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Original Article

Antimicrobial, Antioxidant and Anti-Inflammatory Assessment of a Phytocosmetic Produced with Glycinin Peptides

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Abstract

Phytocosmetics are increasingly gaining attention from consumers who search for alternatives for the maintenance and protection of the skin. This article reports formulation of a phytocosmetic derived from defatted soybean flour, an emulsion base that is hydrolyzed from the glycinin protein (1000 µg/g). Test for stability, microbiological control and biological activities antimicrobial (agar diffusion method), antioxidant (method of ABTS/TEAC radicals) and anti-inflammatory (method of hyaluronidase enzyme) properties were conducted. The results indicated that the phytocosmetic was stable and had low indices of microorganisms, according to Resolution 481/99. The bioactivity of the glycinin peptides was not altered or harmed by the other components of the emulsion. For the antimicrobial activity, the bacteria *E. coli*, *S. aureus* and *P. acnes* had values of 30.5 mm, 28 mm and 25 mm halos, respectively. For the antioxidant activity, the result was of 25.1 TEAC and the anti-inflammatory activity was measured at 83.4% inhibition of hyaluronidase enzyme. This study showed that the formulated phytocosmetic has great potential for topical use; it is comparable to other anti-aging cosmetics for daily skin care with antioxidant, anti-inflammatory and antimicrobial properties.

Keywords: Glycinin, biological activity, antimicrobial, antioxidant, anti-inflammatory, phytocosmetic.

Introduction

The main nutritional components of the soybean are the proteins obtained from its by-product, defatted soybean flour, which is derived from the soybean oil. The main proteins present in the flour are the glycinin and β-conglycinin, corresponding to about 80% of the total proteins in soybeans.[1]

Over the last decades, new molecules have emerged with functions that are specific for skin care.[2] Vegetable proteins like wheat, corn, oatmeal, rice, sunflower and soy have become important sources of peptides in the cosmetic field and can be incorporated into lotions, gels and creams.[3,4]

Several peptides in plants are being identified and characterized and are promising candidates for different applications for human health. Studies have reported that main biological activities of these peptides are antioxidant, antimicrobial, anti-inflammatory, anti-tumor, anticholesterolemic and antihypertensive. Glycinin consists of an acid and basic subunits (molecular mass of 34 and 20 KDa respectively), linked together by a disulfide bond, the basic subunit is both hydrophobic and cationic and may be able to react with bacterial cell wall and with acid subunit are responsible for antioxidant and anti-inflammatory activities. [5,6,7,8]

Nowadays, consumers are on a constant quest for a younger, prettier, healthier appearances and are seeking alternatives for skin care. This has caught the attention of the cosmetic industry. Brazil is the third largest producer of cosmetics in the world, behind the United States and China. In 2014 the revenue from the cosmetic market amounted to R$160 billion. [9]

Phytocosmetics are cosmetics with vegetable active principles that are incorporated into emulsion and are responsible for the biological activity.[10] The cosmetic emulsion can have antimicrobial, antioxidant or anti-inflammatory activities, and used on the face as an adjuvant for the control of acne and premature aging.

Acne is inflammation in the skin, which occurs due to an imbalance in the sexual hormones, it is very common in adolescence, but it can also occur during pregnancy and menopause. The bacteria that normally inhabit the follicles feed on the excess oil, forming colonies that cause swelling, redness and, later, the eruption of the follicles.[11]

On the other hand, aging is a set of irreversible and inevitable physiological alterations, and it is a dynamic process observed on the epidermis resulting from the modifica-

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tions that occur in the conjunctive tissue of the dermis. Skin aging is part of the changes that occur in different sectors of the organism, and it is divided into two types. The intrinsic one, which results from the natural aging of the body with the passage of years and is expected, predictable, inevitable, progressive, with no interference from external agents and equivalent to the aging of all the organs. In addition, the extrinsic type is due to smoking and physical, nutritional and mechanical factors.[12]

Low molecular weight peptides have been used in cosmetics because they have different functions: cicatrizing, depigmentizing, regenerating the extracellular matrix, diminishing wrinkles, improving firmness and elasticity.[13]

Thus, incorporating glycinin peptides an emulsion and assessing the biological potential of this phytocosmetic is highly favorable for innovation in the field of cosmetics, mainly the consumer association against the use of animal-derived ingredients, which allowed vegetable peptides derivatives to become more important. [12]

Materials and Methods

Obtaining the Glycinin Peptides

The peptides were obtained according to Vasconcellos et al. [14]

Developing the Emulsion

The emulsion base was produced according to Table 1. The aqueous phase was heated to 75°C and the oil phase to 80°C. The aqueous phase was transferred into the oil phase with agitation until cooling.

