Toxicology Letters 178 (2008) 71–76

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

# Verminoside- and verbascoside-induced genotoxicity on human lymphocytes: Involvement of PARP-1 and p53 proteins

Antonietta Santoro[a], Giuseppe Bianco[a], Patrizia Picerno[a], Rita Patrizia Aquino[a], Giuseppina Autore[a], Stefania Marzocco[a], Patrizia Gazzerro[a], Maria Brigida Lioi[b], Maurizio Bifulco[a,*]

[a] Department of Pharmaceutical Sciences, University of Salerno, Via Ponte don Melillo, 84084 Fisciano, Salerno, Italy
[b] Department of Animal Production Sciences, University of Basilicata, Viale dell'Ateneo Lucano 10, 85100 Potenza, Italy

ARTICLE INFO

*Article history:*
Received 12 November 2007
Received in revised form 4 February 2008
Accepted 6 February 2008
Available online 23 February 2008

*Keywords:*
Verminoside
Verbascoside
Chromosome aberrations
Sister chromatid exchanges
PARP-1
p53

ABSTRACT

Verminoside and verbascoside are natural compounds present in plants used in traditional medicine. They exhibit several biological activities including anti-inflammatory, anti-bacterial and anti-tumor properties. The potential applications of these compounds as ingredients in pharmaceutical formulations and cosmetics prompted us to investigate on cytotoxic and genotoxic activity of verminoside and verbascoside on human lymphocytes using genetic toxicity assays recommended in preclinical studies by the US Food and Drug Administration (FDA). We analyzed chromosome aberrations (CAs) and sister chromatid exchanges (SCEs) as well as the mitotic index (MI) and cell viability after the treatments with verminoside and verbascoside. This report is the first to clearly demonstrate a significant increase of structural CAs and SCEs on normal human lymphocytes associated with a reduction of the MI in both verminoside- and verbascoside-treated cells. Moreover, we observed enhanced protein expression levels of PARP-1 and p53 that are key regulatory proteins involved in cell proliferation and DNA repair. Interestingly, mass spectrometric analysis of the compounds in the culture supernatants also showed that verminoside remained unchanged during the culture period while verbascoside was hydrolyzed to its derivative, caffeic acid and the last one seems to be responsible for the observed biological activity.



## 1. Introduction

Verminoside, an iridoid derivative, and verbascoside, a phenylethanoid compound, are the major constituents of *Kigelia africana* (Lam.) Benth (syn *Kigelia pinnata* DC, Bignoniaceae), a tropical plant used in folk medicine. Remedies prepared from dried fruits are used for emollient, anti-eczema and anti-psoriasis properties. Remedies from root bark are also used for venereal disease treatment and naphtoquinones extracted from *K. africana* also show anti-trypanosomal (Moideen et al., 1999) and anti-microbial (Akunyili et al., 1991) activities and exhibit anti-tumor activity against melanoma and renal carcinoma cells (Houghton et al., 1994; Jackson et al., 2000). Quantitative High Performance Liquid Chromatogrophy (HPLC) analysis of the dried fruit extract from *K. africana* showed that verminoside and verbascoside represented 2.1% and 0.55% (w/w) of the extract respectively (Picerno et al., 2005). Several studies have demonstrated that verbascoside exhibits a number of biological activities including anti-oxidative (Xiong et al., 1996; Wong et al., 2001) anti-bacterial (Rigano et al., 2006) and anti-tumor actions (Kunvari et al., 1999; Ohno et al., 2002; Lee et al., 2007). Verbascoside has been also shown to modulate nitric oxide (NO) production and the expression of inducible nitric oxide synthase (iNOS) in activated macrophages (Xiong et al., 2000; Lee et al., 2005). It also inhibits histamine, arachidonic acid release and prostaglandin E2 production in RBL-2H3 mast cells suggesting a possible application of the compound as anti-inflammatory remedy (J.H. Lee et al., 2006; K.Y. Lee et al., 2006). More recently it has been reported that verbascoside attenuates glutamate-induced neurotoxicity and mitigates scopolamine-induced memory impairment in mice (Koo et al., 2005; J.H. Lee et al., 2006; K.Y. Lee et al., 2006). Despite the expanding literature on verbascoside, very little is known about verminoside. We have previously demonstrated the anti-inflammatory property of verminoside which is able to inhibit iNOS expression and NO release in LPS-induced J774.A1 macrophage cells (Picerno et al., 2005).

