Original Research

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Journal of Environmental Biology

JEB™

ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

DOI : http://doi.org/10.22438/jeb/38/5/MRN-420

In-vitro studies on antioxidant, anti-melanogenic and anti-wrinkle effects of medicinal plant *Glycyrrhiza uralensis*

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Key words

Anti-melanogenesis, Antioxidant effect, Anti-wrinkle effect, *Glycyrrhiza uralensis*

Publication Info

Paper received : 23.07.2016
Revised received : 19.01.2017
Accepted : 23.03.2017

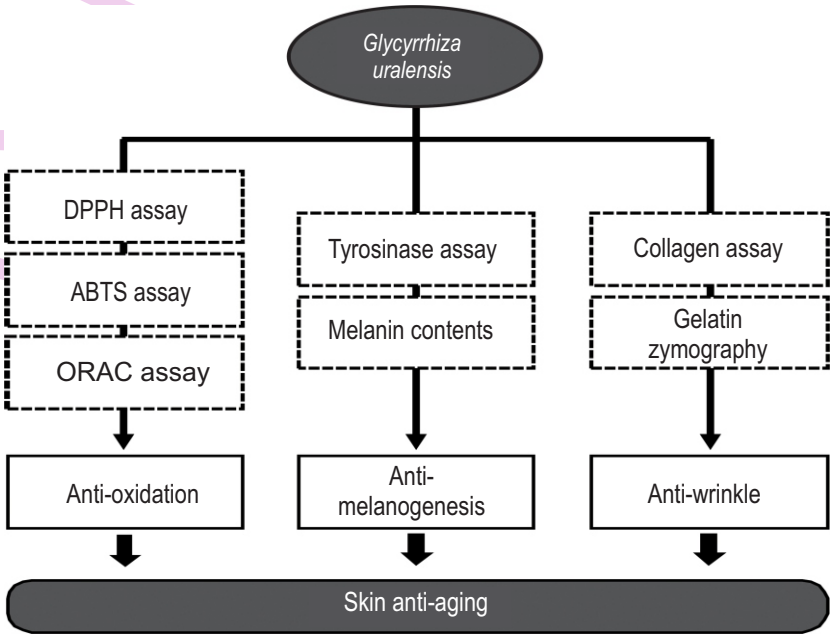
Abstract

Aim: The aim of the present study was to investigate the antioxidant, anti-melanogenic and anti-wrinkle activity of *Glycyrrhiza uralensis (G. uralensis)* for cosmeceutical application.

Methodology: The free radical scavenging activity of 1,3-butylene glycol (1,3-BG) extract of *G. uralensis* was analyzed using 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and oxygen radical absorbance capacity (ORAC) assays. The anti-melanogenesis and anti-wrinkle effects of *G. uralensis* extract were also analyzed by tyrosinase, zymography and collagen assays.

Results: The EC_{50} of 1,3-BG extract of *G. uralensis,* determined by DPPH and ABTS assays, was 0.59% and 1.17%, respectively.The ORAC value of the extract was 41.56 µM trolox equivalent. The *G. uralensis* extract inhibited *in vitro* mushroom tyrosinase activity with an IC_{50} of 0.6%, and it also reduced melanin contents in mouse melanoma cells. In addition, the *G. uralensis* extract notably inhibited matrix metalloproteinase-2 activity *via* zymography. Approximately, 60% collagen production enhancement was observed upon treatment with the extract, suggesting its anti-wrinkle potential.

Interpretation: The 1,3-BG extract of *G. uralensis* may be utilized as a cosmeceutical ingredient, offering anti-oxidant, brightening and anti-wrinkle effects.



