UNIVERSIDADE ESTADUAL DE CAMPINAS

Instituto de Biologia

LETICIA CARAMORI CEFALI

ESTUDO DO FATOR DE PROTEÇÃO SOLAR DE EXTRATO CONTENDO FLAVONÓIDES INCORPORADO EM FORMULAÇÃO DE USO TÓPICO

STUDY OF SUN PROTECTION FACTOR OF EXTRACTS RICH IN FLAVONOIDS INTO TOPICAL FORMULATION

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutora em Ciências, na área de Fármacos, Medicamentos e Insumos para Saúde.

Thesis presented to Institute of biology of University of Campinas in partial fulfillment of requirements for the PhD degree in Sciences, in area of Drugs, Medicine and Health Supplies.

Orientadora: PROFª DRª PRISCILA GAVA MAZZOLA

Co-Orientadora: PROFª DRª MARY ANN FOGLIO

Este exemplar corresponde à versão final da tese defendida pela aluna Letícia Caramori Cefali, orientada pela Profª Drª Priscila Gava Mazzola e co-orientada pela Profª Drª Mary Ann Foglio.

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Os membros da Comissão Examinadora acima assinaram a Ata de Defesa, que se encontra no processo da vida acadêmica do aluno.

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RESUMO

CEFALI, L.C. **Estudo do fator de proteção solar de extrato contendo flavonoides incorporado em formulação de uso tópico.**

O objetivo do estudo foi desenvolver fitocosmético contendo uma associação de extratos ricos em flavonóides obtidos das espécies *Dirmophandra mollis* Benth, *Ginkgo biloba* L., *Ruta graveolens* L. e *Vitis vinífera* L. Os extratos alcoólicos individualmente e em associação foram analisados por cromatografia líquida de alta eficiência para a quantificação dos flavonóides rutina e quercetina. Ensaios *in vitro* como viabilidade celular, proteção solar e eficácia antioxidante foram avaliados. Emulsão óleo em água foi desenvolvida, a mistura de extratos (1:1:1:1) foi incorporada à formulação (200 µg/ml), denominada de FPS, e ensaios para determinar o fator de proteção solar, atividade antioxidante, estabilidade fisico-química, fotoestabilidade, reologia, textura, permeação e retenção cutâneas foram avaliados. Os extratos também foram nanoparticulados, via gelificação iônica, e suas propriedades fisico-químicas foram avaliadas como morfologia, tamanho de partícula, potencial zeta e eficiência de encapsulação dos extratos. Outra emulsão óleo em água foi obtida contendo a dispersão de nanopartículas (200 µg/ml), denominada de FPS-nano. Resultados mostraram que o método cromatográfico utilizado para a quantificação dos flavonóides apresentou linearidade, precisão, exatidão e robustez. Nenhum dos extratos afetou a viabilidade celular na faixa de concentração utilizada, todos apresentaram fator de proteção solar e atividade antioxidante, sendo que *Ginkgo biloba* L. e a mistura dos extratos apresentaram resultados mais expressivos. A mistura dos extratos apresentou proteção solar UVA/UVB e proteção solar contra radiação UVA de 6,92 ± 0.1. A formulação FPS apresentou estabilidade quando estocada em baixas temperaturas (5ºC), boa espalhabilidade, baixas força de ruptura e adesividade, fragilidade e comportamentos pseudo-plástico e visco-elásticos, sendo parâmetros

coerentes para um produto tópico. A formulação FPS apresentou porcentagem de inibição frente à solução de DPPH de 17,96 ± 0,03%, proteção UVA de 2,4 ± 0,5, comprimento de onda crítico de 387,0 nm e razão UVA/UVB de 0,78, determinando que a formulação pode ser usada como filtro solar de proteção UVA/UVB. Fotoestabilidade também foi avaliada e o fator de proteção solar referente à proteção UVA diminui 13,04%, sendo considerado um produto fotoestável. Rutina permeou a barreira da pele e também foi encontrada no estrato córneo (3,27 ± 1,92 μg/ml) e retida nas camadas da pele (114,68 ± 8,70 μg/ml). Nanopartículas apresentaram coloração amarelada, formato esférico e uniforme, tamanho de partícula de 557,11 ± 3,1 nm, índice de polidispersividade de 0,39 ± 0,27, potencial zeta de -11,54 ± 2,1 mV e eficiência de encapsulação de 71,43% referente à rutina. A formulação FPS-nano apresentou estabilidade físico-quimica, fotoestabilidade e parâmetros de textura apropriados para aplicação tópica. Rutina também permeou a barreira da pele e foi encontrada no estrato córneo (1,26 ± 0,20 μg/ml), como para a formulação FPS, e retida nas camadas da pele (91,52 ± 3,94 μg/ml). O fator de proteção solar referente à radiação UVA foi de 2,3 ± 0,4, comprimento de onda crítico de 387,0 nm e razão UVA/UVB de 0,69. Assim, a mistura de extratos pode ser uma alternativa em potencial de filtros solares químicos e os fitocosméticos apresentaram fator de proteção solar e características físicas promissoras para um produto a ser utilizado como protetor solar.

Palavras chaves: antioxidante, filtro solar, fitocosmético, flavonóides, fotoestabilidade, nanopartículas.

ABSTRACT

CEFALI, L.C. **Study of sun protection factor of extracts rich in flavonoids into topical formulation.**

The aim of this study was to develop phytocosmetic containing a blend of enriched flavonoids extracts from *Dirmophandra mollis* Benth, *Ginkgo biloba* L., *Ruta graveolens* L. and *Vitis vinífera* L. Individual alcoholic extracts and a mixed extract were analyzed by validated high performace liquid chromatography for quantification of rutin and quercetin. *In vitro* cell viability, sun protection factor and antioxidant efficacy studies were carried out. Oil-in-water emulsion was development, mixed extracts (1:1:1:1) was incorporated to formulation (200 µg/ml), named SPF and sun protection factor, antioxidant activity, physicochemical stability, photostability, rheology, texture assay, cutaneous permeation and retention of flavonoids were evaluated. Vegetable extracts were also loaded in nanoparticles, by ionic gelation and its physicochemical properties were evaluated such as morphology, mean particle size, zeta potential and encapsulation efficiency for flavonoids-enriched vegetable extracts. Another oil-in-water emulsion was obtained by dispersing nanoparticles in emulsion (200 µg/ml), named SPF-nano. Results showed that the HPLC method used for the quantification of flavonoids in extracts exhibited linearity, precision, accuracy and robustness. All extracts did not affect cell viability at the evaluated concentration range and exhibited sun protection effect and antioxidant activity. *Ginkgo biloba* L. and mixed extracts depicted the most expressive results. Mixed extract exhibited sunscreen protection against UVA/UVB and UVAPF value of 6.92 ± 0.1. SPF formulation was stable when stored at low temperatures (5ºC), spreadability, low rupture strength and adhesiveness, higher brittleness, pseudo-plastic and viscoelastic behaviors been that parameters were in accordance for a topical product. SPF formulation presented 17.96 ± 0.03% of inhibition percentage against DPPH

solution, 2.4 ± 0.5 value referent to UVA protection, critical wavelength equal 387.0 nm and UVA/UVB rate equal 0.78, determining that the formulation can be used as sun filter with UVA/UVB protection. Photostability was evaluated and UVAPF value decreased 13.04%, being considered a photostable product. Rutin permeated skin barrier and was also found in stratum corneum (3.27 ± 1.92 μg/ml) and retention test (114.68 ± 8.70 μg/ml). Nanoparticles were prepared and presented yellowish color, spherical shape and uniform in their appearance. Extract-loaded nanoparticles showed a mean particle size of 557.11 ± 3.1 nm, polydispersity index (PDI) of 0.39 ± 0.27, zeta potential of -11.54 ± 2.1 mV and encapsulation efficiency of 71.43% in rutin. SPF-nano showed physicochemical stability, photostability and texture parameters appropriate for skin application. Rutin was also permeated skin barrier and was found in stratum corneum (1.26 ± 0.20 μg/ml) and retention test (91.52 ± 3.94 μg/ml), such as occurred to SPF formulation. The recorded UVAPF value was of 2.3 ± 0.4, with a critical wavelength of 387.0 nm, and UVA/UVB rate of 0.69. Then, mixed extract may be a potential alternative over chemical sun filter and phytocosmetics containing reported herein provide with sun protection and promising physical characteristics for a potential sunscreen formulation.

Keywords: antioxidant, flavonoids, nanoparticles, photostability, phytocosmetic, sunscreen.

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LISTA DE ABREVIATURAS E SIGLAS

AAPH: 2,2'-azinobis(2-amidinopropano) dihidrocloreto

Abs: Absorbância

ABTS: Radical 2,2'-azino-bis (ácido 3-etillbenziltiazolino-6-sulfonico)

ANVISA: Agência Nacional de Vigilância Sanitária

A/O: Água em óleo

BMDBM: *Butyl Methoxydibenzoylmethane*

CFU: *Colony forming unit*

CIE: *International Commission on Illumination*

COLIPA: *The European Cosmetic and Perfumery Association*

DAD: Diiodo

DL: *Detection limit*

DLS: *Dynamic light scattering*

DMSO: Dimetilsulfóxido

DPPH: 1,1-difenil-2-picrilhidrazila

DSC: Calorimetria exploratória diferencia*l/ Differential scanning calorimetry*

EE: *Encapsulation efficiency*

EMEA: *European Medicines Agency*

EROs: Espécies reativas de oxigênio

FBS: *Fetal bovine serum*

FDA: *Food and Drug Administration*

FPS ou SPF: Fator de proteção solar*/ Sun protection factor*

FTIR: *Fourier-transform infrared spectroscopy*

G: *Stocking module*

G': *Elastic module*

G": *Viscous module*

HaCat: *Human keratinocytes*

HPLC ou CLAE: Cromatografia líquida de alta eficiência/ *High performance liquid chromatography*

HPLC-DAD ou CLAE-DAD: Cromatografia líquida de alta eficiência acoplada ao detector de diiodo

IC_{50}: Concentração inibitória 50%

IL: *Interleukin*

IR: *Infrared radiation*

ISO: *International Organization for Standardization*

NR: *Neutral Red*

NRU: *Neutral Red Uptake*

$O_2^{\bullet-}$: Ânion superóxido

$^1O_2$: Oxigênio singleto

O/A: Óleo em água

OCT: *Octocrylene*

$OH^{\bullet}$: Radical hidroxila

ORAC: *Oxigen Radical Absorbance Capacity*

O/W: *Oil in water*

PA: Para análise

PABA: Ácido para-aminobenzóico

PDI: *Polydispersity index*

pH: potencial hidrogêniônico

PMMA: *Polymethylmetacrylate*

p/p: Peso por peso

QL: *Quantification limit*

ROS: *Radical Oxygen Specie*

RPMI: *Roswell Park Memorial Institute*

SEM: *Scanning electron microscopy*

TG: Termogravimetria/ Thermogravimetry

TiO_2: Dióxido de titânio

TNF: *Tumor necrosis factor*

TPP: *Tripolyphosphate*

US: *United States*

USP: *United States Pharmacopeia*

UV: Ultravioleta

UVA: Ultravioleta A

UVA/UVB: Proteção contra radiação UVA e UVB

UVAPF ou PF-UVA: Fator de proteção referente à radiação UVA

UVB: Ultravioleta B

UVC: Ultravioleta C

UV/vis: Ultravioleta/visível

v/v: Volume por volume

w/v: Weight for volume

ZnO: Óxido de zinco

SUMÁRIO

1. Introdução

Desde a década de 30, o corpo bronzeado tornou-se uma tendência marcante na população brasileira e um novo padrão de beleza no país (GONTIJO, 1987). Porém, nos últimos anos, a prática de expor-se ao sol durante longo período, tornou-se menos frequente, pois a preocupação com a saúde da pele é maior, tornando, portanto, mais comum a ida às piscinas e praias durante os períodos de menor exposição solar, sendo no início da manhã e após às dezesseis horas da tarde, preconizados pela Organização Mundial da Saúde (OMS) como horários mais seguros.

O Brasil apresenta alta incidência de raios ultravioleta, os quais podem provocar danos aos seres humanos, ocasionando o surgimento de diversas doenças de pele, além da aceleração do envelhecimento cutâneo. Assim sendo, o uso de filtros solares apresentou um grande crescimento nos últimos anos, de acordo com a Associação Brasileira da Indústria de Higiene Pessoal, Perfumaria e Cosméticos – ABIHPEC (ABIHPEC, 2018) demonstrando, dessa maneira, maior esclarecimento da população sobre os riscos provocados pela exposição excessiva ao sol.

Apesar do fator positivo em relação ao crescimento do consumo de produtos cosméticos com fator de proteção solar no Brasil (ABIHPEC, 2018) e no mundo (WHO, 2018), houve um acréscimo do número de casos de reações adversas, como dermatites, decorrentes do uso desses produtos (GOLZALEZ, FERNÁNDEZ-LORENTE, GILABERTE-CALZADA, 2008). Fenômeno ocasionado devido à presença de filtros solares de ação química, os quais são comumente relacionados a casos de hipersensibilidade em alguns usuários (BALOGH *et al.*, 2011; GOLZALEZ, FERNÁNDEZ-LORENTE, GILABERTE-CALZADA, 2008; NELSON e COX, 2006).

Dessa maneira, há grande interesse dos centros de pesquisas em desenvolver formulações cosméticas, objetivando a proteção solar, contendo componentes menos

irritantes para a pele, como por exemplo, compostos de origem vegetal, pois são vistos pelos pesquisadores, empresas e, consequentemente, pela população, como produtos menos agressivos (ACEVEDO *et al*., 2005; GUARATINI *et al*., 2009; VELASCO *et al*., 2008; VIOLANTE *et al*., 2008). Isso se deve principalmente a capacidade de absorção da radiação ultravioleta pelos cromóforos da molécula de compostos vegetais, associada à possível atividade antioxidante de extratos constituídos, especialmente, de flavonóides (GUARATINI *et al*., 2009).

Portanto, extratos vegetais, em especial os ricos em flavonóides passam a ser estudados como alternativas de compostos capazes de agir como protetores solares, protegendo a pele contra os efeitos danosos da radiação solar (CEFALI *et al.,* 2016).

Outra importante característica dos flavonóides é sua atividade antioxidante, sendo um fator contribuinte para serem utilizados como produtos tópicos, como minimizar os efeitos do envelhecimento precoce, ocasionado pela exposição solar (ADHIKARI *et al.,* 2017; HUBINGER *et al.*, 2010). Porém podem estar sujeitos à instabilidade físico-química quando são incorporados em formulações cosméticas e sujeitos ao calor e⁄ou à luz (HUBINGER *et al.*, 2010).

Assim, para minimizar esse fenômeno, técnicas como micro ou nanoencapsulação podem ser utilizadas, pois, de acordo com a literatura (AJAZUDDIN, 2010; SCALIA e MEZZENA, 2010), a encapsulação de compostos pode protegê-los de danos físicos e químicos, otimizando sua estabilidade, assim como sua biodisponibilidade, a liberação do ativo, entre outros fatores, contribuindo para a eficácia do produto final.

Muitos materiais podem ser utilizados para o desenvolvimento de micro ou nanopartículas, sendo a quitosana, polímero semi-sintético derivado da quitina, um composto bastante empregado como material capaz de promover a encapsulação de

compostos em combinação com uma espécie negativamente carregada atuando com um poliânion (KASPAR *et al.*, 2013).

Um outro fator importante no desenvolvimento de um produto a ser aplicado topicamente é avaliar sua capacidade de permitir ou evitar a permeação cutânea de um componente ativo e sua retenção na região da epiderme/derme, proporcionando sua atividade ou, até mesmo, o seu potencial tóxico. Para formulações com atividade de proteção solar, é desejável que os filtros solares químicos e físicos permaneçam no estrato córneo para que haja a proteção efetiva da pele contra a radiação solar. Porém, a presença de compostos com ação antioxidante retidos na pele pode ser desejável, proporcionando o sequestro de radicais livres provenientes do sol, minimizando os efeitos do fotoenvelhecimento, por exemplo.

A utilização rotineira e concomitante de demais formulações tópicas ao uso do filtro solar, como repelentes e especialmente formulações contendo compostos com finalidade rejuvenescedora, podem ocasionar a promoção de maior absorção de ativos, sendo fator indesejável para a ação fotoprotetora e risco de toxicidade ao usuário, tornando, dessa maneira, o estudo de permeação cutânea indispensável para o desenvolvimento de um produto com fator de proteção solar.

Em adição, uma formulação de ação tópica em que o composto ativo esteja incorporado deve apresentar estabilidade química e físico-química, e ser fotoestável, após exposição à radiação solar durante seu uso, mantendo o grau de proteção independente do tempo de exposição solar (FORESTIER, 2008; GASPAR e MAIA CAMPOS, 2006), garantindo, contudo, sua qualidade.

Portanto, esse trabalho teve como objetivo desenvolver formulação cosmética estável, com características físico-químicas desejáveis para ser aplicado sobre a pele, na forma de emulsão, contendo extrato de origem vegetal na forma livre ou nanoparticulado,

e avaliar sua atividade referente à proteção solar e atividade antioxidante, através de métodos *in vitro*, tornando-se, contudo, uma alternativa para o uso no combate aos danos provocados pela radiação proveniente do sol.

Contudo, o presente trabalho encontra-se dividido em capítulos redigidos sob a forma de artigos, a saber: **CAPÍTULO I:** Artigo de revisão da literatura "*Plant compounds as active photo protectants in sunscreens". International Journal of Cosmetic Science*, v.1, p.1- 8, 2016; **CAPÍTULO II:** Artigo experimental "*Flavonoids enriched extract: validation of quantification method, in vitro SPF, antioxidant activity and cell viability*". Submetido em periódico científico; **CAPÍTULO III:** Artigo experimental *"Topical formulation with blend of enriched flavonoids plant extracts: photostability, rheology and cutaneous permeation assays".* Submetido em periódico científico; **CAPÍTULO IV:** Artigo experimental *"In vitro SPF and photostability assays of emulsion containing nanoparticles with vegetable extracts rich in flavonoids".* Submetido em periódico científico.

Dessa forma, determinadas informações que constam em um capítulo poderão ser repetidas nos capítulos seguintes, assim como na discussão e na conclusão.

2. Objetivo

2.1 Objetivo Geral

Estudar o fator de proteção solar *in vitro* de extrato contendo flavonóides em formulação para uso tópico.

2.2 Objetivos específicos

- Obtenção das espécies vegetais *Dimorphandra mollis* Beth (faveiro), *Ginkgo biloba L.* (ginkgo biloba), *Ruta graveolens* L. (arruda) e cascas de *Vitis vinifera* L. (uva), *in natura* e em pó;
- Obtenção de extratos contendo flavonóides a partir das espécies vegetais estudadas;
- Identificar e quantificar rutina e quercetina do extratos ricos em flavonóides;
- Validar o método analítico de quantificação do extrato (Mix), referente aos flavonóides rutina e quercetina;
- Avaliar a atividade de proteção solar do extrato (Mix) *in vitro*;
- Avaliar a viabilidade celular e a atividade antioxidante do extrato (Mix) para o combate ao envelhecimento precoce *in vitro;*
- Desenvolver a formulação de uso tópico;

- Avaliar a estabilidade da formulação sem e com o extrato (Mix);
- Desenvolver formulação com nanopartículas contendo o extrato (Mix);
- Avaliar o fator de proteção solar e a atividade antioxidante do extrato (Mix) incorporado nas formulações *in vitro;*
- Verificar a capacidade de retenção do extrato (Mix) na pele através de ensaios de liberação e permeação cutânea;
- Verificar a fotoestabilidade das formulações cosméticas frente à radiação UV artificial.

CAPITULO I

"*Plant compounds as active photo protectants in sunscreens*. International Journal of Cosmetic Science, v.1, p.1-8, 2016"

Letícia Caramori Cefali, Janaína Artem Ataíde, Patrícia Moriel, Mary Ann Foglio, Priscila Gava Mazzola.

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Abstract

Excessive exposure to the sun's radiation is the major exogenous mediator of skin damage, which accelerates skin aging and increases the risk of developing skin cancer. Compounds with photoprotectant activity are extremely useful for decreasing the effect of ultraviolet (UV) radiation on the skin; however, numerous sun filters, especially organic sunscreens, are allergenic. Therefore, the development of formulations containing plant extracts, which may be potentially safer, is extensively being explored. Plant-based cosmetics are commonly used to avoid skin aging because they contain antioxidant agents that minimize free radical activity, and numerous studies have investigated the skin-protectant effects of related plant species. In addition to their antioxidant properties, plant-based cosmetics protect the skin against solar radiation because they contain polyphenols such as flavonoids and carotenoids. Therefore, this study aims to present a review of plant species commonly used in sunscreens to protect the skin against damage due to sunlight exposure.

Keywords: emulsions, flavonoids, formulations, plant-based cosmetics, spectroscopy, sunscreen

Introduction

Sunscreens are topical products used to protect the skin against damage caused by ultraviolet (UV) rays. The deleterious effects of UV solar radiation on the skin, which include irreversible skin aging and dermal pathologies, are cumulative, irreversible and currently a major health concern [1]. Solar radiation is capable of producing biochemical and physiological changes including alterations in collagen and elastic fibres, loss of subcutaneous adipose tissue and photocarcinogenic changes [2, 3]. Sunscreens not only protect against skin cancer, but they also prevent the development of other solar radiation-mediated skin conditions such as aging, wrinkle formation, undesired pigmentation and collagen loss [4, 5]. The use of sunscreens in regions with high solar radiation levels is an effective precautionary measure against developing these problems [1].

New compounds, especially from plant materials, with the ability to absorb UV radiation have been extensively researched for development as products for protecting the skin against harmful solar radiation. Therefore, we aimed to perform a comprehensive literature review of plant compounds that are reported to protect the skin against solar radiation when incorporated into topical formulations.

Conclusions

Sunscreen use is essential to combat skin damage, and therefore, numerous studies are being conducted, aimed at developing new formulations that are safe and efficacious. Additionally, scientists are exploring natural compounds of plant origin that are capable of absorbing UV radiation and possess photoprotective activity. Polyphenols, especially flavonoids, have been widely explored for radiation absorptive properties. Furthermore, carotenoids are currently being analyzed for potential antioxidant activity. Studies on açai and the combination of *H. arenarium*, *C. monogyna* and *S. nigra* demonstrated one of the

highest SPF values. However, although numerous studies have demonstrated that the use of these plant compounds improved the SPF values and efficacy of commercial UV filters, the research into and discovery of new sunscreens that contain plant compounds, which can protect the skin effectively, according to ANVISA and FDA guidelines, is still necessary.

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References

1. Ortega, P.A., Guzman, M.E., Vera, L.R. and Velazquez, J.A. Dihydroeuparin as sunscreen. Bol. Soc. Chil. Qu ım. 45, 629–636 (2000).

2. Bihhimer, W.L. Avaliação de filtros solares em seres humanos: proteção contra a queimadura solar. Cosmet. Toil. 1, 41–48 (1989).

3. Rieger, M.M. The chemistry of tanning. Cos- met. Toil. 98, 47–50 (1983).

4. Duale, N., Olsen, A.K., Christensen, T., Butt, S.T. and Brunborg, G. Octyl methoxycinnamate modulates gene expression and pre- vents cyclobutane pyrimidine dimer formation but not oxidative DNA damage in UV-exposed human cell lines. Toxicol. Sci. 114, 272–284 (2010).

5. Seite, S., Colige, A., Vivenot, P.P., Mon- tastier, C., Fourtanier, A., Lapi ere, C. and Nusgens, B. A full-UV spectrum absorbing daily use cream protects human skin against biological changes occurring in photoaging. Photodermatol. Photoimmunol. Photomed. 16, 147–155 (2000).

6. Duthie, M.S., Kimber, I. and Norval, M. The effects of ultraviolet radiation on the human immune system. Br. J. Dermatol. 140, 995– 1009 (1999).

7. Gonzalez, S., Fernandez-Lorente, M. and Gilaberte-Calzada, Y. The latest on skin photo- protection. Clin. Dermatol. 26, 614–626 (2008).

8. Palm, M.D. and Donoghue, M.N. Update on photoprotection. Dermatol. Ther. 20, 360–376 (2007).

9. Pathak, M.A. Photoprotection against harmful effects of solar UVB and UVA radiation: an update. In: Sunscreens: Development, Evaluation and Regulatory Aspects (Lowe, N.J., Shaath, N.A. and Pathak, M.A., eds), pp. 59– 82. Marcel Dekker, New York, NY (1997).

10. Romanhole, R.C., Ataide, J.A., Moriel, P. and Mazzola, P.G. Update on ultraviolet A and B radiation generated by the sun and artificial lamps and their effects on skin. Int. J. Cosm. Sci. 37, 1–5 (2015).

11. Sgarbi, F.C., Carmo, E.D. and Rosa, L.E.B. Radiação ultravioleta e carcinogênese. Rev. Cienc. Med. 16, 245–250 (2007).

12. Klug, H.L.P., Tooze, J.A., Cherry-Graff, C. et al. Sunscreen prevention of melanoma in man and mouse. Pigm. Cell Melanoma Res. 23, 835–837 (2010).

13. Wang, S.K., Balagula, I. and Osterwalder, U. Photoprotection: a review of the current and future technologies. Dermatol. Ther. 23, 31–47 (2010).

14. Murphy, G.M. The acute effects of ultraviolet radiation on the skin. In: Photodermatology (Hawk, J.L.M., ed.), pp. 43–52, Oxford University Press, London (1999).

15. Wlaschek, M., Tantcheva-Poor, I., Naderia, L. et al. Solar UV irradiation and dermal photoaging. J. Photochem. Photobiol., B 63, 41–51 (2001).

16. Tarozzi, A., Marchesi, A., Hrelia, A. et al. Protective effects of cyanidin-3-O-b-glucopyranoside against UVA-induced oxidative stress in human keratinocytes. Photochem. Photobiol. 81, 623–629 (2005).

17. Pourzand, C. and Tyrrell, R.M. Apoptosis, the role of oxidative stress and the example of solar UV radiation. Photochem. Photobiol. 70, 380–390 (1999).

18. Zanatta, C.F., Mitjans, M., Urgatondo, V., Rocha-Filho, P.A. and Vinardell, M.P. Photo protective potential of emulsions formulated with Buriti oil (*Mauritia flexuosa*) against UV irradiation on keratinocytes and fibroblasts cell lines. Food Chem. Toxicol. 48, 70–75 (2010).

19. Packer, L. and Sies, H. Singlet oxygen, UVA and ozone. Methods Enzymol. 319, 3–682 (2000).

20. Giacomoni, P.U. Understanding reactive oxygen species. Cosm. Toil. 122, 36–40 (2008).

21. Morita, A. and Krutmann, J. Ultraviolet A radiation-induced apoptosis. Methods Enzymol. 319, 302–309 (2000).

22. Liochev, S.I. and Fridovich, I. The role of O_2 in the production of OH *in vitro* and *in vivo*. Free Radic. Biol. Med. 16, 29–33 (1994).

23. Flor, J., Davolos, M.R. and Correa, M.A. Protetores solares. Quım. Nova, 30, 153–158 (2007).

24. Jain, S.K. and Jain, N.K. Multiparticulate carriers for sunscreening agents. Int. J. Cosmet. Sci. 32, 89–98 (2010).

25. Manaia, E.B., Kaminiski, R.C.K., Correa, M.A. and Chiavacci, L.A. Inorganic UV filters. Braz. J. Pharm. Sci. 49, 201–209 (2013).

26. Lowe, N.J., Shaath, N.A. and Pathak, M.A. Sunscreens: Development: Evaluation, and Regulatory Aspects, 2nd, v.15, 744p. Marcel Dekker, New York, NY (1997).

27. BRASIL. Agência Nacional de Vigilância Sanitária. Resolução RDC nº 47, de 16 de março de 2006. Lista de filtros ultravioletas permitidos para produtos de higiene pessoal,

cosméticos e perfumes. Diário Oficial da União, nº 53, 17 de março de 2006. Seção 1. p.60 (2006).

28. Salgado, C., Galante, M.C. and Leonardi, G.R. Filtros solares: mecanismos de ação e metodologias em preparações magistrais. Int. J. Pharm. Comp. 6, 224–236 (2004).

29. Ribeiro, Claudio de Jesus. Fotoproteção e Fotoprotetores. In: Cosmetologia Aplicada a Dermoestetica. 2 ed., Pharmabooks, São Paulo (2006).

30. Antoniou, C., Kosmadaki, M.G., Stratigos, A.J. and Katsambas, A.D. Sunscreens – what's important to know. J. Eur. Acad. Dermatol. Venereol. 22, 1110–1118 (2008).

31. Jin, C.Y., Zhu, B.S., Wang, X.F. and Lu, Q.H. Cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells. Chem. Res. Toxicol. 21, 1871–1877 (2008).

32. van der Pols, J.C., Williams, G.M., Pandeya, N., Logan, V. and Green, A.C. Prolonged prevention of squamous cell carcinoma of the skin by regular sunscreen use. Cancer Epidemiol. Biomarkers Prev. 15, 2546–2548 (2006).

33. Mileyeva-Biebesheimer, O.N., Zaky, A. and Gruden, C.L. Assessing the impact of titanium dioxide and zinc oxide nanoparticles on bacteria using a fluorescent-based cell membrane integrity assay. Environ. Eng. Sci. 27, 329–335 (2010).

34. Serpone, N., Dondi, D. and Albini, A. Inorganic and organic UV filters: their role and efficacy in sunscreens and suncare products. Inorg. Chem. Acta 360, 794–802 (2007).

35. Balogh, T.S., Velasco, M.V.R., Pedriali, C.A., Kaneko, T.M. and Baby, A.R. Ultraviolet radiation protection: current available resources in photoprotection. An. Bras. Dermatol. 86, 732–742 (2011).

36. Nelson, D.L. and Cox, M.M. Metabolismo do DNA. In: Princípios de Bioquímica do Lehninger (Lehninger, A.L., ed.), pp. 940–984, Sarvier, Sao Paulo (2006).

37. Lautenschlager, S., Wulf, H.C. and Pittelkow, M.R. Photo protection. Lancet 370, 528–537 (2007).

38. Foley, P., Nixon, R., Marks, R., Frowen, K. and Thompson, S. The frequency of reactions to sunscreens: results of a longitudinal population-based study on the regular use of sunscreens in Australia. Br. J. Dermatol. 128, 512–518 (1993).

39. Bissonnette, R. Update on sunscreens. Skin Ther Lett. 13, 5–7 (2008).

40. Isaac, V.L.B., Cefali, L.C., Chiari, B.G., Oliveira, C.C.L.G., Salgado, H.R.N. and Correa, M.A. Protocolo para ensaios físico-químicos de estabilidade de fitocosméticos. Rev. Ciênc. Farm. Básica Apl. 29, 81–96 (2008).

41. Isaac, V.L.B. Desenvolvimento, estabilidade e liberação *in vitro* de preparações lipolíticas. 1998, 158f. (Doutorado em Ciências Farmacêuticas) – Faculdade de Ciências Farmacêuticas – Universidade de São Paulo, São Paulo (1998).

42. Cooman, L., Everaert, E. and de Keukeleire, D. Quantitative analysis of hop acids, essential oils and flavonoids as a clue to the identification of hop varieties. Phytochem. Anal. 9, 145–150 (1998).

43. BRASIL. Agência Nacional de Vigilância Sanitária. Resolução RDC nº 30 de 1° de junho de 2012. Regulamento Técnico Mercosul sobre Protetores Solares em Cosméticos. Brasília, p. 1–7 (2012).

44. FDA. Food and Drug Administration. Sunscreen drug products for over-the-coun- ter human use. Code of Federal Regulations. Title 21, v. 5 (2013).

45. Domas, W.C., Oliveira, T.T., Rodrigues-Das- dores, R.G., Santos, A.F. and Nagem, T.J. Flavonoides: potencial terapêutico no estresse oxidativo. Rev. Ciênc. Farm. Básica Apl. 28, 241–249 (2007).

46. Ahlenstiel, T., Burkhardt, G., Kohler, H. and Kuhlmann, M.K. Bioflavonoids attenuate renal proximal tubular cell injury during cold preservation in Euro-Collins and University of Wisconsin solutions. Kidney Int. 63, 554–563 (2003).

47. Behling, E.B., Sendao, M.C., Francescato, H.D.C., Antunes, L.M.G. and Bianchi, M.L.P. Flavonoide quercetina: aspectos gerais e ações biológicas. Alim. Nutr. 15, 285–292 (2004).

48. Hertog, M.G.L., Hollman, P.C.H. and Katan, M.B. Contend of potentially anticarcinogenic flavonoids in 28 vegetables and 9 fruits commonly consumed in the Netherlands. J. Agric. Food Chem. 40, 2379–2883 (1992).

49. Su, J.F., Guo, C.J., Wei, J.Y., Yang, J.J., Jiang, Y.G. and Li, Y.F. Protection against hepatic ischemiare perfusion injury in rats by oral pretreatment with quercetin. Biomed. Environ. Sci. 16, 1–8 (2003).

50. Huber, L.S., Hoffmann-Ribani, R. and Rodriguez-Amaya, D.B. Quantitative variation in Brazilian vegetable sources of flavonols and flavones. Food Chem. 113, 1278–1282 (2009).

51. Nijveldt, R.J., van Nood, E., van Hoom, D.E., Boelens, P.G., van Norren, K. and van Leeuwen, P.A. Flavonoids: a review of probable mechanisms of action and potential applications. Am. J. Clin. Nutr. 74, 418–425 (2001).

52. Choquenet, B., Couteau, C., Paparis, E. and Coiffard, L.J.M. Quercetin and rutin as potential sunscreen agents: determination of efficacy by an *in vitro* method. J. Nat. Prod. 71, 1117–1118 (2008).

53. Velasco, M.V.R., Balogh, T.S., Pedriali, C.A., Sarruf, F.D., Pinto, C.A.S.O., Kaneko, T.M. and Baby, A.R. Associação da rutina com p-metoxicinamato de octila e benzofenona-3: avaliação *in vitro* da eficácia fotoprotetora por espectrofotometria de refletância. Lat. Am. J. Pharm. 27, 23–27 (2008).

54. Verschooten, L., Claerhout, S., Van Laethem, A., Agostinis, P. and Garmyn, M. New strategies of photoprotection. Photochem. Photobiol. 82, 1016–1023 (2006).

55. Chiu, A. and Kimballl, A.B. Topical vita- mins, minerals and botanical ingredients as modulators of environmental and chronological skin damage. Br. J. Dermatol. 149, 681–691 (2003).

56. Maclennan, K.M., Darlington, C.L. and Smith, P.F. The CNS effects of *Ginkgo biloba* extracts and ginkgolide B. Prog. Neurobiol. 67, 235–257 (2002).

57. Mahady, G.B. *Ginkgo Biloba*: a review of quality, safety, and efficacy. Nutr. Clin. Care 4, 140–147 (2001).

58. van Beek, T.A. and Montoro, P. Chemical analysis and quality control of *Ginkgo biloba* leaves, extracts, and phytopharmaceuticals. J. Chromatogr. A 1216, 2002–2032 (2009).

59. Katiyar, S.K., Matsui, M.S., Elmets, C.A. and Mukhtar, H. Polyphenolic antioxidant epigallocatechin-3-gallate from green tea reduces UVB-induced inflammatory responses and infiltration of leukocytes inhuman Skin. Photochem. Photobiol. 69, 148–153 (1999).

60. Dal Belo, S.E., Gaspar, L.R. and Campos, P.M.B.G.M. Photo protective effects of topical formulations containing a combination of *Ginkgo biloba* and green tea extracts. Phyt Res. 25, 1854–1860 (2011).

61. Matsuda, A.H., Machado, L.B. and del Mastro, N.L. Thermal analysis applied to irradiated propolis. Radiat. Phys. Chem. 63, 353– 355 (2002).

62. Park, Y.K., Ikegak, M., Abreu, J.A.S. and Alcici, N.M.F. Estudo da preparação dos extratos de propolis e suas aplicações. Ciênc. Tecnol. Aliment 18, 313–318 (1998).

63. Rocha, L., dos Santos, L.R., Arcenio, F. et al. Otimização do processo de extração de propolis através da verificação da atividade antimicrobiana. Rev. Bras. Farmacogn. 13, 71–74 (2006).

64. Nascimento, C.S., Nunes, L.C.C., de Lima, A.A.N., Grangeiro Junior, S. and Rolim Neto, P.J. Incremento do FPS em formulação de protetor solar utilizando extratos de pro polis verde e vermelha. Rev. Bras. Farm. 30, 334–339 (2009).

65. Howitt, C.A. and Pogson, B.J. Carotenoid accumulation and function in seeds and non-green tissues. Plant, Cell Environ. 29, 435–445 (2006).

66. Pinto, J.T. Efeito da estocagem da goiabada a diversas temperaturas sobre os teores de carotenoides e de acido ascórbico. 2006. 59f. (Master in Pharmaceutical Sciences) – Faculty of Pharmaceutical Science, Sao Paulo State University, Araraquara (2006).

67. Sies, H. and Stahl, W. Lycopene: a biologically important carotenoid for humans? Arch. Biochem. Biophys. 336, 1–9 (1996).

68. Costa, P.A., Ballus, C.A., Teixeira Filho, J. and Godoy, H.T. Fatty acids profile of pulp and nuts of Brazilian fruits. Ciênc. Tecnol. Aliment 31, 950–954 (2011).

69. Garcia-Quiroz, A., Moreira, S.G.C., de Morais, A.V., Silva, A.S., da Rocha, G.N. and Alcantara, P. Physical and chemical analysis of dieletric properties and differential scanning calorimetry techniques on buriti oil. Instrum Sci. Technol. 31, 93–101 (2003).

