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Evaluation of *in vivo* efficacy of topical formulations containing soybean extract

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Abstract

In the present study it was evaluated the: (i) functional stability of the soybean extract as a raw material and dispersed in two different topical formulations, (ii) skin retention using modified Franz diffusion cells, and (iii) *in vivo* activity of these formulations to inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) increases in the skin of hairless mice. The physico-chemical stability was evaluated by pH, globule size and centrifugation test. Furthermore, functional stability was also evaluated by antilipoperoxidative activity. The two topical formulations were stored at 4 °C, 30 °C/60% RH and 40 °C/70% RH for 6 months. The evaluation of the antiperoxidative stability of soybean extract itself and incorporated in formulations did not demonstrate loss of activity by storage at 4 °C/6 months. During 6 months of the study in different storage conditions the formulations 1 and 2 added or not with soybean extract were stable to physico-chemical tests. The effect of antioxidant compounds detected by the inhibition of MDA formation was time-dependent for formulation 2 as detected in the skin retention study. Pretreatment with formulation 1 or 2 significantly diminished TPA-induced H_2O_2 and MDA generation. In conclusion, the present results suggest for the first time that formulations containing soybean extract may be a topical source of antioxidant compounds that decrease oxidative damages of the skin.

Keywords: Antioxidant; Isoflavonoid; *In vitro* retention; Stability test; Skin; Topical formulation

1. Introduction

Skin is a highly metabolic tissue that presents the largest surface area in the body and serves as the protective layer for internal organs (Kohen and Gati, 2000). It is designed to give both physical and biochemical protection and is equipped with a large number of defense mechanisms (Halliwell and Gutteridge, 1990).

On the other hand, the skin is very susceptible to oxidative stress, because it presents susceptible biological targets for such reactions. It is exposed to a variety of damaging oxidative species, from outer environment; skin itself, and various endogenous sources (Kohen and Gati, 2000). Elevation in cellular concentration of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radicals characterizes the oxidative stress (Salem et al., 1999). These ROS seem deleterious since they were related to many skin disorders such as cancer, cutaneous autoimmune diseases and skin aging (Lopez-Torres et al., 1998; Fuchs and Packer, 1991).

Researches have focused on the potential use of some enzymes and secondary compounds of higher plants as free radical scavengers to prevent oxidative skin damage (Casagrande et al., 2006a), and thus, their topical application has been of considerable interest (Saija et al., 1998). In this sense, antioxidants from natural products provide novel possibilities

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for the treatment and prevention of oxidative stress-mediated skin diseases (Aquino et al., 2002).

Among the natural products, flavonoids, a group of polyphenolic compounds possessing broad biological properties, exert beneficial effects due to their capability to interact with protein phosphorylation, and the antioxidant, iron-chelant and free radical scavenging activities (Saija et al., 1998). Furthermore, flavonoids are claimed to be free of toxicity and side effects, and particularly, harmless to the skin (Bonina et al., 1996; Fuchs and Packer, 1991). In this context, the subclass of the more ubiquitous flavonoids, isoflavonoids (Messina, 2000) include genistein and daidzein. There are evidences suggesting that these compounds may prevent cancer (Wang et al., 2002) and ROS-induced human skin damages. In fact, genistein and daidzein as well as polyphenols present antioxidant activity and are found in the extract of soybean (Messina, 2000). The use of the extract of soybean instead of the purified genistein and daidzein can increase the antioxidative potential of the purified molecules alone. Furthermore, plant extracts acquisition is less expensive than the purification of specific substances.

The evaluation of topical formulations added with soybean extract by antioxidant activity is a crucial issue in the study of new pharmaceutical products for skin oxidative damage treatment. Furthermore, there is no evidence on *in vivo* use of topical formulation containing soybean extract to prevent oxidative damages. Thus, the present study was designed to evaluate both physico-chemical and antioxidant activity stability of different formulations containing soybean extract as well as *in vitro* percutaneous absorption. Finally, the *in vivo* protection against TPA-induced oxidative stress was assessed. The TPA model was used since it is recognized as an inflammatory agent (Sultana and Saleem, 2004) and tumor promoter that has been shown to generate superoxide anion, hydrogen peroxide and lipid hydroperoxides leading to oxidative damage (Sharma and Sultana, 2004).

