UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

DISCIPLINA DE TRABALHO DE CONCLUSÃO DE CURSO

EXTRATO DE SOJA ENRIQUECIDO EM GENISTEÍNA: INCORPORAÇÃO EM NANOEMULSÕES E ESTUDOS DE CARACTERIZAÇÃO, PERMEAÇÃO CUTÂNEA E ATIVIDADE ANTIOXIDANTE

Patricia Inês Back

Porto Alegre, dezembro de 2016

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE FARMÁCIA

DISCIPLINA DE TRABALHO DE CONCLUSÃO DE CURSO

EXTRATO DE SOJA ENRIQUECIDO EM GENISTEÍNA: INCORPORAÇÃO EM NANOEMULSÕES E ESTUDOS DE CARACTERIZAÇÃO, PERMEAÇÃO CUTÂNEA E ATIVIDADE ANTIOXIDANTE

Profº Drº Helder Ferreira Teixeira

Orientador

Mestre Marina Cardoso Nemitz

Coorientadora

Porto Alegre, dezembro de 2016

AGRADECIMENTOS

Agradeço a Deus por ter me concedido o dom da vida e pelas oportunidades a mim destinadas.

À Universidade Federal do Rio Grande do Sul pelas oportunidades e ensinamentos concedidos através dos grandes mestres que tive.

Ao meu orientador Profº Drº Helder Teixeira por ter me acolhido no Laboratório de Desenvolvimento Galênico. Agradeço pelo apoio, conselhos, entusiasmo e orientação nesse último ano.

A minha coorientadora Marina Nemitz pela incansável ajuda, paciência, dedicação, compreensão e pelas valiosas sugestões e contribuições. Obrigada por todo o carinho e suporte.

À Lucélia Balestrin, obrigada pela ajuda, contribuições e disponibilidade.

Aos meus colegas e amigos do Laboratório de Desenvolvimento Galênico, agradeço por todo o carinho, apoio, conselhos e amizade. Agradeço em especial à Flávia pela disposição, ajudas e conselhos.

Aos colegas do Departamento de Bioquímica, obrigada pela disponibilidade e ajuda.

Aos amigos que a Faculdade de Farmácia me deu, agradeço por toda a parceria, apoio acadêmico e emocional. Em especial, agradeço à Lúcia Meirelles pelos anos de convívio, companheirismo e amizade incondicional. Agradeço também à Graziela Rigo e à Júlia Biz por terem me acolhido tão bem após meu retorno à faculdade em 2014 e me mostrado uma amizade verdadeira.

A todos meus amigos de Santa Cruz que se mostraram companheiros e que sempre me deram apoio e torceram por mim, seja para o vestibular, provas difíceis, TCC ou processos seletivos.

Ao programa Ciências Sem Fronteiras pela oportunidade de agregar valores à minha formação e pelos amigos que me deu.

As meninas que aceitaram dividir moradia comigo, agradeço pela companhia e por tornarem a minha estadia em Porto Alegre mais leve e prazerosa.

As minhas madrinhas, obrigada pelo amor e por terem compreendido minha ausência.

A minha família, agradeço por todo o amor, carinho, ombro amigo e apoio.

RESUMO

As isoflavonas da soja têm sido estudadas como promissores compostos anti-envelhecimento, sendo as formas agliconas, principalmente a genisteína, frequentemente citadas como responsáveis por diversos efeitos benéficos para a pele. Nesse sentido, o objetivo deste estudo foi desenvolver nanoemulsões contendo extrato de soja enriquecido em genisteína (NE_{SHE}) destinado a aplicações dermatológicas. Para isso, primeiramente foram obtidos extratos de soja, os quais foram preparados por diferentes protocolos hidrolíticos visando a hidrólise das isoflavonas conjugadas até agliconas. Nanoemulsões contendo extrato de soja hidrolisado por procedimento enzimático (NE_{SHE}) e genisteína pura (NE_{Ge}) foram preparadas pelo método de emulsificação espontânea. As gotículas de ambas as nanoemulsões apresentaram diâmetro entre 150 - 200 nm, e elevado potencial zeta negativo. O ensaio de permeação/retenção cutânea utilizando protocolo com pele de orelha suína demonstrou que a genisteína ficou mais retida na pele a partir de NE_{Ge} em comparação com NE_{SHE}. No entanto, apenas NE_{SHE} permitiu a permeação dessa isoflavona através da pele. As peles foram então tratadas com as formulações e os efeitos fotoprotetores dos produtos foram avaliados após irradiação com lâmpada UVA/UVB. Foi observada uma menor formação de proteínas carboniladas e substâncias reativas ao ácido tiobarbitúrico em peles tratadas com NE_{SHE}, mostrando que esta formulação é um sistema que proporciona a liberação da genisteína na pele e um potencial produto antioxidante a ser aplicado no campo cosmético.

Palavras-chave: antioxidante, genisteína, isoflavonas da soja, nanoemulsão, pele.

SUMÁRIO

Este trabalho é apresentado sob forma de artigo a ser submetido à Revista Química Nova. As normas e instruções aos autores encontram-se em anexo.

SOYBEAN ISOFLAVONE AGLYCONE RICH EXTRACT-LOADED NANOEMULSIONS: PHYSICOCHEMICAL PROPERTIES, SKIN RETENTION, AND ANTIOXIDANT ACTIVITY

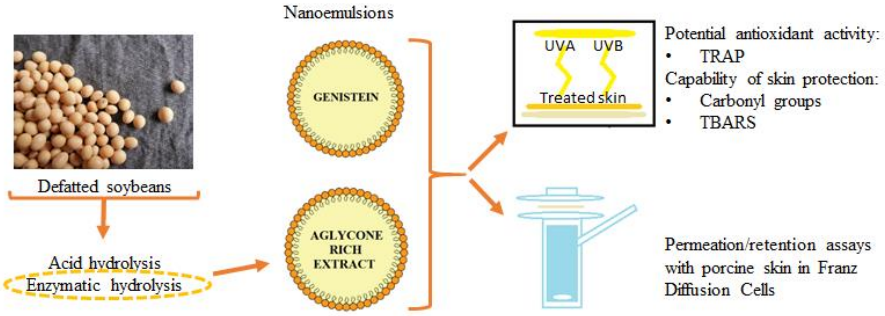
Patricia Inês Back[a], Luisa Furtado[a], Marina Cardoso Nemitz[a], Lucélia Albarello Balestrin[a], Flávia Nathiely Silveira Fachel[a], Henrique Mautone Gomes[b], José Cláudio Moreira[b], Gilsane Lino von Poser[c], and Helder Ferreira Teixeira[a*]

[a] Departamento de Produção e Controle de Medicamentos, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, CEP 90610-000 Porto Alegre - RS, Brazil

[b] Departamento de Bioquímica da Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos 2600, CEP 90035-003 Porto Alegre - RS, Brazil

[c] Departamento de Produção de Matéria-Prima, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Av. Ipiranga 2752, CEP 90610-000 Porto Alegre - RS, Brazil

Graphical abstract



The present study evaluates permeability, skin retention, release rates, and antioxidant potential of nanoemulsions containing soybean extract enriched in genistein aiming a topical delivery system.