70. Vendramini, A.N.A. and Trugo, L.C. Phenolic compounds in acerola fruit (*Malpighia punicifoli*a L.). J. Braz. Chem. Soc. 15, 664–668 (2004).

71. Cavalcante, M.L. and Rodriguez-Amaya, D.B. Carotenoid composition of the tropical fruits *Eugenia uniflora* and *Malpighia glabra*. In: Food Science and Human Nutrition (Charalambous, G., ed.), pp. 643–650. Elseiver, Amsterdan (1992).

72. Rosso, V.V. Composição de carotenoides e antocianinas em acerola. Estabilidade e atividade antioxidante em sistemas-modelo de extratos antociânicos de acerola e açaí [Dissertação]. Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas (2006).

73. Souza, F.P., Campos, G.R. and Packer, J.F. Determinação da atividade fotoprotetora e antioxidante em emulsões contendo extrato de *Malpighia glabra* L.–Acerola. Rev. Ciênc. Farm. Básica Apl. 34, 69–77 (2013).

74. Kuskoski, E.M., Asuero, A.G., Morales, M.T. and Fett, R. Frutos tropicais silvestres e polpas de frutas congeladas: atividade antioxidante, polifenóis e antocianinas. Ciênc. Rural, 36, 1283–1287 (2006).

75. Kang, J., Xie, C., Li, Z., Nagarajan, S., Schauss, A.G., Wu, T. and Wu, X. Flavonoids from açai (*Euterpe oleracea* Mart.) pulp and their antioxidant and anti-inflammatory activities. Food Chem. 128, 152–157 (2011).

76. Gordon, A., Cruz, A.P.G., Cabral, L.M.C. et al. Chemical characterization and evaluation of antioxidant properties of Açai fruits (*Euterpe oleraceae* Mart.) during ripening. Food Chem. 133, 256–263 (2012).

77. Inacio, M.R.C., Lima, K.M.G., Lopes, V.G., Pessoa, J.D.C. and Teixeira, G.H.A. Total anthocyanin content determination in intact açai (*Euterpe oleracea* Mart.) and palmitero - juçara (*Euterpe edulis* Mart.) fruit using near infrared spectroscopy (NIR) and multivariate calibration. Food Chem. 136, 1160–1164 (2013).

78. Daher, C.C., Fontes, I.S., Rodrigues, R.O. et al. Development of O/W emulsions containing *Euterpe oleracea* extract and evaluation of photoprotective efficacy. Braz. J. Pharm. Sci. 50, 639–652 (2014).

79. Niculae, G., Lacatusu, I., Badea, N., Stan, R., Vasile, B.S. and Meghea, A. Rice bran and raspberry seed oil-based nanocarriers with self-antioxidative properties as safe photo protective formulations. Photochem. Photobiol. Sci. 13, 703–716 (2014).

80. Chiari, B.G., Trovatti, E., Pecoraro, T., Correa, M.A., Cicarelli, R.M.B., Ribeiro, S.J.L. and Isaac, V.L.B. Synergistic effect of green coffee oil and synthetic sunscreen for health care application. Ind. Crop Prod. 52, 389–393 (2014).

81. Figueiredo, S.A., Vilela, F.M., da Silva, C.A., Cunha, T.M., dos Santos, M.H. and Fonseca, M.J. *In vitro* and *in vivo* photoprotective/ photochemopreventive potential of *Garcinia brasiliensis* epicarp extract. J. Photochem. Photobiol., B 5, 65–73 (2014).

82. Veberic, R., Jakopic, J., Stampar, F. and Schmitzer, V. European elderberry (*Sambucus nigra* L.) rich in sugars, organic acids, anthocyanins and selected polyphenols. Food Chem. 114, 511–515 (2009).

83. CIE. Erythema Reference Action Spectrum and Standard Erythema Dose. Commission Internationale de l'Eclairage International (CIE) Central Bureau. Publ. CIE S007/E, Vienna, Austria (1998).

84. McKinley, A.F. and Diffey, B.L. A reference action spectrum for ultraviolet induced erythema in human skin. CIE J. 6, 17–22 (1987).

85. Kaur, C.D. and Saraf, S. *In vitro* sun protection factor determination of herbal oils used in cosmetics. Pharmacognosy Res. 2, 22–25 (2010).

86. Gaikwad, M. and Kale, S. Formulation and *in vitro* evaluation for sun protection factor of *Moringa oleifera* Lam (family - Moringaceae) oil sunscreen cream. Inter. J. Pharm. Pharm. Sci. 3, 371–375 (2011).

87. Souza, T.M., Santos, L.E., Moreira, R.R.D. and Rangel, V.L.B.I. Sunscreen activity evaluation of *Achillea millefolium* L. (Asteraceae). Rev. Bras. Farmacogn. 15, 36–38 (2005).

88. Violante, I.M.P., Souza, I.M., Venturini, C.L., Ramalho, A.F.S., Santos, R.A.N. and Ferrari, M. In vitro sunscreens preliminary study in plants extracts from Mato Grosso cerrado. Rev. Bras. Farmacogn. 89, 175–179 (2008).

89. Korac, R.R. and Khambholja, K.M. Potential of herbs in skin protection from ultraviolet radiation. Pharmacogn. Rev. 5, 164–173 (2011).

90. Fernandes, S.C., Alonso-Varona, A., Palo- mares, T., Zubillaga, V., Labidi, J. and Bulone, V. Exploiting mycosporines as natural molecular sunscreens for the fabrication of UV-absorbing green materials. ACS Appl. Mater. Interfaces. 7, 16558–16564 (2015).

91. Yong, Q., Xueqing, Q. and Shiping, Z. Lignin: a nature-inspired sun blocker for broad- spectrum sunscreens. Green Chem. 17, 320– 324 (2015).

92. Karsten, U. and Holzinger, A. Green algae in alpine biological soil crust communities: acclimation strategies against ultraviolet radiation and dehydration. Biodivers. Conserv. 23, 1845–1858 (2014).

93. Kitzing, C., Preoschold, T. and Karsten, U. UV-induced effects on growth, photosynthetic performance and sunscreen contents in different populations of the green alga *Klebsormidium fluitans* (Streptophyta) from alpine soil crusts. Microb. Ecol. 67, 327–340 (2014).

CAPITULO II

"Flavonoids enriched extract: validation of quantification method, in vitro SPF, antioxidant activity and cell viability"

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Abstract

The aim of this study was to evaluate the potential sun protecting effect of extracts containing flavonoids obtained from natural sources i.e. from *Dirmophandra mollis* Benth, *Ginkgo biloba* L., *Ruta graveolens* L. and *Vitis vinífera* L.. Individual extracts (obtained from each species) and a mixed extract (containing the four extracts) were analysed by validated HPLC for the identification of flavonoids and quantification of rutin and quercetin. *In vitro* cell viability study was carried out using neutral red method. The *in vitro* sun protect factor was determined by spectral transmittance and *in vitro* antioxidant efficacy was evaluated against DPPH, ABTS and AAPH radicals. The HPLC method used for the identification and quantification of flavonoids in extracts exhibited linearity, precision, accuracy and robustness. Detection and quantification limits were, respectively, 2.881±0.9 $\mu g.ml^{-1}$ and 0.864±0.9 $\mu g.ml^{-1}$ for quercetin, and 30.09±1 $\mu g.ml^{-1}$ and 9.027±1.1 $\mu g.ml^{-1}$ for rutin. All extracts did not affect cell viability at the evaluated concentration range and exhibited sun protection effect and antioxidant activity. Among evaluated extracts, *Ginkgo biloba* L. and mixed extracts depicted the most expressive antioxidant activity. Mixed extract exhibited sunscreen protection against UVA and UVB and a critical wavelength of 372.7±0.1. Our results translate the enhanced flavonoids

composition of the mixed extract, which may be a potential alternative over sunscreens and antioxidants in pharmaceutic/cosmetic formulations.

Keywords: antioxidant, cell viability, flavonoids, sun protection factor, sunscreen

1. Introduction

Sun is essential to life and its benefits are manifold, such as stimulation of vitamin D production and consequently calcium absorption improvement, mood spur, some skin conditions improvement and, in moderate amounts, the prevention of some types of cancer [1,2]. However, sun radiation, especially UVA and UVB, can stimulate free radicals production [3,4,5] which are able of increasing lipid peroxidation rates, damaging mitochondrial enzymes and plasmatic membranes and causing reduction of antioxidant substances in the skin [6]. Moreover, UVA and UVB radiations can promote directly DNA damage, which is associated to skin aging acceleration, skin cancer in addition to skin sunburns [7,8,9].

World Health Organization epidemiological studies showed that UV radiation exposition, especially during childhood and adolescence, is the main etiological agent of skin cancer [10]. In Brazil, according to National Institute of Cancer, skin cancer is the most prevalent in Brazilian population, estimating 180,000 new cases of non-melanoma skin cancer, which can be avoided by individual protection against sunlight, such as sunscreen use, stimulated by health education for the population [11].

Sunscreens are topical products initially developed to prevent sunburns, which have evolved to protect against other harmful effects of ultraviolet radiation, such as skin cancer, aging, wrinkle formation, undesired pigmentation and collagen loss [12,13,14].

These products usually contain a mixture of sun filters, which can be classified as organic or inorganic filters, according to their chemical nature [14,15].

Ideal UV filters should be non-toxic, non-allergenic, and not be systemically absorbed, as filters should remain onto the skin surface to effectively it protect against radiation [16]. Unfortunately, older substances rely on molecules of low molecular weight, while the loading of inorganic filters in nanoparticles may enhance skin permeation, leading to further dermis absorption and adverse side effects [16,17].

Concerns about UV filters safety is still a major issue, since some adverse effects, including contact sensitivity, estrogenicity [14], allergic reactions [18], deleterious cell damages, biological function disruptions [17], and photogenotoxicity [19], have been reported. Moreover, there are also concerns regarding UV filters accumulation in the environment and potential risks of this accumulation [20,21]. Therefore, research on the use of natural ingredients aiming reduction of skin irritation occurrence and other harmful effects associated with sunscreens is steadily increasing [12].

Among many plant compounds, flavonoids are the most studied in use as sun filters by presenting cyclic and aromatic rings in their structure absorbing in the ultraviolet region, especially at wavelength ranges of 240-285 nm and 300-550 nm [22].

Flavonoids are the third largest class of natural products and exhibit many important effects in plants mainly regarding protection against pathogens and UVB radiation [23]. Rutin (quercetin 3-rutinoside) and quercetin are amongst the most common flavonoids and both have been evaluated for many different biological effects [24,25].

With the aim of searching new, effective sunscreens from natural products, our study focused on the characterization and evaluation of species rich in flavonoids such as

Dimorphandra mollis Benth, *Ginkgo biloba* L., *Ruta graveolens* L. and *Vitis vinifera* L. extracts, alone (individual) and as a mixture, as sunscreen filters using *in vitro* models.

2. Material and Methods

2.1 Material

Benitaka grape (*Vitis vinifera* L. fruits) was purchased as single batch in a local supermarket. *Dimorphandra mollis* Beth fava beans was harvested in Private Biological Reserve (22º18′S/47º11′W), in Mogi Guaçu (São Paulo, Brazil), in May 2015. *Ruta graveolens* leaves were harvested in the Chemical, Biological and Agricultural Pluridisciplinary Research Center – CPQBA (22°48'S/47°0'W) at University of Campinas (UNICAMP) (Campinas, São Paulo, Brazil), in April 2015. *Ginkgo biloba* L. dry extract was purchased at Galena (Campinas, São Paulo, Brazil). Ethyl alcohol, acetic acid, hydrochloric acid, boric acid, oxalic acid, acetone, ether, metallic magnesium, metallic zinc, ferric chloride, aluminum chloride were provided by Synth (São Paulo, Brazil), and 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2-2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and fluorescein by Sigma-Aldrich (São Paulo, Brazil). Quercetin (93.3% purity) and rutin (97.3% purity) standard by Acros (Sao Paulo, Brazil). RPMI-1640 and fetal bovine serum were provided by Gibco (Sao Paulo, Brazil) and Tris[hydroxymethyl]aminomethane and neutral red dye was provided by Sigma-Aldrich (São Paulo, Brazil).

2.2 Flavonoids extraction

Fresh *Dimorphandra mollis* Benth fava beans and *Ruta graveolens* L leaves were dried in stove (Lemaq, Mod LM-EST, Diadema, Brazil), at 40 ± 3°C for 72 hours and

ground in hammer mill [26]. Fresh *Benitaka* grapes were analytically weighed, washed with neutral detergent and water, peeled and grouped peels were dried at 40 ± 2ºC in a stove (Lemaq, Mod LM-EST, Diadema, Brazil) for 72 hours. After weight, dry peels were ground in a blender (Black&Decker, Campinas, Brazil).

All dry extracts, including commercial *Ginkgo biloba* leaves extract, were subjected to physicochemical analysis such as pH, density, granulometry measurement, dry loss, total ash and insoluble ash [27].

For each species, extracts were obtained using ethanol at 1:3 (w:v) (Synth) in a liquid extractor by mechanical stirring at 27 ± 3ºC for 90 minutes. Thereafter, all extracts were submitted to vacuum filtration, solvent elimination (Marconi, MA120, Piracicaba, Brazil) at 40 ± 2 ºC, and freeze-dried (Thermo Scientific, Power Dry PL3000, São Paulo, Brazil) [26].

2.3 Flavonoids identification and quantification

All extracts were submitted to identification reactions [28] such as Shinoda, Taubock, Pew, Ferric chloride and aluminum chloride for flavonoids identification.

For this, Shinoda reaction consisted in add 1ml of each extract and tube containing metallic magnesium and hydrochloric acid drops. After the reaction, it was observed of rose and red color.

For determination the Taubock reaction, 3ml of each extract was evaporated until dryness and were added acetone drops, crystals of boric acid and 5ml of ether in tube. It was identified to fluorescence under UV light.

Flavonoids were also identified by reactions with ferric chloride, adding 1ml of each extract in tubes containing drops of ferric chloride solution at 2%, and with aluminium chloride adding drops of each extracts in tubes containing drops of aluminium

chloride solution at 5%. Thus, it was observed green color and fluorescence under UV light, respectively.

2.4 Flavonoids analysis by HPLC

2.4.1 Sample preparation

Extracts (5 μg.ml^{-1} to *Dimorphandra mollis* Benth, 5 μg.ml^{-1} to *Ginkgo biloba* L., 50 μg.ml^{-1} to *Ruta graveolens* L. and 1000 μg.ml^{-1} to *Vitis vinífera* L.) were suspended in methanol HPLC grade (Merck). A mix sample in 1:1:1:1 volume proportion was prepared containing the four extracts, in previously reported concentrations. Then, all samples were separately filtered in 0.45μm membrane (Merck). Quercetin (50 μg.ml^{-1}) and rutin (500 μg.ml^{-1}) standard were analyzed by HPLC in comparison to extracts results.

2.4.2 HPLC conditions

Aliquots of 5μl of each sample were injected in HPLC-DAD instrument (Agilent, Technologies 1250 infinity), using monomeric chromatographic column C_{18} (Phenomenex), flow rate of 0.3ml.min^{-1} for 10 minutes. Mobile phase used was methanol grade HPLC acidified with 0.1% (v/v) formic acid (Synth), at 27 ± 1 °C and flavonoids quercetin and rutin were identified at 257nm [29].

2.4.3 Validation of analytical method by HPLC

The analytical method was validated accordingly to parameters required by Brazilian Resolution nº 899 [30] for phytotherapeutic agents. The results should follow the acceptance levels stipulated for bioanalytical methods, considering the complexity of the plant raw material. For bioanalytical methods, the linear correlation coefficient (R)

for acceptance of the analytical curve should be at least 0.98, with the acceptance of 15% dispersion at all mid points and 20% near the lower bound of quantification [30,31].

Analytical curves were obtained using concentrations of 50.0, 25.0, 12.5, 6.25 e 3.125 $\mu g.ml^{-1}$ for quercetin and 500.0, 250.0, 125.0, 62.5 e 31.25 $\mu g.ml^{-1}$ for rutin, both registered at 257nm.

The precision assay was performed using intra-day and inter-day repeatability [30,31]. Six samples with a concentration of 50 $\mu g.ml^{-1}$ for quercetin and 500 $\mu g.ml^{-1}$ for rutin were analyzed on the same day and on two consecutive days. Areas of standard peaks were obtained, and variation coefficient percentage was calculated. Mix sample was also subjected to precision assay.

The exactness test was performed through the recovery assay, which consisted of adding a known concentration of flavonoids standards to extracts. Then, different volume (0.75 ml, 1.25 ml and 1.75 ml) of quercetin (50 $\mu g.ml^{-1}$) and rutin (500 $\mu g.ml^{-1}$) were transferred to 5 ml volumetric flasks containing 0.625 ml of *Dimorphandra mollis* Benth (1 $mg.ml^{-1}$), *Ginkgo biloba* L. (1 $mg.ml^{-1}$), *Ruta graveolens* L. (5 $mg.ml^{-1}$), *Vitis vinifera* L. (100 $mg.ml^{-1}$) and mixed extracts, separately. Volumes were completed with methanol HPLC grade, resulting in different concentrations of quercetin and rutin (Cf). Recovery percentage of flavonoids standards (R%) were determined [30,31].

For robustness, six concentrations of mixed sample were analyzed, and the analyzer was varied for data comparison [30].

Detection and quantification limits (DL and QL, respectively) were evaluated to determine and quantify the lowest acceptable concentration of quercetin and rutin in extracts [30]. Therefore, rutin and quercetin solution were prepared containing low concentration, and DL and QL were calculated using Equations I and II.

$$DL = SDx3/SC \quad \text{Equation I}$$

$$QL = SDx10/SC \quad \text{Equation II}$$

in which SD is the standard deviation of intercept with the Y axis of at least three analytical curves constructed, and SC is the slope of analytical curve.

To determine the method selectivity, chromatograms of extracts were compared with analytical standards chromatograms to determine impurities in extracts [30].

2.5 *In vitro* viability cell analysis by neutral red uptake (NRU)

2.5.1 Cell culture conditions

The immortalized human keratinocyte (HaCaT) cell line was kindly provided by prof. Dr. Ricardo Della Coletta (University of Campinas) and was maintained in RPMI 1640 (Gibco, USA) supplemented with 5% (v/v) fetal bovine serum (FBS, Gibco) and 1% (v/v) penicillin:streptomycin (Nutricell, 1000 $U.ml^{-1}$:1000 $g.ml^{-1}$) in a humidified atmosphere with 5% CO_2, at 37°C. For the experiments, HaCaT cells were used between passages 5 to 12.

2.5.2 Samples and solutions

The four individual extracts (*D. mollis* fava beans, *G. biloba* dry extract, *R. graveolens* leaves and *V. vinifera* fruit peels) and the mix extract (5 mg) were initially diluted in DMSO (50 µl) followed by the addition of 950 µl of RPMI 1640/FBS 5% (working solution). Final concentrations (250.0, 125.0, 62.5, 31.25, 15.62 $\mu g.ml^{-1}$) were obtained by a series of dilutions in RPMI 1640/FBS 5%. Neutral red stoke solution was prepared at 33 $\mu g.ml^{-1}$ in deionized water. Neutral red work solution was prepared by mixing 1 ml of stoke solution with 79 ml of RPMI-1640 supplemented with 0.5% (v/v) of fetal bovine serum and 1% (v/v) penicillin/streptomycin followed by 30 minutes at

37°C in water bath and centrifugation (Fanem, São Paulo, Brazil) for 10 minutes at 1000 rpm.

2.5.3 Cell viability assay

The *in vitro* cell viability assay was performed as described by Stoke et al. [32] and OECD [33]. The HaCaT (4 x 104 cells.ml^{-1}), in 96-well plates (100 µl cells.well^{-1}), were exposed to samples final concentrations, in triplicate, for 48 h. The final DMSO concentration (≤ 0.25%) did not affect cell viability. Doxorubicin chloride (0.5 µg.ml^{-1}) was used as positive control and medium culture as negative control. After 48h-exposition, medium was removed and replaced by neutral red work solution (200 µl.well^{-1}). Cells were incubated for 3 h followed by supernatant removal and addition of ethanol/acetic acid solution (1.0%; v/v) (100 µl.well^{-1}). Absorbance values were read at 540nm using a spectrophotometer (Versamax, Molecular Devices, São Paulo, Brazil). Concentration-response curve for each sample was plotted using GraphPad Prism (version 5.02) software for Windows (GraphPad Software, San Diego, CA).

2.6 *In vitro* sun protection factor evaluation

In vitro sun protection factor was determined by ultraviolet-visible spectrophotometry method described by Mansur et al. [34]. Spectrophotometric readings were obtained for each extract (100 µg.ml^{-1}) and Mix sample at 290 to 320nm and SPF values were determined using Equation III:

$$SPF=CF\times\Sigma^{290}_{320}\times EE(\lambda)\times I(\lambda)\times Abs(\lambda) \qquad \text{Equation III}$$

where SPF stands for solar protection factor; CF for correction factor; $EE_{(\lambda)}$ is the erythemogenic effect of wavelength radiation (λ) nm, which was previously calculated by Sayre et al. [35]; $I_{(\lambda)}$ is the intensity of solar radiation in the wavelength (λ) nm; and

$Abs_{(\lambda)}$ is the spectrophotometry reading of the absorbance of sunscreen solution in the wavelength (λ) nm.

In vitro SPF, critical wavelength (λc) and UVA/UVB rate were assessed by spectral transmittance (Labsphere® UV-2000S Ultraviolet Transmittance Analyzer, Sao Paulo, Brazil) at 250-450nm, the instrument bandpass is approximately 1nm and the SPF and UVA/UVB rate values were determined [36].

2.7 *In vitro* antioxidant activity analysis

For *in vitro* antioxidant activity, free radicals as 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzilthiazoline-6-sulfonic acid) (ABTS) were used. For DPPH and ABTS methods, different extract concentrations (75, 125, 200, 400, 500, 750, 1000 $\mu g.ml^{-1}$) were used, mixed with appropriate radical amount and kept in the dark during reaction time [37,38]. Both assays were performed using quercetin in different concentrations (0.25, 0.5, 1.0, 1.75, 2.5 e 5.0 $\mu g.ml^{-1}$) as standard and all assays performed in triplicate.

Antioxidant evaluation by Oxygen Radical Absorbance Capacity (ORAC) was also determined using fluorescein as fluorescent molecule and 2,2'-azo-bis (2-amidino propane) dihydrocloreto (AAPH) radical as the oxidant agent. Plates were read in a microplate fluorometer reader (Fluorolog-3 FL3-122, Horiba Jobin Yvon, EUA) and Trolox™ (Sigma-Aldrich – 93.0% of purity) was used as standard antioxidant [39,40].

2.8 Statistical analysis

All assays were performed in triplicate. Statistical analysis was performed using ANOVA test ($p<0.05$) for independent variables, Origin version 8 and Graph Pad Prism (version 5.02) software for Windows (GraphPad Software, San Diego, CA).

3. Results and Discussion

3.1 *Flavonoids extraction from different plants*

After collecting plant material, plants were dried, milled and physicochemical parameters determined. Results are shown in Table 1. All samples exhibited acceptable plant material parameters, ensuring a high degree of quality.

Table 1. Physical-chemical parameters of *D. mollis* Benth (dry favas), *G. biloba* L. (dry extract), *R. graveolens* L. (dry leaves) and *V. vinifera* L. (dry peels).

Species	**Granulo-metry (mm)**	**Density ($g.ml^{-1}$)**	**pH**	**Dry loss (%)**	**Total ash (%)**	**Total insoluble ash (%)**
D. mollis Benth	1.40±1.1	0.27±0.3	5.01±0.8	2.51±1.2	6.98±1.3	1.55±1.1
G. biloba L.	0.35±1.0	0.58±0.2	4.86±1.1	4.60±1.4	5.78±1.3	1.76±1.1
R. graveolens L.	0.60±1.2	0.91±0.2	5.90±1.1	4.44±1.1	6.76±1.2	1.54±0.9
V. vinifera L.	0.60±0.9	0.45±0.2	3.96±1.1	4.83±1.1	6.92±1.4	1.35±1.0

*Values were presented as an average of three measurements and standard deviation (±SD).

Dry extracts obtained from plants were submitted to identification assays and all extracts presented rose color to Shinoda and Pew reactions, yellow fluorescence to Taubock reaction, fluorescence under UV light and brown color when submitted to aluminum and ferric chloride, respectively, evidencing the presence of flavonoids in the extracts [28].

3.2 *Validation of analytical method by HPLC and extract quantification*

Analytical method by HPLC for flavonoids was validated in this study to ensure the reliability of results and meet analytical applications [30] demands. Therefore, parameter, such as specificity/selectivity, linearity, precision, sensitivity (detection limit and quantification limit), accuracy and robustness were assessed.

The linearity assay was determined by quercetin ($y = 35758x - 58195$; $R^2 = 0.9996$) and rutin ($y = 19862x + 26548$; $R^2 = 0.9997$) analytical curves. For the precision test, the peaks areas obtained by HPLC of quercetin and rutin standards and these flavonoids in mixed sample are displayed in Table 2 (intra-day and inter-day assay). Coefficients of variation less than 5.0% were obtained evidencing precision of our method [30].

Table 2. Intra-day and Inter-day precision values for analytical flavonoids standard and in mixed samples.

	Peaks area		Total variation coefficient (%)
	Inter-day (AU)	Intra-day (AU)	
Quercetin	468.63±0.1	469.81±0.1	0.28±0.0
Rutin	269.65±0.1	270.30±0.1	0.89±0.1
Mix (Quercetin)	600236.80±0.1	602254.30±0.0	1.78±0.0
Mix (Rutin)	4491918.70±0.0	4573531.81±0.1	1.62±0.1

*Values were presented as an average of three measurements and standard deviation (±SD).

During precision assay, the analyzer was changed and a variation coefficient less than 5% was obtained, translating the robustness of our method. A recovery assay was performed to determine the accuracy of the method [30]. Percentage of recovered

quercetin and rutin (R%) was calculated and results demonstrate the accuracy of the HPLC method, as the mean recovery rate was close to 100.0% and variation coefficient less than 5.0%, (Table 3).

Table 3. Recovery assay values referents to flavonoids concentration in extracts.

Extracts		**Standard .(Cr) (ml)**	**Final Coef quercetin (Cf)**	**Final Coef. of rutina (Cf)**	**% quer. rec. (R_q%)**	**% rutin rec. (R_r%)**	**Var. Coef. R_q.%**	**Var. Coef. Rr.%**
***D. mollis* Benth**	R1	0.75	5.44±0.2	85.39±0.1	102.05±0.1	110.22±0.1	0.30±0.2	3.64±0.2
	R2	1.25	9.91±0.1	140.88±0.1	102.58±0.1	104.73±0,1		
	R3	1.75	14.88±0.1	192.83±0.1	102.61±0.1	102.78±0.2		
***G. biloba* L.**	R1	0.75	10.72±0.2	139.22±0.2	102.82±0.1	106.53±0.1	1.79±0.1	1.13±0.2
	R2	1.25	15.43±0.2	204.00±0.1	103.10±0.2	109.10±0.1		
	R3	1.75	18.55±0.1	257.10±0.1	104.98±0.2	110.34±0.1		
***R. graveolens* L.**	R1	0.75	6.01±0.1	91.01±0.1	102.44±0.1	103.29±0.1	2.79±0.1	1.02±0.2
	R2	1.25	10.45±0.1	146.39±0.1	103.00±0.2	108.22±0.2		
	R3	1.75	13.97±0.1	196.82±0.2	104.48±0.1	108.65±0.1		
***V. vinifera* L.**	R1	0.75	6.21±0.2	81.69±0.2	101.46±0.2	106.02±0.2	2.14±0.2	0.41±0.2
	R2	1.25	10.47±0.2	139.92±0.1	102.19±0.2	102.20±0.2		
	R3	1.75	16.21±0.2	177.16±0.1	101.45±0.2	102.16±0.2		
Mix	R1	0.75	7.89±0.1	102.03±0.1	100.52±0.1	112.33±0.1	3.55±0.1	0.65±0.1
	R2	1.25	11.59±0.1	169.60±0.2	101.82±0.1	105.23±0.1		
	R3	1.75	16.99±0.1	220.31±0.1	101.43±0.1	106.25±0.2		

*Values were presented as an average of three measurements and standard deviation (±SD).

Detection and quantification limits are important parameters to validate an analytical method, by assessing the lowest amount that can be identified and quantified in a sample, demonstrating precision and accuracy. Then, DL and QL were calculated from Equations II and III, achieving the following values: DL = 0.86± 0.91 μg.ml^{-1} (quercetin) and 9.02± 1.12 μg.ml^{-1} (rutin); QL = 2.88± 0.92 μg.ml^{-1} (quercetin) and 30.09± 1.01 μg.ml^{-1} (rutin).

To determine selectivity of our method, the chromatograms of the extracts were compared to standard chromatograms [40]. The individual extracts and mixed sample did not present any impurities or other compounds capable of interfering in the identification of quercetin and rutin peaks.

Using the validated method, quercetin and rutin content in each extract was performed. Flavonoids retention times of samples were similar to standards at 3.6 minutes for rutin and 5.1 minutes for quercetin. Flavonoids presence in the mixed sample is showed in Figure 1.

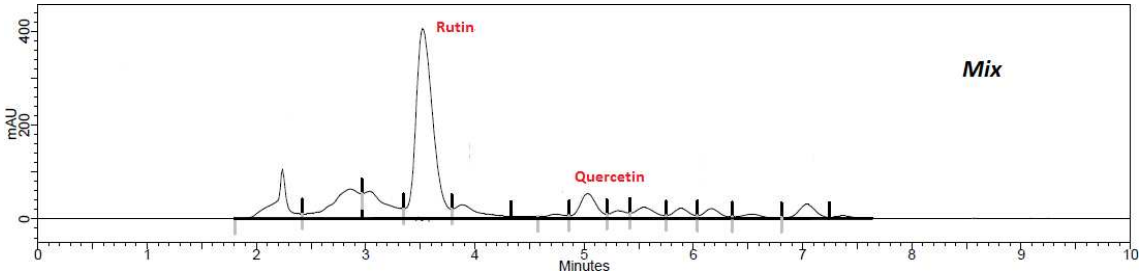

Figure 1. Chromatogram of Mix sample (1:1:1:1) by HPLC assay.

The results showed that *G. biloba* L. extract presented the higher quercetin (47.21 ± 1.1 μg.ml^{-1}) and rutin (406.84 ± 0.8 μg.ml^{-1}) concentrations than *D. mollis* Benth (quercetin = < 2.88 ± 0.9 μg.ml^{-1}; rutin = 108.17 ± 1.2 μg.ml^{-1}), *R. graveolens* L. (quercetin = 11.27 ± 0.7 μg.ml^{-1}; rutin = 94.68 ± 1.1 μg.ml^{-1}) and *V. vinifera* L. (quercetin = < 2.88 ± 0.9 μg.ml^{-1} μg.ml^{-1}; rutin = < 30.09 ± 1.0 μg.ml^{-1}), as expected, according to

literature [42,43,44]. Besides, the mixed sample showed a high concentration of rutin (314.95 ± 0.9 μg.ml^{-1}) followed by a lower concentration of quercetin (7.42 ± 0.9 μg.ml^{-1}).

After flavonoids quantification, individuals extracts and the mixed sample were subjected to cell viability assay and determination of the sun protection factor value and antioxidant activity.

3.3 *In vitro cell viability evaluation by neutral red (NRU)*

According to Stoke et al. [32] neutral red (NR) is a weak cationic dye that readily diffuses through the plasma membrane and concentrates in lysosomes, where the dye electrostatically binds to the anionic lysosomal matrix. The evaluation of neutral red uptake is directly proportional to living cell number [33,45].

In this study, in all tested concentration, any extract or the mix sample have reduced HaCat cell viability to less than 50% (Figure 2). Thus, the concentration required to reduce in 50% the cell viability IC_{50}) was higher than 200 μg.ml^{-1}. We did not tested higher concentrations of the selected extracts to avoid the production of artifacts in culture medium, as already reported for many phenolic compounds [46].

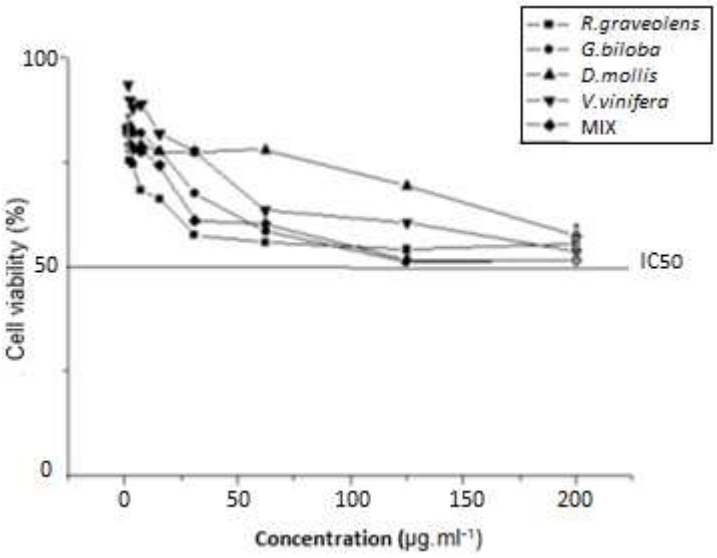

Figure 2. Cell viability curves of samples (*Dimorphandra mollis* Benth, *Ginkgo biloba* L., *Ruta graveolens* L., *Vitis vinifera* L. and Mix (1:1:1:1)) in different concentrations (250.0, 125.0, 62.5, 31.25, 15.62 µg.ml^{-1}) after 48h of HaCat cell line exposition by NRU method.

3.4 *In vitro sun protection factor (SPF) evaluation*

Spectrophotometry in ultraviolet region is an adjuvant and preliminary *in vitro* method to evaluate sun protection factor of compounds [34]. Due to its agility, *in vitro* method has been studied in scientific papers to determined SPF values, especially from vegetal sources [47]. Thus, the four extracts (100 µg.ml^{-1}) and mixed sample were subjected to spectrophotometric analysis and *D.mollis* Benth presented SPF values of 5.04 ± 0.2, *G. biloba* L. of 8.31 ± 0.5, R. *graveolens* L. of 7.08 ± 0.4, *V. vinifera* L. of 3.71 ± 0.5 and mixed sample (1:1:1:1) of 7.72 ± 0.4.

As determined by cell viability assay, both SPF assays were performed at concentrations up to 200 µg.ml^{-1}, which was shown to be the highest non-cytostatic concentration in the cell viability assay.

These preliminary results show that *G. biloba* and *R. graveolens* extracts are the most promising individual extracts besides the mixed sample. To confirm the FPS results, all samples were then evaluated using spectral transmittance [48].

All individual extracts, mixed sample and positive control (Tinosorb S^{TM}) depicted absorption in UVA and UVB regions (Table 4) although in different ways. While *D. mollis* fava beans and *G. biloba* extracts absorbed between 320 and 400 nm range corresponding to UVA radiation, *R. graveolens* leaves and *V. vinifera* fruit peels extracts absorbed around 310 nm corresponding to UVB radiation. The mixed sample and Tinosorb S^{TM} absorbed in a higher range configuring protection in UVA and UVB regions [8].

Table 4. *In vitro* sun protection factor values of extracts by spectral transmittance.

Extracts (200µg.ml^{-1})	**Critical wavelength (nm)**	**UVA***	**UVB***	**SPF**	**UVA/UVB Rate**
D. mollis Benth	398.0 ± 0.1	x		4.96 ± 0.2	0.9 ± 0.0
G. biloba L.	388.1 ± 0.0	x		7.06 ± 0.2	0.9 ± 0.0
R. graveolens L.	309.0 ± 0.2		x	5.34 ± 0.1	0.9 ± 0.0
V. vinifera L.	318.0 ± 0.1		x	3.17 ± 0.2	0.9 ± 0.0
Mix sample	372.7 ± 0.1	x	x	6.92 ± 0.1	0.8 ± 0.0
Tinosorb S^{TM}	369.1 ± 0.1	x	x	21.01 ± 0.2	0.7 ± 0.0

*UVA range equal to 320 to 400nm and UVB equal to 280 to 315nm.

G. biloba L. extract followed by mixed sample presented the higher SPF values (Table 4), attributed to the presence of higher flavonoids concentration. Moreover, *D. mollis* Benth and *V. vinifera* L. extracts, which showed the lowest quercetin concentration, presented low SPF values, corroborating literature [49].

According to literature [22, 50, 51, 52, 53], the SPF values found in our extracts showed lower SPF values than chemical sun filters, such as Tinosorb S^{TM}. Besides, the colorimetric evaluation demonstrated that all individual extracts have flavonoids. Our extracts may therefore be considered a promising source of natural sunscreens.

3.5 *In vitro antioxidant activity of extracts*

Antioxidant activity of phenolic compounds, such as flavonoids, has been widely reported, attributed to their capacity for scavenging free radicals as superoxide anion, hydroxide and peroxide radicals [54,55]. Flavonoids are widely studied as ingredients in cosmetic formulations, used against early skin aging by scavenging reactive oxygen species produced by sun radiation [6,12,26]. Our four extracts were then studied for their antioxidant activity.

In the DPPH and ABTS experiments, the results are expressed as the sample concentration required for 50% of reduction of the radical concentration (Table 7), while in the ORAC protocol, the ability of scavenging peroxyl radicals is expressed as the equivalent concentration of Trolox [39,56]. Once again, the best results were seen for *G. biloba* L. extract and for mixed samples (Table 5) and this can also be attributed to the higher flavonoids concentration on these samples.

Table 5. *In vitro* antioxidant evaluation against DPPH, ABTS and AAPH free radicals of some potential sunscreen natural products.

