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# Bioactivity-guided isolation of cytotoxic secondary metabolites from the roots of *Glycyrrhiza glabra* and elucidation of their mechanisms of action

Dicle Çevik[a], Ş. Burçin Yılmazgöz[b], Yüksel Kan[c], Ece Akhan Güzelcan[d], Irem Durmaz[d], Rengül Çetin-Atalay[d], Hasan Kırmızıbekmez[a,*]

[a] Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, TR-34755, Kayışdağı, İstanbul, Turkey
[b] Faculty of Pharmacy, Yeditepe University, TR-34755, Kayışdağı, İstanbul, Turkey
[c] Department of Medicinal Plants, Faculty of Agriculture, Selçuk University, TR-42070, Konya, Turkey
[d] Cancer Systems Biology Laboratory, Graduate School of Informatics, METU, TR-06800, Ankara, Turkey

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ABSTRACT

Licorice (*Glycyrrhiza glabra* L.) is one of the most widely used plants worldwide for its various pharmacological activities. The aim of this study was to isolate the potential cytotoxic secondary metabolites from the MeOH extract prepared from the roots of *Glycyrrhiza glabra* through bioactivity-guided isolation procedure and to elucidate their mechanisms of action. The crude MeOH extract as well as $CHCl_3$ and EtOAc subextracts significantly inhibited cell proliferation on hepatocelullar (Huh7), breast (MCF7) and colorectal (HCT116) cancer cell lines with $IC_{50}$ values in the range of 5.6 to 33.6 µg/mL. Chromatographic seperations of the $CHCl_3$ and EtOAc subextracts yielded 13 secondary metabolites. Structures were characterized based on NMR and MS data. Amongst isolates, glabridin (**1**), 4′-*O*-methylglabridin (**2**), β-amyrin (3), kanzonol U (4), glabrene (**7**) and tetrahydroxymethoxychalcone (10) were established to be responsible for *in vitro* cytotoxicity of *G. glabra*, exerting the best activity particularly against Huh7 cells. Further mechanistic studies demonstrated that 2 and 7 induced caspase-dependent apoptosis by increasing cytochrome C release and subsequently cleaved caspase-9 level in Huh7 cells. Moreover, both compounds decreased pRb and p21 levels and thus induced the accumulation of Huh-7 cells in subG$_1$ and G$_2$/M phases. Compound 10 which displayed the most potent activity in Hoechst staining and cell cycle assays through G$_2$/M arrest, caused cell death by apoptosis in Huh7 cells.

## 1. Introduction

Cancer is the second leading cause of morbidity and mortality after cardiovascular diseases in industrialized countries. It had been 14.1 million new cancer cases whereas 8.2 million cancer deaths in 2012. Breast cancer has the highest incidence rate among women followed by colorectal cancer. Among men colorectal cancer is the third whereas, liver cancer which has a relatively poor survival and hence mortality is the fifth common sites of cancer (WHO Cancer Report, 2014). Cancer arises from the loss of balance between cell division and cell death. Apoptosis is regulated destruction of cells, plays an important role and is the main target in cancer treatment (Wong, 2011). This is a very complicated process including both intrinsic and extrinsic pathways. Release of cytochrom C from mitochondria into cytosol is occured via intrinsic pathway which subsequently activates caspase-9 and thus caspase-3 (Hengartner, 2000). Activation of these caspases eventually leads to apotosis through caspase cascade by cleaving of poly (ADP-ribose) polymerase (PARP) levels (Mohan et al., 2016; Shyu et al., 2010).

Most of the drugs used in cancer treatment today are not effective enough or have some serious side effects, thus new anticancer drugs are needed to combat cancer. Natural resources particularly medicinal plants play a very significant role for the discovery and development of new drug leads. Clinically used anticancer drugs such as vincristine, vinblastine, vindesine, paclitaxel, etoposide, teniposide, irinotecan and topotecan are natural products or their derivatives which are produced by semi-synthesis from a natural molecule (Saklani and Kutty, 2008).

The genus *Glycyrrhiza* (Fabaceae) commonly known as licorice, contains approximately 30 species distributed worldwide including, *G. glabra*, *G. aspera* and *G. uralensis* (Nomura et al., 2002). In Flora of Turkey, it is represented by six species being *G. glabra*, the most prevalent one (Chamberlain, 1969). *Glycyrrhiza glabra* L. has a long history of use in different folk medicines for the treatment of peptic ulcers, against inflammatory diseases as well as an expectorant agent. In

* Corresponding author.
*E-mail address:* hasankbekmez@yahoo.com (H. Kırmızıbekmez).