2. Materials and methods

2.1. Chemicals

Commercial soybean extract (Isoflavin Beta®) and raw materials for formulations were obtained from Galena (Campinas, SP, Brazil) and are presented in the formulation section. Genistein, daidzein, thiobarbituric acid (TBA), 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), phenol-red and horse radish peroxidase were obtained from Sigma Chemical CO. (St. Louis, MO, USA). 2-deoxy-D-ribose was obtained from Acros (New Jersey, USA) and hydrogen peroxide 30% was purchased from Calbiochem (California, USA). All other reagents used were of pharmaceutical grade or HPLC grade.

2.2. Test formulations

The formulations were developed varying its lipidic content. The non-ionic emulsion with high lipid content (formulation 1) was prepared with commercially available self-emulsifying wax Polawax® (cetostearyl alcohol and polyoxyethylene derivative of a fatty acid ester of sorbitan 20E). The emulsion with low lipid content was prepared with low concentration of Polawax® (formulation 2) and an anionic hydrophilic colloid (carboxypolymethylene, Carbopol® 940) was also added as a stabilizing agent and triethanolamine as neutralized. Both formulation contained macadamia nut oil as emollient and propylene glycol as a moisturizer. The preservative used was a mixture of parabens and imidazolidinyl urea. Deionized water qsp was used for the preparation of all formulations (Table 1). Extract of soybean (2.00%) was firstly solubilized in propylene glycol and next incorporated to the formulations at the room temperature. The control formulations did not contain the extract. All formulations were allowed to equilibrate for 24 h prior to use in the study.

Table 1
Percent composition (w/w) of the emulsions

Components	Formulation 1 (%)	Formulation 2 (%)
Self-emulsifying wax	10.00	2.00
Carbopol® 940	–	0.18
Macadamia nut oil	2.00	2.00
Propylene glycol	6.00	6.00
Soybean lecithin	4.00	4.00
Urea	4.00	4.00
Methylparaben	0.15	0.15
Propylparaben	0.02	0.02
Imidazolidinyl urea	0.30	0.30
Deionized water	73.53	81.35

2.3. Physico-chemical stability

The physico-chemical stability was performed according to World Health Organization. Formulations containing or not the extract were stored at 4 °C, 30 °C/60% relative humidity (RH), and 40 °C/70% RH for 6 months (Casagrande et al., 2007; Singh, 1999).

2.3.1. pH measurements

The pH of formulations diluted 1:10 in deionized water was measured using a Digmed DMPH-2 pHmeter. All measurements were made at room temperature in triplicate for each analyzed sample (Anchisi et al., 2001; Di Mambro and Fonseca, 2007).

2.3.2. Centrifugation assay

The samples were centrifuged at 1660 × *g* for 30 min at room temperature and the phase separation was analyzed visually (Anchisi et al., 2001).

2.3.3. Globule size measurement

The sizes of the emulsions globules were examined microscopically (Olympus microscope fitted with a 40X objective lens). The formulations were diluted 100 times using propylene glycol/water (1:1), and after, the lipofilic stain Sudan II 1% was added. One droplet of the diluted emulsion was put into Neubauer chamber and the average numbers of globules in each square ($\tilde{n}$) were determined. The results were put into Eq. (1) to determine the numbers of globules in each gram of the emulsion

(N_w) (Georgetti et al., 2006).

$$N_w = \frac{\tilde{n}E}{VQp_d} \quad (1)$$

The meanings of symbols are as follow:

$\tilde{n}$ = the mean of globules number per square in the Neubauer chamber (central square);
Q = the amount (g) of internal phase of emulsions after dilution according to E;
E = the amount (g) of the diluted emulsion;
p_d = density of the dilution liquid;
V = the volume of the Neubauer chamber.

