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European Journal of Pharmaceutics and Biopharmaceutics 64 (2006) 99–106
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Evaluation of the antioxidant activity of soybean extract by different in vitro methods and investigation of this activity after its incorporation in topical formulations

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Received 10 February 2006; accepted in revised form 21 April 2006
Available online 16 June 2006

Abstract

Chemoprevention by natural products is an emerging therapeutic approach for free radical-mediated diseases including cancer. This is a consequence of its wide applicability and acceptance. In the present study, the antioxidant activity of the soybean extract (Isoflavin Beta®) and of formulations added with this extract were evaluated using stable free radical 2,2-diphenyl-1-pycrylhydrazyl (DPPH·) and deoxyribose as well as the lipid peroxidation inhibition assays. For all the assays the extract showed a dose-dependent activity, and IC_{50} of 21.03 μg/mL in lipid peroxidation inhibition, 161.8 μg/mL in DPPH·, and 33.5 ng/mL in hydroxyl radical scavenging assay. The antioxidant activity of the extract added in the formulations could not be assessed using the deoxyribose assay. However, the lipid peroxidation inhibition and DPPH· scavenging assays could be successfully applied for the antioxidant activity evaluation of the formulations added with soybean extract to protect the skin against free radicals, which can be generated by the ultraviolet radiation exposure.

Keywords: Soybean extract; Antioxidant; Polyphenols; Topical formulation; DPPH·; Deoxyribose; Lipid peroxidation

1. Introduction

It is well established that the inflammatory response following acute UV light skin irradiation and the degenerative processes related to chronic UV radiation skin exposure are largely mediated by the overproduction of reactive oxygen species (ROS) and free radicals, and by impairment of antioxidant systems [1].

Furthermore, ROS are believed to be involved in many skin disorders such as cancer formation, cutaneous autoimmune diseases, phototoxicity, photosensitivity and skin aging. Two of the early cellular events following UV light exposure are the lipid peroxidation induction and the suppression of replicate DNA synthesis due to DNA damage [2].

Considering that solar UV radiation is the major environmental inducer factor of skin cancer, various efforts have been made to prevent skin cancer caused by sun exposure. For instance, application of sunscreens agents on the body surface is the most used [3]. However, the currently used sunscreens may be sometimes overwhelmed by excessive sun exposure or present several deleterious effects (i.e. allergy, inflammation) on the skin. Therefore, novel materials for chemoprevention of UV-induced skin cancer are needed. In this regard, one of the safest approaches is the prevention by anti-oxidative chemicals or plants materials [3]. Corroborating, soybean-germ oil

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0939-6411/$ - see front matter
doi:10.1016/j.ejpb.2006.04.003

possesses a remarkable protective activity against UVB-induced skin inflammation [4].

Soybeans contain isoflavones that have several known activities, including estrogenic, fungitoxic, and antioxidant [5]. The biologically active components of soy isoflavones include genistein, daidzein and biochanin A [6]. Genistein inhibits protein tyrosine kinase activity, topoisomerases I and II, ribosomal 6S kinase and alters cell proliferation [7]. It also has antioxidant properties and suppresses skin tumorigeneses. In many cases, the combined effect of soy isoflavones might be better than the effect of any single isoflavone compound [8].

Thus, considering that soybeans constituents present potential anti-cancer effects and antioxidant activity, the development of topical formulations added with soybean extracts as well as the correct evaluation of their antioxidant activity by using different methodologies would be very helpful to choose the most adequate one.

Therefore, in the present study it was evaluated the chemical composition, antioxidant and free radical scavenging activities of the soybean extract Isoflavin Beta® alone and added in different topical formulations. The antioxidant activity was evaluated by their ability to inhibit lipid peroxidation induced by Fe^{2+}, H-donor capability and scavenging hydroxyl radical effect. Furthermore, physical-chemical parameters such as centrifugation stability, pH and globule size were also addressed. Therefore, it will be possible to choose the most adequate antioxidant methodology to make the quality control and stability studies of all the formulations in which the soybean extract was added.