Stock solutions of glycinin peptides were into the emulsion in order to obtain a final concentration of 1000 µg/g of sample.

Table 1. Components of the emulsion for producing the phytocosmetic.

Components	Action	Amount (%)
Deionized water	Vehicle	q.s.p. (100)
Disodium EDTA	Chelating	0.10
Propylene	Moistening	2.00
Methylparaben	Preservative	0.50
Cyclomethicone	Emollient	1.00
Glyceryl Stearate	Emulsifier	1.50
Carbomer	Thickening	1.00
Cetyl alcohol	Emulsifier	0.20
Glycerin	Moisturizing	2.50

Stability Study

The formulation was assessed as recommended by the Cosmetic Products Stability Guide from the National Health Surveillance Agency.[15]

Centrifugation Test

Five grams of the phytocosmetic were centrifuged three times at 3,000 rpm for 30 min with temperature of 25°C. After this period, the formulation was assessed as to the separation of phases, indicating stability or not.

Accelerated Stability Testing (AST)

After the centrifugation, the AST was performed. This test consists of applying different temperatures with the goal of accelerating possible reactions among the components, which must be observed. [15]

Twenty grams of the phytocosmetic and the control (emulsion base without glycinin peptides) were put in transparent flasks with wide mouths and screw caps. The formulations were subjected to storage in the following temperature situations:

- room temperature (indirect lighting): 25°C ± 2°C;
- low temperature (cooler): 5°C ± 2°C;
- freezer: -10°C ± 2°C;
- high temperature (heater): 45°C ± 2°C;
- cycles: alternating temperatures -10°C ± 2°C and 45°C ± 2°C (24 h for each temperature).

All samples analyses at 0; 5; 10 and 15 days. The parameters were analyzed involved possible alterations in aspect, color, smell, pH value and separation of phases.[16]

Regarding the parameter aspect, the product must remain intact during all the days of analysis, maintaining the initial appearance, except in high temperatures, freezer or freezing and thawing cycles, when small alterations are acceptable. Regarding the parameters color and smell, they must be stable during the 15 days of testing and small alterations are acceptable only in high temperatures.[15]

The separation of phases was observed visually, and for analyzing the pH values, 1g is taken and homogenized in 9mL of distilled water, determining the pH value at room temperature.

Table 2. Criteria for the assessment of the organoleptic parameters of the cosmetic emulsion.

Symbology	Aspect, Color and Smell
N	normal, no alteration
S	slightly different
M	different
I	intensely different

Table 2 shows the criteria for the assessment of the phytocosmetic emulsion.

Microbiological Control of the Phytocosmetic

Performing biological control on non-sterile products aims determination at microorganisms present in the sample. For counting these microorganisms, 1g of the phytocosmetic was dissolved and homogenized in 9mL of sterile 0.1% peptone water. One mL of this solution was placed on petri dishes containing approximately 20mL of agar nutrient for bacteria and 20mL of sabouraud agar for fungi. The dishes were incubated at 37°C for 24 h for the analysis of the bacteria and at 25°C for 7 days for the analysis of fungi. After this period, the number of colony-forming units (CFU/g sample) was determined.[15]

Biological Activity

For the antimicrobial activity, the agar diffusion test was performed on the strains *Escherichia coli* ATCC 26922,

Staphylococcus aureus ATCC 25923 and *Propionibacterium acnes* ATCC 6919, which inhabit the skin and can cause skin infections as acne.[17,18,19]

The experimental design involve phytocosmetic with glycinin peptides (1000 µg/g), antibiotic tetracycline (30 µg/mL) as a positive control and sterile distilled water and emulsion base without glycinin were negative controls. Briefly, the peptide suspension were sterilized with 0,22 µm membrane (Millipore, Billerica, MA, USA) and bacterial suspension were adjusted by 0,5 MacFarland comparasion. Petri dishes containing culture medium and bacterial suspensions were prepared and after agar solidified, wells were made and 150 µL each sample, positive and negative control were added. Incubated at 37° C for a time appropriate each strain and the antibiosis effects were determinate by measuring inhibited halos (mm).

