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Assays of physical stability and antioxidant activity of a topical formulation added with different plant extracts

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Abstract

In the present investigation the changes on physical stability (pH, viscosity, flow index and tixotropy) of topical formulations were evaluated following inclusion of different plant extracts containing flavonoids. Also, the antioxidant effect of these plant extracts alone and after addition in the formulation was evaluated using chemiluminescence and the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH•) assays, as well as the inhibition of lipid peroxidation. Formulation added with DL-α-tocopherol was used to compare the physical stability and antioxidant activity. Formulations with plant extracts showed pseudoplastic behavior with decreasing on viscosity and tixotropy. The *Glycyrrhiza glabra* (GG) and *Ginkgo biloba* (GB) extracts alone and the formulations containing these extracts showed great antioxidant and free radical scavenging activities while the other extracts studied (mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L and *Arctium majus* root, *Nelumbium speciosum* and soybean) showed lower activity. The results suggest that GG and GB extracts may be used in topical formulations in order to protect skin against damage caused by free radical and reactive oxygen species.

Keywords: Topical formulation; Antioxidant; Flavonoids; Viscosity; Plant extract

1. Introduction

Plants produce a variety of antioxidants against molecular damage from reactive oxygen species (ROS), and phenolic compose the major class of plant-derived antioxidants. Among the various phenolic compounds, the flavonoids are perhaps the most important group [1]. The use of plants containing flavonoids, either alone or in combination, has increased due to both the increasing demand by consumer for compounds of natural origin and by the attention given to dietary plants containing this class of molecule as natural cancer chemopreventive compounds [2]. The capability to interact with protein phosphorylation and the antioxidant, iron chelating and free radical scavenging activity may account for the wide pharmacological profile of flavonoids. Besides scavenging UV-induced radicals and so inhibiting propagation of lipid peroxidative chain reactions, flavonoids might provide their protective effect against UV radiation by acting as strong UV-absorbing screens [3].

Several papers have shown the antioxidant activity in vitro or in vivo of some plant extract as *Glycyrrhiza glabra* and *Ginkgo biloba* extract [4–9]. Both extracts have antioxidant activity against hydroxyl, superoxide and peroxyl radicals, and they may play a role in the treatment of diseases involving free radicals and oxidative damage such as cancer and aging. Topical administration of antioxidants has recently proved to represent a successful strategy for protecting the skin against UV-mediated oxidative damage [10–13], but there is no data in literature about their efficacy following addition in topical formulations and their influence on physical stability of the formulation.

Since flavonoids are claimed to prevent photooxidative stress in the skin, it is important to know if plant extracts

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containing these substances maintain their action after inclusion in topical formulations and how they may interfere with the physical stability of the formulation. In view of the importance of flavonoids in the protection of skin from oxidative injury, and the necessity for correct evaluation of the antioxidant activity in topical formulations containing these substances, we have evaluated the antioxidant activity of different plant extracts alone, and following inclusion in topical formulations, measuring their H-donor capabilities, their free radical scavenging effects by studying changes of the chemiluminescence intensity and their ability of inhibiting lipid peroxidation induced by Fe^{2+}, as well their influence on physical stability of the formulation. Since α-tocopherol is the major lipophilic antioxidant of exogenous origin found in tissues, it was used in order to compare the physical stability and the antioxidant activity.

2. Materials and methods

2.1. Chemicals

Glycyrrhiza glabra extract was a gift from Galena (Campinas, SP, Brazil), *Ginkgo biloba* extract was obtained from a local drugstore, the commercial mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L (comfrey) and *Arctium majus* root extract and soybean (*Glycine soja*) protein were gifts from Extracaps (São Paulo, Brazil). The *Nelumbium speciosum* extract was a gift from Pharmaspecial (São Paulo, Brazil). DL-α-Tocopherol was a gift from BASF (São Paulo, Brazil). Luminol was obtained from Acros Organics (New Jersey, USA), horseradish peroxidase (HRP) and 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); hydrogen peroxide 36% was purchased from Calbiochem (CA, USA). All other chemicals were of reagent grade and were used without further purification.

2.2. Total protein, polyphenol and flavonoid contents

The amount of protein present in all extracts was determined by the biuret assay using bovine albumin as standard [14]. Total polyphenol contents were determined by the Folin-Ciocalteau colorimetric method as described by Kumazawa et al. [15]. Extracts solutions (0.5 mL) were mixed with 0.5 mL of the Folin-Ciocalteau reagent and 0.5 mL of 10% Na_2CO_3, and the absorbance was measured at 760 nm after 1 h incubation at room temperature. Total polyphenol contents were expressed as μg/mg (gallic acid equivalents). Total flavonoid contents were determined using 0.5 mL of extracts solutions and 0.5 mL of 2% $AlCl_3$ ethanol solution. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoid contents were calculated as quercetin (μg/mg) from a calibration curve.