2. Materials and methods

2.1. Chemicals

Isoflavin Beta® extract from France and raw materials for formulations (presented in the formulation section) were obtained from Galena (Campinas, SP, Brazil). Genistein, daidzein, thiobarbituric acid (TBA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH·) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). 2-Deoxy-D-ribose was obtained from Acros (New Jersey, USA). All other reagents were of the highest grade commercially available. The raw materials were dissolved in dimethylsulfoxide for the antioxidant assays. The amount of solvent used had no effects on the assays.

2.2. Total polyphenols and flavonoids contents in the Isoflavin Beta® soybean extract

One hundred milligrams of extract was stirred with 80% ethanol for 15 min. The ethanol suspensions were centrifuged at 1660*g* for 10 min and supernatant fraction collected into 25 mL of the volumetric flask. The precipitate was extracted with 5 mL of 80% ethanol. Finally, the supernatant fraction was combined and the volume adjusted to 25 mL with deionized water.

Total polyphenols contents were determined by Folin-Ciocalteau colorimetric method [9]. In this methodology, 0.5 mL sample was mixed with 0.5 mL of the Folin-Ciocalteau reagent and 0.5 mL of 10% Na_2CO_3, the absorbance was measured at 760 nm after 1 h incubation at room temperature. Total polyphenols contents were expressed as mg/g (gallic acid equivalents).

Total flavonoids contents of the soybean extract were determined using the aluminum chloride colorimetric method. To 0.5 mL of sample, 0.5 mL of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, the absorbance was measured at 420 nm [10]. Total flavonoids contents were calculated as quercetin (mg/g) from an analytical curve.

2.3. HPLC analysis

Genistein and daidzein contents in Isoflavin Beta® extract were determined by reversed-phase HPLC analysis. Isoflavin Beta® was dissolved in 25 mL methanol, and diluted in the mobile phase 1:2, filtered with a 0.45 μm filter, and 20 μL was injected into the HPLC system. The separation of the isoflavonoids genistein and daidzein was performed by employing the SuperPac Sephasil C18 (5 μm) column, 250 × 4 mm attached to a pre column. A mobile phase was employed; it consisted of 0.1% acetic acid in acetonitrile, 0.1% acetic acid (70:30), 1 mL/min. Eluted isoflavonoids were detected by their absorbance at 250 nm [11]. The linearity was obtained with concentrations of 0.05–10 μg/mL. Quantitative data for daidzein and genistein were obtained by comparison to known standards.

2.4. Formulations

The formulations were developed varying the content of lipidic and emulsifying agent. Formulation 1 was prepared with commercially available self-emulsifying wax Polawax® (cetostearyl alcohol + polyoxyethylene derived of a fatty acid ester of sorbitan 20E), formulation 3 with Croda® (mineral oil + petroleum + lanolin alcohol + ethoxylated fatty alcohol) and into formulations 2 and 4 anionic hydrophilic colloid (carboxypolymethylene, Carbopol®) was also added as a stabilizing agent. Macadamia nut oil was added as an emollient, and propylene glycol as a moisturizer. The preservative used was a mixture of parabens and imidazolidinyl urea. Deionized water was used for the preparation of all formulations (Table 1). Extract of soybean (Isoflavin Beta®) 2.0% was firstly solubilized in propylene glycol and then incorporated into the formulations at room temperature.

2.5. Preparation of samples

The extract Isoflavin Beta® was diluted with dimethylsulfoxide (DMSO) to final concentrations of 12.5, 25, 50, 100, 150, 200, 250 and 300 μg/mL, and 31.25, 62.5, 125,

Table 1
Percent composition (w/w) of the emulsion media of the formulations

Components	Formulation 1 (%)	Formulation 2 (%)	Formulation 3 (%)	Formulation 4 (%)
Polawax®	10.0	2.0	–	–
Croda®	–	–	10.0	2.0
Carbopol® 940 (dispersion 3%)	–	6.0	–	6.0
Macadamia nut oil	2.0	2.0	2.0	2.0
Propylene glycol	6.0	6.0	6.0	6.0
Methylparaben	0.15	0.15	0.15	0.15
Propylparaben	0.02	0.02	0.02	0.02
Imidazolidinyl urea	0.30	0.30	0.3	0.3
Deionized water	81.53	83.53	81.53	83.53

