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Calendula officinalis Protection Against Cytotoxicity Effects of Personal Care Products on HaCaT Human Skin Cells

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Abstract

Background: Human skin is normally exposed to ionizing and UV radiations and on occasions it may also be subjected to beauty products or drugs that the host uses; all of which can generate reactive oxygen species (ROS).

Objective: In this study, *Calendula officinalis* extract, which contains antioxidant compounds, was examined for its protective effects against the products that generate ROS- induced cytotoxic activity towards human skin cells.

Methodology: The protection against cytotoxicity by *Calendula officinalis* extract was investigated *in vitro* using a dose-response on HaCaT human skin cells. The proprietary aqueous calendula extract C from biodynamically grown plant was examined. Protection against cytotoxicity was measured via the methyl tetrazolium cytotoxicity (MTT) assay. Cells were exposed to the calendula extracts for 48h before being exposed to personal care products consisting of two head lice treatments -Lice Breaker (Permethrin 1% w/w) and Organix Pyrethrum treatment (4 g/L Pyrethrins, 16 g/LPiperonyl Butoxide) and two beauty products -Nivea Visage Q10 plus Anti-Wrinkle face moisturizer + TiO_2; and Nivea Visage Q10 Plus Anti-Wrinkle face moisturizer (without TiO_2) for 1h.

Results: The effect of different concentrations of calendula extract on HaCaT human skin cells *in vitro* was explored. Doses of 1% (v/v) [1.4 mg dry weight equiv/ml] or less showed no toxicity. Cells were also exposed to the calendula extract for 48 hours before being exposed to the four personal care products for 1h. using the MTT cytotoxicity assay; it was observed that extract of *Calendula officinalis* gave time-dependent and concentration-dependent protection against harmful products that induce cell killing. Pre-incubation with the calendula extract for 48 hours significantly increased survival relative to the population without the extract by 30% and 47.4%, respectively, following treatment with personal care products.

Conclusion: This study demonstrates that calendula extract contains bioactive and free radical scavenging compounds that significantly protect against personal care products that generate oxidative stress cytotoxicity in a human skin cell culture model.

Keywords: Toxicity; *Calendula officinalis*; Beauty products; Lice treatments; Cell culture

Introduction

Human skin exposed to ionizing and UV radiations or drugs and/or personal care products such as head lice treatments or beauty products that could generate reactive oxygen species (ROS) in vast quantities, could oxidize degrading pathways [1]. Uncontrolled ROS can be implicated in many aspects of pathogenesis such as human skin disorders, for example cutaneous neoplasia [1,2]. In human skin there are many agents that can produce oxidative stress including industrial sources, UV radiation, food contaminants, additives, drugs or cosmetic products [1,3]. Also, reactive nitrogen species (RNS) can be formed from exposure to environmental agents like xenobiotics [1]. Oxidative stress interacts with the process of variety of skin diseases [1]. Calendula flower has been shown to protect against cells being killed and chromosomal damage induced by hydrogen peroxide (H_2O_2) in HaCaT human skin cells [4,5]. It was shown that *Calendula officinalis* contains a number of compounds with antioxidants and free radicals with scavenging potential. Also, Calendula plant was used as a skin conditioning agent in cosmetics because of its anti-inflammatory properties [6]. The other use of the Calendula plant includes treatment for first degree burns and rashes [6]. However, chemical head lice treatments and chemical beauty products can induce toxicity and genotoxicity in human skin cells [7]. The hypothesis is that personal care products such as chemical head lice treatments and beauty products induce toxicity effects by producing oxidative stress in human skin cells.

In this study, we examine two head lice treatments (Lice Breaker treatment (Permethrin 1% w/w) and Organix Pyrethrum treatment (4 g/L Pyrethrins plus 16 g/L Piperonyl Butoxide)) and two beauty products (Nivea Visage Q10 plus Anti-Wrinkle face moisturizer (contains mixture of chemicals ingredients) + TiO_2; and Nivea Visage Q10 Plus Anti-Wrinkle face moisturizer (contains mixture of chemicals ingredients) (without TiO_2). The objective of this study is that to determine the ability of the calendula flower extract to protect human skin cells against any detrimental effects of these personal care products on human skin cells *in vitro*.

