Protective effect of *Calendula officinalis* extract against UVB-induced oxidative stress in skin: Evaluation of reduced glutathione levels and matrix metalloproteinase secretion

Yris Maria Fonseca[a], Carolina Dias Catini[a], Fabiana T.M.C. Vicentini[a], Auro Nomizo[a], Raquel Fernanda Gerlach[b], Maria José Vieira Fonseca[a,*]

[a] *Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Avenida do Café s/n, 14040-903 Ribeirão Preto, São Paulo, Brazil*
[b] *Faculdade de Odontologia de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil*

ABSTRACT

Background and purpose: Calendula officinalis flowers have long been employed time in folk therapy, and more than 35 properties have been attributed to decoctions and tinctures from the flowers. The main uses are as remedies for burns (including sunburns), bruises and cutaneous and internal inflammatory diseases of several origins. The recommended doses are a function both of the type and severity of the condition to be treated and the individual condition of each patient. Therefore, the present study investigated the potential use of *Calendula officinalis* extract to prevent UV irradiation-induced oxidative stress in skin.
Methods: Firstly, the physico-chemical composition of marigold extract (ME) (hydroalcoholic extract) was assessed and the *in vitro* antioxidant efficacy was determined using different methodologies. Secondly, the cytotoxicity was evaluated in L929 and HepG2 cells with the MTT assay. Finally, the *in vivo* protective effect of ME against UVB-induced oxidative stress in the skin of hairless mice was evaluated by determining reduced glutathione (GSH) levels and monitoring the secretion/activity of metalloproteinases.
Results and conclusions: The polyphenol, flavonoid, rutin and narcissin contents found in ME were 28.6 mg/g, 18.8 mg/g, 1.6 mg/g and 12.2 mg/g, respectively and evaluation of the *in vitro* antioxidant activity demonstrated a dose-dependent effect of ME against different radicals. Cytoxicity experiments demonstrated that ME was not cytotoxic for L929 and HepG2 cells at concentrations less than or equal to of 15 mg/mL. However, concentrations greater than or equal to 30 mg/mL, toxic effects were observed. Finally, oral treatment of hairless mice with 150 and 300 mg/kg of ME maintained GSH levels close to non-irradiated control mice. In addition, this extract affects the activity/secretion of matrix metalloproteinases 2 and 9 (MMP-2 and -9) stimulated by exposure to UVB irradiation. However, additional studies are required to have a complete understanding of the protective effects of ME for skin.

1. Introduction

Calendula officinalis L. (Asteracee) is an annual herb native to the Mediterranean region. In Europe and America it is cultivated for ornamental and medicinal purposes. It is commonly known as the marigold or maravilla, and its flowers have long been employed in folk therapy (Duke et al., 2002). More than 35 properties have been attributed to decoctions and tinctures from the flowers, and these preparations have been considered valuable remedies for burns, bruises, cuts, rashes, skin wounds and other conditions (Brown and Dattner, 1998).

Calendula officinalis is used mainly for cutaneous and internal inflammatory diseases of several origins. The dosages cited are 2–4 mL of tincture diluted to 250–500 mL with water or 2–5 g of herb in 100 g of ointment. A tea made from 1 to 2 g of the flower in 150 mL of boiling water has also been used up to 3 times a day as an antispasmodic (Re et al., 2009). However, the recommended doses are a function of both the type and severity of the condition to be treated and the individual condition of each patient.

The main chemical constituents of *Calendula officinalis* include steroids, terpenoids, free and esterified triterpenic alcohols, phenolic acids, flavonoids (quercetin, rutin, narcissin, isorhamnetin, kaempferol), and other compounds (Re et al., 2009).

Phytopharmacological studies of different marigold extracts have shown anti-tumoral (Jiménez-Medina et al., 2006), anti-inflammatory, wound healing (Zitterl-Eglseer et al., 1997) and

antioxidant activities (Katalinic et al., 2006). In clinical studies, marigold was highly efficacious in the prevention of acute dermatitis in cancer patients undergoing postoperative irradiation (Pommier et al., 2004).

It is well established that the inflammatory response following acute UV light irradiation of the skin and the degenerative processes related to chronic UV irradiation skin exposure are largely mediated by the overproduction of reactive oxygen species (ROS) and free radicals and by the impairment of antioxidant systems (Aquino et al., 2002). Therefore, due to the deleterious effects of ROS in the skin, many studies have focused on the establishment and evaluation of antioxidants to enrich the endogenous cutaneous protection system, and thus to prevent and/or treat UV irradiation-induced skin damage. In this context, much attention has been paid to antioxidants from natural sources, especially flavonoids and other phenolic compounds (Atoui et al., 2005).

