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Short communication

Anti-melanoma effects of ingenanes isolated from *Euphorbia* species

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Introduction

The Euphorbiaceae family is one of the most widespread plant families containing about 300 genera and more than 8000 species (Webster, 1994). The plants belonging to this genus are well known for the chemical diversity of their terpene constituents. Literature data indicate that metabolites isolated from this genus have analgesic, antipyretic, antimicrobial, antiviral, antiproliferative, and selective antitumor activity (Jadranin et al., 2013, Krstić et al., 2018, Wang et al., 2003). The most important metabolite isolated from the genus is ingenol-mebutate. It was isolated from the latex of *Euphorbia paralias* and *E. peplus*, and it has been registered under phase III clinical trials under the commercial name Picato® for the treatment of actinic keratosis and pre-cancerous and cancerous skin changes (Aditya and Gupta, 2013, Seca and Pinto, 2018).

In this research, from two species, *E. palustris* and *E. lucida*, four ingenane derivatives were isolated. Their anticancer effects were evaluated in the human melanoma – 518A2 cell line and compared with the effects of ingenol-mebutate. Selectivity towards human melanoma cells was determined using normal human keratinocytes – HaCaT.

Materials and methods

Plant materials

The *E. palustris* latex was collected in Besni Fok (Serbia), in May 2011, while the *E. lucida* stems were collected in Makiš (Serbia), in May 2012. The plants were identified by Professor Petar Marin, University of Belgrade – Faculty of Biology, Institute of Botany. Voucher specimens (No. 16,877 for *E. palustris*, and No. 16,879 for *E. lucida*) have been deposited at the Herbarium of Botanical Garden "Jevremovac", University of Belgrade, Belgrade (Serbia).

Isolation and purification

The collected latex of *E. palustris* was first lyophilized and then the obtained lyophilizate was separated by gradient *dry-flash* column chromatography (DF CC). Fractions obtained by elution with 60 and 80% EtOAc were further fractionated by open column chromatography. Selected fractions were finally purified on RP HPLC. From these fractions, ingenanes **1** (9.0 mg) and **2** (6.1 mg) were isolated. *E. lucida* stems were dried and ground nd then extracted with EtOAc/hexane mixture. The resulting extract was further fractionated with DF CC, gradient from 100% hexane to 100% EtOAc. Fractions eluted with 15, 40, and 60% EtOAc were further separated first by column chromatography, and RP HPLC was used for final purification. From these fractions, ingenanes **1** (5.5 mg), 3 (2.2 mg), and **4** (7.5 mg) were isolated.

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Cell lines and assays

518A2 and HaCaT cells were cultured in DMEM supplemented with 10% FBS, 4 g/L glucose, L-glutamine (2 mM), 5000 U/mL penicillin, and 5 mg/mL streptomycin solution. All cell lines were sub-cultured at 72 h intervals using 0.25% trypsin/EDTA and seeded into a fresh medium at the density of 8000 cells/cm^2.

Cell viability was assessed by MTT assay. To determine IC_{50} values of ingenol-mebutate, **1**, **2**, **3**, and **4**, cells were treated with different increasing concentrations of compounds. The absorbance of obtained dye was measured at 570 nm with a reference wavelength of 690 nm. IC_{50} was calculated by non-linear regression analysis.

The percentages of apoptotic, necrotic, and viable cells were assessed by Cell Death Detection Kit and flow cytometry using Annexin-V-FITC and PI staining according to the manufacturer's instructions.

Results and discussion

Ingenanes **1** (3*β*-benzoyloxy-13*α*-dodecanoyloxy-ingenol) (Wang et al., 2003) and **2** (3*β*,13*α*,17-tribenzoyloxy-ingenol) (Lu et al., 2008) were isolated from *E. palustris* latex, while a total of three ingenane derivatives were isolated from *E. lucida* stem: **1**, **3** (3*β*,5*β*-dibenzoyloxy-20-deoxyyingenol) (Gotta et al., 1984) and **4** (3*β*,5*β*,20-tribenzoyloxyingenol) (Opferkuch et al., 1981). All isolated compounds have been described in the literature, but compound **4** was reported as a natural product for the first time. Previously, compound **4** has been described in the literature as the esterification reaction product of the ingenol. The structures of the isolated compounds were determined using data obtained from 1D and 2D NMR spectra, and the molecular formulas were confirmed by HR-ESI MS. In the literature, the biological activities of the isolated compounds have not been described in detail.

Ingenanes **1** and **2** exerted a strong cytotoxic effect in the nanomolar range (IC_{50} values were 5 nM and 45 nM, respectively) in melanoma cells, while normal keratinocytes were not sensitive to these compounds even in the micromolar range. Ingenol-mebutate, **3**, and **4** had a less prominent anti-melanoma effect with IC_{50} values between 20 µM and 30 µM in 518A2 cells. Selectivity towards melanoma cells was considerable for ingenol-mebutate and **4** with IC_{50} values in HaCaT cells > 100 µM. Cell Death analysis confirmed the preferential cytotoxicity of ingenanes in melanoma cells.

Conclusion

Ingenanes isolated from *E. palustris* and *E. lucida* showed significant and selective anti-melanoma effects and further molecular mechanism elucidation *in vitro* and *in vivo* is envisioned.

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