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Quantification of betulinic, oleanolic and ursolic acid as medicinally important triterpenoids in wild and *in vitro* callus culture of *Salvia sahendica* (Lamiaceae): a comparative study

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Key words: *Salvia* sp., Lamiaceae, Pentacyclic triterpenoids, Callus culture, HPLC.

Abstract

In the present study, quantitative determination of betulinic acid (BA), oleanolic acid (OA) and ursolic acid (UA) as well-known medicinal pentacyclic triterpenoids was simultaneously carried out in wild and *in vitro* callus culture of *Salvia sahendica* Boiss. Buhse (Lamiaceae) by reverse-phase high performance liquid chromatography (HPLC). The plant is an endemic medicinal species which is growing in the northwest of Iran. Callus induction (100%) was achieved from young leaf and intermodal explants cultured on Murashige and Skoog (MS) supplemented with 1.0 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.5 mg/L 6-benzylaminopurine (BAP). Our results revealed that the content of BA, OA and UA in the aerial parts of wild *S. sahendica* was 15.05, 645.93 and 112.92 mg per 100g dry weight (DW) (standard deviation: 0.73-3.0), respectively while their content in callus culture of the plant were 17.28, 126.27 and 121.59 mg per 100g DW (standard deviation: 1.6-3.2), respectively. Our findings show the merit of *in vitro* callus culture of *S. sahendica* for production of medicinally important triterpenoids. It can be also provide an ample opportunity to take this plant for extensive research for mass cultivation on plants and enhanced the production of these compounds through different biotechnological strategies like cell suspension cultures and large scale cultivation in bioreactor system.

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Introduction

Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid, BA), oleanolic acid (3β-hydroxyolean-12-en-28-oic acid, OA), and ursolic acid (3β-hydroxyurs-12-en-28-oic acid, UA) are highly sought-after pentacyclic triterpenoids (PTs, Fig. 1) because of their wide spectrum of biological activities (Liu, 1995; Zhang *et al.*, 2014). They are most highly regarded for their antiinflammatory, hepatoprotective, antimicrobial, anti-HIV-1 activity, antiulcer, gastroprotective, hypoglycemic, antihyperlipidemic activity and specific cytotoxicity against a variety of tumor cell lines (Liu, 1995 and 2005; Ghaffari Moghaddam *et al.* 2012; Pertino *et al.* 2013). PTs naturally occur in the raw plant materials such as berries, leaves, flowers, and fruits. Their content has been previously assayed in different plant families (Farina *et al.*, 1998; Zhang *et al.*, 2002; Neto, 2007; Jager *et al.*, 2009; Ayatollahi *et al.*, 2011; Begum *et al.* 2014). Lamiaceae, one of the most important family among the medicinal plants, has been reported as a wide-ranging source for isolation of free BA, OA and UA besides other compounds (Mendes *et al.*, 1989; Tezuka *et al.*, 2000; Tan *et al.*, 2002). So far, isolation and quantitative determination of PTs have been performed in many members of Lamiaceae family such as *Leonurus cardiaca* (Ali *et al.*, 2007), *Rosmarinus officinalis*, *Salvia officinalis*, *Satureja montana*, *Salvia sclarea*, *Salvia glutinosa* (Razboršek *et al.*, 2008) and *Thymus persicus* (Bakhtiar *et al.* 2014).

The genus *Salvia* L. is one of the largest genera in the family Lamiaceae and represents approximately 1000 species (Hedge, 1982). The genus is represented in the flora of Iran by 61 species, 17 of which as *Salvia sahendica* Boiss. & Buhse are endemic (Rechinger, 1982; Mozaffarian, 1996). *S. sahendica* is restricted to some regions around Sahand Mountain in northwest Iran, where is locally named 'Maryamgoli-Sahandi' (Rechinger, 1982; Mozaffarian, 1990). The plant is traditionally used as spasmolytic, astringent, antiseptic and treatment of Dyspepsia (Lotfipour *et al.*, 2007). In addition, the essential oils and various extract of *S. sahendica* were found to have biological properties such as antibacterial and antioxidant activities (Salehi *et al.*, 2004; Esmaeili *et al.*, 2009). Neuroprotective effects of the plant have been recently reported as well (Shaerzadeh *et al.*, 2011). During our ongoing efforts to investigate natural sources of PTs, we found *S. sahendica* as a potent plant species. Due to the importance of BA, OA and UA in clinical medicine, we attempted to simultaneously quantify these compounds in wild mature and callus culture of the plant. Our findings can be provide an ample opportunity to take this plant for extensive research for mass cultivation on plants and enhanced the production of these compounds through different biotechnological strategies like cell suspension cultures and large scale cultivation in bioreactor system.

Materials and methods

Plant material and chemicals

Seeds and one-year-old mature plant aerial parts of *S. sahendica* were collected from regions around Sahand Mountain (37°57′ N, 46°22′ E at an altitude of 1675 m) in northwestern Iran. A voucher specimen of the plant (MPH-1992) has been deposited at the Herbarium of Medicinal Plants and Drugs Research Institute (MPH), Shahid Beheshti University, Tehran, Iran.

Basal media salts, vitamins, sucrose, agar, PGRs, HPLC grade methanol and standards of BA, OA, and UA were purchased from Merck (Darmstadt, Germany) and Sigma (Sigma-Aldrich Corporation, MO, USA). Methanol and phosphoric acid of analytical grade were obtained from Merck (Darmstadt, Germany). HPLC grade water was used throughout the analysis.

