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PROGRAMA DE PÓS-GRADUAÇÃO EM FÁRMACO E MEDICAMENTOS
ÁREA DE PRODUÇÃO E CONTROLE FARMACÊUTICOS

Cutaneous attributes of chlorogenic acid, apigenin, kaempferol and naringenin established by HPLC-TBARS-EVSC protocol and Laser Doppler flowmetry

Atributos cutâneos do ácido clorogênico, apigenina, kaempferol e naringenina estabelecidos pelo protocolo HPLC-TBARS-EVSC e fluxometria Laser Doppler

Nadia Ruscinc

Tese para obtenção do grau de
DOUTOR

Orientador: Prof. Dr. André Rolim Baby

São Paulo

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Prof. Dr. André Rolim Baby

orientador/presidente

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2º. Examinador

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3º. Examinador

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ABSTRACT

RUSCINC, N. **Cutaneous attributes of chlorogenic acid, apigenin, kaempferol and naringenin established by HPLC-TBARS-EVSC protocol and Laser Doppler flowmetry**. 2024. 65f. Thesis (PhD) - Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2024

The integumentary system, a vital organ for organisms, constitutes a multifaceted barrier against pathogens and environmental factors, crucial for maintaining homeostasis. Skin, composed of epidermis, dermis, and hypodermis, plays a pivotal role in preserving life. Intrinsic and extrinsic factors can accelerate skin aging and compromise its homeostatic functions. Solar radiation, particularly ultraviolet (UV) radiation, poses a significant risk for skin cancer and accelerates photoaging. This study explores the in vitro safety and protective effects of polyphenols (chlorogenic acid, apigenin, kaempferol, and naringenin) against UV-induced lipid peroxidation using innovative methodologies, including HET-CAM assay, HPLC-TBARS-EVSC, and laser Doppler flowmetry. The HET-CAM assay confirmed non-irritating profiles for the polyphenols. HPLC-TBARS-EVSC revealed that only naringenin exhibited a significant reduction in lipid peroxidation, while others showed pro-oxidant tendencies. Laser Doppler flowmetry demonstrated anti-inflammatory potential, with naringenin displaying superior efficacy in reducing inflammatory response induced by methyl nicotinate. This comprehensive investigation underscores the diverse protective roles of polyphenols in skin health, emphasizing naringenin's notable anti-radical and anti-inflammatory properties.

Keywords: Polyphenols. TBARS. Lipid peroxidation. Oxidative stress

RESUMO

RUSCINC, N. **Atributos cutâneos do ácido clorogênico, apigenina, kaempferol e naringenina estabelecidos pelo protocolo HPLC-TBARS-EVSC e fluxometria Laser Doppler.** 2024. 65f. Tese (Doutorado) - Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2024.

O sistema tegumentar, órgão vital para os organismos, constitui uma barreira multifacetada contra patógenos e fatores ambientais, crucial para a manutenção da homeostase. A pele, composta por epiderme, derme e hipoderme, desempenha um papel fundamental na preservação da vida. Fatores intrínsecos e extrínsecos podem acelerar o envelhecimento da pele e comprometer as suas funções homeostáticas. A radiação solar, especialmente a radiação ultravioleta (UV), representa um risco significativo de cancer de pele e acelera o fotoenvelhecimento. Este estudo explora a segurança in vitro e os efeitos protetores dos polifenóis (ácido clorogênico, apigenina, kaempferol e naringenina) contra a peroxidação lipídica induzida por UV usando metodologias inovadoras, incluindo ensaio HET-CAM, HPLC-TBARS-EVSC e fluxometria Laser Doppler. O ensaio HET-CAM confirmou os perfis não irritantes para os polifenóis. HPLC-TBARS-EVSC revelou que apenas a naringenina exibiu uma redução significativa na peroxidação lipídica, enquanto outros mostraram tendências pró-oxidantes. A fluxometria Laser Doppler demonstrou potencial anti-inflamatório, com a naringenina apresentando eficácia superior na redução da resposta inflamatória induzida pelo nicotinato de metila. Esta investigação abrangente destaca os diversos papéis protetores dos polifenóis na saúde da pele, enfatizando as notáveis propriedades anti-radicais e anti-inflamatórias da naringenina.