Marigold flowers contain large quantities of antioxidant compounds (flavonoids and polyphenols), suggesting they may possess antioxidants to prevent UV-induced skin damage. In addition, *Calendula officinalis* flowers have long been employed in folk therapy as remedies for diverse burns, including sunburns and skin wounds. This study investigated the potential use of orally administered marigold extract (ME) to prevent UV irradiation-induced oxidative stress in the skin. As a first step, the physico-chemical composition and the antioxidant potential of ME were evaluated. The toxicity of this extract was then investigated in cell culture and its *in vivo* capacity to prevent UV irradiation-induced reduced glutathione (GSH) depletion and the secretion/activity of metalloproteinases the skin of hairless mice was evaluated.

2. Materials and methods

2.1. Chemicals

The *Calendula officinalis* L. dried flowers were a gift from Santos Flora (Sao Paulo, SP, Brazil). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and antibiotic solution containing 5 mg of penicillin, 5 mg of streptomycin and 10 mg of neomycin per mL were purchased from Gibco (Grand Island, NY, USA). Luminol, thiobarbituric acid (TBA), ethylene glycol bis(-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), xanthine, xanthine-oxidase (XOD), protease inhibitor cocktail and rutin (95%) were purchased from Sigma–Aldrich (St Louis, MO, USA) and acetic acid of high-performance liquid chromatography (HPLC) grade and Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany). Ethanol was obtained from Synth (Sao Paulo, Brazil), *o*-phthalaldehyde (OPT), narcissin from Chromadex® (Ivine, CA, USA) and acetonitrile and methyl alcohol were obtained from J.T. Baker (USA). All other chemicals were of reagent grade and were used without further purification.

2.2. Preparation of marigold extract

The *Calendula officinalis* L. dried flowers were ground in a knife mill into fine particles (0.3 mm – mean diameter). The powdered drug was macerated with 50% ethanol (1:9, w/w) at 25 °C for 5 days. This mixture was subjected to mechanical agitation at 870 rpm (Fisatom, model 713 D) for 1 h at the beginning and end of the maceration period. Afterwards, the extract was filtered and dried at 40 °C in a stove with air circulation. Finally, the residue was resuspended into 50% hydroalcoholic solution (200 mL) and stored at −20 °C. The obtained concentrated extract contained 15.7% dry weight.

2.3. Assessment of the physico-chemical composition of marigold extract

2.3.1. Total polyphenol and flavonoid contents

Total polyphenol and flavonoid contents in marigold extract were determined by the colorimetric methods described by Kumazawa et al. (2004).

2.3.2. Evaluation of rutin and narcissin content by HPLC

The rutin and narcissin levels in marigold extract were determined using a Shimadzu (Kyoto, Japan) liquid chromatography system equipped with an LC-10 AT VP solvent pump unit and an SPD-10A VP UV-Visible detector operating at 340 nm. Samples were injected manually through a Rheodyne injector (20 μl loop). Separation was performed in a C18 Hypersyl BDS-CPS cyano column (250 mm × 4.6 mm, 5 μm) (Thermo Electron Corporation, USA) equipped with a precolumn C18 Hypersyl BDS (10 mm × 4 mm, 5 μm) (Thermo Electron Corporation, USA). The mobile phase was acetonitrile–water (15:85, v/v) containing 2% (v/v) acetic acid at a flow rate of 1 mL/min. Data were collected using a Chromatopac C-R6A integrator (Shimadzu, Kyoto, Japan).

Calendula extract solutions were prepared by dilution of 100 μL of concentrated extract into 10 mL of 50% methanolic solution. Next, 1 mL of this solution was diluted into 5 mL of the mobile phase. Finally, this solution was filtered and analyzed by the previously described HPLC method. The results were calculated in relation to the dry weight of extract.

Qualitative and quantitative data for rutin and narcissin were obtained by comparison to known standards of rutin (from Sigma®) and narcissin (from Chromadex®). The HPLC method employed for the determination of rutin and narcissin in ME was previously validated considering the parameters linearity, accuracy and precision. The method was linear over the concentration ranges evaluated and the values obtained for the precision and accuracy of the measurements are in agreement with ICH guidelines.

2.4. Determination of in vitro antioxidant efficacy

The antioxidant activity of ME was evaluated by H-donor activity using DPPH• radical as described by Blois (1958), by inhibition of lipid peroxidation as described by Rodrigues et al. (2002) and scavenging superoxide radicals produced in the chemiluminescence assay using the xanthine/luminol/XOD system (Girotti et al., 2000).