Table 1
Composition of the emulsion added or not with the different active substances tested

Component	Emulsion (g)
Self-emulsifying wax	1.00
Macadamia nut oil	2.00
Propylene glycol	6.00
Phenoxyethanol and parabene	0.40
Carbomer	0.18
Triethanolamine	0.15
Deionized water	90.27

2.3. Test formulation

An emulsion stabilized by an anionic hydrophilic colloid (Carbopol® 940), was developed, based on a commercially available self-emulsifying wax; macadamia nut oil was added as an emollient, and propylene glycol as a moisturizer. The preservative used was a mixture of phenoxyethanol and parabene. Deionized water was used for the preparation of all formulations (Table 1). Preservative and the active substances were incorporated at room temperature. All formulations were allowed to equilibrate for 24 h prior to use in the studies.

2.4. Physical stability evaluation

Physical stability was evaluated by submitting the formulation to storage at 4 and 30 °C and at 40 °C with 70% RH (relative humidity) for a period of 30 days. Samples were collected for the evaluation of rheological behavior, viscosity and pH measurements at the initial time and after 1, 7, 15 and 30 days. The minimum apparent viscosity and rheological behavior were determined using a rotational rheometer with a cone-plate configuration (Brookfield DV-III) with a CP52 spindle, and 0.5 g of the sample. A Brookfield software program, Rheocalc 1.01 was also used. Measurements were made at progressively higher rotation speeds and shear rates (10–100 rpm and 20–200 s^{-1}, respectively) to obtain the ascending curve and the procedure was repeated in reverse with progressively slower rates (100–10 rpm and 200–20 s^{-1}) to obtain the descending curve. The rate was kept constant for 10 s at each shear rate before a measurement was made. The pH of formulations diluted 1:10 in distilled water was measured using a Digmed pH meter. All measurements were made at room temperature in triplicate for each analyzed sample.

2.5. Statistical analysis

The initial values of pH, minimum apparent viscosity and the rheological parameters (flow index and histeresis loop), as well as the changes in viscosity in terms of period of study, storage temperature and presence of the active substances, were analyzed statistically by ANOVA following the Tukey test. The level of significance was set at $P < 0.05$.

2.6. Preparation of samples

DL-α-tocopherol was solubilized in ethanol to final concentrations of 1, 5, 10, 50, 100 and 200 μg/mL. The plant extracts were diluted from 250 to 8000 times in the suitable buffer for each assay in order to obtain final concentrations of 0.125, 0.25, 0.50, 1.0, 2.0 and 4.0 μL/mL. Formulation containing 2% of Vitamin E was diluted 1:5 with the extraction solution (Tween 20/H_2O 1:5 w/w), and the formulations added with plant extracts (2%) was diluted 1:2.5 in the suitable buffer for each assay. All diluted formulations were mixed for 20 minutes prior to the measurement of their antioxidant activity.

2.7. Free radical scavenging assays

In order to evaluate the free radicals scavenging activity of α-tocopherol and plant extracts three methods were used. Changes of chemiluminescence intensity of the H_2O_2–luminol–HRP system by different concentrations of actives ingredients and their formulations, were measured as described by Krol et al. [16]. Chemiluminescence was measured for 10 min at 25 °C with an EG & G Berthold Autolumat LB953 apparatus (Bad Wildbad, Germany). The H-donor ability was evaluated using an ethanol solution of DPPH•, a stable nitrogen-centered free radical. The DPPH• shows maximum absorbency at 517 nm, which decreases in the presence of H-donor molecules, and in our experiments was considered to be correlated to the scavenging action of the test compounds and formulations studied. Lipid peroxidation was assayed as malondialdehyde (MDA) generation using mitochondria (1 mg of protein) as lipid source as described by Rodrigues et al. [17]. Two controls were used for this test, a positive control (sample absent) and a negative control (iron absent). The blank was prepared as reaction mixture without mitochondria. All measurements were made in triplicate for the actives substances and formulations.

3. Results

3.1. Total protein, polyphenol and flavonoid contents

The total protein, polyphenol and flavonoid contents found in each extract are shown in Table 2. The higher amount of protein, polyphenol and flavonoid were found in the *Glycyrrhiza glabra* extract. *Ginkgo biloba* also showed a high polyphenol and flavonoid content while *Nelumbium speciosum* and the commercial mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L and *Arctium majus* root showed lower protein, polyphenol and flavonoid contents.

3.2. Physical stability evaluation

The average pH was about 7.4 for the base and the preparations added with the active substances. The addition of the vitamin or the plant extracts did not change the pH values, except for the formulation containing the commercial mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L and *Arctium majus* root extract, which showed a little decrease on pH. The flow index of all formulations was less than 1, indicating a pseudoplastic behavior. The non-Newtonian behavior can be mathematically determined by applying the model of Oswald–De Waele:

$$T = K\gamma^n$$

where T is the shear stress, γ the shear rate, K the consistency index and n the flow index [18].