250 and 500 μg/mL, and 0.005, 0.01, 0.02, 0.04, 0.2, 0.5 and 1.0 μg/mL, for the following methodologies: inhibition of lipid peroxidation, reduction of 2,2-diphenyl-1-picrylhydrazyl radicals (DPPH·) and deoxyribose assay. The formulations added with 2.0% of soybean extract and extract-free formulations were diluted 1:5 with ethanol for the inhibition of lipid peroxidation and DPPH· reduction. For the deoxyribose assay formulations added to 0.05% of soybean extract were diluted 1:5 with ethanol and after 1:2 with reaction medium. After this, the antioxidant activities found in the formulations were compared to that of the Isoflavin Beta® soybean extract in the same final concentration.

2.6. Lipid peroxidation induced by Fe^{2+}/citrate system

Male Wistar rats weighing approximately 200 g were sacrificed by cervical dislocation. 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, and homogenized three times for 15 s at 1 min intervals in a Potter–Elvehjem homogenizer. Mitochondria were prepared by standard differential centrifugation techniques as previously described [12,13], and protein content determined by the biuret reaction [14].

Ten microliters of different concentrations of the extract Isoflavin Beta® and 37.5 μL of formulations with and without the extract were added to 1.0 mL of a reaction mixture containing sucrose (125 mM), KCl (65 mM) and Tris–HCl (10 mM) (pH 7.4; medium I), and mitochondria (final concentration of 1 mg of protein). Then, 50 μM $(NH_4)_2Fe(SO)_4$ and 2 mM sodium citrate were added, and the samples were incubated at 37 °C for 30 min. For malondialdehyde (MDA) determination, 1 mL of 1% thiobarbituric acid (TBA) (prepared in 50 mM NaOH), 0.1 mL of 10 M NaOH and 0.5 mL of 20% H_3PO_4 were added, followed by incubation for 20 min at 85 °C. The MDA–TBA complex was extracted with 2 mL *iso*-butanol. The samples were then centrifuged at 1660*g* for 10 min. The measurement was performed on the supernatant at 535 nm. The amount of TBA-reactive compounds was expressed as MDA, and calculated from $\varepsilon = 1.56 \times 10^5/\mathrm{M}$.

2.7. Antioxidant activity by DPPH· assay

The antioxidant activities of different concentrations of extract Isoflavin Beta® and formulations were determined by hydrogen-donating ability of isoflavonoids of the soybean extract to free radical stable DPPH· [15]. For radical scavenging measurements, 1 mL of 100 mM acetate buffer, pH 5.5, 1 mL of ethanol, 0.5 mL of 100 μM ethanolic solution of DPPH· were mixed, 10 μL of extract and 50 μL of formulations under study were added, then the absorbance was measured after 15 min at 517 nm [16]. Blank was prepared from the reaction mixture without DPPH· solution. All measurements were performed in triplicate.

2.8. Deoxyribose assay

The degradation of deoxyribose by hydroxyl radical was evaluated as described by Halliwell [17], with some modifications. One milliliter of KH_2PO_3–KOH buffer (20 mM, pH 7.4), 10 μL ascorbate (100 μM), 10 μL of the extracts in different concentrations or of formulations, 10 μL deoxyribose (2.8 mM), 10 μL H_2O_2 (1 mM), 10 μL Fe-EDTA ($FeCl_3$ 50 μM, EDTA 52 μM) were mixed. Reaction mixtures were incubated at 37 °C for 30 min and MDA formation by deoxyribose degradation caused by hydroxyl radical was estimated using the thiobarbituric acid method [18,19].

2.9. Precision of the analytical methods

The precision of the methods was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by assaying samples with the same concentration and during the same day. The intermediate precision was studied by comparing the assays on different days (3 days). The precision of the HPLC and spectrophotometric methods was evaluated using $n = 9$ for intra-day or $n = 15$ for inter-day [20].

2.10. Centrifugation assay and pH measurements

The samples were centrifuged at 3000 rpm for 30 min at room temperature. Phase separation was analyzed visually [21]. Sample pH measurements, after dilution 1:10 in water,

were carried out at room temperature with Digimed DMPH-2 pHmeter.

