Antiproliferative activities of isolated flavone glycosides and fatty acids from *Stachys byzantina*

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ABSTRACT

From the aerial parts of *Stachys byzantina* C. Koch., a new flavone glycoside, was isolated for the first time in addition to known two flavone glycosides. Structures were established by conventional methods of analysis and confirmed by 1H, 13C NMR and mass spectral analysis. Antiproliferative activities of isolated compounds, crude extract and fractions, fatty acids (extracts of hexane and hexane:ethyl acetate, 9:1) of aerial parts of *S. byzantina* were investigated against Vero (African green monkey kidney), HeLa (human uterus carcinoma) and C6 (rat brain tumor) cells in vitro and compared with 5-fluorouracil (5-FU). Antiproliferative effect of the extract, isolated flavonoids and fatty acids were tested at 100, 250, 500 and 1000 μg/ml using BrDU cell proliferation ELISA.

1. Introduction

The genus *Stachys* (Lamiaceae) includes about 200–300 species in the world (Rechinger, 1986). Plants of this genus have been used in folk medicine for centuries to treat genital tumors, sclerosis of the spleen, inflammatory diseases, cough, ulcers and infected wounds (Hartwell, 1982). Medicinal plants have been used in developing countries as alternative treatments to health problems. Many plant extracts and secondary metabolites isolated from plants have been shown to exert biological activity in vitro, which justified research on traditional medicine focused on the characterization of antiproliferative activity of these plants (Martinez et al., 1996). The Lamiaceae family is well-known because of the biological activities of its taxa. The subcosmopolitan genus *Stachys* compromises more than 270 species and is justifiably considered as one of the largest genera of the Lamiaceae (Skaltsa et al., 2001). In recent years, investigations on different taxa of this genus have showed that extracts or components of *Stachys* species exert various pharmacological effects: anti-inflammatory (Khanavi et al., 2005), antitoxic, antibacterial, antioxidant and cytotoxic (Salehi et al., 2005). Phytochemical studies in *Stachys* species have shown the presence of polyphenols including flavonoids (El-Ansari et al., 1995), tannins (Vundac et al., 2007), phenolic acids (Vundac et al., 2005), and phenyl ethanolid glycosides (Miyase et al., 1996). Most of species of this genus has been previously analyzed in numerous studies concerning their chemical composition, pharmacological properties and therapeutic uses. Nevertheless, the literature data on their antiproliferative activities are scarce and little is known about chemical components with antiproliferative activity. It seems that there is a significant relationship between the presence of total phenol and biological activity in *Stachys* species.

To the best of our knowledge, reports on the isolation of flavonoids and fatty acid contents of *S. byzantina* C. Koch and their antiproliferative activities are scant and there is no report on the antiproliferative activities in the literature. Thus, the present research reports (i) the isolation of compounds content of the secondary metabolites and fatty acids of *S. byzantina* C. Koch growing in Turkey, (ii) the antiproliferative activity of the extracts obtained by vacuum column chromatography (iii) testing antiproliferative activities for isolated flavonoid glycosides and fatty acids.

2. Results and discussion

2.1. Identification of compounds 1–3

The present study on the EtOAc/MeOH (3:1) fraction of *S. byzantina* resulted in the isolation and characterization of
compounds 1–3 (Fig. 1). The structures of these compounds – apigenin 7-O-β-D-(6′-trans-p-coumaroyl) glucoside (1), 4′-O-methyl isoscutellarein 7-O-β-D-allopyranosyl(1→2)-6′-O-acetyl-β-D-glucopyranoside (2), isoscutellarein 7-O-[β-D-allopyranosyl(1→2)-6′-O-acetyl-β-D-glucopyranoside] (3) were identified on the bases of spectroscopic methods and comparison with the data reported in the literature. These compounds were isolated for the first time from this plant.

The flavonoid 1 was obtained with the 6′-trans-p-coumaryl moieties. In the 1H NMR spectrum, the coupling constant (J = 7.2 Hz) of H-1′′ (δH 5.12, d) determined the β configuration of o-glucose, and the coupling constant (J = 15.9 Hz) of H-2′′′′ and H-3′′′′ (δH 6.32 and 7.48) was consistent with trans-olefinic protons in the α,β-unsaturated carbonyl system of the coumaryl moiety. From the HMBC spectrum, long-range correlations between H-1′′ (δH 5.12) and C-7 (δC 162.6), H-2′′′′ (δH 4.15 and 4.46) and C = O (δC 166.4) were found, indicating their relationships. Thus, the structure of 1 was assigned as apigenin 7-O-β-D-(6′-trans-p-coumaroyl) glucoside. The spectroscopic data with shifting reagents was indicative of a flavone skeleton for compounds 2

Table 1

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Fig. 1. Isolated compounds from S. byzantina.