2.4. Fe^{2+}/citrate-mediated lipid peroxidation assay

The preparation of reagents and reaction were performed as follows: (1) male Wistar rats weighing approximately 200 g were sacrificed by cervical dislocation. (2) Their livers (10–15 g) were immediately removed, sliced in 50 mL of medium containing 250 mM sucrose, 1 mM EGTA and 10 mM HEPES-KOH, pH 7.4. (3) After, sliced livers were homogenized three times for 15 s at 1 min intervals in a Potter–Elvehjem homogenizer. (4) Mitochondria were prepared by standard differential centrifugation techniques as previously described (Cain and Skilleter, 1987; Casagrande et al., 2006b), and (5) protein content determined by the biuret reaction (Gornall et al., 1949). (6) Mitochondria were used as a source of lipid membranes to evaluate lipid peroxidation. (7) The lipid peroxidation was estimated by the MDA formation based on its first description by Buege and Aust (1978) as follows: (a) it was added to 1.0 mL of medium I (125 mmol/L sucrose, 65 mmol/L KCl and 10 mmol/L Tris–HCl, pH 7.4), (b) 10 μL of soybean extract or 40 μL of each formulation under study (160 μg/mL in medium reaction), (c) mitochondria to produce a final concentration of 1 mg of protein, (d) 50 μmol/L of $(NH_4)_2Fe(SO_4)_2$, and (e) 2 mmol/L of sodium citrate. After, (f) the reaction was kept for 30 min at 37 °C, and (g) MDA formation was estimated by using the thiobarbituric acid method (Rodrigues et al., 2002; Georgetti et al., 2006). (h) Blank was prepared from the medium I without mitochondria. (i) All measurements were performed in triplicate.

2.5. Evaluation of antioxidant activity stability

As in the physico-chemical stability studies, samples were stored at 4 °C, 30 °C/60% RU and 40 °C/70% RU. At predetermined times (initial, 48 h, 1, 2, 3 and 6 months) samples of extract (raw material) and formulations were collected for the evaluation of the functional stability by antiperoxidative activity (see Section 2.4). The extract was diluted in DMSO (dimethylsulfoxide), and 10 μL was utilized for the reaction (160 μg/mL in the reaction mixture). Formulations containing or not the extract were diluted in ethanol to obtain the same concentration (160 μg/mL) used for the analysis of the raw material (soybean extract) in the reaction medium. The following controls were included in the test: one positive control was prepared in the absence of the sample (raw material) and another by adding the formulations without extract (100% of peroxidation), and a negative control in the absence of iron. It was added one positive control for each storage condition. Blanks were prepared from the reaction mixture without mitochondria. All measurements were performed in triplicate. There was no difference between incubated control formulations and fresh control formulations in all tests (data not shown).

2.6. Preparation of pig ear skin

In vivo studies in humans provide the most direct, relevant, and therefore, conclusive information on skin permeation. Nevertheless, the advantages of *in vitro* experiments are lower cost, and the ability to test large numbers of formulations in relatively short time. Moreover, *in vitro* data can also be used to identify the rate-limiting skin layer for a given compound (Zats, 1993). In this regard, pig ear skin is considered as an excellent skin model, because the histological characteristics of pig and human skins have been reported to be very similar in terms of epidermal thickness and composition, pelage density, epidermal lipid biochemistry and general morphology (Bathia and Singh, 1996). Therefore, we used this pig ear skin model for *in vitro* permeation studies. Pig ears were obtained within 2 h after slaughter of the animals. The whole skin membrane was then carefully removed from the underlying cartilage with the help of a scalpel. The subcutaneous tissues were removed and the skin was stored at −4 °C for a maximum period of 30 days before use (Lopez et al., 2003).

2.7. In vitro permeation studies

The skin was mounted on the modified Franz diffusion cell with dermis facing the receptor compartment, and 1 g of the formulation containing 2% of the extract was placed on the membrane surface in the donor compartment while the receptor was filled with 10 mL of receptor medium containing 0.1 M phosphate buffer (pH 7.6), which was in contact with the skin. During the experiments, the receptor solution was continuously stirred at 100 rpm and kept at 37 ± 1 °C. At designated time intervals (3, 6, 9, 10 and 12 h) the receptor medium was removed and the amount of daidzein and genistein were analyzed by HPLC and scavenging of hydroxyl radical activity (see Section 2.9) (Bathia and Singh, 1996; Bentley et al., 1999). All measurements were performed in quintuplicate and formulation without soybean extract was used as control.

