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Original Article

Cytotoxic diterpenoids from the roots of *Salvia lachnocalyx*

Hossein Hadavand Mirzaei[a], Omidreza Firuzi[a], Bernd Schneider[b], Ian T. Baldwin[c], Amir Reza Jassbi[a,*]

[a] *Medicinal and Natural Products Chemistry Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[b] *Forschungsgruppe Biosynthese/NMR, Max-Planck-Institut für chemische Ökologie, Jena, Deutschland (Germany)*
[c] *Abteilung für Molekulare Ökologie, Max-Planck-Institut für chemische Ökologie, Jena, Deutschland (Germany)*

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ABSTRACT

Salvia lachnocalyx Hedge, Lamiaceae, is an endemic sage which grows naturally in the Fars Province of Iran. The phytochemical analyses of the roots of *S. lachnocalyx* led to the isolation of five known diterpenoids: ferruginol (**1**), taxodione (**2**), sahandinone (**3**), 4-dehydrosalvilimbinol (**4**) and labda-7,14-dien-13-ol (**5**). Their chemical structures were elucidated using one (^{1}H and ^{13}C) and two dimensional (COSY, HSQC and HMBC) NMR spectroscopic data as well as electron impact mass spectra. The cytotoxicity of the purified compounds was evaluated against three human cancer cell lines; MOLT-4 (acute lymphoblastic leukemia), HT-29 (colorectal adenocarcinoma) and MCF7 (breast adenocarcinoma) and all of the isolated compounds showed considerable cytotoxic activity against these cell lines. Compounds **2** and **3** (IC_{50} range: 0.41–3.87 μg/ml) with endocyclic α,β-unsaturated carbonyl functional group, exhibited the highest cytotoxic activities compared to the other compounds (IC_{50} range: 6.85–17.23 μg/ml). In conclusion, compounds **2** and **3** are presented as compounds that deserve further investigation of their biological activities.

Introduction

The genus *Salvia*, Laminaceae, consists of more than 900 species that are widely dispersed throughout the world. There are 61 *Salvia* species naturally growing in Iran, seventeen of them are endemic (Jamzad, 2012). Several species of *Salvia* are pharmacologically active and have been widely used in folk medicine (Wu et al., 2013; Firuzi et al., 2013; Jassbi et al., 2017). Bioassay-guided purification of chemical constituents of *Salvia* species has resulted in the isolation of many bioactive phytochemicals such as flavonoids, phenolics, terpenoids and steroids (Jassbi et al., 2017). Among them, more than 550 diterpenoids have been reported from different *Salvia* species (Kabouche and Kabouche, 2008), most of which have shown a broad spectrum of interesting biological activities and ecological roles, that include antimicrobial, anticancer, antiviral, antioxidant and anti-inflammatory activities (González, 2015; Jassbi et al., 2017). Among different classes of anticancer drugs, natural products remain a main source of potential drugs to treat human tumors. According to a recent publication on the role of natural products in the field of anticancer drugs, about 75% of small molecules with anticancer effect have been directly or indirectly derived from natural products (Newman and Cragg, 2016).

Salvia lachnocalyx Hedge is an endemic sage which grows naturally in the Fars Province of Iran (Jamzad, 2012). Recently, three sesterterpene lactones, two sesterterpenoids, three flavonoids, two steroids, one norditerpenoid and one triterpenoid were reported from an acetone extract of the aerial parts of *S. lachnocalyx* (Farimani and Mazarei, 2014). Furthermore, the essential oil of *S. lachnocalyx* has been analyzed by GC–MS and bicyclogermacrene, α-pinene, sabinene and β-pinene have been detected as major constituents (Mirza and Bahernik, 2007). To the best of our knowledge, the present report is the first bioassay-guided phytochemical analyses of the roots of *S. lachnocalyx* for isolation and structural elucidation of its cytotoxic active natural products.