The preparation without actives (formulation A) showed an initial flow index of 0.20 and a range of 0.16–0.22. The formulation with DL-α-tocopherol (formulation B) showed an initial flow index of 0.18 and a range of 0.19–0.22. The initial values of the formulation added with the *Glycyrrhiza glabra* extract, containing the flavonoid glabridin (formulation C), with G*inkgo biloba* extract (formulation D), containing biflavonoids, and with *Nelumbium speciosum* extract, containing the flavonoid rutin (formulation E) were 0.22, 0.23 and 0.21, and the range was 0.19–0.23, 0.21–0.24 and 0.21–0.23, respectively, formulation containing the commercial mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L and *Arctium majus* root extract (formulation F) and soybean protein (formulation G) was the less pseudoplastic formulation, with an initial value of about 0.25 and 0.23 and a range of 0.24–0.26 and 0.23–0.25. The addition of the vitamin or the plant extracts did not affect the flow behavior, except for the formulation F, which showed an increasing of the flow index values.

There were no significant differences in the initial minimum apparent viscosity among formulations supplemented or not with the Vitamin E, but the formulations added with the plant extracts showed a significant decrease in the initial minimum apparent viscosity. In addition to the statistical analyses of the initial values, the minimum apparent viscosity was analyzed in terms of storage time and temperature. Although the extracts decreased the viscosity of the emulsion; the viscosity did not change during the period of study concerning the storage time and temperature. The initial values were 679.6 and 617.6 cP for formulations A and B, and the range were 648.5–742.5 and 636.7–739.2, respectively. Formulations C and E showed a little decrease (initial values of 568.2 and 446.6 cP and a range of 466.9–621.6 and 408.6–476.1, respectively) while formulations D and G showed a higher variation (initial values of 367.3 and 309.7, and range of 326.7–370.6 and 310.0–345.1 cP, respectively). The lowest values were observed for formulation F with initial value of 288.7 and range of 229.4–310.9 cP. The minimum apparent viscosity of the formulations regarding period of study at 4, 30 and 45 °C/70%RH are shown in Figs. 1–3.

All formulations were found to be thixotropic. The hysteresis loop was calculated by the area under the curve for the rheograms obtained for each formulation. There were no significant differences in the initial values of the hysteresis loop between the formulations containing or not the Vitamin E

Table 2
Total protein, polyphenol and flavonoid contents of each plant extract

Plant extracts	Protein (μg/mg of plant extract)	Polyphenol (μg/mg of plant extract)	Flavonoid (μg/mg of plant extract)
Glycyrrhiza glabra	115.7 ± 2.4	7.42 ± 0.480	0.88 ± 0.015
Ginkgo biloba	33.2 ± 1.6	0.99 ± 0.006	0.44 ± 0.006
Nelumbium speciosum	10.2 ± 0.4	0.61 ± 0.017	0.20 ± 0.006
Glycyrrhiza glabra, *Symphytum officinale* L and *Arctium majus* root	17.7 ± 1.0	0.58 ± 0.010	0.14 ± 0.006
Soybean protein	44.2 ± 0.3	0.46 ± 0.028	0.19 ± 0.006

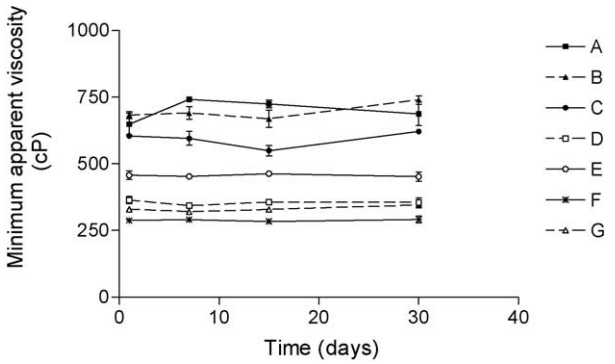

Fig. 1. Minimum apparent viscosity of formulations when stored at 4 °C during 30 days: (A) base, (B) DL-α-tocopherol, (C) *Glycyrrhiza glabra* extract, (D) *Ginkgo biloba* extract, (E) *Nelumbium speciosum* extract, (F) mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L and *Arctium majus* root extract, (G) soybean protein.

(11483 and 11582 Dinas/cm^2 s, respectively). However, the formulations supplemented with the plant extracts showed a significant decrease in the histeresis loop with values of 6345.7, 3415.0, 4114.3, 1769.5 and 1643.0 Dinas/cm^2 s for formulations C, D, E, F and G, respectively. Again, formulation F and G were the ones with the higher variation.