Extracts	**IC_{50} – DPPH ($\mu g.ml^{-1}$)**	**IC_{50} - ABTS ($\mu g.ml^{-1}$)**	**IC_{50} - AAPH ($\mu g.ml^{-1}$)**
D. mollis Benth	174.51 ± 1.10	596.73 ± 1.66	15.43 ± 1.21
G. biloba L.	8.12 ± 0.81	109.09 ± 1.06	25.55 ± 0.44
R. graveolens L.	281.02 ± 1.09	587.98 ± 0.81	17.87 ± 0.38
V. vinífera L.	296.90 ± 1.21	643.13 ± 0.92	16.08 ± 1.30
Mix	28.73 ± 0.70	325.08 ± 0.83	23.79 ± 0.11
Quercetin	1.75 ± 0.40	2.00 ± 1.20	0.97 ± 0.93

Extracts and mixed sample exhibited antioxidant activity against DPPH, ABTS and AAPH radicals, which is an indicative of premature aging protection. Then, considering the samples' potential as sunscreens, all individual extracts and mixed samples can be incorporated into a cosmetic formulation, aiming the development of a new sunscreen containing chemical sun filter from plant material.

4. Conclusions

All extracts presented flavonoids, were not cytotastic, and exhibited antioxidant activity and sun protecting effects by *in vitro* assays. The mixed sample composed of four plants showed the most promising results. The quantification method exhibited linearity, precision, accuracy, robustness and no impurities or other compounds capable of interfering in the identification peak of flavonoids were found. This work is the first

study until now reporting the sun protection effect of *Dimorphandra mollis* Benth and *Ruta graveolens* L., while we demonstrate the added value of using plant extracts from these species, together with *Ginkgo biloba* L., and *Vitis vinífera* L. as a sunscreen in pharmaceutical/cosmetic formulations.

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6. References

[1] Holick MF, Chen TC, Lu Z, Sauter E. Vitamin D and skin physiology: a D-lightful story. J Bone Min Res. 2007;22:28–3.

[2] Lindqvist PG, Epstein E, Nielsen K, Landin-Olsson M, Ingvar C, Olsson HJ. Avoidance of sun exposure as a risk factor for major causes of death: a competing risk analysis of the Melanoma in Southern Sweden cohort. J Int Med. 2016;280:375-87.

[3] Peres DD, De Oliveira CA, Da Costa MS, Tokunaga VK, Mota JP, Rosado C, Consiglieri VO, Kaneko TM, Velasco MV, Baby AR. Rutin increases critical wavelength of systems containing a single UV filter and with good skin compatibility. Skin Res Technol. 2016;22:325-332.

[4] Rojas J, Londono C, Ciro Y. The health benefits of natural skin UVA photopretective compounds found in botanical sources. Int J Pharm Pharm Sci. 2016;8:13-3.

[5] Lowe NJ, Shaath NA, Pathak MA. Sunscreens, development, evaluation and regulatory aspects. Marcel Dekker: New York; 1997.

[6] Giacomoni PU. Understanding reactive oxygen species. Cosm Toil. 2008;122:1-5.

[7] Baron ED, Kirkland EB, Domingo DS. Advances in photo protection. Derm Nur. 2008;20:265-72.

[8] Palm MD, O'Donoghue MN. Update on photoprotection. Derm Ther. 2007;20:360-76.

[9] Lautenschlager S, Wulf H, Pittelkow MR. Photoprotection. The Lancet. 2007;370:528-37.

[10] WHO. Fact sheet no. 261: protecting children from ultraviolet radiation. Geneva (Switzerland): World Health Organization. 2001.

[11] INCA. Estimativa 2016: Incidência de Câncer no Brasil. Rio de Janeiro: Instituo Nacional do Câncer 2016.

[12] Cefali LC, Ataide JA, Foglio MA, Moriel P, Mazzola PG. Plant-based active photoprotectans for sunscreens. Inter J Cosm Sci. 2016;1:1-8.

[13] Romanhole RC, Ataide JA, Cefali LC, Moriel P, Mazzola P. Photostability study of commercial sunscreens submitted to artificial UV irradiation and/or fluorescent radiation. J Photochem Photobiol B: Biol. 2016;162:45-9.

[14] Sambandan DR, Ratner D. 2011. Sunscreens: An overview and update. J Am Acad Dermatol. 2011;64:748-58.

[15] Wang SQ, Balagula Y, Osterwalder U. Photoprotection: a review of the current and future technologies. Derm Ther. 2010;23:31-7.

[16] Skotarczak K, Osmola-Mańkowska A, Lodyga M, Polańska A, Mazur M, Adamski Z. Photoprotection: facts and controversies. Eur Rev Med Pharmacol Sci. 2015;19:98-12.

[17] Gilbert E, Pirot F, Bertholle V, Roussel L, Falson F, Padois K. Commonly used UV filter toxicity on biological functions: review of last decade studies. Inter J Cosm Sci. 2013;35:208-19.

[18] Wong T, Orton D. Sunscreen allergy and its investigation. Clin Dermatol. 2011;29:306-10.

[19] Amar SK, Goyal S, Dubey D, Srivastav AK, Chopra D, Singh J, Shankar J, Chaturvedi RK, Ray RS. Benzophenone 1 induced photogenotoxicity and apoptosis via release of cytochrome c and smac/diablo at environmental UV radiation. Toxicol Let. 2015;239:182-93.

[20] Ramos S, Homem V, Alves A, Santos L. Advances in analytical methods and occurrence of organic UV-filters in the environment — A review. Sci Total Environ. 2015;526:278-11.

[21] Ramos S, Homem V, Alves A, Santos L. A review of organic UV-filters in wastewater treatment plants. Environ Int. 2016;86:24-4.

[22] Korac RR, Khambholja KM. Potential of herbs in skin protection from ultraviolet radiation. Pharmacog Rev. 2011;5:164-73.

[23] Tohge T, Souza LP, Fernie AR. Current understanding of the pathways of flavonoid biosynthesis in model and crop plants. J Exp Bot. 2017;68:4013–28.

[24] Abbas S, Saleem H, Gill MSA, Bajwa AM, Sarwar A, Omer MO. Physicochemical, Phytochemical and Nutritional values determination of Suaeda fruticosa (Chenopodiaceae). Acad J Med Plant. 2016;4:1-9.

[25] Santos IS, Ponte BM, Boonme P, Silva AM, Souto EB. Nanoencapsulation of polyphenols for protective effect against colon-rectal cancer. Biotechnol Adv. 2013;31:514-23.

[26] Hubinger SZ, Cefali LC, Vellosa JCR, Salgado HRN, Isaac VLB, Moreira RRD. *Dimorphandra mollis*: an alternative as a source of flavonoids with antioxidant action. Latin Am J Pharm. 2010;29: 271-74.

[27] Mello JCP, Petrovick PR. Quality control of Baccharis trimera (Less.) DC.(Asteraceae) hydroalcoholic extracts. Acta Farm Bonaer. 2000;19:211-15.

[28] Jouahri D, Boudarene L, Sabaou N. Flavonoids. Inter J Pharmacog Phytochem Res. 2013;5:45-50.

[29] Seal TJ. Quantitative HPLC analysis of phenolic acids, flavonoids and ascorbic acid in four different solvent extracts of two wild edible leaves, Sonchus arvensis and Oenanthe linearis of North-Eastern region in India. Appl Pharm Sci. 2016;6:10-9.

[30] Anvisa. Resolução RDC nº26, de 13 de maio de 2014. Dispõe sobre o registro de medicamentos fitoterápicos e o registro e a notificação de produtos tradicionais fitoterápicos. Diário Oficial da União. 2014.

[31] Anvisa. Consolidado de normas da COFID. Versão IV. Brasília: Agência Nacional de Vigilância Sanitária. 2014.

[32] Stoke WS, Casati S, Strickland J, Paris M. Neutral red uptake cytotoxicity tests for estimating starting doses for acute oral toxicity tests. Cur Protocol Toxicol. 2008;20:1-20.

[33] OECD. Organization for Economic Co-operation and Development. Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests. n.129. 2010.

[34] Mansur MCPPR, Leitão SG, Cerqueira-Coutinho C, Vermelho AB, Presgrave OAF, Leitão AAC, Leitão GG, Ricci-Junior E, Santos EP. *In vitro* and *in vivo* evaluation of efficacy and safety of photoprotective formulations containing antioxidant extracts. Rev Bras Farmacog. 2016;26:251-58.

[35] Sayre RM, Agin PP, Levee GJ, Marlowe E. Comparison of *in vivo* and *in vitro* testing of sunscreening formulas. Photochem Photobiol. 1979;29:559-66.

[36] Rai R, Srinivas CR. Photoprotection. Ind J Dermatol. 2007;73:73-9.

[37] Rufino MSM, Alves RE, Brito ES, Jimenez JP, Calixto FS. Bioactive compounds and antioxidant capacities of 18 non-traditional tropical fruits from Brazil. Food Chem. 2010;121:996-02.

[38] Floegel A, Kim DO, Chung SJ, Koo SI, Chun OK. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. J Food Comp Anal. 2011;24: 1043-48.

[39] Ou B, Hampsch-Woodill M, Prior RL. Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe. J Agr Food Chem. 2001;49:4619-26.

[40] Huang D, Ou B, Prior RL. The Chemistry behind Antioxidant Capacity Assays. J Agr Food Chem. 2005;53:1841-56.

[41] Casedey ECL, Salgado HRN. Development and validation of UV spectrophotometric method for orbifloxacin assay and dissolution studies. Braz J Pharm Sci. 2014;50:457-66.

[42] Dal Belo SE, Gaspar LR, Campos PMBGM. Photoprotective effects of topical formulations containing a combination of *Ginkgo bilob*a and Green Tea extracts. Phytother Res. 2011;25:1854-60.

[43] Maclennan KM, Darlington CL, Smith PF. The CNS effects of *Ginkgo biloba* extracts and ginkgolide *B.* Progr Neurobiol. 2002;67:235-57.

[44] Pastrana-Bonilla E, Akoh CC, Sellappan S, Krewer G. Phenolic content and antioxidant capacity of Muscadine grapes. J Agr Food Chem. 2003;51:5497-03.

[45] Chamberlain M, Parish WE. Hazard and risk based on *in vitro* test data. Toxicolol in Vitro. 1990;4:694-97.

[46] Halliwell B. The antioxidant paradox. Lancet. 2000;355:1179–80.

[47] Wagemaker TAL, Carvalho CRL, Maia NB, Baggio SR, Filho OG. Sun protection factor, content and composition of lipid of green coffee beans. Ind Crops Prod. 2011;33:469-73.

[48] Springsteen A, Yurek R, Frazier M, Carr KF. *In vitro* measurement of sun protection factor of sunscreens by diffuse transmittance. Anal Chim Acta. 1999;380:155-64.

[49] Choquenet B, Couteau C, Paparis E, Coiffard LJM. Quercetin and rutin as potential sunscreen agents: determination of efficacy by an *in vitro* method. J Nat Prod. 2008;71:1117–18.

[50] Chiari BG, Trovatti E, Pecoraro T, Corrêa MA, Cicarelli RMB, Ribeiro SJL, Isaac VLB. Synergistic effect of green coffee oil and synthetic sunscreen for health care application. Ind. Crops Prod. 2014;52:389-93.

[51] Ebrahimzadeh A, Gharaei A, Saryazdi K. Allelic diversity of polymorphic AMA-1 vaccine candidate antigen of *Plasmodium falciparum* in two population of imported and indigenous cases in South-East of Iran using vested-PCR and RFLP. J Trop Dis Public Health. 2014;2:149-55.

[52] Figueiredo SA, Vilela FMP, Silva CMP, Cunha TM, Dos Santos MH, Fonseca MJV. *In vitro* and *in vivo* photoprotective/photochemopreventive potential of *Garcinia brasiliensis* epicarp extract. J Photochem Photobiol B: Biol. 2014;131:65-3.

[53] Zanatta CF, Mitjans M, Urgatondo V, Rocha-Filho PA, Vinardell MP. Photo protective potential of emulsions formulated with Buriti oil (*Mauritia flexuosa*) against UV irradiation on keratinocytes and fibroblasts cell lines. Food Chem Toxicol. 2010;48:70-5.

[54] Floegel A, Kim DO, Chung SJ, Koo SI, Chun OK. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. J Food Comp Anal. 2011;24:1043-48.

[55] Wen L, Zhao Y, Jiang Y, Yu L, Zeng X, Yang J, Tian M, Liu H, Yang B. Identification of a flavonoid C-glycoside as potent antioxidant. Radic Biol Med. 2017;110:92-01.

[56] Cao G, Alessio HM, Cutler RG. Oxygen-radical absorbance capacity assay for antioxidants. Free Radic Biol Med. 1993;14:303-11.

CAPITULO III

"Topical formulation with blend of enriched flavonoids plant extracts: photostability, rheology and cutaneous permeation assays".

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Abstract

The aim of this study was to develop a phytocosmetic sunscreen formulation containing a blend of enriched flavonoids plant extracts. Sun protection factor, *in vitro* antioxidant activity, formulation stability, photostability, cutaneous permeation and retention of flavonoids were evaluated. Formulations reported herein were stable with one of them chosen as formulation vehicle after sensorial analysis incorporated with extracts's blend. The final formulation was stable when stored at low temperatures (5ºC), presenting concentration above the quantification limit (2.8µg/ml) of quercetin and (30.39±0.39 µg/ml) rutin after 120 days of storage. Furthermore spreadability, low rupture strength and adhesiveness formulation parameters were in accordance for a topical product. Higher brittleness, pseudo-plastic and viscoelastic behaviors were also determined. Formulation presented 17.96 ± 0.03% of inhibition percentage against DPPH solution, 2.4 ± 0.5 value referent to UVA protection, critical wavelength equal 387.0 nm and UVA/UVB rate equal 0.78, determining that the formulation can be used as sun filter with UVA/UVB protection. Photostability was evaluated and SPF value decreased 12.50%, being considered a photostable product. Rutin permeated skin barrier and was

also found in stratum corneum (3.27 ± 1.92 μg/ml) by tape stripping and retention test (114.68 ± 8.70 μg/ml). Results reported herein provide with antioxidant activity, sun protection and promising physical characteristics for a potential sunscreen formulation.

Keywords: antioxidant, flavonoids, permeation, photostability, rheology, sunscreen.

1. INTRODUCTION

Ultraviolet (UV) radiation from sun causes damage to skin, including alterations in collagen and elastic fibers, and photocarcinogenic changes, promoting directly DNA damage, which is associated to acceleration of skin aging and skin cancer (Baron et al., 2008; Palm and O'donoghue, 2007). Therefore, these risks can be prevented by solar radiation protection, using e.g. sunscreens (Arts and Hollman, 2005; FDA, 2013; Romanhole et al., 2015).

Sunscreens can be composed of physical and chemical filters. Chemical filters are known to cause allergic reactions, contact sensitivity, photogenotoxicity among other disorders (Amar et al., 2015; Gilbert et al., 2013; Ramos et al., 2016; Sambandan and Ratner, 2011; Wong and Orton, 2011). Potential endocrine disruptors of typical UV filters including benzophenones, camphor derivatives and cinnamate derivatives (Ruszkiewicz et al., 2017).

Therefore, research of natural ingredients aiming the reduction of skin irritation and other harmful effects associated with sunscreens is steadily increasing (Cefali et al., 2016). Plant materials with ability to absorb or block UV radiation have been extensively studied in the development of sunscreen products against harmful solar radiation (Cefali et al., 2016). Among examples of these ingredients are flavonoids, phenolic compounds,

which can absorb UV rays, especially UVA and UVB, in wavelengths between 200 nm and 400 nm (De Cooman et al., 1998).

Formulation development requires stability on the chemical integrity with maintenance of their physicochemical properties since formulation stability is associated to quality control, consumer acceptance and product efficacy, wherein this is an essential test in new cosmetic formulations development (Correa et al., 2005). Rheological behavior, texture and thermal analysis are also important parameters to be considered during product development. These parameters assist in the assessment of formulation physicochemical nature allowing early signs of instability detection (Barry, 1993; Ribeiro et al., 2004).

Cutaneous permeation and retention of sunscreen components are relevant once sun filters besides being topical products should also remain on skin surface to protect against sun radiation, ensuring products efficacy (Montenegro and Puglisi, 2013). Sun filters cannot undergo modification when exposed to sunlight, therefore the photostability assay becomes necessary. According to Huong et al. (2008) and Romanhole et al. (2016), the decrease in sunscreens' absorptive capacity, due to instability, results in an increasing amount of radiation directly incident on the skin with an accompanying increase in the deleterious effects of these rays on the human body.

Therefore, the aim of this study was to develop a topical phytocosmetic formulation, to be used as sunscreen, containing a blend of plant extracts rich in flavonoids to evaluate sun protection factor, *in vitro* antioxidant activity, stability, photostability and cutaneous permeation/retention of flavonoids.

2. MATERIAL AND METHODS

2.1 Material

Freeze-dried extracts rich in flavonoids from four different plant materials (*Ginkgo biloba* L., *Dimorphandra mollis* Beth, *Ruta graveolens* and *Vitis vinifera* L.) and a blend of extracts containing equal parts of each extracts (1:1:1:1) with previously determined SPF values of 5.04 ± 0.2 to *D. molli* Benth, 8.31 ± 0.5 to *G. biloba* L., 7.08 ± 0.4 to R. *graveolens* L., of 3.71 ± 0.5 to *V. vinifera* L. and 7.72 ± 0.4 to mixed sample (1:1:1:1) by *in vitro* assay.

Emulsions were formulated using raw materials, such as tribehenin, sorbitan stearate/sucrose cocoate, sucrose palmitate glyceryl stearate/glyceryl stearate citrate/ sucrose/manna/xanthan gum, caprylic/capric triglyceride, hydrolyzed wheat protein/PVP cross-polymer, *Persea Gratissima* (avocado) oil, glycerin and talc provided by Croda (Campinas, São Paulo, Brazil) and PharmaSpecial (São Paulo, São Paulo, Brazil). Isopropanol and ethyl alcohol were provided by Synth (São Paulo, Brazil), 1,1-diphenyl-2-picrylhydrazyl (DPPH), methanol (HPLC grade) and formic acid by Sigma-Aldrich (São Paulo, São Paulo, Brazil), and quercerin (93.3% of purity) and rutin (97.3% of purity) analytical standards by Acros (Itu, Brazil).

2.2 Development and stability study of base formulations

Nine (F1 to F9) oil-in-water (O/W) emulsions were development (Table 1) using protocol technique, which consists in heat aqueous and oily phases up to 70 ± 3°C and homogenize until 25 ± 3°C.

Table 1. Components of oil-in-water base emulsions.

Components (INCI NAME)	Concentration (%)								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
Tribehenin	5.0	6.0	5.0	6.0	-	-	8.0	8.0	-
Sorbitan stearate/sucrose cocoate	8.0	8.0	8.0	8.0	8.0	8.0	-	-	8.0
Sucrose palmitate glyceryl stearate/ glyceryl stearate citrate/ sucrose/ manna/xanthan gum	5.0	5.0	3.0	3.0	5.0	2.0	5.0	2.0	0.5
Caprylic/capric triglyceride	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Hydrolyzed wheat protein/PVP cross-polymer	3.0	3.0	5.0	5.0	3.0	2.0	3.0	2.0	2.0
Persea Gratissima (Avocado) oil	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Phenoxyethanol	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Glycerin	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Talc	1.0	1.0	1.0	1.0	1.0	1.0	1.5	1.5	1.5
Aqua qs	100	100	100	100	100	100	100	100	100

Formulations were subjected to stability study according to EMEA (2003), COLIPA (2004) and ISO (2013). Therefore, approximately 5.0g of emulsions were subjected to three centrifuge cycles (3000 rpm) for 30 minutes/cycle at 27 ± 2°C to evaluate physical stability. Organoleptic characteristics (color, odor and appearance), pH (Quimis pHmeter, São Paulo, Brazil), density and viscosity values were also studied.

Density was calculated by the difference between pycnometers weight (with 5mL of formulation and empty), dividing by the sample's volume (Idson, 1993).

Apparent viscosity determination was evaluated using a rotational viscometer (Brookfield, Mod LV-T, São Paulo, Brazil) with spindle number 4 and 1.5 rpm rotation for 30 seconds at 27 ± 2°C (Corrêa et al., 2005).

2.3 Sensorial Analysis

Formulations with better results during stability tests were subjected to sensorial analysis (number: 59552216.3.0000.5404 – approved by the Ethics Committee of University of Campinas), by sensory difference and preference tests (Macfie et al., 1988).

Fifty volunteers (37 women and 13 men) among 20 and 50 years of age, after signing the Consent Term, selected emulsion randomly designated 272 (F5), 348 (F6), 476 (F8) and 612 (F9). Volunteers were given a 0.1g cream sample, and each cream was assessed in terms of 6 aspects (speed absorption, residual fatty sensorial, speed drying, stickiness, spreading and dry touch) rated on a scale (like and dislike) (Almeida et al., 2008; Dutcosky, 2007; Isaac et al. 2012a).

2.4 Development and stability study of phytocosmetic

A phytocosmetic was prepared using the blend of plant extract (blend of extracts containing equal parts of each extract (1:1:1:1) - *Ginkgo biloba* L., *Dimorphandra mollis* Beth, *Ruta graveolens* and *Vitis vinifera* L.) and a was added to the formulation (200 µg/ml) chosen as preferred in sensorial analysis. Phytocosmetic was subjected to stability study (EMEA, 2003; COLIPA, 2004; ISO, 2013).

Stability study was also conducted using the multisample analytical centrifuge LUMiSizer (LUM, GmbH, Germany). Vehicle and phytocosmetic were diluted in

distilled water (1:5; w/w) and evaluated for 2 hours at 3000 rpm and 27.5 ±0.5°C (Badolato et al., 2008).

Flavonoids quercetin and rutin concentrations were evaluated by High Liquid Pressure Chromatography (HPLC) with diode array UV/vis detector. Emulsions submitted to stability study were dissolved in isopropanol (1:10, w/v) and filtered in 0.45µm membrane (Merck, Darmstadt, Germany). Quercetin (50 µg/mL) and rutin (500 µg/mL) analytical standard were analyzed by HPLC in comparison to results.

Aliquots of 5µL of each sample were injected in HPLC-DAD instrument (Agilent, Technologies 1250 infinity, Santa Clara, United States), using monomeric chromatographic column C_{18} (Phenomenex), flow rate of 0.3mL/min for 10 minutes. Mobile phase was methanol grade HPLC acidified with 0.1% (v/v) formic acid (Synth, São Paulo, Brazil), at 27±1°C, with flavonoids identification at 257nm (Seal, 2016).

2.5 Zeta potential of formulations

Vehicle and phytocosmetic were diluted in distilled water (1:500; w/w) and subjected to Zetasizer® equipment (Malvern Instruments Ltd., Malvern, Worcestershire, United Kingdom). Approximately 1 mL of diluted samples was placed in a disposable cuvette and the zeta potential was determined. The intensity of scattered light was transformed into the diffusion factor; the mean value of 10 measurements was obtained for each formulation and was repeated three times, at 25.0 ± 0.1°C (Roland et al., 2003; Hejjaji et al., 2017).

2.6 Drop size distribution

Vehicle and phytocosmetic' drop size distribution was carried out by laser diffraction using particles analyzer (MasterSizer®, Malvern, United Kingdom) and large

volume sample dispersion units (Malvern Hydro 2000 MU, Malvern, Germany). Study was carried out at 750rpm using obscuration range of 10-20% and water as dispersant liquid. Assays were performed in triplicate.

2.7 Mechanical analysis: texture, spreadability and rheological behavior

The textural analysis were performed in a texturometer (Stable Micro Systems TA-XT2i, United Kingdom), using compression mode. Penetration test was carried out using a load cell of 5kg, a cylindrical probe (SMS P/1R), penetration depth of 5mm, test speed of 3 mm/s and a trigger force of 0.050 N. Rupture strength (g), brittleness (mm) and adhesiveness (gsec) were calculated. All measurements were performed in triplicate.

Extrusion evaluation was also performed. In this test, the extrusion cell (HDP/FE) measured the compression force required for a piston disc to extrude a product through a standard size outlet in the base of the sample container. Samples were deposited on cup and compressed by cylinder probe (5mm). Firmness (g) was determined and all measurements were performed in triplicate (Savary et al., 2013; Estanqueiro et al., 2014).

Spreadability assay of vehicle and phytocosmetic were performed in triplicate according to methodology described by Borguetti and Knorst (2006). One gram of each sample was introduced to glass plate and other glass plate of known weight (420 ± 1g) was placed over the sample. After 1 minute, diameter was read with the aid of millimetric graph paper scale. This procedure was repeated successively adding other plates of the same weight in one-minute intervals until spreading stopped. Results were expressed as spreadability of samples due to the applied weight.

Rheological behavior was assessed using a Modular Compact Rheometer (ANTON PAAR, MCR 102, São Paulo, Brazil), sensor cone/plate (C35/2º Ti) and the data were analyzed with RheoCompass^{TM} Software. Rheological behavior was

determined using flow curve assays with sheering rates ranging from 0 to 100 Pa/s for the upward slope for 120 seconds and from 100 to 0 Pa/s for the downward slope for 120 seconds. After, the sheering tension at which the sample exhibited limited flow (underwent deformation) was determined through the flow limit tension ramp test with a tension range of 0 to 50 Pa for 120 seconds (Isaac et al., 2013).

Tension and frequency scanning assays were performed to analyze samples' dynamic viscosity (η) and stocking module (G). For the tension scanning test, a sheering tension range of 0 to 100 Pa/s and frequency of 10 rad/s were used. The frequency scanning test was performed using a frequency range of 0.1 to 600 rad/s at 0.1 Pa/s (Cefali et al., 2015; Dávila and D'ávila, 2017).

The creep and relaxation assay was performed to determine the samples' viscoelasticity using a sheering tension of 7 Pa for 45 seconds for fluency, 70 Pa for 60 seconds for resting, 7 Pa for 100 seconds for relaxation (Cefali et al., 2015; Dávila and D'ávila, 2017). All assays were performed in triplicate, at 25 ± 0.5 ºC using approximately 1 g of sample for each test.

2.8 Thermal analysis

The phytocosmetic was subjected to differential scanning calorimetry (DSC) and thermogravimetry (TG) analysis using the thermal analysis equipment TA-50WSI (SHIMATZU, 50WSI, Japan) carried out in triplicate.

Approximately 10mg of sample was deposited in aluminum straw for DSC analysis, at a heating rate of 15ºC/min in N_2 atmosphere, using 25 to 350 ± 1ºC temperature. For TG analysis, approximately 10mg of sample was deposited in alumina straw, 10ºC/min heating rate in N_2 atmosphere with a temperature range of 25 to 500 ± 1ºC (Ribeiro et al., 2004) were used.

2.9 Microbiologic control

One gram of sample was diluted in 9mL of phosphate buffer solution at pH 7.2. One mL of this solution was pipetted, diluted and added to 20 mL of thioglycolate agar for bacteria and Sabouraud for yeasts in Petri dishes, which were subsequently incubated at 35°C for 24 hours and at 25°C for seven days for the examination of bacteria and fungi growth, respectively.

The presence of *Salmonella* sp, *Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa* were determined. The diluted samples were added into xylose-lysine-desoxycholate agar, bismuth sulfite agar, MacConkey agar, Vogel and Johnson agar and dishes containing cetrimide agar. The dishes were incubated at 35°C for 24 hours and the characteristics of colonies were assessed (ISO 17516, 2014; USP 36, 2013). The assays were performed in triplicate.

2.10 *In vitro* sun protection factor (SPF) evaluation of the phytocosmetic

In vitro solar protection factor (290 to 320nm) of phytocosmetic was determined by ultraviolet-visible spectrophotometry using Equation I (Mansur et al., 2016).

$$\boldsymbol{SPF = CF \times \Sigma_{290}^{320} \times EE_{(\lambda)} \times I_{(\lambda)} \times Abs_{(\lambda)}} \qquad \text{Equation I}$$

Where, SPF stands for solar protection factor; CF for correction factor where 10 was used; $EE_{(\lambda)}$ is the erythemogenic effect of wavelength radiation (λ) nm, which was previously calculated by Sayre et al. (1979); $I_{(\lambda)}$ is the intensity of solar radiation in the wavelength (λ) nm; and $Abs_{(\lambda)}$ is the spectrophotometry reading of the absorbance of sunscreen solution in the wavelength (λ) nm.

In vitro UVAPF, critical wavelength (λc) and UVA/UVB rate were also assessed according to ISO24443:2012 protocol (ISO, 2012) by spectral transmittance (Labsphere® UV-2000S Ultraviolet Labsphere Halma Company, Sao Paulo, Brazil) at 290-400nm.

2.11 *In vitro* antioxidant activity analysis of the phytocosmetic

For *in vitro* antioxidant activity, DPPH radical was used. One gram of formulation was diluted in 10mL of isopropanol. A volume of 2.5mL of this solution was placed in test tubes and then 2.5ml of 0.004% DPPH solution (w/v) in ethanol was added. The reaction was carried out for 30 minutes stored in the absence of light. Analysis was also performed for quercetin as an antioxidant standard at concentrations 0.25, 0.5, 1.0, 1.75 and 2.5µg/mL. Ethanol was used instead of sample as blank for maximal absorbance determination at 513 nm. In the presence of DPPH radical scavengers, absorbance intensity decreased, and the percentage of inhibition (% Inhibition) was calculated according to Rufino et al. (2010).

2.12 Skin permeation

To determine flavonoids release from formulation, synthetic cellulose membranes (Millipore, 0.45 µm) were placed on diffusion cells in contact with receiving medium (phosphate buffer, pH 7.2). Approximately two-hundred milligrams of formulation containing extract was added on membranes and the receiving solution was stirred at 200 rpm at 37ºC. For the skin permeation assay, pig skin including hair were cleaned, stored at -5°C and used after 24 hours. Skins were placed on diffusion cells with dermis in contact with the receiving medium. The receiving medium was collected after 1, 2, 4 and

6 hours for release assay and after 2, 4, 8, 12 and 24 hours for permeation assay and rutin was determined by HPLC/DAD (Seal, 2016).

At the end of experiment, skins were removed from the diffusion cells, the excess of formulation was removed using distilled water and skins were dried with absorbent paper. Skins were place on surface and the stratum corneum was removed using tape-stripping method, which consists of 20 stripping using adhesive tape (Dsquame D100, 22mm - Monaderm, Monaco).

Tapes were transferred to test tubes containing 4mL of methanol and tubes were subjected to three cycles of shaking on agitator for one minute and sonication for 30 minutes in ultrasound bath. The supernatant was analyzed by HPLC/DAD. Skin samples without stratum corneum were cut and transferred to tubes containing 4mL of methanol and tubes were subjected to the same shaking and sonication process than tapes, and the supernatant was analyzed by HPLC/DAD in triplicate.

All assays were carried out in sextuplicate using vehicle as the blank control (Alencastre et al., 2006).

2.13 Photostability study

The assay was performed using a solar simulator (CPS+, 1012014, Atlas Material Testing Solutions), a sensor for calibration of irradiance (SunCal BB 300-400 BST, 1603001, Atlas Material Testing Solutions) and a UV transmittance analyzer (Labsphere® UV-2000S).

PMMA substrate (HELIOPLATE HD 6 - 50 mm x 50 mm, 6 microns) containing the samples (vehicle and phytocosmetic) were irradiated for 90 min in the solar simulator with radiation intensity of 1.2 J/cm^2 (ISO, 2012). Plates were stored in the absence of light, without being irradiated, as negative control. The test was performed in

quadruplicate and the approved criteria was ≤ 17%, according to ISO24443:2012 protocol (ISO, 2012).

2.14 Statistical analysis

All assays were performed in triplicate or more replicates. Statistical analysis was performed using ANOVA test (p<0.05) for independent variables and Origin version 8 software for Windows.

3. RESULTS AND DISCUSSION

3.1 Development, stability and sensorial analysis of vehicle

Emulsions containing low fatty material concentration are considered smooth with minimal oily sensation.

Formulations were white, creamy, pH around 6.6 ± 0.5, density around 1.0 ± 0.01 g/ml. However, formulations showed different skin sensation and viscosity, due to different components concentration in each formulation.

When subjected to stability study F1, F2, F3, F4 and F7 presented significant viscosity increase (p>0.05) and were excluded from this study. This behavior may be attributed to the presence of tribehenin that is a long-chain fatty acid (Pubchem, 2018).

Formulations stability was evaluated using an analytical centrifuge Lumisizer® allowing stability parameters such as sedimentation velocity and shelf life prediction to be directly calculated (Lerche, 2002).

This device also determined sedimentation information (Paul et al., 2013). After assay, all formulations (F5, F6, F8 and F9) showed sedimentation signs. However, F5 and F8 were more stable, which may be explained by the presence of a thickening agent Tribehenin (Sorbitan stearate/sucrose cocoate) in F8 composition, and higher

concentration of polymeric-based oil-in-water emulsifier (sucrose palmitate/glyceryl stearate/glyceryl stearate citrate/sucrose/manna/xanthan gum) in formulation F5. Those four formulations continued to sensorial analysis.

Affective and discriminative methods were the most used methods in sensorial analysis in cosmetic industries (Almeida et al., 2008; Isaac et al., 2012a; Meilgaard et al., 1991; Munoz et al., 1993; Silva et al., 2004), and thus they were applied in this study. In affective method, volunteers graded formulations from 0 to 9.

Sample F9 had the best outcome, with 6.8 ± 1.7 grade, followed by F6 (6.7 ± 1.7), F5 (6.1 ± 1.9) and F8 (5.3 ± 2.0). Formulations were evaluated by sensorial characteristics and F9 presented the best results in absorption speed and drying, easy spreading, low residual fatty sensorial and stickiness, and dry touch. F6 presented similar results to F9, whereas F8 had the worst evaluation (Fig 1).

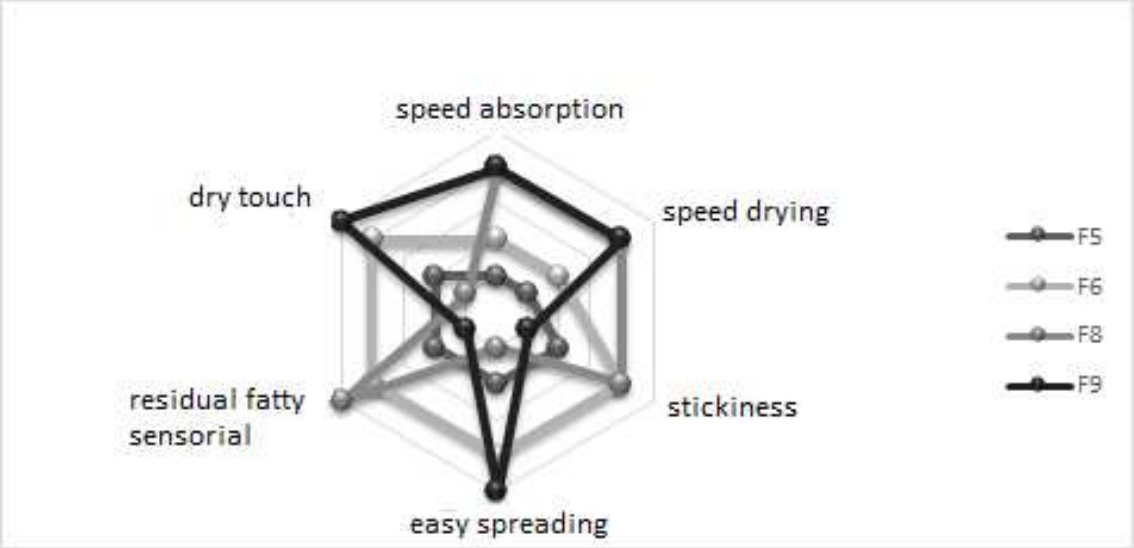

Figure 1: Evaluation of aspects such as speed absorption, residual fatty sensorial, speed drying, stickiness, spreading and dry touch of base formulations (F5, F6, F8 e F9) by sensorial analysis.

According to these results, F8 was pointed out as the most stickiest of all formulations, due to tribehenin presence; F5 received median grades from volunteers and therefore was discarded from the next steps of this study. However, F6 was shown to have stickier than F9, due to samples higher concentration of sucrose palmitate glyceryl stearate/ glyceryl stearate citrate/ sucrose/ manna/xanthan gum (5.0%) and lower talc concentration (1.0%). Based on this, F9 was chosen to be used as the formulation to incorporate plant extracts rich in flavonoids.

3.2 Development and stability study of the phytocosmetic

Phytocosmetics (represented by F9) presented creamy, shiny appearance, yellowish color, and characteristic odor, due to extracts presence. The sample presented pH value equal 6.80 ± 0.11, compatible with the skin (Raab and Kindl, 1999), density of 0.96 ± 0.01 g/ml, being close to 1.00 g/ml evidencing low air quantity incorporated in the formulation during its preparation, and viscosity 46,000 ± 1.21 mPa.s. All desirable characteristics for a topical use formulation (EMEA, 2003; COLIPA, 2004; Isaac et al., 2008; ISO, 2013).

Formulation did not present phase separation after centrifugation assay and then was submitted to stability study (EMEA, 2003; COLIPA, 2004; ISO, 2013). During preliminary study, the phytocosmetic was stable without significant (p<0.05) changes in pH, density and viscosity in all storage conditions. The incorporation of freeze-dried extracts provided more stability of flavonoids promoting formulation stability (Liapis and Bruttini, 2006).

After 90 days of stability study, phytocosmetic color darkened when stored in oven, which was expected and previously described by other authors (Figueiredo et al.,

2014; Hubinger et al., 2010; Veberic et al., 2009). Parameters such as pH, density and viscosity did not show significant changes (p<0.05).