Palavras-chave: Polifenois. TBARS. Peroxidação Lipídica. Estresss Oxidativo.

SUMMARY

Introduction

1. Introduction

The integumentary system constitutes an organ, featuring a specialized structure that culminates in the formation of a protective barrier enveloping the external aspect of the organism. This barrier serves as a robust defense mechanism against pathogenic agents and environmental factors. Moreover, it plays a pivotal role in the regulation of water and electrolyte balance, contributes to the synthesis of vitamin D, and actively participates in temperature regulation, thereby striving to achieve and maintain homeostasis within the organism (JUNQUEIRA; CARNEIRO, 2013).

Comprising the epidermis, dermis, and hypodermis, the skin manifests a tri-layered architecture, each layer endowed with distinctive characteristics and functions (HARRIS, 2003). The maintenance of structural integrity and functionality of these layers is paramount due to the vital role the skin plays in preserving life.

The skin is subject to intrinsic and extrinsic processes that can hasten cellular senescence, disrupting its homeostatic functions (TOBIN, 2017). A proactive approach to sustain skin health involves the utilization of products bolstering protection against solar radiation, particularly ultraviolet (UV) radiation, as unprotected exposure poses a significant risk for skin cancer development (IARC, 2012) and other degenerative processes such as actinic elastosis (REINEHR, 2019). Continuous exposure to solar radiation expedites photoaging (SINOVÁ *et al*., 2021).

Skin cancer, a prevalent malignancy in the Brazilian population, inflicts substantial harm, characterized by the aberrant proliferation of skin cells (ZHAO *et al*., 2016). Projections from the National Cancer Institute (INCA) anticipate 220,490 new cases of non-melanoma skin cancer annually between 2023 and 2025, corresponding to 101.95 new cases per 100,000 inhabitants (INCA, 2022).

UV radiation exhibits varying behaviors contingent on its wavelength range (nm) (LAUTENSCHLAGER *et al*., 2007). Despite endogenous defense mechanisms, cumulative exposure to harmful UV effects can disrupt skin homeostasis, predisposing the skin to pathologies and accelerating aging processes. Natural protective mechanisms, such as the inhibition of free radicals and thickening of the horny layer, have limits in the face of prolonged exposure (JANSEN *et al*., 2013).

Polyphenols are molecules that have the property of easily donating hydrogen or electrons so that their intermediate radicals effectively prevent the oxidation of substances, such as lipids, for example (VELLOSA *et al*., 2021). Due to these properties, they act by inhibiting the formation of reactive oxygen species (ROS). By preventing their formation, they can also inhibit the harmful effects caused by UV radiation (SUN *et al*., 2022). This skin protection mechanism of action of such compounds against diverse unfavorable contexts justifies the present investigation, which established the in vitro safety of the topical application of chlorogenic acid, apigenin, kaempferol, and naringenin, in addition to the innovation through the use of the *ex vivo* assay developed by this Research Group, the HPLC-TBARS-EVSC (high-performance liquid chromatography-thiobarbituric acid reactive substances-*ex vivo* stratum corneum) (SAUCE *et al*., 2021a) and the *in vivo* determination (research participants) of the anti-inflammatory potential of the aforementioned polyphenols by laser Doppler flowmetry.

Literatura review

2. Literature review

2.1 An overview of oxidative stress

The endogenous protection of the skin against UV radiation may be insufficient when there is excessive production of free radicals due to metabolism or unprotected exposure to environmental factors. The excessive formation of reactive oxygen species (ROS), constantly produced by the body, can cause damage to proteins and oxidize lipids present in cells. Cutaneous oxidative stress can trigger modifications in the normal functions of cells, leading to inflammation, erythema, premature aging, and the development of skin cancer, particularly associated with frequent exposure of unprotected skin to such radiation (CHEN *et al*., 2012; QUINTON, 2012).