3.3. Free radicals scavenging activity assays

Based on the measurements of the areas under the time-course curves of chemiluminescence in the presence of respectively, DL-α-tocopherol (TOC), *Glycyrrhiza glabra* extract (GG), *Ginkgo biloba* extract (GB), *Nelumbium speciosum* extract (NS), mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L and *Arctium majus* root extract (ME) and soybean protein (SB), we estimated the relative inhibitory activity of each sample at different concentrations. The percent inhibition caused by each sample was calculated as:

$$\text{inhibition}\,(\%) = \frac{100 \times \text{AUC}_1}{\text{AUC}_0}$$

where AUC_0 and AUC_1 represent the areas under the curve observed for the control (ethanol, phosphate buffer pH 7,4 and the active-free control formulation) and experimental samples, respectively.

The percent inhibition of chemiluminescence as a function of the concentration of GG, GB, NS, ME and SB, respectively, is shown in Fig. 4. TOC showed an inhibition of 15.7, 51.7 and 87.2% for the concentrations of 1, 5 and 10 μg/mL. At the concentration of 50 μg/mL, the inhibition was about 97%. Higher concentrations caused no further inhibition. Fig. 5 shows the percent inhibition achieved for each formulation.

The change in absorbency produced by reduced DPPH$^\bullet$ was used to evaluate the antioxidant ability of the compounds tested. Figs. 6 and 7 show the percent decrease of absorbance, caused by different concentrations of GG, GB, ME, NS and SB alone or after addition to the formulations, respectively. The vitamin showed a concentration-dependent antioxidant

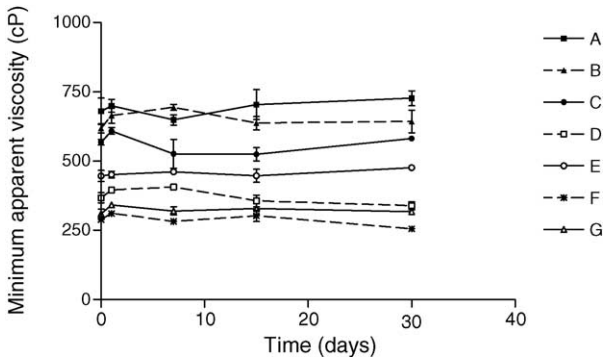

Fig. 2. Minimum apparent viscosity of formulations when stored at 30 °C during 30 days: (A) base, (B) DL-α-tocopherol, (C) *Glycyrrhiza glabra* extract, (D) *Ginkgo biloba* extract, (E) *Nelumbium speciosum* extract, (F) mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L and *Arctium majus* root extract, (G) soybean protein.

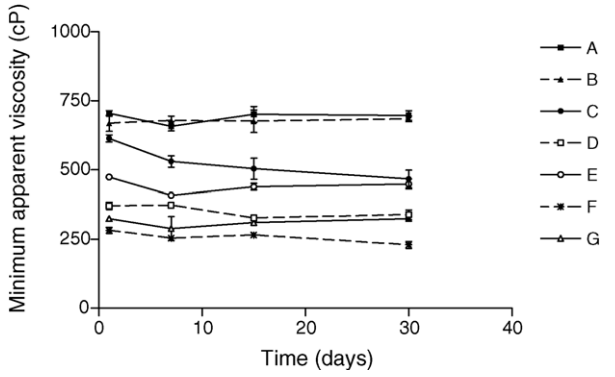

Fig. 3. Minimum apparent viscosity of formulations when stored at 40 °C/70% RH during 30 days: (A) base, (B) DL-α-tocopherol, (C) *Glycyrrhiza glabra* extract, (D) *Ginkgo biloba* extract, (E) *Nelumbium speciosum* extract, (F) mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L and *Arctium majus* root extract, (G) soybean protein.

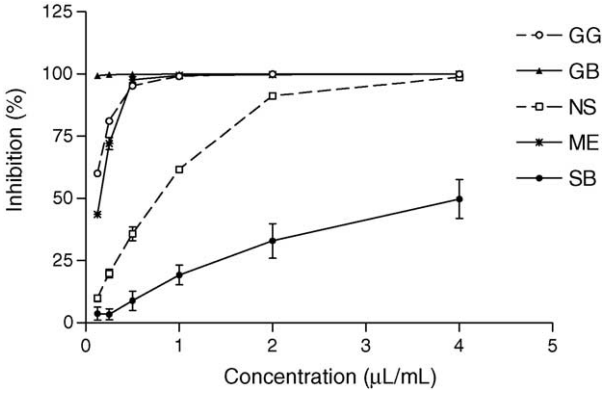

Fig. 4. Percent inhibition of light emission from the HRP – catalyzed luminescent reactions with luminol. Results are means ± S.D. of three measurements run in parallel. GG: *Glycyrrhiza glabra* extract, GB: *Ginkgo biloba* extract, NS: *Nelumbium speciosum* extract, ME: mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L and *Arctium majus* root extract, SB: soybean protein.