Marigold extract was first solubilized with ethyl alcohol (50%, v/v) and diluted using the medium of each reaction to the following final concentration ranges: 30–180 μg/mL for H-donor activity using the DPPH• radical assay, 75–600 μg/mL for the lipid peroxidation assay, and 1–18 μg/mL for the chemiluminescence assay using the xanthine/luminol/XOD system.

For all the three different methodologies employed, the percentage inhibition was plotted against different concentrations of ME and the concentration that caused 50% inhibition of the system was reported as the IC_{50} value.

The percentage inhibition was calculated using the following equation:

$$\text{inhibition } (\%) = 100 - \left[\frac{100 \times A_s}{A_0} \right]$$

where A_s is the absorbance (spectrophotometric methods) or area under curve (chemiluminescent method) observed when the experimental sample was added, and A_0 is the absorbance (spectrophotometric methods) or area under curve (chemiluminescent method) of the positive control (ME absence).

2.5. Determination of cytotoxicity

2.5.1. Cell culture and treatment

L929 and HepG2 cells were routinely grown in $150 \, cm^2$ tissue culture flasks in DMEM supplemented with 1% (v/v) of an antibiotic solution containing 5 mg of penicillin, 5 mg of streptomycin and 10 mg of neomycin per mL, and 7.5% or 10.0% (v/v) heat-inactivated FBS at 37 °C under 5% CO_2.

Marigold extract was evaporated to dryness using a Speed-Vac concentrator (model SPD 2010, Thermo Electron Corporation) at 45 °C. The dry residue was dissolved in dimethyl sulfoxide (DMSO) and diluted using PBS to a final concentration range of 1.5–37.5 mg/mL in relation to dry weight of extract. The final concentration of DMSO was less than 0.1%. MTT assays used 20 μL of each dilution.

2.5.2. MTT assay

The sensitivity of cells to ME was determined by a standard spectrophotometric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Cells were seeded at a density of 10^5 cells/well into 96-well plates and incubated for 24 h at 37 °C in atmosphere of 95% air and 5% CO_2. Then, 20 μL of ME, at different concentrations in PBS was added to the culture plates for 24 h. After treatment, cells were rinsed once with PBS and serum-free culture medium without phenol red was replaced in all wells. Cells were then incubated for 4 h with MTT solution (5 mg/mL). The yellow tetrazolium salt was metabolized by viable cells to form purple crystals of formazan. The crystals were solubilized overnight (12 h) in a mixture consisting of 20% sodium dodecyl sulfate (SDS) in HCl (0.01 M). The product was quantified spectrophotometrically by measuring absorbance at 570 nm using a microplate reader (μQuant™, BioTek Instruments Inc., USA). The cellular viability was expressed as the percentage of viable cells compared to the control group.

2.6. Assessment of the in vivo protective effect against UVB-induced oxidative stress

2.6.1. Animals and experimental protocol

In vivo experiments were performed on 3-month-old, sex-matched hairless mice of the HRS/J. The animals, weighing 20–30 g, were housed in a temperature-controlled room, with access to water and food *ad libitum* until use. They were housed within cages with a 12-h light and 12-h dark cycle. All experiments were conducted in accordance with National Institutes of Health guidelines for the welfare of experimental animals and with the approval of the Ethics Committee of the Faculty of Pharmaceutical Science of Ribeirao Preto (University of Sao Paulo).

The animals were divided in five groups ($n = 3$): Group 1 = non-irradiated control (water treatment), Group 2 = irradiated control (water treatment), Group 3 = irradiated and treated with a solution containing 150 mg/kg of ME, Group 4 = irradiated and treated with a solution containing 300 mg/kg of ME and Group 5 = irradiated and treated with a solution containing 600 mg/kg of ME, in relation to the dry weight of the extract. The concentration of ME used in this assay was selected based on an anti-inflammatory activity study reported by Núñez Figueredo et al. (2007). The treatment protocol consisted of applying 100 μL of the test solutions orally 30 min and 18 h before the irradiation.

2.6.2. Irradiation

The UV irradiation source was a Philips TL/12RS 40W lamp (Medical-Holand). This source emits in the range of 270–400 nm with an output peak at 313 nm, resulting in an irradiation of $0.27 \, mW/cm^2$ at a distance of 20 cm as measured by an IL 1700 radiometer (Newburyport, MA, USA) equipped with UVB and UV

detectors. The minimal dose which induces GSH depletion and gelatinase activity ($2.46 \, J/cm^2$) was determined by Casagrande et al. (2006). The mice were killed with an overdose of carbon dioxide 6 h after the start of UVB exposure, and full dorsal skins were removed and stored at −80 °C until analysis.

