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Original Article

Preliminary stability study of dry and glycolic extract of *Ginkgo biloba* L. incorporated in phytocosmetic formulations

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Abstract

Introduction: The use of *Ginkgo biloba* L. (Gb) extracts have been proposed in topical formulations for prevention and treatment of the damages caused by free radicals, due this extract has high flavonoid content, adding benefit to the compounding product. Such advantages justify their use by the cosmetic industry, however, there is a need for scientific studies to ensure that these extracts have activity and therapeutic efficacy after incorporation into creams. Thus, the aim of this research was determining in vitro the antioxidant activity of Gb glycolic and dry extract after incorporation in the dosage form cream by the free radical scavenging activity method, DPPH (2,2-diphenyl-1-picrylhydrazyl), and the preliminary stability study through physical-chemical tests (pH, spreadability, viscosity, centrifugation and microscopic analysis) to determine its behavior against environmental conditions.

Methods: The formulations were stable against the physical-chemical stability tests. However, after incorporation in the creams, the extracts of Gb presented instability against antioxidant efficacy, being observed a greater loss in those containing Gb glycolic extract.

Results: The results indicate the feasibility of using Gb dry extract in cosmetics of topical use since all the formulations added to this extract, even after thermal stress, presented antioxidant activity within the pharmacopeia parameters.

Conclusion: Therefore, it is suggested the microencapsulation of the extracts, in order to ensure greater stability of the active after incorporation in cosmetic products, as well as during storage.

Keywords: Cosmetic stability, Emulsions, Antioxidants, Plant extracts

Introduction

The cosmetics industry has increasingly used plant extracts in formulations, given the growing demand on the world market for these products. A fact related both to the appeal of "natural marketing" that attracts consumers, but also to the need to replace animal derivatives that are not fully replaced by synthetic substances in cosmetic products.[1,2]

Ginkgo biloba L. (Gb) is a tall tree that can reach 30 meters high, native to Japan, China and Korea. It is the only living plant of the Ginkgoacecae family, being considered a "living fossil" due to the approximate age of 250 million years of some of its fossils. The use of its extracts have been proposed in topical formulations in the prevention and treatment of free radical damage, since these extracts have high content of phenolic constituents, such as flavonoids, phenolic acids and polyphenolic compounds that neutralize free radicals, adding benefit to the manipulated product.[3-5]

Due to its high flavonoids content, Gb has shown significant role in the aging treatment as well as in the stimulation of collagen synthesis. In a study by Chuarienthong et al,[6] the gel preparation containing Gb increased skin moisture (27.88%) and smoothness (4.32%) reduced roughness (0.4%) and wrinkles (4.63%). Kim et al[7] demonstrated in vitro effects of Gb extract and its isolated flavonoids (quercetin, canferol, sciadopitisin, ginkgetin, isoginkgetin) on fibroblast proliferation in vitro and on the production of collagen and fibronectin. For the same purpose, the association of Gb extract with vitamins A, C and E derivatives in cosmetic formulations also showed antioxidant activity in vitro and protection against damage induced by ultraviolet radiation, such as protection against the formation of sunburn cells.[3,8]

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These benefits justify the use of Gb by the cosmetic industry, however, there is a need for scientific studies that can guarantee that Gb extracts have activity and therapeutic efficacy after incorporation into creams, being indispensable the care in the acquisition, reception and manipulation of the raw material thus allowing the development of effective and safe formulations.[1,9,10]

Studies involving the stability of extracts of Gb incorporated in cosmetic emulsions are rare in the literature, being a large part of the studies referring to oral administration phytotherapy, which limits the amount of reference data for comparison.[3,11,12]

Given the above, the aim of this research was determining *in vitro* the antioxidant activity of Gb glycolic and dry extract after incorporation in the dosage form cream by the free radical scavenging activity method, DPPH (2,2-diphenyl-1-picrylhydrazyl), and the preliminary stability study through physical-chemical tests (pH, spreadability, viscosity, centrifugation and microscopic analysis) to determine its behavior against environmental conditions.

Material and Methods

Preparation of the Samples

The raw materials were purchased from different compounding pharmacies: *Ginkgo biloba* L. glycolic extracts from providers A and B and *Ginkgo biloba* L. dried extracts from providers C and D. All the glycolic extracts used had a concentration of 20% w/v.

