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

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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.
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1. Introduction

Calendula officinalis L. (Asteraceae) 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

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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).

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