Introduction

Progressive loss of structural integrity and physiological function of the skin occur with aging by intrinsic and extrinsic factors (Farage *et al*., 2008). The inevitable intrinsic skin aging is due to physiological aging (Bergfeld, 1997), while extrinsic skin aging is due to diverse determinants such as sun exposure, external pollutants (Cha *et al*., 2015), smoking (Kennedy *et al.,* 2003) and diet (Draelos, 2013). Among the various extrinsic skin aging determinants, the most prominent extrinsic factor is UV-induced photoaging.Several reactive oxygen species (ROS); namely hydrogen peroxide (H_2O_2), superoxide anion radical (O_2-), hydroxyl radical (·OH) and singlet oxygen ($^1O_2$), are produced *in vitro* and *in vivo* upon exposure to UV (Herrling *et al*., 2006; Lee and Lee, 2016; Ryu *et al*., 2016) which occurs near or within the cell surface membrane of human skin (Yasui *et al*., 2000; Xu *et al*., 2005). In particular H_2O_2, one of the major reactive oxygen species, is produced in significant amounts during normal aerobic metabolism of skin cells (Dash *et al*., 2008).The accumulation of ROS leads to enhanced matrix metalloproteinases (MMPs) activity and collagen breakdown in the dermis.

Numerous researches using various medicinal plants have been performed to overcome skin aging and some of the results have been applied and utilized in developing cosmeceuticals for whitening, anti-wrinkle or anti-aging purposes.

Glycyrrhiza uralensis, a licorice species of plant, also known as gancao, sweet root or kanzoh, is used in traditional Chinese medicine. *G.uralensis* has been used as a"harmonizing"ingredient in Korean traditional medicine, Hanyak. Glycyrrhizin, the main sweet-tasting constituent of *G. uralensis,* has been reported to scavenge free radicals and block the chain reaction of lipid peroxidation (Binic *et al*., 2013). Moreover, its antioxidant, anti-inflammatory, chemopreventive, and antiproliferative activity have been reported (Rahman *et al*., 2007; Visavadiya *et al.,* 2009; Binic *et al.,* 2013).However, information on the cosmeceutical activity of *G. uralensis* is limited. *G. uralensis* extract in most research has been mainly prepared in methanol. Recently, glycol, including 1,3-butylene glycol (1,3-BG) has been widely used in the cosmetic industry and personal care products.It is used as a preservative, emulsifier, solidifier, plasticizer in hair and bath products, eye and facial makeup, fragrances and skin care preparations (Antoce *et al.,* 1998). The aim of this study was to investigate the *in vitro* cosmeceutical effect of 1,3-BG extract of *G. uralensis* in focusing on its antioxidative, anti-melanogenic and anti-wrinkle efficacy.

Materials and Methods

Antioxidative activity assay : DPPH radical cation scavenging activity of *G. uralensis* was estimated according to the method of Blois (1958) with some modification. A 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Sigma-Aldrich, MO, USA; 50 µl) was added into each extract, and absorbance was then measured at 520 nm with a spectrophotometer (Sunrise, Tecan, Männedorf, Switzerland). ABTS radicals scavenging activity was assayed by the method of Leelarungrayub *et al.* (2006) with some modification. For the estimation of decrease in $ABTS^{\cdot+}$ radicals, $ABTS^{\cdot+}$ solution was added rapidly to the extract and the absorbance was measured at 620 nm. The ORAC assay was modified based on Wu *et al.* (2008). Various concentrations of Trolox calibration solution and fluorescein solution were added to a well in black 96-well microplate. And then 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) solution was added to each well as peroxyl generator to the start reaction. The microplate reader from Tecan (Männedorf, Switzerland) was programmed to record the fluorescence reading with an excitation wavelength of 485 nm and an emission wavelength of 530 nm at 4 min interval for 40 min.