2.11. Globule size measurement

The sizes of the emulsion globules were examined microscopically (Olympus microscope fitted with a 40× objective lens). The formulations were diluted 100 times using propylene glycol/water (1:1), and after, the lipophilic stain Sudan II 1% was added. One droplet of the suspensions was put into Neubauer chamber and the average numbers of globules in each square ($\tilde{n}$) were determined. The results were applied into the following equation to determine the numbers of globules in each gram of the emulsion (N_w) [22].

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

where $\tilde{n}$ is the number of particles in a size range whose midpoint

Q = the amount (g) of internal phase of emulsions after dilution according to E
E = the amount (g) of the diluted emulsion which contains Q_g of internal phase
P_d = density of the dilution liquid
V = the volume of the chamber.

2.12. Statistical analysis

Data were expressed as means ± SE determined of triplicate analysis. The percentage which caused 50% of inhibition of the systems assessed (IC_{50}) by the Isoflavin Beta® extract was determined using GraphPad Prism® software. Data were statistically analyzed by one-way ANOVA, followed by Bonferroni's multiple comparisons *t*-test for evaluation of the formulations influence in the antioxidant activity assays. Results were presented as means ± SEM (standard error mean) and considered significantly different when $P < 0.05$ was obtained.

3. Results and discussion

The skin is considered to be our biological interface with the surroundings and it is the first line of defense from toxic external stimuli like ultraviolet and visible radiations, pro-oxidant chemical compounds, infections and ionizing radiation. Skin exposure to ultraviolet radiation induces the formation of lipid peroxidation products, lipid radicals, melanin radicals, endogenous antioxidants depletion, and ROS overproduction [23]. Soybeans contain a number of important phenolic compounds, including free phenolic acid, phenolic acid esters, isoflavones, genistein and daidzein, their glycosides genistein and daidzein and coumesterol [24]. Recent work highlighted the role of the polyphenolic components of the high plants as antioxidants, antimutagenic, anti-inflammatory and antimicrobial [25]. Moreover, the antioxidant activity may be related to polyphenol and flavonoid content since it has been reported that these phenolic compounds can act breaking the chain reaction of lipid by scavenging several ROS [26], and inhibiting chemiluminescence reactions [27].

Total polyphenol present in the Isoflavin Beta® extract was determined using the Folin-Ciocalteau method coloration [9]. Total flavonoids contents were measured by the aluminum chloride colorimetric method [10]. Table 2 shows that polyphenols contents were about 11 times higher than total of flavonoids contents. In agreement, the HPLC method indicated that genistein and daidzein are the main components of the soybean extract. These isoflavonoids may complex with $AlCl_3$, but with low absorption in 415 nm or even do not form complex with $AlCl_3$ [10]. Consequently, the smaller amount of total flavonoids obtained in Isoflavin Beta® extract was due to the low concentration of flavonoids other than genistein and daidzein. Corroborating, the quantitative analysis of isoflavonoids by HPLC-UV (254 nm) using Super Pac®C_{18} (5 μm) column and isocratic elution showed the separation of 5 peaks of the Isoflavin Beta® methanol extract (Fig. 1). Two peaks were identified as daidzein and genistein on the basis of their retention times. Standard curves of genistein and daidzein concentration versus peak height were linear as indicated by the correlation coefficient for $r > 0.999$. The inter-assay coefficients of variation for genistein (0.87 μg/mL) and daidzein (1.97 μg/mL) were 0.38% and 1.48%, respectively. Additionally, the flavonol quercetin was not detected in the extract.

The antioxidant activity of the Isoflavin Beta® extract was evaluated by different methods since the oxidative stress depends on the type of generated ROS, how it is generated, where it is generated, and the oxidative target evaluated. Furthermore, the total antioxidant activities of vegetables cannot be evaluated by any single method, due to the complex nature of phytochemicals. Two or more methods should always be employed in order to evaluate the total antioxidant effects of vegetables [28].

Table 2
Polyphenol, flavonoid total and isoflavonoids (daidzein and genistein) content in Isoflavin Beta® soybean extract

Extract	Total flavonoids (mg/g)	Isoflavonoids (mg/g)		Total polyphenol (mg/g)
		Daidzein	Genistein	
Isoflavin Beta®	6.8 ± 2.2[a]	196.0 ± 1.7[a]	87.0 ± 1.2[a]	75.0 ± 1.8[a]

[a] Results are represented by means ± SEM.