Material and Methods

Materials

1640 RPMI medium, foetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco, Invitrogen, Barcelona. Phosphate buffered saline (PBS) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from (Sigma™). Media for growth and treatment were 1640 RPMI medium with 10% FBS. All the reagents used in this study were purchased from Sigma-Aldrich unless otherwise stated.

Plant extract and characterization

The characterization of calendula extract C and its ability to protect human skin cells HaCaT against hydrogen peroxide (H_2O_2) induced oxidative stress cell killing and genetic damage was published previously [4,5]. Briefly, dried flowers of *Calendula officinalis* were supplied by an industry partner (Jurlique International, Mount Barker, South Australia) from biodynamically grown plants. Calendula extract C was prepared as a proprietary aqueous extract. It was diluted in RPMI media to make the treatment solution. The determination of density for extract C (1.036) was carried out at 20°C from the average of 10 independent measurements. The dry weight of extract C was 1.4 mg/ml as determined after extracts samples were freeze dried at -56°C at 3.4 Pa for 24h. The Folin–Ciocalteu assay was used to estimate polyphenol composition and the gallic acid equivalent of extract C was 10.83 mg GAE/g dry weight. Also, DPPH assay was used in previously published work to perform the free radical inhibition of extract C. Extract C showed 47.7% radical inhibitions at concentration 2.5% (3.5 mg/ml dry weight equivalent).

Cell culture

A human non-cancer keratinocytes cell line HaCaT was like a gift from the Department of Haematology & Genetic Pathology - Flinders Medical Centre, School of Medicine at Flinders University, Adelaide, South Australia. It was maintained in RPMI 1640 medium, with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Thermo Scientific, Australia). Cells were seeded in tissue culture flasks and incubated at 37°C in a 5% CO_2 fully humidified incubator. HaCaT cells were subcultured when they reached 60–80% confluence.

Cell treatment

HaCaT cells were treated to allow toxicity exposure before the protection assay being conducted. Microplates 96 wells -flat bottom were seeded with104 cells/well and incubated for 19 h to allow cells to adhere at a temperature of 37°C in 5% CO_2. The media were aspirated and replaced with 200 µl of the treatment solution which was 0.125, 0.5, 1.0, 2.0 and 5% (v/v) extract C per well for 48 h.Then follow up with 100 µl products treatments (head lice treatments or titanium dioxide or beauty products) for 1 h before MTT assay analysis. The negative or zero control was media.

MTT cell survival assay

To measure cell viability of HaCaT human skin, the methyl tetrazolium cytotoxicity assay was performed as published [4,8,9]. Briefly, the treatment solution was aspirated, and wells washed twice with PBS, cells were incubated with 200 µl MTT (0.5 mg/ml)/well for 4 h at 37°C. Formazan crystals were then dissolved in SDS (20% sodium dodecyl sulphate in 0.02 M HCL) for overnight. The absorbance was measured on an ELISA plate reader with the background reference wavelength of 630 nm and a test wavelength of 570 nm.

Statistical analysis

The experiments were done in triplicate and data is presented as the mean ± S.E.M. One-way ANOVA with Tukey's posthoc test was carried out using SPSS software, version 22. Also, one-tailed t-test was also used. Differences were considered to be statistically significant when the P-value was less than 0.05.