----------*marque uma alternativa, não apague o texto em azul*----------

( ) Manuscrito com material suplementar

(x) Manuscrito sem material suplementar

---

*email: helder.teixeira@ufrgs.br

SOYBEAN ISOFLAVONE AGLYCONE RICH EXTRACT-LOADED NANOEMULSIONS: PHYSICOCHEMICAL PROPERTIES, SKIN RETENTION, AND ANTIOXIDANT ACTIVITY

Abstract

Soybean isoflavones have been studied as promising anti-aging compounds, being their aglycone forms, mainly genistein, frequently cited as responsible for several skin benefic effects. In this sense, the goal of this study was to develop nanoemulsions containing soybean extract enriched in genistein intended to dermatological applications. For that, it was firstly obtained soybean extracts by different hydrolysis protocols. Nanoemulsions containing soybean extract hydrolyzed by enzymatic procedure (NE_{SHE}) or pure genistein (NE_{Ge}) were prepared by spontaneous emulsification procedure. Both nanoemulsions showed droplets sizes within 150 – 200 nm, and high negative zeta-potential. Permeation/retention assay using porcine ear skin revealed a higher accumulation of genistein in skin from NE_{Ge} in comparison to NE_{SHE}. However, only NE_{SHE} allowed the permeation of this isoflavone through the skin. Finally, porcine skin was treated with formulations and the photoprotective effects of products on skin were evaluated after UVA/UVB irradiation. Lower formation of protein carbonyls and thiobarbituric acid reactive substances was observed in skin treated with NE_{SHE}, demonstrating that this formulation is an encouraging system for skin delivery of genistein and a promising antioxidant product to be applied in the cosmetic field.

Keywords: antioxidant, genistein, nanoemulsions, soybean isoflavones, skin.

INTRODUCTION

Soybean is a rich source of isoflavones. These compounds are classified as phytoestrogens due to its similarity to 17β-estradiol, which enables them to bind into estrogenic receptors.[1] Because of that, one of the applications being studied is the use of isoflavones as skincare raw materials for anti-aging products.[2-5] Besides, isoflavones have demonstrated other skin benefices, such as antioxidant and photoprotective activities. These effects were proved by several *in vitro* and *in vivo* studies, as well as by some clinical trials.[4,6,7]

Skin is the organ with the largest surface in the body and acts as a barrier to avoid external particles and substances to get in contact with internal organs.[8,9] Due to its composition, it is very susceptible to oxidative stress, which may be caused by external and endogenous environment.[8] As potential external sources, it can be cited air pollutants, natural gases, irradiation, pathogens, chemicals, and toxins. Internal sources include reactive oxygen species produced by enzymes, activated white cells, and ischemic processes, for example.[8] In addition, it is also believed that aging processes comes out of accumulation of oxidation products, which may include oxidized proteins, DNA, and lipid metabolites; the reduction of natural antioxidant defense from cells; and the decrease of the mitochondria function.[8]

One of the most important ways to prevent biological damage of skin is through antioxidant pathways. Exposure to ultraviolet (UV) radiation induces a variety of free radical and oxidative molecules, which alter molecular structures and cause significant damages on cells. Antioxidant compounds, such as isoflavones, are substances capable of scavenging free radicals, acting as metal chelating agents, or stimulating and inhibiting enzymes involved in oxidative processes.[7] When topically applied, they are able to protect the skin cells from damages caused by UV radiation. Several studies have been constantly conducted to prove antioxidant and photoprotection effects of isoflavones.[7,10-13] Most of them have conducted assays with the isolated forms; however, the usage of soybean extract containing more than one isoflavone has showed a higher activity than the individual ones.[11,14]

Isoflavones found in soybeans include the aglycone forms daidzein, glycitein, and genistein, their glucosides daidzin, glycitin, and genistin, as well as their malonylglucosides and acetylglusides.[15] The conjugated forms are the isoflavones predominantly present in soybean seeds. However, the main responsible for the therapeutic and promising effects of isoflavones are

the aglycone compounds, especially genistein.[16-18] When applied on the skin, isoflavone's mechanism of action, after penetration and/or permeation, may include inhibition of enzymes and binding to receptors. Some studies reported that if the glucose is still attached to the structure, the molecule will not be able to be absorbed or fit its binding site, and will present a low cellular uptake.[19] Therefore, obtaining firstly a soybean extract through a catalyst system to hydrolyze the conjugated forms, and then incorporating the isoflavone aglycone extract into formulations may present a good alternative to apply into the development of pharmaceutical and cosmetic products.

During the development of topical products, the incorporation of isoflavone aglycones into hydrophilic formulations represents a technological challenge due to their low water-solubility. Topical products with hydrophilic characteristics are more acceptable by consumers though, and because of that, studies have proposed the use of nanotechnology to produce final aqueous products containing isoflavones, such as micro and nanocapsules,[20,21] nanoemulsions,[22,23] liposomes,[19,24] and lipid solid nanoparticles.[25] However, most of these studies have been performed with pure isoflavones, and not with hydrolyzed soybean extracts.

Considering all the highlighted points, the aim of this study was to develop o/w nanoemulsions containing soybean extract enriched in genistein (hydrolyzed extract), and to perform the release rates and skin permeation of this isoflavone from the formulations. In addition, their potential to protect the skin from oxidative damage generated by exposure to UVA/UVB radiation was also assessed.

EXPERIMENTAL PART

Materials and chemicals

Soybean seeds (EMBRAPA BRS262 cultivar) used in this study were kindly donated by SEMEL seeds (São Paulo, Brazil). The isoflavones genistein, daidzein, and glycitein were acquired from Cayman Chemical Company (Ann Arbor, MI, USA). Egg lecithin and medium chain triglycerides (MCT) used for nanoemulsions development were purchased from Lipoid GmbH (Ludwigshafen, Germany). The reagents for chromatography and antioxidants assays were purchased from Merck (Darmstadt, Germany), Sigma-Aldrich (St. Louis, MO, USA), and

Dynamics (Diadema, SP, Brazil). Porcine ears were obtained at a local abattoir (Slaughterhouse Ouro do Sul, Harmony, RS, Brazil), and release membranes from Merck Millipore (Darmstadt, Germany).

Analysis of genistein and other compounds

To analyze the genistein presented in soybean extracts, nanoemulsion products, and permeation assays, an ultra-fast liquid chromatography (UFLC) method was used following the same conditions reported by Nemitz et al. (2015).[26] Additionally, the same method was utilized during the analysis of soybean extracts to estimate the content of daidzein and glycitein, and to evaluate the presence or absence of sugar degradation products, such as 5-hydroxymethylfurfural (HMF) and 5-ethoxymethyl-2-furfural (EMF).

UFLC conditions

A Shimadzu Prominence system device coupled to a photodiode array (PDA) detection with an automatic injector controlled by LC-Solution Multi-PDA software (Kyoto, Japan) was used. The stationary phase was a Shim-pack XR ODS column (Shimadzu, 100 x 2.0 mm i.d.; particle size, 2.2 µm) guarded by a pre-column. The mobile phase consisted of (A) trifluoroacetic acid 0.1 % (v/v) and (B) acetonitrile. The gradient elution was 20 – 25% B (0 - 2 min), 25 – 30% B (2 - 3 min), 30 – 35% B (3 - 4 min), and 35 – 20% B (4 - 6 min) and 20% B (6 - 8 min). The flow rate was a gradient of 0.35 mL min^{-1} (0 - 1.5 min), 0.35 - 0.5 mL min^{-1} (1.5 - 2 min), 0.5 mL min^{-1} (2 - 3 min), 0.5 - 0.35 mL min^{-1} (3 – 4 min), and 0.35 mL min^{-1} up to 8 minutes. The analysis were carried out at 55 °C, with 3 µL of injection volume and wavelength at 260 nm.

Preparation of hydroethanolic soybean extract

Soybeans were previously grinded with a knife mill and defatted by exhaustive extraction with n-hexane in a Soxhlet apparatus. The extraction of 10.0 g of defatted soybean material (DS) was performed in a Soxhlet using 200 mL of ethanol 80% (v/v), during 8 h at 70 - 80 °C. Several extraction processes were performed and the extracts obtained were pooled, filtered, and conserved at 4 ^{o}C.

Preparation of aqueous extract

The ethanol of the hydroethanolic soybean extract (HSE) was evaporated under reduced pressure. Each 200 mL of HSE was reduced to approximately 20 mL, and then, the volume was adjusted to 40 mL with purified water. Numerous processes were performed, pooled, and stored at – 20 ºC. Each 4 mL of aqueous extract corresponded to the extracted from 1.0 g of DS.

