, **11**, and **14** were the most active against the HT-29 cell line with IC_{50} values of 192.7, 36.8, 38.2, and 12.8 μg/mL, respectively. Compounds **14**, **11**, **5**, and **10** were 6.4, 2.8, 2.6, and 1.4 times, respectively, more selective than methotrexate. Compound **5** was the most active against the K-562 cell line (IC_{50} = 50.2 μg/mL), with a selectivity exceeding that of methotrexate 13.3 times. The results of the cytotoxic assay confirmed that growth and proliferation of the cancer cell lines are predominantly influenced by triterpene derivatives and sterols of this plant.

Key words: Lythrum salicaria, Phytochemistry, Cytotoxic Effect

Introduction

Cancer has become one of the most devastating diseases of humankind in the world (Ma *et al.*, 2009). Natural products or their related components constitute more than half of the drugs used in cancer therapy (Newman and Cragg, 2007). Discovery of efficacious anticancer agents has provided convincing evidence that plants could be a source of chemotherapeutic agents (Kostova, 2005).

Lythrum salicaria belongs to the plant family Lythraceae comprising 30 species, seven of which are found in Iran (Rechinger, 1968). The plant is known as "Turbinkwash" and "Surmankhal" in Persian (Soltani, 2011). Phytochemical investigations on this plant have revealed the presence of tannins, anthocyanins, phenolic aids, and flavon-*C*-glycosides (Rauha *et al.*, 2000, 2001; Zhou *et al.*, 2011). Additionally, biologically active compounds have been isolated from the plant such as oleanolic acid and ursolic acid as antifungal compounds and the hexahydroxydiphenoyl ester vescalagin as an antibacterial agent (Becker *et al.*, 2005). Antioxidant activity of various extracts of the plant has been tested, and polar extracts were found to be the most active ones (Coban *et*

al., 2003; Lopez *et al*., 2008; Tunalier *et al*., 2007). The plant extract decreases the blood glucose level in both hyperglycaemic and normoglycemic rats and rabbits (Lamela *et al*., 1986; Torres and Suarez, 1980). Flowering aerial parts of the plant have been traditionally used for the treatment of diarrhea, chronic intestinal catarrh, haemorrhoids, eczema, varicose veins, bleeding of the gums, and vaginitis (Mantle *et al*., 2000; Rauha *et al*., 2000). Evaluation of the antidiarrheal effect of Salicairine®, a formulated medication, demonstrated that there was either a significant increase in the colon net fluid absorption or a decrease in the net fluid secretion (Brun *et al*., 1997).

We have previously analysed the extract and fractions of *L. salicaria* for cytotoxic activity against cancer cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Ethyl acetate and chloroform fractions of the plant exhibited cytotoxicity toward the T47D cancer cell line with IC_{50} values of 63.1 and 108.2 μg/mL, respectively (Khanavi *et al*., 2011). The objective of the present study was the isolation and characterization of major constituents of *L. salicaria* through chromatographic and spectroscopic methods, respectively. We also demonstrated cytotoxic effects of the purified compounds using the MTT assay against the three cancerous cell lines HT-29 (colon carcinoma), K-562 (leukemia), and T47D (breast ductal carcinoma), and the normal cell line NIH-3T3 (Swiss mouse embryo fibroblast).

Material and Methods

General

Column chromatography (CC) was carried out using silica gel (70–230 mesh; Merck, Darmstadt, Germany) and Sephadex LH-20 (Fluka, Buchs, Switzerland) as stationary phases. Pre-coated silica gel 60 F254 plates (Merck) were used for thin-layer chromatography (TLC). Spots on TLC plates were visualized under UV light (at 254 and 366 nm) and also after spraying the developed plates with anisaldehyde followed by heating for 5 min. All solvents used in this study were distilled prior to use. Nuclear magnetic resonance (NMR) spectra were obtained on a Bruker (Billerica, MA, USA) DRX 500 instrument (500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR) with tetramethylsilane (TMS) as an internal standard. EI-mass spectra were recorded on an Agilent Technology instrument (New York, NY, USA) (HPTM) with a 5973 Network mass selective detector (MS model). UV spectra were measured on an Optizen (Daejeon, Korea) model 2021 UV plus, IR spectra on a Nicolet (New York, NY, USA) 550-A spectrometer using NaCl pellets, and melting points on a Büchi (Flawil, Switzerland) melting point B-545 apparatus.

Plant material

Aerial parts (stems, leaves, flowers) of *L. salicaria* were collected in May 2011 from Lahidjan city, Guilan province (North of Iran) and deposited in the Central Herbarium of Medicinal Plants, Karaj, Iran (Ajani 313). They were cleaned and dried in the shade at room temperature.

Isolation of compounds

Aerial, shade-dried parts of the plant (1800 g) were crushed and extracted with 80% aqueous MeOH (3 × 48 h). The extracts were concentrated in a vacuum evaporator to give a crude extract (330 g), which was fractionated to three main fractions, *i.e*. chloroform, ethyl acetate, and methanol, yielding 1.17, 7.97, and 161.83 g, respectively.