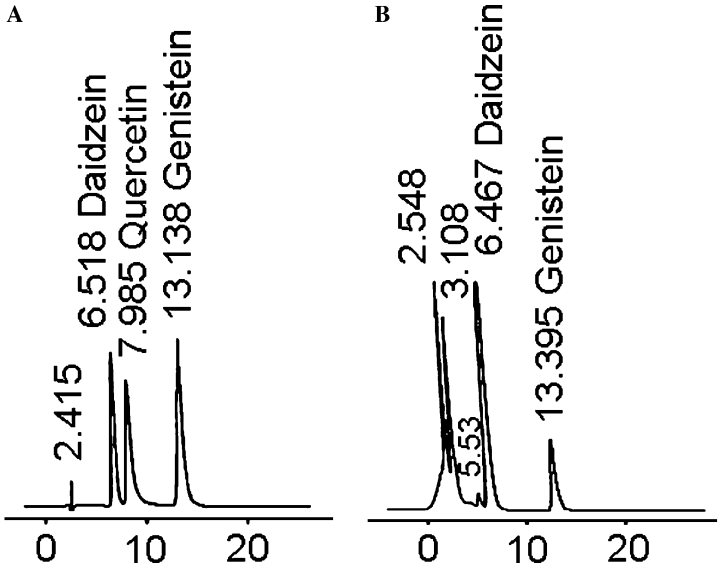

Fig. 1. Chromatograms of daidzein, quercetin and genistein standards (A) and Isoflavin Beta® extract (B). Chromatographic conditions: SuperPac Sephasil C18 (5 μm) column, 250 × 4 mm attached to a pre column, mobile phase: 0.1% acetic acid in acetonitrile, 0.1% acetic acid (70:30), 1 mL/min. Detection at 250 nm.

Therefore, the lipid peroxidation-inhibiting activity of Isoflavin Beta® was evaluated using the Fe^{2+}-citrate mitochondria system. Iron, either in an inorganic form or as a heme complex, is likely to form an important part of the catalytic system in tissue homogenates [29] and lipid peroxidation in the mitochondrial fraction is stimulated by iron addition [30]. In Fig. 2 the percent of peroxidation inhibition is plotted against different concentrations of the Isoflavin Beta® extract. The IC_{50} value was obtained by the concentration that caused 50% inhibition. The results were expressed using this same procedure for all the employed methodologies.

Thus, the extract showed significant inhibitory activity of lipid peroxidation compared to the positive control (100% complex MDA–TBA). This effect was concentration-dependency and the IC_{50} was 21.03 μg/mL.

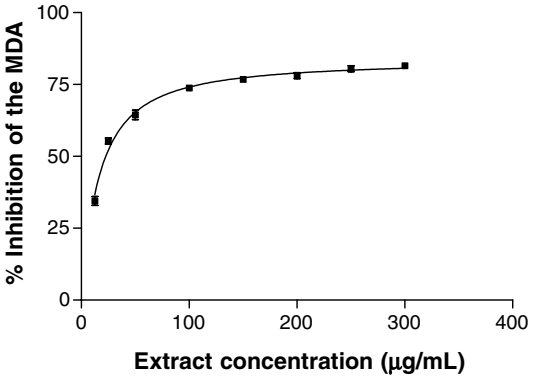

Fig. 2. Inhibition by Isoflavin Beta® extract of lipid peroxidation induced by Fe^{2+}/citrate by different concentrations of Isoflavin Beta® soybean extract. Mitochondria was utilized as lipidic material. MDA was determined as described in Materials and methods. Results are represented by means ± SEM.

The maximum lipid peroxidation inhibition by Isoflavin Beta® (150 μg/mL) was approximately 80% in which a plateau effect was observed. A 3.07% intra-assay precision and 5.03% inter-day repeatability were obtained. Therefore, the results suggest that effects of the extract on lipid peroxidation can be mainly due to alkoxyl and peroxyl radicals scavenging and/or iron chelating activities.