In vitro callus induction

S. sahendica seeds were soaked in 70% ethanol for 1 min and surface-sterilized with 1% (v/v) of commercial bleach (5% sodium hypochlorite) for 8 min, followed by three rinses in sterile distilled water. The seeds were aseptically sown in glass Petri dishes (10-cm inner diameter) on half-strength MS medium

(Murashige & Skoog 1962) containing 1% (w/v) sucrose and incubated in the dark at 25±2 ºC. *In vitro* callus induction and culture maintenance have been performed as described before (Santos-Gomes *et al.*, 2003). Callus tissues were harvested and washed with distilled water three times to remove any residual medium. Then, the calli were lyophilised (Lyophilizer, CHRiST, Germany) at – 40 °C until a constant weight was achieved.

Extraction and HPLC analysis

Dried aerial parts and *in vitro* calli of *S. sahendica* were extracted for the HPLC analyses according to the method previously reported by Srivastava and Chaturvedi (2010). The extract was dissolved in HPLC grade methanol (10 mL), filtered through a Millipore filter (0.45 mm) and stored in a refrigerator until analysis. A Knauer liquid chromatography apparatus consisting of a 1000 Smartline Pump, a 5000 Smartline Manager Solvent Organizer and a 2800 Smartline Photo- diode Array Detector was used for the HPLC analysis (Wang *et al.*, 2008).

Results and discussion

The simultaneous quantitative HPLC determination of BA, OA and UA in the aerial parts and *in vitro* callus culture of *S. sahendica* is reported here for the first time. According to Wang *et al.* (2008), a mobile phase consisting of MeOH-phosphoric acid-water (87:0.05:12.95, v/v/v, isocratically) gave peaks at a retention time (RT) of 20.1, 21.9, and 22.9 min for BA, OA and UA, respectively (Table 1). Identification of these PTs in extracts was based on the comparison of their retention time with their authentic references, spike of standards and their UV spectra. Our results revealed that the content of BA, OA and UA in the aerial parts of wild *S. sahendica* was 15.05, 645.93 and 112.92 mg per 100g dry weight (DW) (standard deviation: 0.73-3.0), respectively while their content in callus culture of the plant were 17.28, 126.27 and 121.59 mg per 100g DW (standard deviation: 1.6-3.2), respectively (Table 2). Simultaneous determination and quantification of BA, OA, and UA from the parent plant and *in vitro*-raised cultures of *Lanata camara* (Verbenaceae) have been also reported by Srivastava and Chaturvedi (2010). Razboršek *et al.* (2008) reported detection and quantification of BA, OA, and UA in some members of Lamiaceae by gas chromatography-mass spectrometry (GC-MS) and they obtained 0.6% BA, 0.09–0.9% OA, and 0.09–1.6% UA in dried plant materials. Jäger *et al.* (2009) studied PTs distribution in various plants and found that the Lamiaceae family is an especially good source for BA, OA and UA, reaching the highest concentration measured within *Rosmarinus officinalis* leaves. Due to the importance of BA, OA and UA as natural compounds with potent antitumor activities (Zhang *et al.* 2014; Radwan and Alanzi, 2014), there is a need for further investigations on the other plant source as well as optimization of their in vitro cultures. Our results indicate that *in vitro*-raised cultures as callus culture can be produce PTs as well as wild mother plant. Thus, in vitro cultures provide an ample opportunity to take this plant for extensive research for mass cultivation on plants and enhanced antitumor compounds production through different biotechnological strategies like cell suspension cultures and large scale cultivation in bioreactor system.

Table 1. Standard curves and retention times of triterpene acids.

Compound	Retention time	Standard equation	R^2
Betulinic acid	20.1	Y=6953.7416x+1074.3353	0.9991
Oleanolic acid	21.9	Y=9623.8718x–22522.2671	0.9994
Ursolic acid	22.9	Y=8365.6058x–16086.3008	0.9994

Y peak area, x concentration (mg), R^2 correlation coefficient.

Table 2. Content, and recovery percentage of pentacyclic triterpenoids in wild aerial parts and callus culture of *Salvia sahendica*.

Compound	Content (mg 100g $^{-1}$ DW±*SD*)		Recovery (%)
	Wild plant	Callus culture	
Betulinic acid	15.05±0.73	17.28±1.68	99.8
Oleanolic acid	645.93±1.33	126.27±3.21	99.6
Ursolic acid	112.92±3.05	121.59±1.66	103.6

SD standard deviation; *n*=3.

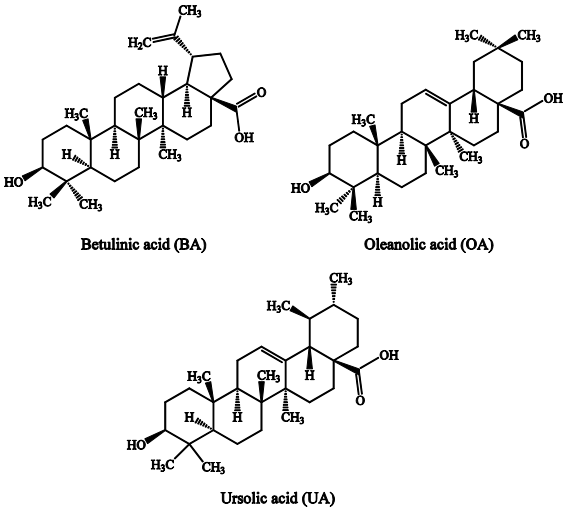

Fig. 1. Chemical structures of pentacyclic triterpenoids (PTs).

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