The methanol fraction was subjected to column chromatography on silica gel using MeOH/$CHCl_3$ (2:8–10:0, v/v) as eluent to yield eight fractions, M_1–M_8. Fraction M_2 (280 mg) was subjected to chromatography on a silica gel C_{18}-reverse phase (C_{18}-RP) column eluted with MeOH/H_2O (5:5–0:10), which yielded compound **1** (5 mg). Fraction M_3 (12 g) was subjected to silica gel column chromatography eluted with MeOH/$CHCl_3$ (1:19–0:20), which gave nine sub-fractions, M_{31}–M_{39}. On the basis of analytical TLC, sub-fraction M_{34} (761 mg) was chromatographed on a C_{18}-RP column, eluted with MeOH/H_2O (3:7–5:5), and then on a Sephadex LH-20 column using MeOH, yielding compound **2** (5.5 mg). Sub-fraction M_{35} (538 mg) was purified on a C_{18}-RP column eluted with MeOH/H_2O (3:7–10:0) to afford three new fractions, of which each for final purification was subjected to Sephadex LH-20 column chromatography using MeOH to produce compounds **3** (10 mg), **4** (1.4 mg), and **5** (10 mg).

For purification of the ethyl acetate fraction, it was applied to a silica gel column eluted with $CHCl_3$/EtOAc (7:3–0:10) and produced five fractions, E_1–E_5. Fraction E_1 (600 mg) was subjected to column chromatography over silica

gel eluted with *n*-hexane/EtOAc (9:1–0:10) and then with $CHCl_3$/MeOH (10:0–2:8) to produce compounds **6** (4.2 mg) and **7** (12.4 mg). Fraction E_2 (700 mg) was subjected to a silica gel column eluted with *n*-hexane/EtOAc (8:2–6:4) and re-chromatographed on a Sephadex LH-20 column eluted with $CHCl_3$/MeOH (5:5) to produce compound **8** (73 mg). Fraction E_3 (143 mg) was chromatographed on a silica gel column eluted with *n*-hexane/EtOAc (8:2–0:10) and then on a Sephadex LH-20 column using MeOH to produce compound **9** (4 mg). Fraction E_4 (342 mg) was purified on a silica gel column eluted with $CHCl_3$/EtOAc (7:3–0:10) to produce new sub-fractions. Sub-fraction E_{42} was chromatographed on a silica gel column eluted with $CHCl_3$/MeOH (19:1–0:20) to yield compound **10** (12 mg).

Purification of the chloroform fraction was carried out using column chromatography on silica gel eluted with $CHCl_3$/EtOAc (1:0–9:1), producing eight new fractions, C_1–C_8. Fraction C_5 (800 mg) was re-chromatographed as above, using *n*-hexane/EtOAc (19:1–5:5), and produced new sub-fractions. Sub-fraction C_{54} was purified on a silica gel column eluted with *n*-hexane/EtOAc (9:1–7:3) to produce compounds **11** (8.1 mg), **12** (3.4 mg), and **13** (5 mg). Fraction C_7 was subjected to column chromatography over silica gel eluted with *n*-hexane/EtOAc (8:2–0:10) and produced new sub-fractions. Sub-fraction C_{75} was subjected to a silica gel column eluted with *n*-hexane/EtOAc (19:1–5:5) and yielded compound **14** (8.2 mg).

5-Hydroxypyrrolidin-2-one (**1**): ^{1}H NMR (500 MHz, MeOD): δ = 5.5 (1H, dd, $^3J_{5,4eq}$ = 1.7 Hz, $^3J_{5,4ax}$ = 6.35 Hz, H-5), 2.5 (1H, m, $^2J_{3ax,3eq}$ = 17.15 Hz, $^3J_{3ax,4ax}$ = 9.15 Hz, $^3J_{3ax,4eq}$ = 8 Hz, H-3_{ax}), 2.37 (1H, m, $^2J_{4ax,4eq}$ = 13.5 Hz, $^3J_{4ax,3eq}$ = 9.8 Hz, $^3J_{4ax,3ax}$ = 9.15 Hz, $^3J_{4ax,5}$ = 6.35 Hz, H-4_{ax}), 2.2 (1H, m, $^2J_{3eq,3ax}$ = 17.15 Hz, $^3J_{3eq,4ax}$ = 9.8 Hz, $^3J_{3eq,4eq}$ = 3.6 Hz, H-3_{eq}), 1.90 (1H, m, $^2J_{4eq,4ax}$ = 13.5 Hz, $^3J_{4eq,3ax}$ = 8 Hz, $^3J_{4eq,3eq}$ = 3.6 Hz, $^3J_{4eq,5}$ = 1.7 Hz, H-1). – ^{13}C NMR (125 MHz, MeOD): δ = 180.8 (C-2), 80.9 (C-5), 31.2 (C-4), 29.5 (C-3). – MS (EI, 70 eV): *m/z* (%) = 101 (54) [M$^+$], 84 (100), 83 (50), 81 (15), 57 (70), 55 (50), 53 (10).