In the present study, the antioxidant activity was evaluated in different formulations added or not with extract. We observed that the capacity to inhibit the lipid peroxidation induced by Fe^{2+} was kept in the formulations with different content of lipidic (Fig. 3 F1 *versus* F2, and F3 *versus* F4), and emulsifying agents (Fig. 3, F1 *versus* F3, and F2 *versus* F4). In agreement with previous data, there was no significant influence of the formulations constituents in the MDA production as determined using the $\varepsilon = 1.56 \times 10^5\ M^{-1}$ (Fig. 3). The inhibition of lipid peroxidation by soybean extract was similar to that found for different formulations in which the extract was added (no significant difference). Therefore, suggesting that this methodology can be used to analyze the capability of inhibiting lipid peroxidation of antioxidants compounds incorporated in topical formulations.

The DPPH· assay [15] is widely used for the measurement of free radical scavenging capacity in phytotechnology, food technology, and pharmacology/toxicology. The DPPH· is a free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule [31]. The soybean extract tested in the present study for their H-donor ability, measured by the stable free radical DPPH· assay, showed lower antioxidant activity compared to the capability of inhibiting lipid peroxidation. The IC_{50} for the DPPH· assay was 161.8 μg/mL. The highest H-donor capability was achieved with 250 μg/mL of the extract (approximately 75% of DPPH· reduced), and after this

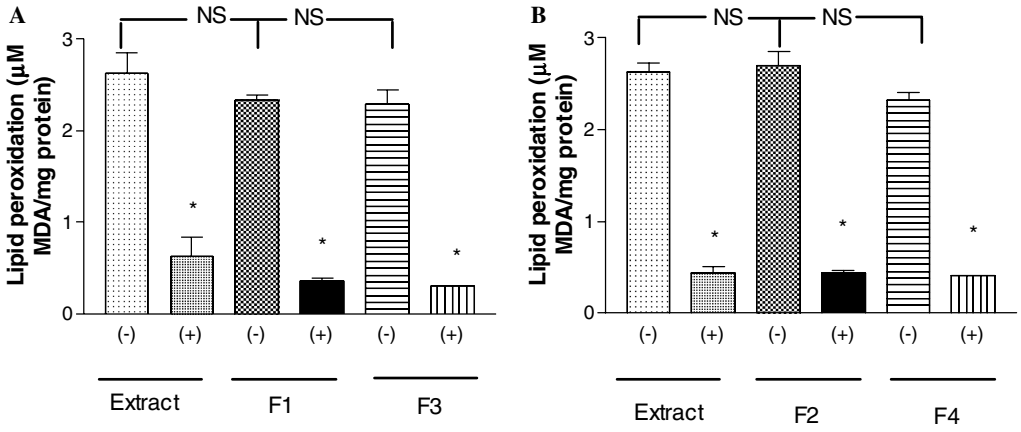

Fig. 3. Inhibition of lipid peroxidation induced by Fe^{2+}/citrate by formulations containing soybean extract (Isoflavin Beta®). Panel A: formulations 1(F1) and 3(F3). Panel B: formulations 2(F2) and 4(F4). Results are represented as concentration of MDA/mg of protein ± SEM ($\varepsilon = 1.56 \times 10^5\ M^{-1}$). Statistical analysis was performed by one-way ANOVA followed by Bonferroni's *t*-test. *Significantly different relative to respective control ($P < 0.05$).

concentration a plateau effect was observed (Fig. 4). The precision of this method was also evaluated, showing 1.8% intra-assay precision and 2.35% inter-day repeatability. Fig. 5 shows that the capacity to scavenge DPPH· was maintained in the formulations with different lipidic content of the self-emulsifying wax Polawax® compared with extract solution in the same concentration in the reaction medium. However, this result was not observed with self-emulsifying wax Croda® that showed lower H-donor capability (right bars of Fig. 5A-B). These may be due to interferences of formulation components in the reaction medium. Therefore, these results suggested that even though DPPH· assay is a fast and easy methodology to be used in quality control of formulations containing soybean extract, depending on the self-emulsifying base used this could interfere in the spectrophotometry reading which is used to determine the antioxidant activity [32].