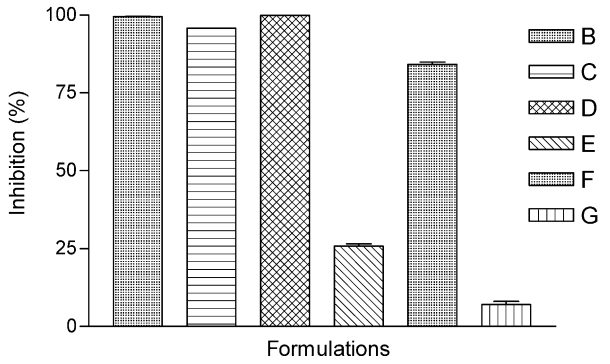

Fig. 5. Percent inhibition of light emission from the HRP-catalyzed luminescent reactions with luminol by the diluted formulations. Results are means ± S.D. of three measurements run in parallel. Formulation B: DL-α-tocopherol, C: *Glycyrrhiza glabra* extract, D: *Ginkgo biloba* extract, E: *Nelumbium speciosum* extract, F: mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L and *Arctium majus* root extract, G: soybean protein.

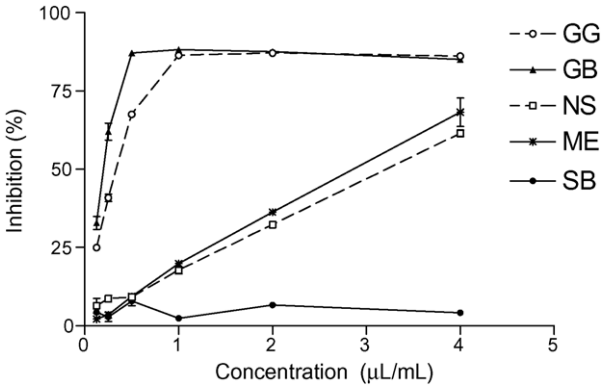

Fig. 6. Inhibition of hydrogen donor activity measured by the DPPH• method. Results are means ± S.D. of three measurements run in parallel. GG: *Glycyrrhiza glabra* extract, GB: *Ginkgo biloba* extract, NS: *Nelumbium speciosum* extract, ME: mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L and *Arctium majus* root extract, SB: soybean protein.

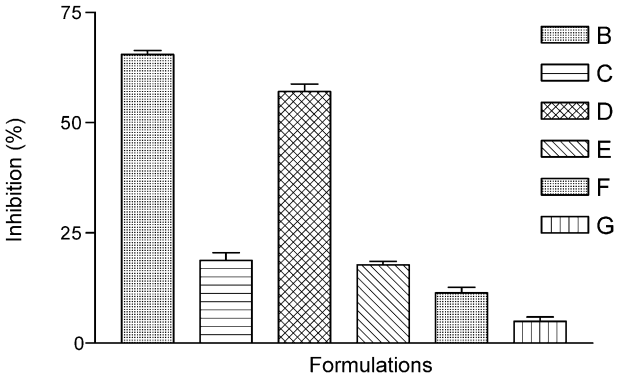

Fig. 7. Inhibition of hydrogen donor activity by the formulations measured by the DPPH• method. Results are means ± S.D. of three measurements run in parallel. Formulation B: DL-α-tocopherol, C: *Glycyrrhiza glabra* extract, D: *Ginkgo biloba* extract, E: *Nelumbium speciosum* extract, F: mixture of *Glycyrrhiza glabra*, *Symphytum officinale* L and *Arctium majus* root extract, G: soybean protein.

activity in the range of 0.4–20 μg/mL (3.4–74.8%). Again, higher concentrations caused no further decrease.

We investigated whether the extracts, the Vitamin E used and the studied preparations were effective in inhibiting lipid peroxidation. Lipid peroxidation is the parameter most extensively used to verify the involvement of free radicals in cell damage. The inhibitory effects of GG and GB extracts in different concentrations on lipid peroxidation in isolated rat liver mitochondria, estimated by the amount of TBA-reactive compounds accumulated 30 min after incubation of the mitochondria with Fe^{2+}/citrate is shown in Fig. 8. NS and ME extracts showed a low inhibition, about 22 and 6%, respectively only when 4 μL/mL was used. Lower concentrations showed no inhibition. SB caused no inhibition on lipid peroxidation. Vitamin E showed a great inhibition from 78 to 96% from the concentrations of 1–200 μg/mL. The inhibitory effect observed for the preparations is shown in Fig. 9.