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The flavonoids obtained as the 6’-acetylated derivative of the diglucose moiety was attached to the oxygen at C-7. The molecular formulas of 2 and 3 were determined to be C_{30}H_{35}O_{17} and C_{29}H_{35}O_{17}, respectively. The IR spectra indicated absorption bands for a hydroxyl, carbonyl, ester carbonyl and aromatic rings. The 3’,4’-disubstitutions on the B ring were indicated by a pair of ortho-coupled (J = 9.0 Hz) doublets at (δH 8.08 and 7.12) for compound 2. The ¹H-NMR and ¹³C-NMR spectrum of compound 2 were similar to those of 3, except for the methoxy substitution at C-4’ (Table 1).

2.2. The results of antiproliferative activities

2.2.1. Antiproliferative activities of fraction 1 and 2 against C6, HeLa and Vero cell lines

The antiproliferative activity of the fraction 1 (hexane) and 2 (10% EtOAc/hexane) were evaluated at the concentration of 500, 250 and 100 µg/mL. The antiproliferative activity of the fraction were compared with 5-fluorouracil used as standards.

According to Fig. 2A, antiproliferative activity of fraction 2 was obtained to increase depend on increase of dose against C6 cell line. However, antiproliferative activity of fraction 2 was determined to have the similar activity with 5-fluorouracil (5-FU) at 500 µg/mL. On the contrary fraction 2, at antiproliferative activity of fraction 1 was shown to decrease depend on increase of dose against C6 cell line (Fig. 2A).

According to Fig. 2B, antiproliferative activity of fraction 2 increased with increasing the dose against HeLa cell line. But, fraction 1 has weak activity against HeLa cell line.

At the antiproliferative activities of fraction 1 and 2 were observed to increase dose dependent manner against Vero cell line (Fig. 2C). In addition, antiproliferative activity of fraction 2 was determined to have the similar activity with 5-fluorouracil (5-FU) at 500 and 250 µg/mL.

2.2.2. Antiproliferative activities of crude extract, fractions and isolated compounds against Vero cell line

According to Table 2A, either crude extract or fractions 3–9 have been shown strong antiproliferative activity against Vero cell line. The each dose (500 and 250 µg/mL) has inhibited above 80% proliferation of Vero cell line. The antiproliferative activity of crude extract (fraction 7) was higher than the antiproliferative activity of 5-FU (Table 2B). However, the antiproliferative activities of compounds 1–3 were higher than 5-FU at 500 µg/mL (Table 2B).

2.3. Determination of fatty acids at fraction 1 and 2 from S. byzantina

Total sixteen fatty acids were determined at fraction 1 and 2. The fraction 1 was shown five saturated fatty acids and six unsaturated fatty acids (Table 3). Main components were palmitic acid (31.30%) and γ-linolenic acid (19.66%) at fraction 1.
fraction 2 was determined with nine saturated fatty acids and seven unsaturated fatty acids (Table 3). The main components were stearic acid (31.29%) and linoelaidic acid (27.15%) at fraction 2.

2.4. HPLC–TOF chromatograms of S. byzantina

HPLC–TOF chromatograms of the fractions were recorded. In the chromatograms, compounds 1 and 2 were determined at fraction 5 and 6 (Fig. 3 and Table 2A). Compounds 2 and 3 were observed at fractions 7, 8 and 9. Compounds 1–3 were observed in the HPLC–OF chromatogram of the crude extract.

In conclusions, cell proliferation involves complex combinations of many biochemical processes, and different chemicals might influence different biochemical processes or stages in different manners. The evaluation of antiproliferative capacity of individual compounds, fractions and fatty acids, with the employment of sophisticated assays, stands as a challenging perspective in order to further pinpoint the pharmacological efficacy and safety of S. byzantina.

3. Materials and methods

3.1. General experimental procedures

The mass spectral analyses were carried out using a Micromass Quattro LC–MS/MS spectrometer. 1H NMR and 13C NMR spectra were obtained in DMSO solution using 400 MHz H-1 and 100 MHz C-13 Bruker Avance-III NMR instrument. Infrared spectra were obtained using IR Jasco 430 FT/IR spectrophotometer. Melting points were determined by using a Barnstead Elektrotermal melting points apparatus. UV–Vis spectral analyses were carried out by using JASCO V 530 spectrophotometer at 25 °C. Thin-layer
chromatography (TLC) was carried out on Merck precoated 60 Kieselgel F254 analytical aluminum plates.