Results

The toxicity of Calendula extract C on HaCaT cells *in vitro* was explored by incubating cells with extract for 48 h. Figure 1 showed the toxicity of extract *C. calendulas* extract C showed toxicity only at a high dose (5%) for the 48h treatment time. At the lowest doses, the extract (0.125, 0.5, 1.0 and 2.0 % (v/v)) was not toxic on HaCaT skin cells for any of the treatment periods. This effect could involve a period of contact between these components and the cells, such that these toxic components would be taken up by cells, thus reducing the cellular viability. Therefore, in contrast, lower concentrations of such extracts would not be toxic if incorporated into products at a safe dose, which is difficult to ascertain on human skin. The toxicity at the higher doses of calendula extracts that has been found in this study needs to be translated into a recommendation for use of calendula extract on human skin.

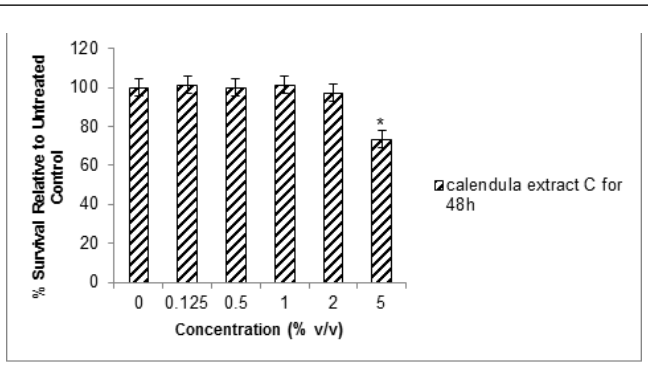

Figure 1: Relative survival at 48 h for calendula extract. HaCaT cells were treated for the time indicated then assayed by MTT assay (see section 2.4). Data are shown as mean survival relative to the untreated control ± SEM; n=3. * = treatments are significantly different from the 0 untreated controls at $p<0.05$.

Head lice treatments can induce cell killing act as apoptosis or necrosis [7]. Figure 2 showed the toxicity results of personal care products on HaCaT human skin cells. HaCaT human skin cells were pre-treated for 48h with 1% calendula extract C then followed by 1 h treatment with personal care products (beauty products or head lice treatments). Permethrin doses of 10% and 100%, Pyrethrum doses of 10% and 50% and Nivea visage at doses 0.3% and 3% with or without TiO_2 proved to be significantly toxic in HaCaT cells when measured by MTT assay.