2.6.3. GSH assay

The GSH skin levels were determined using a fluorescence assay as previously described (Hissin and Hilf, 1976). The total skin of hairless mice (1:3, w/w dilution) was homogenized in 100 mM NaH_2PO_4 (pH 8.0) containing 5 mM EGTA using a Turrax TE-102 (Turratec, Sao Paulo). Whole homogenates were treated with 30% trichloroacetic acid and centrifuged at $1900 \times g$ for 6 min and the fluorescence of the resulting supernatant was measured in a Hitachi F-4500 fluorescence spectrophotometer. Briefly, 100 μL of sample was mixed with 1 mL of 100 mM NaH_2PO_4 (pH 8.0) containing 5 mM EGTA and 100 μL of OPT (1 mg/mL in methanol). The fluorescence was determined after 15 min ($\lambda_{exc} = 350 \, nm$; $\lambda_{em} = 420 \, nm$).

2.6.4. Qualitative analyses of skin proteinases by substrate-embedded enzymography

SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) substrate-embedded enzymography (zymography) was used to detect enzymes with gelatinase activity. Assays were carried out as previously described by Gerlach et al. (2007).

Total skins of hairless mice (1:4, w/w dilution) were homogenized in 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM $CaCl_2$ and 1% protease inhibitor cocktail in a Turrax TE-102 (Turratec). Whole homogenates were centrifuged at $12,000 \times g$ for 10 min at 4 °C. The Lowry method was used to measure protein levels in skin homogenates (Lowry et al., 1951). Supernatant aliquots (50 μL) were mixed with 10 μL of 100 mM Tris–HCl buffer (pH 7.4) containing 4% SDS, 20% glycerol and 0.001% bromophenol blue. For electrophoresis, 30 μL of the mixture (40 μg of protein) was used. SDS-PAGE was performed using 10% acrylamide gels containing 0.25% gelatin. After electrophoresis, the gels were washed twice for 30 min with 2.5% Triton X-100 under constant shaking, incubated overnight in 50 mM Tris–HCl (pH 7.4) 10 mM $CaCl_2$ and 0.02% sodium azide at 37 °C, and stained the following day with Coomassie Blue 350-R (Phast gel blue R-Pharmacia Biotech). After destaining in 20% acetic acid, zones of enzyme activity were detected as regions of negative staining against a dark background. The proteolytic activity was qualitatively analyzed by comparing controls and marigold extract-treated animals.

2.7. Statistical analysis

Data were expressed as means ± SE determined by triplicate analysis. The ME levels causing 50% inhibition of the system assessed (IC_{50}) were determined using GraphPad Prism® software. Data were statistically analyzed by Student's *t*-test or one-way ANOVA followed by Bonferroni's test of multiple comparisons and the level of significance was set to $p < 0.05$.

3. Results and discussion

3.1. Preparation of marigold extract

Five extractions of marigold were performed in the same day and under the same experimental conditions to evaluate the repeatability of the proposed extraction process. The extraction precision was evaluated by determination of the dry weight (14.9 ± 3.2%), H-donor activity in the DPPH• radical assay (56.6 ± 1.5%) and polyphenol content (26.5 ± 3.2 mg/g) of the five extracts. The results demonstrated the efficiency and reliability

of this method of obtaining marigold extract, as the average relative standard deviation (RSD) from the three different parameters determined was no more than 3.2%.

3.2. Physico-chemical composition of marigold extract

ME contained 28.6 ± 2.3 mg/g and 18.2 ± 0.5 mg/g of polyphenol and flavonoid content relative to the dry weight of extract, respectively. Furthermore, HPLC analysis (Fig. 1) of ME showed several chromatographic peaks, revealing a wide chemical diversity. Among the substances present, rutin and narcissin were identified. These flavonoids are widely distributed in medicinal plants. The identification and quantification of rutin and narcissin in ME were done by comparing the obtained retention time and area with known standards of rutin (Sigma®) and narcissin (Chromadex®). The rutin and narcissin contents found were 1.6 ± 0.1 mg/g and 12.2 ± 0.5 mg/g relative to the dry weight of extract, respectively.

3.3. Determination of in vitro antioxidant efficacy

The *in vitro* antioxidant activity of ME was evaluated against several free radicals using different methods. ME showed antioxidant activity against various radicals. It was possible to build a dose–response curve for ME using all of the methodologies employed, demonstrating that these methods are adequate to evaluate the antioxidant activity of ME. The IC_{50} values were 97.1 ± 2.1 µg/mL, 350.0 ± 13.1 µg/mL and 4.4 ± 0.9 µg/mL for the DPPH• system, lipid peroxidation assay and xanthine/luminol/XOD assay, respectively.