Lanette® cream (FARMOS LTDA) was used as the base (P-placebo) for the formulations where 1% of each extract from the mentioned suppliers were named: Formulation with dry extract 1 (FES-1); Formulation with dry extract 2 (FES-2); Formulation with glycolic extract 1 (FEG-1) and Formulation with glycolic extract 2 (FEG-2).

For the FES-1 and FES-2 formulations, a sufficient amount of extractive liquid (20% w/v), consisting of water and propylene glycol (Codossal Química LTDA) was used to solubilize the dry extract in order to favor its incorporation into the cream.

Preliminary Stability Test

Stability Against Different Temperature Conditions

The formulations were submitted to different temperature conditions, at elevated temperature 40°C ± 3°C/24 hours and the low temperature was 4°C ± 2°C/24 hours, thereby completing the 24 alternating hours of freeze-thaw cycles for 30 days.[13]

Visual Assessment and Organoleptic Characteristics

The test formulations were visually analyzed for appearance, color and odor using the following evaluation criteria: Appearance (Normal, unchanged, Slightly separated or precipitated, Separated, cloudy or precipitated), Color (Normal, unchanged, Slightly modified Modified and Intensely Modified), Odor (Normal, unchanged, Slightly Modified and Intensely Modified).[13]

Microscopic Analysis

Slides prepared with sample of the formulations were observed, taking into account the size, shape and distribution of the droplets, whether or not homogeneous; Presence of clusters, clumps, and their sizes, presence of air bubbles and oil droplets. The analysis was performed in triplicate, using optical microscope (Physis) in the 10x and 40x objective.[14]

Centrifuge Test

About 5 g of the samples were centrifuged (CentriBio®) at 3000 rpm for 30 minutes. The same evaluation criterion was used for the aspect, previously mentioned, to classify the physical instabilities detected.[8]

Determination of pH

The determination of the pH was performed in pH meter (HANNA PH 21), calibrated with buffer solutions of pH 4.0 and 7.0, inserting the electrode directly into the formulation.[15]

Determination of Viscosity

The viscosity was determined in rotary viscometer (QUIMIS Q400MT). According to the viscosity of the emulsions, spindle n° 4 and rotation of 6 rpm (rotations per minute) were chosen.[15]

Determination of Spreadability

A plastic mold of 1.1 cm in diameter was used, and this was placed on a glass plate (20 cm x 20 cm). A 1 mL sample (determined in a syringe) was introduced into the plastic mold and then carefully removed, after that, a glass plate of predetermined mass was placed on the sample. After 1 minute, the area covered by the measurement with a ruler of the diameter was calculated in two opposite positions (d1 = horizontal diameter and d2 = vertical diameter), with subsequent calculation of the mean diameter (d), according to equation.[16]

$$d = \frac{d1 + d2}{2}$$

The spreadability (Ei), determined at 25°C, was calculated using the equation:

$$Ei = \frac{(d)^2 \times \pi}{4}$$

where (Ei) is the sample spreadability for mass (mm^2), (d) is the mean diameter (mm) and π = 3.14.

Evaluation of the Antioxidant Activity by the DPPH Method

The antioxidant activity (AA%) dosage was adapted from Gettens and Frasson,[17] using the *in vitro* photocolorimetric method of the stable free radical, DPPH (2,2-diphenyl-

1-picrylhydrazyl), obtained from SIGMA. A solution of concentration 1.25 mg/mL Gb was prepared from each formulation, which were carried out a series of dilutions to obtain concentrations of 1, 0.5, 0.25, 0.125 and 0.05 mg/mL. This procedure was performed with the formulations before and after freeze-thaw cycle. From each dilution was used 2.5 mL, which was added 1 mL of DPPH solution (0.3 mM). The time required for the reaction to occur was 30 minutes, and then, the absorbance was measured at 516 nm. The ability to eliminate the DPPH radical (% of antioxidant activity) was calculated using equation:

$$AA(\%) = \frac{A_{control(-)} - A_{sample}}{A_{control(-)}} \times 100$$

where $A_{control(-)}$ is the absorbance of the DPPH solution without the sample and A_{sample} is the absorbance of the sample with the DPPH.

Statistical Analysis

The data obtained were evaluated by the paired student *t* test. GraphPad Prism® 7.0 software was used to evaluate the results, with a confidence interval of 95%. Values of $P < 0.05$ were considered significant.

Results

After the visual and organoleptic evaluation it was verified that the emulsions submitted to the stability tests did not show changes in color, odor and appearance, remaining unchanged at times 0 and 30.