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Traditional Chinese Medicine licorice which is known as Gancao, is used for its nourishing, analgesic, spleen and stomach tonifying, expectorant and relieving cough effects (Yang et al., 2015). It is one of the most widely used plants in phytotherapy and food industry due to its miscellaneous pharmacological activities and sweet taste. The secondary metabolites, including triterpene saponins, flavonoids and isoflavonoids have been isolated from licorice as the major chemical classes (Hosseinzadeh and Nassiri-Asl, 2015). Previous bioactivity studies on licorice and some of its secondary metabolites revealed antiulcer (Wittschier et al., 2009), antimicrobial (Kırmızıbekmez et al., 2015), hepatoprotective (Ji et al., 2016), anti-inflammatory (Thiyagarajan et al., 2011), immunomodulatory, memory enhancing, antiprotozoal, cytotoxic and antitumor activities (Yang et al., 2015).

In recent years, biological activities particularly anticancer and cytotoxic effects of *Glycyrrhiza* extracts and isolated secondary metabolites have attracted much interest. Previous *in vitro* studies demonstrated that *Glycyrrhiza* extracts have cytotoxic activites against several cancer cells in different degrees (Aydemir et al., 2011; Park et al., 2014; Vlaisavljević et al., 2018). Moreover, the secondary metabolites obtained from several *Glycyrrhiza* species were shown to possess significant cytotoxic and anticancer activities in recent years (Tang et al., 2015; Ji et al., 2016; Li et al., 2017; Lin et al., 2017). Considering the wide utilization of *G. glabra* in folk medicines and phytotherapy as well as the recently documented bioactivities of several *Glycyrrhiza* species, we aimed to isolate the cytotoxic secondary metabolites from roots of *G. glabra* through *in vitro* cytotoxicity-guided isolation techniques. Furthermore, the underlying mechanisms behind this cytotoxic activites of the bioactive isolates were also elucidated.

## 2. Materials and methods

### 2.1. General procedures

Chromatography: Column chromatography (CC) was performed on silica gel 60 (0.063-0.200 mm; Merck, Darmstadt, Germany), Sephadex LH-20 (25–100 µm; Sigma-Aldrich, St. Louis, MO, USA) and Polyamide (50–160 µm; Fluka Analytical, Sigma-Aldrich, St. Louis, MO, USA). Medium-pressure liquid chromatography (MPLC) was performed by Sepacore® Flash Systems X10/X50 (Buchi Labortechnik AG, Flawil, Switzerland) on Redi sep columns LiChroprep $C_{18}$ and $SiO_2$ (Teledyne Isco, Lincoln, Nebraska, USA). Thin layer chromatography (TLC) analyses were carried out on silica gel 60 $F_{254}$ plates (9.5–11.5 µm; Merck, Darmstadt, Germany) on aluminum, visiualization was established by spraying with 1% vanillin/sulphuric acid solution followed by heating at 105 °C for 2–3 min, detected with a UV at 254 and 365 nm. HPLC system, Agilents Technologies HP1100 (Agilent Technologies, Waldbronn, Germany) equipped with a Vacuum degasser G1379 A, quaternary pump G1311 A, an auto-sampler G1313 A, a thermo-stated column compartment G1316 A and a diode array detector G1315B. The separation was performed on an Agilent Zorbax Eclipse Plus $C_{18}$ ODS column (5 µm, 250 mm × 9.4 mm, i.d.). UV Spectra: HP Agilent 8453 spectrophotometer (Agilent Technologies, Santa Clara, CA, USA); λmax in nm. IR Spectra (KBr): PerkinElmer 2000 FT-IR spectrometer (PerkinElmer, Waltham, Massachusetts, USA); $\upsilon$ in $cm^{-1}$. NMR Spectra: $^1H$ (400 MHz), $^{13}C$ (100 MHz), COSY, HSQC, HMBC and NOESY NMR spectra were recorded on a Varian Mercury FT spectrometer (Palo Alto, CA, USA) in $CD_3OD$ or $CDCl_3$; $\delta$ in ppm rel. $Me_4Si$ as internal standard, $J$ in Hz. Mass Spectroscopy: Agilent G6530B TOF/Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) in MeOH; positive-ion mode; in $m/z$.

### 2.2. Plant material

The roots of *Glycyrrhiza glabra* L. were collected from Medicinal Plant Experimental Garden, Faculty of Agriculture, Selçuk University, Konya, Turkey, where the plant species was cultivated. Voucher specimen (YEF 15005) has been deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Yeditepe University, İstanbul, Turkey.