2.8. In vitro skin retention studies

At the end of the experiment described above (12 h), the skin was removed, cleaned with cotton soaked in methanol and homogenized in methanol. The solvent was evaporated with compressed air, and precipitate suspended in medium II (20 mM KH_2PO_4–KOH buffer, pH 7.4), which was used to determine the inhibition of MDA (malondialdehyde) formation-induced by hydroxyl radical (see Section 2.9). All measurements were performed in quintuplicate and formulation without extract was used as control.

2.9. Scavenging effect on hydroxyl free radical

10 μL of each sample were added to 1.0 mL of medium II plus 50 μM $FeCL_3$, 52 μM EDTA, 1 mM H_2O_2, 2.8 mM deoxyribose and, 100 μM ascorbate and reactions mixtures were incubated at 37 °C for 30 min. MDA formation caused by deoxyribose degradation (which occurred due to attack of hydroxyl radical) was estimated using thiobarbituric acid method (Aruoma, 1994; Georgetti et al., 2006). In this way, after the incubation 1 mL of 1% thiobarbituric acid (TBA), prepared in 50 mmol/L of NaOH plus 0.1 mL of 10 mol/L NaOH and 0.5 mL of 20% H_3PO_4 were added to medium II, followed by a 20 min incubation at 85 °C, and reading at 535 nm. The following controls were included in the test: (i) absence of sample, (ii) a positive control of the skin in the presence of formulations without extract and, (iii) a negative control in the absence of iron. The blank was prepared with the medium II alone. Thus, the positive control indicates the maximum MDA formation, which was considered 100% of deoxyribose degradation to calculate the scavenging of hydroxyl radical by the extract retained in the skin. All measurements were performed in triplicate.

2.10. In vivo evaluation of the efficacy of the formulations against damage induced by TPA

2.10.1. Animals and experimental protocol

In vivo experiments were performed in hairless male mice of the HRS/J strain (30 g) at 3 months of age. To study the effect of different formulations containing soybean extract on TPA-mediated cutaneous oxidative stress, 21 mice were randomly allocated to 7 groups of three mice each. Groups: (1) naïve control mice; (2) 0.2 mL acetone (TPA vehicle control); (3) TPA; (4) formulation 1 without soybean extract (0.5 g) + TPA; (5) formulation 1 with soybean extract (0.5 g) + TPA; (6) formulation 2 without soybean extract (0.5 g) + TPA; and (7) formulation 2 with soybean extract (0.5 g) + TPA. Mice were treated with formulations by topical application and after 4 h the groups 3–7 received the topical applications of TPA (20 nmol/0.2 mL acetone/animal). Mice were sacrificed after 12 h of TPA stimulus, and the skin tissues were excised and washed with ice-cold 0.9% NaCl. The tissues were homogenized in cold phosphate buffer (0.1 M, pH 7.4) by Polytron® homogenizer (PT3100). After that, homogenates were centrifuged at 12,100 × *g* for 20 min and the resulting supernatant assayed as described below (Section 2.10.2). The results are representative of two experiments with 3 mice per group.

2.10.2. Estimation of hydrogen peroxide

Hydrogen peroxide was estimated by the method described by Sharma and Sultana (2004). Briefly, a 0.5 mL solution of phenol-red (0.1 mg/mL phosphate buffer, 0.1 M pH 7.4) and horseradish peroxidase (50 μg/mL phosphate buffer, 0.1 M pH 7.4) was mixed with 0.5 mL of supernatants and incubated at 37 °C for 10 min. Then, 1 mL NaOH (1 M) was added and the absorbance was read at 610 nm. Hydrogen peroxide was calculated as nmol H_2O_2/g tissue using a molar extinction co-efficient of 43,600.