Microscopic analysis revealed that all formulations containing the active substance (Figure 1) showed clear droplet formation without homogeneity. Only the placebo cream (Figure 1 - I/J) showed well-formed and homogeneous globules, but without uniformity throughout the slide.

When subjected to centrifugation, none of the samples showed any modification at the end of the experimental procedure at both times.

There was no significant difference between the pH values obtained in formulations containing the active substance ($P > 0.05$) (Table 1).

The viscosity was also evaluated considering the results presented in (Figure 2). At time 0, it is observed that there is no difference in viscosity results after incorporation of the active principle into the formulation ($P > 0.05$). There was no change in viscosity when compared to placebo after the ice-defrost cycle. After incorporation of the active principle a three-fold increase in the viscosity value was observed comparing T0 and T30 of each

Table 1. pH Values for the Different Storage Times ($P > 0.05$)

	Time	
	T0	**T30**
P	6.8±0.05	6.2±0.17
FES-1	6.4±0.68	6.4±1.14
FES-2	6.4±0.68	6.4±1.14
FEG-1	6.4±0.68	6.4±1.14
FEG-2	6.4±0.6	6.4±1.14

Results are showed as mean ± standard deviation. Placebo (P), Formulation with dry extract 1 (FES-1), Formulation with dry extract 2 (FES-2), Formulation with glycolic extract 1 (FEG-1), Formulation with glycolic extract 2 (FEG-2). Hydrogen ionic potential (pH).

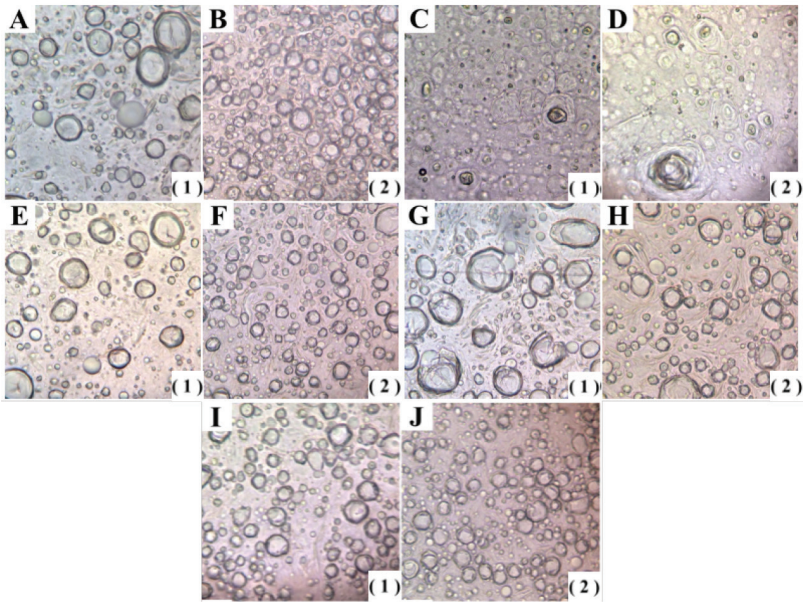

Figure 1. Microscopy of the Pre and Post Freeze-Thaw Cycle Formulations. Results are showed as cycles (1:Time 0 e 2: Time- 0); A/B formulation with dry extract 1 (FES-1); E/F Formulation with dry extract 2 (FES-2); C/D formulation with glycolic extract 1 (FEG-1); G/H formulation with glycolic extract 2 (FEG-2) e I/J Placebo (P); Objective used: 40x.

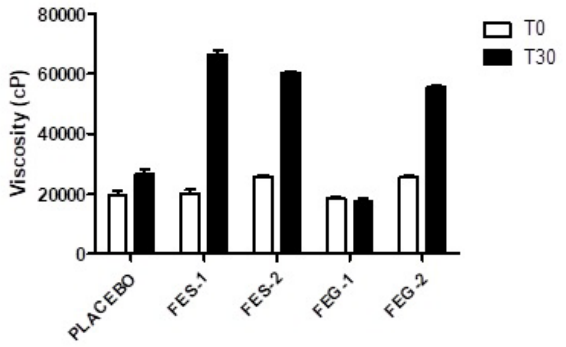

Figure 2. Graph With Viscosity Values at Time Zero (T0) and Time 30 (T30). Results are expressed in Centipoise (cP). Placebo (P), Formulation with dry extract 1 (FES-1), Formulation with dry extract 2 (FES-2), Formulation with glycolic extract 1 (FEG-1), Formulation with glycolic extract 2 (FEG-2).

