Photoprotective effect of verbascoside from *Buddleja cordata* in SKH-1 mice exposed to acute and chronic UV-B radiation

[Enfoco fotoprotector del verbascósido de *Buddleja cordata* en ratones SKH-1 expuestos a radiación UV-B]

Adriana Montserrat Espinosa-González1,2, Ana María García-Bores1, José del Carmen Benítez-Flores3, César Enoc Sandoval-Pérez2, María del Rosario González-Valle3, Carlos L. Céspedes4 & José Guillermo Avila-Acevedo1

1Laboratorio de Fitoquímica, UBIPRO, Facultad de Estudios Superiores-Iztacala, Universidad Nacional Autónoma de México, Estado de México, México
2Posgrado en Ciencias Biológicas, Universidad Nacional Autónoma de México, Coyoacán, Distrito Federal, México
3Laboratorio 1, UMF, Facultad de Estudios Superiores-Iztacala, Universidad Nacional Autónoma de México, Estado de México, México
4Laboratorio de Fitoquímica Ecológica, Departamento de Ciencias Básicas, Universidad del BioBio, Chillán, Chile

Abstract: Ultraviolet radiation (UVR) is involved in both sunburn and the development of skin cancer, which has a high incidence worldwide. Strategies to reduce these effects include the use of photoprotective substances. The aim of this work was to investigate the photoprotective effect of verbascoside isolated from the methanolic extract of *Buddleja cordata* (BCME) in SKH-1 mice exposed to acute and chronic UV-B radiation. The mouse dorsal area was evaluated macroscopically and microscopically for diagnosis; verbascoside penetration into mouse skin was investigated in vivo by the tape stripping method. After acute UV-B exposure, 100% of irradiated mice that had been protected with verbascoside showed no signs of sunburn or of inflammatory processes. After chronic exposure, 100% of unprotected mice showed skin carcinomas; in contrast, in mice topically treated with either BCME or verbascoside, the presence of lesions was decreased by 90%. These results prove that verbascoside penetrates through the skin of mice and suggest that verbascoside and BCME may potentially prevent photodamage on mice’s skin after acute and chronic UVR exposure.

Keywords: edema, erythema, photocarcinogenesis, sunburn, verbascoside.

Resumen: La radiación ultravioleta (RUV) provoca quemaduras solares y el desarrollo de cáncer de piel. El objetivo de este trabajo fue investigar el efecto fotoprotector del verbascósido obtenido del extracto metanólico de *Buddleja cordata* (EMBC) en ratones SKH-1 expuestos a RUV-B de manera aguda y crónica. El diagnóstico histológico se llevó a cabo en la piel de la zona dorsal de los ratones. La penetración del verbascósido fue cuantificada mediante la técnica de la cinta adhesiva. En el experimento agudo, el 100% de los ratones protegidos con verbascósido no evidenciaron signos de quemadura ni procesos inflamatorios. En el experimento crónico los ratones sin protección e irradiados presentaron carcinomas cutáneos. En contraste en los ratones protegidos con EMBC o verbascósido las lesiones disminuyeron un 90% en ambos grupos. El verbascósido penetró en la piel del ratón. Los resultados sugieren que el EMBC y el verbascósido previenen el fotodaño en la piel de ratones expuestos de forma aguda o crónica a la RUV.

Palabras clave: edema, eritema, fotocarcinogénesis, quemadura solar, verbascósido.
INTRODUCTION

The skin is the largest organ of the body and, among other functions, acts as a barrier to protect organisms from potentially harmful physical, environmental and biological factors (Randhawa et al., 2013). One of the main physical factors that damage human skin is UV radiation. UVR can be classified according to its wavelength as UV-C (200-280 nm), UV-B (280-320 nm) and UV-A (320-400 nm). UV-C radiation does not reach the surface of the Earth, while UV-B has been identified as the primary etiological agent of skin cancer development (Maverakis et al., 2010).