For the chromatographic analysis of the negative control extract (extract without hydrolysis), 4 mL of the aqueous extract were diluted up to 40 mL with ethanol. Then, this solution was diluted 1:10 in 50% acetonitrile (v/v) and filtered through a 0.22 µm membrane. The results were expressed as the mean of three independent procedures.

Hydrolysis procedures

Acid hydrolysis

Hydrolysis was performed adding hydrochloric acid to 4 mL of aqueous extract, aiming a final concentration of 1.3 M. The volume was completed up to 40 mL with ethanol and the procedure was maintained under reflux at 80 °C for 2 h. For the chromatographic analysis, the extract was neutralized, diluted 1:10 in 50% acetonitrile (v/v), and filtered through a 0.22 µm membrane. The results were expressed as the mean of three independent hydrolytic procedures.

Enzymatic hydrolysis

Firstly, an enzyme solution of β-glucosidase from almonds (MP Biomedicals, 2500 U/mg) was prepared in order to obtain the theoretical concentration of 250 units in each milliliter of pH 6.8 phosphate buffer. Then, the enzymatic procedure was performed by adding 16 mL of the enzyme solution in 4 mL of aqueous extract. The mixture was taken to a water bath at 37 °C for 24 h. At the end of the procedure, 20 mL of ethanol was added to the hydrolyzed extract to promote the enzyme denaturation and solubilize the isoflavones aglycone precipitated in the media. For the chromatographic analysis, the extract was diluted 1:10 in 50% acetonitrile (v/v) and filtered through a 0.22 µm membrane. The results were expressed as the mean of three independent hydrolytic procedures.

Preparation of Nanoemulsions

Nanoemulsions containing genistein (NE_{Ge}) and soybean hydrolyzed extract (NE_{SHE}), as well as blank nanoemulsions (NE_B) were prepared by the spontaneous emulsification technique. The final composition of the formulations is presented in Table 1. The chemical marker was genistein, and both NE_{Ge} and NE_{SHE} were developed with a final concentration of 0.03%. The oil phase was composed by medium chain triglycerides (MCT) stabilized with binary systems of surfactants.

To produce NE_{Ge}, firstly, it was prepared an organic phase with MCT, egg lecithin, genistein (1.5 mg), and ethanol. This mixture was added, under constant stirring, into the aqueous phase formed by polysorbate 80 and water. The emulsion formed was kept under constant stirring for 15 minutes, and the organic solvent was removed by evaporation under reduced pressure. The evaporation was performed up to the desired final volume (5 mL).

A similar process was performed to obtain NE_{SHE}. Primarily, 65 mL of enzymatically hydrolyzed soybean extract was rotaevaporated to remove its ethanol content. The extract was then diluted with water and lyophilized to obtain a dry extract with the desired concentration of genistein (1.5 mg). To the lyophilized extract, it was added 15 mL of an ethanol: acetone (1:1) mixture, maintaining constant stirring for 30 minutes. Then, a previously prepared solution containing MCT, lecithin, and 15 mL of ethanol was added to it. The suspension was filtered and poured under constant stirring to the aqueous phase, formed by water and polysorbate 80. The mixture was stirred for 15 minutes and rotaevaporated until the final volume of 5 mL.

Table 1. Final composition of nanoemulsions

Formulation	Components (%)				
	MCT	Egg lecithin	Polysorbate 80	Chemical marker (genistein)	Water Qsp
NE_{Ge}	8	2	1	0.03	100
NE_{SHE}	8	2	1	0.03	100
NE_B	8	2	1	-	100

Where, NE_{Ge}: nanemulsion with genistein; NE_{SHE}: nanoemulsion with soybean hydrolyzed extract; NE_B: blank nanoemulsion.

Characterization of Nanoemulsion

pH determination

The analysis of pH was determined without prior dilution of nanoemulsions, using a calibrated potentiometer (Model 21 pH, Hanna, Brazil). The results were expressed as the average of three independent determinations.

Size and polydispersity index determination

The formulations were characterized by dynamic light scattering analysis with monochromatic laser diffusion, which crosses the colloidal dispersion, using Zetasizer (Zetasizer 3000HS, Malvern Instruments, Malvern, England). This analysis was made by observing the 90^{o} scattering after diluting the samples in water (1:1000) previously filtered through a 0.22 µm membrane. The results were expressed as the average of three independent determinations.

Zeta potential determination

Zeta potential was determined by electrophoretic mobility of the droplets in Zetasizer (Zetasizer 3000HS, Malvern Instruments, Malvern, England). All analyzes were performed after diluting the samples in 1 mM NaCl (1:1000), previously filtered through a 0.22 µm membrane. The results were expressed as the average of three independent determinations.

Genistein content

For chromatographic analysis, the formulations were diluted in the proportion 1:100 in 50% acetonitrile (v/v) and filtered through a 0.22 µm membrane.

In vitro release assay

Franz diffusion cells were used with an internal circular interface area of 1.77 cm^2 and acceptor phase with 10 mL of volume. Release assays were performed using cellulose ester membranes (50 nm pore diameter, Milipore®) between the donor compartments and the Franz

cell receptors. The membranes were pre-hydrated with pH 7.4 phosphate buffer and the bath temperature was adjusted to 32 ± 1.0 °C. The receptor compartment was filled with a mixture of pH 7.4 phosphate buffer and ethanol (70:30 v/v) and maintained under constant stirring at 450 rpm. On the membranes was added 400 µL of nanoemulsions. Control suspensions with genistein in propylene glycol was also used. The experiment was carried out keeping *sink* conditions, using the same concentration of isoflavones and fluid receptor as reported by Nemitz et al. (2015).[26] Aliquots were removed every hour and replaced by same amounts of fresh receptor media. The total duration of the experiment was 8 h. The genistein content in each aliquot was analyzed by the UFLC method.

Skin permeation studies

For permeation/skin retention studies, Franz diffusion cells were also used. The porcine skin was cut in circles using a scalpel aid. The subcutaneous tissue was removed and the skin was stored in aluminum foil for one month at the lasted in a -20 °C freezer.

Before using, the skin was left in contact with pH 7.4 phosphate buffer for 10 minutes. Then, the skin was placed on a Franz Cell, maintaining the dermis in contact with the acceptor fluid. The experiment was carried out keeping *sink* conditions. The bath temperature was adjusted to 32 ± 1.0 °C and the acceptor compartment was filled with fluid (pH 7.4 phosphate buffer with 30% of ethanol) and remained under constant stirring at 450 rpm. On the top of the skin was added 400 µL of nanoemulsions or control propylene glycol suspension. After 8 h of experiment, a fluid sample was collected from the acceptor compartment and the skin was removed from the Franz Cell. The excess of formulation was removed from the skin, which was then cut into small pieces. Those were transferred to a test tube, to where 2 mL of methanol were added. The samples were kept in an ultrasound bath for 30 minutes for the extraction of isoflavones. The samples were then filtered with a 0.22 µm membrane and analyzed by the UFLC method.

Antioxidant studies

Evaluation of antioxidant activity of formulations

Total reactive antioxidant potential (TRAP) was performed in order to evaluate the potential antioxidant activity of the formulations.[27] As a positive control, Trolox 40 μM was used. For the test, a free radical solution of AAPH (2,2-azobis (2-amidinopropane) hydrochloride) with luminol was prepared, and maintained under room temperature (22 °C) for 2 h for stabilization matters. Then, to 20 μL of formulation were added 180 μL of the AAPH solution. The amount of chemiluminescence intensity of luminol was read by a liquid scintillation counter (Wallac TriLux Micobeta® 1450, Perkin-Elmer, Boston, MA, USA). A chemiluminescence intensity vs. time graph was plotted from the data area under the curve in order to determine TRAP.