Stability of phytocosmetic was evaluated using an analytical centrifuge Lumisizer®. Although sedimentation started lightly later in phytocosmetic when compared to vehicle, both presented similar sedimentation behavior, and transmission percentage was around 88% at position 115mm and around 8% at position 120mm. Therefore, blend extract presence did not influence on stability behavior by analytical centrifuge analysis.

Phytocosmetic was evaluated by HPLC/DAD for quercetin and rutin concentrations, that determined 8.75 ± 0.19µg/ml and 54.67 ± 0.11µg/ml concentrations, respectively. When phytocosmetic was stored in oven during stability study, a significant (p>0.05) decrease in flavonoids content was detected, with quercetin concentration below quantification limit (2.8µg/ml) and 30.39 ± 0.39µg/ml of rutin after 120 days (Fig. 2 A and B). Thus, phytocosmetic was stable when stored protected from heat.

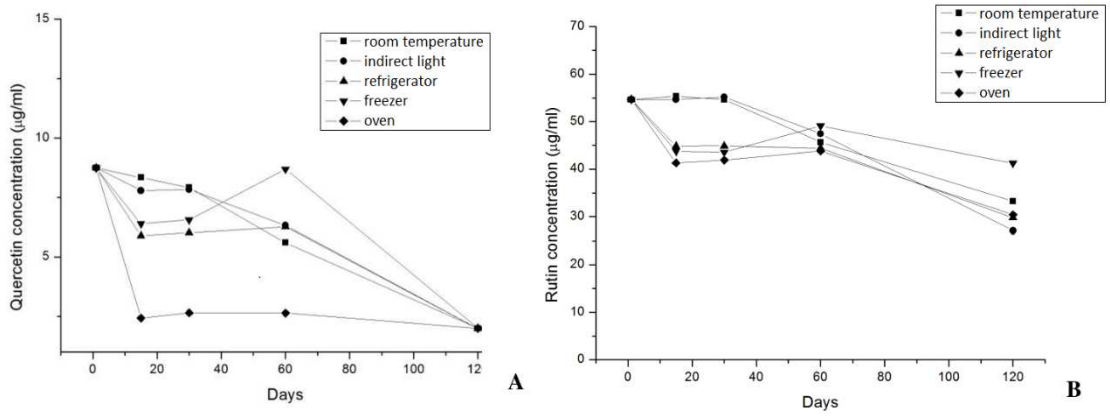

Figure 2: Quercetin concentration (µg.ml^{-1}) (A) and Rutin concentration (µg.ml^{-1}) (B) during stability study of phytocosmetic by HPLC-DAD method.

3.3 Formulations Zeta Potential

In this study, vehicle and phytocosmetic presented zeta potential values of + 35.73 ± 1.6 and + 30.76 ± 1.9 mV, respectively. According to Jeong et al. (2001) and Wiacek and Chibowski (2002), a surface zeta potential of approximately |30mV| is necessary to prevent flocculation and coalescence, and thus desirable emulsion behavior was observed.

The loading of extracts decreased the zeta potential of the formulation, attributed to the presence of solvent residues found in formulation. This result was similar to a result found by Zanatta et al. (2010), in which there was difference between zeta potential values of emulsion with and without plant components.

3.4 Drop size distribution

Laser diffraction method was used to determine drop size distribution and D_{10}, D_{50} and D_{90} values were around 6.84, 23.34 and 57.25μm, respectively for formulations without significant difference between them (p<0.05). Furthermore, only one drop size population was observed, with a constant profile of drop size distribution, without different drop sizes which could cause coalescence in emulsion (Ivanov et al., 1998). This result suggests emulsion stability containing extracts incorporation not influencing emulsions behavior.

3.5 Mechanical evaluation: texture analysis, spreadability and rheological behavior

Phytocosmetic and vehicle were submitted to texture analysis set-up to quantify the mechanical properties, as rupture strength, brittleness, adhesiveness and firmness (Table 2) (Tai et al., 2014).

Table 2. Texture analysis of vehicle and phytocosmetic.

Formulations	Rupture Strength (G)	Brittleness (mm)	Adhesiveness (G.s)	Firmness (G)
Vehicle	3.62 ± 1.31	19.05 ± 0.76	-50.18 ± 0.63	633.26 ± 0.11
Phytocosmetic	3.07 ± 0.33	16.57 ± 2.90	-43.01 ± 2.53	487.08 ± 0.15

Phytocosmetic showed significant (p>0.05) lower values of rupture strength, brittleness and firmness than base formulation. This decrease was attributed to the presence of the extract, which contribute to make the product texture lighter.

According to Almeida and Bahia (2006), topical formulations should exhibit acceptable mechanical characteristics, such as easy application and suitable adhesiveness, enabling skin adhesion. In this study, formulations presented low rupture strength and adhesiveness, and higher brittleness values, representing a lower force needed to promote their uniform application on skin surface.

Nevertheless, formulations showed high firmness values due to polymeric-based oil-in-water emulsifier presence. This result was similar to that found by Tai et al. (2014) in an anti-inflammatory cream. Although formulation had high firmness, emulsifiers containing polymers can enhance product stability.

Phytocosmetic was subjected to spreadability assay presenting value equal to 6079.04±0.44mm^2 when subjected to first plate and 13677.84±0.43mm^2 after the sixth plate. Vehicle was also evaluated and no significant difference was observed (p<0.05).

Data was compared to other studies (Borguetti and Knorst, 2006; Cefali et al., 2015) and phytocosmetic presented higher spreadability value compared to other formulas containing different consistency, surfactants and thickener agents and sun filters. Volunteers in sensorial analysis also ranked F9 as the easiest spreading.

Rheology is the study of flow and deformation behavior of materials under applied forces being frequently used during development of cosmetics products (Almeida and Bahia, 2003; Correa et al., 2005).

According Ansel et al. (2000) fluids can be classified as Newtonian and non-Newtonian, in which emulsions are constituted by asymmetric particles presenting non-Newtonian behavior, available for cosmetic product that suffer deformation to be applied in skin.

Phytocosmetic presented non-Newtonian, shear thinning and thixotropic behaviors, hysteresis area equal 522.28 ± 0.4 Pa/s, exhibited deformation after to 30.9±0,2 Pa and then suffered deformation under tension force, allowing spreadability when applied in skin (Isaac et al., 2013).

Elastic and viscous behaviors of formulation were assessed by amplitude and frequency range tests (Ribeiro et al, 2004). Data showed G' (elastic module) value higher than G" (viscous module) value, determining elastic behavior commonly observed in oil–in–water emulsions (Cefali et al., 2015; Isaac et al., 2013).

In this test, formulation suffer deformation at 9.78 ±0.4 Pa/s when G' and G" Cross (crossover range), providing the fluidity of sample.

Creep/relaxation assay that determines the viscoelasticity of samples (Isaac et al., 2013) was observed the resistance of sample to flow and recovery of its elasticity when the shearing tension ceased (Fig. 3).

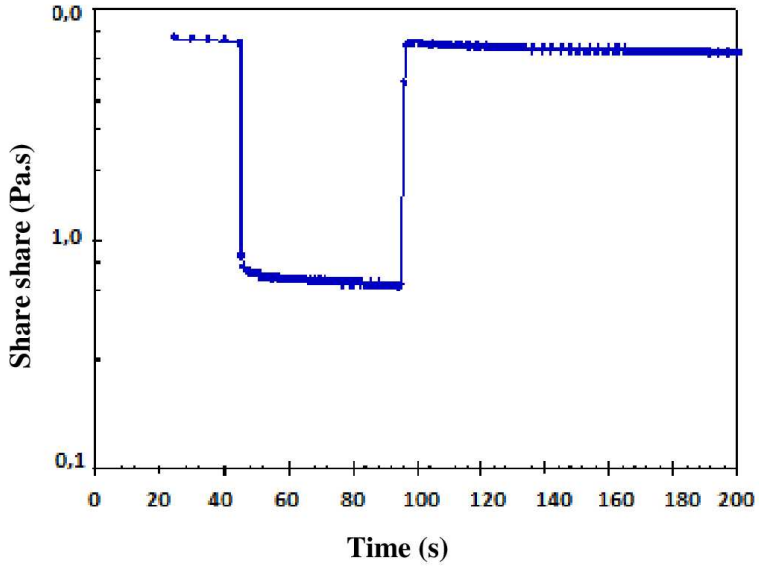

Figure 3: Rheogram of fluency and relaxation assay of phytocosmetic using sheering tension of 7 Pa for 45 seconds for fluency, 70 Pa for 60 seconds for resting 7 Pa for 100 seconds for relaxation.

3.6 Thermal analysis

Thermal methods of analysis such as DSC and TG were applied in this study for determination of physic-chemical stability of emulsions according to temperature variation (Casimiro et al., 2005).

According to Table 3, vehicle and phytocosmetic presented similar behavior towards the presence of endothermic events approximately 52ºC (stage 1) and 115ºC (stage 2), demonstrating, according to Daneluti et al. (2015), loss of water and volatilization of other emulsions components.

Table 3: Temperature values (ºC) presented during enthalpy variation of components from vehicle and phytocosmetic.

Formulations	Enthalpy variation	
	Stage 1 (ºC)	**Stage 2 (ºC)**
Vehicle	51.66	120.93
Phytocosmetic	52.65	113.77

Similar results were found for Schnitzler et al. (2001) and Cefali et al. (2015) in which O⁄W emulsions were also subjected to DSC analysis and showed endodermic peaks in the same temperature ranges, demonstrating change of compound's physical state present in formulation with temperature variation.

Furthermore, according to data, both formulations presented similar values where endothermic peaks were identified.

Termogravimetry assay presented three events that occurred due to formulations' weight loss (Table 4) showing the loss of free and arrested water from sample and of other components around 86ºC (stage 1), 172ºC (stage 2) and 280ºC (stage 3), presenting weight loss around 80%, 4% and 5%, respectively.

Table 4: Percentage of weight loss of components from vehicle and phytocosmetic during the three stages presented by medium temperatures (T_M) determined.

Formulas	T_M (ºC) stage 1	Weight loss (%)	T_M (ºC) stage 2	Weight loss (%)	T_M (ºC) stage 3	Weight loss (%)
Vehicle	87.89	86.64	167.83	3.94	278.22	4.21
Phytocosmetic	82.76	79.87	166.97	4.53	273.20	5.82

Similar results were found for Daneluti et al. (2015) in which emulsions after thermogravimetry analysis presented weight loss of 86% and 95%.

In comparison to DSC data, formulations presented similar results in weight loss and extracts presence in emulsion did not influence formula's thermal behavior.

3.7 Microbiologic control

Phytocosmetic analysis did not show pathogenic microorganisms growth. Total count of microorganisms was lower than 10 colony forming unit (CFU) of bacteria, fungi and yeasts in each gram of the sample, thereby demonstrating final product's quality (ISO 17516, 2014; Usp 36, 2013).

3.8 *In vitro* solar protection factor (SPF) evaluation of phytocosmetic

Formulation containing blend extracts presented SPF equal to 2.94 ± 0.4 using Mansur method (Mansur et al., 2016), and 2.4 ± 0.5 referent do UVA protection by diffuse reflectance spectroscopy method. The product presented critical wave-length equal to 387.0nm and UVA/UVB rate equal 0.78, determining the formulation developed as sunscreen with UVA/UVB protection, protecting skin against damages causes by ultraviolet radiation (COLIPA, 2006; Flora and Ferguson, 2005).

These *in vitro* methods are preliminary assays although formulation presented value above 2.0 required by FDA *in vivo* assay (FDA, 2013). Therefore, formulation containing blend extracts is a promising product to be used as chemical sun filter with UVA/UVB protection that can be associated with physical sun filters increasing the activity.

Vehicle was evaluated and did not present SPF value by methods used.

3.9 *In vitro* antioxidant activity analysis of phytocosmetic

Phytocosmetic was subjected to *in vitro* antioxidant assay against DPPH free radical. Formulation presented 17.96±0.03% inhibition against DPPH solution. This result is similar to that found by Cefali et al. (2015) in which cosmetic containing carotenoid presented 19.51% of inhibition percentage.

Results of antioxidant activity were lower than single extracts against DPPH (IC_{50} of 174.51 ± 1.1 µg/mL to *D. molli* Benth; 8.12 ± 0.8 µg/mL to *G. biloba* L.; 281.02 ± 1.0 µg/mL to *R. graveolens* L.; 296.90 ± 1.2 µg/mL to *V. vinifera* L., and 28.73 ± 0.7 µg/mL to mix sample) (data not shown).

These results indicate a promising antioxidant ability that could aggregate anti-aging property to the formulation.

3.10 Permeation test

Phytocosmetic allowed the release of rutin though a synthetic membrane (after 1, 2, 4 and 6 hours) in a receiving medium collected and analyzed using HPLC/DAD, according Figure 4A.

Rutin was also found in receiving medium in permeation test using pig skin as membrane. This assay was performed for 24 hours and rutin was determined in all aliquots obtained during study (Fig.4B) and in ascending amounts until 12 hours.

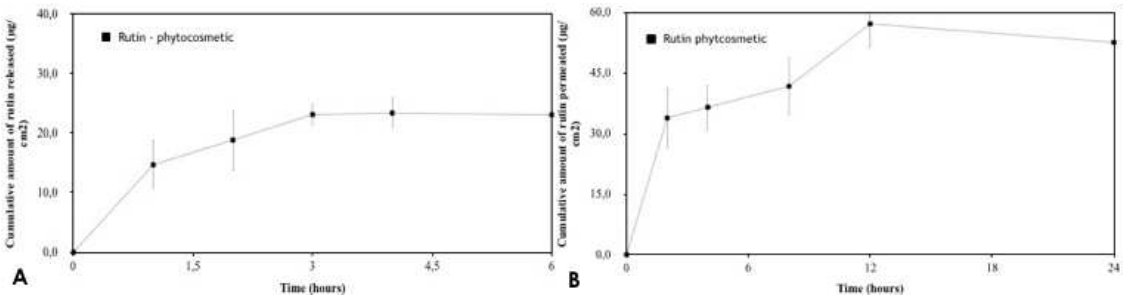

Figure 4: Rutin concentration of µg.ml^{-1} from phytocosmetic by release test (A) and by permeation test (B).

Although rutin permeated the skin, the compound was also found in stratum corneum (3.27 ± 1.92 µg/ml) by tape stripping test and in higher concentration in the retention test (114.68 ± 8.70 µg/ml).

Studies performed by Chuang et al. (2017) and Isaac et al. (2012) showed release and deposit of flavonoids when incorporated in topical formulations. Moreover, Chung et al. (2017), in their study, determined that the viable skin, in especial the stratum corneum, could be a barrier retarding flavonoid permeation by enhancing compound deposition.

Rutin's amphiphilic characteristic (Grinberg et al., 1994) retained the compound in the skin (stratum corneous and epidermis/dermis) allowing antioxidant activity and solar protection which can be a promising and desirable specification for anti-aging and sunscreen protection.

3.11 Photostability study

Photoproducts generated during absorption of UV radiation process (Gaspar and Maia Capos, 2006; Mayer et al., 2005; Perugini et al., 2002) can cause reactive and causes damages such as dermatitis and photo-allergies or be ineffective as sun filters (Gaspar and Maia Campos, 2006; Antoniou et al., 2008).

Therefore, photostability evaluation is mandatory for formulations containing new sun filters, ensuring product's efficacy and safety issues (COLIPA, 2006; Herzog et al., 2009).

Product's photostability assay demonstrated a decrease in SPF value to 2.1±0.4 after 90 min of irradiation (loss of 12.50%). Thus, the product can be considered photostable. According to Hojerova et al. (2011), since the protocol considers that a formulation can lose up to 20% of the SPF after being subjected to artificial UV radiation.

That was an expected result for the formula containing antioxidant materials such as flavonoids.

Studies performed by Jarzycka et al. (2013) and Choquenet et al. (2008) found similar results reported (Alencar Filho et al., 2016; Scalia et al., 2010) that quercetin is capable to reduce the photodegradation of chemical sun filters without any changes in formula effectiveness.

Moreover, according to Jarzycka et al. (2013), the multiple effects of polyphenolic compounds (antioxidants) incorporated into sunscreen formulations represent a promising strategy for the development of broad-spectrum sunscreen products with enhanced efficacy and safety.

4. CONCLUSION

The final formulation presented stability when stored protected from heat with high spreadibility, low rupture strength and adhesiveness and higher brittleness, shear shinning and viscoelastic behaviors, being characteristics desirable for topical formulations. Also, antioxidant activity and UVA protection by *in vitro* assays, and photostability measured quality the formulation as sun filter with UVA/UVB protection. Futhermore, rutin permeated in the skin and was also found in stratum corneum and in

other layers of epidermis/dermis, allowing antioxidant activity and solar protection that is promising and desirable for anti-aging and sunscreen actions.

5. ACKNOWLEDGMENT

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6. REFERENCES

1) ABIHPEC. Associação Brasileira de Indústrias de Higiene Pessoal, Perfumaria e Cosméticos. Available in: https://www.abihpec.org.br/2012/04/brasil-tem-maior-taxa-de-crescimento-percentual-entre-os-top-10-mercados-mundiais-de-hppc/. Access in Jan 20, 2018.
2) ALENCASTRE, J.B.; BENTLEY, M.V.L.B.; GARCIA, F.S.; MORAGAS, M.; VILADOR, J.L.; MARCHETTI, J.M. A study of the characteristics and *in vitro* permeation properties of CMC/ chitosan microparticles as a skin delivery system for vitamin E. *Rev. Bras. Ciênc. Farm.*, v. 42, n.1, p. 69-76, 2006.
3) ALENCAR FILHO, J. M. T.; SAMPAIO, P. A.; PEREIRA, E. C. V.; DE OLIVEIRA JÚNIOR, R. G.; SILVA, F. S.; ALMEIDA, J. R. G. S.; ROLIM, L. A.; NUNES, X. P.; ARAÚJO, E. C. C. Flavonoids as photoprotective agents: A systematic review. *J. Med. Plants Res.* v.10, n.47, p.848-864, 2016.
4) ALMEIDA, I.F.; BAHIA, M.F. Evaluation of the physical stability of two oleogels. *Int. J. Pharm*., v.327, n.1-2, p.73-77, 2006.
5) ALMEIDA, I.F.; GAIO, A.R.; BAHIA, M.F. Hedonic and descriptive skin feel analysis of two oleogels: comparison with other topical formulations. *J. Sens. Stud*. v.23, n.1, p.92-113, 2008.
6) AMAR, S.K.; GOYAL, S.; DUBEY, D.; SRIVASTAV, A.K.; CHOPRA, D.; SINGH, J.; SHANKAR, J.; CHATURVEDI, R.K.; RAY, R.S. Benzophenone 1 induced photogenotoxicity and apoptosis via release of cytochrome c and smac/diablo at environmental UV radiation. *Toxicol Let*. v.239, n.1, p.182-193, 2015.
7) ANSEL, H.C.; POPOVICH, N. G.; ALLEN Jr., L.V. Formas farmacêuticas e sistema de liberação de fármacos. 6. ed. São Paulo: Editorial Premier, 2000. 568 p.

8) ANTONIOU, C.; KOSMADAKI, M.; KOSMADAKI, G.; STRATIGOS, A.J.; KATSAMBAS, A.D. Sunscreens – what's important to know? *J Eur Acad Dermatol Venereol*. v.22, n.1, p.1110-1119, 2008.

9) ARTS, I.C.W.; HOLLMAN, P.C.H. Polyphenols and disease risk in epidemiologic studies. *Am J Clin Nutr*. v.81, p.317-325, 2005.

10) BADOLATO, G.G.; AGUILAR, F.; SCHUCHMANN, H.P.; SOBISCH, T.; LERCHE, D. Evaluation of long term stability of model emulsions by multisample analytical centrifugation. *Progr. Colloid Polym. Sci.* v.134, n.1, p. 66 – 73, 2008.

11) BARON, E.D.; KIRKLAND, E.B.; DOMINGO, D.S. Advances in photoprotection. *Dermatol Nurs.* v.20, n.1, p.265-272, 2008.

12) BARRY, B.W. Rheology of dermatological vehicles. In: Dermatological formulations. New York: Marcel Dekker, p. 351-439, 1993.

13) BORELLA, J.C.; RIBEIRO, N.S.; TEIXEIRA, J.C.L.; CARVALHO, D.M.A. Avaliação da espalhabilidade e do teor de flavonoides em forma farmacêutica semissólida contendo extratos de *Calendula officinalis* L. (Asteraceae). *Rev Ciênc Farm Básica Apl*., v.31, n.2, p.193-197, 2010.

14) BORGHETTI, G.S.; KNORST, M.T. Desenvolvimento e avaliação da estabilidade física de loções O/A contendo filtros solares. *Rev. Bras. Ciên. Farm*., v.42, p.531-537, 2006.

15) CASIMIRO, M. H.; LEAL, J. P.; GIL, M. H.; CASTRO, C. A. N. Análise calorimétrica aplicada a polímeros biológicos. Parte I: Fundamentos teóricos. *Bol. Soc. Port. Quím.,* v.98, n.1, p. 29-35, 2005.

16) CEFALI, L.C.; SOUZA-MOREIRA, TATIANA MARIA DE; CORRÊA, M. A.; SALGADO, H. R. N.; ISAAC, V. L. B. Development and evaluation of an emulsion containing lycopene for combating acceleration of skin aging. *Braz. J. Pharm. Sci*. v.51, n.3, p. 579-590, 2015.

17) CEFALI, L.C.; ATAIDE, J.A.; FOGLIO, M.A., MORIEL, P.; MAZZOLA, P.G. Plant compounds as active photo protectants in sunscreens. *Inter. J. Cosm. Sci*., v.1, p.1-8, 2016.

18) CHANAMAI, R.; HERRMAN, N.; MCCLEMENTS, D. J. Ultrasonic spectroscopy study of flocculation and shear-induced floc disruption in oil-in-water emulsions. *J. Coll. Int. Sci.* v.204, n.2, p.268-276, 1998.

19) CHUANG, S.Y.; LIN, C.F.; WANG, P.W.; CHEN, E.L.; FANG, J.Y. Elucidating the Skin Delivery of Aglycone and Glycoside Flavonoids: How the Structures Affect Cutaneous Absorption. *Nutrients*, v.9, n.12, p.1304-1310, 2017.

20) CHOQUENET, B., COUTEAU, C., PAPARIS, E.; COIFFARD, L.J.M. Quercetin and rutin as potential sunscreen agents: Determination of efficacy by an *in vitro* method. *J. Nat. Prod.* v.71, n.6, p.1117–1118, 2008.

21) COLIPA. Colipa Project Team IV. Method for the in vitro determination of UVA protection provided by sunscreen products. Guideline, 2006.

22) COOMAN, L. DE; EVERAERT, E.; DE KEUKELEIRE, D. Quantitative analysis of hop acids, essential oils and flavonoids as a clue to the identification of hop varieties. *Phytochem. Anal.,* v.9, n.1, p.145-150, 1998.

23) CORREA, N. M.; CAMARGO JUNIOR, F. B.; IGNÁCIO, R. F.; LEONARDI, G. R. Avaliação do comportamento reológico de diferentes géis hidrofílicos. *Braz.J. Pharm. Sci.* v. 41, n. 1, p. 73-78, 2005.

24) DANELUTI, A.L.M.; VELASCO, M.V.R.; BABY, A.R.; MATOS, J.R. Thermal Behavior and Free-Radical-Scavenging Activity of Phytic Acid Alone and Incorporated in Cosmetic Emulsions. *Cosmetics.* v.2, n.1, p.248-258, 2015.

25) DÁVILA, J.L.; DÁVILA, M.A. Laponite as a rheology modifier of alginate solutions: Physical gelation and aging evolution. *Carboh. Polymers*, v. 157, n.1, p. 1-8, 2016

26) DAVIS, H.M. Analysis of creams and lotion. In: Newburguer's manual of cosmetic analysis. Washington: Association of Official Analytical Chemists, 1977, p.32.

27) DUTCOSKY, S.D. Análise Sensorial de Alimentos. 2 ed. Curitiba: Champagnat; 2007.

28) EMEA. Guideline on Stability Testing: Stability Testing of Existing Active Substances and Related Finished Products. Committee for Proprietary Medicinal Products (CPMP). European Medicines Agency – Inspections (EMEA), 2003.

29) ESTANQUEIRO, M.; CONCEIÇÃO, J.; AMARAL, M.H.; SANTOS, D.; SILVA, J.B.; LOBO, J.M.S. Characterization and stability studies of emulsion systems containing pumice. *Braz. J. Pharm. Sci*. v.50, n.2, p.361-369, 2014.

30) FDA. Food and Drug Administration. Sunscreen drug products for over-the-counter human use. Code of Federal Regulations. Title 21, v. 5. 2013.

31) FIGUEIREDO, S.A.; VILELA, F.M.; DA SILVA, C.A.; CUNHA, T.M.; DOS SANTOS, M.H.; FONSECA, M.J. *In vitro* and *in vivo* photoprotective/ photochemopreventive potential of Garcinia brasiliensis epicarp extract. *J Photochem Photobiol B.* v.5, n.31, p.65-73, 2014.

32) FLORA, S.DE; FERGUSON, L.R. Overview of mechanisms of cancer chemopreventive agents. *Mutat. Res*. v.11, n.1-2, p.8-15, 2005.

33) GASPAR, L.R.; MAIA CAMPOS, P.M.B.G. Rheological behavior and the SPF of sunscreens. *Int. J. Pharm.* v. 250, n. 1, p. 35-44. 2003.

34) GIACOMONI, P. U. Understanding reactive oxygen species. *Cosm. & Toil.,* v.122, n. 5, p.36-40, 2008.

35) GILBERT, L.; PICARD, C.; SAVARY. G.; GRISEL, M. Rheological and textural characterization of cosmetic emulsions containing natural and synthetic polymers: relationships between both data, *Coll. Sur. A*. v.421, n.1, p.150-163, 2013.

36) GRINBERG, L. N.; RACHMILEWITZ, E. A.; NEWMAN, H. Protective effects of rutin against hemoglobin oxidation. *Biochem. Pharmacol.* v.48, n.1, p.643-649, 1994.

37) HEJJAJI, E.M.; SMITH, A.M.; MORRIS, G.A. Designing chitosan-tripolyphosphate microparticles with desired size for specific pharmaceutical or forensic applications. *Int. J. Biol. Macromol.* v.95, n.1, p.564-573, 2017.

38) HERZOG, B.; WEHRLE, M.; QUASS, K. Photostability of UV absorber systems in sunscreens. *J. Photochem. Photobiol. B*. v.85, n.1, p.869-878, 2009.

39) HOJEROVÁ, A.; MEDOVCÍKOVÁ, A.; MIKULA M. Photoprotective efficacy and photostability of fifteen sunscreen products having the same label SPF subjected to natural sunlight. *Inter. J. Pharm*. v.408, p.27–38, 2011.

40) HUBINGER, S. Z.; CEFALI, L. C.; VELLOSA, J. C. R.; SALGADO, H. R. N.; ISAAC, V. L. B.; MOREIRA, R. R. D. *Dimorphandra mollis*: an alternative as a source of flavonoids with antioxidant action. *Lat. Am. J. Pharm.* v. 29, n. 2, p. 271-274, 2010.

41) HUONG, S.P.; ROCHER, E.; FOURNERON, J.D.; CHARLES, L.; MONNIER, V.; BUN, H.; ANDRIEU, V. Photoreactivity of the sunscreen butylmethoxydibenzoylmethane under various experimental conditions. *J. Photochem. Photobiol. A.*, v.196, n.1, p.106–112, 2008.

42) IDSON, B. Stability testing of emulsions, I. *Drug & Cosmetics Industry,* v.151, n.1, p.27-28,30, 1993.

43) INCA. Instituto Nacional de Câncer. Estimativas 2016: Incidência de Câncer no Brasil. Rio de Janeiro: Instituto Nacional do Câncer; 2016.

44) ISAAC, V.L.B.; CEFALI, L.C.; CHIARI, B.G.; OLIVEIRA, C.C.L.G.; SALGADO, H.R.N.; CORRÊA, M.A. Protocolo para ensaios físico-químicos de estabilidade de fitocosméticos. *Rev Ciênc Farm Básica Apl.* v.29, n.1, p.81-96, 2008.

45) ISAAC, V.L.B.; CHIARI, B. G.; MAGNANI, C.; CORRÊA, M. A. Análise sensorial como ferramenta útil no desenvolvimento de cosméticos. *Rev. Ciên. Farm. Básica. Apl.* v.33, n.4, p. 479-488, 2012a.

46) ISAAC, V.L.B.; CHIARI, B.G.; MIGLIOLI, K.; MOREIRA, R.; OLIVEIRA, J.R.S.; SALGADO, H.R.N.; RELKIN, P.; CORRÊA, M.A.; SALGADO, A.; RIBEIRO, H.M. Development of a topical formulation containing *S. lutea* extract: Stability, i*n vitro* studies and cutaneous permeation. *J. Appl. Pharm. Sci.* v.2, n.8, p.174-179, 2012b.

47) ISAAC, V.L.B.; CEFALI, L.C.; CHIARI, B.G.; ALMEIDA, J.M.G.; RIBEIRO, H.M.; CORREA, M.A. Effect of various thickening agents on the rheological properties of oil-in-water emulsions containing nonionic emulsifier. *J. Disp. Sci. Tech*. v. 34, n.6, p.880-885, 2013.

48) ISO. International Organization of Standardization. ISO 24443:2012. Determination of sunscreen UVA photoprotection *in vitro*. Geneva, Switzerland, 2012.

49) ISO. International Organization of Standardization. ISO/TR 13097. Guidelines for the Characterization of Dispersion Stability. Geneva, Switzerland, 2013.

50) ISO. International Organization of Standardization. ISO 17516:2014. Cosmetics - Microbiology - Microbiological limits. Geneva, Switzerland, 2014.

51) JARZYCKA, A. Assessment of extracts of *Helichrysum arenarium*, *Crataegus monogyna*, *Sambucus nigra* in photoprotective UVA and UVB; photostability in cosmetic emulsions. J. Photochem. Photobiol. B: Biol., v.128, n.1, p. 50-57, 2013.

52) JEONG, Y. I.; CHO, C. S.; KIM, S. H.; KO, K. S.; KIM, S. I.; SHIM, Y. H.; NAH, J. W. Preparation of poly (DL-lactide –co-glycolide) nanoparticles without surfactant. *J. Appl. Poly. Sci*. v.80, n.1, p.2228-2236, 2001.

53) KANG, J.; XIE, C.; LI, Z.; NAGARAJAN, S.; SCHAUSS, A.G.; WU, T.; WU, X. Flavonoids from acai (*Euterpe oleracea* Mart.) pulp and their antioxidant and anti-inflammatory activities. *Food Chem.,* v.128, 1n., p.152-157, 2011.

54) LERCHE, D. Dispersion Stability and Particle Characterization by Sedimentation Kinetics in a Centrifugal Field. *J. Disp. Sci. and Technol*. v.23, n.5, p.699 - 709, 2002.

55) LIAPIS, A.I.; BRUTTINI, R. “Freeze Drying” in Handbook of Industrial Drying, LLC, pp. 257, 2006.

56) MACFIE, H.J.H.; THOMSON, D.M.H. Preference mapping and multidimensional scaling. In: Piggot JR, editor. Sensory analysis of foods. 2 ed. Londres; Elsevier; 1988. 389 p.

57) MANSUR, M.C.P.P.R.; LEITÃO, S.G.; CERQUEIRA-COUTINHO, C.; VERMELHO, A.B.; PRESGRAVE, O.A.F.; LEITÃO, A.A.C.; LEITÃO, G.G.; RICCI-JUNIOR, E.; SANTOS, E.P. *In vitro* and *in vivo* evaluation of efficacy and safety of photoprotective formulations containing antioxidant extracts. *Rev Bras Farmacog*. v.26, n.1, p.251-58, 2016.

58) MAYER, L.; QUINN, T.; WADSLEY, J.; STADEL, J. Gravitational instability in binary protoplanetary discs: new constraints on giant planet formation, *MNRAS,* v.363, n.1, p.641–648, 2005.

59) MEILGAARD, M.; CIVILLE, G.V.; CARR, B.T. Sensory evaluation techniques. Florida: CRC Press; 1991. p. 142-47.

60) MONTENEGRO, L.; PUGLISI, G. Evaluation of sunscreen safety by *in vitro* skin permeation studies: effects of vehicle composition. *Pharmazie.* v.68, n.1, p.34-40, 2013.

61) MUÑOZ, A.M.; CIVILLE, G.V.; CARR, B.T. Sensory evaluation in quality control. New York: Van Nostrand Reinhold; 1993. p. 240.

62) PALM, M.D.; O`DONOGHUE, M.N. Update on photoprotection. *Dermatol Ther*. v.20, n.1, p.360-376, 2007.

63) PAUL, N.; BIGGS, S.; EDMONDSONB, M.; HUNTER, T.N.; HAMMONDA, R.B. Characterizing highly active nuclear waste simulates. *Chem. Eng. Res. Des.* v.91, n.1, p.742-751, 2013.

64) PERUGINI D., POLLI, G.; GATTA, D. Analysis and simulation of magma mixing and processes in 3D. *Lithos*. v.65, n.1, p. 313–330, 2002.

65) PINTO, T.J.A.; KANEKO, T.M.; OHARA, M.T. C*ontrole Biológico de Qualidade de Produtos Farmacêuticos, Correlatos e Cosméticos.; São Paulo:* Atheneu. 2003, p. 75-96.

66) RAAB, W.; KINDL, U. Pflegekosmetik. 3.ed. Stuttgart: Wissenschaflliche, 1999. 371 p.

67) RAMOS, S.; HOMEM, V.; ALVES, A.; SANTOS, L. A review of organic UV-filters in wastewater treatment plants. *Environ Int.* v.86, p.1, p:24-40, 2016.

68) RIBEIRO, H. M.; MORAIS, J.A.; ECCLESTON, G.M. Structure and rheology of semisolid O/W creams containing cetyl alcohol/ non-ionic surfactant mixed emulsifier and different polymers. *Inter. J. Cosm. Sci.*, v.26, n.1, p.47-59, 2004.

69) ROLAND, I.; PIEL, G.; DELATTRE, L.; EVRARD, B. Systematic characterization of oil-in- water emulsions for formulation design. *Int. J. Pharm.* v.16, n.1-2, p.85-94, 2003.

70) ROMANHOLE, R.C.; ATAIDE, J.A.; CEFALI, L.C.; MORIEL, P.; MAZZOLA, P. Photostability study of commercial sunscreens submitted to artificial UV irradiation and/or fluorescent radiation. *J Photochem Photobiol B: Biol*. v.162, n.1, p.45-49, 2016.

71) RUFINO, M.S.M.; ALVES, R.E.; BRITO, E.S.; JIMENEZ, J.P.; CALIXTO, F.S. Bioactive compounds and antioxidant capacities of 18 non-traditional tropical fruits from Brazil. *Food Chem.* v.121, n.1, p.996-1002, 2010.

72) RUSZKIEWICZ, J.A.; PINKAS, A.; FERRER, B.; PERES, T.V.; TSATSAKIS, A.; ASCHNER, M. Neurotoxic effect of active ingredients in sunscreen products, a contemporary review. *Toxicol. Reports.* v.4, n.1, p.245–259, 2017.

73) SAMBANDAN, D.R.; RATNER, D. Sunscreens: An overview and update. *J. Am. Acad. Dermatol.* v.64, p.748-758, 2011.

74) SAVARY, G.; GRISEL, M.; PICARD, C. Impact of emollients on the spreading properties of cosmetic products: a combined sensory and instrumental characterization. C*oll. Surf. B Bioint.* v.102, n.1, p.371-378, 2013.

75) SAYRE, R. M.; AGIN, P. P.; LEVEE, G. J.; MARLOWE, E. Comparison of *in vivo* and in vitro testing of sunscreening formulas. *Photochem. Photobiol.*, v. 29, p. 559-566, 1979.

76) SCALIA, S.; MEZZENA, M. Photostabilization effect of quercetin on the UV filter combination, butyl methoxydibenzoylmethane-octyl methoxycinnamate. *Photochem. Photobiol.* v.86, n.2, p.273-278, 2010.

77) SCHNITZLER, E.; CARVALHO-FILHO, M.A.S.; STADLER, C.C.; VOLPATO, A.M.; IONASHIRO, M. Aplicação da calorimetria exploratória diferencial (DSC) na caracterização térmica do acetato de dexametazona, excipientes e do creme de dexametazona. *Eclet.Quím.* v. 26, n.1, p.1-18, 2001.

78) SEAL, T. Quantitative HPLC analysis of phenolic acids, flavonoids and ascorbic acid in four different solvent extracts of two wild edible leaves, Sonchus arvensis and Oenanthe linearis of North-Eastern region in India. *J. Appl. Pharm. Sci*. v.6, n.2, p. 157-166. 2016.

79) SILVA, T.M.A.; MIGLIATO, K.F.; SALGADO, H.R.N.; ISAAC, V.L.B. Quality comparison of W/O and O/W photo-protection creams. *Cosmet Toil*. v.119, n.7, p.41-46, 2004.

80) STONE, H.S.; SIDEL, J.L. Sensory evaluation practices. San Diego, CA: Academic Press; 1992.

81) TAI, A.; BIANCHINI, R.; JACHOWICZ, J. Texture analysis of cosmetic/pharmaceutical raw materials and formulations. *Int. J. Cosmet. Sci.* v.36, n.4, p.291-304, 2014.

82) USP - United States Pharmacopeia. 36th.ed. The United States Pharmacopeial Convention: Rockville, 2013.

83) VEBERIC, R.; JAKOPIC, J.; STAMPAR, F.; SCHMITZER, V. European elderberry (*Sambucus nigra* L.) rich in sugars, organic acids, anthocyanins and selected polyphenols. *Food Chem.* v.114, p.511–515, 2009.