Harmful effects of UV on the skin can be classified as acute or chronic. These effects depend on the wavelength, skin penetration, and particularly radiation exposure time. Acute effects include dermatitis, immunosuppression, hyperplasia and sunburn involving erythema, edema, blistering and pain; later, flaking and peeling of the skin appears. Sunburn is associated with the classic signs of inflammation: redness, heat, pain and swelling (Kullavanijaya & Lim, 2005). Acute exposure to UV induces various disorders in the skin tissues that can be assessed histologically: epidermal thickening (hyperplasia), the presence of keratinocytes with some degree of disturbance, loss of cell polarity, spongiosis (epithelial edema) and increased melanin synthesis. In the dermis, acute UV exposure induces vasodilation, edema and inflammatory infiltration (Matsumura & Ananthaswamy, 2004).

The dose of UV that causes sunburn is sufficient to cause DNA damage directly by inducing the formation of cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts in the epidermal cells. These mutations often appear in genes with pivotal functions in cell proliferation and differentiation control, such as proto-oncogenes and tumor suppressor genes, and they are the leading long-term cause of skin cancer (Matsumura & Ananthaswamy, 2004).

Chronic effects associated with UV include photocaging and photocarcinogenesis. The reduced levels of collagen, elastic net degradation and moisture loss that characterize the former are collectively known as elastosis (Kircik, 2012). At the macroscopic level, very fine wrinkles and skin thinning are observed due to atrophy of the dermis and loss of subcutaneous adipose tissue; blotches on the skin also appear due to the reorganization of melanocytes (Matsumura & Ananthaswamy, 2004).

Photocarcinogenesis is a complex phenomenon that involves three distinct stages: initiation, promotion and progression. Each is mediated by molecular, biochemical and cellular changes initiated by the fixation of mutations following genetic defects; they lead to cell cycle abnormalities and the consequent formation of malignant, invasive and metastatic tumors (Matsumura & Ananthaswamy, 2004; Afaq et al., 2005). Skin cancer can be differentiated based on the cell type involved: melanoma cancer (MC) arises from transformed melanocytes and non-melanoma cancer (NMC) from keratinocytes. NMC is divided into two types: basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). Both have low mortality and metastasis rates, although they are the most common skin cancers and can lead to the destruction of the surrounding tissues (Afaq & Mukhtar, 2006; Hong et al., 2008).

In recent years, some compounds of both natural and synthetic origin have gained considerable attention as photoprotective agents. The use of sunscreens made from natural products is an option for skin care to prevent UV damage (Afaq, 2011). It has been shown that substances produced by plants such as phenylpropanoids, flavonoids and polyphenolic compounds have the ability to absorb UV (Kostyuk et al., 2008). Verbascoside is a phenylpropanoid isolated from various plants, belonging mainly to the Buddleja genus (Avila et al., 2005; Adedapo et al., 2009). Buddleja cordata Kunth (Scrophulariaceae) is a shrub or tree that is widespread throughout Mexico. This plant is distributed from northern Mexico to Guatemala at altitudes between 1500 and 3000 meters above sea level. People have utilized its leaves as a poultice to treat tumors, abscesses, sores and burns. Decoctions of the roots, leaves, and bark of B. cordata have diuretic effects when administered orally, whereas topical application is used to heal wounds and rheumatic pains (Adedapo et al., 2009; Alonso-Castro et al., 2011).

Phytochemical studies of Buddleja species have shown the presence of compounds such as sesquiterpenes, flavonoids, phenylethanoids, phenylpropanoids and notably verbascoside (Figure...
Previous studies have shown that verbascoside has several biological activities, including anti-inflammatory (Martínez-Vázquez et al., 1998; Esposito et al., 2010; Seo et al., 2013), neuroprotective (Esposito et al., 2010; Kurisu et al., 2013), anti-tumor (Alipieva et al., 2014), antibacterial (Avila et al., 1999) and antioxidant (Alipieva et al., 2014). As verbascoside absorbs light in the UV region, it can also be considered a sunscreen (Kostyuk et al., 2013; Alipieva et al., 2014).

**Figure 1**

Chemical structure of verbascoside

Previously, our group showed that methanolic extract of *Buddleja cordata* (BCME) exerts photochemoprotective properties in an animal model using UV-B radiation acute challenge experiments (Avila et al., 2014). In this work, we report the photoprotective effect of verbascoside in acute and chronic challenge experiments, as well as the photoprotective effect of BCME at a chronic level.