Umbelliferone-6-carboxylic acid (**2**): M.p. 199.6 – 200.6 °C. – IR (NaCl): ν = 3420, 3296, 1743 cm^{-1}. – UV/Vis (MeOH): λ_{max} (log ε) = 250 (1.06), 305 (1.5, sh), 330 nm (2.32). – ^{1}H NMR (500 MHz, DMSO-d_6): δ = 8.3 (OH), 7.96 (1H, s, H-5), 7.92 (1H, d, J = 9.35 Hz, H-4), 6.45 (1H, s, H-8), 6.03 (1H, d, J = 9.35 Hz, H-3). – ^{13}C NMR (125 MHz, DMSO-d_6): δ = 169.86 (COOH), 169.58 (C, C-7), 160.74 (CO, C-2), 157.2 (C, C-8a), 145.43 (CH, C-4), 130.47 (CH, C-5), 117.04 (C, C-4a), 109.06 (CH, C-3), 108.3 (C, C-6), 102.46 (CH, C-8). – MS (EI, 70 eV): *m/z* (%) = 206 (10) [M$^+$], 111 (100), 110 (68), 109 (93), 83 (81).

*3,3',4'-Tri-O-methylellagic acid-4-O-β-*D*-(2"-acetyl)-glucopyranoside* (**3**): IR (NaCl): ν = 3394, 2925, 2854, 1739, 1456, 1252, 1032 cm^{-1}. – UV/Vis (MeOH): λ_{max} (log ε) = 237.5 (2.5, sh), 260 (2.68), 365 nm (0.88). – ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.86 (1H, s, H-5'), 7.65 (1H, s, H-5), 4.05 (3H, s, 3'-OMe), 4.01 (3H, s, 3-OMe), 3.97 (3H, s, 4'-OMe); sugar protons: 5.1 (1H, d, H-1"), 3.7 (1H, Ha-6"), 3.5 (1H, Hb-6"), 3.47 (1H, H-5"), 3.45 (1H, H-2"), 3.44 (1H, H-3"), 3.17 (1H, H-4"), 2.06 (s, 3H, 2"-$COCH_3$). – ^{13}C NMR (125 MHz, DMSO-d_6): δ = 158.35 (C-7), 158.12 (C-7'), 154.45 (C-4), 151.37 (C-4'), 141.47 (C-3'), 141.37 (C-2'), 140.9 (C-2), 140.8 (C-3), 114.16 (C-1'), 112.76 (C-1), 112.55 (C-6), 112.19 (C-6'), 112.19 (C-5'), 107.67 (C-5), 61.59 (3-OMe), 61.39 (3'-OMe), 56.83 (4'-OMe); sugar carbon atoms: 169.47 (2"-**C**OCH$_3$), 98.59 (C-1"), 77.38 (C-5"), 73.54 (C-3"), 73.46 (C-2"), 69.52 (C-4"), 60.33 (C-6"), 20.89 (2"-CO**C**H$_3$).

*3,3',4'-Tri-O-methylellagic acid-4-O-β-*D*-glucopyranoside* (**4**): UV/Vis (MeOH): λ_{max} (log ε) = 230 (2.5, sh), 267 (2.86), 350 nm (1.78). – ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.85 (1H, s, H-5'), 7.67 (1H, s, H-5), 4.1 (3H, s, 3'-OMe), 4.05 (3H, s, 3-OMe), 4.02 (3H, s, 4'-OMe); sugar protons: 5.18 (1H, d, H-1"), 3.79 (1H, Ha-6"), 3.5 (1H, Hb-6"), 3.47 (1H, H-5"), 3.47 (1H, H-4"), 3.45 (1H, H-2"), 3.44 (1H, H-3"). – ^{13}C NMR (125 MHz, DMSO-d_6): δ = 158.23 (C-7), 157 (C-7'), 154 (C-4), 151.92 (C-4'), 141.2 (C-2'), 141 (C-2), 140.8 (C-3'), 140 (C-3), 113.73 (C-1'), 112.94 (C-6), 112.94 (C-1), 112 (C-5'), 111.95 (C-6'), 108 (C-5), 61.72 (3-OMe), 61.38 (3'-OMe), 56.82 (4'-OMe); sugar carbon atoms: 101 (C-1"), 77.29 (C-5"), 76.48 (C-3"), 73.34 (C-2"), 69.48 (C-4"), 60.53 (C-6").