Material and methods

Reagents and chemicals

Fetal bovine serum (FBS), phosphate buffered saline (PBS), RPMI 1640 and trypsin were purchased from Biosera (Ringmer, UK). Acetonitrile (ACN, HPLC grade), dichloromethane (DCM), dimethyl sulfoxide (DMSO), methanol (MeOH), silica gel (70–230 mesh) for open column chromatography and pre-coated silica gel F_{254} TLC aluminum sheets were obtained from Merck (Darmstadt,

* Corresponding author.
E-mail: jassbiar@sums.ac.ir (A.R. Jassbi).

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Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma–Aldrich (St. Louis, MO, USA), while cisplatin and penicillin/streptomycin were provided from EBEWE Pharma (Unterach, Austria).

Instrumentation

The purified compounds were subjected to NMR spectroscopy measurements on a Bruker Avance 400 spectrometer (BioSpin GmbH, Rheinstetten, Germany) at resonance frequencies of 400.13 for ^{1}H and 100.6 MHz for ^{13}C. ^{1}H and ^{13}C NMR were measured in $CDCl_3$ with TMS as an internal standard. Mass spectra (EI-MS) were recorded on an Agilent 5975C inert GC/MSD instrument.

The optimization of separation conditions and the evaluation of purity percentage of the isolated compounds were assessed by analytical HPLC using different gradients of ACN and H_2O on a RP C18 HPLC column (Eurospher-100, 250 × 4.6 mm, Knauer, Berlin, Germany) using a Knauer system (Knauer), that consisted of a K-1800 pump and a K-2500 UV–Vis detector.

Separations on preparative HPLC (prep. HPLC) were developed on a Knauer system, that consisted of a K-1800 pump, with an RP C18 (Eurospher II 100-5 C18, 250 × 20 mm ID with pre-column 30 × 20 mm ID) column, eluted isocratically with ACN/H_2O (85:15) at 20 ml/min. The K-2500 UV–vis detector was set at 210 nm

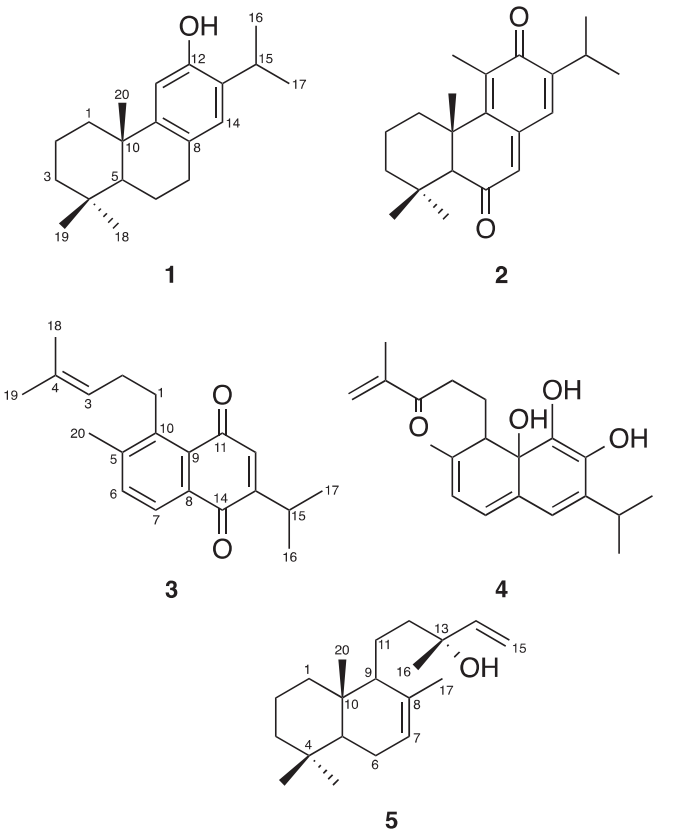
Optical rotations were measured on a Krüss P8000 polarimeter.