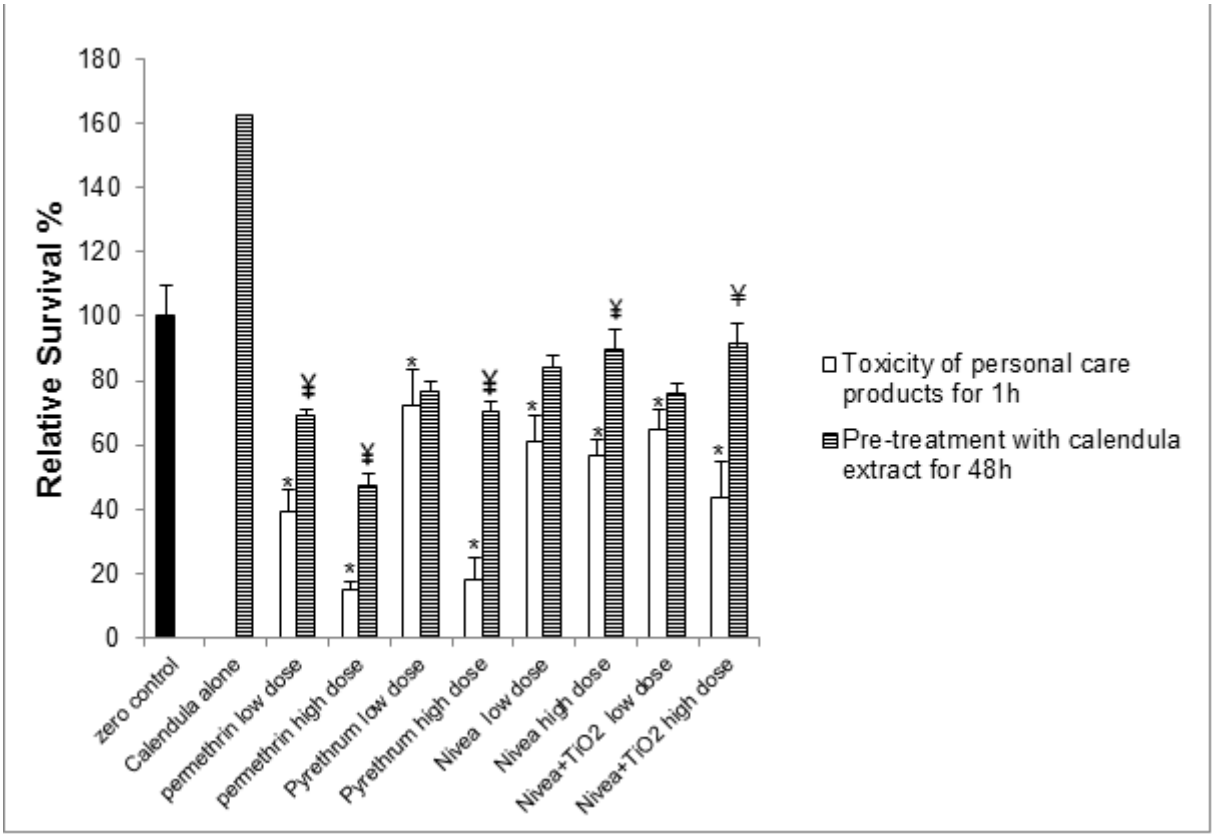

Figure 2: Toxicity and Calendula protection of personal care products on HaCaT human skin cells measured by MTT assay. Permethrin (low dose; 10%, high dose; 100%), Pyrethrum (low dose; 10%, high dose; 50%), Nivea visage without TiO_2 (low dose; 0.3%; high dose; 3%) and Nivea visage with TiO_2 (low dose; 0.3%; high dose 3%). Data shown as mean ± SEM, n=3. * = treatments are significantly different from the 0 untreated control at $p<0.05$. ¥= pre-treatment with calendula extract significantly different from products toxicity (protection).

The lower concentration of Calendula extract C 1% chosen as pre-treatment for HaCaT cells due to its safety treatment for HaCaT cells for all treatments period. *Calendula officinalis* extract showed the ability to protect significantly against toxicity effects of personal care products including TiO_2 treatment after treated HaCaT cells with Calendula extract C for 48h as illustrated in Figure 2. Calendula treatment for 48h offers more protection than 24h treatment time (Data not shown).

Sample	Toxicity for 1 h	Pre-treatment with calendula extract for 48 h
Medium control	100 ± 1.3	100 ± 1.5
TiO_2 low dose (150 µg/ml)	*67.8 + 1.4	73.6 ± 10.9
TiO_2 high dose (200 µg/ml)	*58.2+3.2	**73.3 ± 5.2

Table 1: Untreated control and Titanium Dioxide (TiO_2) the positive control of beauty products. Data shown as mean ± SEM, n=3. * = treatments are significantly different from the 0 untreated control at $p<0.05$. **= treatment is significantly different from TiO_2 dose at $p<0.03$ carried out using one- tailed T-Test.

Table 1 shows the untreated and positive controls of Titanium dioxide TiO_2 in the experiments. Cells were incubated for 48 h with calendula extract C alone there was a mean increase of 62% in relative survival. This is likely due to some cell growth during the incubation period.

It is important that the protection observed for the personal care products is real. The reason is that the positive control TiO_2 challenge still results in cells being killed and only a small level of protection. Of note, the treatment of calendula alone lead to an increase in cell number. This was not used as the untreated control as the correct control was the untreated cells.