Tyrosinase activity inhibition assay : Tyrosinase activity was determined by spectrophotometry, as described by Joo *et al*. (2015) with modifications. A 10 µl of 5 mM L-3,4-dihydroxyphenylalanine (L-DOPA) solution (Sigma-Aldrich, MO,USA), 70 µl of 100 mM phosphate buffer (pH 6.8) and 10 µl of *G. uralensis* extract were mixed. Then, 10 µl of mushroom tyrosinase (500 U ml^{-1}, Sigma-Aldrich, MO, USA) was added. The increase in absorption at 475 nm due to the formation of DOPA chrome was determined with a spectrophotometer from Tecan (Männedorf, Switzerland) at 1 min interval for 2 min. The initial rate was used as a measure for the tyrosinase activity. The percent inhibition of tyrosinase activity was calculated as follows :

Tyrosinase inhibition activity (% of control) = [(As (2 min) – As (0 min)) / (A0 (2 min) - A0 (0 min))] × 100 where, A_0 is the absorbance of tyrosinase without sample, as is the absorbance of sample and tyrosinase.

Cell culture : Human dermal fibroblast (HDF-n) cell line and mus musculus skin melanoma (B16F10) was obtained from the American Type Culture Collection (ATCC, Rockville, USA). Cells were maintained in DMEM [with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (PS)] in a humidified atmosphere containing 5% CO_2 at 37°C (Kim *et al.*, 2016).

MTT cell viability assay : B16F10 cell viability was measured by 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay (Kim and Lee, 2011). The cells were cultured in 96-well plates at a density of 1×10^4 cells per well. The cells were then treated with varying concentrations of *G. uralensis* extract for 72 hrs. The cells were washed and treated with MTT, after which the plates were incubated at 37°C in dark for 3 hrs. After the formation of formazan, 50 µl of DMSO was added and the absorbance was measured at 570 nm using a microtiter plate reader.

Inhibition of melanin production : Extracellular released melanin production was estimated following the method of Jeong *et al.* (2013), with slight mordifications. B16F10 cells were incubated at a density of 2×10^5 cells in 6-well plates for 24 hrs. After incubation, the cells were treated with phenol red free

DMEM including 50 nM α-melanin stimulating hormone (α-MSH), 1% FBS, 1% PS, and 0.1% plant extracts for 5 days. The supernatants were transferred to 96-well plates and optical densities (OD) were measured at 405 nm with an ELISA reader. Inhibiting activity of melanin production was exhibited as percentage of α-MSH treated controls.

Collagen production-promoting assay : Collagen contents in the ECM on HDF-n cells were determined by Sircol collagen assay (Biocolor, UK) according to the manufacturer's protocols. HDF-n cells were seeded 1.5×10^5 in 6-well plates and incubated for 24 hrs at 37 °C. After incubation, *G. uralensis* extract was diluted in DMEM (with 1% PS) to 0.3125% and treated for 24 hrs at 37 °C. After removing the medium, the cells were incubated in exchanged new DMEM (with 1% PS) for 24 hrs at 37 °C. The supernatant was collected and added to Sircol dye reagent, and collagen dye complexes formed were precipitated out from the soluble unbound dye. After centrifugation, the pellet was washed with ice-cold acid-salt wash reagent and then reacted with alkali reagent. The samples were transferred to 96-well plate and the absorbance was read at 570 nm. The amount of collagen was calculated based on a standard curve obtained with the standard bovine type I collagen supplied with the kit.

Gelatin zymography : The MMP activity on HDF-n cells was analyzed using gelatin zymography, which was slightly modified based on Buzoglu *et al.* (2009). The MMP activity inhibition by *G. uralensis* extract against oxidative stress by H_2O_2 was investigated. A 8% sodium dodecyl sulfate (SDS)–polyacrylamide running gel containing 0.1% (wt vol^{-1}) gelatin was overlaid with 5% acrylamide stacking gel. The sample was mixed with an equal volume of Laemmli buffer (10% SDS; 125 mM Tris-HCl, pH 6.8, 10% glycerol and 0.002% bromophenol blue) and then the gel was run at 100 V for 2 hrs. After electrophoresis, gels were soaked and washed twice in 2.5% Triton X-100 for 30 min at room temperature. And then incubated in 50 mM Tris buffer, pH 7.6, containing 10 mM $CaCl_2$, 50 mM NaCl, and 0.05% Brij 35 (wt vol^{-1}) with gentle shaking at room temperature for 30 min. Following incubation, the gels were stained for 2 hrs with 0.5% coomassie brilliant blue (CBB) R-250 in a solution of 20% acetic acid and 20% methanol, and were then destained for 30 min in the same solution without CBB R-250. CBB R-250-stained gel was scanned and analyzed by image analysis software (ImageMaster 2D Platinum 6.0 software, GE Healthcare, Fairfield, CT, USA).