The same *in vitro* methodologies employed in the present work to evaluate the antioxidant activity of ME were previously used by our group to determine the IC_{50} values of quercetin, a flavonoid that has well-known antioxidant activity. Thus, quercetin is used as a reference antioxidant compound in order to evaluate the activity of different extracts. As demonstrated by Vicentini et al. (2007) quercetin showed an IC_{50} of 0.2 µg/mL for the inhibition of lipid peroxidation and of 0.8 µg/mL in the DPPH• assay. For the xanthine/luminol/XOD assay, the IC_{50} value was 11.3 µg/mL (unpublished data). Therefore, based on the IC_{50} values found for ME and by comparing with the IC_{50} value obtained for quercetin,

it can be concluded that ME showed higher activity in scavenging superoxide radicals produced in the xanthine/luminol/XOD system, but quercetin showed higher activity against the hydroxyl, peroxyl and alkoxyl radicals produced during lipid peroxidation and by the DPPH• radical.

3.4. Determination of cytotoxicity

The viability of L929 mouse fibroblasts and HepG2 human hepatoma cells was determined in order to evaluate the cytotoxicity of ME. These experiments demonstrated that small concentrations of ME are capable of stimulating the proliferation of mouse fibroblasts, with an approximately 27% increase of viability observed in cells treated with 11.25 and 15 mg/mL of ME. However, when higher concentrations of ME were applied, a cytotoxic effect of this extract was observed, so that the treatment with 37.5 mg/mL of ME significantly reduced the viability of mouse fibroblasts by 21.6% ($p < 0.05$ using Student's t-test).

On the other hand, no stimulating effect on HepG2 human hepatoma cells was observed after treatment with ME in the concentration range of 1.5–15 mg/mL when compared with the control group. Treatment of HepG2 cells with 30 and 37.5 mg/mL of ME significantly reduced the viability by approximately 84% and 93%, respectively ($p < 0.05$ using Student's t-test).

These results support the observations of Matysik et al. (2005), who showed that ME in small concentrations can stimulate the proliferation of human fibroblasts (HSF), but at high concentrations it can be toxic. In addition, ME did not stimulate the cellular proliferation of human breast cancer cells T47D.

3.5. Assessment of the in vivo protective effect against UVB-induced oxidative stress

The redox status of glutathione has been confirmed as an early and sensitive sensor of UVB-induced epidermal oxidative stress, suitable for testing the protective antioxidant effects of a substance (Meloni and Nicolay, 2003).

Consistent with Casagrande et al. (2006), who detected a dose-dependent depletion of GSH in the skin of hairless mice after UVB irradiation (0.96–2.87 J/cm²), in this study a UVB dose of 2.87 J/cm² induced a 44.5% decrease in GSH levels compared to non-irradiated control mice (Fig. 2).
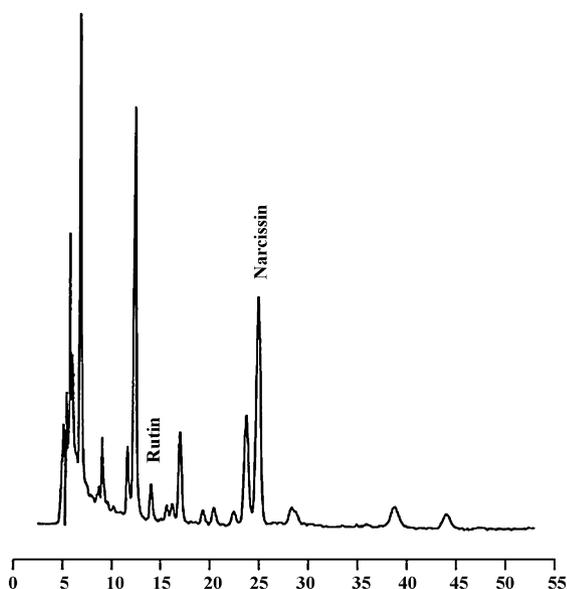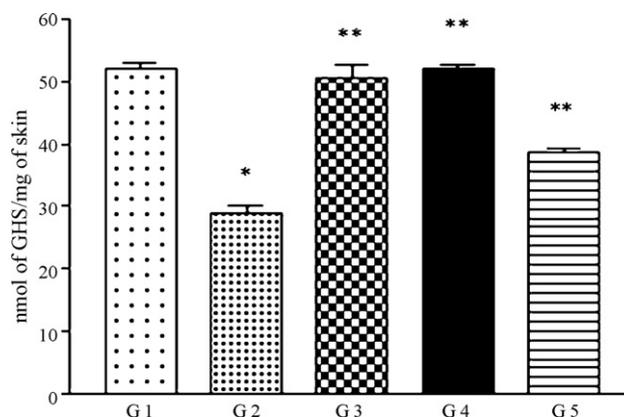

Fig. 1. HPLC Chromatogram of marigold extract. Chromatographic conditions: reversed-phase C_{18} column, with a mobile phase of acetonitrile–water (15:85, v/v) containing 2% (v/v) acetic acid (flow rate of 1 mL/min) and UV detection at 340 nm.