Daucosterol (**5**): ^{1}H NMR (500 MHz, DMSO-d_6): δ = 5.3 (1H, bs, H-6), 1.2 (3H, s, H-19), 0.64 (3H, s, H-18); sugar protons: 4.2 (1H, bs, H-1'), 3.62 (1H, Ha-6'), 3.39 (1H, Hb-6'), 3.11 (1H, H-3'), 3.05 (1H, H-5'), 3 (1H, H-4'), 2.88 (1H, H-3'). – ^{13}C NMR (125 MHz, DMSO-d_6): δ = 140.32 (C-5), 121.26 (C-6), 76.95 (C-3), 56.22 (C-17), 55.46 (C-14), 49.63 (C-8), 45.17 (C-24), 41.89 (C-13), 40 (C-12), 39.83 (C-4), 38.33 (C-10), 36.25 (C-9), 35.53

(C-20), 33.37 (C-22), 31.45 (C-7), 29.30 (C-16), 28.72 (C-27), 27.85 (C-2), 25.43 (C-23), 23.91 (C-15), 22.63 (C-25), 20.64 (C-11), 19.76 (C-21), 19.14 (C-19), 18.97 (C-28), 18.66 (C-29), 11.71 (C-26); sugar carbon atoms: 100.81 (C-1'), 76.75 (C-5'), 76.75 (C-3'), 73.49 (C-2'), 70.1 (C-4'), 61.11 (C-6').

Phytol (**6**): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.42 (1H, d, J = 6.8, 1.3 Hz, H-2), 4.16 (2H, d, J = 6.8 Hz, H-1), 1.99 (2H, m, H-4), 1.67 (3H, bs, H-20). – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 140.32 (C, C-3), 123.06 (CH, C-2), 59.4 (CH_2, C-1), 39.58 (CH_2, C-4), 39.34 (CH_2, C-5), 37.4 (CH_2, C-9), 37.33 (CH_2, C-6), 37.26 (CH_2, C-8), 36.63 (CH_2, C-10), 32.78 (CH, C-11), 27.97 (CH, C-15), 25.12 (CH_2, C-12), 24.78 (CH_2, C-13), 24.77 (CH_3, C-19), 24.46 (CH_2, C-14), 24.44 (CH_3, C-20), 23.68 (CH, C-7), 22.6 (CH_3, C-18), 19.72 (CH_3, C-16), 16.15 (CH_3, C-17).

Dodecanoic acid (**7**): ^{1}H NMR (500 MHz, pyridine-d_5): δ = 2.5 (2H, t, J = 7.35 Hz, H-2), 1.77 (2H, m, J = 7.35 Hz, H-3), 1.36 (2H, m, H-11), 0.84 (3H, t, J = 6.8 Hz, H-12). – ^{13}C NMR (125 MHz, pyridine-d_5): δ = 176 (COOH), 34.91 (C-2), 32.11 (C-10), 25.6 (C-3), 29.4 – 29.95 (C-4, C-5, C-6, C-7, C-8, C-9), 22.92 (C-11), 14.27 (C-12).

Oleanolic acid (**8**): ^{1}H NMR (500 MHz, pyridine-d_5): δ = 5.47 (1H, H-12), 3.42 (1H, dd, J = 10.3, 6.1 Hz, H-3), 3.29 (1H, dd, J = 13.8, 4.4 Hz, H-18), 1.26 (3H, s, H-27), 1.22 (3H, s, H-23), 1 (3H, s, H-30), 1 (3H, s, H-24), 0.98 (3H, s, H-29), 0.92 (3H, s, H-25), 0.87 (3H, s, H-26). – ^{13}C NMR (500 MHz, pyridine-d_5): δ = 180.16 (C-28), 144.8 (C-13), 122.5 (C-12), 79.76 (C-3), 55.8 (C-5), 48.12 (C-9), 46.67 (C-17), 46.48 (C-19), 42.17 (C-14), 42.01 (C-18), 39.7 (C-8), 39.4 (C-4), 38.93 (C-1), 37.38 (C-10), 34.21 (C-21), 33.37 (C-22), 33.26 (C-29), 33.19 (C-7), 30.53 (C-20), 28.7 (C-23), 28.3 (C-15), 28.1 (C-2), 26.16 (C-27), 23.81 (C-30), 23.75 (C-16), 23.7 (C-11), 18.79 (C-6), 17.44 (C-26), 16.5 (C-24), 15.54 (C-25).

3,3',4'-Tri-O-methylellagic acid (**9**): UV/Vis (MeOH): λ_{max} (log ε) = 237.5 (2.6, sh), 267 (2.77), 372 (0.88), 402 nm (0.7, sh). – ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.62 (1H, s, H-5'), 7.52 (1H, s, H-5), 4.05 (3H, s, 3'-OMe), 4.04 (3H, s, 3-OMe), 3.99 (3H, s, 4'-OMe). – ^{13}C NMR (125 MHz, DMSO-d_6): δ = 141.06 (C-2), 111.8 (C-5), 61.42 (3'-OMe), 56.79 (4'-OMe). The signals for the other carbon atoms could not be detected because of the small sample size.