84) WIACEK, A.E.; CHIBOWSKI, E. Zeta potential and droplet size of n-tetradecane/ethanol (protein) emulsions. *Coll. Surf. B: Biointer.* v.25, n.1, p.55-67, 2002.

85) WONG, T.; ORTON, D. Sunscreen allergy and its investigation. *Clin Dermatol*. v.29, p.306-310, 2011.

86) ZANATTA, C.F.; MITJANS, M.; URGATONDO, V.; ROCHA-FILHO, P.A.; VINARDELL, M.P. Photo protective potential of emulsions formulated with Buriti oil (*Mauritia flexuosa*) against UV irradiation on keratinocytes and fibroblasts cell lines. *Food Chem. Toxicol.*, v.48, n.1, p. 70-75, 2010.

CAPITULO IV

"In vitro SPF and photostability assays of emulsion containing nanoparticles with vegetable extracts rich in flavonoids".

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ABSTRACT

The aim of study was to determine the *in vitro* sun protection factor (SPF) and the photostability profile of a topical formulation composed of nanoparticles loaded with vegetable extracts and to assess its physicochemical properties. Chitosan/tripolyphosphate (TPP) nanoparticles loaded with flavonoids-enriched vegetable extracts (*Ginkgo biloba* L., *Dimorphandra mollis* Benth, *Ruta graveolens* and *Vitis vinifera* L.) were produced and characterized for their morphology, mean particle size, zeta potential, and encapsulation efficiency. A final topical formulation was obtained by dispersing chitosan/TPP nanoparticles in an o/w emulsion. Results showed that nanoparticles dispersion presented yellowish color, spherical shape and uniform appearance. Extract-loaded chitosan/TPP nanoparticles showed a mean particle size of 557.11 ± 3.1 nm, polydispersity index of 0.39 ± 0.27, zeta potential of -11.54 ± 2.1 mV and encapsulation efficiency of 71.43% in rutin. Formulation containing nanoparticles showed texture parameters appropriate for skin application. The SPF obtained was 2.3 ± 0.4, with a critical wavelength of 387.0 nm and 0.69 UVA/UVB ratio. The developed formulation was shown to be photostable and allowed the release of flavonoids from nanoparticles while retaining rutin into the skin in a higher extension.

Keywords: flavonoids, nanoparticles, permeation, photostability, rutin, sunscreen.

1. INTRODUCTION

Flavonoids are polyphenolic compounds found in most plants, concentrated in seeds, fruits, husks, roots, leaves and flowers (Feldman, 2001; Mann, 1987) and they are the third largest class of natural products and exert many important effects on plants mainly regarding protection against pathogens and UVB radiation (Balogh et al., 2011; Tohge et al., 2017).

Attributed to their capacity to absorb UV rays, especially UVA and UVB, in wavelengths between 200 nm and 400 nm (De Cooman et al., 1998), flavonoids have been extensively exploited for development of products for protecting the skin against harmful solar radiation (Cefali et al., 2016; Chiari et al., 2014; Dal Belo et al., 2011).

Other important property of these compounds is their antioxidant activity, which may compromise the chemical stability in formulations when subjected to heat or light (Hubinger et al., 2010). To minimize this phenomenon, techniques, such as micro and nanoencapsulation of compounds can be used, since encapsulation can protect these compounds from physical and chemical damage (Ajazuddin, 2010), contributing to improve efficacy and increase the quality of the final product (Scalia and Mezzena, 2009).

Photostability is an important parameter to be studied in the development of a sunscreen formulation. The selected sun filter cannot undergo modification when exposed to sunlight because in addition to losing protective properties and exposing the skin directly to incident radiation, it can promote serious deleterious effects on the human body (Huong et al., 2007; Romanhole et al., 2016).

The aim of study was to determine the sun protection factor (SPF) and the photostability profile of a topical formulation composed of nanoparticles loaded with vegetable extracts.

2. MATERIAL AND METHODS

2.1 Material

Freeze-dried extracts rich in flavonoids from four different vegetable materials (*Ginkgo biloba* L., *Dimorphandra mollis* Beth, *Ruta graveolens* and *Vitis vinifera* L.) and a blend of extracts contained equal quantity of each plant (1:1:1:1) obtained previously were used in this paper for presenting *in vitro* SPF values of 5.04 ± 0.2 to *D. molli* Benth, 8.31 ± 0.5 to *G. biloba* L., 7.08 ± 0.4 to R. *graveolens* L., of 3.71 ± 0.5 to *V. vinifera* L. and 7.72 ± 0.4 for the blend of extracts (Mix; 1:1:1:1).

The emulsion was produced using raw materials such as sorbitan stearate and sucrose cocoate, sucrose palmitate glyceryl stearate and glyceryl stearate citrate and sucrose and manna and xanthan gum, caprylic and capric triglyceride, hydrolyzed wheat protein and PVP cross-polymer, *Persea gratissima* (Avocado) oil, glycerin and talc provided by Croda (Campinas, São Paulo, Brazil) and PharmaSpecial (São Paulo, São Paulo, Brazil). The reagents ethyl alcohol, sodium acetate and acetic acid were provided by Synth (São Paulo, Brazil), chitosan, sodium tripolifosfate (TPP), methanol and formic acid by Sigma-Aldrich (São Paulo, São Paulo, Brazil), and quercerin (93.3% of purity) and rutin (97.3% of purity) standards by Acros (Itu, Brazil).

2.2 Preparation of nanoparticles containing vegetable extracts rich in flavonoids

Chitosan solution (2.0 mg/ml) and TPP solution (0.84 mg/ml) were prepared by dissolving the powders separately in acetate buffer (pH equal 5.2) using a magnetic stirrer (Fisatom, 735A, São Paulo, Brazil) at 25 ± 3ºC (Hejjaji et al., 2017; Morris et al., 2011).

To prepare the chitosan and TPP nanoparticles, 10 ml of TPP solution were added drop wise to 20 ml of chitosan solution at 600 rpm for 60 min, at 25 ± 3ºC.

To prepare the nanoparticles containing vegetable extracts rich in flavonoids, 2 ml of extracts diluted in ethanol (0.28 ml of extract in 2 ml of ethanol) were added in 10 mL of TPP and 20 ml of chitosan, stirred at 600 rpm for 60 min, at 25 ± 3ºC.

Nanoparticles solution without (blank) and with flavonoids-enriched vegetable extracts were stored at 5°C and their physical and chemical characteristics were evaluated.

2.3 Determination of average particle size, zeta potential and encapsulation efficiency (EE)

The average particle size was measured directly by dynamic light scattering (DLS) using a Zetasizer® equipment (Malvern Instruments Ltd., Malvern, United Kingdom). About 1 ml of the suspension was placed in a disposable cuvette and the particle size determined. The intensity of scattered light was transformed into the diffusion factor, the mean value of 10 measurements was obtained and the assay was repeated in triplicate, at 25.0 ± 0.1◦C.

Zeta potential was measured for each volume ratio using a Zetasizer® and all measurements were performed in acetate buffer in triplicate, at 25.0 ± 0.1ºC (Azofeifa et al., 2012; Hejjaji et al., 2017).

Rutin was quantified submitting nanoparticles containing flavonoids-enriched vegetable extracts to ultracentrifugation (Eppendorf, 5417R, Hamburgo, Germany) at 14000 rpm for 30 min (Katas et al., 2013). Supernatant recovered from centrifugation was decanted. The amount of rutin in the supernatant was measured by UV-Vis spectrophotometer (Thermo Scientific, Genesis 10S, Massachussets, United States) at 257 nm in triplicate. The encapsulation efficiency expressed as the percentage of encapsulation efficiency was calculated using the following equation:

$$\text{Encapsulation Efficiency}(\%)=\frac{\text{Total mass of drug}(g)\text{-Mass of drug in supernatant (g)}}{\text{Total mass of drug (g)}}\times 100$$

2.4 Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra of nanoparticles with and without extracts were recorded using a Fourier transform infrared spectrophotometer (Shimadzu Scientific Instruments, Model 8300, Japan), operating from 4000 to 650 cm^{-1}, at resolution of 4 cm^{-1}. Chitosan and TPP powders also characterized by FTIR technique for comparison of data and all measurements were performed in triplicate (Martins et al., 2012).

2.5 Scanning electron microscopy (SEM)

The morphology of particles was analyzed by scanning electron microscopy (SEM) (Tescan, Vega 3 SBH, Shanghai, China). Particles were sputter-coated with a thin layer of gold for allowing the SEM visualization. Images were taken by applying an electron beam accelerating voltage of 5 kV. The particles average diameters were calculated by analysis of SEM images using the software VegaTC©.

2.6 Development and stability study of topical formulation containing extracts nanoparticles

An o/w emulsion (Table 1) was developed using protocol technique consisting of heating both aqueous and oily phases up to 70 ± 3°C, following manual homogenization until 25 ± 3°C.

Table 1: Components of oil-in-water base emulsion (vehicle).

Ingredients (INCI NAME)	**Concentration (%, w/w)**
Sorbitan stearate and sucrose cocoate	8.0
Sucrose palmitate glyceryl stearate and glyceryl stearate citrate and sucrose and manna and xanthan gum	0.5
Caprylic and capric triglyceride	2.0
Hydrolyzed wheat protein and PVP crosspolymer	2.0
Persea Gratissima (Avocado) oil	0.5
Phenoxyethanol	0.5
Glycerin	3.0
Talc	1.5
Aqua	82.0

Vehicle and formulation containing flavonoids-enriched vegetable extracts (200 µg/ml) were subjected to stability study according to Anvisa (2004). Then, approximately 5.0 g of emulsion was submitted to three centrifuge cycles (3000 rpm) for 30 minutes/cycle at 27±2 °C to evaluate physical stability. Organoleptic characteristics (color, odor and appearance), pH, density and viscosity values were also determined.

The pH values were determined using digital pHmeter (Quimis, São Paulo, Brazil). (Davis, 1977). For the density evaluation, an empty pycnometer with 5 ml capacity and a pycnometer with 5 ml of the sample had their weight analytically verified. Density was calculated by the difference between pycnometers weight, dividing by the sample volume

(Idson, 1993). Apparent viscosity was evaluated using a rotational viscometer (Brookfield, Mod LV-T, São Paulo, Brazil) with spindle number 4 and 1.5 rpm rotation for 30 seconds at 27±2 °C (Corrêa et al., 2005). Results are expressed in mPa.s.

Formulations were stored in transparent glass bottles with lid and submitted to stability study applying the following stress conditions: at 25 ± 3ºC; exposure to indirect light; refrigeration (5 ± 3ºC); thermostatic oven (45 ± 0.5°C); freezer (-5 ± 3°C) and alternating cycles of cooling and heating (24 hours at 45 ± 2°C, and 24 hours at -5 ± 2°C), for 15 days (preliminary stability) and 90 days (accelerated stability) (Anvisa, 2004).

Organoleptic (appearance, color and odor) and physicochemical (pH, viscosity and density) characteristics were also evaluated.

Stability study was also conducted using the multisample analytical centrifuge LUMiSizer (LUM, GmbH, Germany). Formulations were diluted in distilled water (1:5; w/w) and evaluated for 2 hours at 3000 rpm, 27.5±0.5°C (Badolato et al., 2008).

2.7 Zeta potential of formulation with and without nanoparticles containing flavonoids-enriched vegetable extracts

Vehicle and formulation containing nanoparticles with flavonoids-enriched vegetable extracts were diluted in distilled water (1:500; w:w) prior to analysis by Zetasizer® (Malvern Instruments Ltd., Malvern, United Kingdom). About 1 ml of samples diluted was placed in a disposable cuvette and the zeta potential was determined. The intensity of scattered light was transformed into the diffusion factor, the mean value of 10 measurements was obtained and this assay was also repeated in triplicate, at 25.0 ± 0.1◦C (Roland et al., 2003; Hejjaji et al., 2017).

2.8 Texture analysis

Textural analysis was performed in the compression mode using a texture analyzer from Stable Micro Systems TA-XT2i (United Kingdom) by carrying out a penetration test using a load cell of 5kg, a cylindrical probe (SMS P/1R), a penetration depth of 5mm, test speed of 3 mm/s and a trigger force of 0.050 N. Gel strength (G), Rupture strength (G), Brittleness (mm) and Adhesiveness (g.sec) were calculated. All measurements were performed in triplicate.

Extrusion evaluation was also performed. In this test, the extrusion cell (HDP/FE) measures the compression force required for a piston disc to extrude a product through a standard size outlet in the base of the sample container. Samples were deposited on cup and compressed by cylinder probe (5 mm). Firmness (G) was determined and all measurements were performed in triplicate (Savary et al., 2013; Estanqueiro et al., 2014).

2.9 *In vitro* sun protection factor (SPF)

In vitro UVAPF, critical wavelength (λc) and UVA/UVB ratio were determined according to ISO24443:2012 protocol (ISO, 2012) by spectral transmittance (Labsphere® UV-2000S Ultraviolet Labsphere Halma Company, Sao Paulo, Brazil) at 290-400 nm.

2.10 *In vitro* release and permeation studies

For the release assay, synthetic cellulose membranes (Millipore, 0.45 μm) were placed on Franz cells in contact with receptor medium (phosphate buffer, pH 7.2). An amount of 200 mg of formulation (nanoparticles dispersed in emulsion O/W) were added on membranes and the receptor medium was stirred at 200 rpm at 37±1ºC.

For the skin permeation assay, pig skin including hair was cleaned immediately following acquisition, stored at -5±2ºC and used after 24 hours.

Skins were placed on Franz cells with dermis in contact with the receptor medium. The assay was conducted following the same procedure as described for the release assay.

The receptor medium was sampled after 1, 2, 4 and 6 hours for the release assay and after 2, 4, 8, 12 and 24 hours for the permeation assay and rutin was determined by HPLC. For the quantification of rutin, aliquots of 5 µl of each sample were injected in HPLC-DAD instrument (Agilent, Technologies 1250 infinity), using monomeric chromatographic column C_{18} (Phenomenex), flow rate of 0.3ml.min^{-1} for 10 minutes. Mobile phase used was methanol grade HPLC acidified with 0.1% (v/v) formic acid (Sigma), at 27 ± 1°C and rutin was identified at 257nm (Seal, 2016).

At the end of experiment, skins were removed from the diffusion cells, the excess of formulation was removed using distilled water and skins were dried with absorbent paper. Skins were place on surface and the *stratum corneum* (SC) was removed using tape stripping method, which consists of 20 stripping using adhesive tape (Dsquame D100, 22mm - Monaderm, Mônaco).

The tapes were transferred to test tubes containing 4 ml of methanol and tubes were subjected to three cycles of shaking on agitator for one minute and sonication for 30 minutes in ultrasound bath. The supernatant was analyzed by HPLC-DAD.

Skin samples without *stratum corneum* were cut and transferred to tubes containing 4 ml of methanol and tubes were submitted to the same shaking and sonication process to tapes and the supernatant was analyzed by using HPLC-DAD in triplicate.

All assays were carried out in sextuplicate using vehicle as the blank control (Alencastre et al., 2006).

2.11 Photostability study

The assay was performed using a solar simulator (CPS+, 1012014, Atlas Material Testing Solutions), a sensor for calibration of irradiance (SunCal BB 300-400 BST, 1603001, Atlas Material Testing Solutions) and a UV transmittance analyzer (Labsphere® UV-2000S).

PMMA substrate containing formulations (HELIOPLATE HD 6 - 50 mm x 50 mm, 6 microns) were irradiated for 90 min in the solar simulator with radiation intensity of 1.2 J cm^2 (ISO, 2012; Mansur et al., 2016).

Plates were stored in the absence of light without being irradiated, as a negative control. The test was performed in quadruplicate and the approved criteria was confidence interval ≤ 17% according ISO24443:2012 protocol (ISO, 2012).

2.12 Statistical analysis

All assays were performed in triplicate. Statistical analysis was performed using ANOVA test (p<0.05) for independent variables and Origin version 8 software for Windows.

3. RESULTS AND DISCUSSION

3.1 Preparation of nanoparticles containing vegetable extracts rich in flavonoids

Chitosan is receiving increasing attention by the pharmaceutical and cosmetic industries owing to its biocompatibility, low toxicity, and biodegradability and the degradation products of chitosan are nontoxic, non-immunogenic, and non-carcinogenic (Sinha, 2004; Sezer et al., 1999).

In addition, according Nallamuthu et al. (2015), chitosan is one of the widely used encapsulating agent, and several reports on chitosan-based encapsulation have been published for polyphenolic compounds including quercetin (Kumari et al., 2010), gallic acid (Rosa et al., 2013), catechin and epigallocatechin gallate (Dube et l., 2012), procyanidins (Zou et al., 2012), ferulic acid (Woranuch et al., 2013) and tea polyphenols (Liang et al., 2011).

According to literature (Dyer et al., 2002; Janes et al., 2001; Morris et al., 2011; Souto et al., 2014), the nanoparticles formation can be achieved by ionic cross-linking between chitosan (being cationic), and either negatively charged macromolecules or anionic cross-linking agents. For this, acidic solution of chitosan is prepared, and the ionic cross-linker such as tripolyphosphate (TPP), a non-toxic multivalent polyanion, is added dropwise along with stirring and sonication.

This process is called ionic gelation, also known as ionotropic gelation. Ionic cross-linking can occur inside the interactions between the negative charges of the cross-linker such as TPP and the positively charged amino groups of chitosan molecules (Morris et al., 2011; Shu and Zhu, 2000). In addition, this method has several advantages as the reaction is simple, the conditions are relatively mild and do not require the use of organic solvents or high temperatures (Rampino et al., 2013; Sailaja et al.; 2010). This method has also been recommended for the loading of small hydrophilic peptides as insulin (Jose et al., 2012; Jose et al., 2013).

The temperature was also reported to influence the degree of cross-linking and loading capacity of chitosan microparticles (Ibezim et al (2011). Indeed, when increasing the temperature, a decrease on the degree of cross-linking was observed which compromised the loading capacity of the particles for the tested drug. The encapsulation

efficiency was shown to improve when cross-linking occurred at ambient temperature rather than at higher temperatures.

Chitosan/TPP ratio is also an important parameter to obtain micro or nanoparticles, because an increase in the zeta potential, size and polydispersity can be observed when increasing the volume ratio of chitosan/TPP solutions (Hejjaji et al., 2017; Gan et al., 2005; Li and Huang, 2012, Hassani et al., 2015).

According to Hassani et al. (2015), when increasing the concentration of chitosan, the increase of zeta potential was observed, attribute to the increased number of positively charged groups on the surface leading to a strong electrostatic repulsion between particles (Li and Huang, 2012). The opposite occurs when increasing the concentration of TPP, which decreases the zeta potential values attributed to the neutralizing effect of the negatively charged phosphate ions which react with chitosan amino groups. The mean nanoparticle size increased when increasing TPP concentration (Hassani et al., 2015).

In our study, a chitosan/TPP ratio of 2:1 was used, according to the optimization of chitosan/extract ratio, using TPP proportion was determined based on Chabib et al. (2011), and assay was performed at 25 ± 3ºC. Solution containing chitosan/TPP in buffer acetate showed uniform appearance, contributing for the production of nanoparticles of relatively low polydispersion, as shown by Sawtarie et al., 2017, who reported polydispersity index (PDI) values of 0.38 ± 0.25. When loaded with flavonoids-enriched vegetable extracts, nanoparticles suspension exhibited yellowish color attributed to the presence of flavonoids in extracts (Mann, 1987), uniform appearance and PDI of 0.39 ± 0.27, thus indicating a narrow particle size distribution (PDI < 0.5).

3.2 Determination of average particle size, zeta potential and encapsulation efficiency (EE)

Non-loaded chitosan/TPP nanoparticles and chitosan/TPP nanoparticles containing flavonoids-enriched vegetable extracts exhibited zeta potential values of -13.14 ± 2.9 mV and -11.54 ± 2.1 mV, respectively. These results show that flavonoids-enriched vegetable extracts lead to a minor reduction of the surface electrical charge of the particles. Previous studies report similar data in which natural materials decrease zeta potential of chitosan/TPP particles (Kailaku et al., 2014; Nallamuthu et al., 2015; Mohammadpour Dounighi et al., 2012).

The mean particle size was also evaluated and the results obtained by dynamic light scattering (DLS) non-loaded chitosan/TPP nanoparticles and chitosan/TPP nanoparticles containing flavonoids-enriched vegetable extracts were 932 ± 3.4 nm and 557.11 ± 3.1 nm, respectively. The decrease of particles size when loading vegetable compounds has been previously reported by Nallamuthu et al. (2015).

The encapsulation efficiency (EE), which translates the amount of rutin (flavonoid found in flavonoids-enriched vegetable extracts) encapsulated in the nanoparticles over the total amount of rutin weighted for the production, was found to be 71.43%, which is in line with values reported in the literature Chabib et al. (2012).

3.3 Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra of nanoparticles with and without flavonoids-enriched vegetable extracts are shown in Figures 1 and 2, respectively. Similar peaks have been recorded in both spectra (Table 2).

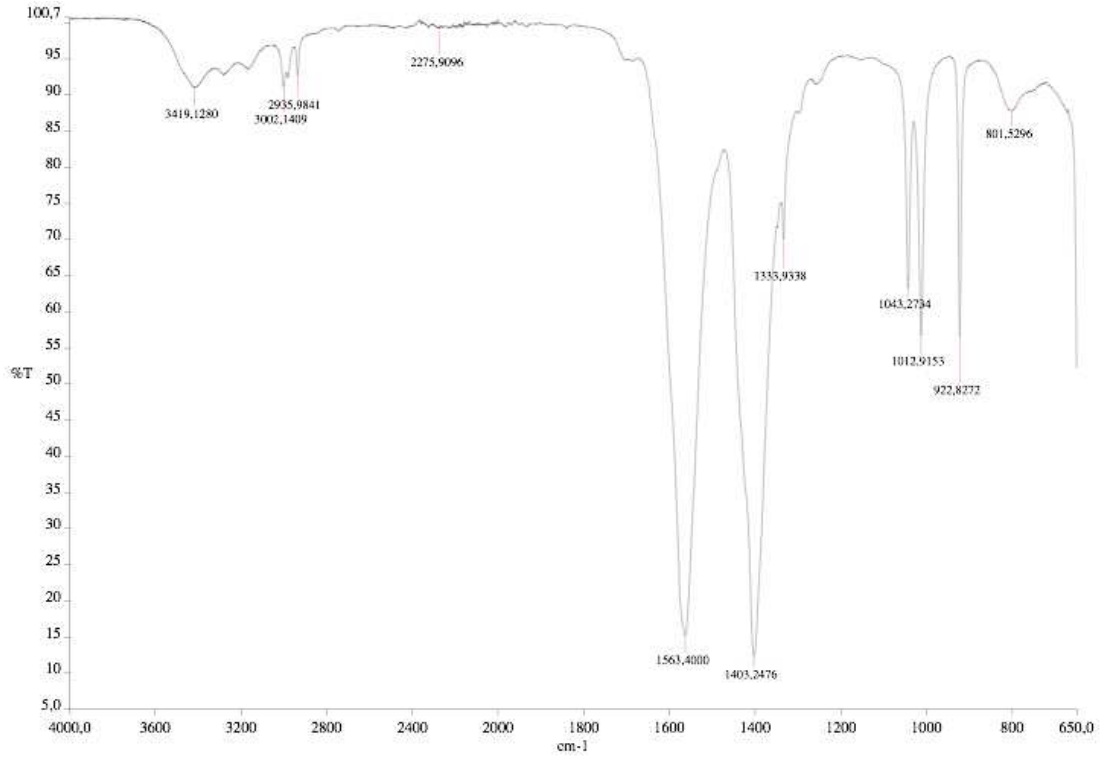

Figure 1: FTIR spectra of nanoparticles without flavonoids-enriched vegetable extracts.

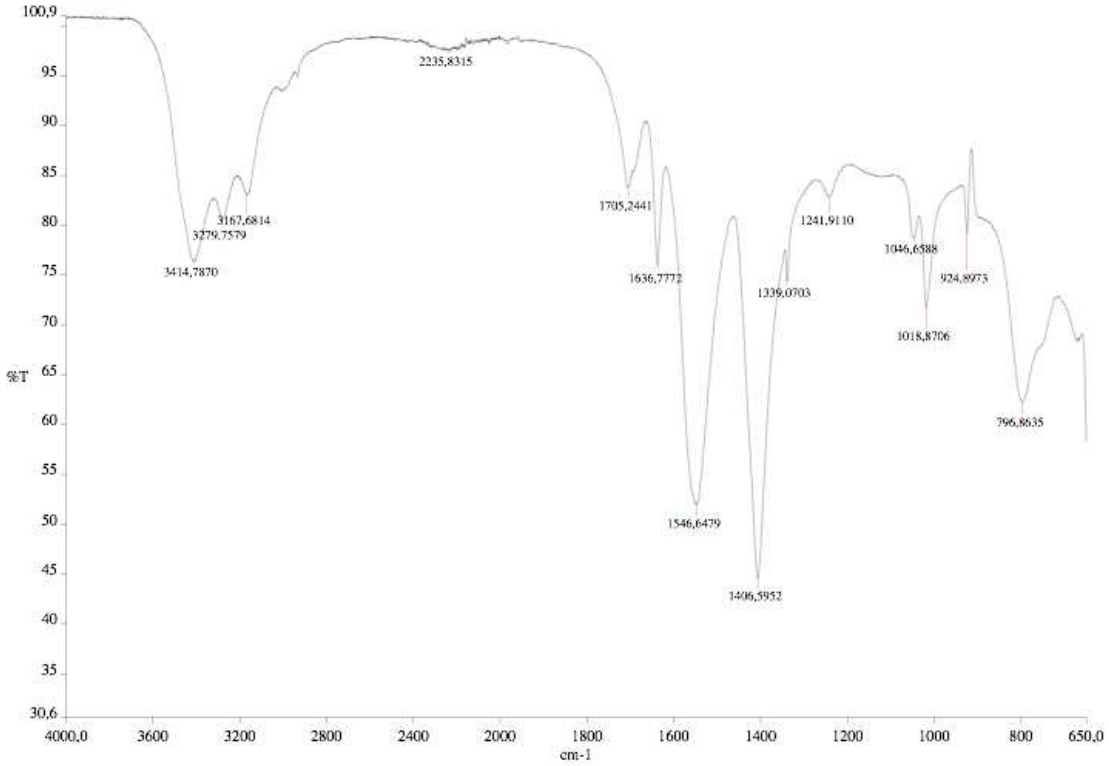

Figure 2: FTIR spectra of nanoparticles containing flavonoids-enriched vegetable extracts.

Table 2: FTIR spectra peaks of nanoparticles with and without flavonoids-enriched vegetable extracts.

	O-H	C-O-C	C-N	N-H
Nanoparticles without extracts	3419.12	1043.27	1333.90	1563.40
Nanoparticles with extracts	3414.78	1046.65	1339.07	1546.64

The peak recorded in the 3500-3300 range is attributed to hydrogen-bonded O-H stretching vibration. According Yu et al. (1999), peaks of N-H stretching from primary amine and type II amide are overlapped in the same region.

Peaks for C-N stretching vibration of type I amine are found at around 1317 cm^{-1}. In nanoparticles with flavonoids-enriched vegetable extracts the tip of the peak of 3438 cm^{-1} has a shift to 3279 cm^{-1} and becomes wider with increased relative intensity indicating an enhancement of hydrogen bonding. In nanoparticles, the peaks for N-H bending vibration of amine I at 1600 cm^{-1} shifted to 1563 cm^{-1} (without extracts) and 1546 cm^{-1} (with extracts). These results are similar to those found in the literature (Mohammadpour DounighiIn et al., 2012).

Our results allow confirming the formation of nanoparticles and their interaction with flavonoids-enriched vegetable extracts.

3.4 Scanning electron microscopy (SEM)

The morphology of chitosan/TPP nanoparticles without (A) and with (B) flavonoids-enriched vegetable extracts is shown in Fig. 3.

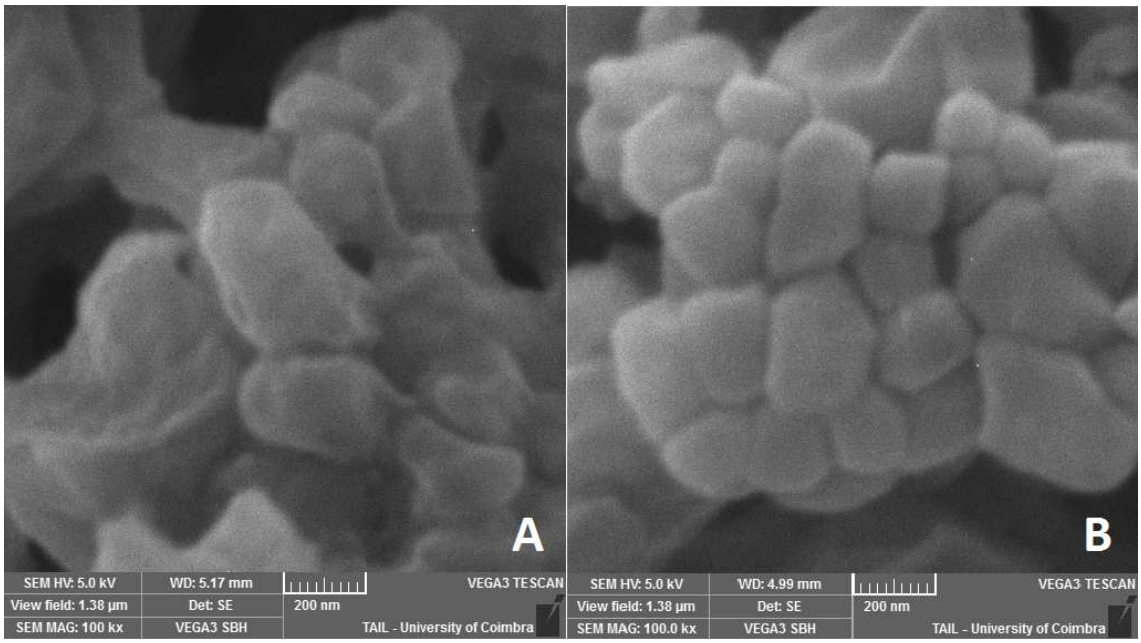

Figure 3: Morphological characteristics of nanoparticles without (A) and with (B) flavonoids-enriched vegetable extracts by SEM at 100kx.

Loading flavonoids-enriched vegetable extracts in chitosan/TPP nanoparticles contributed to the production of particles with a more spherical shape (Figure 3B), than non-loaded particles (Figure 3A). Similar results have also been reported in literature (Shende et al., 2014; Sangeetha et al., 2017; Martins et al., 2012; Salar and Kumar, 2016).

SEM analysis confirmed the results obtained by DLS (section 3.2), i.e. the loading of flavonoids-enriched vegetable extracts contributed to generate particles of lower size (ca. 500 nm) against non-loaded particles (ca. 800 nm).

3.5 Development and stability study of topical formulation containing extracts nanoparticulate

Emulsions containing low fatty material concentration can be considered a smooth formulation, minimizing the oil sensation when applied onto the skin.

Emulsion (vehicle) presented creamy aspect, white color, pH 6.62 ±0.5, density of 1.0 ± 0.1 g/ml and viscosity of 46,000±1.56 mPa.s.

Formulation containing nanoparticles with flavonoids-enriched vegetable extracts presented creamy, shiny appearance and slightly yellowish color, owing to the presence of extracts. It showed characteristic odor, pH 6.67±0.10, compatible with the skin (Raab and Kindl, 1999), density of 0.97±0.00 g/ml, being close to 1.00 g/ml evidencing low air quantity incorporated in formulation during its preparation, and viscosity of 46,000±1.09 mPa.s, which are desirable characteristics for the topical use (Anvisa, 2004).

The presence of loaded nanoparticles did not influence significantly (p<0.05) the physicochemical properties of the emulsion such as pH, density and viscosity.

Formulation did not suffer phase separation by centrifugation assay and then was subjected to stability study.

After 90 days of storage under stress conditions (room temperature; exposure to indirect light; at 5 ± 3ºC, 45 ± 0.5°C and -5 ± 3°C), formulation presented stable (p<0.05), pH 6.66±0.48, density 0.97±0.10 g/ml and viscosity around 46,000±2.3 mPa.s in all conditions. Color and odor did not change evidencing that nanoencapsulation of flavonoids-enriched vegetable extracts protected extracts from oxidation by heat when stored in oven (at 45 ± 0.5°C). It is commonly reported that, when stored at high temperatures, formulations containing vegetable compounds may darken due to the presence of antioxidant molecules (Figueiredo et al., 2014; Hubinger et al., 2010; Veberic et al., 2009).

Stability of formulation was also evaluated using an analytical centrifuge Lumisizer allowing stability parameters such as sedimentation velocity and shelf life prediction to be directly calculated (Lerche, 2002). Both formulations exhibited similar

sedimentation behavior and the transmission percentage was around 88% at position 115 mm and around 8% at position 120 mm after the measuring time.

3.6 Zeta potential of formulations with and without nanoparticles containing flavonoids-enriched vegetable extracts

Samples presented zeta potential values of +35.73 ± 1.6 and +36.53 ± 2.3, respectively, before and after loading the particles with flavonoids-enriched vegetable extracts. These results are in accordance with the literature (Wiacek and Chibowski, 2002; Jeong et al., 2001), which report that the surface potential of about |30 mV| for emulsions is recommended to prevent flocculation and coalescence, contributing to the stabilization of nanoparticles.

The loading of nanoparticles with flavonoids-enriched vegetable extracts did not influence their surface electrical charge.

3.7 Texture analysis

Freshly prepared emulsion without particles and the topical formulation containing nanoparticles with flavonoids-enriched vegetable extracts were subjected to texture analysis to characterize the mechanical and physical properties of semisolid systems (Gilbert et al., 2013; Toksoy et al., 2013) such as strength, brittleness, adhesiveness and firmness. Results are showed in Table 3.

Table 3: Texture analysis of vehicle and formulation containing flavonoids-enriched vegetable extracts encapsulated.

Formulations	Rupture Strength (G)	Brittleness (mm)	Adhesiveness (G.s)	Firmness (G)
Vehicle	3.62 ± 1.31	19.05 ± 0.76	-50.18 ± 0.63	633.26 ± 0.11
Formulation with nanoparticles	2.94 ± 2.43	14.11 ± 1.17	-55.69 ± 1.53	508.32 ± 0.10

Semi-solid formulations exhibited high firmness attributed to presence of polymeric sucrose palmitate glyceryl stearate/ glyceryl stearate citrate/ sucrose/ manna/xanthan gum, enhancing the stability of the semisolid and offering the desirable properties for topical application (as low rupture strength and adhesiveness). In addition, higher brittleness values representing a lower force needed to promote their application on skin.

3.8 *In vitro* sun protection factor (SPF)

In vitro sun protection factor of semi-solid formulation containing nanoparticles with flavonoids-enriched vegetable extracts was determined following the method described by Mansur (Mansur et al., 2016) and by diffuse reflectance spectroscopy (ISO, 2012). SPF values were 2.94 ± 0.4 and 2.3 ± 0.4 (UVAPF), respectively. Nanoparticles-free emulsion was used as blank and not presented SPF value by methods performed.

In this assay, the critical wave-length and UVA/UVB ratio of formulation were also determined. Results showed values of 387.0 nm and 0.69, respectively, which confirm that, according to COLIPA (2006), our developed formulation can be used as sun filter for UVA/UVB protection.

3.9 *In vitro* release and permeation studies

The release profile and permeation capacity of the particles were evaluated by quantifying the amount of rutin by HPLC-DAD. Topical formulation released rutin in all time points (1, 2, 4 and 6 hours) (Fig.4A).

After 8 hours of assay, the permeated amount of rutin decreased (Fig. 4B) being retained in the upper layer of the skin. The limited capacity of glycosides flavonoids such as rutin in permeating the skin has been is attributed to their amphiphilic character, as described by Chuang et al. (2017).

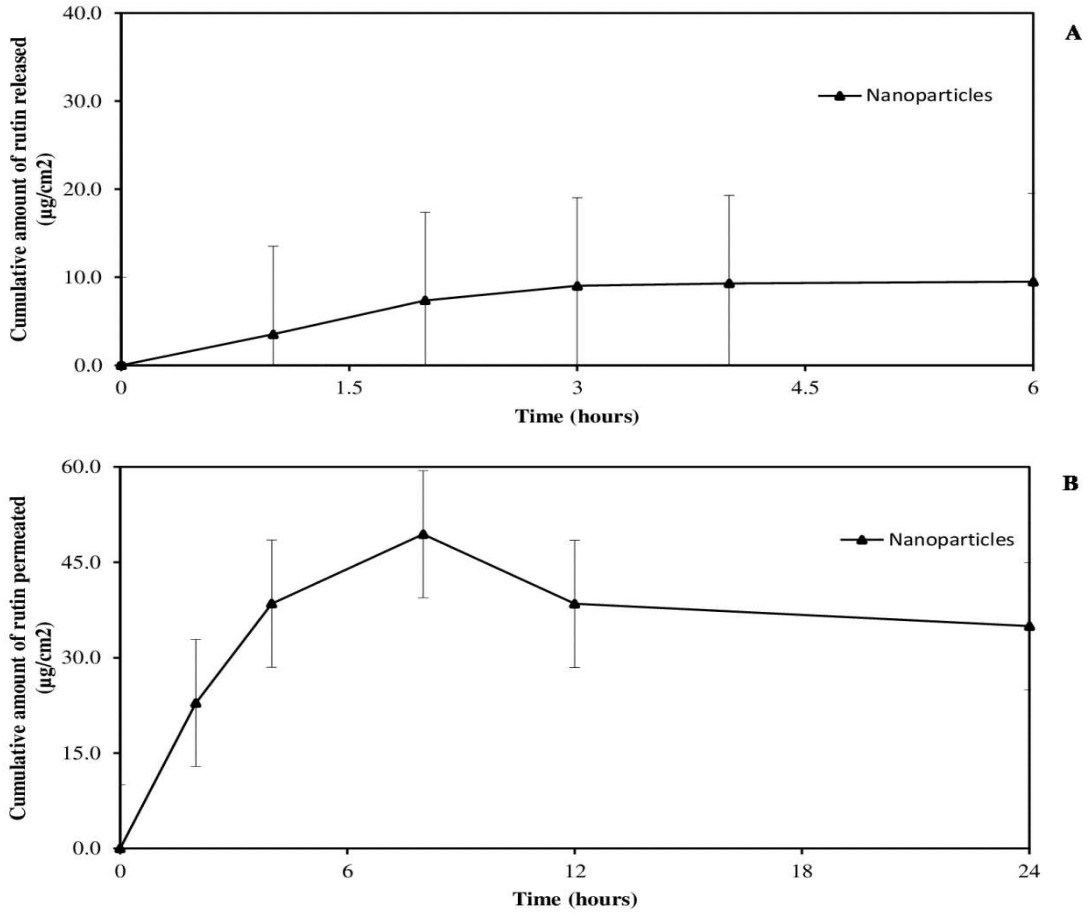

Figure 4: Release (A) and permeation (B) assays of formulation containing nanoparticles of chitosan/TPP with flavonoids-enriched vegetable extracts rich in flavonoids during 1, 2. 3, 4 and 6 hours using synthetic membrane to release test and 2, 4, 8, 12 and 24 hours using pig skin as membrane to permeation test by cell Franz method.