Capability of formulation protection on skin

The skin treatment protocol was based on Balestrin et al. (2016).[28] The skin was placed on a glass plate and in order to maintain its moisture, cotton soaked in pH 7.4 phosphate buffer was added. It was added 500 μL of formulations on top of each porcine ear skin piece measuring 2.54 cm^2. The plates were then placed inside of a chamber and exposed to an UVA lamp Exo Terra Sun Glo Neodymium 100 W (41 $mWcm^{-2}$ dose; wavelength 365 nm) and to an UVB lamp Exo Terra Repti Glo 15 W (1.8 $mWcm^{-2}$ dose; wavelength 290 nm) for 6 hours. It was ensured skin moisture and coverage with formulation during the whole experimental time. At the end, skin was washed, the boarders were cut off, and the rest of the skin cut into small pieces, and homogenized in an Ultra-Turrax® with 3.5 mL of phosphate buffer. They were centrifuged and the supernatant analyzed. The treated skin was compared with two controls: skin that was not exposed to radiation (NIS) and skin that was not treated with formulations before exposure to radiation (IS). The results were expressed in terms of total protein content.[29]

Thiobarbituric Acid Reactive Substances Assay (TBARS) was performed according to the method described by Draper and Hadley (1990) to analyze lipoperoxidation of the skin.[30] 200 μL of trichloroacetic acid 15% were added to the homogenate skin, mixed, and centrifuged at 10,000 rpm for 10 min. A 100 μL aliquot was taken from the supernatant and mixed with 100 μL thiobarbituric acid 0.67 %. Then, they were heated to 100 ^oC in a dryblock for 40 min and the intensity of the color formed was analyzed by a spectrophotometer at 532 nm.

The method used to analyze protein carbonyls was the one described by Levine et al. (1990).[31] The samples and a blank tube without samples were prepared with 1 mg of protein, volume completed to 200 µL of phosphate buffered saline (PBS), and 200 µL of trichloroacetic acid (TCA) 20%. The tubes were taken to a vortex and an ice bath for 5 min, and centrifuged at 4,000 rpm for 5 min. To the pellet was added 100 µL of NaOH 0.2 N and mixed. Then, 100 µL of hydrochloric acid was added to the blank control tube and 100 µL of 2,4-dinitrophenilhidrazine (DNPH) 10 mM was added to the formulation samples. Both were incubated for 1h at room temperature and mixed every 15 min. Then, 100 µL of TCA 20% were added and the tubes were centrifuged at 16,000 g/5 min. The pellet was washed 3 times with 500 µL of ethanol/ethyl acetate (1:1), centrifuged at 16,000 rpm/5 min, and re-suspended again with 1 mL of pH 2,3 urea 8 M. The results were analyzed at 370 nm through a spectrophotometer.

Statistical analysis

Statistical analysis were performed using GraphPad Prism software version 6.1. Data were analyzed by parametric test one-way ANOVA and complemented by a Tukey test for multiple comparisons. The significance level was $\alpha = 0.05$.

RESULTS AND DISCUSSIONS

Soybean hydrolytic processes

The main objective of this study was the development of nanoemulsions containing soybean extract enriched in genistein to be topically applied, mainly as a photoprotective and antioxidant product. However, it is important to highlight that to obtain the desired extract, it is recommended a soybean extraction followed by hydrolysis process, since the isoflavone glycosides are the predominant forms in "*in natura*" soybeans.[4,19]

Aiming to obtain an enriched genistein extract, an extensive literature search was conducted to select a rich cultivar in isoflavones. Among the analyzed data, Ávila et al. (2011)[32] reported that the Brazilian cultivar EMBRAPA BRS 262 represented a good source of isoflavones and, for this reason, this cultivar was chosen for the development of the present study.

The soybean seeds were firstly grinded, defatted, and extracted with a hydroethanolic media. The ethanol was evaporated and the aqueous extract was submitted to hydrolysis processes, which were performed using acid and enzymatic protocols. The acid hydrolysis was performed based on conditions previous optimized by Nemitz et al. (2015).[33] The authors reported that by using this method, it is possible to produce a high content of isoflavone aglycones. However, under acid conditions, a high level of furanic compounds (HMF and EMF) is also produced. These substances are formed by the degradation of sugars under extreme pH conditions, and have been showing genotoxic effects, being related to possible harmful effects to health.[34] Because of this, the enzymatic hydrolysis was also performed aiming to be an alternative method to obtain an enriched isoflavone aglycone extract without the presence of furanic compounds in the final product.

The chromatograms obtained after analysis of the aqueous extract, extract hydrolyzed by acid process, and extract obtained by enzyme hydrolysis are shown in Figure 1. As it can be seen, the aqueous extract without hydrolysis procedure presents peaks of isoflavone aglycone daidzein (peak 1), glycitein (peak 2), and genistein (peak 3), but the areas are significantly smaller than the peaks observed after hydrolysis procedures. Also, as expected, it can be seen that the acid hydrolysis presents peaks related to furanic impurities (peak 1' and 2'). On the other hand, the enzymatic hydrolysis did not lead to the formation of sugar degradation compounds, indicating the selectivity of the process only in hydrolyzing isoflavones. The results found in this study are broadly advantageous for the development of skincare products rich in bioactive isoflavones and free from furanic impurities.

The quantitative analysis of isoflavones were also performed. The total content of genistein, glycitein, and daidzein for each extract is presented in Figure 2. It is worth noting that all the extracts corresponded to 1.0 g of the defatted soybean and have their final volume adjusted to 40 mL with ethanol in order to compare results. Moreover, Table 2 shows the content of each isoflavone aglycone evaluated in extracts. The results demonstrate that the enzymatic hydrolysis procedure shows a yield of total aglycone equivalent to the found in the acid hydrolyzed extract. Thus, the enzymatic conditions herein performed are able to completely transform conjugated forms of isoflavones into aglycone forms in the soybean extract.

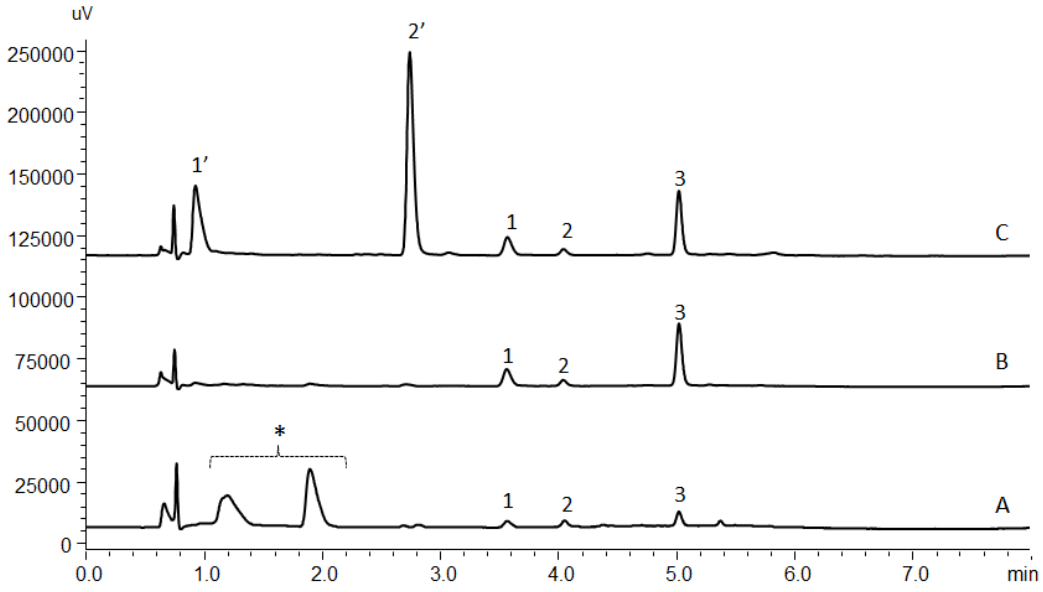


Figure 1. *Chromatogram obtained at 260 nm after analysis by UFLC of (A) aqueous extract, (B) aqueous extract after enzymatic hydrolysis with 4000 units of enzyme, and (c) aqueous extract after acid hydrolysis. Interest chemicals and their corresponding retention times are: * possible conjugated isoflavones; 1': HMF (~0.90 min); 2': EMF (~2.80min); 1: Daidzein (~3.60 min); 2: Glycitein (~4.00 min); 3: Genistein (~5.00 min)*