Fig. 2. Effect of marigold extract on the decrease of endogenous GSH levels induced by UVB irradiation. G1 = non-irradiated control; G2 = irradiated control; G3 = irradiated and treated with ME (150 mg/kg), G4 = irradiated and treated with ME (300 mg/kg) and G5 = irradiated and treated with ME (600 mg/kg). Bars represent means ± SE of three separate experiments (n = 3–4 animals per group). Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test of multiple comparisons. *$p < 0.05$ compared to the non-irradiated control and **$p < 0.05$ compared to the irradiated control.
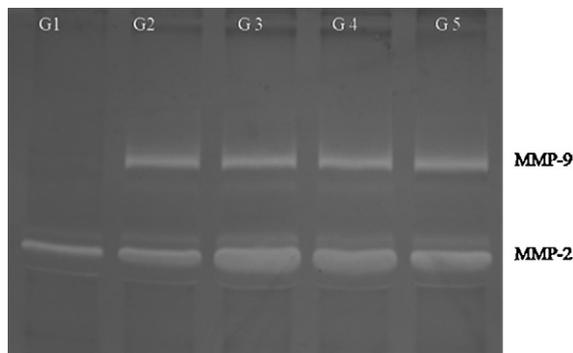
Fig. 3. Zymographic patterns of metalloproteinase secretion/activity induced by UVB irradiation of the skin of hairless mice. G1 = non-irradiated control; G2 = irradiated control; G3 = irradiated and treated with ME (150 mg/kg), G4 = irradiated and treated with ME (300 mg/kg) and G5 = irradiated and treated with ME (600 mg/kg). The data are representative of three separate experiments (n = 3–4 animals per group).

The oral treatment of hairless mice with 150 and 300 mg/kg of ME maintained GSH levels close to those of the non-irradiated control, however, at the highest dose of ME administered (600 mg/kg), an approximately 43% depletion of GSH levels was observed (Fig. 2). A possible explanation is that high concentrations of antioxidants can stabilize, rather than eliminate, radicals in the cell (Schreck et al., 1992). The protective effective against UVB irradiation-induced GSH depletion by the oral treatment with 150 and 300 mg/kg of ME was similar to that observed by our group through the topical treatment of hairless mice with a quercetin-loaded microemulsion (Vicentini et al., 2008).

The antioxidant enzymes system works cooperatively, so that changes in one component can affect the state of balance of ROS. Thus, the ROS not eliminated by the biological system can cause cellular damage and biochemical alterations, such as oxidation of proteins and lipids, inflammation, damage to DNA, and activation and inactivation of enzymes (Shindo et al., 1993). Therefore, the prevention of UVB irradiation-induced GSH depletion by the oral treatment with ME might be an important strategy for protection against UVB-induced skin damage.

The effectiveness of the ME in modifying the proteinase secretion/activity induced by UVB irradiation was also investigated in this study. Consistent with Vicentini et al. (2008), a significant increase in the expression/activity of gelatinases in the skin of hairless mice was observed after UVB irradiation in this study. The analysis of the gelatinases (MMP-2 and MMP-9) in the skin showed that MMP-9 only appeared in irradiated skin. Interestingly, samples from irradiated skin in animals not treated with ME only displayed the higher molecular weight form of MMP-9 (92 kDa, the pro-enzyme), while animals treated with 150, 300 and 600 mg/kg of ME showed both the 92 kDa- and the 87 kDa MMP-9, the active enzyme. Furthermore, there was also an increase in the 68-kDa MMP-2 form in irradiated skin from animals treated with 150, 300 and 600 mg/kg of ME, indicating an increase in both gelatinases elicited by the ME (Fig. 3).

Recent clinical data indicate that the relationship between MMPs and disease is not simple; for example, increased MMP activity can either enhance or inhibit tumor progression. This complex relationship between MMP expression and cancer has increased the basic and clinical interest in understanding MMP function *in vivo*, but it has also focused attention on MMPs and pathology, and relatively less attention has been focused on the normal roles of these enzymes (Page-McCaw et al., 2007).