Although rutin permeated the skin, a higher concentration was observed in retention test (91.52 ± 3.94 μg/ml) and in *stratum corneum* (1.26 ± 0.20 μg/ml) by tape stripping test. This result emphasizes the added value of our formulation for topical application of sunscreens, for which no absorption should be observed. Flavonoids should remain on the skin and/or retained onto the surface layers of the skin, protecting it against UV radiation and the damage action of free radicals.

Gold nanoparticles (Gupta and Rai, 2016) and lipid nanoparticles (Han et al., 2014) have been reported to increase the skin permeation of flavonoids (e.g. quercetin). Our formulation showing higher permeation may be however recommended for anti-aging, for which neutralizing free radicals is required.

3.10 Photostability study

To ensure efficacy and safety of sun filters, photostability study was performed and formulation containing nanoparticles, after to subjection of UVA radiation showed decrease of UVAPF value (2.0 ± 0.3) down to 13.04%.

This outcome was expected because of the antioxidant properties of flavonoids, however it is considered a positive result because sunscreen should remain around 80% of its SPF value, classifying the product as photostable (Hojerova et al., 2011).

4. CONCLUSIONS

Flavonoids-enriched vegetable extracts can be loaded by chitosan/TPP nanoparticles and formulation with nanoparticles showed SPF value and photostability by *in vitro* tests being a promising to product to use to protecting skin against damage from solar radiation.

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6. REFERENCES

1) AJAZUDDIN, S.S. Applications of novel drug delivery system for herbal formulations. *Fitoter*. v. 81, n.7, p. 680-689, 2010.

2) ALENCASTRE, J.B.; BENTLEY, M.V.L.B.; GARCIA, F.S.; MORAGAS, M.; VILADOR, J.L.; MARCHETTI, J.M. A study of the characteristics and *in vitro* permeation properties of CMC/ chitosan microparticles as a skin delivery system for vitamin E. *Rev. Bras. Ciênc. Farm.***,** v. 42, n.1, p. 69-76, 2006.

3) AVRANAS, A.; STALIDIS, G.; RITZOULIS, G. Demulsification rate and zeta potential of O/ W emulsions. *Colloid Polym Sci*, v.266, n.1, p.937-940,1988.

4) AZOFEIFA, D.E.; ARGUEDAS, H.J.; VARGAS, W.E. Optical Properties of Chitin and Chitosan Biopolymers with Application to Structural Color Analysis. *Opt. Mater*. v.35, n.1, p. 175–183, 2012.

5) BALOGH, T.S.; VELASCO, M.V.R.; PEDRIALI, C.A.; KANEKO, T.M.; BABY, A.R. Ultraviolet radiation protection: current available resources in photoprotection. *An. Bras. Dermatol.*, v. 86, n.4, p.732-742, 2011.

7) BADOLATO, G.G.; AGUILAR, F.; SCHUCHMANN, H.P.; SOBISCH, T.; LERCHE, D. Evaluation of long term stability of model emulsions by multisample analytical centrifugation. *Progr. Colloid Polym. Sci.* v.134, n.1, p. 66 – 73, 2008.

8) BONNABRY, C. Mise au point et validation de modeles d'etude *in vitro* du passage percutane, Universite Dauvergne – UFR de Pharmacie, 1999.

9) BRASIL, *Guia de estabilidade de produtos cosméticos***.** Brasília: Núcleo de Assessoramento em Comunicação Social e Institucional, 2004, p.12-19, 26,27.

10) CEFALI, L.C.; SOUZA-MOREIRA, TATIANA MARIA DE; CORRÊA, M. A.; SALGADO, H. R. N.; ISAAC, V. L. B. Development and evaluation of an emulsion

containing lycopene for combating acceleration of skin aging. *Braz. J. Pharm. Sci*. v.51, n.3, p. 579-590, 2015.

11) CEFALI, L.C.; ATAIDE, J.A.; FOGLIO, M.A., MORIEL, P.; MAZZOLA, P.G. Plant compounds as active photo protectants in sunscreens. *Inter. J. Cosm. Sci*., v.1, p.1-8, 2016.

12) CHABIB, L.; MARTIEN, R.; ISMAIL, H. Formulation of nanocurcumin using low viscosity chitosan polymer and its cellular uptake study into T47D cells. *Indonesian J. Pharm*. v. 23, n.1, p. 27 –35, 2011.

13) CHIARI, B.G.; TROVATTI, E.; PECORARO, T.; CORRÊA, M.A.; CICARELLI, R.M.B.; RIBEIRO, S.J.L.; ISAAC, V.L.B. Synergistic effect of green coffee oil and synthetic sunscreen for health care application. *Ind. Crop. Prod.* v.52, n.1, p.389-393, 2014.

14) CHUANG, S.Y.; LIN, C.F.; WANG, P.W.; CHEN, E.L.; FANG, J.Y. Elucidating the skin delivery of aglycone and glycoside flavonoids: How the structures affect cutaneous absorption. *Nutrients*, v.9, n.12, p.1304, 2017.

15) COLIPA. Colipa Project Team IV. Method for the in vitro determination of UVA protection provided by sunscreen products. Guideline, 2006.

16) COOMAN, L. DE; EVERAERT, E.; DE KEUKELEIRE, D. Quantitative analysis of hop acids, essential oils and flavonoids as a clue to the identification of hop varieties. *Phytochem. Anal.,* v.9, n.1, p.145-150, 1998

17) CORREA, N. M.; CAMARGO JUNIOR, F. B.; IGNÁCIO, R. F.; LEONARDI, G. R. Avaliação do comportamento reológico de diferentes géis hidrofílicos. *Braz.J. Pharm. Sci.* v. 41, n. 1, p. 73-78, 2005.

18) DAL BELO, S.E., GASPAR, L.R. e CAMPOS, P.M.B.G.M. Photo protective Effects of Topical Formulations Containing a Combination of Ginkgo biloba and Green Tea Extract*s. Phyt. Res.* v.25, n.1, p.1854–1860. 2011.

19)DAVIS, H.M. Analysis of creams and lotion. In: Newburguer's manual of cosmetic analysis. Washington: Association of Official Analytical Chemists, 1977, p.32.

20) DUBE, A.; NICOLAZZO, J.A.; LARSON, I. Chitosan nanoparticle enhance the plasma expo- sure of e epigallocatechin gallate in mice through an enhancement in intestinal stability. *Eur. J. Pharm. Sci.* v.44, n.1, p.422-426, 2011.

21) DYER, A.M.; HINCHCLIFFE, M.; WATTS, P.; CASTILE, J.; JABBAL-GILL, I.; NANKERVIS, R.; SMITH, Q.; ILLUM. L. Nasal delivery of insulin using novel chitosan-based formulations: a comparative study in two animal models between simple chitosan formulations and chitosan nanoparticles. *Pharm. Res. v.*19, n.7, p.998–1008, 2002.

22) ECOCERT. Organismo de inspeção e certificação. Ecocert Brasil. Available in: http://www.brazil.ecocert.com/politicas-e-diretrizes-ecocert. Access in Jun 01st, 2017.

23) ESTANQUEIRO, M.; CONCEIÇÃO, J.; AMARAL, M.H.; SANTOS, D.; SILVA, J.B.; LOBO, J.M.S. Characterization and stability studies of emulsion systems containing pumice. *Braz. J. Pharm. Sci.* v.50, n.2, p.361-369, 2014.

24) FELDMAN, K.A. Cytochrome P450s as genes for crop improvement. *Curr Opin Plant Biol* v.4, n.1, p.162-167, 2001.

23) FIGUEIREDO, S.A.; VILELA, F.M.; DA SILVA, C.A.; CUNHA, T.M.; DOS SANTOS, M.H.; FONSECA, M.J. *In vitro* and *in vivo* photoprotec tive/photochemopreventive potential of *Garcinia brasiliensis* epicarp extract. *J. Photochem. Photobiol. B.* v.5, n.31, p.65-73, 2014.

24) GAN, Q.; WANG, T.; COCHRANE, C.; MCCARRON, P. Modulation of surface charge, particle size and morphological properties of chitosan-TPP nanoparticles intended for gene delivery. *Coll. Surf B Biointer.* v.44, n.2-3, p.65-73, 2005.

25) GILBERT, L.; PICARD, C.; SAVARY. G.; GRISEL, M. Rheological and textural characterization of cosmetic emulsions containing natural and synthetic polymers: relationships between both data, *Coll. Sur. A*. v.421, n.1, p.150-163, 2013.

26) GUPTA, R.; RAI, B. Penetration of Gold Nanoparticles through Human Skin: Unraveling Its Mechanisms at the Molecular Scale. *J. Phys. Chem. B*. v.120, n.1, p.7133−7142, 2016.

27) HAN, S.B.; KWON, S.S.; JEONG, Y.M.; YU, E.R.; PARL, S.N. Physical characterization and in vitro skin permeation of solid lipid nanoparticles for transdermal delivery of quercetin. *Int. J. Cosmet. Sci.* v.36, n.6, p.588-597, 2014.

28) HASSANI, S.; LAOUINI, A.; FESSI, H.; CHARCOSSET, C. Preparation of chitosan–TPP nanoparticles using micro engineered membranes – Effect of parameters and encapsulation of tacrine. *Coll. Surf. A Phys. Eng. Asp*. v.482, n.1, p.34-43, 2015.

29) HEJJAJI, E.M.; SMITH, A.M.; MORRIS, G.A. Designing chitosan-tripolyphosphate microparticles with desired size for specific pharmaceutical or forensic applications. *Int. J. Biol. Macromol.* v.95, n.1, p.564-573, 2017.

30) HOJEROVÁ, A.; MEDOVCÍKOVÁ, A.; MIKULA, M. Photoprotective efficacy and photostability of fifteen sunscreen products having the same label SPF subjected to natural sunlight. *Inter. J. Pharm.* v.408, n.1, p.27–38, 2011.

31) HUBINGER, S. Z.; CEFALI, L. C.; VELLOSA, J. C. R.; SALGADO, H. R. N.; ISAAC, V. L. B.; MOREIRA, R. R. D. *Dimorphandra mollis*: an alternative as a source of flavonoids with antioxidant action. *Lat. Am. J. Pharm.* v. 29, n. 2, p. 271-274, 2010.

32) HUONG, S.P.; ROCHER, E.; FOURNERON, J.D.; CHARLES, L.; MONNIER, V.; BUN, H.; ANDRIEU, V. Photoreactivity of the sunscreen butylmethoxydibenzoylmethane under various experimental conditions. *J. Photochem. Photobiol. A*. v.196, n.1, p.106–112, 2008.

33) IBEZIM, E.C.; ANDRADE, C.T.; MARCIA, C.; BARRETTO, B.; ODIMEGWU, D.C.; DE LIMA, F.F. Ionically cross-linked chitosan/tripolyphosphate microparticles for the controlled delivery of pyrimethamine. *J. Med. Biom. Sci.* v.3, n.1, p.77–88, 2011.

34) IDSON, B. Stability testing of emulsions, I. *Drug & Cosmetics Industry,* v.151, n.1, p.27-28,30, 1993

35) ISO. International Organization of Standardization. ISO 24443:2012. Determination of sunscreen UVA photoprotection *in vitro*. Geneva, Switzerland, 2012.

36) JANES, K.A.; CALVO, P.; ALONSO, M.J. Polysaccharide colloidal particles as delivery systems for macromolecules. *Adv. Drug. Deliv. Rev.* v.47, n.1, p.83–97, 2001.

37) JEONG, Y. I.; CHO, C. S.; KIM, S. H.; KO, K. S.; KIM, S. I.; SHIM, Y. H.; NAH, J. W. Preparation of poly (DL-lactide–co-glycolide) nanoparticles without surfactant. *J. Appl. Poly. Sci*. v.80, n.1, p.2228-2236, 2001.

38) JOSE, S.; FANGUEIRO, J.F.; SMITHA, J.; CINU, T.A.; CHACKO, A.J.; PREMALETHA, K.; SOUTO, E.B. Cross-linked chitosan microspheres for oral delivery of insulin: Taguchi design and *in vivo* testing, *Coll. Surf. B Biointer.* v.92, n.1, p.175–179, 2012.

39) JOSE, S.; FANGUEIRO, J.F.; SMITHA, J.; CINU, T.A.; CHACKO, A.J.; PREMALETHA, K.; SOUTO, E.B. Predictive modeling of insulin release profile from cross-linked chitosan microspheres. *Eur. J. Med. Chem.* v.60, n.1, p. 249–253, 2013.

40) KAILAKU, S.I.; MULYAWANTI, I.; ALAMSYAH, A.N. Formulation of nanoencapsulated catechin with chitosan as encapsulation material. *Proc. Chem*. v.9, n.1, p.235 – 241, 2014.

41) KUMARI, A.; YADAV, S.K.; PAKADE, Y.B.; SINGH, B.; YADAV, S.C. Development of biodegradable nanoparticle for delivery of quercetin. *Coll. Surf. B.* v.80, n.1, p.184-192, 2010.

42) LERCHE, D. Dispersion Stability and Particle Characterization by Sedimentation Kinetics in a Centrifugal Field. *J. Disp. Sci. and Technol*. v.23, n.5, p.699 - 709, 2002.

43) LI, J.; HUANG, Q. Rheological properties of chitosan–tripolyphosphate complexes: From suspensions to microgels. *Carb. Polym.* v. 87, n.2, p.1670-1677, 2012.

44) LIANG, D.; ZHOU, Q.; GONG, W.; WANG, Y.; NIE, Z.; HE, H.; LI, J.; WU, J.; WU, C.; ZHANG, J. Studies on the antioxidant and hepatoprotective activities of polysaccharides from Talinum triangulare. *J. Ethnopharmacol.* v. 136, n. 1, p. 316-321, 2011.

45) MANN, J. Secondary metabolism. Oxford: Clarendon Press; 1987. p.374.

46) MARTINS, J.T; CERQUEIRA, M.A.; BOURBON, A.I.; PINHEIRO, A.C.; SOUZA, B.W.S.; VICENTE, A.A. Synergistic effects between k-carrageenan and locust bean gum on physicochemical properties of edible films made thereof. *Food Hydrocoll*. v.29, n.1, p.280-289, 2012.

47) MOHAMMADPOUR DOUNIGHI, N.; ESKANDARI, R.; AVADI, M.R.; ZOLFAGHARIAN, H.; MIR MOHAMMAD SADEGHI, A.; REZAYAT, M. Preparation and *in vitro* characterization of chitosan nanoparticles containing *Mesobuthus eupeus* scorpion venom as an antigen delivery system. *J. Venom. Anim. Toxins. Incl. Trop. Dis*. v.18, n.1, p.44-52, 2012.

48) MORRIS, G.A.; CASTILE, J.; SMITH, A.; ADAMS, G.G.; HARDING, S.E. The effect of pro- longed storage at different temperatures on the particle size distribution of tripolyphosphate (TPP)-chitosan nanoparticles. C*arbohydr. Polym*. v.84, n.1, p.1430–1434, 2011.

49) NALLAMUTHU, I.; DEVI, A.; KHANUM, F. Chlorogenic acid loaded chitosan nanoparticles with sustained release property, retained antioxidant activity and enhanced bioavailability. *Asian J. Pharm. Sci.* v.10, n.3, p.203-211, 2015.

50) PAUL, N.; BIGGS, S.; EDMONDSONB, M.; HUNTER, T.N.; HAMMONDA, R.B. Characterizing highly active nuclear waste simulates. *Chem. Eng. Res. Des.* v.91, n.1, p.742-751, 2013.

51) RAMPINO, A.; BORGOGNA, M.; BLASI, P.; BELLICH, B.; CESARO, A. Chitosan nanoparticles: preparation, size evolution and stability. *Int. J. Pharm*. v.455, n.1-2, p.219–228, 2013.

52) ROLAND, I.; PIEL, G.; DELATTRE, L.; EVRARD, B. Systematic characterization of oil-in- water emulsions for formulation design. *Int. J. Pharm.* v.16, n.1-2, p.85-94, 2003.

53) ROMANHOLE, R.C.; ATAIDE, J.A.; CEFALI, L.C.; MORIEL, P.; MAZZOLA, P. Photostability study of commercial sunscreens submitted to artificial UV irradiation and/or fluorescent radiation. *J. Photochem. Photobiol. B: Biol*. v.162, n.1, p.45-49, 2016.

54) ROSA, C.G.; BORGES, C.D.; ZAMBIAZI, R.C.; NUNES, M.R.; BENVENUTTI, E.V.; DA LUZA, S.R.; D'AVILA, R.F.; RUTZ, J.K. Microencapsulation of gallic acid in chitosan, b-cyclodextrin and xanthan. *Ind. Crops Prod.* v.46, n.1, p.138-146, 2013.

55) SAILAJA, A.K.; AMARESHWAR, P.; CHAKRAVARTY, P. Chitosan nanoparticles as a drug delivery system. *Res. J. Pharm. Biol. Chem. Sci.* v.1, n.3, p. 474–484, 2010.

56) SALAR, R.K.; KUMAR, N. Synthesis and characterization of vincristine loaded folic acid– chitosan conjugated nanoparticles. *Resour. Efficien. Technol.* v.2, n.1, p.199–214, 2016.

57) SANGUETHA, J.; THANGADURAI, D.; HOSPET, R.; PURUSHOTAHAM, P.; KAREKALAMMANAVAR, G.; MUNDARAGI, A.C.; DAVID M.; SHINGE, M.R.; THIMMAPPA, S.C.; PRASAD, R.; HARISH, E.R. Agricultural nanotechnology: concepts, benefits and risks. Springer Nature Singapore Pte Ltd., Singapore, pp.1-17, 2017.

58) SAVARY, G.; GRISEL, M.; PICARD, C. Impact of emollients on the spreading properties of cosmetic products: a combined sensory and instrumental characterization. C*oll. Surf. B Bioint.* v.102, n.1, p.371-378, 2013.

59) SAWTARIE, N.; CAI, Y.; LAPITSKY, Y. Preparation of chitosan/tripolyphosphate nanoparticles with highly tunable size and low polydispersity. *Coll. Surf. B. Bioint.* v.157, n.1, p. 110-117, 2017.

60) SCALIA, S.; MEZZENA, M. Photostabilization effect of quercetin on the UV filter combination, butyl methoxydibenzoylmethane-octyl methoxycinnamate. *Photochem. Photobiol.* v.86, n.2, p.273-278, 2010.

61) SEAL, T. Quantitative HPLC analysis of phenolic acids, flavonoids and ascorbic acid in four different solvent extracts of two wild edible leaves, *Sonchus arvensis* and *Oenanthe linearis* of North-Eastern region in India. *J. Appl. Pharm. Sci*. v.6, n.2, p. 157-166. 2016.

62) SEZER, A.D.; AKBUGA, J. Release characteristics of chitosan treated alginate beads: I. Sustained release of a macromolecular drug from chitosan treated alginate beads. *J. Microencapsul. v.*16, n.2, p.195 – 203, 1999.

63) SHENDE, P.T.; YADAVA, S.K.; PATIL, P.S. Development and characterization of chitosan nanoparticles containing erthromycin estolate. *Int. J. Pharm. Appl.* v.5, n.1, p 1-7, 2014.

64) SHU, X.Z.; ZHU, K.J. Novel approach to prepare tripolyphosphate/chitosan complex beads for controlled release drug delivery. *Int. J. Pharm.* v.201, n.1, p.51-58, 2000.

65) SINHA, V.R.; SINGLA, A.K.; WADHAWAN, S.; KAUSHIK, R.; KUMRIA, R.; BANSAL, K.; DHAWAN, S. Chitosan microspheres as a potential carrier for drugs. *Int. J. Pharm*. v.274, n. 1-2, p.1-33, 2004.

66) SOUTO, E.B.; SANTANA, M.H.A. Chitosan cross-linked pentasodium tripolyphosphate micro/nanoparticles produced by ionotropic gelation. *Sugar Tech.* v.18, n.1, 49-54, 2014.

67) TOHGE, T.; SOUZA, L.P.; FERNIE, A.R. Current understanding of the pathways of flavonoid biosynthesis in model and crop plants. *J. Exp. Bot*. v.68, n.1, p.4013–4028, 2017.

68) TOKSOY, M.O.; TUGCU-DEMİRÖZ, F.; TIRNAKSIZ FABAD, F. Evaluation of Rheological and Textural Properties of Toothpastes. *J. Pharm. Sci*. v.38, n.3, p.135-141, 2013

69) VEBERIC, R.; JAKOPIC, J.; STAMPAR, F.; SCHMITZER, V. European elderberry (*Sambucus nigra* L.) rich in sugars, organic acids, anthocyanins and selected polyphenols. *Food Chem.* v.114, n.1, p.511–515, 2009.

70) WIACEK, A.E.; CHIBOWSKI, E. Zeta potential and droplet size of n-tetradecane/ethanol (protein) emulsions. *Coll. Surf. B: Biointer.* v.25, n.1, p.55-67, 2002.

71) WORANUCH, S.; YOKSAN, R. Preparation, characterization and antioxidant property of water-soluble ferulic acid grafted chitosan. *Carbohydr Polym.* v.96, n.2, p.495-502, 2013.

72) YU, G.; MORIN, F.G.; NOBES, G.A.R.; MARCHESSAULT, R.H. Degree of acetylation of chitin and extent of grafting PHB on chitosan determined by solid state NMR. *Macromol*. v.32, n.1, p. 518-520, 1999.

73) ZOU, T.; PERCIVAL, S.S.; CHENG, Q. LI, Z.; ROWE, C.A.; GU, L. Preparation, characterization, and induction of cell apoptosis of cocoa procyanidins gelatin-chitosan nanoparticle. *Eur. J. Pharm. Biopharm.* v.82, n.1, p.36-42, 2012.

Discussão geral

O uso de formulações com ação protetora contra a radiação ultravioleta é cada vez mais frequente, devido ao conhecimento da população sobre os efeitos danosos dos raios UVA e UVB na pele, em particular o câncer cutâneo (INCA, 2016), e por isso, há o aumento do interesse dos pesquisadores em encontrarem novas moléculas e/ou produtos que possam ser eficazes e seguros para os consumidos.

A pesquisa em torno dos flavonóides tem sido amplamente encontrada em artigos científicos, explorando o seu uso como filtros solares por apresentarem estrutura molecular favorável para a absorção da radiação ultravioleta, e, consequentemente, favorecendo a proteção à pele (CEFALI *et al*., 2016; VELASCO *et al*., 2008; VIOLANTE *et al*., 2008).

Ante o exposto, espécies vegetais como *Dimorphandra mollis* Beth, *Ginkgo biloba* L., *Ruta graveolens* L. e cascas de *Vitis vinífera* L. foram escolhidas para o trabalho por possuírem flavonóides em seus frutos e folhas (HECKER *et al*., 2002; KATO *et al*, 2012; SOUSA *et al*. 1991).

De acordo com a literatura (DAL BELO *et al.,* 2008; MACLENNAN *et al.*, 2002; TESCH, 2002; VAN BEEK e MONTORO, 2009), *Ginkgo biloba* L. é uma das espécies vegetais que possuem concentração mais expressiva dos flavonóides quercetina e rutina em sua composição, justificando, portanto, sua aplicação em diversos tratamentos terapêuticos e/ou cosméticos.

Dessa forma, os dados teóricos foram equivalentes aos observados no presente estudo, e é importante ressaltar o uso do *Ginkgo biloba* L. no formato de extrato seco, em contrapartida ao uso das outras espécies, as quais foram adquiridas *in natura*.

Para a espécie *Dimorphandra mollis* Benth, a presença de rutina foi, da mesma maneira, bem expressiva, corroborando com os dados da literatura (BRANDÃO, 1992;

FRANCO *et al.,* 1996; SOUSA *et al.,* 1991; LORENZI, 2000), e a utilização de dois flavonóides, rutina e quercetina, para a identificação e quantificação de ativos nos extratos utilizados no trabalho, é justificada, pois o uso de apenas um dos padrões analíticos poderia provocar resultados equivocados ao estudo, referente à presença de flavonóides nessas espécies vegetais.

De acordo com GRINBERG e colaboradores (1994), a molécula de rutina apresenta porção glicídica na molécula, diferenciando-se da quercetina, porém, ao ser submetida à hidrólise por ação enzimática, por exemplo, a molécula de rutina, pode perder sua porção glicídica, dando origem apenas à quercetina (KREFT *et al.,* 2006; MANACH *et al*., 1997). Por esse motivo, é compreensível a presença dos dois flavonóides ou de apenas um em uma determinada espécie vegetal.

Semelhantemente à espécie *Ginkgo biloba* L., *Ruta graveolens* L. também apresentou os dois flavonóides rutina e quercetina em sua composição, sendo que a presença de rutina foi mais significativa, de acordo com o encontrado na literatura (SKIDMORE-ROTH, 2004; LORENZI e MATOS, 2002).

O extrato contendo cascas de *Vitis vinifera* L., da espécie Benitaka, apresentou menor concentração de rutina, em relação aos demais extratos analisados, e, em adição, a presença de quercetina não foi possível de ser identificada e quantificada, devido à baixa concentração do ativo na amostra. Resultado coerente com a literatura, pois, de acordo com PASTRANA-BONILLA e colaboradores (2003) e FALCÃO e colaboradores (2007), o flavonóide majoritário encontrado nas espécies de uvas é a antocianina, ativo não quantificado no presente estudo.

Assim, conforme os dados mencionados no Capítulo II, a amostra Mix apresentou-se como promissora em relação à concentrações de rutina e quercetina, devido

à presença dos flavonóides nos extratos estudados, sendo resultado confirmado pela análise cromatográfica.

Em relação à eficácia protetora dos extratos frente à radiação solar, a técnica espectrofotométrica utilizando a região ultravioleta consiste em um método auxiliar e *in vitro* para a determinação do fator de proteção solar de compostos. De acordo com MANSUR e colaboradores (1986), os valores de FPS encontrados, oriundos da análise *in vitro*, corroboram com os dados obtidos por método *in vivo*, sendo, portanto, uma primeira análise de eficácia aplicada anteriormente aos estudos em seres humanos.

Devido à sua praticidade, agilidade para a obtenção de resultados, e correlação com estudos *in vivo* (BARTH, 2000; GARCIA, 1990; SANTOS *et al*., 1999), o método de Mansur tem sido amplamente empregado em estudos científicos, para a determinação de FPS, especialmente, de novos compostos obtidos de extratos vegetais (FERRARI *et al.*, 2007; KAUR e SARAF, 2010; VIOLANTE *et al*., 2008; WAGEMAKER *et al*., 2011).

Diante dos resultados obtidos, os extratos oriundos das espécies *Ginkgo biloba* L, *Ruta graveolens* L. e a amostra Mix apresentaram fator de proteção solar *in vitro*, pelo método de Mansur, sendo considerados promissores para serem utilizados como ativos de proteção solar, sendo que a espécie *Ginkgo biloba* L., seguido do Mix, apresentaram os maiores valores de FPS, devido à presença de maiores concentrações de flavonoides nas amostras, como observado no ensaio de quantificação por CLAE.

Todavia, os extratos obtidos da *Dimorphandra mollis* Benth e cascas de *Vitis vinifera* L., da espécie Benitaka, apresentaram valores menores em comparação com os demais extratos estudados. É possível observar que a menor eficácia encontrada nas espécies mencionadas pode estar relacionada à ausência do flavonóide quercetina nos extratos, conforme resultado também apresentado pelo ensaio de quantificação, pois o

dado obtido no presente trabalho pode ser comparado ao encontrado por CHOQUENET e colaboradores (2008), ao determinar o fator de proteção solar *in vitro* de quercetina e rutina isoladas, e verificar maior fator de FPS para quercetina.

Em contrapartida, os dados de FPS *in vitro*, obtidos pelo método de Mansur, são preliminares, sendo necessária a realização de demais ensaios, como a utilização de espectroscopia de reflectância difusa com esfera integrada, em que, de acordo com VELASCO e colaboradores (2011), um feixe de luz de xenônio com energia espectral, em torno de 250 a 450nm, atinge a amostra em estudo, sendo possível, dessa maneira, medir a transmitância hemisférica total pelo uso de uma esfera de integração, a qual coleta a luz dispersa em todos os ângulos, convertendo os valores encontrados em parâmetros de eficácia fotoprotetora (SPRINGSTEEN *et al.,* 1999). Assim, utilizando dois métodos distintos para a avaliação do FPS da amostra, o estudo torna-se mais confiável, atingindo melhor conclusão dos resultados.

Com isso, diante dos resultados obtidos, foi possível observar que os quatro extratos analisados, juntamente com a amostra Mix, apresentaram absorção da radiação ultravioleta nas regiões UVA e UVB, também como o controle positivo Tinosorb S®, haja vista a capacidade dos extratos de *Dimorphandra mollis* Benth e *Ginkgo biloba* L. absorverem radiação na faixa de 320 a 400nm, correspondendo à radiação UVA, e dos extratos de *Ruta graveolens* L. e *Vitis vinífera* L. absorverem na região em torno de 310nm, resultado característico de filtros solares com absorção UVB (MOTA *et al*., 2009; PALM, O'DONOGHUE, 2007).

Os ensaios propostos pelos órgãos reguladores ANVISA e FDA exigem ensaios *in vivo* para a determinação do fator de proteção solar dos compostos em estudo, porém de acordo com os ensaios *in vitro* realizados no presente trabalho, foi possível observar diante de ensaios preliminares, que os extratos obtidos podem ser promissores para uso

como protetores solares de ação química e, por apresentarem baixo valor de fator de protetor solar, podem estar associados a outros filtros químicos e físicos.

Dessa maneira, semelhantemente aos resultados obtidos pelo método de Mansur, foi possível observar que os extratos apresentam fator de proteção solar pelo método espectrométrico por reflectância em esfera integrada e, em adição, também foi possível constatar que as metodologias para determinação de fator de proteção solar *in vitro*, de acordo com VELASCO e colaboradores (2011), podem ser utilizadas na seleção de compostos ou formulações de melhor desempenho com agilidade, facilidade de manuseio, menor custo e com respostas úteis na escolha das preparações que servirão de base para o estudo em humanos, visando oferecer um produto de eficácia fotoprotetora comprovada.

A atividade antioxidante de flavonóides e de outros derivados fenólicos é amplamente conhecida por atuarem na captura e neutralização de espécies oxidantes como o ânion superóxido, radical hidroxilo ou radical peróxido (ZUANAZZI e MONTANHA, 2007).

De acordo com MARTINEZ-FLORES e colaboradores (2002), a capacidade antioxidante dos flavonóides aplica-se, sobretudo, na neutralização das espécies reativas de oxigênio (EROs), além de desempenharem uma função preventiva na oxidação das lipoproteínas de baixa densidade (LDL). Também podem exercer efeito antioxidante através de outros mecanismos como: inibição de enzimas responsáveis pela produção do ânion superóxido envolvidas na geração de espécies reativas de oxigênio (MUSCHIETTI e MARTINO, 2007), além de atuarem como quelantes de metais divalentes, os quais desempenham um papel importante no metabolismo do oxigênio, como captadores de EROs e como participantes da regulação da atividade de enzimas antioxidantes, de acordo com VAN ACKER e colaboradores (1996).

Diante da atividade dos compostos, os flavonóides, como também outros ativos provenientes de material vegetal, são amplamente estudados como componentes de formulações cosméticas, com o objetivo de auxiliarem no combate ao envelhecimento cutâneo precoce, agindo como sequestrante de espécies reativas de oxigênio, como o oxigênio singleto, proveniente do sol (ALENCAR FILHO *et al*., 2016; CEFALI *et al*., 2015; GIACOMONI, 2008; HUBINGER *et al*., 2010).

Dessa forma, os extratos em estudo no presente trabalho foram submetidos aos ensaios de atividade antioxidante *in vitro*, frente a diferentes radicais livres, e foi observada a atividade antioxidante de todos os extratos, frente aos radiais DPPH, ABTS e AAPH, sendo que a espécie *Ginkgo biloba* L. e a amostra Mix se destacaram novamente, apresentando os resultados mais expressivos, em acordo com os dados apresentados na quantificação de flavonóides e valores de FPS.

Após a análise química, de eficácia e citostática dos extratos, e a constatação da qualidade da amostra Mix, a formulação cosmética foi desenvolvida, estudando, dessa maneira, uma forma de aplicação para o produto.

Emulsão que possui fase interna oleosa e fase externa aquosa, do tipo O/A, e contendo baixas concentrações de material graxo, pode ser considerada uma emulsão leve (ISAAC *et al.*, 2008), sendo atributos desejáveis para seu uso como filtro solar, não permitindo e/ou minimizando a sensação de pegajosidade e oleosidade sobre a pele após a aplicação.

Diante disso e apoiando as diretrizes da ECOCERT, organismo de inspeção e certificação, fundado na França, em 1991, e estabelecido no Brasil desde 2001, responsável por contribuir ativamente para o desenvolvimento da agricultura orgânica e apoiar o desenvolvimento sustentável no país e na Europa (ECOCERT, 2017), nove emulsões do tipo O/A foram desenvolvidas contendo matérias primas certificadas como

orgânicas, conforme o referencial ECOCERT (CRODA, 2017), as quais se diferenciaram pela presença de apenas um ou a combinação de dois agentes de consistência, alterações nas concentrações do emulsionante polimérico, do agente espessante e do agente secativo talco.

Assim, todas as formulações apresentaram aspecto cremoso, coloração branca, odor característico de formulação base e sensação diferente ao toque devido à alteração de composição e concentração dos seus componentes.

Porém, para tornar a seleção da formulação cosmética a ser escolhida para dar continuidade ao trabalho, foi realizado o estudo de análise sensorial, o qual, de acordo com STONE e SIDEL (1992), é responsável por interpretar, evocar, avaliar e medir reações às características de um produto, após estímulos ao ser humano como visão, tato, odor e sabor, e como esses estímulos são percebidos pelos órgãos do sentido. Por isso, a técnica é amplamente utilizada nas indústrias alimentícia e cosmética (ALMEIDA *et al*., 2008; ISAAC *et al*., 2008), exercendo importante função no desenvolvimento de produtos, sendo os métodos sensoriais afetivo e descritivo, os mais utilizados (ISAAC *et al*., 2012; MEILGAARD *et al*., 1991; MUÑOZ *et al*., 1993; SILVA *et al.,* 2004).

Após a escolha da formulação base, o fitocosmético (FPS) foi finalmente desenvolvido apresentando a mistura de extratos livres ricos em flavonoides e nanoparticulados (FPS-nano), sendo ambas formulações submetidas aos estudo de estabilidade físico-química, determinação de parâmetros físicos capazes de fornecer informações sobre o comportamento do produto, em determinado intervalo de tempo, frente a condições ambientais a que possa ser submetido, desde a fabricação até o término da validade (ANVISA, 2004), e sobretudo, características físicas como textura e espalhabilidade, as quais contribuem para uma maior compreensão sobre a sua aplicação como um produto tópico.

A nanotecnologia foi empregada no estudo, pois, devido ao resultado de instabilidade físico-química apresentado pela formulação contendo os extratos livres quando submetido a temperaturas altas (45ºC) durante o ensaio de estabilidade, foi pertinente o uso da técnica, pois de acordo com a literatura (AJAZUDDIN, 2010; SCALIA e MEZZENA, 2009), a encapsulação de ativos pode proteger os compostos de danos físicos e químicos, otimizando sua estabilidade, assim como sua biodisponibilidade, a liberação do ativo, entre outros fatores, contribuindo para a eficácia do produto final.

O ensaio de permeação cutânea também foi realizado para as formulações propostas, permitindo avaliar a cedência e permeação/retenção do flavonóide rutina incorporado nas formulações na pele de porco. E foi constatado, frente aos resultados apresentados, que o flavonóide, embora ter permeado pela pele, também foi encontrado no estrato córneo e em maiores concentrações retido nas camadas da pele.

Esse resultado foi considerado desejável para o estudo, pois as formulações tópicas desenvolvidas (FPS e FPS-nano) são destinadas ao uso para proteção solar e, portanto, a mistura de extratos capaz de absorver a radiação ultravioleta deve permanecer sobre ou retida nas camadas da pele, protegendo-a dos danos causados pela radiação solar e pela formação de radicais livres, podendo ser, dessa maneira, formulações utilizadas como filtro solar e no combate à aceleração do envelhecimento cutâneo.