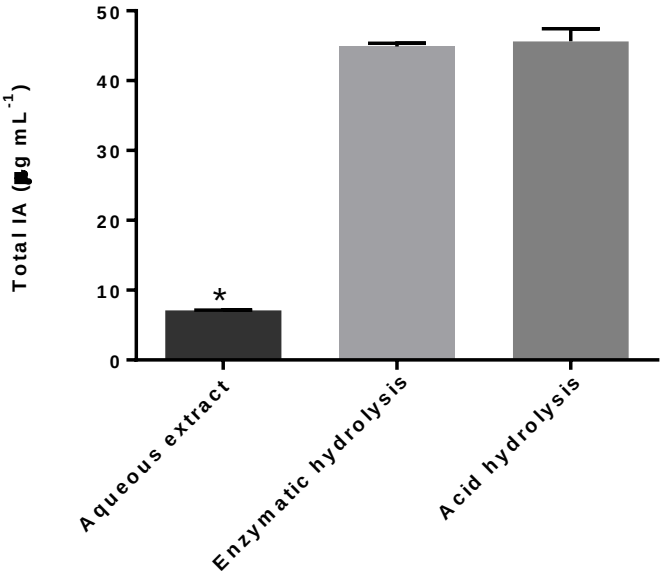


Figure 2. *Content of total isoflavone aglycones (IA) obtained in extract without hydrolysis and in extracts after two different hydrolysis processes (acid or enzymatic protocols), where: * is statistically different from other groups*

Table 2. Content of isoflavones daidzein, genistein, and glycitein in different extracts evaluated

Sample	Isoflavones (μg mL^{-1} of extract, average of triplicate ± SD)			
	Daidzein	Glycitein	Genistein	Sum of isoflavone aglycones
Aqueous extract (AE)	2.82 ± 0.02*	1.87 ± 0.07*	2.42 ± 0.004*	7.11 ± 0.05*
AE after acid hydrolysis	17.34 ± 0.63	4.38 ± 0.02	23.94 ± 1.12	45.66 ± 1.77
AE after enzymatic hydrolysis with 4000 U	16.16 ± 0.15	5.01 ± 0.18	23.73 ± 0.20	44.90 ± 0.44

Where: * means statistical difference ($p < 0.05$) between the values on each column. SD: standard deviation.

Nanoemulsions containing soybean extract

It is important to highlight that the isoflavone aglycones (IA) are poorly hydrosoluble and this may limit their incorporation into traditional topical delivery systems.[4] To allow better usage of IA in formulations and enhance their penetration, some alternative technologies, like nanoemulsions, have been studied.

Our research group previously reported the development of lecithin-based nanoemulsions, obtained by spontaneous emulsification, as a genistein topical delivery system.[22,23] Following up, the present study investigated the feasibility of soybean extract enriched in genistein in nanoemulsions intended to photoprotection and antioxidant dermal use.

To produce NE_{SHE}, a volume of 65 mL of enzymatically hydrolyzed soybean extract was lyophilized and used for the development of the products in order to obtain 5 mL of a final product, containing approximately 300 μg mL^{-1} of genistein. For comparison purposes, NE_{Ge} and NE_B were also prepared. The physicochemical properties of nanoemulsions are shown in Table 3. Droplets sizes were within 150 – 200 nm, and the zeta-potential was negative for all formulations. This last property could be related to the negatively-charged phospholipids of egg-lecithin present in the nanoemulsions. The zeta-potential of NE_{SHE} was significantly higher in module when compared to the NE_{Ge} and NE_B, which is a possible indicator that other compounds present in the soybean extract may be located on the oil-water interface of the

nanoemulsions.[35,36] Such property could also indicate a higher stability of the formulation when compared to the others,[36,37] since it creates a higher energy barrier, helping to avoid coalescence.[38] Genistein was probably associated with nanoemulsions since its partition coefficient is 2.98 (when estimated by online ACD/I-Lab service), favoring its incorporation into the oil phase of nanoemulsions. In addition, the pH value for both NE_{Ge} and NE_B was between 4.0 and 5.0, which is in accordance with topical formulation containing these surfactants.[39] The pH of NE_{SHE} was higher than other formulations possible due to other compounds present in the extract. The polydispersity index was below 0.25, showing a narrower distribution of the droplets, and is in accordance with other studies.[23,35] The content of genistein in NE_{SHE} was slightly lower than the theoretical concentration, which is probably due to the greater number of steps necessary to obtain the formulation that can lead to some losing during the process.

Table 3. Physicochemical properties of nanoemulsions

	Size (nm)	Zeta potential (mV)	Polydispersity index	pH	Genistein content (mg mL^{-1})
NE_B	168.70 ± 6.1	-15.2 ± 1.3	0.124 ± 0.06	4.0 – 5.0	-
NE_{Ge}	165.49 ± 7.6	-17.9 ± 1.0	0.108 ± 0.01	4.0 – 5.0	0.29 ± 0.01*
NE_{SHE}	153.68 ± 10.9	-34.0 ± 8.7*	0.175 ± 0.02	6.5 – 7.5 *	0.25 ± 0.01

Where, NE_{Ge}: nanemulsion with genistein; NE_{SHE}: nanoemulsion with soybean hydrolyzed extract; NE_B: blank nanoemulsion. * means higher statistically ($p < 0.05$) compared to the values on the same column.

During the development of skincare products, it is necessary to perform studies to evaluate the release profile of the compound of interest from the formulations, and analyze its permeation/retention ability after skin application. Considering this requirement, the release of genistein from the nanoemulsions (NE_{Ge} and NE_{SHE}) was studied and compared to a control suspension in propylene glycol. As can be seen in Figure 3, genistein was almost fully released from the control suspension in approximately 6 h. On the opposite, a progressive release of genistein was observed from both nanoemulsions, and only 57.64 ± 4.61% and 59.27 ± 0.78% were released after 8 h from NE_{Ge} and NE_{SHE}, respectively. It was not observed a significant

difference in the release profile of genistein between nanoemulsions. The genistein flow across the membrane was 7.29 ± 0.13, 4.64 ± 0.37, and 4.08 ± 0.05 μg cm^{-2} h for the control, NE_{Ge}, and NE_{SHE}, respectively, showing that incorporating genistein into nanoemulsions decreases the flux in comparison to a propylene glycol suspension ($p < 0.05$).