**Table 1**
Cytotoxic activity results of crude MeOH extract, subextracts and the main fractions of the active subextracts of *G. glabra* against Huh7, MCF7 and HCT116 cancer cell lines.

| Extracts/Fractions | Huh7 | | MCF7 | | HCT116 | |
|---|---|---|---|---|---|---|
| | $IC_{50}$(µg/ml)[a] | $R^2$ | $IC_{50}$(µg/ml)[a] | $R^2$ | $IC_{50}$ (µg/ml)[a] | $R^2$ |
| MeOH | 18.9 | 0.6 | 28.8 | 0.6 | 33.6 | 0.7 |
| $CHCl_3$ | 13.3 | 0.9 | 29.3 | 0.6 | 8.2 | 0.9 |
| Fr. B | 14 | 0.8 | 14.6 | 0.8 | 18.5 | 0.8 |
| Fr. C | 9.2 | 0.7 | 8.9 | 0.6 | 14.9 | 0.6 |
| EtOAc | 5.6 | 0.9 | 9.1 | 0.9 | 7.4 | 0.9 |
| Fr. 2 | NI | – | NI | – | NI | – |
| Fr. 3 | NI | – | NI | – | NI | – |
| Fr. 4 | 4.9 | 0.9 | 2.5 | 0.9 | 1.3 | 0.7 |
| *n*-buOH | NI | | NI | | NI | |
| $H_2O$ | NI | | NI | | NI | |

NI: No Inhibition.
[a] $IC_{50}$ values were calculated from the cell growth inhibition curves obtained from the treatments with increasing concentrations of extracts or fractions (30, 15, 7.5, 3.25 and 1.875 µM) for 72 h. Experiments were done in triplicate.

### 2.3. Extraction and isolation

The dried and powdered roots of *G. glabra* (370 g) were extracted with MeOH (2 × 2.5 L, 4 h). The combined extracts were concentrated under reduced pressure to afford crude MeOH extract (87.2 g) which was suspended in 200 mL of $H_2O$ and partitioned three times with equal volumes (each 200 mL) of $CHCl_3$, EtOAc and *n*-buOH, respectively to yield subextracts. The crude MeOH extract and subextracts were evaluated for their cytotoxic activities by Sulphorodamine B (SRB) assay (Table 1). The cytotoxically active subextracts, $CHCl_3$ and EtOAc were purified by successive chromatographic methods. $CHCl_3$ subextract (3.92 g) fractionated over Sephadex LH-20 (90 g) CC eluted with $CH_2Cl_2$:MeOH (1:1, 400 mL) and MeOH (200 mL), respectively to yield three main fractions (fr. A–C). EtOAc subextract (5.4 g) was loaded onto poliamide column (40 g) eluting with $H_2O$ and stepwise gradient of MeOH in $H_2O$ (25–100% in steps of 25%, each 100 mL) to obtain frs.1-4. These main fractions were also evaluated in the same cell panel (Table 1). Active fractions (frs. B and C from $CHCl_3$, fr. 4 from EtOAc) were subjected to chromatographic seperations. Fr. B (2.6 g) was separated by $SiO_2$-MPLC (120 g) eluting with stepwise *n*-hexane:EtOAc gradient (0–100% EtOAc) to obtain $B_{1\text{-}18}$ subfractions. Subfraction $B_8$ (34 mg) was further purified by $SiO_2$-MPLC (12 g), eluted with the same mobile phase (0–20% EtOAc) to isolate **2** (9 mg). Subfraction $B_{11}$ (147 mg) was applied to Sephadex LH-20 (10 g) column with $CH_2Cl_2$:MeOH (1:1) to give **3** (3 mg). Subfraction fr. $B_{13}$ (409 mg) was submitted to MPLC ($SiO_2$, 40 g) eluting with stepwise gradient of *n*-hexane:EtOAc (0–20% EtOAc) to yield **1** (13 mg) and fr. $B_{13\text{-}b}$ (100 mg) which was purified by $SiO_2$-MPLC (40 g) with *n*-hexane:EtOAc mixture (0–60%, EtOAc) to obtain **4** (7 mg). Fractionation of subfraction $B_{14}$ (199 mg) by MPLC ($SiO_2$, 12 g) eluting with stepwise of *n*-hexane:EtOAc gradient (20–100% EtOAc) yielded fr. $B_{14\text{-}a}$ (101 mg) which was further applied to Sephadex LH-20 (8 g) with MeOH to obtain fr. $B_{14\text{-}a\text{-}1}$ (20 mg). Compound **11** (4 mg) was purified from fr. $B_{14\text{-}a\text{-}1}$ by semi-preparative HPLC on Zorbax Eclipse XDB-$C_{18}$ column (9.4 × 250 mm) eluted with $H_2O$:MeCN mixture (10–50%, MeCN). Fr. C (100 mg) was fractionated over $SiO_2$-MPLC (24 g) eluting with *n*-hexane:EtOAc (10–50% EtOAc) to afford subfractions, $C_{1\text{-}4}$. Compound **2** (2 mg) was isolated from subfraction $C_1$ (20 mg) by Sephadex LH-20 CC (35 g) eluted with MeOH. Fr. $C_2$ (20 mg) was separated by Sephadex LH-20 (6 g, MeOH) followed by MPLC ($SiO_2$, 24 g, *n*-hexane-EtOAc, 90:10 and