Based on the part of the curves, which showed concentration-dependent activity, obtained for each plant ex-

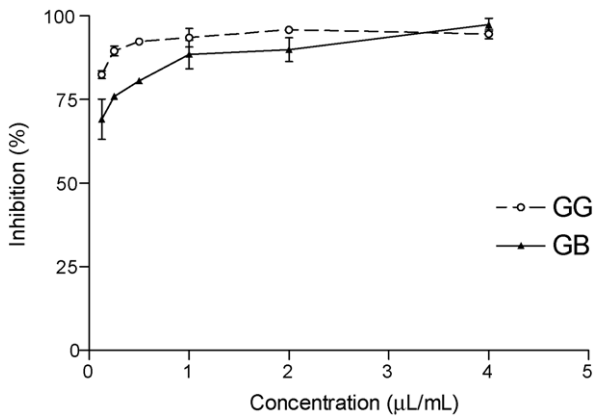

Fig. 8. Fe^{2+} induced lipid peroxidation inhibitory activity by GG (*Glycyrrhiza glabra*) and GB (*Ginkgo biloba*) extracts. Each point is the mean ± S.D. of the triplicate.

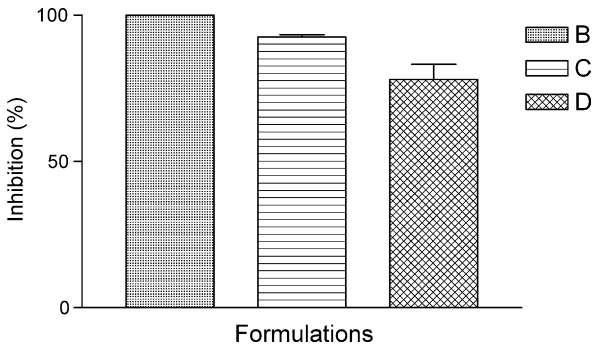

Fig. 9. Fe^{2+} induced lipid peroxidation inhibitory activity by formulation B (DL-α-tocopherol), C (*Glycyrrhiza glabra* extract) and D (*Ginkgo biloba* extract). Each point is the mean ± S.D. of the triplicate.

tract in the different antioxidant assays, it was possible to calculate the concentration that would show 50% of antioxidant activity (IC_{50}). The IC_{50} values found for each plant extracts in the antioxidant assays are shown in Table 3.

4. Discussion

The skin, as our major external barrier, is continually exposed to the oxidative injury caused by free radicals. It is considered that topical use of antioxidant vitamins and other antioxidants can protect and possibly correct oxidative skin damage by neutralizing free radicals [19]. Thus, it is necessary to use appropriate vehicles and excipients in order to obtain good formulation stability and the maintenance of the antioxidant activity. An emulsion stabilized by hydrophilic colloid was developed and studied for stability when the plant extracts and DL-α-tocopherol were incorporated to obtain a preparation where the antioxidant activity of the active substances could be measured. The formulations were stored at 4, 30 and 40 °C with 70% of RH for 30 days. Temperature is one of the most important factors for the rheology of a system. Temperature can change molecular bonds and thereby have different effects [20]. The interfacial rheological properties usually need long time to set in equilibrium hence the stability studies were carried out after 24 h.

As a rule, the rheological study and the evaluation of thixotropic properties allow to obtain a correct picture of the physical properties and the structural stability of semisolid systems [22]. The rheograms of all formulations indicated non-Newtonian behavior, with flow index less than 1, this reflects their pseudoplastic tendency, a desirable rheological behavior in these preparations. Formulations with a pseudoplastic flow produce a coherent film covering the skin surface, and this is important for a better antioxidant protection of the skin surface. The cause of pseudoplastic flow may be due to the progressive breakdown of the internal structure of the preparation, under increasing shear, and its later reconstruction by means of Brownian movement [23]. Formulation F, containing the mixture of plant extracts was the less pseudoplastic formulation. Although the plant extract mixture addition decreased the flow index, it did not show any significant changes during the period of study. Thus, the addition of these extracts does not alter the flow behavior of the preparations even when they were stored at 40 °C/70% RH.

Despite the decrease/reduction in the viscosity by the addition of the plant extracts, no significant changes were observed during the study. The viscosity was constant concerning storage time and temperature. This viscosity reduction/decrease may be due to the extract, since the added formulations with the lower viscosity extracts showed the higher decrease in viscosity (formulations D–G). When plant extracts with lower viscosity are added to the aqueous phase, they dilute the system and probably destabilize the cream structure. Anchisi et al. found a decrease in viscosity about 20% in formulations added with 2% *Salvia officinalis*, *Centella asiatica* or *Calendula* [24].