Oxidative stress is a biological condition responsible for various damages to the organism. The main harmful free radicals to humans are derived from three elements: oxygen, nitrogen, and sulfur, giving rise to reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species (RSS) (CAROCHO *et al*., 2013). The primary targets of these reactive species are proteins, DNA, RNA, sugar molecules, and lipids (LU *et al*., 2010; CRAFT *et al*., 2012).

Although a significant amount of free radicals is naturally produced by the body and is essential for life, their high reactivity can lead to unwanted reactions resulting in cellular damage. Human organisms have endogenous defenses capable of suppressing oxidative actions (CAROCHO *et al*., 2013). Enzymatic defenses, such as glutathione peroxidase and catalase, eliminate hydrogen peroxide. Another essential enzyme is superoxide dismutase, responsible for converting superoxide anion into hydrogen peroxide for subsequent degradation by the catalase-superoxide dismutase

system, protecting the organism from the harmful effects of superoxide anion. Non-enzymatic defense systems, such as L-ascorbic acid, vitamin A compounds, alpha-tocopherol, and ubiquinol (coenzyme Q_{10}), suppress and prevent the formation of free radicals (CAROCHO *et al*., 2013).

Another important process that promotes cellular damage is lipid peroxidation, resulting from a cascade of biochemical events caused by oxidative deterioration of lipids generated by oxidative stress. Lipid peroxidation occurs when free radicals react with the unsaturated fatty acids of phospholipids in cell membranes, altering their biophysical properties and allowing the entry of these highly reactive species into intracellular structures (HALLIWELL; GUTTERIDGE, 1989; JOHNSON, 2017). The reaction results in the formation of lipid peroxides, leading to deleterious actions in cells such as the rupture of cell membranes, affecting organelles, mutations in DNA, oxidation of unsaturated lipids, formation of chemical residues, and impairment of extracellular matrix components such as proteoglycans, collagen, and elastin (HALLIWELL; GUTTERIDGE, 1989; DEVASAGAYAM *et al*., 2003). A relevant means of evaluating formation of lipid peroxides and oxidative stress is TBARS method, which is highly versatile for evaluating lipoperoxidation in models of varios pathologies, in the food area and topical use.

Conditions induced by oxidative damage are associated with various severe diseases, including autoimmune ones, inflammatory and degenerative disorders associated with aging. Diabetes mellitus and its complications, cataracts, cardiovascular diseases, such as atherosclerosis and stroke, neurological disorders, renal and hepatic disorders, rheumatoid arthritis, adult respiratory distress syndrome, Alzheimer's and Parkinson's diseases, Huntington's disease, lupus erythematosus,