Assim, ante o exposto, é possível constatar que as formulações em estudo, apesar de apresentarem valores baixos de FPS, são promissoras para serem utilizadas como filtros solares tópicos e, especialmente, em associação com filtros solares físicos, aumentando, consequentemente, a sua proteção contra a radiação solar.

Conclusões

Diante dos resultados apresentados, foi possível concluir que os extratos obtidos das espécies vegetais *Dimorphandra mollis* Benth, *Ginkgo biloba* L., *Ruta graveolens* L. e cascas de *Vitis vinifera* L., da espécie Benitaka, possuem flavonóides e, consequentemente, atividade antioxidante perante aos radicais livres estudados e fator de proteção solar *in vitro*, sendo a espécie *Ginkgo biloba* L. a que apresentou resultados mais expressivos no presente trabalho. No entanto, a amostra Mix, constituída dos quatro extratos estudados, também apresentou resultados promissores frente aos ensaios realizados.

Em adição, o método de quantificação apresentou linearidade, precisão, exatidão, robustez e ausência de impurezas ou outros compostos capazes de interferirem na identificação de picos referentes aos flavonóides quercetina e rutina estudados.

A formulação base, escolhida como preferida durante o estudo de análise sensorial, foi utilizada como veículo para a mistura de extratos livres e nanoparticulados. O fitocosmético contendo extratos livres (FPS) apresentou estabilidade físico-química durante 90 dias de análise quando estocado protegido do calor, boa espalhabilidade, baixa força de ruptura e adesividade e alta fragilidade, pseudoplasticidade e viscoelasticidade, sendo características desejáveis favorável para uma formulação de uso tópico.

A formulação também apresentou atividade antioxidante *in vitro* frente ao radical DPPH, proteção contra radiação UVA/UVB *in vitro* e fotoestabilidade ao ser submetida à radiação UVA. Além disso, o flavonóide rutina foi permeado pela pele, porém foi encontrado no estrato córneo e em maior quantidade nas camadas da pele (epiderme/derme).

A formulação com nanopartículas (FPS-nano) contendo a mistura de extratos forneceu maior estabilidade físico-química ao produto. Como apresentado pelo

fitocosmético contendo extratos livres, a formulação apresentou fator de proteção solar, fotoestabildade e permitiu a retenção de rutina no estrato córneo e camadas da pele de acordo com o ensaio *in vitro* realizado.

Frente ao exposto, as formulações tornam-se promissoras para serem utilizadas no combate à aceleração do envelhecimento cutâneo e proteção solar contra os danos provocados pela radiação ultravioleta.

Bibliografia

1) ABBAS S.; SALEEM H.; GILL M.S.A.; BAJWA, A.M.; SARWAR, A.; OMER, M.O. Physicochemical and nutritional values determination of *Suaeda fruticosa* (Chenopodiaceae). *Acad. J. Med. Plant*. v.4, p.1-9, 2016.

2) ABIHPEC. Associação Brasileira de Indústrias de Higiene Pessoal, Perfumaria e Cosméticos. Disponível em: https://www.abihpec.org.br/2012/04/brasil-tem-maior-taxa-de-crescimento-percentual-entre-os-top-10-mercados-mundiais-de-hppc/. Acessado em 20 de janeiro de 2018.

3) ACEVEDO, JGA; CASTANEDA, C.M.C.; BENITEZ, F.J.C.; DURÁN, D.A.; BARROSO, V.R.; MARTINEZ, C.G.; MUNOZ, L.J.L.; MARTINEZ, C.A.; ROMO de VIVAR, A. Photoprotective activity of Buddleja scordioides. *Fitoterapia*.v.76, n. 3-4, p.301-309, 2005.

4) ADHIKARI, D.; PANTHI, V.K.; PANGENI, R.; KIM, H.J.; PARK, J.W. Preparation, characterization, and biological activities of topical anti-aging ingredients in a *Citrus junos* callus extract. *Molecules*. v.22, n.1, p. 2198-2213, 2017.

5) AJAZUDDIN, S.S. Applications of novel drug delivery system for herbal formulations. *Fitoterapia.* v. 81, n.7, p. 680-689, 2010.

6) AHLENSTIEL, T., BURKHARDT, G., KOHLER, H., KUHLMANN, M.K. Bioflavonoids attenuate renal proximal tubular cell injury during cold preservation in Euro-Collins and University of Wisconsin solutions. Kidney Int. v.63, p.554–563, 2003.

7) ALENCAR FILHO, J. M. T.; SAMPAIO, P. A.; PEREIRA, E. C. V.; DE OLIVEIRA JÚNIOR, R. G.; SILVA, F. S.; ALMEIDA, J. R. G. S.; ROLIM, L. A.; NUNES, X.

P.; ARAÚJO, E. C. C. Flavonoids as photoprotective agents: A systematic review. *J. Med. Plants Res.* v.10, n.47, p.848-864, 2016.

8) ALENCASTRE, J.B.; BENTLEY, M.V.L.B.; GARCIA, F.S.; MORAGAS, M.; VILADOR, J.L.; MARCHETTI, J.M. A study of the characteristics and *in vitro* permeation properties of CMC/chitosan microparticles as a skin delivery system for vitamin E. *Rev. Bras. Ciênc. Farm.***,** v.42, n.1, p. 69-76, 2006.

9) ALMEIDA, I.F.; BAHIA, M.F. Evaluation of the physical stability of two oleogels. *Int. J. Pharm*., v.327, n.1-2, p.73-77, 2006.

10) ALMEIDA, I.F.; GAIO, A.R.; BAHIA, M.F. Hedonic and descriptive skin feel analysis of two oleogels: comparison with other topical formulations. *J. Sens. Stud*. v.23, n.1, p.92-113, 2008.

11) AMAR, S.K.; GOYAL, S.; DUBEY, D.; SRIVASTAV, A.K.; CHOPRA, D.; SINGH, J.; SHANKAR, J.; CHATURVEDI, R.K.; RAY, R.S. Benzophenone 1 induced photogenotoxicity and apoptosis via release of cytochrome c and smac/diablo at environmental UV radiation. *Toxicol. Let*. v.239, 182-193, 2015.

12) ANSEL, H.C.; POPOVICH, N. G.; ALLEN Jr., L.V. Formas farmacêuticas e sistema de liberação de fármacos. 6. ed. São Paulo: Editorial Premier, 2000. 568 p.

13) ANTONIOU, C.; KOSMADAKI, M.; KOSMADAKI, G.; STRATIGOS, A.J.; KATSAMBAS, A.D. Sunscreens – what's important to know? *J. Eur. Acad. Dermatol. Venereol*. v.22, n.1, p.1110-1119, 2008.

14) ANVISA. Guia para validação de métodos analíticos e bioanalíticos conforme o anexo desta resolução: Diário Oficial da União. Resolução RE nº 899. 2003.

15) ANVISA. Agência Nacional de Vigilância Sanitária. Resolução RDC nº 47, de 16 de março de 2006. Lista de filtros ultravioletas permitidos para produtos de higiene pessoal, cosméticos e perfumes. Diário Oficial da União, nº53, 17 de março de 2006. Seção 1. p.60.

16) ANVISA. Agência Nacional de Vigilância Sanitária. Resolução RDC nº 30 de 1° de junho de 2012. Regulamento Técnico Mercosul sobre Protetores Solares em Cosméticos. Brasília, 2012. p. 1-7.

17) ANVISA. Consolidado de normas da COFID. Versão IV. Brasília: Agência Nacional de Vigilância Sanitária. 2014.

18) ARTS, I.C.W.; HOLLMAN, P.C.H. Polyphenols and disease risk in epidemiologic studies. *Am. J. Clin. Nutr*. v.81, p.317-325, 2005.

19) AVRANAS, A.; STALIDIS, G.; RITZOULIS, G. Demulsification rate and zeta potential of O/ W emulsions. *Coll. Polym. Sci*. v.266, n.1, p.937-940,1988.

20) AZOFEIFA, D.E.; ARGUEDAS, H.J.; VARGAS, W.E. Optical Properties of Chitin and Chitosan Biopolymers with Application to Structural Color Analysis. *Opt. Mater*. v.35, n.1, p. 175–183, 2012.

21) BADOLATO, G.G.; AGUILAR, F.; SCHUCHMANN, H.P.; SOBISCH, T.; LERCHE, D. Evalu- ation of long term stability of model emulsions by multisample analytical centrifugation. *Progr. Colloid Polym. Sci.* v.134, n.1, p. 66 – 73, 2008.

22) BALOGH, T.S.; VELASCO, M.V.R.; PEDRIALI, C.A.; KANEKO, T.M.; BABY, A.R. Ultraviolet radiation protection: current available resources in photoprotection. *An. Bras. Dermatol.*, v.86, n.4, p.732-742, 2011.

23) BARON, E.D.; KIRKLAND, E.B.; DOMINGO, D.S. Advances in photoprotection. *Dermatol. Nurs.* v.20, n.1, p.265-272, 2008.

24) BARRY, B.W. Rheology of dermatological vehicles. In: Dermatological formulations. New York:Marcel Dekker, p. 351-439, 1993.

25) BARTH, A.L. Fator de proteção solar versus coeficiente de carga de filtros solares químicos: avaliação fotobiológica de uma mistura de filtros solares químicos. 2000. 89f. (Dissertação- Mestrado em Farmácia). Rio de Janeiro: Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, 2000.

26) BEHLING, E.B., SENDAO, M.C., FRANCESCATO, H.D.C., ANTUNES, L.M.G. BIANCHI, M.L.P. Flavonoide quercetina: aspectos gerais e ações biológicas. *Alim. Nutr.* v.15, p.285–292, 2004.

27) BIHHIMER, W.L. Avaliação de filtros solares em seres humanos: proteção contra a queimadura solar. *Cosmet. Toil.* v.1, p.41–48, 1989.

28) BISSONNETTE, R. Update on sunscreens. *Skin Ther Lett.* v.13, p.5–7, 2008.

29) BONNABRY, C. Mise au point et validation de modeles d'etude *in vitro* du passage percutane, Universite Dauvergne – UFR de Pharmacie, 1999.

30) BORELLA, J.C.; RIBEIRO, N.S.; TEIXEIRA, J.C.L.; CARVALHO, D.M.A. Avaliação da espalhabilidade e do teor de flavonoides em forma farmacêutica semissólida contendo extratos de *Calendula officinalis* L. (Asteraceae). *Rev. Ciênc. Farm. Básica Apl*., v.31, n.2, p.193-197, 2010.

31) BORGHETTI, G.S.; KNORST, M.T. Desenvolvimento e avaliação da estabilidade física de loções O/A contendo filtros solares. *Rev. Bras. Ciên. Farm*., v.42, p.531-537, 2006.

32) BRANDÃO, M. Plantas produtoras de tanino nos cerrados mineiros. *Inf. Agrop.* v.16, p. 33-35, 1992.

33) BRAVO, L. Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. *Nutr Rev.* v.56, n.1, p.317-33, 1998.

34) BRONAUGH, R.L.; STEWART, R.F.; Methods for in vitro percutaneous absorption studies IV: preparation of the barrier layer. *J. Pharm. Sci.,* v. 75, n. 5, p. 487-491, 1986.

35) CABRAL, L.D.S; PEREIRA, S.O.; PARTATA, A.K. Filtros solares e fotoprotetores mais utilizados nas formulações no Brasil. *Rev. Cien. ITPAC*. v.4, n.3, p.1-9, 2011.

36) CAO, G.H.; ALESSIO, H.M.; CUTLER, R.G. Oxygen-radical absorbency capacity assay for antioxidants. *Free Radical Biol. Med.* v.14, n.1, p.303- 311, 1993.

37) CASEDEY, E.C.L. e SALGADO, H.R.N. Development and validation of UV spectrophotometric method for orbifloxacin assay and dissolution studies. *Braz. J. Pharm. Sci.* v. 50, n.3, p.457-466. 2014.

38) CASIMIRO, M. H.; LEAL, J. P.; GIL, M. H.; CASTRO, C. A. N. Análise calorimétrica aplicada a polímeros biológicos. Parte I: Fundamentos teóricos. *Bol. Soc. Port. Quím.,* v.98, n.1, p. 29-35, 2005.

39) CAVALCANTE, M.L., RODRIGUEZ-AMAYA, D.B. Carotenoid composition of the tropical fruits *Eugenia uniflora* and *Malpighia glabra*. In: Food Science and Human Nutrition (Charalambous, G., ed.), pp. 643–650. Elseiver, Amsterdan, 1992.

40) CEFALI, L.C.; SOUZA-MOREIRA, TATIANA MARIA DE; CORRÊA, M. A.; SALGADO, H. R. N.; ISAAC, V. L. B. Development and evaluation of an emulsion containing lycopene for combating acceleration of skin aging. *Braz. J. Pharm. Sci*. v.51, n.3, p. 579-590, 2015.

41) CEFALI, L.C.; ATAIDE, J.A.; FOGLIO, M.A., MORIEL, P.; MAZZOLA, P.G. Plant compounds as active photo protectants in sunscreens. *Inter. J. Cosm. Sci*., v.1, p.1-8, 2016.

42) CHANAMAI, R.; HERRMAN, N.; MCCLEMENTS, D. J. "Ultrasonic Spectroscopy Study of Flocculation and Shear-Induced Floc Disruption in Oil-in-Water Emulsions". *J. Coll. Int. Sci.* v.204, n.2, p.268-276, 1998.

43) CHAMBERLAIN, M.; PARISH, W.E. Hazard and risk based on in vitro test data. *Toxicol. In vitro.* v.4, n.4/5, p.694-697. 1990.

44) CHIARI, B.G.; TROVATTI, E.; PECORARO, T.; CORRÊA, M.A.; CICARELLI, R.M.B.; RIBEIRO, S.J.L.; ISAAC, V.L.B. Synergistic effect of green coffee oil and synthetic sunscreen for health care application. *Ind. Crop. Prod.* v.52, n.1, p.389-393, 2014.

45) CHIU, A.; KIMBALLL, A.B. Topical vitamins, minerals and botanical ingredients as modulators of environmental and chronological skin damage. *Br. J. Dermatol.* v.149, p.681–691, 2003.

46) CHOQUENET, B.; COUTEAU, C.; PAPARIS, E.; COIFFARD, L.J.M. Quercetin and rutin as potential sunscreen agents: Determination of efficacy by an *in vitro* method. *J. Nat. Prod. v.*71, p.1117–1118, 2008.

47) CHUANG, S.Y.; LIN, C.F.; WANG, P.W.; CHEN, E.L.; FANG, J.Y. Elucidating the skin delivery of aglycone and glycoside flavonoids: How the structures affect cutaneous absorption. *Nutrients*, v.9, n.12, p.1304, 2017.

48) CIE. Erythema reference action spectrum and standard erythema dose. Commission Internationale de l'Eclairage International (CIE) Central Bureau. CIE S007/E, Vienna, Austria, 1998.

49) COLIPA. Colipa Project Team IV. Method for the in vitro determination of UVA protection provided by sunscreen products. Guideline, 2006.

50) COOMAN, L. DE; EVERAERT, E.; DE KEUKELEIRE, D. Quantitative analysis of hop acids, essential oils and flavonoids as a clue to the identification of hop varieties. *Phytochem. Anal.,* v.9, n.1, p.145-150, 1998.

51) CORREA, N. M.; CAMARGO JUNIOR, F. B.; IGNÁCIO, R. F.; LEONARDI, G. R. Avaliação do comportamento reológico de diferentes géis hidrofílicos. *Braz.J. Pharm. Sci.* v. 41, n. 1, p. 73-78, 2005.

52) COSTA, P.A.; BALLUS, C.A.; TEIXEIRA FILHO, J.; GODOY, H.T. Fatty acids profile of pulp and nuts of Brazilian fruits. *Ciênc. Tecnol. Aliment.* v.31, n.4, p.950-954. 2011.

53) CRODA. Croda do Brasil – Personal Care – Brasil. Disponível em: http://www.crodapersonalcare.com. Acessado em 1 de junho de 2017.

54) DAHER, C.C.; FONTES, I.S.; RODRIGUES, R.O. Development of O/W emulsions containing *Euterpe oleracea* extract and evaluation of photoprotective efficacy. *Braz. J. Pharm. Sci*. v.50, p.639–652, 2014.

55) DAL BELO, S.E.; GASPAR, L.R.; CAMPOS, P.M.B.G.M. Photo protective effects of topical formulations containing a combination of *Ginkgo biloba* and green tea extract*s. Phyt. Res.* v.25, n.1, p.1854–1860. 2011.

56) DANELUTI, A.L.M.; VELASCO, M.V.R.; BABY, A.R.; MATOS, J.R. Thermal behavior and free-radical-scavenging activity of phytic acid alone and incorporated in cosmetic emulsions. *Cosmetics.* v.2, n.1, p.248-258, 2015.

57) DÁVILA, J.L.; DÁVILA, M.A. Laponite as a rheology modifier of alginate solutions: Physical gelation and aging evolution. *Carboh. Polymers*, v. 157, n.1, p. 1-8, 2016

58) DAVIS, H.M. Analysis of creams and lotion. In: Newburguer's manual of cosmetic analysis. Washington: Association of Official Analytical Chemists, 1977, p.32.

59) DOMAS, W.C.; OLIVEIRA, T.T.; RODRIGUES-DAS-DORES, R.G.; SANTOS, A.F.; NAGEM, T.J. Flavonoides: potencial terapêutico no estresse oxidativo. *Rev. Ciênc. Farm. Básica Apl*. v.28, p.241–249, 2007.

60) DUALE, N.; OLSEN, A.K.; CHRISTENSEN, T.; BUTT, S.T.; BRUNBORG, G. Octyl methoxycinnamate modulates gene expression and prevents cyclobutane pyrimidine dimer formation but not oxidative DNA damage in UV-exposed human cell lines. *Toxicol. Sci*. v.114, n.1, p.272–284, 2010.

61) DUTCOSKY, S.D. Análise Sensorial de Alimentos. 2 ed. Curitiba: Champagnat; 2007.

62) DUTHIE, M.S.; KIMBER, I.; NORVAL, M. The effects of ultraviolet radiation on the human immune system. *Br. J. Dermatol*. v.140, n.1, p.995– 1009, 1999.

63) EBRAHIMZADEH, A.; GHARAEI, A.; SARYAZDI, K. Allelic diversity of polymorphic AMA-1 (Apical membrane antigen 1) vaccine candidate antigen of plasmodium falciparum in two population of imported and indigenous cases in South-East of Iran using nested-PCR and RFLP. *J Trop Dis* v.2, n.1, p.149-155, 2014.

64) ECOCERT. Organismo de inspeção e certificação. Ecocert Brasil. Disponível em http://www.brazil.ecocert.com/politicas-e-diretrizes-ecocert. Acessado em 01 de junho de 2017.

65) EMEA. Guideline on Stability Testing: Stability testing of existing active substances and related finished products. Committee for proprietary medicinal products (CPMP). European Medicines Agency – Inspections (EMEA), 2003.

66) ESTANQUEIRO, M.; CONCEIÇÃO, J.; AMARAL, M.H.; SANTOS, D.; SILVA, J.B.; LOBO, J.M.S. Characterization and stability studies of emulsion systems containing pumice. *Braz. J. Pharm. Sci.* v.50, n.2, p.361-369, 2014.

67) FDA. Food and Drug Administration. Sunscreen drug products for over-the-counter human use. Code of Federal Regulations. Title 21, v. 5. 2013.

68) FELDMAN, K.A. Cytochrome P450s as genes for crop improvement. *Curr Opin Plant Biol* v.4, n.1, p.162-167, 2001.

69) FERNANDES, S.C.; ALONSO-VARONA, A.; PALOMARES, T.; ZUBILLAGA, V.; LABIDI, J.; BULONE, V. Exploiting mycosporines as natural molecular sunscreens for the fabrication of UV-absorbing green materials. *ACS Appl. Mater. Interfaces.* v.7, n.1, p.16558–16564, 2015.

70) FERRARI, M.; OLIVEIRA, M.S.C.; NAKANO, A.K.; ROCHA-FILHO, P.A. Determinação do fator de proteção solar (FPS) *in vitro* e *in vivo* de emulsões com óleo de andiroba (*Carapa guianensis*). *Rev Bras Farmacogn* v.17, n.1, p.626- 630, 2007.

71) FIGUEIREDO, S.A.; VILELA, F.M.; DA SILVA, C.A.; CUNHA, T.M.; DOS SANTOS, M.H.; FONSECA, M.J. *In vitro* and *in vivo* photoprotective/ photochemopreventive potential of Garcinia brasiliensis epicarp extract. *J. Photochem. Photobiol. B.* v.5, n.31, p.65-73, 2014.

72) FLOEGEL, A.; KIM, D.O.; CHUNG, S.J.; KOO, S.I.; CHUN, O.K. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *J. Food Comp. Anal*. v.24, n.1, p.1043-1048, 2011.

73) FLOR, J.; DAVOLOS, M. R.; CORREA, M. A. Protetores Solares. *Quím. Nova.* v.30, n.1, p.153-158, 2007.

74) FLORA, S.; FERGUSON, L.R. Overview of mechanisms of cancer chemopreventive agents. *Mutat. Res*. v.591, n.1, p.8-15, 2005.

75) FOLEY, P.; NIXON, R.; MARKS, R.; FROWEN, K.; THOMPSON, S. The frequency of reactions to sunscreens: results of a longitudinal population-based study on the regular use of sunscreens in Australia. *Br. J. Dermatol.* v.128, n.1, p.512–518, 1993.

76) FORESTIER, S. Rationale for sunscreen development. *J. Am. Acad. Dermatol*. v.58, n.1, p.133-138, 2008.

77) FRANZ, T.J. Percutaneus absorption on relevance of *in vitro* data. *J. Invest. Dermatol.,* v. 64, p.190, 1975.

78) GAIKWAD, M.; KALE, S. Formulation and in vitro evaluation for sun protection factor of *Moringa oleifera* Lam (family - Moringaceae) oil sunscreen cream. *Inter. J. Pharm. Pharm. Sci.* v.3, n.1, p.371–375, 2011.

79) GARCIA-QUIROZ, A.; MOREIRA, S.G.C.; DE MORAIS, A.V.; SILVA, A.S.; DA ROCHA, G.N.; ALCANTARA, P. Physical and chemical analysis of dieletric properties and differential scanning calorimetry techniques on buriti oil. *Instrum Sci. Technol*. v.31, n.1, p.93–101, 2003.

80) GASPAR, L.R.; MAIA CAMPOS, P.M.B.G. Rheological behavior and the SPF of sunscreens. *Int. J. Pharm.,* v. 250, n. 1, p. 35-44. 2003.

81) GASPAR, L.R.; MAIA CAMPOS, P.M.B.G. Evaluation of the photostability of different UV filters associations in a sunscreen. *Int. J. Pharm.,* v.307, n.2, p.123-128, 2006.

82) GIACOMONI, P. U. Understanding reactive oxygen species. *Cosm. Toil.,* v.122, n. 5, p.36-40, 2008.

83) GILBERT, E.; PIROT, F.; BERTHOLLE, V.; ROUSSEL, L.; FALSON, F.; PADOIS, K. Commonly used UV filter toxicity on biological functions: review of last decade studies. *Inter J Cosm Sci*. v.35, n.1, p.208-219, 2013.

84) GONTIJO, S. 80 anos de moda no Brasil. Rio de Janeiro: Editora Nova Fronteira; 1987.

85) GONZÁLEZ, S.; FERNÁNDEZ-LORENTE, M.; GILABERTE-CALZADA, Y. The latest on skin photoprotection. *Clin. Dermatol.*, v.26, n.6, p.614-26, 2008.

86) GORDON, A.; CRUZ, A.P.G.; CABRAL, L.M.C. Chemical characterization and evaluation of antioxidant properties of Açai fruits (*Euterpe oleraceae* Mart.) during ripening. *Food Chem*. v.133, n.1, p.256–263, 2012.

87) GUARATINI, T.; CALLEJON, D.R.; PIRES, D.C.; LOPES, J.N.C.; LIMA, L.M.; GIANNELLA NETO, D.; SUSTOVICH, C.; LOPES, N.P. Fotoprotetores derivados de produtos naturais: perspectivas de mercado e interações entre o setor produtivo e centros de pesquisa. *Quim Nova.* v.32, n.3, p.717-721, 2009.

88) GRINBERG, L. N.; RACHMILEWITZ, E. A.; NEWMAN, H. Protective effects of rutin against hemoglobin oxidation. *Biochem. Pharmacol.,* v.48, n.1, p.643-649, 1994.

89) HALLIWELL B. The antioxidant paradox. *Lancet*. v.355, n.1, p.1179–1180, 2000.

90) HECKER, H.; JOHANNISSON, R.; KOCH, E.; SIEGERS, C.P. *In vitro* evaluation of cytotoxic potential of alkylphenols from *Ginkgo biloba* L. *Toxicol.,* v.177, n.2-3, p.167-77, 2002.

91) HEJJAJI, E.M.; SMITH, A.M.; MORRIS, G.A. Designing chitosan-tripolyphosphate microparticles with desired size for specific pharmaceutical or forensic applications. *Int. J. Biol. Macromol.* v.95, n.1, p.564-573, 2017.

92) HERTOG, M.G.L.; HOLLMAN, P.C.H.; KATAN, M.B. Contend of potentially anticarcinogenic flavonoids in 28 vegetables and 9 fruits commonly consumed in the Netherlands. *J. Agric. Food Chem*. v.40, p.2379–2883, 1992.

93) HERZOG, B.; WEHRLE, M.; QUASS, K. Photostability of UV absorber systems in sunscreens. *J. Photochem. Photobiol. B*. v.85, n.1, p.869-878, 2009.

94) HOJEROVÁA, A.; MEDOVCÍKOVÁ, A.; MIKULA, M. Photoprotective efficacy and photostability of fifteen sunscreen products having the same label SPF subjected to natural sunlight. *Inter. J. Pharm.* v.408, n.1, p.27–38, 2011.

95) HOLICK, M.F.; CHEN, T.C.; LU, Z.; SAUTER, E. Vitamin D and skin physiology: a D-lightful story. *J. Bone Min. Res*. v.22, n.1, p.28–33, 2007.

96) HOWITT, C.A.; POGSON, B.J. Carotenoid accumulation and function in seeds and non-green tissues. *Plant, Cell Environ*. v.29, n.1, p.435–445, 2006.

97) HUANG, D.; OU, B.; PRIOR, R.L. The Chemistry behind Antioxidant Capacity Assays. J. *Agric. Food Chem.* v.53, n.1, p.1841−1856, 2005.

98) HUBER, L.S.; HOFFMANN-RIBANI, R.; RODRIGUEZ-AMAYA, D.B. Quantitative variation in Brazilian vegetable sources of flavonols and flavones. *Food Chem*. v.113, n.1, p.1278–1282, 2009.

99) HUBINGER, S. Z.; CEFALI, L. C.; VELLOSA, J. C. R.; SALGADO, H. R. N.; ISAAC, V. L. B.; MOREIRA, R. R. D. *Dimorphandra mollis*: an alternative as a source of flavonoids with antioxidant action. *Lat. Am. J. Pharm.* v. 29, n. 2, p. 271-274, 2010.

100) HUONG, S.P.; ROCHER, E.; FOURNERON, J.D.; CHARLES, L.; MONNIER, V.; BUN, H.; ANDRIEU, V. Photoreactivity of the sunscreen butylmethoxydibenzoylmethane under various experimental conditions. *J. Photochem. Photobiol. A.* v.196, n.1, p.106–112, 2008.

101) IDSON, B. Stability testing of emulsions, I. *Drug & Cosmetics Industry,* v.151, n.1, p.27-28,30, 1993.

102) INACIO, M.R.C.; LIMA, K.M.G.; LOPES, V.G.; PESSOA, J.D.C.; TEIXEIRA, G.H.A. Total anthocyanin content determination in intact açai (*Euterpe oleracea* Mart.) and palmitero - juçara (*Euterpe edulis* Mart.) fruit using near infrared spectroscopy (NIR) and multivariate calibration. *Food Chem*. v.136, n.1, p.1160–1164, 2013.

103) INCA. Instituto Nacional de Câncer. Estimativas 2016: Incidência de Câncer no Brasil. Rio de Janeiro: Instituto Nacional do Câncer; 2016.

104) ISAAC, V. L. B. Desenvolvimento, estabilidade e liberação *in vitro* de preparações lipolíticas. 1998, 158f. (Doutorado em Ciências Farmacêuticas) – Faculdade de Ciências Farmacêuticas – Universidade de São Paulo, São Paulo, 1998.

105) ISAAC, V.L.B.; CEFALI, L.C.; CHIARI, B.G.; OLIVEIRA, C.C.L.G.; SALGADO, H.R.N.; CORRÊA, M.A. Protocolo para ensaios físico-químicos de estabilidade de fitocosméticos. *Rev. Ciênc. Farm. Básica Apl.* v.29, n.1, p.81-96, 2008.

106) ISAAC, V.L.B.; CHIARI, B. G.; MAGNANI, C.; CORRÊA, M. A. Análise sensorial como ferramenta útil no desenvolvimento de cosméticos. *Rev. Ciên. Farm. Básica. Apl.* v.33, n.4, p. 479-488, 2012.

107) ISAAC, V.L.B.; CEFALI, L.C.; CHIARI, B.G.; ALMEIDA, J.M.G.; RIBEIRO, H.M.; CORREA, M.A. Effect of various thickening agents on the rheological properties of oil-in-water emulsions containing nonionic emulsifier. *J. Disp. Sci. Tech*. v. 34, n.6, p.880-885, 2013.

108) ISO. International Organization of Standardization. ISO 24443:2012. Determination of sunscreen UVA photoprotection *in vitro*. Geneva, Switzerland, 2012.

109) ISO. International Organization of Standardization. ISO/TR 13097. Guidelines for the Characterization of Dispersion Stability. Geneva, Switzerland, 2013.

110) ISO. International Organization of Standardization. ISO 17516:2014. Cosmetics - Microbiology - Microbiological limits. Geneva, Switzerland, 2014.

111) JAIN, S.K.; JAIN, N.K. Multiparticulate carriers for sun-screening agents. *Int. J. Cosmet. Sci.* v.32, n.1, p.89–98, 2010.

112) JARZYCKA, A. Assessment of extracts of H*elichrysum arenarium, Crataegus monogyna, Sambucus nigra* in photoprotective UVA and UVB; photostability in cosmetic emulsions. J. Photochem. Photobiol. B: Biol. v.128, n.1, p. 50-57, 2013.

113) JIN, C.Y.; ZHU, B.S.; WANG, X.F.; LU, Q.H. Cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells. *Chem. Res. Toxicol.* v.21, n.1, p.1871–1877, 2008.

114) JOUAHRI, D.; BOUDARENE, L.; SABAOU, N. Flavonoids. *Inter J Pharmacog Phytochem Res*. v.5, n.1, p.45-50, 2013.

115) KANG, J.; XIE, C.; LI, Z.; NAGARAJAN, S.; SCHAUSS, A.G.; WU, T.; WU, X. Flavonoids from acai (*Euterpe oleracea* Mart.) pulp and their antioxidant and anti-inflammatory activities. *Food Chem.,* v.128, n.1, p.152-157, 2011.

116) KARSTEN, U.; HOLZINGER, A. Green algae in alpine biological soil crust communities: acclimation strategies against ultraviolet radiation and dehydration. *Biodivers. Conserv*. v.23, n.1, p.1845–1858, 2014.

117) KASPAR, O.; JACUBEC, M.; STEPANEK, F. Characterization of spray dried chitosan-TPP microparticles formed by two and three fluid nozzles. *Powder Tech*. v.240, n.1, p.31-40, 2013.

118) KATIYAR, S.K.; MATSUI, M.S.; ELMETS, C.A.; MUKHTAR, H. Polyphenolic antioxidant epigallocatechin-3-gallate from green tea reduces UVB-induced

inflammatory responses and infiltration of leukocytes inhuman Skin. *Photochem. Photobiol.* v.69, n.1, p.148–153, 1999.

119) KATO, C.G.; TONHI, C.D.; CLEMENTE, E. Antocianinas de uvas (*Vitis vinífera L.*) produzidas em sistema convencional. *Rev. Bras. Tecnol. Agroind.* v. 6, n.2, p. 809-821, 2012.

120) KAUR, C.D.; SARAF, S. *In vitro* sun protection factor determination of herbal oils used in cosmetics. *Pharmacog. Res*. v.2, n.1, p.22–25. 2010.

121) KITZING, C.; PREOSCHOLD, T.; KARSTEN, U. UV-induced effects on growth, photosynthetic performance and sunscreen contents in different populations of the green alga *Klebsormidium fluitans* (Streptophyta) from alpine soil crusts. *Microb. Ecol*. v.67, n.1, p.327–340, 2014.

122) KLUG, H.L.P.; TOOZE, J.A.; CHERRY-GRAFF, C. Sunscreen prevention of melanoma in man and mouse. *Pigm. Cell Melanoma Res*. v.23, p.835–837, 2010.

123) KORAC, R.R.; KHAMBHOLJA, K.M. Potential of herbs in skin protection from ultraviolet radiation. *Pharmacogn Rev.* v.5, n.10, p.164-173, 2011.

124) KREFT, I; FABJAN, N; YASUMOTO, K. Rutin content in buckwheat (*Fagopyrum esculentum* Moench) food materials and products. *Food Chem.* v.98, n.1, p.508-512, 2006.

125) KUSKOSKI, E.M.; ASUERO, A.G.; MORALES, M.T.; FETT, R. Frutos tropicais silvestres e pol- pas de frutas congeladas: atividade antioxidante, polifenois e antocianinas. *Ciênc. Rural*. v.36, n.1, p.1283–1287, 2006.

126) LAUTENSCHLAGER, S.; WULF, H.C.; PITTELKOW, M.R. Photoprotection. *Lancet*. v.370, n.1, p.528-537, 2007.

127) LIAPIS, A.I.; BRUTTINI, R. “Freeze Drying” in Handbook of Industrial Drying, LLC, pp. 257, 2006.

128) LINDQVIST, P.G.; EPSTEIN, E.; NIELSEN, K.; LANDIN-OLSSON, M.; INGVAR, C.; OLSSON, H.J. Avoidance of sun exposure as a risk factor for major causes of death: a competing risk analysis of the Melanoma in Southern Sweden cohort. *J. Int. Med*. v.280, n.1, p.375-387, 2016.

129) LIOCHEV, S.I.; FRIDOVICH, I. The role of O_2 in the production of HO^1: *in vitro* and *in vivo*. *Free Radic. Biol. Med.* v.16, n.1, p.29-33, 1994.

130) LORENZI, H. Árvores brasileiras: manual de identificação e cultivo de plantas arbóreas nativas do Brasil. 3 ed. Nova Odessa: Plantarum, v.1, 2000.

131) LORENZI, H.; ABREU MATOS, F.J. Plantas medicinais no Brasil nativas e exóticas. Nova Odessa: Instituto Plantarum, 2002.

132) LOWE, N.J.; SHAATH, N.A.; PATHAK, M.A. Sunscreens: Development: Evaluation, and Regulatory Aspects, 2nd, v.15, 744p. Marcel Dekker, New York, NY,1997.

133) MACFIE, H.J.H.; THOMSON, D.M.H. Preference mapping and multidimensional scaling. In: Piggot JR, editor. Sensory analysis of foods. 2 ed. Londres; Elsevier; 1988. 389 p.

134) MACLENNAN, K.M.; DARLINGTON, C.L.; SMITH, P.F. The CNS effects of *Ginkgo biloba* extracts and ginkgolide *B. Prog. Neurobiol.* v.67, p.235-257, 2002.

135) MAGALHAES, J. Cosmetologia. Rio de Janeiro, Rubio, 2000. p. 115-118.

136) MAHADY, G.B. *Ginkgo Biloba*: a review of quality, safety, and efficacy. *Nutr. Clin. Care.* v.4, n.1, p.140–147, 2001.

137) MANACH, C.; MORAND, C.; DEMIGNE, C.; TESIER, O.; REGERAT, F.; REMESY, C., Bioavailability of rutin and quercetin in rats. Fed. *Eur. Biochem. Soc*. v. 409, n.1, p.12-16, 1997.

138) MANAIA, E.B.; KAMINISKI, R.C.K.; CORREA, M.A.; CHIAVACCI, L.A. Inorganic UV filters. *Braz. J. Pharm. Sci*. v.49, n.1, p.201–209, 2013.

139) MANN, J. Secondary metabolism. Oxford: Clarendon Press; 1987. p.374.

140) MANSUR, J.S.; BREDER, M.N.R.; MANSUR, M.C.A.; AZULARY, R.D. Determinação do fator de proteção solar por espectrofotometria. *An. Bras. Dermatol*. v.61, n.3, p.121-124, 1986.

141) MANSUR, M.C.P.P.R.; LEITÃO, S.G.; CERQUEIRA-COUTINHO, C.; VERMELHO, A.B.; PRESGRAVE, O.A.F.; LEITÃO, A.A.C.; LEITÃO, G.G.; RICCI-JUNIOR, E.; SANTOS, E.P. *In vitro* and *in vivo* evaluation of efficacy and safety of photoprotective formulations containing antioxidant extracts. *Rev Bras Farmacog*. v.26, n.1, p.251-258, 2016.

142) MARTINEZ-FLÓREZ, S.; GONZÁLEZ-GALLEGO, J.; CULEBRAS, J.M.; TUÑON, M.J. Los flavonoides: propiedades y acciones antioxidantes. *Nutr. Hosp.,* v.17, n.6, p.271-278, 2002.

143) MATSUDA, A.H.; MACHADO, L.B.; DEL MASTRO, N.L. Thermal analysis applied to irradiated propolis. *Radiat. Phys. Chem*. v.63, n.1, p.353– 355, 2002.