Corosolic acid (**10**): ^{1}H NMR (500 MHz, pyridine-d_5): δ = 5.44 (1H, m, J = 3.5 Hz, H-12), 4.09 (1H, td, J = 9.4, 4.4 Hz, H-2), 3.39 (1H, d, J = 9.4 Hz, H-3), 2.61 (1H, d, J = 11.35 Hz, H-18), 1.26 (3H, s, H-27), 1.19 (3H, s, H-23), 1.03 (3H, s, H-25), 1 (3H, s, H-26), 0.97 (3H, d, J = 4.9 Hz, H-30), 0.96 (3H, s, H-24), 0.93 (3H, d, J = 5.5 Hz, H-29). – ^{13}C NMR (500 MHz, pyridine-d_5): δ = 179.89 (COOH, C-28), 139.27 (C, C-13), 125.53 (CH, C-12), 83.8 (CH, C-3), 68.58 (CH, C-2), 55.89 (CH, C-5), 53.5 (CH, C-18), 48.14 (C, C-17), 47.73 (CH, C-9), 46.64 (CH_2, C-1), 41.97 (C, C-14), 39.83 (C, C-8), 39.46 (C, C-4), 39.46 (CH, C-19), 39.38 (CH, C-20), 38.41 (C, C-10), 37.42 (CH_2, C-22), 33.18 (CH_2, C-7), 31.05 (CH_2, C-21), 29.36 (CH_3, C-23), 28.26 (CH_2, C-15), 24.88 (CH_2, C-16), 23.89 (CH_3, C-27), 23.71 (CH_2, C-11), 21.39 (CH_3, C-30), 18.82 (CH_2, C-6), 17.7 (CH_3, C-29), 17.46 (CH_3, C-26), 16.96 (CH_3, C-25), 16.82 (CH_3, C-24).

β-Sitosterol (**11**): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.36 (1H, bs, H-6), 3.5 (1H, m, J = 6.3, 4.5 Hz, H-3), 1.02 (3H, s, H-19), 0.93 (3H, d, J = 5.8 Hz, H-21), 0.84 (3H, t, J = 7.2 Hz, H-29), 0.83 (3H, d, J = 6.8 Hz, H-27), 0.81 (3H, d, J = 7.2 Hz, H-26), 0.69 (3H, s, H-18). – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 140.95 (C-5), 121.73 (C-6), 71.79 (C-3), 56.76 (C-14), 56.02 (C-17), 50.1 (C-9), 42.28 (C-13), 42.28 (C-4), 39.75 (C-12), 37.23 (C-1), 36.45 (C-10), 36.13 (C-20), 33.92 (C-22), 31.88 (C-7), 31.88 (C-8), 31.66 (C-2), 29.43 (C-25), 28.23 (C-16), 26.04 (C-23), 24.29 (C-15), 23.04 (C-24a), 21.06 (C-11), 19.38 (C-19), 19.82 (C-26), 19.01 (C-27), 18.76 (C-21), 11.96 (C-24b), 11.84 (C-18).

Peucedanin (**12**): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.72 (1H, d, J = 9 Hz, H-4), 7.5 (1H, s, H-5), 7.19 (1H, s, H-8), 6.30 (1H, d, J = 9 Hz, H-3), 3.87 (3H, s, 9-OMe), 3.18 (1H, sept, J = 7 Hz, H-1'), 1.28 (6H, J = 7 Hz, H-2', H-3'). – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 161.13 (C-2), 153.68 (H-6), 152.69 (H-10), 151.65 (C-8a), 144.07 (C-4), 136.35 (C-9), 121.99 (C-7), 116.5 (H-5), 114.75 (H-4a), 114.48 (C-3), 100.02 (H-8), 61.68 (9-OMe), 26.07 (C-1'), 20.73 (C-2'), 20.73 (C-3').

Buntansin (**13**): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 11.21 (6-COOH, s), 8.04 (1H, s, H-5), 7.63 (1H, d, J = 9.5 Hz, H-4), 6.9 (1H, s, H-8), 6.30 (1H, d, J = 9.5 Hz, H-3), 3.99 (3H, s, 6-OMe). – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 169.56 (6-COOH), 164.4 (C-7), 160.13 (C-2), 158.98 (C-8a), 143 (C-4), 130.74 (C-5), 114.26 (C-3), 110.05 (C-6), 111.97 (C-4a), 104.83 (C-8), 52.54 (7-OMe). – MS (EI,

70 eV): *m/z* (%) = 221 (53) [M^+ + 1], 171 (53), 153 (92), 101 (38), 87 (53), 78 (100), 59 (69).