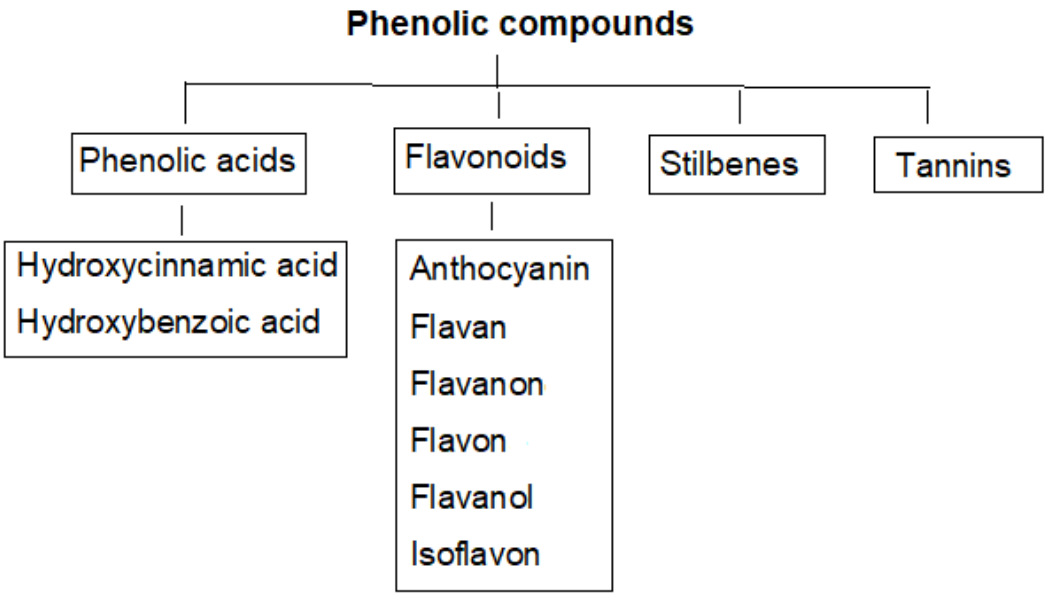
and cancer among others are characterized as degenerative diseases (RAHMAN, 2007; LOBO *et al*., 2010; LU *et al*., 2010; SINGH *et al*., 2010).

2.2 Phenolic Compounds and Antioxidant Activity

Secondary metabolite compounds from plants exhibit a wide range of biological properties and can be characterized by one or more hydroxyl groups (OH) attached to an aromatic ring, which play a crucial role in protecting against damage induced by UV radiation and microorganisms due to their chromophore groups and aromatic rings (CANTUÁRIA *et al*., 2023; SARAF; KAUR, 2010).

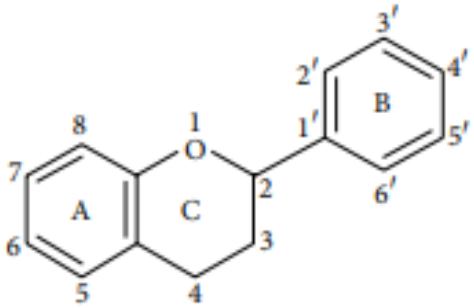
Phenolic compounds can be classified into simple phenolics and polyphenols, including phenolic acids, tannins, flavonoids, and stilbenes (NIEDZWIECKI *et al*., 2016). These substances can also be classified based on substituents in their structures, such as hydroxyls, methoxyls, acyls *etc.*, leading to categories like flavones, flavonols, anthocyanins, chalcones, aurones, flavanones, and isoflavonoids, among others, as shown in **Figure 1** (SIMÕES *et al*., 2010).

Figure 1 - Classification of phenolic compounds (DE LA ROSA *et al*., 2019).



The biochemical activities of phenolic compounds depend on their chemical structure, particularly the hydrogen atoms of adjacent hydroxyl groups (ortho-diphenols) located in various positions of rings A, B, and C, as can be seen in **Figure 2**. Additionally, the presence of double bonds in benzene rings and the keto (-C=O) function of some flavonoid molecules contribute to their high antioxidant activity. The activities can also vary with substitutions such as hydrogenation, hydroxylation, methylation, sulfation, glycosylation (BIRT; HENDRICH; WANG, 2011).

Figure 2 - Flavonoid basic structure (PIETTA, 2000).