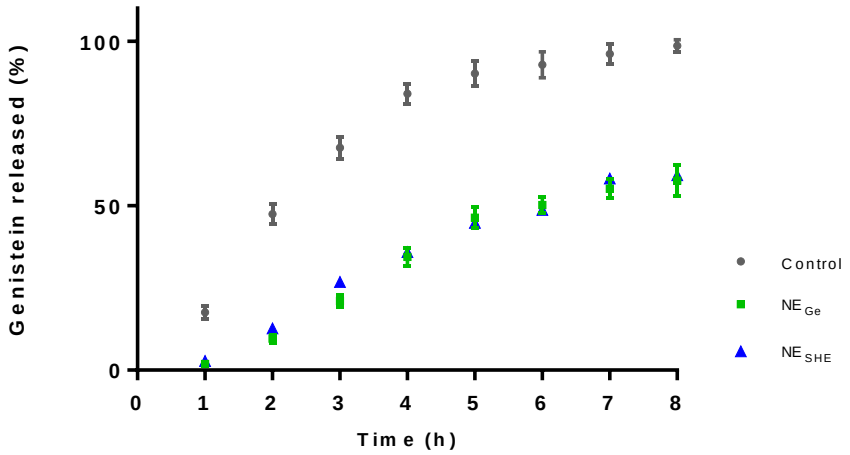


Figure 3. *Release profile of genistein from propylene glycol (control) and nanoemulsions (NE_{Ge} and NE_{SHE}) through synthetic cellulose membranes. Data shown are the mean ± SD of three replicates*

The profile retention of genistein in porcine ear skin after 8 h of contact in nanoemulsions (NE_{Ge} and NE_{SHE}) and in suspension in propylene glycol (control) are shown in Figure 4. The retention of genistein was significantly higher when NE_{Ge} was applied on the skin ($p < 0.05$), and no genistein was detected in the acceptor fluid after 8 h of study. On the other hand, an amount close to 0.52 μg cm^{-2} of genistein permeated to the acceptor fluid from NE_{SHE}. It is important to highlight that although the genistein retention from NE_{SHE} was lower than NE_{Ge}, only NE_{SHE} allowed the permeation of the isoflavone through the skin. Such fact suggests that other extract components might be influencing and facilitating the permeation of genistein.

The literature shows that plant extracts can cause a positive or synergic effect on drug behavior when topically applied, increasing, the drug permeation through the skin.[40,41] The genistein retention on the skin was higher when NE_{Ge} was applied. However, the permeation of this isoflavone was higher when NE_{SHE} was used. This could probably be occurring due to the synergistic effect of all compounds present in the extract incorporated in the NE. The same

behavior was found by Zorzi et al. (2016)[36] when comparing nanoemulsions containing pure quercetin to an extract containing this flavonoid. The authors suggest that some compounds of the extract could be interacting with the skin layers, acting, therefore, as possible permeation enhancers, increasing the permeability of the flavonoid. Moreover, permeation may be getting higher due to extract compounds that could be disfavoring the binding of the flavonoid into the nanostructure.[36]

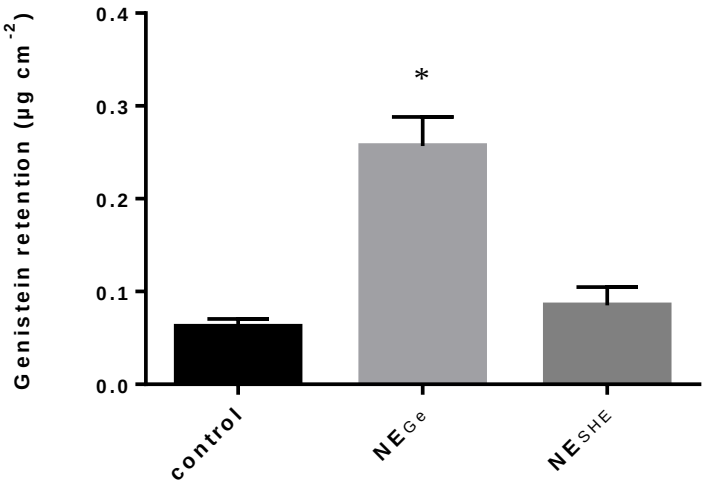


***Figure 4**. Genistein retention from propylene glycol (control) and nanoemulsions (NE_{Ge} and NE_{SHE}) through porcine skin after 8h of study. The values are means ± SD of 5 experiments. * means higher statistically ($p < 0.05$)*

Aiming a topical delivery, the compound has to reach the adequate skin layer, penetrating at a sufficient rate and amount.[42] In order to choose a formulation though, the desired effect also needs to be pre-established. For a transdermal delivery, the formulation needs to be able to permeate skin, achieving deeper layers to reach the circulatory system. Such facts allow the compound to possible perform a systemic action, being commonly used as an alternative to oral delivery.[43] Considering these characteristics, NE_{SHE} may look more promising, since it was able to permeate through the skin, being detected in the acceptor fluid of the Franz Cells after 8 h of assay. On the other hand, if a local effect is desired, NE_{Ge} showed a better profile, being capable to retained more genistein within the skin layers. However, since the objective of this study was

to identify the best formulation aiming an antioxidant protection, those results also need to be taken in consideration when choosing the best formulation.

Antioxidant potential of nanoemulsions

Antioxidant potential was analyzed through the determination of TRAP. This test indicates the antioxidant capability of the formulations by a non-enzymatic mechanism. A high antioxidant potential is shown by a low chemiluminescence (AUC), being the amount of chemiluminescence proportional to the oxidation reaction formed by the reaction of AAPH radical with luminol. As it can be seen in Figure 5, NE_{SHE} showed a better antioxidant protection compared to NE_{Ge}, since the chemiluminescence was significantly lower ($p < 0.05$), and the most comparable to Trolox, a potent antioxidant and analog of vitamin E. These results suggest a positive effect of other extract components over the antioxidant potential of the formulation.

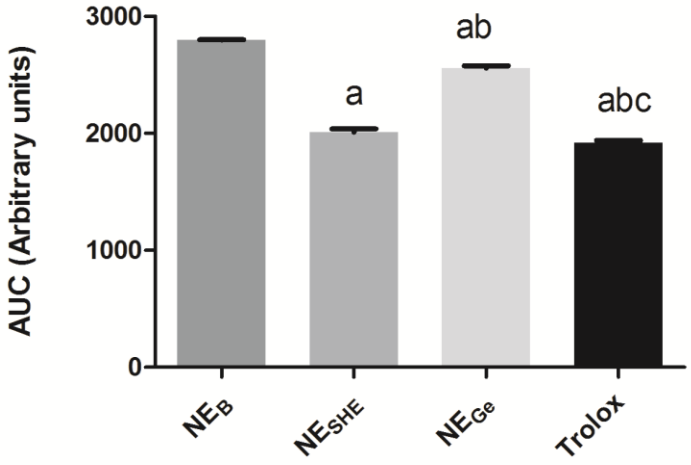


Figure 5. *Amount of chemiluminescence formed by all the formulations analyzed reflecting their antioxidant potential; where Trolox is the positive control, the blank nanoemulsion is the negative control; and AUC means area under the curve. (a): statistically different to NE_B, (b): statistically different to NE_{SHE}; (c): statistically different to NE_{Ge}, (d): statistically different to IS*

The ability of the formulations to protect the skin against UVA and UVB radiation was evaluated through the analysis of carbonyl groups and TBARS (Figure 6). Damage to protein groups in skin was analyzed by quantifying the carbonylation occurrence when reactive oxygen species (ROS), attacking amino acids, produce carbonyls groups. In turn, the new groups formed

are resistant to degradation, provoking skin damage by leading to a protein dysfunction.[44] Carbonyl groups were quantifying when reacting with 2.4-dinitrophenylhydrazine (DNPH), generating colored substances: hydrozones,[27] which can be quantified by spectrophotometry.