Erythrodiol (**14**): ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.2 (1H, bs, H-12), 3.5 (1H, d, *J* = 11 Hz, H-28a), 3.22 (1H, d, *J* = 11 Hz, H-28b), 3.2 (1H, m, H-3), 1.17 (3H, s, H-27), 1 (3H, s, H-30), 0.94 (3H, s, H-29), 0.94 (3H, s, H-26), 0.89 (3H, s, H-25), 0.88 (3H, s, H-24), 0.79 (3H, s, H-23). – ^{13}C NMR (125 MHz, $CDCl_3$): δ = 144.19 (C-13), 122.36 (C-12), 79 (C-3), 69.71 (C-28), 55.14 (C-5), 47.56 (C-9), 46.44 (C-19), 42.32 (C-18), 41.7 (C-14), 39.8 (C-8), 38.77 (C-4), 38.57 (C-1), 36.92 (C-10), 36.92 (C-17), 34.07 (C-21), 33.2 (C-29), 32.55 (C-7), 31.05 (C-22), 30.96 (C-20), 28.08 (C-23), 27.19 (C-2), 25.53 (C-15), 25.94 (C-27), 23.58 (C-11), 23.52 (C-30), 21.97 (C-16), 18.34 (C-6), 16.72 (C-26), 15.58 (C-24), 15.52 (C-25). – MS (EI, 70 eV): *m/z* (%) = 443 (31) [M^+], 425 (50), 424 (100), 411 (56).

Cytotoxicity assay

Cytotoxic effects of the compounds were assessed using the modified MTT assay (Rahman *et al*., 2001). HT-29 (colon carcinoma), K-562 (leukemia), and T47D (breast ductal carcinoma) cell lines were harvested in RPMI 1640 cell culture medium (PAA, Piscataway, NJ, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, Hamburg, Germany), to produce a stock cell suspension. The NIH-3T3 (Swiss mouse embryo fibroblast) cell line was kept in Dulbecco's modified Eagle's medium (DMEM; PAA) supplemented with 10% FBS. One hundred IU/mL penicillin and 100 μg/mL streptomycin (Roche, Penzberg, Germany) were added to all media. All cell lines were cultured at 37 °C in an air/CO_2 (95:5) atmosphere. For assessment of cytotoxic effects, 10^4 cells/well were plated into 96-well plates (Nunc, Apogent, Denmark) and incubated for 24 h before the addition of drugs. After 72 h and 96 h of incubation of HT-29 and T47D cells, respectively, 20 μL of the MTT (Merck) reagent (5 mg/mL) in phosphate-buffered saline (PBS) were added to each well. The plates were incubated at 37 °C for 4 h, and then the medium was aspirated. In each well, the formed formazan crystals were dissolved in 100 μL dimethylsulfoxide (DMSO). After incubation at 37 °C for 10 min, absorbance of formazan was detected at 570 nm by a microplate reader (Anthos, Salzburg, Austria). The cell viability according to the MTT assay was calculated as percentage of the control value. Cytotoxicity was expressed as the concentration of compounds inhibiting the cell growth by 50% (IC_{50}). All tests and analyses were done in triplicate. The selectivity index (SI; expressed selectivity of the sample compared to the cell lines tested) was calculated from the IC_{50} ratio in NIH-3T3 cells versus cancerous cell lines. Any sample demonstrating an SI value > 2 was considered to have selectivity for the cancerous cell line.

Statistical analysis

IC_{50} values were calculated by sigma plot 10 software. Data representative of three independent experiments with similar results were presented as means ± standard deviation (SD).

Results and Discussion

Successive chromatographic separations of the methanol, ethyl acetate, and chloroform fractions of *L. salicaria* aerial parts yielded compounds **1**–**5**, **6**–**10**, and **11**–**14**, respectively (Fig. 1). The structures of these compounds were determined on the basis of spectroscopic data compared with those reported in the literature. The compounds included three coumarins: umbelliferone-6-carboxylic acid (**2**) (Zhao *et al*., 2012), the furanocoumarin peucedanin (**12**) (Alavi *et al*., 2008), and buntansin (**13**) (Huang *et al*., 1989); three ellagic acid derivatives: 3,3',4'-tri-*O*-methylellagic acid-4-*O*-*β*-D-(2"-acetyl)-glucopyranoside (**3**), 3,3',4'-tri-*O*-methylellagic acid-4-*O*-*β*-D-glucopyranoside (**4**), and 3,3',4'-tri-*O*-methylellagic acid (**9**) (Ndukwe *et al*., 2008; Li *et al*., 1999); three triterpenes: oleanolic acid (**8**), erythrodiol (**14**) (Mahato and Kundu, 1994), and the ursan-type triterpene corosolic acid (**10**) (Seo *et al.*, 1975); two steroids: daucosterol (**5**) (Kim *et al*., 2006) and *β*-sitosterol (**11**) (Kim *et al*., 2005); together with 5-hydroxypyrrolidin-2-one (**1**) (Chen *et al*., 2008), phytol (**6**) (Kim *et al*., 2005), and dodecanoic acid (**7**). Compounds **8** and **11** had been previously isolated from this plant but the others were found for the first time. To the best of our knowledge, coumarins and pyrrolidin derivatives have not previously been reported from this plant.