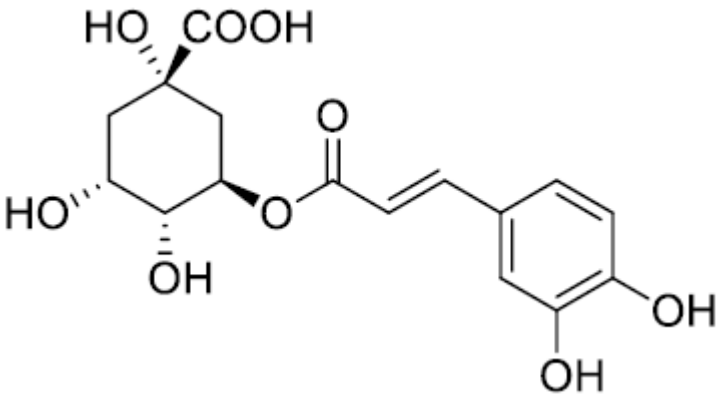


These compounds, both flavonoids and non-flavonoids, possess diverse properties such as anti-inflammatory, antioxidant, adjuvant in photoprotection, and antimicrobial, among other benefits for human health. Due to their structural diversity and the use of nanotechnology and biotechnology, these substances find relevant applications in the cosmetic industry for maintaining skin health and improving cutaneous cosmetic attributes (HWANG, 2012; OLIVEIRA; DARIO, 2017).

2.2.1 Chlorogenic Acid

Chlorogenic acid (ACLOR) [1,4, 5-trihydroxyclohexanecarboxylic acid 3-(3,4 dihydroxycinnamate)], illustrated in **Figure 3**, is an ester of hydroxycinnamic and quinic acids, found widely in the plant kingdom, including coffee beans, apples, and blueberries (SCHIASSI *et al*., 2018).

Figure 3 - Chlorogenic acid chemical structural formula (SIMÕES *et al.*, 2010).



Among the biological properties of ACLOR, its anti-inflammatory, antioxidant, and immunomodulatory actions have been extensively studied (XU, *et al*., 2010; NAVEED et al., 2018). In terms of antioxidant activity, ACLOR plays a crucial role in maintaining cellular homeostasis, preventing photoaging of the skin by slowing down this process when exposed to UVA radiation (GIRSANG *et al*., 2021).

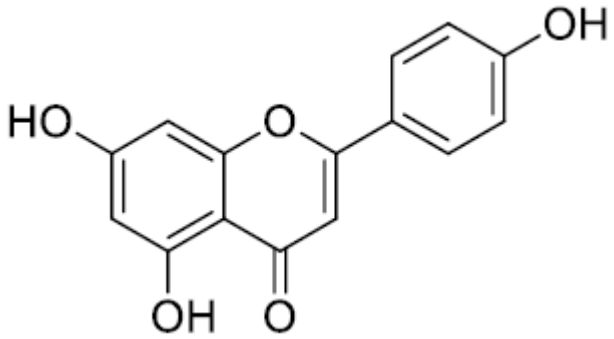
Studies by Xue *et al*. (2022) revealed the protective effect of ACLOR in preventing damage to human dermal fibroblasts under UVA irradiation. ACLOR was observed to enhance the expression of collagen type 1, reducing levels of oxidative stress, promoting cell repair, and reducing apoptosis in human dermal fibroblasts exposed to UVA radiation.

ACLOR also demonstrates the ability to suppress melanogenesis in melanoma cells by inhibiting the activity of the tyrosinase enzyme, preventing excessive pigment formation, and reducing excessive coloration in skin hyperpigmentation (LI *et al*., 2014). Moreover, ACLOR has been also shown to protect cells from oxidative stress induced by UVB radiation, eliminating superoxide anions, hydroxyl radicals, and intracellular ROS generated by hydrogen peroxide and UVB radiation in human HaCaT cells (CHA *et al*., 2014).

2.2.2 Apigenin

Apigenin (API) [4', 5, 7-trihydroxyflavone, 5, 7-Dihydroxy-2-(4-hydroxyphenyl)-4-benzopyrone)] is found in fruits, vegetables, flowers, such as chamomile, and spices (ZHOU *et al*., 2017). It has antioxidant, anti-inflammatory, and antimicrobial properties (MADUNIC *et al*., 2018). Apigenin has been shown to protect Caco-2 cells against potential damage caused by UVA and UVB radiation (**Figure 4**).

Figure 4 - Apigenin chemical structural formula (DE LA ROSA *et al.*, 2019).