**MATERIAL AND METHODS**

**BCME preparation and HPLC analysis**

*Buddleja cordata* Kunth (Scrophulariaceae) leaves (253 g) were dried, ground and successively extracted with hexane and methanol. The methanolic extract was evaporated under reduced pressure at 55°C to obtain a syrup. The resulting dry residue (29.55 g) was stored at 4°C and is hereafter referred to as *Buddleja cordata* methanolic extract (BCME).

BCME was characterized by HPLC in reverse phase, in agreement with previous work (Avila et al., 2014). Verbascoside (98.75 mg/g), linarin (36.45 mg/g) and syringin are the main compounds present in BCME and served as standards.

**Verbascoside isolation**

A 300 g sample of BCME was subjected to column chromatography (CC) on silica gel using CH₂Cl₂–MeOH (19:1) as an eluent, with increasing concentrations of MeOH. Fractions eluted with CH₂Cl₂–MeOH (9:1 and 8:2) gave verbascoside (11 g) as a pure amorphous powder, [α]D⁺22° =–41.5 (MeOH; c 1.1). UV λ<sub>max</sub> (nm): 208 (4.30), 217 (4.45), 292 (4.10), 329 (4.25). IR ν<sub>KBr</sub> (cm⁻¹): 3400 (OH), 2932 (C-H), 1701 (conj. ester), 1631, 1604, 1521 (aromatic ring).

**1H NMR**

(DMSO-d6, TMS) δ ppm: 0.97 (d, J= 6 Hz, 3H, Me of Rha), 2.70 (t, J=7Hz, 2H, Ar-CH₂-CH₂), 4.36 (d, J= 7.2 Hz, 1H, H-1 of Glc), 4.72 (t, J= 9.6 Hz, 1H, H-4 of Glc), 5.03 (d, J= 1.1 Hz, 1H, H-1 of Rha), 6.21 (d, J= 15.8 Hz, 1H, Ar-CH-CH), 6.49–7.01 (6H, aromatic H), 7.47 (d, J= 15.8 Hz, 1H, Ar-CH-CH), 8.69, 8.75, 9.19, 9.62 (4 x OH). Analytical data were identical to the results published for previously isolated verbascoside (Liu & Jia, 1991).

**Experimental animals**

The SKH-1 hairless mouse strain is a widely used model for human photocarcinogenesis. Female 5–6
week old, SKH-1 mice (weighing 26 ± 5 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) and maintained in a climate-controlled environment with a 12 h light/dark cycle. Five mice were housed per cage and were acclimatized for two weeks before the start of the experiment. Throughout the experimental period, mice had free access to food and water provided through the food chamber on top of the cages. The Institutional Biosecurity and Bioethical Committee approved all animal protocols.

**In vivo verbascoside penetration study: tape stripping**

Mice were placed in a laminar flux chamber at 21°C and 62% relative humidity for 30 min; an application zone measuring 2x2 cm was marked on the dorsal area of each. Verbascoside was applied to the surface of the skin at 2 mg/cm². Fifteen min after application, the excess substance on the application area was wiped off with a cotton swab; the area was then washed with ethanol 96% and dried. The stratum corneum of the treated areas was removed by four successive tape stripings using Scotch tape strips (19 mm x 32.9 m, 3M, MN, USA). Each stripping was performed in a controlled way, i.e., a 10 g rubber weight was rolled over the tape 10 times prior to removal. The first strip was discarded, and the three subsequent ones were collected and deposited in a 20 mL beaker; 5 mL of ethanol was added to each sample and then stirred with a magnetic bar for 30 min. The absorbance was measured at 320 nm using a double-beam spectrophotometer (Perkin-Elmer, Lambda 2S UV/VIS). Verbascoside penetration was calculated from concentration data obtained using a regression analysis performed on absorbance measurements (Wissing & Müller, 2002; Lademann et al., 2009).

**Acute UV-B exposure bioassay**

For acute experiments, the mice were randomly divided into five groups of five animals: untreated (U); negative control treated with ethanol (C-); positive control, treated with ethanol and irradiated UV (C+); experimental group 1, treated with verbascoside (E1-ac); and experimental group 2, treated with verbascoside and irradiated with UV (E2-ac). Control groups were treated topically on the dorsal skin with 200 µL of ethanol. Experimental groups were treated topically on the dorsal skin with 200 µL of verbascoside dissolved in ethanol at a concentration of 2 mg/mL (Avila et al., 2014).