*Abbreviations:* CA, chromosome aberration; FDA, US Food and Drug Administration; ESIMS, electrospray-ionization mass spectrometry; HPLC, High Performance Liquid Chromatogrophy; iNOS, nitric oxide synthase; MI, mitotic index; PARP-1, poly(ADP-ribose) polymerase; PHA, phytohemagglutinin; SCE, sister chromatid exchange.

* Corresponding author. Tel.: +39 089 969742; fax: +39 089 969602.
*E-mail address:* maubiful@unisa.it (M. Bifulco).

0378-4274/$ – see front matter © 2008 Published by Elsevier Ireland Ltd.
doi:10.1016/j.toxlet.2008.02.006

On the basis of the overall observations, suggesting the potential applications of verbascoside and verminoside as ingredients of pharmaceutical formulations and cosmetics, here we investigated the cytotoxic and genotoxic activity of both compounds on human lymphocytes. We used chromosome aberration (CA) and sister chromatid exchange (SCE) analyses as genetic end-points and mitotic index (MI) and exclusion of trypan blue dye as markers of cell proliferation and viability. To characterize the observed effects at molecular level we also determined the expression level of poly(ADP-ribose) polymerase (PARP-1) and p53. Indeed, both proteins are required for maintaining genomic integrity (Lane, 1992; Wang et al., 1997), synergize in suppressing chromosomal rearrangements and regulate DNA double strand break repair in primate cells (Kastan et al., 1991; Nelson and Kastan, 1994; Süsse et al., 2004). Finally, we also performed mass spectrometric analysis of verminoside and verbascoside in the culture supernatants in order to assess whether the genotoxic as well as cytotoxic activity of the compounds was due to intact molecules or to their derivatives.

## 2. Materials and methods

### 2.1. Chemicals

Verminoside (β-D-glucopyranoside, 6[[3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl]oxy]-1a,1b,2,5a,6,6a-hexahydro-1a-(hydroxymethyl)oxireno[4,5]cyclopenta[1,2-*c*]pyran-2-yl, [1aS-[1aα,1bβ,2β,5aβ,6β(E),6aα]]) and verbascoside ([2-(3,4-dihydroxyphenylethyl)-1-*O*-α-L-rhamnopyranosyl-(1 → 3)-β-D-(4-*O*-caffeyl)-glucopyranoside]) were extracted and purified as reported by Picerno et al. (2005) (purity of each compound >98%) and dissolved in sterile water. Each compound was added immediately after phytohemagglutinin (PHA) stimulation and left throughout the culture period. Chemical structures of verminoside and verbascoside are shown in Fig. 1.

### 2.2. Lymphocyte cultures

Blood samples were obtained from three healthy unrelated donors, aged 25–30 years, none of them was receiving drugs or had a history of smoking/drinking. Lymphocytes were separated by Ficoll–Hypaque gradient density. $2 \times 10^6$ cells were cultured in 9 ml of RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 10 μg/ml phytohemagglutinin (M form). Cell culture reagents were all obtained from Gibco, Invitrogen. Cells were cultured in a humidified atmosphere containing 5% $CO_2$ and 95% air at 37 °C. Cell survival was determined by the Trypan-blue exclusion method.

### 2.3. CA, SCE, and MI determinations

Cells were cultured for 72 h at 37 °C for cytogenetic studies. After 70 h of incubation, colcemid (Sigma, 0.2 μg/ml) was added. Lymphocytes were collected by centrifugation, resuspended in prewarmed hypotonic solution (0.075 M KCl) for 20 min, fixed in methanol/acetic acid (3:1) for 10 min, and stained with a 5% Giemsa solution. For SCE analysis, 30 h prior harvesting, BrdU (Sigma) was added to each culture (10 μg/ml) and samples were protected from light. Colcemid was added during the final 2 h of cell growth. Harvested cells were treated with hypotonic solution and fixed with methanol/acetic acid (3:1). Air-dried slides were stained with a 0.2% acridine orange solution in phosphate buffer (pH 6.8) and sealed with paraffin. From each concentrations and from each subject, 50 well-spread metaphases were analyzed for both structural chromosome aberrations and sister chromatid exchanges. Gaps were recorded but not included either in the percentage of aberrant cells or in the aberration frequency. Mitotic index was determined as the percentage of cells (prophases and metaphases) over a total of 1000 nuclei analyzed at random (Lioi et al., 1998).