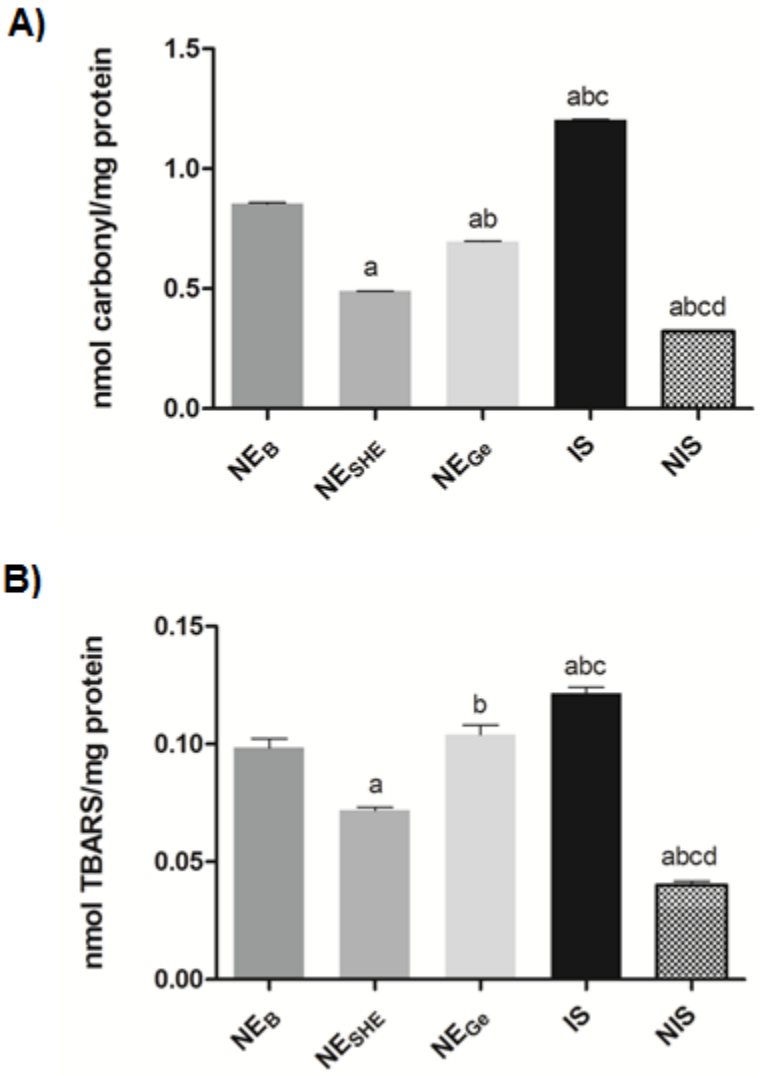


Figure 6. *Evaluation of oxidative damages after porcine ear skin treatments with different formulations followed by exposure with UVA and UVB (A) Amount of carbonyl groups formed/mg of protein analyzed for all treatments. (B) Amount of TBARS formed/mg of protein analyzed for all all treatments. Where, Positive control: irradiated skin without formulation (IS); Negative control: non-irradiated skin (NIS),* NE_B*: blank nanoemulsion;* NE_{Ge}*: nanoemulsion containing genistein;* NE_{SHE}*: nanoemulsion containing soybean hydrolyzed extract; (a): statistically different to* NE_B*, (b): statistically different to* NE_{SHE}*; (c): statistically different to* NE_{Ge}*, (d): statistically different to IS*

Figure 6A shows the results for carbonylation after skin being exposed to UV radiation and treatments. As expected, irradiated skin not treated with any formulation (IS) presented a higher level of carbonyl groups, showing a higher oxidative damage in proteins, while non-irradiated skin (NIS) maintained its levels low. The results showed that both formulations were able to protect skin when UV radiation was irradiated over it, since the amount of carbonyl groups formed was lower when compared to IS. A better skin protection was shown with NE_{SHE} than with NE_G, suggesting that other extract components may be increasing the protection of skin.

In addition, lipid oxidative damage was evaluated by the reaction with thiobarbituric acid to malondialdehyde, where the main lipid oxidation product was quantified, generating a colored compound. Results are represented in Figure 6B. NE_{SHE} offered better skin protection, being the damage lower when compared to skin fully exposed to UV radiation without formulation (IS). However, NE_{Ge} protection was not statistically different from the protection offered by $NE_{B.}$ This result shows that genistein did not present activity in this concentration for this parameter, and the decrease on the oxidative damage could be associated with other components of the formulation, such as polysorbate and egg lecithin.[45] Moreover, this result could also be attributed to the physical protection of the nanodropplets due to their shape, size, and formulation composition, leading to a light scattering.[46] NE_{SHE}, on the other hand, showed a higher skin protection though, indicating that other extract components might also be protecting the skin.

Some studies have been reporting antioxidant activity of soybean isoflavones on their isolated forms and extracts, but the comparison between them is still very limited.[16,47-50] Kao et al. (2006)[11] reported that soybean extracts present a higher scavenging potential than isolated isoflavones. Such behavior may happen due to synergic effects between isoflavones, as well as to other substances presented in the extract. The authors suggest that soybean cake extract may contain saponins and other phenolic compounds that might be increasing its antioxidant capability, and well as other isoflavones, like daidzein, which is also described as a potential antioxidant.[11] Synergic effects also have been describing by the literature when isolated isoflavones are combined.[50] Such results are, therefore, in accordance with the ones obtained in our study.

CONCLUSION

The present study showed that enzymatic hydrolysis is a good process to produce a soybean extract with high content of isoflavone aglycones and without the presence of genotoxic impurities, such as furanic compounds. The hydrolyzed extract was standardized in genistein and it was incorporated into nanoemulsions. Physicochemical properties, genistein permeation/retention potential, and antioxidant potential of extract-loaded nanoemulsions were compared to nanoemulsions containing only genistein. Zeta potential was higher in module for nanoemulsions containing extract, while other physicochemical characteristics showed to have similar properties. The presence of the extract enabled higher skin permeation, while pure genistein in nanoemulsions favored the retention of the compound in the skin. Both nanoemulsions presented a sustained release rate. Antioxidant studies showed a greater antioxidant capability and activity for nanoemulsions containing extract when compared to the pure isoflavone. Taking into consideration all results herein found, nanoemulsions containing soybean extract enriched in genistein may present a better formulation aiming skin protection from UVA/UVB oxidative damage.

ACKNOLEDGMENT

The authors want to thank the National Council for Scientific and Technological Development (CNPq) (grant agreement nº 459619/2014-4). H.F.T., G.V.P., and J.C.M are recipients of CNPq research fellowship.

REFERENCES

1. Piovesan, A.C; Soares Júnior, J.M.; Mosquette R.; Simões, M.J.; Simões, R.S.; Baracat, E.C.; *Rev. Bras. Ginecol. Obstet.* **2005,** 27(4), 204.
2. Schmid, D.; Zulli, F.; *Cosmet. Toiletries*. **2002**, 117, 45.
3. Accorsi-Neto, A.; Haidar, M.; Simões, R.; Simões, M.; Soares-Jr, J.; Baracat, E.; *Clinics* **2009**, 64(6), 505.
4. Nemitz, M.C.; Moraes, R.C.; Koester, L.S.; Bassani, V.L.; Von Poser, G.L.; Teixeira, H.F.; *Phytochem. Rev.* **2015**, 14, 849.