Fifteen minutes after application of the substances, the C+ and E2-ac groups were irradiated with UV-B lamps (302 nm, UVP, UVM-26, 6 W) positioned 15 cm above their backs for ten minutes. Irradiation at this distance produced a dose of 6 mJ/cm², which was measured using a Spectrolino model DM-300HA research radiometer. The parameters evaluated were as follows: erythema, an indicator of sunburn, was observed in unprotected animals 24 h post-irradiation. Animals were subsequently sacrificed by carbon dioxide, and the dorsal skin was dissected and fixed for histological study and diagnosis.

**Chronic UV-B exposure bioassay**

For chronic experiments, the mice were divided into seven groups: untreated (U); negative control (C-); positive control (C+); verbascoside (E1 ch); verbascoside with UV irradiation (E2 ch); BCME (E3 ch); and BCME with UV irradiation (E4 ch).

The experiment lasted 33 weeks, and the mice were administered ethanol, verbascoside and BCME in similar manner to the procedure in the acute experiment. Two UV-B regimens were used. For the first two weeks, the C+, E2 ch and E4 ch groups were irradiated with UV-B daily for one minute at 6 mJ/cm² (promotion time). After this time, the mice were irradiated 3 times at week for one minute for 31 weeks (Bissett et al., 1987; Katiyar et al., 1997; Kundoor et al., 2007).

**Assessment of photocarcinogenesis**

The time of appearance and location of tumors were mapped for each mouse. Skin tumor formation, as evidenced by the presence of outgrowths with a diameter of 1 mm or greater, was expressed both as tumor incidence and as average tumor multiplicity (average number of tumors per mouse) for each experimental group. All UV-B irradiated mice bore tumors by week 29; tumor numbers were recorded for 33 weeks. After this time, some groups developed very high tumor numbers; these tumors tended to coalesce, and accurate counting of smaller lesions was no longer possible.

After week 30, tumors progressing to carcinoma (Ca) could still be distinguished from the
larger mass of more benign lesions and could be counted at week 33, after which the study ended. The final tumors were macroscopically classified and confirmed by histological diagnosis as benign wart-like pedunculated, broader-based sessile papillomas, or malignant carcinoma, according to previously described characteristics (Gallagher et al., 1984; Canfield et al., 1988; Hong et al., 2008).

**Histological study**

The dorsal skin samples from mice subjected to acute and chronic UV-B exposure bioassays were divided into regions without apparent injury and injured regions and/or tumors. Each skin sample was fixed with 2% paraformaldehyde buffer solution (pH 7.2) for 24 h in a tissue embedding cassette, dehydrated with a sequence of ethanol solutions (70, 80, 95 and 100%, v/v) and embedded in paraffin. Serial sections were cut to a thickness of 5 µm, deparaffined, and stained with hematoxylin-eosin (H&E). Histological changes in each section were observed using multiple microscopic fields and photographed with a photomicroscope (Nikon). Measurements included the epidermal thickness and the number of sunburned cells (400X). Histological diagnosis was performed, comparing the untreated (U) and C- mouse skin samples with the ones from the UV-B irradiated groups. UV-derived histological damage in the epidermis and dermis was classified based on the degree (mild, moderate or severe) and extent (focal, multifocal or diffuse) of the lesions (González et al., 2000). Damage induced by acute UV-B exposure was assessed in both the epidermis and the dermis, including sunburn signs and inflammatory processes. Damage induced by chronic UV-B exposure was assessed in both the epidermis and the dermis. Each injury was described and classified as carcinoma, hyperplasia or actinic keratosis (Rigel et al., 2006).

**Statistical analysis**

Statistical analysis was performed on the collected data. The mean values of C-, C+ and the experimental groups were obtained from descriptive analyses. After histological descriptions, the incidence of damage, injury and/or tumors in both the acute and chronic study were expressed in terms of frequency. The results were analyzed using Student’s t test with p ≤ 0.05 in Microsoft Excel 2010.