Statistical analysis : All experiments were carried out in triplicate and analyzed by SPSS package for Windows version 13 (SPSS Inc., Chicago, IL). The analytical data were shown as mean ±SD. The results were then subjected to one-way analysis of variance (ANOVA), the significance of mean differences was determined by Duncan's multiple range test at $p< 0.05$.

Results and Discussion

The DPPH and ABTS radical scavenging activity of *G. uralensis* extract is shown in Table 1. The *G. uralensis* extract showed prominent radical scavenging activity in a concentration dependent manner. The EC_{50} value of *G. uralensis,* representing the concentration of *G. uralensis* capable of scavenging 50% of the DPPH radical,was 0.59%. The EC_{50} value of *G. uralensis* for ABTS radical was 1.17%. An ORAC assay, the most sensitive method to determine a substance's antioxidative activity, revealed that *G. uralensis* extract had a value of 41.56 μM trolox equivalent (TE). The ORAC values were determined by TE curve. These results showed that *G. uralensis* extract had prominent antioxidant activity.

A higher melanogenic activity of melanocytes and deeper skin pigmentation were commonly found in skin of older than in younger people (Lock-Andersen *et al.*, 1998). In addition, the generation of ROS can induce α-MSH and ACTH to trigger excess melanin production in melanocytes. Tyrosinase plays a pivotal role in the regulation of melanogenesis, as well as catalyzes three distinct reactions in the melanogenic pathway: hydroxylation of monophenol, dehydrogenation of catechol and dehydrogenation of 5,6-dihydroxyindole (DHI) (Slominski *et al*., 2004).

As shown in Fig. 1, tyrosinase activity was inhibited by *G. uralensis* extract in a concentration dependent manner. Approximately, 30% and 50% of tyrosinase activity were inhibited at 0.59% and 1.17% of *G. uralensis*, respectively. The notable inhibition of *in vitro* tyrosinase activity by *G. uralensis* may be due to its antioxidant activity.

The inhibitory effect of *G. uralensis* extract on melanin production in B16F10 cells was also examined (Fig. 2). The cytotoxicity of *G. uralensis* extract on B16F10 cells was first checked before carrying out the anti-melanogenesis

Table 1 : Antioxidant activity of *G. uralensis* extract

	Antioxidant concentration (%, EC_{50})		
Sample	**DPPH**	**ABTS**	**ORAC (μM TE)**
G. uralensis	0.59	1.17	41.56

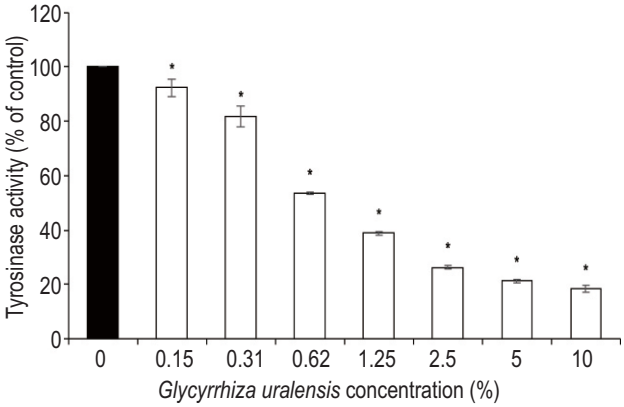


Fig. 1: Inhibition of tyrosinase activity by *G. uralensis* extract in a concentration dependent manner