The hyaluronidase enzyme method was used for the anti-inflammatory activity.[20,21,22] The phytocosmetic with glycinin peptides (1000 µg/g) was homogeneized with 500 µ/L hyaluronic acid solution (1,2 mg hyaluronic acid/mL 0,1 M in acetate buffer, pH 3,6 and 0,15 M in NaCl) was incubated at 37°C with 50µL hyaluronidase enzyme for 40 min. After 0,8 M potassium tetraborate was added and incubated at boiling for 3 min, 0,3 mL p-dimethylaminobenzaldehyde was homogeneized for 20 min at 37°C. The positive control was dimethylsulfoxide, a topic anti –inflammatory and negative control were sterile distilled water and emulsion base without glycinin. The results were mesuared in 585nm absorbance and the anti-inflammatoy activity was mensured percentage of enzyme inhibition.

The antioxidant activity was used the ABTS free radical method.[23,24]

ABTS solution (200 µL), Trolox (50 µL) and suspensions of phytocosmetic with glycinin peptides (1000 µg/mL) and emulsion base without glycinin were added to 96 well-plate and incubated in the dark for 6 minutes at room temperature, after the absorbance at 734 nm was mensured with microplate reader (Power Wave XS, Biotek, Winooski, VT, USA). The antioxidant activity expressed as Trolox equivalent antioxidant capacity (TEAC).

Statistical Analysis

The results obtained in this study were expressed by the mean ± standard deviation of triplicates. Differences were analyzed using ANOVA followed by the Tukey's test and the Student-t test for comparison between means when necessary. Differences were considered significant with p value < 0.05.

Results and Discussion

Centrifugation Test

This procedure is efficient for determining the instability of emulsified products, since the simulation of an increase in the force of gravity can separate the components that have different densities.[25]

In this study, there was no separation of phases in the control group and the phytocosmetic group, suggesting the emulsion is stable when facing gravitational stress.

The absence of separation of phases demonstrates that in normal conditions of gravity and at room temperature, the phytocosmetic is physically stable; this occurred in this study.[26]

Accelerated Stability Test

As to the organoleptic parameters aspect, color, smell and separation of phases, there were no relevant alterations in all stress conditions during the 15 days of analysis. The variables high temperature and cycles caused subtle changes only in the aspect of the phytocosmetic and the control, as can be seen on Table 3. This change may have occurred due to the evaporation of water when the phytocosmetic and the control were in the heater.[16,10]

Table 3. Assessment of the organoleptic characteristics (color, smell and aspect) and separation of the phases of the phytocosmetic and control.

Variables/ Days	0	5	10	15
Room temperature	N	N	N	N
Low temperature	N	N	N	N
Freezer	N	N	N	N
High temperature	N	N	N	S
Cycles	N	N	N	S

For the parameter pH stability was observed from the 10th day of analysis, as shown on Figure 1. The control behaved similarly to the phytocosmetic, which demonstrates that adding glycinin did not alter the properties of the components of the emulsion. The formulation presented values between 5.8-5.9 compatible with the skin pH which is around 5.0-6.0.[27] All the conditions tested during the 15 days did not present significant differences ($p < 0.05$), which confirms the stability of the phytocosmetic proposed in this study.

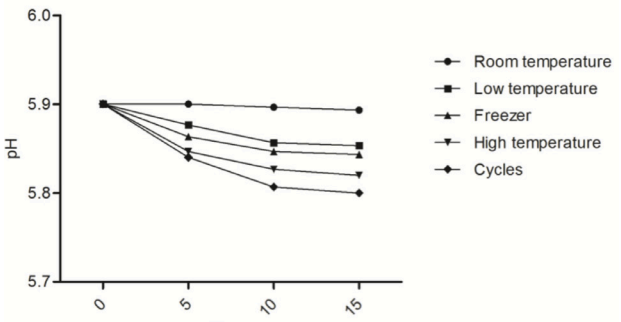

Figure 1. pH variation in the phytocosmetic and control during the 15 days of analysis in the accelerated stability test.

Microbiological Control of the Phytocosmetic

The Resolution — RDC 481[28] establishes the limits for the microbiological control in personal hygiene products, perfumes and cosmetics. The phytocosmetic produced in this study, according to this resolution, is on level II, that is, products that have specific indications and have characteristics that demand safety and/or efficiency proof, as well as information, warnings, instructions for use and restrictions. Thus, the maximum allowed contamination by aer-

Table 4. Antimicrobial activity of the phytocosmetic and tetracycline antibiotic*

Strains Halos (mm)	Phytocosmetic	Tetracycline
E.coli	30.5±0.50Aª	32.5±0.50Aa
S. aureus	28.0±0.20Bb	30.0±0.10Bb
P. acnes	25.0±0.30Cc	27.5±0.20Cc

*Mean ± standard deviation of the triplicates
Uppercase letters show significant differences between phytocosmetic and antibiotic.
Lowercase letters show significant differences between the strains.
Tukey's range test (p <0.05)

obic microorganisms is of 5 x 103 CFU/g or mL of sample. The total number of microorganisms in the phytocosmetic and the control was absent for bacteria and with indices lower than 4 CFU/g for fungi. These results are inside the limit recommended by the resolution, and there was no need to isolate the colonies and identify them.