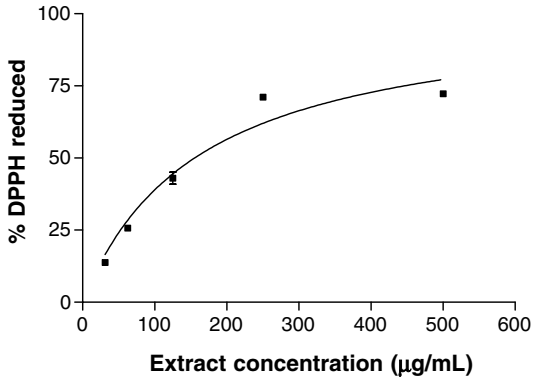

Fig. 4. H-donor ability soybean extract using stable radical DPPH· in 100 μM ethanolic solution found for different concentrations of Isoflavin Beta® soybean extract. Results are represented by means ± SEM.

The interaction of iron ions with hydrogen peroxide in biological systems can lead to formation of a highly reactive tissue-damaging species that is thought to be the hydroxyl radical (·OH) [31]. Thus, the scavenging hydroxyl radical effect of soybean extract added in different topical formulations was evaluated using deoxyribose assay. The sugar deoxyribose (2-deoxy-D-ribose) is degraded on exposure to hydroxyl radicals generated by irradiation or by Fenton systems (iron salt-dependent decomposition) [17].

Therefore, the ability of Isoflavin Beta® to scavenge hydroxyl radical was determined by the inhibition of

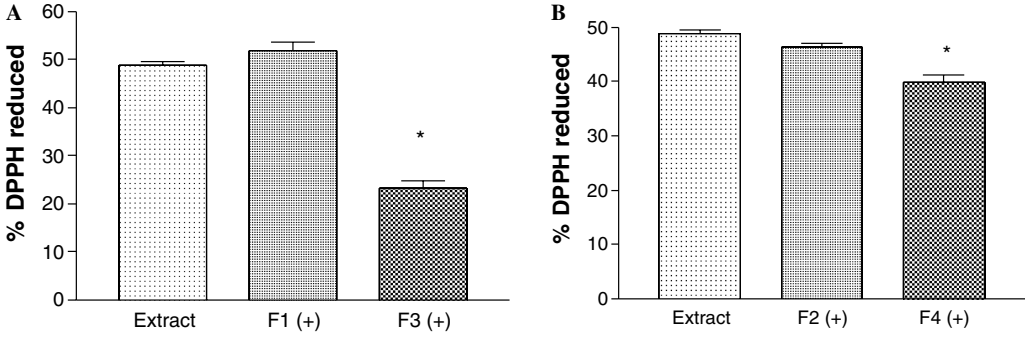

Fig. 5. Inhibition H-donor activity using DPPH· assay by the different topical formulations added with Isoflavin Beta® soybean extract. Panel A: formulations 1(F1) and 3(F3). Panel B: formulations 2(F2) and 4(F4). Results are represented as DPPH· reduced (%) comparing with absorbance at 517 nm of the control (100% of radical). Statistical analysis was performed by one-way ANOVA followed by Bonferroni's *t*-test. *Significantly different relative to respective control ($P < 0.05$).

thiobarbituric acid-reactive substances formation (MDA). MDA originates from the degradation of deoxyribose by Fenton reaction (iron (III)-EDTA and H_2O_2 in the presence of ascorbate). This degradation decreased with increasing concentrations of Isoflavin Beta® within the range of 0.005–1.0 μg/mL (Fig. 6). The level of inhibition reached a plateau of 90% at a concentration of 1.0 μg/mL Isoflavin Beta®. The IC_{50} of the extract was 33.5 ng/mL as calculated from the concentration-activity curve. This indicates that the isoflavonoids daidzein and genistein present in high concentration in Isoflavin Beta® possess a strong scavenging effect on hydroxyl radical. The precision of this method was also evaluated, showing 1.12% intra-assay precision and 2.37% inter-day repeatability.

However, the different topical formulations added with soybean extract did not display as effective scavenging hydroxyl radical activity as the extract solution in the same concentration, i.e. all the formulations added with extract showed the same amount of MDA formation as the formulations without the extract. Therefore, the deoxyribose assay is not adequate to evaluate the antioxidant activity of soybean extract Isoflavin Beta® added in topical formulations containing different lipid contents (data not shown). The interference of formulation components might occur in the development of the reactions involved with deoxyribose method as well as the possible inhibition of this activity by these components.