Discussion

The human body is exposed to variety of pro-oxidants in the environment including drugs, chemicals, mixtures, radiation, pollutants or cosmetics. These agents can target lipid-rich membranes, cellular DNA or proteins to produce toxicity. Personal care products such as beauty products can generate oxidative stress in human skin cells [1]. The increases in ROS play a role in a variety of skin diseases and carcinogenesis [1,10]. Beauty products and head lice treatments

examined in this study induced significant toxicity in HaCaT human skin cells. The hypothesis was that personal care products such as beauty products or head lice treatments could generate oxidative stress in human skin cells. As a result, there was significant toxicity induced after treating HaCat cells with these products for 1h. The toxicity results of personal care products on HaCaT cells were measured by MTT assay which is a relative survival assay. *Calendula officinalis* showed protection activity against hydrogen peroxide (H_2O_2) induced oxidative stress cell killing and chromosomal damage [4,5]. Therefore, HaCaT cells treated with Calendula extract C to produce bioactivity antioxidant compounds did protect against toxicity of personal care products (beauty products and head lice treatments) that caused oxidative stress cell killing. The protection offered by Calendula extract C was time-dependent as explored early [4,5]. It was noted that there was significantly higher protection after pre-treatment with calendula extract with compounds that displayed higher toxicity when induced by the treatment of compounds alone. This was seen in the protection with the high of Pyrethrum, Nivea visage and Nivea visage with TiO_2. Further work needs to be done to understand the mechanism of action of calendula plant components and how they work to protect human skin cells. TiO_2 was classified as a carcinogenic nanoparticle used in cosmetics. Titanium dioxide (TiO_2) plays a role in the induction of apoptosis as well as oxidative stress [11-13]. One study indicated that Titanium dioxide TiO_2 can affect the mitochondria [14].

Calendula extract contains bioactives and free radical scavenging compounds that can interact with ROS to either eliminate them or minimise their deleterious effects [1]. These antioxidants or bioactive compounds including ascorbic acid, vitamin E, Vitamin C, alpha-tocopherol, quercetin, beta-carotene and amino acid [1,4]. Oxidative stress also drives the production of oxidation products, e.g. 4-hydroxy-2-nonenal or malonaldehyde that can denature proteins and alter apoptosis or influence the release of pro-inflammatory mediators such as cytokines in inflammatory skin diseases [1]. Moreover, ROS in the induction of many biological responses can act as second messengers such as in the generation of cytokines [15]. The alterations in cellular proteins or peroxidation of lipid-rich membranes caused by ROS can contribute to a range of skin diseases or cancer [1]. Experimental evidence supports the role played by ROS in the cancer process [1,10].

The increases of ROS in the cell, through either endogenous or exogenous factors, contribute to the carcinogenesis process. This could occur via genotoxic effects resulting in oxidative DNA adducts or via modification of gene expression [10]. MTT assay was employed in this study as a colorimetric assay for mammalian cell growth and survival, and it depends on the ability of viable cells to metabolize the yellow and water-soluble tetrazolium salt in the mitochondria of living cells. It can be used for mitochondrial dysfunction [9,16]. Mitochondrial damage is linked to the induction of mutations [17]. Moreover, mitochondrial DNA mutation and alteration in gene expression (mutation in the gene encoding for complexes I, II, IV and V) has been identified in many types of cancers and human tumours [17]. The mutation rate in mitochondrial is reported to be greater than in nuclear DNA [18].

In conclusion, it is evident that beauty products and head lice treatments examined in this study induced significant toxicity on HaCaT human skin cells, and that could be due to generating oxidative stress in skin cells. This process contributes to skin disorders such as inflammation and carcinogenesis or cancer. Also, the positive control of TiO_2 beauty product which is a carcinogen nanoparticle does play a role in the oxidative stress by damaging the mitochondria in the cell [11-14]. Finally, this study indicated that *Calendula officinalis* extract C does protect HaCaT human skin cells against the toxicity caused by beauty products and head lice treatments. Further work could be done to determine the potential protection of calendula extract against the chromosome damage induced by personal care products also fluorescent cell viability dyes to specify the damaged chromosome and cancer type on Human skin cells.

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