5. Nemitz, M.C.; Argenta, D.F.; Koester, L.S.; Bassani, V.L.; Von Poser, G.L.; Teixeira, H.F.; *Trends Food Sci. Technol.* **2016**, 49, 85.
6. Wei, H.; Saladi, R.; Lu, Y.; Wang, Y.; Palep, S.R.; Moore, J.; Phelps, R.; Shyong, E.; Lebwohl, M.G.; *J. Nutr.*. **2003**, 133, 3811S.
7. Mazumder, Md.A.R.; Hongsprabhas, P. *Biomed. Pharmacother.* **2016**, 86, 379.
8. Kohen, R.; Gati, I.; *Toxicology* **2000**, 148, 149.
9. Pinnell, S.R.; *J. Am. Acad. Dermatol.* **2003**, 48(1), 1.
10. Mitchell, J.H.; Gardner, P.T.; McPhail, D.B.; Morrice, P.C.; Collins, A.R.; Duthie, G.G.; *Arch. Biochem. Biophys.* **1998**, 360(1), 142.
11. Kao, T.H; Chen, B.H.; *J. Agric. Food Chem*. **2006**, 54, 7544.
12. Huang, Z.R.; Huang, C.F.; Lin, Y.K.; Fang, J.Y.; *Int. J. Pharm.* **2008**, 364, 36.
13. Iovine, B.; Iannella, M.L.; Gasparri, F.; Giannini, V.; Monfrecola, G.; Bevilacqua, M.A.; *Int. J. Mol. Sci.* **2012**, 13, 16444.
14. Chiu, T.M.; Huang, C.C.; Lin, T.J.; Fang, J.Y.; Wu, N.L.; Huang, C.F. J.; *Ethnopharmacol.* **2009**, 126, 108.
15. Yuan, D.; Pan, Y.; Chen, Y.; Uno, T.; Zhang, S.; Kano, Y.; *Chem. Pharm. Bull.* **2008,** 56(1), 1.
16. Georgetti, S.R.; Casagrande, R.; Verri Jr., W.A.; Lopeza, R.F.V.; Fonseca, M.J.V.; *Int. J. Pharm.* **2008**, 352, 189.
17. Poluzzi, E.; Piccinni, C.; Raschi, E.; Rampa, A.; Recanatini, M.; Ponti, F.; *Curr. Med. Chem.* **2014**, 21, 417.
18. McCue, P.P; Shetty, K. *Process Biochem.* **2005**, 40, 1791.
19. Schmid, D.; Zulli, F.; Nissen, H.P.; Prieur, H. *Cosmet. Toiletries* **2003**, 118(9), 71.
20. Sansone, F.; Picerno, P.; Mencherini, T.; Russo, P.; Gasparri, F.; Giannini, V.; Lauro, M.R.; Puglisi, G.; Aquino, R.P.; *J. Food Eng.* **2013**, 115, 298.
21. Zampiere, A.L.T.C.; Ferreira, F.S.; Crispim, E.; Gaeti, M.P.N.; Diniz, D.G.A.; Taveira, S.F.; Lima, E.M.; *J. Biomed. Nanotechnol.* **2013**, 9(3), 627.
22. Silva, A.P.C.; Nunes, B.R.; Oliveira, M.C.; Koester, L.S.; Mayorga, P.; Bassani, V.L.; Teixeira, H.F.; *Pharmazie* **2009**, 64, 32.
23. Vargas, B.A.; Bidone, J.; Oliveira, L.K.; Koester, L.S.; Bassani, V.L.; Texeira, H.F.; *J. Biomed. Nanotechnol.* **2012**, 8, 1.

24. Kang, K.H.; Kang, M.J.; Lee, J.; Choi, Y.W.; 2010; *J. Dispersion Sci. Technol.* **2010**, 31(8), 1061.
25. Andrade, L.M.; Reis, C.F.; Maione-Silva, L.; Anjos, J.L.V.; Alonso, A.; Serpa, R.C.; Marreto, R.N.; Lima, E.M.; Taveira, S.F.; *Eur. J. Pharm. Biopharm.* **2014**, 88, 44.
26. Nemitz, M.C.; Yatsu, F.K.J.; Bidone, J.; Koester, L.S.; Bassani, V.L.; Garcia, C.V.; Mendez, A.S.L.; von Poser, G.L.; Teixeira, H.F.; *Talanta* **2015**, 134, 182.
27. Dresch, M.T.Z.; Rossato, S.B.; Kappel, V.D.; Biegelmeyer, R.; Hoff, M.L.M.; Mayorga, P.; Zuanazzi, J.A.S.; Henriques, A.T.; Moreira, J.C.F.; *Anal. Biochem.* **2009**, 385, 107.
28. Balestrin, L.A.; Bidone, J.; Bortolin, R.C.; Moreira, J.C.; Teixeira, H.F.; *J. Photochem. Photobiol.* **2016**, 163, 269.
29. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J.; *J. Biol. Chem.* **1951**, 193(1), 265.
30. Draper, H.H.; Hadley, M.; *Methods Enzymol.* **1990**, 186, 421.
31. Levine, R.L.; Garland, D.; Oliver, C.N.; Amici, A.; Lenz, A.G.; Ahn, B.W.; Shaltiel, S.; Stadtman, E.R.; *Methods Enzymol.* **1990**, 186, 464.
32. Ávila, M.R.; Braccini, A.L; Albrecht, L.P.; Scapim, C.A.; Mandarino, J.C.G.; Bazo, G.L.; Cabral, Y.C.F.; *Revista Brasileira de Sementes* **2011**, 33(1), 149.
33. Nemitz, C.M.; Teixeira, H.F.; von Poser, G.L.; *Ind. Crops Prod.* **2015**, 67, 414.
34. Toker, O.S.; Dogan, M.; Ersöz, N.B.; Yilmaz, M.T. *Ind. Crops Prod.* **2014**, 137, 137.
35. Zorzi, G.K; Carvalho, E.L.S.; von Poser, G.L.; Teixeira, H.F.; *Rev. Bras. Farmacogn.* **2015**, 25(4), 426.
36. Zorzi, G.K.; Caregnato, F.; Moreira, J.C.F.; Teixeira, H.F.; Carvalho, E.L.S. *AAPS PharmSciTech* **2016**, 17, 844.
37. Tsai, Y.J.; Chen, B.H.; *Int. J. Nanomed.* **2016**, 11, 1907.
38. Ribeiro, R.C.A.; Barreto, S.M.A.G.; Ostrosky, E.A.; Rocha-Filho, P.A.; Veríssimo, L.M.ç Ferrari, M.; *Molecules* **2015**, 20(2), 2492.
39. Ali, S.M.; Yosipovitch, G.; *Acta Derm.-Venereol.*, **2013**, 93, 261.
40. Pawankumar, G.; Shiradkar, M.; *Adv. Appl. Sci. Res.* **2012**, 3(1), 261.
41. Chen, F.; Li, S.; Li, D.; Ding, J.S.; *Fitoterapia* **2012**, 83, 954.
42. Vicentini, F.T.M.C.; Georgetti, S.R.; Bentley, M.V.L.B.; Fonseca, M.J.V.; *Braz. J. Pharm. Sci.* **2009**, 45 (2), 357.
43. Azagury, A.; Khoury, L.; Enden, G.; Kost, J.; *Adv. Drug Delivery Rev.* **2014**, 72, 127.

44. Dalle-Donne, I.; Aldini, G.; Carini, M.; Colombo, R.; Rossi, R.; Milzani, A. J.; *Cell. Mol. Med.* **2006**, 10(2), 389.
45. Pan, Y.; Tikekar, R.V.; Nitin, N.; *Int. J. Pharm.* **2013**, 450, 129.
46. Morabito, K.; Shapley, N.C.; Steeley, K.G.; Tripathi, A.; *Int. J. Cosmet. Sci.* **2011**, 33, 385.
47. Chiang, H.S.; Wu, W.B.; Fang, J.Y.; Chen, B.H.; Kao, T.H.; Chen, Y.T.; Huang, C.C.; Hung, C.F.; *Int. J. Sci.* **2007**, 8, 651.
48. Georgetii, S.R.; Casagrande, R.; Vicentini, F.T.M.C.; Verri, Jr., W.A.; Fonseca, M.J.; *Eur. J. Pharm. Biopharm.* **2006**, 64, 99.
49. Lee, C.H.; Yang, L.; Xu, J.Z.; Yeung, S.Y.V.; Huang, Y.; Chen, Z.Y.; *Food Chem.* **2005**, 90, 735.
50. Record, I.R.; Dreosti, I.E.; McInerney, J.K.; *Nutritional Biochemistry*, **1995**, 6, 481.
51. Iovine, B.; Iannella, M.L.; Gasparri, F.; Monfrecola, G.; Bevilacqua, M.A.; *J. Biomed. Biotechnol.* **2011**, 2011, 1.