2.10.3. Estimation of lipid peroxidation

The assay for lipid peroxidation was done according to the method of Iqbal et al. (1996). The reaction mixture consisted of 0.58 mL phosphate buffer (0.1 M, pH 7.4), 0.2 mL of the supernatants, 0.2 mL ascorbic acid (100 mM) and 0.02 mL ferric chloride (100 mM) in a total volume of 1 mL. The mixture was incubated at 37 °C for 1 h. The reaction was stopped by the addition of 1 mL of 10% trichloroacetic acid. All the tubes were placed in a boiling water bath for 20 min. Then, tubes were placed in an ice bath and centrifuged at 2500 × *g* for 10 min. The amount of malondialdehyde (MDA) formed in each of the samples was assessed by measuring the absorbance in 535 nm. The results were expressed as nmol MDA formed/h/g tissue at 37 °C using a molar extinction co-efficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ (Salem et al., 1999).

2.11. Statistical analyses

Data were statistically analyzed by one-way ANOVA, followed by Bonferroni's multiple comparisons *t*-test for evaluation of the effect of different formulations against damage oxidative in mice skin induced by TPA. Results were presented as mean ± S.E.M. (standard error mean) and considered significantly different when $P < 0.05$ was obtained.

3. Results

3.1. Physico-chemical stability

Table 2 shows the range values of pH, globule size and centrifugation assay data for formulations with high and low lipid content (formulation 1 and formulation 2, respectively) without and with 2% soybean extract. There was no significant difference for pH and globule size after addition of the soybean extract as well as there was no phase's separation. The pH values showed minimal changes within 5.55–5.40 and 5.47–5.30 range for formulation 1 and formulation 2 containing soybean extract, respectively, using accelerated storage conditions (40 °C/70%UR). The globule size values were between 2.55–2.45 and 2.55–2.50 μm range for formulation 1 and formulation 2 containing soybean extract, respectively, for all storage conditions. During 6 months of the study in different storage conditions the formulations 1 and 2 added or not with soybean extract were stable to centrifugation test.

Table 2
Range values of pH, globule size and centrifugation found for formulations added or not with soybean extract

Formulation	pH	Globule size (μm)	Centrifugation
F1	6.20–6.20	2.50–2.53	No separation
F1 + extract	5.55–5.40	2.55–2.45	No separation
F2	6.15–6.10	2.50–2.55	No separation
F2 + extract	5.47–5.30	2.55–2.50	No separation

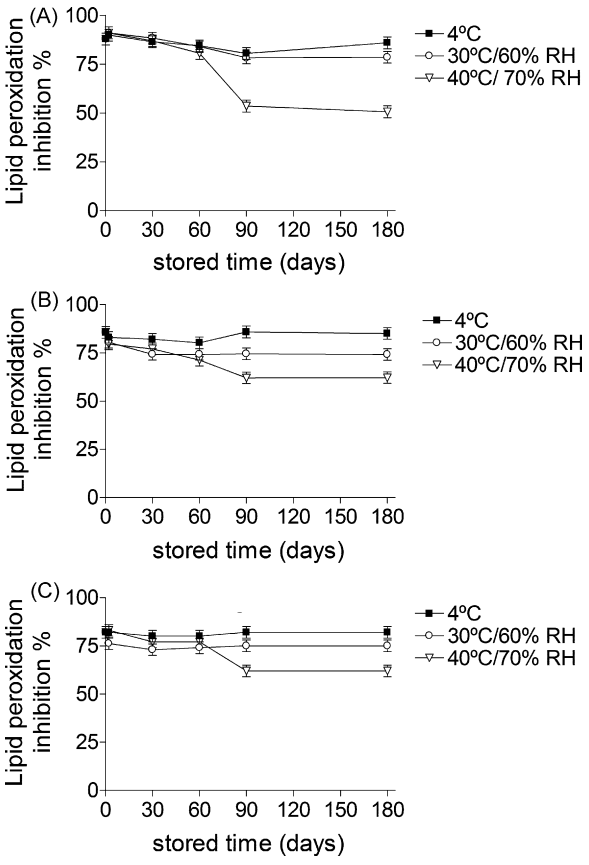

Fig. 1. Antiperoxidative activity evaluation of Isoflavin Beta® extract (A), formulation 1 (B) and formulation 2 (C) stored at 4 °C (■); 30 °C/60% RH (◯) and 40 °C/70% RH (▽) during 6 months.