Plant material

The roots of *Salvia lachnocalyx* Hedge, Lamiaceae, were collected from Eghlid in the Fars Province, Iran, in May 2015 and identified by Mojtaba Asadollahi, plant taxonomist at the Medicinal and Natural Products Chemistry Research Center (MNCRC). A voucher specimen (No. 94-3-8-4) was deposited at the MNCRC, Shiraz University of Medical Sciences, Fars, Iran.

Extraction, fractionation and purification

The air-dried, ground roots of *S. lachnocalyx* (400 g) were extracted with DCM (3 × 2 l) at room temperature for two days. The extracts were filtered and the solvent was evaporated under reduced pressure at 40 °C. The concentrated extract (6 g; yield = 1.5%, w/w) was subjected to open column chromatography (100 × 5 cm) over silica gel (110 g, 70–230 mesh). The elution of the column was performed using an *n*-hexane with gradient of DCM up to 100%, and then followed by increasing the polarity of the mobile phase to pure MeOH. This chromatographic separation afforded 49 fractions. After checking the purity of fractions by TLC, similar fractions were pooled to afford overall 22 fractions (F1–F22). Fraction F15, eluted with hexane-DCM (80:20), was evaporated to dryness (420 mg), reconstituted in MeOH, centrifuged at 5000 × *g* for 10 min and filtered through a 0.45 μm syringe filter before HPLC analyses. The residue (70 mg) and the upper layer (350 mg) were stored separately. A part of the methanol soluble fraction (20 μl, 200 μg/ml) was repeatedly analyzed by analytical HPLC with various solvent systems to optimize the separation conditions. The optimal conditions were used for the prep. HPLC and the fractions were collected manually according to the chromatographic profiles. Then, 20 μl of each of the fractions obtained by prep. HPLC were injected onto the analytical HPLC column to evaluate their purity. The purity was measured by HPLC peak-area normalized method. According to the analytical HPLC results, the fractions with identical retention times were combined and evaporated under reduced pressure to afford the pure natural products. Purification by prep. HPLC afforded compound **1** (13 mg, HPLC purity ≥ 98%), **2** (43 mg, HPLC purity ≥ 97%), **3** (144 mg, HPLC purity ≥ 95%), **4** (17 mg, HPLC purity ≥ 97%), and **5** (9 mg, HPLC purity ≥ 97%). The pure phytochemicals were stored at −20 °C until performing the spectroscopic experiments and cytotoxicity assays.



Cell lines and culture

HT-29 (human colorectal adenocarcinoma, Cell bank number: IBRC C10097) cell line was purchased from Iranian Biological Resources Center, Tehran, Iran, while MCF7 (human breast adenocarcinoma, Cell bank number: C135) and MOLT-4 (human lymphoblastic leukemia, Cell bank number: C149) cells were obtained from National Cell Bank of Iran, Pasteur Institute, Tehran, Iran. The cells were cultured in sterile T25 flasks in RPMI 1640 medium supplemented with 10% v/v fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). HT-29 and MCF7 cell lines were grown in monolayer cultures, while MOLT-4 cells were grown in suspension cultures in humidified air containing 5% CO_2 at 37 °C.

Cytotoxicity assay

The inhibitory effect of purified compounds on cancer cell proliferation was assessed by the MTT reduction assay (Mosmann, 1983). This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the purple formazan by the action of mitochondrial dehydrogenase enzymes in viable cells. The purified compounds were dissolved in DMSO, and then diluted in growth medium at least 400 times. Cells were seeded in 96-well plates at the density of 50,000 cells/ml in 100 μl of growth medium. After 24 h of incubation at 37 °C, 50 μl of the medium was exchanged with 50 μl of test compounds diluted in fresh growth medium (3–4 different concentrations). After 72 h of further incubation, the medium of each well was replaced by RPMI without phenol red containing MTT 0.5 mg/ml and incubated at 37 °C for 4 h. DMSO was used to solubilize the formed formazan crystals. The absorbance of wells was measured at 570 nm, with background

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