**RESULTS AND DISCUSSION**

**In vivo verbascoside penetration study**

Verbascoside penetrated 0.465% ± 0.2% (9.3 ± 1.3 µg/mL, 14.4 ± 2.0 nmol/cm²) into the mouse skin. In a recent study, Thitilertdecha et al., (2014) using a lotion preparation of Clerodendrum petasites, determined that verbascoside has a low penetration in human skin 0.5 ± 0.03 nmol/cm². Our results suggest that SKH-1 mouse skin is more permeable to verbascoside than human skin, probably because of the thin stratum corneum present in the dorsal area of SKH-1 mice.

**Acute photoprotection**

Analysis of the histological diagnosis showed that 20% of untreated (U) mice developed edema, which is normal in this animal model. Hyperplasia was observed in 20% of mice of the C- group. Hyperplasia and edema were found in 100% of the histological samples from the irradiated group (C+) and hemorrhage in 20%. Topical application of verbascoside to mouse skin caused a slight increment in hyperplasia (40%). However, edema was lower than in C+ (Figure 2). Therefore, verbascoside protects mice from inflammation generated by UV-B radiation. Figure 3 shows the most representative results of the histological evaluation of SKH-1 hairless mouse skin. The skin from the U, C-, and E1-ac groups showed no histological damage (Figure 3a, Figure 3b and Figure 3c). Skin from UV-B irradiated mice (C+ group) showed severe histological alterations, as previously described for acute UV-B skin damage (González et al., 2000). The histological results revealed edema, apoptotic cells, necrosis, and paraqueratosis. In addition, we found erythrocytes, which are mainly related to congestion in blood vessels (Figure 3d). Furthermore, sunburn cells were observed, namely basal keratinocytes with pyknotic nuclei having an eosinophilic cytoplasm. However, for animals topically treated with verbascoside (E2 ac), skin damage was markedly lower than in the control. Thus, the erythema was eliminated, the collagen fibers were normal, and the vascular damage was reduced (Figure 3e and Figure 3f). Erythema and edema are specific markers of acute UV-B exposure because of the inflammatory reaction. Our results were consistent with the findings reported by Regalado et al., 2011. After acute exposure to UV-B, they found the same histological damage in albino...
mouse skin as we did in SKH-1 mice. When they used the lipophilic extract from the seaweed *Thalassia testudinum* Banks & Sol. Ex Koening (Hidrocharitaceae) as a photoprotector, the level of protection was also similar to what we obtained in the verbascoside tests.

**Figure 2**
Incidence of lesions according to histological examination in acute bioassay. U: Untreated group, C-: group with ethanol, C+: group mice without protection and irradiated, E1 ac mice with verbascoside and E2 ac mice with verbascoside and irradiated.

**Chronic photoprotection: Inhibition of carcinogenesis**

**Incidence and multiplicity**
The photoprotective effects of verbascoside and BCME against photocarcinogenesis are shown in Figure 4a. In the C+ group, irradiated without protection, tumor onset occurred at week 29 and reached 100% by week 33. Mice treated with verbascoside and BCME before exposure to UV-B showed identical appearance at the onset of tumorigenesis. The occurrence of tumors initiated after 29 weeks, reaching 50% incidence at week 33 in the verbascoside-treated group. In the case of mice treated with BCME, the tumors were also evident from week 29 and reached 40% incidence at week 33. Verbascoside and BCME treatments significantly reduced the number of tumors per mouse compared to C+ throughout the experiment. The mean numbers of tumors at the end of the experiment in the C+, E2 ch (verbascoside+UV) and E4 ch (BCME+UV) treated groups were found to be 15, 8 and 6, respectively (Figure 4b). These results indicated that verbascoside and BCME inhibited skin tumor development in terms of both tumor incidence, to a lesser extent, and tumor multiplicity.

**Histological diagnosis**
Moreover, histological study showed that 100% of mice in group C+ presented with lesions, corresponding to carcinoma (Ca 100%), hyperplasia (Hp 100%) and actinic keratosis (80%). In E2 ch and E4 ch animals, only 10% of the mice showed carcinomas in both groups. Mice treated with verbascoside (E2 ch) showed lesions classified as hyperplasia (60%) and actinic keratosis (40%), whereas mice administered BCME (E4 ch) topically showed values of 40% and 10%, respectively (Figure 5).
Figure 3

Histological sections representing each group in the acute experiment. a) U: Untreated group, b) C-: group with ethanol, c) E1 ac: group with topical application of verbascoside, d) C+: group without skin protection and irradiated, e) and f) E2 ac: group with verbascoside and irradiated.