MMPs can be both pro-inflammatory and anti-inflammatory. MMPs facilitate inflammatory cell recruitment and clearance of inflammatory cells by cleaving inflammatory mediators, result-ing in a tightly regulated inflammatory response (Page-McCaw et al., 2007). MMPs can regulate chemokine activity, either by direct proteolysis or by affecting the formation of chemokine gradients (Gill and Parks, 2008). Thus, several chemokines, including C-C motif ligand-7 (CCL7) and CXCL12, are substrates of MMP-2. MMP-2 cleavage of CXCL-12 results in the loss of its ability to bind its cognate receptor (CXCR4). CCL7 is cleaved by MMP-2, and the removal of the four (N)-terminal amino acids from the active CCL7 chemokine molecule converts it to a truncated form that can still bind to its CC chemokine receptor, but cannot activate it, thus functioning as a receptor antagonist (Manicone and McGuire, 2008). In addition, MMP-9 cleaves and activates CXCL6 and CXCL8, whereas it inactivates CXCL1 and CXCL4 (Page-McCaw et al., 2007).

Studies showed that elevated levels of degraded collagen observed in photodamaged skin act to down-regulate type I procollagen synthesis. Of the different MMPs, MMP-1 was the most effective collagenase, followed by MMP-8 and MMP-13. Gelatinolytic enzymes (MMP-2 and MMP-9) did not degrade intact collagen, but the combination of MMP-1 and MMP-9 broke down collagen into small peptides. These small fragments did not inhibit procollagen synthesis, but the larger breakdown fragments of type I collagen negatively regulated its synthesis (Varani et al., 2001).

Since inflammatory cells need to cross the extracellular matrix during skin repair following UV irradiation, it may be that the increase in gelatinases, which degrade most matrix extracellular molecules, may be beneficial for skin healing. Furthermore, the increase in both gelatinases (MMP-9 and MMP-2) induced by the ME in irradiated skin may be beneficial for procollagen synthesis, regulation of the inflammatory response and rearrangement of damaged skin. However, future studies addressing these possibilities are required.

In conclusion, the present study suggests the potential applicability of marigold extract against UV-induced skin damage, as this extract showed relevant *in vitro* antioxidant activity against different radicals and prevented the UVB irradiation-induced GSH depletion in the skin of hairless mice after oral administration. This extract affects the activity/secretion of proteinases stimulated by exposure to UVB irradiation, and this increase in gelatinase activity may be beneficial for skin healing and procollagen synthesis. However, additional studies are required in order to have a complete understanding of protective effects of ME for the skin.

Acknowledgments

References

Aquino, R., Morelli, S., Tomaino, A., Pellegrino, M., Saija, A., Grumetto, L., Puglia, C., Ventura, D., Bonina, F., 2002. Antioxidant and photoprotective activity of a crude extract of *Culcitium reflexum* H.B.K. leaves and their major flavonoids. Journal of Ethnopharmacology 79, 183–191.

Atoui, A.K., Mansouri, A., Boskou, G., Kefalas, P., 2005. Tea and herbal infusions: their antioxidant activity and phenolic profile. Food Chemistry 89, 27–36.

Blois, M.S., 1958. Antioxidant determinations by the use of a stable free radical. Nature 26, 1199–1200.

Brown, D.J., Dattner, A.M., 1998. Phytotherapeutic approaches to common dermatologic conditions. Archives of Dermatology 134, 1401–1404.

Casagrande, R., Georgetti, S.R., Verri Jr., W.A., Dorta, D.J., dos Santos, A.C., Fonseca, M.J.V., 2006. Protective effect of topical formulations containing quercetin against UVB-induced oxidative stress in hairless mice. Journal of Photochemistry and Photobiology B: Biology 84, 21–27.

Duke, J.A., Bogenschutz-Godwin, M.J., Du Celliar, J., Duke, P.A.K., 2002. Hand Book of Medicinal Herbs, 2nd ed. CRC Press, Boca Raton, pp. 139–140.

Gerlach, R.F., Demacq, C., Jung, K., Tanus-Santos, J.E., 2007. Rapid separation of serum does not avoid artificially higher matrix metalloproteinase (MMP)-9 levels in serum versus plasma. Clinical Biochemistry 40, 119–123.

Gill, S.E., Parks, W.C., 2008. Metalloproteinases and their inhibitors: regulators of wound ealing. International Journal of Biochemistry & Cell Biology 40, 1334–1347.

Girotti, S., Fini, F., Ferri, E., Budini, R., Piazzi, S., Cantagalli, D., 2000. Determination of superoxide dismutase in erythrocytes by a chemiluminescent assay. Talanta 51, 685–692.

Hissin, P.J., Hilf, R., 1976. A fluometric method for determination of oxidized and reduced glutathione in tissues. Analytical Biochemistry 74, 214–226.