Biological Activity

Antimicrobial Activity

The results of the agar diffusion test for the bacteria *S. aureus*, *E. coli* and *P. acnes* can be seen on Table 4. Glycinin peptides showed antibiotic activity, all strains were susceptible. The positive control showed halos with media 30,0 mm and negative control did not effect without halos. The concentration 1000 µg/g glycinin peptides was similar effect that tetracycline antibiotic (30 µg/mL). Futhermore, the high concentrations of glycinin peptides are required to produce like effect to that commercial antibiotic.

Peptides kill bacteria by permeabilization through the formation of stable pores, as well as by membrane thinning or micellization in a detergent-like manner and positive charges peptides facilitate interaction with negative charges bacteria phospholipid containing.[29,30]

The gram- negative bacteria are susceptible peptides, because the lipopolysaccharides that have high negative charge and facilitate linkage to cationic peptides and gram- positive bacteria have negative charge in teichoic and lipoteichoic acids in peptidoglycans, therefore, both bacterial were susceptible to peptides.[31,32]

Anti-inflammatory Activity

The phytocosmetic has 83.4% of anti-inflammatory activity. The peptides action in the anti-inflammatory process inhibiting hyaluronidase enzyme degrades hyaluronic acid, present in the skin, in fragments in which high potential process inflammation, increasing permeability and decreasing the firmness and elasticity of skin. [33,34]

Antioxidant Activity

The antioxidant activity found in the phytocosmetic with the ABTS radical method was of 25.1±1.75 ABTS. The standard curve of the trolox is on Figure 2. Peptides originating from milk and soybeans have been shown to exhibit antioxidant activity due abilities to inactivate reactive oxygen species (ROS), scavenge free radicals, chelate

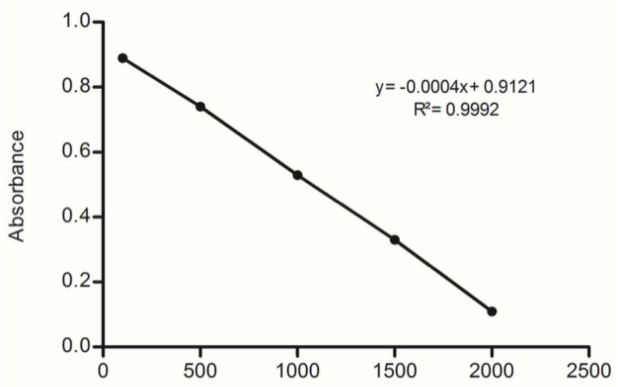

Figure 2. Standard curve of the trolox against the ABTS radical.

pro-oxidative transition metals, reduce hydroperoxides.[35] These peptides act on the stratum corneum of the skin (superficial layer), improving hydration, elasticity, expression lines, improving the antioxidant ability, regenerating fibroblasts and reducing acne inflammation. There is evidence that ROS are involved in the process of skin aging, and the topical application of cosmetics with antioxidant properties has certified biological effects on the ROS. [36,37,38,4]

Alanine, aspartic acid, phenylalanine, histidine, tyrosine, methionine, cysteine are responsible antioxidant capacity peptides.[39,40] And the glycinin constituted in aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine and arginine amino acids.[14]

However, the amino acids arginine, lysine, histidine (cationic), alanine, leucine, isoleucine and methionine (hydrophobic) are responsible for the connections with the epithelial cells of the stratum corneum, which have negative charges, increasing their permeability and easing the passage of the phytocosmetic.[41,42,43]

The phytocosmetics play an important part in the cosmetic field due to their relevance and high efficacy.[44] The use of bioactive peptides in this field is promising, due to the great versatility and diversity of biological activities.[45]

As was observed in study that demonstrated the capacity of the use of defatted soybean flour as a source of proteins for the formulation of a phytocosmetic with great bioactive power.

Conclusion

The stability study showed that the phytocosmetic was stable when it came to environmental changes during the period of analysis and it did not present relevant indices of external contamination by microorganisms.

The phytocosmetic was efficient regarding the antimicrobial, antioxidant and anti-inflammatory biological activities. It demonstrated the great potential of peptides derived from glycinin as a source of vegetal raw material to be used in the cosmeceutical field.

However, this phytocosmetic requires more research and tests before its topical use.

Competing Interests

The authors declare no competing interests.

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