3.2. Plant material

*Stachys byzantina* was collected from campus area of Tokat, Turkey during June–July 2006 and was identified by Ass. Prof. Dr. H. Askın Akpulat from Cumhuriyet University, faculty of Science and Literature, Department of Biology. A voucher specimen has been deposited (CUFH 4316) at the department of Botany, Faculty of Science and Art, Cumhuriyet University. Three human cell lines were used for cytotoxicity screening, Vero cells (African green monkey kidney), C6 cells (Rat Brain tumor cells) and HeLa cells (human uterus carcinoma) were kindly provided by Assist. Prof. Dr. Nazli Arda (Department of Molecular Biology, Istanbul University, Turkey).

3.3. Gas chromatography (GC) analysis

Oil obtained from aerial parts of *S. byzantina* was analysed by gas chromatography (GC) on Perkin Elmer Clarus 500 with BPX-20 capillary column (30 m × 0.25 mm, 0.25 μm film thickness) 5% phenyl polysilphenylene-siloxane at an ionization voltage of 70 eV equipped with an FID detector. Oven temperature was programmed from 50 to 120 °C at 5 °C/min and from 120 °C to 240 °C at 10 °C/min and hold for 5 min in the final temperature. Diluted samples of 1.0 μL were injected in the 300:1 split mode. Injector and detector temperatures were set at 220 °C and 290 °C, respectively. Helium was used as carrier gas at a flow rate of 1 mL/min, and diluted samples (1/1000).

3.4. Gas chromatography/mass spectrometry (GC/MS) analysis

GC/MS analyses were obtained on Perkin Elmer mass spectrometer with built-in-Autosampler using BPX-20 column (30 m × 0.25 mm × 0.25 μm film). For GC/MS detection, an electron ionization system, ionization energy of 70 eV, was used. Helium was the carrier gas, at a flow rate of 1.3 mL/min. The column temperate was operated under the same conditions as described above.

3.5. Fatty acid separation

Dried and finely powdered aerial parts (963 g) were extracted with 1:1 methanol:chloroform (3 × 5 L) at room temperature for 2 weeks. After removal of the solvent with evaporator at 40 °C, the residue (207 g, 20%, w/w) was subjected to vacuum column chromatography, eluting with hexane and 1:9 ethyl acetate:hexane (1) to give lipophilic extract as 1.74 g and 3.2 g hexane and ethyl acetate:hexane fractions, respectively. All chemicals used were of purified reagents or higher grade.

3.6. Identification of fatty acids

Identification of the individual components was based on: (a) comparison of their GC retention indices (RI) with those of authentic compounds or literature data; (b) computer matching with a mass spectral library and commercial libraries (WILLEY and NIST database/ChemStation data system). The composition of the fatty acid fractions from aerial parts of *S. byzantina* is listed in Table 3 according to their retention indices on a PBX-20 column.

3.7. Analysis of isolated compounds from fraction 7 by HPLC–TOF/MS

Phenolic content of the plant extract determined by using Agilent Technologies 1260 Infinity HPLC System coupled with 6210 Time of Flight (TOF) LC/MS detector and Agilent Poroshell 120EC-18 (3.0 × 50 mm, 2.7 μm) column (Demirtas et al., 2013). Mobile phases A and B were ultra-pure water solution with 0.1% formic acid and acetonitrile, respectively. Flow rate was 0.4 mL/min and column temperature was 35 °C. Injection volume was 10 μL. Solvent program was as follow: 0 min 10% B; 0–1 min 10% B; 1–10 min 80% B; 10–19 min 80% B; 19–19.10 min 10% B; 19.10–29.10 min 10% B. Retention times and m/z values of standard

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**Fig. 3.** HPLC–TOF chromatograms of crude extract and fractions from *S. byzantina*. 
compounds were used on the determination step. Ionization mode of MS–TOF instrument was negative with gas temperature of 325 °C, gas flow of 11.0 L/min, and nebulizer of 45 (psi).