Jiménez-Medina, E., Garcia-Lora, A., Paco, L., Algarra, I., Collado, A., Garrido, F., 2006. A new extract of the plant calendula officinalis produces a dual in vitro effect: cytotoxic anti-tumor activity and lymphocyte activation. BioMed Central Cancer 6, 119.

Katalinic, V., Milos, M., Kulisic, T., Jukic, M., 2006. Screening of 70 medicinal plant extracts for antioxidant capacity and total phenols. Food Chemistry 94, 550–557.

Kumazawa, S., Hamasaka, T., Nakayama, T., 2004. Antioxidant activity of propolis of various geographic origins. Food Chemistry 84, 329–339.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265–275.

Manicone, A.M., McGuire, J.K., 2008. Matrix metalloproteinases as modulators of inflammation. Seminars in Cell & Developmental Biology 19, 34–41.

Matysik, G., Wójciak-Kosior, M., Paduch, R., 2005. The influence of Calendula officinalis flos extracts on cell cultures, and the chromatographic analysis of extracts. Journal of Pharmaceutical and Biomedical Analysis 38, 285–292.

Meloni, M., Nicolay, J.F., 2003. Dynamic monitoring of glutathione redox status in UV-B irradiated reconstituted epidermis: effect of antioxidant activity on skin homeostasis. Toxicology In Vitro 17, 609–613.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods 65, 55–63.

Núñez Figueredo, Y., Montero Alarcón, C., Agüero Fernández, S., Muñoz Cernuda, A., 2007. Efecto antiinflamatorio preclínico del polvo seco de Calendula officinalis. Latin American Journal of Pharmacy 26, 548–552.

Page-McCaw, A., Ewald, A.J., Werb, Z., 2007. Matrix metalloproteinases and the regulation of tissue remodelling. Nature Reviews Molecular Cell Biology 8, 221–233.

Pommier, P., Gomez, F., Sunyach, M.P., D'Hombres, A., Carrie, C., Montbarbon, X., 2004. Phase III randomized trial of Calendula officinalis compared with trolamine for the prevention of acute dermatitis during irradiation for breast cancer. Journal of Clinical Oncology 22, 1447–1453.

Re, T.A., Mooney, D., Antignac, E., Dufour, E., Bark, I., Srinivasan, V., Nohynek, G., 2009. Application of the threshold of toxicological concern approach for the safety evaluation of calendula flower (Calendula officinalis) petals and extracts used in cosmetic and personal care products. Food and Chemical Toxicology 47, 1246–1254.

Rodrigues, T., Santos, A.C., Pigoso, A.A., Mingatto, F.E., Uyemura, A.S., Curti, C., 2002. Thioridazine interacts with the membrane of mitochondria acquiring antioxidant activity toward apoptosis-potentially implicated mechanisms. British Journal of Pharmacology 136, 136–142.

Schreck, R., Albermann, K., Baeuerle, P.A., 1992. Nuclear factor Kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). Free Radical Research Communications 17, 221–237.

Shindo, Y., Witt, E., Packer, L., 1993. Antioxidant defense mechanisms in murine epidermis and dermis and their responses to UV light. Journal of Investigative Dermatology 100, 260–265.

Varani, J., Spearman, D., Perone, P., Fligiel, S.E.G., Datta, S.C., Wang, Z.Q., Shao, Y., Kang, S., Fisher, G.J., Voorhees, J.J., 2001. Inhibition of type I procollagen synthesis by damaged collagen in photoaged skin and by collagenase-degraded collagen in vitro. American Journal of Pathology 158, 931–942.

Vicentini, F.T.M.C., Casagrande, R., Georgetti, S.R., Bentley, M.V.L.B., Fonseca, M.J.V., 2007. Influence of vehicle on antioxidant activity of quercetin: a liquid crystalline formulation. Latin American Journal of Pharmacy 26, 805–810.

Vicentini, F.T.M.C., Simi, T.R.M., Del Ciampo, J.O., Wolga, N.O., Pitol, D.L., Iyomasa, M.M., Bentley, M.V.L.B., Fonseca, M.J.V., 2008. Quercetin in w/o microemulsion: in vitro and in vivo skin penetration and efficacy against UVB-induced skin damages evaluated in vivo. European Journal of Pharmaceutics and Biopharmaceutics 69, 948–957.

Zitterl-Eglseer, K., Sosa, S., Jurenitsch, J., Schubert-Zssilavecz, M., Della Loggia, R., Tubaro, A., Bertoldi, M., Franz, C., 1997. Anti-oedematous activities of the main triterpenodiol esters of marigold (Calendula officinalis L., Asteraceae). Journal of Ethnopharmacology 57, 139–144.