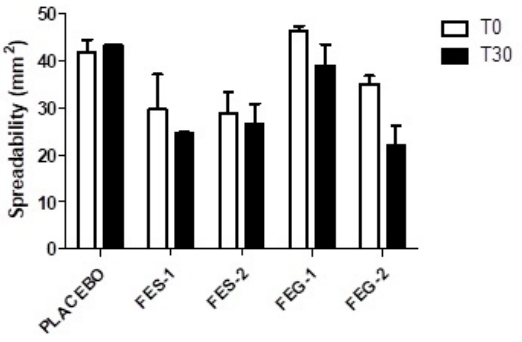

Figure 3. Graph With the Values of the Spreadability for the Samples Submitted to the Conditions of the Preliminary Stability Study (T0 and T30). Results are expressed as mean.; Placebo (P), Formulation with dry extract 1 (FES-1), Formulation with dry extract 2 (FES-2), Formulation with glycolic extract 1 (FEG-1), Formulation with glycolic extract 2 (FEG-2).

formulation. Only the FEG-1 and P formulation showed different behavior, requiring a more detailed evaluation to determine these results.

The placebo and FEG-1 formulations had the same spreadability behavior (Figure 3). However, after incorporation of the active principle, there was a reduction in the spreadability values, with no significant changes occurring after the freeze-thaw cycle ($P > 0.05$).

In relation to the antioxidant activity, formulations containing dry extracts (FES-1 and FES-2) presented a higher antioxidant potential compared to the formulations containing glycolic extract. Even after thermal stress, they continued to present antioxidant potential, however, only at concentrations of 0.5 and 1 mg/mL.

At time 0, FES-1 reached 54.8% AA and FES-2 a total of 38.0%, both reduced to 38.7% and 22.0%, respectively, at time 30, at the concentration of 1 mg/mL.

In both times, FEG-1 did not show activity, as shown in the data presented in (Figure 4), where this formulation obtained similar behavior to placebo. FEG-2 presented a small antioxidant activity (13.6%), but not as intense, only at the concentration of 1 mg/mL at time 0.

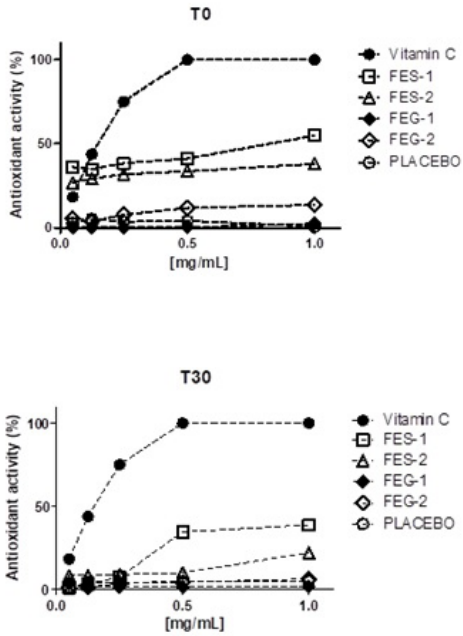

Figure 4. Antioxidant Activity (%) of the Formulations Tested by the Free Radical DPPH Methodology Time 0 (T0) and 30 Days (T30). Results are expressed as AA% ± standard deviation; Placebo (P), Vitamin C, Formulation with dry extract 1 (FES-1), Formulation with dry extract 2 (FES-2), Formulation with glycolic extract 1 (FEG-1), Formulation with glycolic extract 2 (FEG-2).

Discussion

In preliminary stability studies of cosmetics, it is expected that the organoleptic characteristics of a product remain the same until the end of the tests, even in the face of the different stress conditions in the sample, since these characteristics may influence the efficacy and quality of the product and its acceptance by the consumer.[18, 19]

The force of gravity acts on the products, causing their particles to move inside them. Centrifugation is used as a preliminary analysis of the stability of the formulation, producing stress in the sample, simulating an increase in the gravity force, increasing the mobility of the particles, separating the components that have different densities and anticipating possible instabilities (formation of compact sediment, flocculation, coalescence, precipitation and separation of phases). Even after 30 days of submission to thermal stress, the results showed that the incorporation of the dry and glycolic extracts did not interfere with the stability of the emulsion in relation to this parameter.[15,18,20]

The pH should ensure the stability of the formulation excipients, the efficacy and safety, as well as their compatibility with the biological fluids in accordance with the intended route of administration. The systems present greater stability when the pH values are kept within a small variation, which was visualized in the formulations studied. Thus, the progressive decrease of stability occurs when the pH moves away from its optimal limit.[2,21,22]