3.2. Evaluation of antioxidant activity stability

Initially, possible interferences of the formulations components in the lipid peroxidation assay were investigated. The MDA production in the presence or absence of extract was compared with the formulations 1 and 2 with or without extract. It was detected that soybean extract significantly inhibited the MDA production, which was not altered by the formulations constituents (data not shown). These results further confirm the adequacy of the assay to evaluate the extract functional stability in those formulations as we previously demonstrated (Casagrande et al., 2006b; Georgetti et al., 2006).

The evaluation of the antiperoxidative stability of soybean extract did not demonstrate loss of activity when stored at 4 °C in the study period, while the extract kept at 30 °C/60% RH (relative humidity) or 40 °C/70% RH for 6 month, lost 10 and 42% of the initial (zero time) antiperoxidative activity, respectively (Fig. 1A).

The results presented in Fig. 1B and C demonstrate the antiperoxidative activity of the formulations 1 and 2 during the stored period at 4 °C; 30 °C/60% RH or 40 °C/70% RH. The antiperoxidative activity remained stable for 6 months at 4 °C in both formulations. However, after 6 month at 30 °C/60% RH or 40 °C/70% RH the formulation 1 lost 13.3 and 26.5% of the antiperoxidative activity, respectively. In the same conditions, the loss of activity for formulation 2 was 7.0 and 23.8%, respectively.

The stability data of antioxidant activity obtained from soybean extract and formulations 1 and 2 containing this extract showed that at 40 °C/70% RH for 6 months the extract alone lost about 1.5-fold the antiperoxidative activity compared to the extract added in formulations.

3.3. In vitro retention studies

The retention studies were initially done by measuring the genistein and daidzein amounts in the skin methanol extract by HPLC. However, there was significant interference of skin components in the chromatographic method (data not shown). Thus, the amount of soybean extract (Isoflavin Beta®) retained into pig ear skin was evaluated determining the amount of antioxidant substances (μg/cm^2, polyphenols) by the deoxyribose method using a curve of soybean extract concentration versus % inhibition of MDA-TBA formation ($y = 967.9x + 23.01$). The results obtained were 0.17 ± 0.02 and 0.24 ± 0.02 μg/cm^2 for formulations 1 and 2, respectively, demonstrating no difference between the retention of antioxidant substances yielded by the formulations.

3.4. In vivo evaluation of the efficacy of the formulations containing soybean extract against TPA-induced oxidative skin damage

The production of H_2O_2 was evaluated in the skin of hairless mice induced by application of TPA (20 nmol/0.2 mL acetone/animal). As shown in Fig. 2, a single topical application

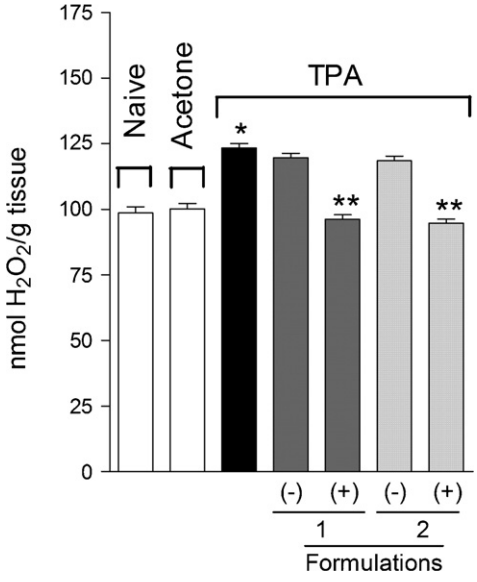

Fig. 2. Formulations containing soybean extract inhibits the TPA-induced generation hydrogen peroxide in mice. Bars represent means ± S.E.M. of 2 separates experiments, 3–5 mice per group. Formulations lacking (−) or containing (+) Isoflavin Beta® extract were used. Acetone is the vehicle of TPA. *Significant ($P < 0.05$), when compared with acetone treated control group. **Significant ($P < 0.05$), when compared with TPA treated control group and formulations 1 and 2.