3.8. Extraction and isolation of flavonoids

Dried and finely powdered aerial parts (963 g) were extracted with methanol:methylene chloride (1:1, 3 × 5 L) at room temperature for 2 weeks. After removal of the solvent in vacuum at 40 °C, the residue (207 g, 20%, w/w) was subjected to vacuum column chromatography, eluting with respectively hexane (Fraction 1), 1/9 ethyl acetate/hexane (Fraction 2), 1/3 ethyl acetate/hexane (Fraction 3), 1/1 ethyl acetate/hexane (Fraction 4), 3/1 ethyl acetate/hexane (Fraction 5), ethyl acetate (Fraction 6), 3/1 ethyl acetate/methanol (Fraction 7), 1/1 ethyl acetate/methanol (Fraction 8), methanol (Fraction 9) (see Table 2A).

The ethyl acetate/methanol (3:1) fraction (Fraction 7) was subjected to column chromatography and eluted with methanol and a gradient of methanol/chloroform. The fraction eluted with methanol/chloroform (20/80) yielded compound 1 (35 mg). The fraction eluted with methanol/chloroform (1/3) yielded compound 2 (55 mg) and the fraction eluted with methanol/chloroform (1:1) yielded compound 3 (52 mg).

3.8.1. Apigenin 7-O-β-D-(6'-trans-p-coumaryl) glucoside (1)

Mp 244 °C, compound was isolated as a dark white solid with a molecular formula of C_{36}H_{34}O_{12}, based on its LC–MS/MS, m/z 578.52 [M⁺] and HPLC–TOF, m/z 577.1315 (calculated m/z 577.1346) in negative mode (see Table 2, S13); its UV spectrum, with λ_{max} (nm) CH_{3}OH: 289, 367; IR ν_{max} cm⁻¹ KBr: 3546, 2933, 1639, 1617, 1079; ¹H and ¹³C NMR (Table 1).

3.8.2. 4'-O-Methyl isoscutellarein 7-O-[β-D-allopyranosyl-(1→2)⟩6'-O-acetyl-β-D-O-glucopyranoside] (2)

Mp 247 °C, compound was isolated as a pale yellow, amorphous powder, molecular formula of C_{39}H_{38}O_{13}, based on its LC–MS/MS m/z 666.18 [M⁺] and HPLC–TOF m/z 665.1685 (calculated m/z 665.1718) in negative mode (see Table 2, S13); UV λ_{max} (nm) CH_{3}OH: 306, 326; IR ν_{max} cm⁻¹ KBr: 3417, 3237, 1639, 1617, 1222; ¹H and ¹³C NMR (Table 1).

3.8.3. Isoscutellarein 7-O-[β-D-allopyranosyl-(1→2)-6'-O-acetyl-β-D-O-glucopyranoside] (3)

Mp 235 °C, compound was isolated as a pale yellow, amorphous powder, molecular formula of C_{36}H_{32}O_{17}, based on its LC–MS/MS m/z 652.55 [M⁺] and HPLC–TOF/MS m/z 651.1538 (calculated m/z 651.1561) in negative mode (see Table 2, S13); UV λ_{max} (nm) CH_{3}OH: 305, 328; IR ν_{max} cm⁻¹ KBr: 34,133,236, 1617, 1637, 1218; ¹H and ¹³C NMR (Table 1).

3.9. Antiproliferative activity

3.9.1. Cell lines and cell culture

HeLa, Vero and C6 cancer cell lines were grown in Dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% penicillin streptomycin. The medium was changed twice a week.

3.9.2. Cell proliferation assay

Antiproliferative effect of the plant was investigated on HeLa, Vero and C6 cell lines using proliferation BrdU ELISA assay (Demirtas et al., 2009; Demirtas and Sahin, 2013). Cultured cells were grown in 96-well plates (COSTAR, Corning, USA) at a density of 3 × 10⁴ cells/well. The cell lines were exposed to two concentrations of crude extract and fractions from S. byzantina for 24 h at 37 °C in a humidified atmosphere of 5% CO₂. 5-Fluorouracil was used as standard compound. Cells were then incubated for overnight before applying the BrdU cell proliferation ELISA assay reagent (Roche, Germany) according to manufacturer’s procedure. The amount of cell proliferation was assessed by determining the A450 nm of the culture media after addition of the substrate solution by using a microplate reader (Ryto, China). Results were reported as percentage of the inhibition of cell proliferation, where the optical density measured from vehicle-treated cells was considered to be 100% of proliferation. Percentage of inhibition of cell proliferation was calculated as follows: [1-(A_{treatment}/A_{vehicle control})] × 100.

3.10. Statistical analysis

The results of investigation in vitro are means ± SD of nine measurements. Differences between groups were tested by analysis of variance (ANOVA). p values of < 0.05 were considered significant.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.02.001.

References