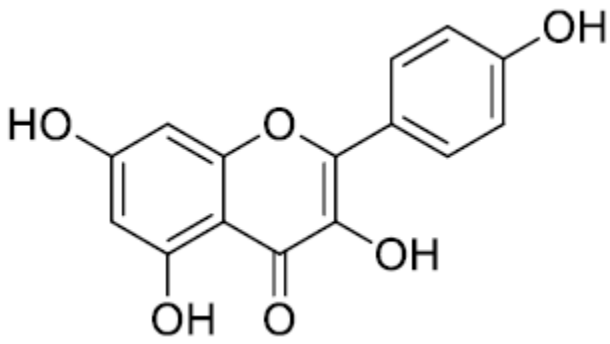


Studies demonstrated that apigenin significantly increased the viability of HaCaT cells and reduced the generation of ROS when exposed to UVA radiation. Pretreatment of HaCaT cells with apigenin before UVA exposure resulted in the suppression of matrix metalloproteinase (MMP-1) production, preventing skin damage when exposed to UV radiation (LI *et al*., 2014).

2.2.3 Kaempferol

Kaempferol (KAPF) [3,4', 5, 7-Tetrahydroxyflavone, 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one)], illustrated in **Figure 5**, is a flavonoid present in various plants, including broccoli, kale, beans, strawberries, *Ginkgo biloba* L., and *Equisetum* spp. It exhibits antioxidant and anti-inflammatory activities, acting on superoxide radicals, combating the formation of ROS, and preserving antioxidant enzymes, such as catalase, glutathione peroxidase, and S-transferase (CALDERÓN-MONTAÑO *et al*., 2011).

Figure 5 - Kaempferol chemical structural formula (DE LA ROSA *et al.*, 2019).



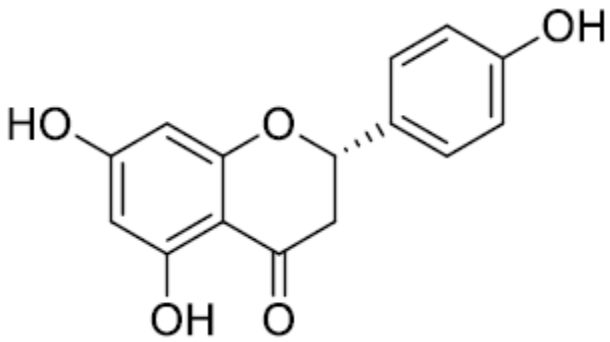
Kaempferol acts as an antioxidant by eliminating ROS produced during oxidative stress and has a pronounced protective effect on cell membranes against these harmful substances. These properties make kaempferol beneficial for application in cosmetic formulations for skin protection (PARK *et al*., 2012; TAIWO *et al*., 2019). In mouse ear inflammation models, kaempferol demonstrated inhibitory activity against enzymes such as hyaluronidase, elastase, and collagenase, responsible for breaking

down the extracellular matrix, along with a photoprotective effect on human dermal fibroblasts (TAVARES *et al*., 2023; TAKEDA *et al*., 2018).

2.2.4 Naringen

Naringenin (NAR) [(2, 3-Dihydro-5-7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one, 4', 5, 7-Trihydroxyflavanone], illustrated in **Figure 6**, is another flavonoid with significant antioxidant, antiviral, and anti-inflammatory activities.

Figure 6 - Naringenin chemical structural formula (DE LA ROSA *et al.*, 2019).



Naringenin belongs to the flavanone class and is a glycosylated polyphenol, commonly found in citrus fruits and tomatoes, with a higher concentration in the solid part of fruits than in the juice (CHTOUROU *et al*., 2015).

The hydroxyl groups in naringenin contribute to its remarkable antioxidant activity, eliminating free radicals and promoting an increase in antioxidant system components and the expression of antioxidant enzymes, including superoxide dismutase, catalase, glutathione transferase, and peroxidase. This compound effect helps preventing cellular damage (ARAÚJO *et al*., 2018; LUTU *et al*., 2019).