AP: apoptotic cells; C: cyst; D: dermis; ED: edema; EP: epidermis; F: follicle; H: hypodermis; N: necrosis; P: paraqueratosis; SC: stratum corneum; SG: sebaceous gland; V: blood vessel. H&E staining, magnification: panel a 100X, panel b-f 400X
Figure 4
Multiplicity and incidence of skin tumors in mice protected with verbascoside and BCME against chronic UV-B irradiation. Each point represents the mean number of tumors per mouse. C+: group without UV protection and irradiated; E2 ch: group with verbascoside and irradiated; E4 ch: group with BCME and irradiated.
Figure 5
Histological diagnosis of macroscopic lesions. Data refer to total platelets observed per group. C+: group without protection and irradiated, E2 ch: group with verbascoside and irradiated, E4 ch: group with BCME and irradiated (* p≤0.05). The images show the progression and different types of injuries sustained in C+ group mice during 33 weeks of the experiment.

Figure 6 shows histological sections of untreated and C- groups considered healthy skin (Panel a and b). The histological sections of types of UV-B injury are shown in Figure 6c, Figure 6d, Figure 6e and Figure 6f. The main feature is the loss of the basal layer and invasion of epithelial cells into the dermis. Furthermore, non-protected mice irradiated chronically with UV-B showed hyperplasia, actinic keratosis and carcinomas, with indicators of carcinoma: angiogenesis, mitotic and pleomorphic cells, mast and neutrophils, the latter in blood vessels. For mice treated with verbascoside and BCME, the main lesion type was benign hyperplasia (Figure 6g and Figure 6h). The difference between these two groups is likely due to the synergistic action of the components of BCME. BCME contains an appreciable quantity of linarin, a compound with anti-inflammatory properties (Martinez-Vazquez et al., 1998), in addition to syringin, a glucoside phenylpropanoid with immunomodulating properties (Sharma et al., 2012). UV-B radiation-induced inflammatory responses lead to increased blood flow and vascular permeability, resulting in edema, erythema, hyperplastic responses, and the activation of cyclooxygenase-2 (COX-2) and prostaglandin (PG) metabolites. Inflammation results in the recruitment of infiltrating leukocytes secreting a variety of proinflammatory cytokines at the UV-irradiated sites and is therefore considered an early event in tumor promotion. Chronic inflammation plays a crucial role in all three stages of tumor development: initiation, promotion, and progression (Kim & He, 2014). Thus, the linarin and syringin present in BCME could inhibit chronic inflammation and thereby act synergistically along with verbascoside, which has sunscreen, antioxidant and anti-inflammatory properties (Alipieva et al., 2014). In experiments with human keratinocyte cultures, Kostyuk et al. (2013) showed that verbascoside’s molecular characteristics confer the following photoprotective qualities: photostability, direct free radical scavenging and antioxidant effect, rescue of skin antioxidants, and inhibition of UV-derived inflammatory and metabolic responses. Our results indicate that BCME and verbascoside have sunscreen and anti-inflammatory properties in vivo. Molecular characteristics of verbascoside contribute to their photoprotection against chronic UV exposure.
Figure 6
Histological sections representing each group in chronic experiment. a) U: Untreated group, b) C-: groups E1 ch and E3 ch, c), d), e) and f) C+: group without skin protection and irradiated, g) E2 ch: group with verbascoside and irradiated, h) E4 ch: group with BCME and irradiated

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CONCLUSIONS
Our results showed that verbascoside protects against UV-derived damage at both acute and chronic levels. In the acute assay, verbascoside prevented sunburn and histological damage; in the chronic experiments, the results revealed a delay in the onset of tumorigenesis and reduced multiplicity of tumors in BCME- and verbascoside-treated animals. Likewise, lesions in mice treated with BCME and verbascoside were fewer and more benign than in the unprotected controls. This paper is the first report on the photoprotective activity of verbascoside and BCME in a chronic animal model.

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