The determination and control of the pH, from a

cosmetic and/or dermatological point of view, is extremely useful as it helps to avoid the use of inappropriate topical products. Low pH values can lead to cumulative dermal irritation, and promote instability of the cosmetic formulation.[23,24]

Viscosity can be understood as the internal resistance to the flow that a fluid presents, resulting from the application of a force that causes temporary or permanent deformation of matter; or simply, the resistance of the fluid to flow or movement. Therefore, the higher the viscosity, the higher the resistance to flow. Although informative and does not constitute a factor for batch reprobation in physico-chemical quality control analyzes, from the pharmacotechnical point of view, it becomes essential to know the apparent viscosity of the system, since the appropriate rheological behavior is required to ensure the therapeutic activity or the cosmetic functions of the product.[21,25]

In the present study, the formulations showed an increase in viscosity values with a consequent decrease in spreadability values, a fact similar to the studies carried out by Pianovski et al,[26] who observed a reduction of this parameter for the samples stored at high temperature during the days due to the loss of water by evaporation, with consequent alteration of the consistency of the product.

Similar to viscosity, the spreadability determination is used to evaluate changes in rheological characteristics of the formulation during the study. In the case of topical semisolids, the quantification of this parameter is important to monitor changes in the formulations ability to spread or cover a particular area, which may facilitate or hinder its application.[27]

The presence of droplets without uniformity, visualized in the 40x objective, allows smaller ones to be added larger ones, destabilizing the system more quickly. However, no physical-chemical instability was observed in the formulations.

The results obtained in the analyzes of the antioxidant activity of the formulations, corroborate with the studies carried out by Gettens and Frasson,[17] where they affirm that the dry extract of Gb presents a greater antioxidant action in comparison with the glycolic extract, where the maximum antioxidant activity in creams with dry extract were at concentrations of 0.5 and 1 mg/mL.

The glycolic extract of Gb also presents antioxidant activity, but not so intense, probably due to the decrease of the concentration of compounds with antioxidant action after the preparation process of this extract, or even due to the conditions proposed in this study, such as the use of high temperatures, which may have compromised the chemical stability of the glycolic extract.[4,17]

In a study comparing the antioxidant activity of Gb in relation to the dosage of the flavonoid content in commercial extracts, it was observed that when the flavonoid content is very low, the sample shows little antioxidant activity, in contrast, when the flavonoid contents are higher high, the sample shows an excellent activity against free radicals, confirming the antioxidant potential of Gb.[28]

In order to solve the reduction of AA, which can be justified by the formation of degradation products, as well as by interferences of the components of the cosmetic formulation or even incompatibility of the extract with them, the microencapsulation technique of the active is suggested.

Microencapsulation is a widely used technique in the cosmetic field and the domain of its applications have been extensively extended including, fundamentally, microencapsulation of highly volatile compounds such as fragrances and vegetable extracts for personal care. At the cosmetics level, microencapsulation has several benefits, such as: protecting the encapsulated substance from oxidation, the action of environmental factors (light and heat) and possible interactions with other substances; to preserve the bioactivity of the active during storage (before its incorporation in cosmetic products) but also during their shelf life; to increase the stability of unstable components; to increase the shelf-life of encapsulated substances, among others.[29,30]

Therefore, it is also necessary to prove interferences in the formulation by assaying the samples.

Conclusion

In the experimental conditions of this study, it was possible to conclude that phytocosmetic formulations were stable compared to the physical-chemical stability tests. However, it is pointed out the possibility of the extracts have been purchased from compounding pharmacies in the state of degradation, since they presented instabilities in therapeutic efficacy throughout the study process.

The present work points to the feasibility of using the dry extract of Gb in cosmetics of topical use since all the formulations added of this extract, even after thermal stress, presented considerable antioxidant activity.

An optimization for the reduction of AA of the formulations presented would be the use of microencapsulation, since it is advantageous not only to preserve the bioactivity of the Gb extract incorporated in the cosmetic emulsion, but also during the storage period, still in the raw state as well as during its shelf life ensuring a higher bioavailability of the active when used by the consumer.

In view of these aspects, it is important to carry out a strict quality control of raw materials and finished products, as well as greater supervision of the suppliers of these raw materials, thus avoiding the risk of producing a drug or cosmetic product that instead of bringing benefits could lead to damages to the consumer.

Competing Interests
None.

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