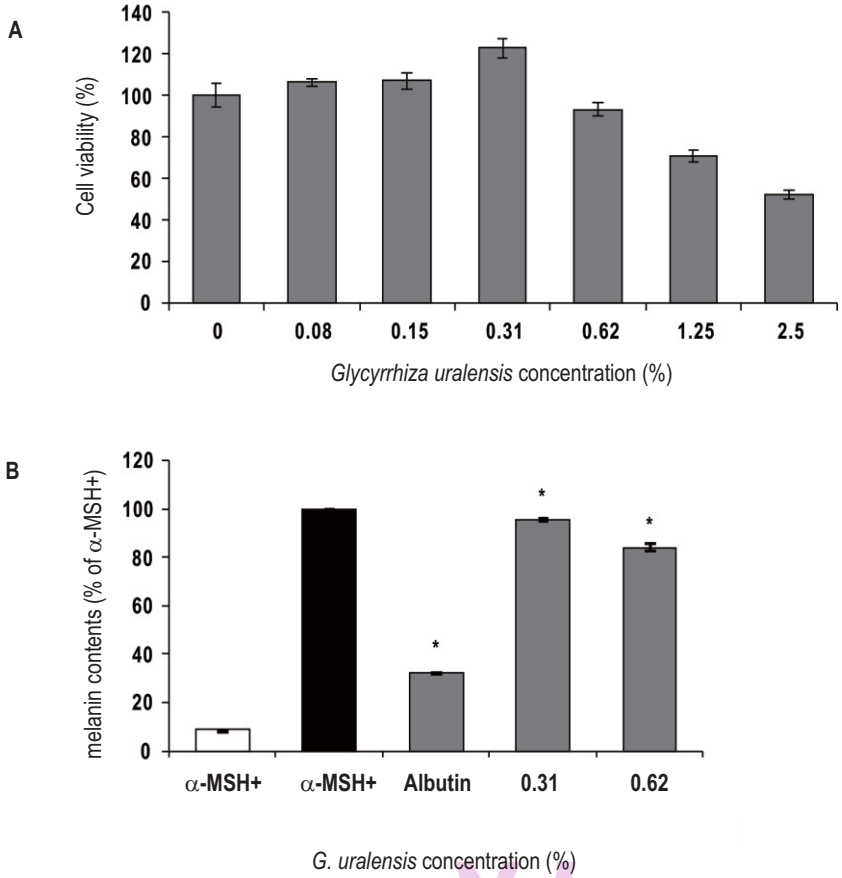


Fig. 2 : The effect of *G. uralensis* extract on (A) cell viability of B16F10 cells and (B) melanin production of B16F10 cells