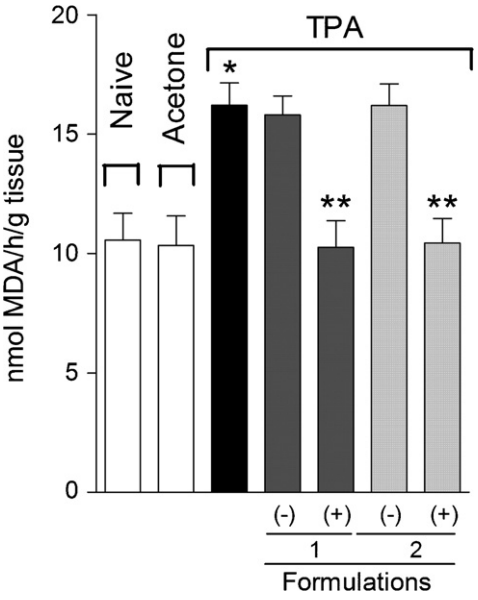

Fig. 3. Formulations containing soybean extract inhibits the TPA-induced enhance in the susceptibility of cutaneous for iron-ascorbate-induced lipid peroxidation. Bars represent means ± S.E.M. of 2 separates experiments, 3–5 mice per group. Formulations lacking (−) or containing (+) Isoflavin Beta® extract were used. Acetone is the vehicle of TPA. *Significant ($P < 0.05$), when compared with acetone treated control group. **Significant ($P < 0.05$), when compared with TPA treated control group and formulations 1 and 2.

of TPA significantly enhanced the production of H_2O_2 by 1.25-fold in the mice skin comparing to naïve or vehicle (acetone) controls. This TPA-mediated production of H_2O_2 was abolished when the skin was pretreated with formulation 1 and formulation 2 containing soybean extract (Fig. 2).

The effect of pretreatment with topical formulations containing soybean extract was also tested on TPA-mediated susceptibility of cutaneous membrane to iron-ascorbate-induced lipid peroxidation (MDA formation). The treatment with TPA alone increased the MDA formation to approximately 1.6-fold comparing to naïve and vehicle controls. Further supporting the possible usefulness of the present formulations, their topical application in the mice skin abolished the MDA formation induced by TPA (Fig. 3). Furthermore, confirming that the abolishing effect of these formulations is related with the active principle (soybean extract), none of the formulations without active principle affected the TPA-induced H_2O_2 and MDA formation (Figs. 2 and 3).

4. Discussion

There are evidences suggesting that topical application of antioxidants might diminish the oxidative damages of the skin with special emphasis on plant-derived drugs (Casagrande et al., 2006a). In this sense, we previously evaluated the chemical composition, and antioxidant and free radical scavenging activities of the soybean extract Isoflavin Beta® alone and added in different topical formulations. Thus, this *in vitro* study suggests the possible usefulness of formulation containing soybean extract to control oxidative stress (Georgetti et al., 2006). Therefore, two formulations containing soybean extract were developed and their physical stability (pH, centrifugation, globule size) functional stability (by antioxidant activity), percutaneous absorption and *in vivo* activity on TPA-induced hydrogen peroxide production and MDA formation were evaluated.

The formulations used in this study are emulsions, which are inherently unstable systems and phases may ultimately completely separate (Pather et al., 1995). Physical instability of disperse systems in general and of emulsions in particular is caused by physical phase separation of some type, which leads to change in appearance, consistency, redispersability and performance (Idson, 1993). The emulsions with high lipid content (formulation 1) and with low lipid content (formulation 2) containing soybean extract stored in different conditions of the temperature and relative humidity were stable after 6 months when centrifugation test was applied.

The measurement of pH of the formulations is necessary to detect pH alterations during the time of storage ensuring that pH value is compatible with the components of formulation and with the application place, which avoids irritation for instance, of the skin. There was no significant variation of the pH of the different formulations containing or not soybean extract during the time storage in different conditions of the temperature and relative humidity. Another parameter evaluated was the sizes of the globules during the storage, which provides an indication of the stability of the system. It is a consensus that the globules increase in size negatively correlates with the stability (Georgetti et al., 2006). It was detected that the globule size remained unaltered during the storage period, thus, suggesting that there was no coalescence.