Formulation added with *Glycyrrhiza glabra* extract showed the less reduction in the viscosity. This result may be due to either the extract higher viscosity or the amount of protein present in this extract (Table 2). Proteins spontaneously adsorb to oil–water interfaces, essentially due to the hydrophobic properties of these interfaces. Adsorption then results in the decrease of the interfacial tension and a formation of a large interface area in emulsion. Upon adsorption, globular proteins unfold to an extension depending on their intrinsic structural stability, and constitute progressively an interfacial film exhibiting viscoelastic properties [25]. The structure of proteins and their physical and chemical properties (as net electric charge) may contribute for the viscoelastic properties of oil–water interface and thus reducing the cream structure destabilization caused by the extract addition. Thus, when emulsions for topical use are prepared, the chemical composition of the plant extracts should be considered in order to obtain a formulation with the intended viscosity.

Table 3
IC_{50} values found for each plant extracts

Plant extracts	Chemiluminescence assay (μL/mL)	Stable free radical scavenging activity (μL/mL)	Lipid peroxidation assay (μL/mL)
Glycyrrhiza glabra	0.09 ± 0.004	0.32 ± 0.007	0.07 ± 0.002
Ginkgo biloba	0.04 ± 0.001	0.16 ± 0.013	0.05 ± 0.015
Nelumbium speciosum	0.73 ± 0.044	3.14 ± 0.023	–
Glycyrrhiza glabra, *Symphytum officinale* L and *Arctium majus* root	0.12 ± 0.008	2.83 ± 0.160	–
Soybean protein	3.20 ± 0.650	–	–

The plant extract formulations showed a decrease in the shear stresses when compared with the formulation without plant extract or formulation added with the Vitamin E (data not shown). This indicates that the deformation of these networks required lower shear stresses than those needed to deform the base and the formulation added with Vitamin E.

Tixotropy (hysteresis loop) was observed for the formulations and the presence of plant extracts statistically decreases the tixotropy values. Tixotropy is desirable in topical formulations because it helps to maintain the suspending components stable, moreover it can influence the active substances release to the skin due to the structural disarrangement of the system, where the active substances diffusion is facilitated. Although the tixotropy and viscosity values have decreased for formulations added with the plant extracts, the rheograms obtained showed no instability signals (data not shown). These results show that these formulations could be considered stable since the rheological parameters were constant during the period of study.

Flavonoids can interfere not only with the propagation reactions of the free radical, but also with the formation of the radicals. Recently, flavonoids have gained great interest as potential therapeutic agents against a wide variety of diseases, most of which involve radical damage [26]. Since the formulations with the vitamin or plant extracts were stable, the antioxidant activity was evaluated. To increase effectiveness, three analytical methods were used to evaluate the antioxidant activity of the active substances alone and after addition in the formulations.

Several recent reviews have reported on the wide applicability of chemiluminescence as a sensitive assay to monitor levels of free radicals and reactive metabolites as well as for the screening of antioxidant activity [27–30]. Among antioxidant activity assays, chemiluminescence presents advantages due to its high sensitivity and speed [31]. The peroxidase-catalyzed chemiluminescent oxidation of luminol involves the formation of a complex between oxidant (H_2O_2) and peroxidase to produce a luminol radical. Luminol radicals then undergo further reaction resulting in the formation of an endoperoxide which decomposes to yield an electronically excited 3-aminophthalate dianion emitting light on return to its ground-state [32].

The GG, NS, ME and SB extracts inhibited chemiluminescence intensity in a concentration-related manner, GG and ME inhibited over an antioxidant range from 0.125 to 0.5 μL/mL. Above 0.5 μL/mL, these samples reached a plateau corresponding to a 99% inhibition of chemiluminescent intensity. NS caused 99% of inhibition only when higher concentration was used (4 μL/mL), while the maximum of inhibition caused by SB was about 49%. GB was the extract with the higher antioxidant activity even when lower concentrations were employed (IC_{50} 0.04 μL/mL). DL-α-Tocopherol (α-TOC) showed a great inhibition of the chemiluminescence reaction with an IC_{50} about 3.3 μg/mL.

In order to evaluate the antioxidant activity of the formulations following addition of these extracts or the DL-α-tocopherol, they were diluted 1:2.5 (plant extracts formulations) or 1:5 (vitamin formulation) in the extraction solution (phosphate buffer or Tween 20/H_2O 1:5) and 50 or 10 μL were added to the reaction mixture, yielding a final concentration of 0.4 μL or 40 μg of plant extracts and vitamin respectively per mL of reaction mixture. The use of formulations with the vitamin and GB extract led to a 99% inhibition of the chemiluminescence intensity relative to that of the base without antioxidant. Formulations with GG and ME showed an inhibition about 96 and 84%, respectively, while formulations added with NS and SB caused low inhibition, about 25 and 7%, respectively. These results are similar to those found for the raw material alone. In the conditions employed, the formulation components do not interfere with the antioxidant activity measurements by chemiluminescence [33]. These results together suggest that the formulation components do not interfere with the antioxidant activity of these substances, since they inhibited the chemiluminescence reaction in the same way of the respective raw material. There was no significant difference in the antioxidant activity of DL-α-tocopherol, GG and GB extracts showing these materials to be equally potent when added to the topical formulation. Moreover, these actives substances showed greater antioxidant activity than the other extracts studied.