Emulsions are two-phase systems, consisting of liquid globules dispersed in an immiscible liquid. These systems are inherently unstable and, ultimately, the phases will separate completely [33]. 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 [34].

The centrifugation test is of major interest since it provides fast information about comparable stability properties of different emulsions. It is commonly accepted that shelf life under normal storage conditions can be rapidly predicted by observing the separation of the dispersed phase due to either creaming or coalescence when the emulsion is exposed to centrifugation [34]. This test is employed especially to evaluate emulsions stability during pre-formulation stage, however, can be used to detect alterations during storage [35]. Formulations 1, 2 and 4 were stable when centrifugation test was applied while visually phase separation was detected in formulation 3 after centrifugation (Table 3).

The measurement of pH of the formulations is necessary to detect pH alterations during the time storage, ensuring that pH value is compatible with the components of formulation and with the application place, avoiding irritation. There was no variation in the different pH of formulations during the time of analysis, which were compatible with topical use (Table 3).

The measurement of the sizes of the globules initially and during storage provides an indication of the stability of the system: the globules increase in size negatively correlates with the stability. Globule size can be measured by several methods being optical microscopy a common way of measuring the sizes of emulsion globules [33]. Formulations 3 and 4 showed globules with higher size than formulations 1 and 2. Furthermore, formulation 3 showed phases separation demonstrating that the addition of soybean extract in this type of formulation could compromise its structure.

Thus, this work demonstrated that the soybean extract presented concentration-dependent antioxidant activity in all methodologies studied. These assays may be adequate in evaluating the antioxidant activity of this natural product. Nevertheless, depending on the type of radical generated, the soybean extract showed different IC_{50} values.

Furthermore, it was noticed that depending on the lipidic content and the emulsifying agent used in the formulation interferences in the assays measurements might occur as well as, in the development of the reactions involved in these methodologies. However, in lipid peroxidation and stable free radical DPPH· assays there was no interference which means that they are able to evaluate the antioxidant activity of these formulations.

The addition of vegetable extract did not compromise the structure of the topical formulations, which were confirmed stable in formulations 1, 2 and 4 by the centrifuge assay. Formulations prepared with self-emulsifying wax Polawax® showed the size of the globules lower than the formulations prepared with self-emulsifying wax Croda®.

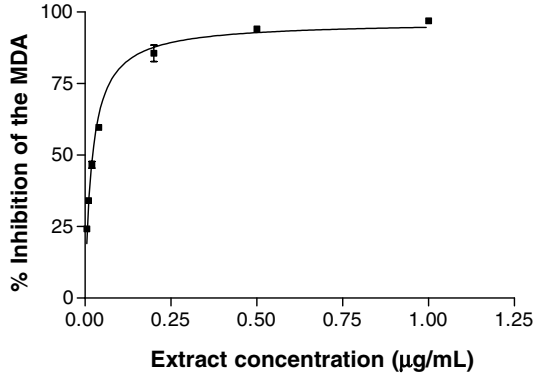

Fig. 6. Inhibition of deoxyribose degradation by different concentration of the Isoflavin Beta® soybean extract. Results are represented as inhibition of MDA formation (%). Results are represented by means ± SEM.

Table 3
Centrifuge, pH and globule size test data of functionalized different topical formulations added with Isoflavin Beta® soybean extract

Formulations	Centrifugation	pH	Size globule (μm)
F1	No separation	5.40	2.60
F2	No separation	6.25	2.50
F3	Separation	5.30	3.78
F4	No separation	6.00	2.90

For the pH test all the formulations showed values acceptable to be used in skin.

The results observed in this work suggest that soybean extract (Isoflavin Beta®) could be active against free radicals and it can be added to topical formulations in order to protect skin against damage caused by reactive oxygen species. Nevertheless, depending on the lipidic content and the emulsifying agent used it is necessary to choose the most adequate methodology to evaluate the antioxidant activity.

Acknowledgments

This work was supported by grants from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support.

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