Data were expressed as mean ± S.E. of three independent experiments. Statistical differences between the treatments and the control were evaluated by one-way analysis of variance (ANOVA). In the case of a significant result in the ANOVA, Student's *t*-test was performed (Lioi et al., 1998; Sarnataro et al., 2006). A *P*-value less than 0.05 was considered statistically significant.

### 2.4. Western blotting

Mitogen activated lymphocytes were incubated with verminoside (0.05–0.1 mM) and verbascoside (0.05–0.1 mM) for 24 h and 48 h. For protein detection, cells were washed with PBS and resuspended in Tris–HCl 20 mM pH 7.5, 10 mM NaF, 150 mM NaCl, 1% Nonidet P40, 1 mM phenylmethylsulphonylfluoride, 1 mM $Na_3VO_4$, leupeptin (10 μg/ml), and trypsin inhibitor (10 μg/ml). After 40 min, cell lysates were obtained by centrifugation at 13,000 rpm for 15 min at 4 °C.

Protein concentrations were estimated by the Bio-Rad protein assay using bovine serum albumin as standard. Equal amounts of protein (40 μg) of cell lysates were dissolved in Laemmli's sample buffer, boiled for 5 min, and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (8% polyacrylamide). Proteins were then transferred to nitrocellulose membrane at 100 mA for 45 min at room temperature.

Filters were blocked with TBS, 5% (w/v) non-fat dry milk for 40 min at room temperature and then skinned overnight at 4 °C with the anti-PARP-1 (Santa Cruz Biotechnology 1:1000) or anti-p53 (Santa Cruz Biotechnology 1:1000) antibody, diluted in TBS, 5% (w/v) non-fat dry milk and 1% tween-20. Blots were then incubated, after four washes in TBS containing 5% (w/v) non-fat dry milk and 1% tween-20, with horseradish peroxidase conjugated goat anti-mouse IgG (1:5000) for 1 h at room temperature. Filter was also incubated in the presence of the antibody against the GAPDH protein.

Immunoreactive bands were visualized using ECL detection system (Amersham, GE Healthcare) according to the manufacturer's instructions and exposed to Kodak X-Omat film. The protein bands of PARP-1 and p53 on X-Omat films were quantified by scanning densitometry (Imaging Densitometer GS-700 Bio-Rad, U.S.A.).

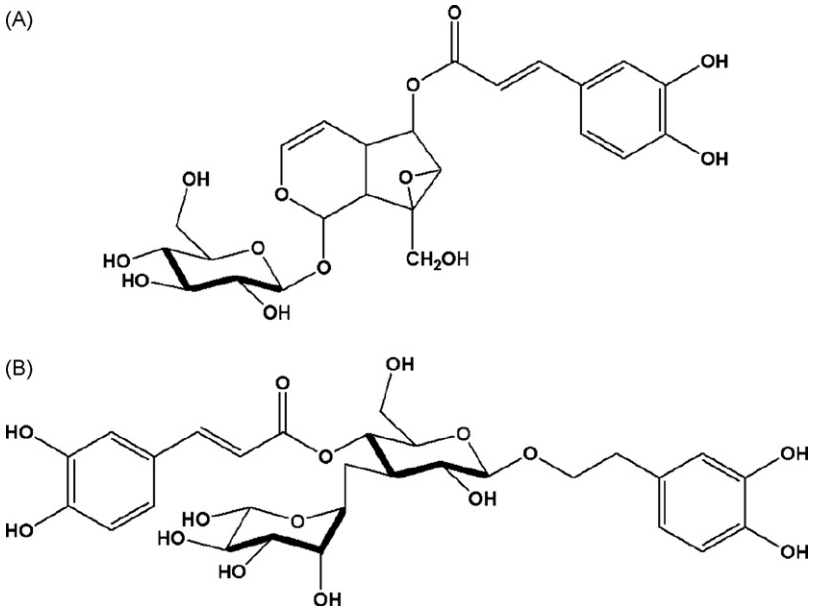


**Fig. 1.** Chemical structures of verminoside (A), and verbascoside (B).