The DPPH• assay, originally developed by Blois [34] is widely used for the measurement of free radical scavenging capacity in phytotechnology, food technology, and pharmacology/toxicology. DPPH• is a free radical that easily accepts an electron or hydrogen radical to become a stable diamagnetic molecule. It can accommodate a large number of samples within a short period, and is sensitive enough to detect low concentrations of the active principles [35].

The extract samples tested in the present study for their H-donor ability, measured by the stable free radical DPPH• assay, showed a lower antioxidant activity when compared to their capability to inhibit the chemiluminescence reaction. At the concentration of 1 μL/mL, GG and GB showed the highest H-donor capability decreasing the absorbency at 517 nm by about 88%. Higher concentrations caused no further decrease. NS and ME showed the same antioxidant profile with a maximum of absorbency decreasing about 63%, while SB showed no H-donor ability. α-TOC showed a maximum of inhibition (74.8%) when 20 μg was employed in the reaction mixture (IC_{50} 8.3 μg/mL).

The reaction of both vitamin and flavonoids with DPPH• is very fast. Tocopherol compounds behave as effective scavengers of free radicals and its hydrogen donor capacity, most probably accounts in large part for these compounds' protective effect against UVB cytotoxicity to human skin fibroblasts and against intracellular-flavin-induced photo-sensitization [36]. Since GG and GB extracts showed a great hydrogen donor capacity, as well as free radical scavenging activity (chemiluminescence assay), these extracts may be used for skin damage caused by UVB irradiation.

Although the tocopherol formulation showed the same activity profile as its respective raw material, the extract for-

mulations showed lower H-donor capability. In contrast with the results obtained by the chemiluminescence assays, a good correlation between the DPPH• scavenging activity of the extracts as such, and of the formulations containing these active principles, could not be found. These results may be due to the presence of the formulation components in the reaction mixture. Since the DPPH• scavenging is measured by spectroscopy, the formulation components may interfere with the antioxidant measurement. This kind of interference does not occur when the formulation with vitamin was employed because it was more diluted than the plant extract formulations leading to low formulation interference.

The α-TOC and GG and GB extracts showed to be able to inhibit lipid peroxidation on the mitochondrial membrane, while the other extract cause no inhibition. α-TOC showed an IC_{50} value about 0.48 μg/mL. These results confirm the radical scavenging activity found in the chemiluminescence assay for GG and GB extracts. This is of pharmacological relevance because this event is implicated in several pathological processes related to oxidative stress [37]. Lipid peroxidation is an autocatalytic process that is a common consequence of cell death. This process may cause peroxidative tissue damage in inflammation, cancer, toxicity of xenobiotics and aging. The membrane polyunsaturated fatty acids are peroxidized by free radical-mediated reactions. The thiobarbituric acid test for lipid peroxides in animal and plant tissues has been widely used during the past decades. Malondialdehyde (MDA) is one of the end products in the lipid peroxidation process [38]. Factors that determine the amount and extend of MDA formation from peroxidized polyunsaturated fatty acids are: the degree of fatty acid unsaturation, the presence of metals, pH, and the temperature and duration of heating [39].

The GG and GB formulations showed the same antioxidant profile as their raw material and an antioxidant activity similar to that found for vitamin formulation. These results confirm the ones obtained by chemiluminescence method and indicate that *Glycyrrhiza glabra* and *Ginkgo biloba* extracts are potent antioxidant and free radical scavengers agents. The greater antioxidant activity found for these extracts may be due to their higher polyphenol and flavonoid contents. Due to the good antioxidant activity of both extract, they could be added in topical formulations in order to protect skin against damage caused by free radical and reactive oxygen species. Nevertheless, evaluation of cutaneous permeation and in vivo efficacy of formulations added with plant extract are necessary in order to confirm their use for skin protection.

The antioxidant activity of the other extracts studied was different among the assays employed. This result suggests that plant extracts could be active against one free radical but fail to protect the skin against other reactions mediated by free radical, since they showed antioxidant activity in the chemiluminescence assay but did not inhibit the lipid peroxidation.

Since the formulations tested showed the same activity profile as their respective raw materials in the chemiluminescence and lipid peroxidation assays, and a good correlation between antioxidant activity of the samples alone, and of the formulations containing these active principles, was found, both methods are suitable for antioxidant activity evaluation of formulations added with these active substances.

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