Three cancerous (HT-29, K-562, and T47D) and one normal (NIH-3T3) cell lines were exposed to various concentrations of the isolated compounds. In the MTT assay, **5**, **10**, **11**, and **14** were the most active compounds against the HT-29 cell line with

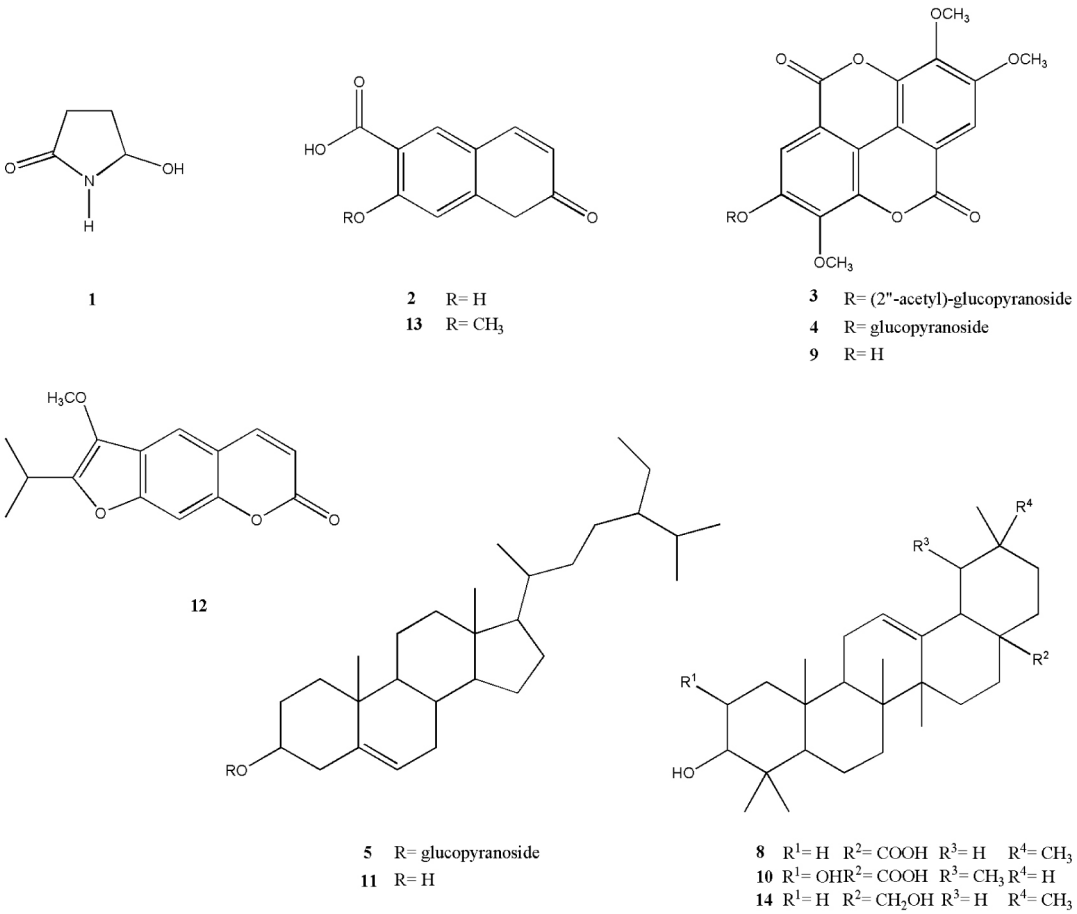

Fig. 1. Chemical structures of the compounds isolated from *L. salicaria*.

IC_{50} values of 192.7, 36.8, 38.2, and 12.8, μg/mL, respectively (Table I). Based on the SI values of these compounds against the HT-29 cancerous cell line, **14**, **11**, **5**, and **10** were 6.4, 2.8, 2.6, and 1.4 times, respectively, more selective than the positive control, methotrexate.

Compound **5** was the most active against the K-562 cell line (IC_{50} = 50.2 μg/mL) and was 13.3 times more selective than methotrexate. It was also active against the T47D cancerous cell line (IC_{50} = 229.8 μg/mL) and was 1.5 times more selective than methotrexate (Table I). All other compounds were less cytotoxic to any of the tested cancer cell lines compared with the normal cell line (NIH-3T3).

Thus, erythrodiol (**14**), corosolic acid (**10**), and β-sitosterol (**11**) inhibited the HT-29 cell line most strongly, and of these, compound **14** affected the growth of the HT-29 cell line (colon carcinoma cells) with relatively high selectivity (SI 6.7). Our results corroborate an earlier finding holding erythrodiol (**14**) to be responsible for the cytotoxic effect of *Viscum coloratum* against HO-8910 and SMMC-7721 cells (Yang *et al*., 2009). Furthermore, its mechanism of action has revealed that it has antiproliferative and pro-apoptotic activity toward the HT-29 cell line (Juan *et al*., 2008). Corosolic acid (**10**) has also been found to have potent cytotxic activity against several human cancer cell lines and to inhibit protein kinase C in a dose-dependent manner (Ahn *et al*., 1998). The major compound of *L. salicaria*, oleanolic acid (**8**), is a significant antitumour agent against the HCT15 cell line (Li *et al*., 2002). Finally, triterpene derivatives were found to inhibit the growth of HepG2 cells in a concentration-dependent manner (Ma *et al*., 2009). Antiproliferative and apoptosis-inducing activities of β-sitosterol (**11**) in human leuke-