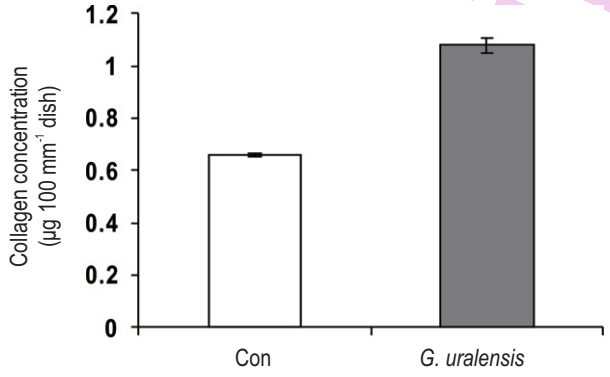


Fig. 3 : Collagen production by *G. uralensis* extract in HDF-n cells

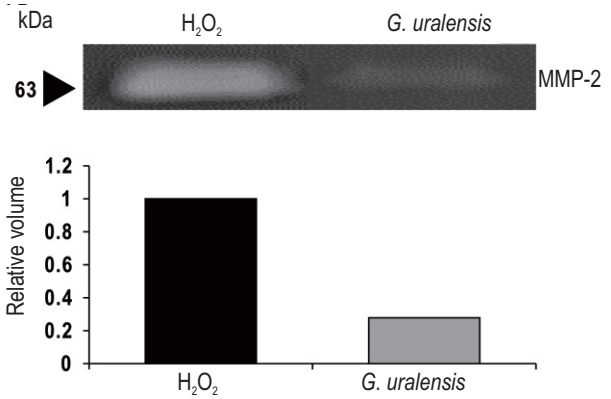


Fig. 4 : The effect of *G. uralensis* extract on H_2O_2-induced MMP-2 activity on zymographic gel

experiment (Fig. 2A). Nearly, all the cells were viable until the concentration of *G. uralensis* extract reached 0.62%, beyond which the cell survival declined rapidly. Thus,0.31% and 0.62% *G. uralensis* extract were used in theanti-melanogenesis test. Upon treatment with 0.31% and 0.62% *G. uralensis,* the melanin levels in α-MSH-induced B16F10 cells were significantly reduced compared with control (α-MSH without *G. uralensis*). This result suggests that *G. uralensis* extract possess skin-brightening activity, as evident by its anti-melanogenic activity.

Human skin is a main target of ROS produced via extrinsic and intrinsic factors, such as solar exposure and metabolically generated pro-oxidants like H_2O_2 (Quan and Fisher,

2015). ROS-exposed human skin through several factors, including exposure to UV, has been reported to enhance matrix metalloproteinases (MMPs) activity associated with notable breakdown of collagen fibers (Ham *et al.*, 2014; Lee *et al.*, 2016). MMPs are among the major enzymes responsible in the degradation of extracellular matrix which contain diverse biomolecules including collagen and gelatin (Sardy, 2009). Among the several types of MMPs, gelatinases (MMP-2 and -9) cleave soluble type-IV and type-I collagen (Yu *et al.*, 2013).

The collagen production-promoting effect of *G. uralensis* extract was determined by Sircol collagen assay kit (Fig. 3).The collagen production value was 0.66 µg without any cell proliferator on HDF-n cells cultured for 24 hrs. However, *G. uralensis* extract-treated cells showed approximately 63% augmentation in collagen production compared to the control.The collagen content in cells upon treatment with *G. uralensis* extract was 1.07 µg, while the control value was 0.66µg.

Gelatin zymography was performed to examine the inhibitory activity of *G. uralensis* extract against active MMP-2 on the gel (Fig. 4).Treatment with *G. uralensis* extract significantly reduced the MMP-2 activity of the active MMP-2 enzyme (62 kDa) in 20 µM H_2O_2-treated HDF-n cells compared to untreated control, as shown by the clear band, indicating gelatin degradation against the blue background.

The present study demonstrated that 1,3-BG extract of *G. uralensis* reduced H_2O_2-induced MMP-2 activity along with notably enhanced collagen production (Fig. 3, 4). The results also indicate that *G. uralensis* extract can induce suppressive effect against H_2O_2-induced MMP-2 expression, probably due to its antioxidative capacity (Lee *et al.*, 2013; Liu *et al.*, 2014). The results clearly revealed that *G. uralensis* extract suppressed MMP-2 via attenuating ROS formation in HDF-n cells.

In conclusion, this study suggests that 1,3-BG extract of *G. uralensis* showed profound anti-wrinkle and anti-melanogenic effect *in vitro*, which may be related to its antioxidant activity. It appears that *G. uralensis* extract may be utilized as a multifunctional ingredient with anti-oxidant, brightening and anti-wrinkle effects in developing better cosmetic products.

Acknowledgment

This work was supported by the Soonchunhyang University research fund.

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