It has been reported that the stability profile of the biological activity of isoflavonoids from soy extracts is strongly affected by processing and storage conditions such as temperature and light exposure. These last two factors are the major ones influencing the antioxidant activity of extracts during storage and they affect different compounds at different extents (Singletary et al., 2000).

Therefore, in the present study the functional stability was evaluated at different climatic conditions and periods by the antioxidant activity. In agreement with previous data, there was no significant influence of the formulations constituents in the MDA production as determined using the $\varepsilon = 1.56 \times 10^5\ M^{-1}$ (Georgetti et al., 2006). On the other hand, the functional stability study demonstrated that temperature and humidity affected the stability of formulations, suggesting that enzymatic hydrolytic reactions may be involved.

The results of functional stability suggest a decrease in the antilipoperoxidative activity of soybean extract in both formulations at high temperature. It has been demonstrated that products from soy such as Isoflavin Beta® (soybean extract), may contain besides isoflavonoids (e.g., biochanin A, daidzin, genistin, daidzein and genistein) other important phenolic compounds (e.g., *p*-hydroxybenzoic, gentistic, vanillic, caffeic, syringic, *p*-coumaric, ferulic, sinapic, salicylic, isoferulic, and *o*-coumaric acids) that could also exhibit antioxidant activity (Soobrattee et al., 2005). These polyphenols are susceptible to temperature and humidity action, and their stability profile and biological activity

are strongly related and affected by processing and storage conditions (Ungar et al., 2003). Thus, the reduction in activity could be related to the polyphenol content that include for instance, daidzein and genistein.

There is no doubt that the release of a drug from a topical formulation can be effectively influenced by the vehicle in which it is applied. An appropriate formulation of the topical agent will ensure that it exerts its maximal activity on the skin. Therefore, the percutaneous absorption of the isoflavonoids daidzein and genistein were evaluated to further substantiate the possible usefulness of extracts containing isoflavonoids (e.g., Isoflavin Beta®) as active principles in formulations.

Considering that genistein and daidzein are constituents of the Isoflavin Beta® extract, the permeation studies were initially done by measuring the genistein and daidzein amounts by HPLC. Although the HPLC is the most used analytic methodology in percutaneous absorption studies, it did not detect isoflavonoids in the receptor medium (data not shown). In addition, isoflavonoids antioxidant activity was also not detected in the receptor medium (data not shown). These results might suggest that soybean extract did not permeate through the pig ear skin or even that the amount permeated was below the HPLC methodology limit detection (0.1 μg/mL).

Moreover, the HPLC analyses did not detect retained daidzein or genistein in the pig ear skin. This negative result could be related to the limit detection of the method or interference of the tissue that was not completely eliminated by the extraction procedure (data not shown). Therefore, deoxyribose assay was used as an alternative approach to evaluate the possibly retained antioxidant substances in the skin. In fact, it was detected significant antioxidant activity in the tissue, suggesting that the antioxidant components of the soybean extract in the topical formulations were released from it and reached the viable epidermis in sufficient amount to present antioxidant activity. This antioxidant activity seems to be local and not systemic since it was not detected in the receptor medium in the permeation studies.

Thus, the next step was to evaluate whether these formulations could protect from oxidative damages *in vivo*. Corroborating the permeation/retention studies that demonstrated similar results for both formulations, the pretreatment with these topical formulations containing soybean extract abolished the TPA-induced generation of hydrogen peroxide and lipid peroxidation (MDA formation). The treatment with TPA has been reported to induce a variety of changes in murine skin, including dark basal keratinocytes and sustained epidermal hyperplasia, reactive oxygen species formation in epidermis, elevated epidermal ciclooxygenase and lipoxygenase activities (Sultana and Saleem, 2004) and increase the susceptibility of cutaneous membrane lipid peroxidation (Salem et al., 1999). Further supporting the present data, it has been previously demonstrated that purified genistein also inhibits TPA-mediated hydrogen peroxide formation and inflammatory responses *in vivo* and *in vitro* (Wei et al., 2001).

Concluding, the main finding of the present study is that formulations containing soybean extract (Isoflavin Beta®) protect the skin against TPA induced oxidative stress. Thus, suggesting the importance of further studies to ascertain the clinical applicability of these formulations.

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