144) MCKINLEY, A.F., DIFFEY, B.L. A reference action spectrum for ultraviolet induced erythema in human skin. *CIE J.* v.6, n.1, p.17–22, 1987.

145) MEILGAARD, M.; CIVILLE, G.V.; CARR, B.T. Sensory evaluation techniques. Florida: CRC Press; 1991. p. 142-47.

146) MELLO, J.C.P.; PETROVICK, P.R. Quality control of *Bacharis trimera* (Less) DC (Asteraceae) hydroalcoholic extracts. *Acta Farm. Bonaerense*, v.19, n.1, p.211-215, 2000.

147) MILEYEVA-BIEBESHEIMER, O.N.; ZAKY, A.; GRUDEN, C.L. Assessing the impact of titanium dioxide and zinc oxide nanoparticles on bacteria using a

fluorescent-based cell membrane integrity assay. *Environ. Eng. Sci.* v.27, n.1, p.329–335, 2010.

148) MORITA, A.; KRUTMANN, J. Ultraviolet A radiation-induced apoptosis. *Methods Enzymol*. v.319, n.1, p.302–309, 2000.

149) MOTA, A.C.V.; FREITAS, Z.M.F.; TODESCHINI, A.R.; CABRAL, L.M.; SANTOS, E.P. Inclusão do filtro solar p-metoxicinamato de octila em β-ciclodextrina e avaliação da purificação dos complexos de inclusão por RMN 1H. *Lat. Am. J. Pharm.* v.28, n.1, p. 97-99, 2009.

150) MUÑOZ, A.M.; CIVILLE, G.V.; CARR, B.T. Sensory evaluation in quality control. New York: Van Nostrand Reinhold; 1993. p. 240.

151) MURPHY, G.M. The acute effects of ultraviolet radiation on the skin. In: Photodermatology (Hawk, J.L.M., ed.), pp. 43–52, Oxford University Press, London,1999.

152) MUSCHIETTI, L.V.; MARTINO, V.S. Atividades biológicas dos flavonóides naturais. In: YUNES, R.A.; CECHINEL FILHO, V. Química de produtos naturais, novos fármacos e a moderna farmacognosia. Itajaí: Univali, 2007. p. 183-207.

153) NASCIMENTO, C.S.; NUNES, L.C.C., LIMA, A.A.N.; GRANGEIRO JUNIOR, S.; ROLIM NETO, P.J. Incremento do FPS em formulação de protetor solar utilizando extratos de pro polis verde e vermelha. *Rev. Bras. Farm*. v.30, n.1, p.334–339, 2009.

154) NELSON, D.L.; COX, M.M. Metabolismo do DNA. In: Lehninger, A.L. Princípios de Bioquímica do Lehninger. São Paulo: Sarvier. 2006. p. 940-84.

155) NICULAE, G.; LACATUSU, I.; BADEA, N.; STAN, R.; VASILE, B.S.; MEGHEA, A. Rice bran and raspberry seed oil-based nanocarriers with self-

antioxidative properties as safe photo protective formulations. *Photochem. Photobiol. Sci*. v.13, n.1, p.703–716, 2014.

156) NIJVELDT, R.J.; VAN NOOD, E.; VAN HOOM, D.E.; BOELENS, P.G.; VAN NORREN, K.; VAN LEEUWEN, P.A. Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr*. v.74, n.1, p.418–425, 2001.

157) OECD. Organization for Economic Co-operation and Development. Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests. n°129. 2010.

158) ORTEGA, P.A.; GUZMAN, M.E.; VERA, L.R.; VELAZQUEZ, J.A. Dihydroeuparin as sunscreen. *Bol. Soc. Chil. Quım.* v.45, n.1, p.629–636, 2000.

159) OU, B.; HAMPSCH-WOODILL, M.; PRIOR, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem*. v.49, n.1, p.4619-4926. 2001.

160) PACKER, L.; SIES, H. Singlet oxygen, UVA and ozone. *Methods Enzymol.* v.319, n.1, p.673–682, 2000.

161) PALM, M.D.; O`DONOGHUE, M.N. Update on photoprotection. *Dermatol Ther*. v.20, n.1, p.360-376, 2007.

162) PARK, Y.K.; IKEGAK, M.; ABREU, J.A.S.; ALCICI, N.M.F. Estudo da preparação dos extratos de propolis e suas aplicações. *Ciênc. Tecnol. Aliment.* v.18, n.1, p.313–318, 1998.

163) PASTRANA-BONILLA, E.; AKOH, C.C.; SELLAPPAN, S.; KREWER, G. Phenolic content and antioxidant capacity of Muscadine grapes. *J. Agric. Food Chem.,* v.51, n.18, p.5497-5503, 2003.

164) PATHAK, M.A. Photoprotection against harmful effects of solar UVB and UVA radiation: an update. In: Sunscreens: Development, evaluation and regulatory aspects

(Lowe, N.J., Shaath, N.A. and Pathak, M.A., eds), pp. 59– 82. Marcel Dekker, New York, NY (1997).

165) PERES, D.D.A.; OLIVERIA, C.; COSTA, M.S.; Tokunaga V. K.; MOTA, J. P.; ROSADO, C.; CONSIGLIERI, V. O.; KANEKO, T. M.; BABY, A.R. Rutin increases critical wavelength of systems containing a single UV filter and with good skin compatibility. *Skin Res. Technol.,* v.22, n.3, p.325-333, 2016.

166) PINTO, J.T. Efeito da estocagem da goiabada a diversas temperaturas sobre os teores de carotenóides e de acido ascórbico. Araraquara: Universidade Estadual Paulista, 2006. 93f. Dissertação (Mestrado em Ciências Farmacêuticas) – Faculdade de Ciências Farmacêuticas, Universidade Estadual Paulista, Araraquara.

167) PINTO, T.J.A.; KANEKO, T.M.; OHARA, M.T. C*ontrole Biológico de Qualidade de Produtos Farmacêuticos, Correlatos e Cosméticos.; São Paulo:* Atheneu. 2003, p. 75-96.

168) POURZAND, C.; TYRRELL, R.M. Apoptosis, the role of oxidative stress and the example of solar UV radiation. *Photochem. Photobiol*. v.70, n.1, p.380–390, 1999.

169) RAAB, W.; KINDL, U. Pflegekosmetik. 3.ed. Stuttgart: Wissenschaflliche, 1999. 371 p.

170) RAI, R.; SRINIVAS, C.R. Photoprotection. *Indian. J. Dermatolol. Venereol. Leprol.* v.73, n.2, p.73-79, 2007.

171) RAMOS, S.; HOMEM, V.; ALVES, A.; SANTOS, L. Advances in analytical methods and occurrence of organic UV-filters in the environment — A review. S*ci. Total Environ*. v.526, n.1, p.278-311, 2015.

172) RAMOS, S.; HOMEM, V.; ALVES, A.; SANTOS, L. A review of organic UV-filters in wastewater treatment plants. *Environ Int*. v.86, n.1, p.24-34, 2016.

173) RIBEIRO, C. J. Envelhecimento Cutâneo e Cosméticos. In: Cosmetologia Aplicada a Dermoestética. São Paulo: Pharmabooks, 2006. p. 203-240.

174) RIBEIRO, H. M.; MORAIS, J.A.; ECCLESTON, G.M. Strucuture and rheology of semisolid O/W creams containing cetyl alcohol/ non-ionic surfactant mixed emulsifier and different polymers. *Inter. J. Cosm. Sci.*, v.26, n.1, p.47-59, 2004.

175) RIEGER, M.M. The chemistry of tanning. *Cosmet. Toil.* v.98, n.1, p.47–50, 1983.

176) ROCHA, L.; DOS SANTOS, L.R.; ARCENIO, F. Otimização do processo de extração de propolis através da verificação da atividade antimicrobiana. *Rev. Bras. Farmacogn*. v.13, n.1, p.71–74, 2006.

177) ROJAS, J.; LONDONO, C.; CIRO, Y. The health benefits of natural skin UVA photopretective compounds found in botanical sources. *Int J Pharm Pharm Sci.* v.8, n.1, p.13-23, 2016.

178) ROMANHOLE, R.C.; ATAIDE, J.A.; MORIEL, P.; MAZZOLA, P.G. Update on ultraviolet A and B radiation generated by the sun and artificial lamps and their effects on skin. *Int. J. Cosm. Sci*. v.37, n.1, p.1–5, 2015.

179) ROMANHOLE, R.C.; ATAIDE, J.A.; CEFALI, L.C.; MORIEL, P.; MAZZOLA, P. Photostability study of commercial sunscreens submitted to artificial UV irradiation and/or fluorescent radiation. *J. Photochem. Photobiol. B: Biol*. v.162, n.1, p.45-49, 2016.

180) ROSSO, V.V. Composição de carotenoides e antocioninas em acerola. Estabilidade e atividade antioxidante em sistemas-modelo de extratos antociânicos de acerola e açaí [Dissertação]. Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, 2006.

181) RUFINO, M.S.M.; ALVES, R.E.; BRITO, E.S.; JIMENEZ, J.P.; CALIXTO, F.S. Bioactive compounds and antioxidant capacities of 18 non-traditional tropical fruits from Brazil. *Food Chem.* v.121, n.1, p.996-1002, 2010.

182) SALGADO, C; GALANTE, MC; LEONARDI, GR. Filtros solares: Mecanismos de ação e metodologias em preparações magistrais. *Inter. J. Pharm. Comp.,* v.6, n.4, p.224-236, 2004.

183) SAMBANDAN, D.R.; RATNER, D. Sunscreens: An overview and update. *J. Am. Acad. Dermatol.* v.64, n.1, p.748-758, 2011.

184) SANTOS, E.P.; FREITAS, Z.M.; SOUZA, K.R.; GARCIA, S.; VERGNANINI, A. *In vitro* and *in vivo* determinations of sun protection factors of sunscreen lotions with octylmethoxycinnamate. *Int. J. Cosmet. Sci.* v.21, n.1, p.1-5, 1999.

185) SANTOS, I.S.; PONTE, B.M.; BOONME, P.; SILVA, A.M.; SOUTO, E.B. Nanoencapsulation of polyphenols for protective effect against colon-rectal cancer. *Biotechnol. Adv*. v.31, n.1, p.514-523, 2013.

186) SAYRE, R. M.; AGIN, P. P.; LEVEE, G. J.; MARLOWE, E. Comparison of *in vivo* and in vitro testing of sunscreening formulas. *Photochem. Photobiol.* v.29, n.1, p. 559-566, 1979.

187) SCALIA, S.; MEZZENA, M. Photostabilization effect of quercetin on the UV filter combination, butyl methoxydibenzoylmethane-octyl methoxycinnamate. *Phtochem. Photobiol.* v.86, n.2, p.273-278, 2010.

188) SCHNITZLER, E.; CARVALHO-FILHO, M.A.S.; STADLER, C.C.; VOLPATO, A.M.; IONASHIRO, M. Aplicação da calorimetria exploratória diferencial (DSC) na caracterização térmica do acetato de dexametazona, excipientes e do creme de dexametazona. *Eclet.Quím.* v. 26, n.1, p.1-18, 2001.

189) SEAL, T. Quantitative HPLC analysis of phenolic acids, flavonoids and ascorbic acid in four different solvent extracts of two wild edible leaves, *Sonchus arvensis* and *Oenanthe linearis* of North-Eastern region in India. *J. Appl. Pharm. Sci*. v.6, n.2, p. 157-166. 2016.

190) SEITE, S.; COLIGE, A.; VIVENOT, P.P.; MON- TASTIER, C.; FOURTANIER, A.; LAPI ERE, C.; NUSGENS, B. A full-UV spectrum absorbing daily use cream protects human skin against biological changes occurring in photoaging. *Photodermatol. Photoimmunol. Photomed.* v.16, n.1, p.147–155, 2000.

191) SERPONE, N.; DONDI, D.; ALBINI, A. Inorganic and organic UV filters: their role and efficacy in sunscreens and suncare products. *Inorg. Chem. Acta.* v.360, n.1, p.794–802, 2007.

192) SGARBI, F.C.; CARMO, E.D.; ROSA, L.E.B. Radiação ultravioleta e carcinogênese. *Rev Cienc Med.* v.16, n.1, p.245-250, 2007.

193) SHAATH, N.A. The chemistry of sunscreens. In: LOWE NJ, SHAATH NA, PATHAK MA. Sunscreens, development, evaluation and regulatory aspects. 2nd. ed. New York: Marcel Dekker; 1997.

194) SIES, H.; STAHL, W. Lycopene: a biologically important carotenoid for humans? *Arch. Biochem. Biophys*. v.336, n.1, p.1–9, 1996.

195) SILVA, T.M.A.; MIGLIATO, K.F.; SALGADO, H.R.N.; ISAAC, V.L.B. Quality comparison of W/O and O/W photo-protection creams. *Cosmet Toil*. v.119, n.7, p.41-46, 2004.

196) SKIDMORE-ROTH, L. Handbook of herbs and natural supplements. 2°. ed. St. Louis: Mosby, 2004.

197) SKOTARCZAK, K.; OSMOLA-MAŃKOWSKA, A.; LODYGA, M.; POLAŃSKA, A.; MAZUR, M.; ADAMSKI, Z. Photoprotection: facts and controversies. *Eur. Rev. Med. Pharmacol. Sci.* v.19, n.1, p.98-112, 2015.

198) SOUSA, M.P.; MATOS, M.E.O.; MATOS, F.J.A.; MACHADO, M.I.L.; CRAVETRO, A.A. Constituintes químicos ativos de plantas medicinais brasileiras. Fortaleza: Universidade Federal do Ceará, 1991.

199) SOUZA, T.M.; SANTOS, L.E.; MOREIRA, R.R.D.; RANGEL, V.L.B.I. Sunscreen activity evaluation of *Achillea millefolium* L. (Asteraceae) *Rev. Bras. Farmacogn*. v.15, n.1, p.36-38, 2005.

200) SOUZA, F.P.; CAMPOS, G.R.; PACKER, J.F. Determinação da atividade fotoprotetora e antioxidante em emulsões contendo extrato de *Malpighia glabra* L. – Acerola. *Rev. Ciênc. Farm. Básica Apl.* v.34, n.1, p.69-77, 2013.

201) SPRINGSTEEN, A.; YUREK, R.; FRAZIER, M.; CARR, K.F. *In vitro* measurement of sun protection factor of sunscreens by diffuse transmittance. *Anal Chim Acta.* v.380, n.2-3, p.155-64, 1999.

202) STONE, H.S.; SIDEL, J.L. Sensory evaluation practices. San Diego, CA: Academic Press; 1992.

203) STOKE, W.S.; CASATI, S.; STRICKLAND, J.; PARIS, M. Neutral red uptake cytotoxicity tests for estimating starting doses for acute oral toxicity tests. *Curr. Protocol. Toxicol.* v.20, n.4, p.1-20, 2008.

204) SU, J.F.; GUO, C.J.; WEI, J.Y.; YANG, J.J.; JIANG, Y.G.; LI, Y.F. Protection against hepatic ischemiare perfusion injury in rats by oral pretreatment with quercetin. *Biomed. Environ. Sci*. v.16, n.1, p.1–8, 2003.

205) TAROZZI, A.; MARCHESI, A.; HRELIA, A. Protective effects of cyanidin-3-O-b-glucopyranoside against UVA-induced oxidative stress in human keratinocytes. *Photochem. Photobiol*. v.81, n.1, p.623–629, 2005.

206) TESCH, B.J. Herbs commonly used by women: an evidence-based review. *Disease-a-Month,* v.48, n.10, p.671-96, 2002.

207) TOHGE, T.; SOUZA, L.P.; FERNIE, A.R. Current understanding of the pathways of flavonoid biosynthesis in model and crop plants. *J Exp Bot*. v.68, n.1, p.4013–4028, 2017.

208) VAN ACKER, S.A.; VAN DER BERG, D.J.; TROMP, M.N.; GRIFFIOEN, D.H.; VAN BENNEKON, W.P.; VAN DER VIJGH, W.J.; BAST, A. Structural aspects of antioxidants activity of flavonoids. *Free Radic. Biol. Med.* v.20, n.3, p.331-342, 1996.

209) VAN BEEK, T.A.; MONTORO, P. Chemical analysis and quality control of *Ginkgo biloba* leaves, extracts, and phytopharmaceuticals. *J Chromatogr A.* v.1216, n.11, p.2002-2032. 2009.

210) VAN DER POLS, J.C.; WILLIAMS, G.M.; PANDEYA, N.; LOGAN, V.; GREEN, A.C. Prolonged prevention of squamous cell carcinoma of the skin by regular sunscreen use. *Cancer Epidemiol. Biomarkers Prev.* v.15, n.1, p.2546–2548, 2006.

211) VEBERIC, R.; JAKOPIC, J.; STAMPAR, F.; SCHMITZER, V. European elderberry (*Sambucus nigra* L.) rich in sugars, organic acids, anthocyanins and selected polyphenols. *Food Chem.* v.114, n.1, p.511–515, 2009.

212) VELASCO M.V.R.; BALOGH, T.S.; PEDRIALI, C.A.; SARRUF, F.D.; PINTO, C.; KANEKO, T.M.; BABY, A.R. Associação da Rutina com *p*-Metoxicinamato de Octila e Benzofenona-3: Avaliação *In Vitro* da Eficácia Fotoprotetora por Espectrofotometria de Refletância. *Lat Amer J Pharm.* v.27, n.1, p.23-27, 2008.

213) VELASCO, M.V.R.; BALOGH, T.S.; PEDRIALI, C.A.; SARRUF, F.D.; PINTO, C.A.S.O.; KANEKO, T.M.; BABY, A.R. Novas metodologias analíticas para avaliação da eficácia fotoprotetora (*in vitro*) - revisão. *Rev. Ciênc. Farm. Básica Apl*., v.32, n. 1, p.27-34, 2011.

214) VENDRAMINI, A.N.A.; TRUGO, L.C. Phenolic compounds in acerola fuit (*Malpighia punicifoli*a L.). *J. Braz. Chem. Soc*. v.15, n.1, p.664–668, 2004.

215) VERSCHOOTEN, L.; CLAERHOUT, S.; VAN LAETHEM, A.; AGOSTINIS, P.; GARMYN, M. New strategies of photoprotection. *Photochem. Photobiol*. v.82, n.1, p.1016–1023, 2006.

216) VIOLANTE, I.M.P.; SOUZA, I.M.; VENTURINI, C.L.; RAMALHO, A.F.S.; SANTOS, R.A.N.; FERRARI, M. Estudo preliminar da atividade fotoprotetora in vitro de extratos vegetais do cerrado de Mato Grosso. *Rev Bras Farm.* v.89, n.3, p.175-179, 2008.

217) USP - United States Pharmacopeia. 36th.ed. The United States Pharmacopeial Convention: Rockville, 2013.

218) WAGEMAKER, T.A.L.; CARVALHO, C.R.L.; MAIA, N.B.; BAGGIO, S.R.; FILHO, O.G. Sun protection factor, content and composition of lipid of green coffee beans. *Ind. Crop. Prod*, v.33, n.1, p.469-473. 2011.

219) WANG, S.Q.; BALAGULA, Y.; OSTERWALDER, U. Photoprotection: a review of the current and future technologies. *Derm Ther*. v.23, n.1, p.31-37, 2010.

220) WEN, L.; ZHAO, Y.; JIANG, Y.; YU, L.; ZENG, X.; YANG, J.; TIAN, M.; LIU, H.; YANG, B. Identification of a flavonoid C-glycoside as potent antioxidant. *Radic. Biol. Med. v.*110, n.1, p.92-101, 2017.

221) WHO. World Health Organization. Fact sheet nº 261: protecting children from ultraviolet. Genebra: World Health Organization; 2001.

222) WHO. World Health Organization. Ultraviolet radiation (UV) – Sun protection. Available in: http://www.who.int/uv/sun_protection/en/. Accessed in June 26th, 2018.

223) WLASCHEK, M.; TANTCHEVA-POOR, I.; NADERIA, L. Solar UV irradiation and dermal photoaging. *J. Photochem. Photobiol. B*, v.63, n.1, p.41–51, 2001.

224) WONG, T.; ORTON, D. Sunscreen allergy and its investigation. *Clin Dermatol*. v.29, n.1, p.306-310, 2011.

225) YONG, Q.; XUEQING, Q.; SHIPING, Z. Lignin: a nature-inspired sun blocker for broad- spectrum sunscreens. *Green Chem*. v.17, n.1, p.320– 324, 2015.

226) ZANATTA, C.F.; MITJANS, M.; URGATONDO, V.; ROCHA-FILHO, P.A.; VINARDELL, M.P. Photo protective potential of emulsions formulated with Buriti oil (*Mauritia flexuosa*) against UV irradiation on keratinocytes and fibroblasts cell lines. *Food Chem. Toxicol.*, v.48, n.1, p.70-75, 2010.

227) ZUANAZZI, J.A.S.; MONTANHA, J.A. Flavonóides. In: SIMÕES, C.M.O.; SCHENKEL, E.P.; GOSMANN, G.; PALAZZO de MELLO, J.C.; MENTZ, L.A.; ROS PETROVICK, P. Farmacognosia da planta ao medicamento. 6 ed. Porto Alegre/ Florianópolis: Ed. Universidade UFRGS/ Ed. Universidade UFSC, 2007. p.577-611.

ANEXO A - Certificado CGEN referente ao uso de patrimônio genético.

Autorização de Acesso e de Remessa de Componente do Patrimônio Genético O Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, nos termos Deliberação 246/2009, do Conselho de Gestão do Patrimônio Genético, autoriza a instituição identificada no verso deste documento a acessar e remeter componente do Patrimônio Genético com a finalidade de pesquisa científica. Brasília, 05 de junho de 2015 Marcelo Marcos Morales Diretor de Ciências Agrárias, Biológicas e da Saúde PO 161/2010	Processo: 010376/2015-1 Validade: 05/06/2015 a 05/06/2016 Instituição: UNIVERSIDADE ESTADUAL DE CAMPINAS CNPJ: 460.684.250/0001-33 Pesquisador: Priscila Gava Mazzola CPF: 292.033.668-14 RG: 306978775 - SSP / SP *Para visualizar a versão digital da Autorização de Acesso e de Remessa de Componente do Patrimônio Genético, V.Sa. poderá utilizar a ferramenta disponibilizada pelo CNPq para esse fim na página http://servicosweb.cnpq.br/visualizador/ e informar o número do protocolo 1913868442670753 para recuperá-la do banco de dados do CNPq*

ANEXO B - Parecer do Comitê de Ética em Pesquisa referente ao estudo de análise sensorial em seres humanos

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: DESENVOLVIMENTO E AVALIAÇÃO DO FATOR DE PROTEÇÃO SOLAR DE EMULSÃO CONTENDO EXTRATO RICO EM FLAVONÓIDES

Pesquisador: Letícia Caramori Cefali

Área Temática:

Versão: 2

CAAE: 59552216.3.0000.5404

Instituição Proponente: Instituto de Biologia - Unicamp

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.898.502

Apresentação do Projeto:

Resumo:

O uso de filtros solares é necessário para evitar doenças de pele, especialmente o câncer de pele, além de prevenir contra o envelhecimento cutâneo precoce. O trabalho tem como objetivo estudar o potencial de fator de proteção solar de extrato contendo flavonóides para ser incorporado em formulação de uso tópico. Será utilizado material vegetal contendo flavonóides para obtenção de um extrato rico. Após a avaliação da concentração de flavonóides no extrato e da verificação da absorção na região do ultravioleta realizado em espectrofotômetro, o extrato será avaliado quanto à sua citotoxidade e atividade antioxidante que contribuirá para o combate ao envelhecimento precoce da pele. O extrato será incorporado em uma emulsão cosmética que será desenvolvida, previamente analisada sensorialmente, e sua estabilidade físico-química será avaliada. A formulação base escolhida através do ensaio de análise sensorial e estável terá o extrato incorporado e será submetida aos testes de

espalhabilidade, análise térmica, controle microbiológico, avaliação da atividade antioxidante e do fator de proteção solar in vitro, e ao teste de permeação cutânea para a avaliação da sua capacidade de permanência no estrato córneo, epiderme e derme, para a efetiva proteção da pele

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frente aos raios solares. Com isso, a formulação contendo extrato rico em flavonóides poderá ser utilizada como um produto destinado à proteção solar, sendo, portanto, uma alternativa a mais para o combate aos efeitos deletérios provenientes da exposição à radiação ultravioleta.

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar as características sensoriais de quatro formulações cosméticas sem ativo ou também denominadas de formulações base, e a escolhida como preferida pelos voluntários será utilizada posteriormente como veículo de material vegetal rico em flavonóides para a utilização como protetor solar.

Objetivo Secundário:

• Desenvolver e avaliar a estabilidade físico-química de quatro formulações base contendo matérias-primas conhecidas e amplamente utilizadas em produtos cosméticos;

• Realizar a análise sensorial com as quatro formulações base;

• Escolher a formulação que apresente maior aceitação entre os voluntários, de acordo com os critérios de preferência, utilizando a escala gostei muitíssimo até desgostei muitíssimo e escala de características sensoriais;

• A formulação escolhida pela análise sensorial será utilizada como veículo para material vegetal rico em flavonóides que apresente fator de proteção solar.

Avaliação dos Riscos e Benefícios:

Riscos:

Se ocorrer algum desconforto, como coceira, na região aplicada, o voluntário será orientado a lavar a região com água e sabão e será suspensa a realização do teste. Caso o voluntário apresente quadro alérgico ao produto, como coceira ou vermelhidão na região, após 48 horas da realização do teste, ele será encaminhado ao serviço de urgência e emergência do Hospital das Clínicas da UNICAMP para ser atendido. Portanto, em caso de qualquer dano decorrente da pesquisa, está garantida a assistência integral e imediata, de forma gratuita, pelo tempo que for necessário.

Benefícios:

Ao participar da pesquisa, o voluntário não terá nenhum benefício direto e/ou a algum tipo de pagamento. Entretanto, o estudo fornecerá informações importantes sobre o uso de cremes corporais, de forma que o conhecimento que será construído a partir desta pesquisa possa contribuir para a evolução do desenvolvimento de produtos cosméticos.

Comentários e Considerações sobre a Pesquisa:

projeto de Doutorado da aluna Pesquisador Letícia Caramori Cefali

Orientador: Profª Drª Priscila Gava Mazzola

O projeto de pesquisa prevê a elaboração de uma formulação cosmética contendo filtro solar, obtido de um material vegetal rico em flavonoides, porém o filtro não será submetido à análise sensorial. Para o estudo de análise sensorial, serão utilizadas apenas as formulações cosméticas sem ativo, ou também denominadas formulações base.

Trata-de de uma pesquisa com o intuito de realizar uma análise sensorial, baseada nas definições de Sensory Evaluation Division of the Institute of Food Techonologists, pode ser entendida como a disciplina que interpreta, evoca, avalia e mede reações às características de um produto, após estímulos ao ser humano em relação à visão, ao tato, ao odor e ao sabor e como esses estímulos são percebidos pelos órgãos do sentido. Portanto, o estudo se baseia em avaliar sensorialmente formulações cosméticas de acordo com a preferência dos voluntários. Pois, para o desenvolvimento de um produto cosmético, a análise sensorial tem se mostrado uma ferramenta útil no sentido de se obter produtos não só seguros e eficazes, assegurados pelos testes específicos de segurança e eficácia, mas também aceitáveis pelos consumidores potenciais. Com isso, para a realização do estudo, o voluntário será convidado a espalhar na região posterior do antebraço com sua própria mão, 2 g de cada um dos quatro tipos de cremes utilizados na pesquisa. Após o espalhamento de cada produto, será oferecido papel absorvente para a limpeza da região e, assim, será realizada a análise do próximo creme até a finalização do teste. Após a experimentação dos cremes, o voluntário responderá um questionário sobre a preferência entre as formulações analisadas.

Considerações sobre os Termos de apresentação obrigatória:

1. Folha de rosto: adequada;
2. Vínculo com a instituição: adequado;
3. Projeto detalhado: adequado;
4. Cronograma: adequado;
5. Orçamento: adequado;foi incluído um total de R$ 673,22 no documento PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_751815.pdf de 20/12/2016
6. TCLE: adequado.

- ANEXO_C_modelo_cartaz.pdf: cartaz

Conclusões ou Pendências e Lista de Inadequações:

- Foi apresentado o modelo do cartaz a ser utilizado; contém local, endereço, horário e título da pesquisa.

- O cronograma foi apresentado detalhadamente na carta resposta e no projeto completo PROJETO_DE_PESQUISA_Leticia_Cefali_reescrito.pdf de 20/12/2016 ; mas não no projeto plataforma Brasil (PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_751815.pdf - 20/12/2016).

Todas as pendências foram respondidas e estão descritas nos itens acima. O projeto encontra-se aprovado.

Considerações Finais a critério do CEP:

- O sujeito de pesquisa deve receber uma via do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado.

- O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado.

- O pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado. Se o pesquisador considerar a descontinuação do estudo, esta deve ser justificada e somente ser realizada após análise das razões da descontinuidade pelo CEP que o aprovou. O pesquisador deve aguardar o parecer do CEP quanto à descontinuação, exceto quando perceber risco ou dano não previsto ao sujeito participante.

- O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo. É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido e enviar notificação ao CEP junto com seu posicionamento.

- Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas.

- Relatórios parciais e final, em formulário próprio do CEP, devem ser apresentados ao CEP, inicialmente seis meses após a data deste parecer de aprovação e ao término do estudo.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_751815.pdf	20/12/2016 10:43:00		Aceito
Projeto Detalhado / Brochura Investigador	PROJETO_DE_PESQUISA_Leticia_Cefali_reescrito.pdf	20/12/2016 10:41:53	Letícia Caramori Cefali	Aceito
Outros	ANEXO_C_modelo_cartaz.pdf	20/12/2016 10:40:20	Letícia Caramori Cefali	Aceito
Recurso Anexado pelo Pesquisador	CARTA_RESPOSTA_CEP_Leticia_Cefali.pdf	20/12/2016 10:39:16	Letícia Caramori Cefali	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	termo_de_consentimento_livre_e_esclarecido_reescrito.pdf	20/12/2016 10:38:44	Letícia Caramori Cefali	Aceito
Orçamento	ORCAMENTO.pdf	20/12/2016 10:38:24	Letícia Caramori Cefali	Aceito
Cronograma	CRONOGRAMA.pdf	20/12/2016 10:37:54	Letícia Caramori Cefali	Aceito
Folha de Rosto	folha_de_rosto.pdf	02/09/2016 21:49:06	Letícia Caramori Cefali	Aceito
Outros	comprovante_de_matricula.pdf	02/09/2016 21:48:51	Letícia Caramori Cefali	Aceito
Outros	questionario.pdf	10/08/2016 10:11:33	Letícia Caramori Cefali	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

CAMPINAS, 25 de Janeiro de 2017

Assinado por:
Maria Fernanda Ribeiro Bittar
(Coordenador)

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ANEXO C - Termo de Consentimento Livre e Adquirido referente ao estudo de análise sensorial em seres humanos.

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Análise sensorial para a escolha de um cosmético de preferência para ser utilizado como filtros solar para o corpo

Letícia Caramori Cefali, Priscila Gava Mazzola

Número do CAAE: 59552216.3.0000.5404

Você está sendo convidado a participar como voluntário de um estudo. Este documento, chamado Termo de Consentimento Livre e Esclarecido, visa assegurar seus direitos e deveres como participante e é elaborado em duas vias, uma que deverá ficar com você e outra com o pesquisador.

Por favor, leia com atenção e calma, aproveitando para esclarecer suas dúvidas. Se houverem perguntas antes ou mesmo depois de assiná-lo, você poderá esclarecê-las com o pesquisador. Se preferir, pode levar para casa e consultar seus familiares ou outras pessoas antes de decidir participar. Se você não quiser participar ou retirar sua autorização, a qualquer momento, não haverá nenhum tipo de penalização ou prejuízo.

Justificativa e objetivos:

O estudo tem como finalidade determinar o melhor cosmético a ser utilizado como um filtro solar para o corpo dentre os quatro tipos de formulações base (sem ativo) analisados.

Procedimentos:

Participando do estudo você está sendo convidado a: espalhar na região posterior do antebraço com sua própria mão, 2 gramas de cada um dos quatro tipos de cremes utilizados na pesquisa. Após o espalhamento de cada produto, será oferecido papel absorvente para a limpeza da região e, assim, será realizada a análise do próximo creme até a finalização do teste. Após a experimentação dos cremes, você responderá um questionário sobre a preferência entre as formulações analisadas.

Observações:

- O teste será realizado durante duas semanas, no Laboratório de Aulas Práticas da Farmácia (FCM 11, Rua Alexander Fleming – UNICAMP – Cidade Universitária, Campinas – SP) e terá duração de 15 a 30 minutos para a execução do teste e do preenchimento do questionário.

Desconfortos e riscos:

Você **não** deve participar deste estudo se apresentar alergia (dermatite de contato) a produtos cosméticos. Se ocorrer algum desconforto, como coceira, na região aplicada, lavar a região com água e sabão e suspender a realização do teste. Caso o voluntário apresente quadro alérgico ao produto, como coceira ou vermelhidão na região, após 48 horas da realização do teste, ele será encaminhado ao serviço de urgência e emergência do Hospital das Clínicas da Unicamp para ser atendido.

Benefícios:

Ao participar desta pesquisa, você não terá nenhum benefício direto. Entretanto, esperamos que este estudo forneça a você informações importantes sobre o uso de cremes

Rubrica do pesquisador:_____ Rubrica do participante:_____

corporais, de forma que o conhecimento que será construído a partir desta pesquisa possa contribuir para a evolução do desenvolvimento de produtos cosméticos.

Acompanhamento e assistência:

Como o estudo será realizado em poucos minutos, não haverá a necessidade de acompanhamento posterior ao teste.

Sigilo e privacidade:

Você tem a garantia de que sua identidade será mantida em sigilo e nenhuma informação será dada a outras pessoas que não façam parte da equipe de pesquisadores. Na divulgação dos resultados desse estudo, seu nome não será citado.

Ressarcimento:

No estudo realizado, você não terá nenhum tipo de despesa para participar desta pesquisa, bem como nada será pago por sua participação.

Em caso de dano decorrente da pesquisa, está garantida a assistência integral e imediata, de forma gratuita, pelo tempo que for necessário. Você também tem direito a indenização em caso de danos.

Contato:

Em caso de dúvidas sobre o estudo, você poderá entrar em contato com Letícia Caramori Cefali, Laboratório de Aulas Práticas da Farmácia (FM 11 Rua Alexander Fleming – UNICAMP – Cidade Universitária, Campinas – SP) ou nos telefones 19 35217486 ou no celular 19 991020829. Em caso de denúncias ou reclamações sobre sua participação no estudo, você pode entrar em contato com a secretaria do Comitê de Ética em Pesquisa (CEP): Rua: Tessália Vieira de Camargo, 126; CEP 13083-887 Campinas – SP; telefone (19) 3521-8936; fax (19) 3521-7187; e-mail: cep@fcm.unicamp.br

Consentimento livre e esclarecido:

Após ter sido esclarecimento sobre a natureza da pesquisa, seus objetivos, métodos, benefícios previstos, potenciais riscos e o incômodo que esta possa acarretar, aceito participar:

Nome do(a) participante: _____

_____ Data: ____/_____/_____.
(Assinatura do participante ou nome e assinatura do responsável)

Responsabilidade do Pesquisador:

Asseguro ter cumprido as exigências da resolução 466/2012 CNS/MS e complementares na elaboração do protocolo e na obtenção deste Termo de Consentimento Livre e Esclarecido. Asseguro, também, ter explicado e fornecido uma cópia deste documento ao participante. Informo que o estudo foi aprovado pelo CEP perante o qual o projeto foi apresentado. Comprometo-me a utilizar o material e os dados obtidos nesta pesquisa exclusivamente para as finalidades previstas neste documento ou conforme o consentimento dado pelo participante.

_____ Data: ____/_____/_____.
(Assinatura do pesquisador)

Rubrica do pesquisador:_____ Rubrica do participante:_____

ANEXO D - Termo de Bioética/Biossegurança.

UNICAMP
COORDENADORIA DE PÓS-GRADUAÇÃO
INSTITUTO DE BIOLOGIA
Universidade Estadual de Campinas
Caixa Postal 6109. 13083-970, Campinas, SP, Brasil
Fone (19) 3521-6378. email: cpgib@unicamp.br
IB

DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Tese de Doutorado, intitulada "***ESTUDO DO FATOR DE PROTEÇÃO SOLAR DE EXTRATO CONTENDO FLAVONÓIDES INCORPORADO EM FORMULAÇÃO DE USO TÓPICO***", desenvolvida no Programa de Pós-Graduação em Biociências e Tecnologia de Produtos Bioativos do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

Assinatura: _____
Nome do(a) aluno(a): Letícia Caramori Cefali

Assinatura: _____
Nome do(a) orientador(a): Profª Drª Priscila Gava Mazzola

Profa. Dra. Priscila G. Mazzola
CRF 33311 - Matr. 293994
Faculdade de Ciências Farmacêuticas
UNICAMP

Data: 31 de agosto de 2018

ANEXO E - Declaração de direitos autorais.

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **ESTUDO DO FATOR DE PROTEÇÃO SOLAR DE EXTRATO CONTENDO FLAVONÓIDES INCORPORADO EM FORMULAÇÃO DE USO TÓPICO**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 31 de agosto de 2018

Assinatura : Letícia C. Cefali

Nome do(a) autor(a): **Letícia Caramori Cefali**

RG n.° 34.995.214-0

Profa. Dra. Priscila G. Mazzola
CRF 33311 - Matr. 293994
Faculdade de Ciências Farmacêuticas
UNICAMP

Assinatura : _____

Nome do(a) orientador(a): **Profª Drª Priscila Gava Mazzola**

RG n.° 30697277-5