mic U937 cells have been well documented (Park *et al*., 2007). In the present study daucosterol (**5**) showed a strong cytotoxicity against the K-562 cells with greatest tumour-specific cytotoxicity (SI 10.5). A cytotoxic effect of daucosterol at $>200\,\mu$g/mL has been reported previously against the human gastric cancer cell lines AGS and SNU638 (Jeong *et al*., 2009). Moreover, this result suggested that compound **5**, which possesses a sugar moiety, acts more selectively on the K-562, T47D, and HT-29 cell lines compared to its aglycone β-sitosterol (**11**). It seems that the sugar moiety is a determining factor for the higher selectivity and cytotxicity of **5** toward the K-562 and T47D cell lines. An interesting phenomenon has been observed with 5-hydroxypyrrolidin-2-one (**1**), *i.e.* a relatively weaker cytotoxicity to NIH-3T3 (normal cell line) as compared to the HT-29 and T47D cell lines.

Coumarin derivatives have been reported as cytotoxic agents (Kostova, 2005; Kawase *et al*., 2005), although the coumarins we isolated in our study had no significant cytotoxicity. However, peucedanin (**12**), with a furan ring, was more inhibitory to T47D than buntansin (**13**). A literature review revealed that some furanocoumarins exhibit a marked inhibitory effect on tumour cell lines (Kostova, 2005). The potential cytotoxic and antiproliferative activities of ellagic acid have been verified in human cancer cell lines. Prevention of tumour growth was attributed to the ability of ellagic acid to induce apoptosis through the intrinsic mitochondrial pathway (Sepulveda *et al*., 2011). Nevertheless, in our hands ellagic acid derivatives were not active against the cancerous cell lines ($IC_{50} > 150\,\mu$g/mL), which is comparable to the inactivity of two ellagic acid derivatives from *Turpinia formosana* against MCF-7, Daoy, WiDr, and Hep2 cancer cell lines (Huang *et al*., 2012).

Based on the SI values, some of the compounds were more selective than the positive control, methotrexate. Triterpenes and sterols appear to be responsible for the anticancer activity of the nonpolar extracts. Nonpolar fractions of the plant had shown cytotoxicity against the T47D cancer cell line in our previous study (Khanavi *et al*., 2011). According to our results, polar extracts of the plant, which are used in traditional medicine, are not toxic, while potent cytotoxic metabolites are present in the ethyl acetate and chloroform fractions.

In conclusion, the *L. salicaria* triterpene derivatives and sterols, respectively, **10**, **11**, and **14**, due to their low IC_{50} values against the HT-29 cell line, and compound **5** against K-562 cells, respectively, could protect against colon cancer development and leukemia.

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Table I. Cytotoxic activities of the *L. salicaria* compounds against three cancerous cell lines and the normal NIH-3T3 cell line determined by the MTT assay.

Sample	NIH-3T3	T47D		K-562		HT-29	
	IC_{50}[a] [μg/mL]	SI[b]	IC_{50}[a] [μg/mL]	SI[b]	IC_{50}[a] [μg/mL]	SI[b]	IC_{50}[a] [μg/mL]
Methotrexate	0.24 ± 0.013	1.5	0.16 ± 0.09	0.75	0.32 ± 0.04	1.0	0.23 ± 0.02
1	> 1000	–[c]	482.1 ± 7.98	–	–	–	472.3 ± 6.1
2	–	–	551.7 ± 5.8	–	> 1000	–	> 1000
3	711.7 ± 34.1	1.5	473.2 ± 9.12	1.2	563.2 ± 11.2	–	–
5	530.4 ± 6.7	2.3	229.7 ± 8.8	10.5	50.2 ± 1.12	2.6	192.7 ± 11.2
9	420.5 ± 10.7	1.1	374.5 ± 16.55	–	–	1.0	410 ± 12.1
10	54.4 ± 9.0	0.4	116.2 ± 14.27	0.2	231.4 ± 22.1	1.4	36.7 ± 4.0
11	109.5 ± 6.9	0.3	359.4 ± 4.7	0.2	573.8 ± 12.2	2.8	38.2 ± 1.4
12	42.3 ± 15.2	0.5	85.0 ± 7.07	0.3	123.2 ± 3.5	–	> 1000
13	50.4 ± 8.7	–	> 1000	–	–	–	> 1000
14	86.6 ± 1.2	0.5	164.9 ± 4.9	–	–	6.4	12.8 ± 3.4

[a] IC_{50}, concentration causing 50% growth inhibition ± standard deviation; it was calculated based on different concentrations (1.9, 3.9, 7.8, 15.6, 31.2, 62.5, 125, 250, 500, and 1000 μg/mL).
[b] SI (selectivity index) = IC_{50} for normal cell line/IC_{50} for cancerous cell line.
[c] Not determined.

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