Naringenin has demonstrated antiviral effects against the yellow fever virus (ESPINAL et al., 2019), dengue virus (FRABASILE et al., 2017), and Zika virus (CATANEO et al., 2019). Additionally, naringenin has exhibited antiviral action against herpes simplex virus, reducing the toxicity associated with compounds present in the medium (POHJALA *et al*., 2011; SUROENGRIT *et al*., 2017).

Considering the actual necessity to improve the skin mechanisms of defense against the inevitable exposition to UV radiation that stimulates the generation of free radicals, and scientific evidence that highlights the benefits of the use of chlorogenic acid, apigenin, kaempferol, and naringenin, this investigation established the *in vitro* safety profile of those compounds and originally determined *ex vivo* and *in vivo* the potential of them against the stratum corneum lipid peroxidation and topical anti-inflammatory property, respectively.

Objectives

3 Objectives

The current investigation elucidated the *in vitro* safety profile through the utilization of the HET-CAM assay (Hen's Egg Test – Chorioallantoic Membrane), and evaluated the characteristics of topical efficacy in both *ex vivo* and *in vivo* settings. The *ex vivo* assessment employed HPLC-TBARS-EVSC methodology (High-Performance Liquid Chromatography–Thiobarbituric Acid Reactive Substances–*Ex Vivo* Stratum Corneum), while *in vivo* efficacy was assessed using laser Doppler flowmetry. The investigation focused on the polyphenols chlorogenic acid, apigenin, kaempferol, and naringenin incorporated into an aqueous gel at 0.1% w/w (isolated).

Material and Methods

4 Material and Methods

4.1 Material and Equipment

Chlorogenic acid (≥95%), apigenin (≥95%), kaempferol (≥97%), naringenina (98%), methyl nicotinate (MN) (99%), 2-thiobarbituric acid, 1,1,3,3-tetramethoxypropane (TEP), phosphoric acid (85%) and BHT were acquired from Sigma Aldrich® (St. Louis, MO, USA). Methanol and n-butanol were obtained from Merck® (Darmstadt, Hesse, Germany). Ammonium acryloyldimethyltaurate/VP copolymer - Aristoflex AVC® (ARX) was obtained from PharmaSpecial® (Santana do Parnaíba, São Paulo, Brazil). All chemicals were of analytical grade and used without prior purification. Water used in this experiment was purified using Merck Millipore Mili-Q® Simplicity UV – 0.22 µm Millipore®, Millipak®. Additionally, polypropylene transparent adhesive tape 750-19 mm, Scotch® (3M®, Sumaré, São Paulo, Brazil) was used.

Among the equipment used in this study were the high-performance liquid chromatograph (HPLC) - Model: RF-20A – Shimadzu® Prominence (Kyoto, Japan) composed of a diode array spectrophotometric detector SPD-M20A, CTO-20A column oven, and C18 column (250 × 4.6 mm, LC Column nº. H15-082795 Allcrom®, particle size 5 mm, Shimadzu, Japan), preceded by a pre-column (10 × 4.6 mm). The flowmetry equipment used was the Laser Doppler PeriFlux® System 5000 (Perimed®, Stockholm, Sweden). Atlas Suntest® CPS+ Solar Light® with a xenon lamp (1500 W) was the chamber used to simulate solar radiation energy (artificial UV), allowing the passage of wavelengths above 290 nm. Analytical balance – Model AUY220, Shimadzu®; Ultrasonic bath – UltraCleaner, Model: USC-1600A, Unique®; Thermostatic bath –

N480, Nova Ética®; Vacuum pump – MaximaDry – Fisher Scientific®; Centrifuge – Hitachi®, RX2; Vortex Genius 3 – IKA®; Thermo-hygrometer – Hygro-meter®; Eppendorf® automatic single-channel micropipettes 1.0 – 10.0 mL and 100.0 – 1000.0 µL; Nylon Syringe Filter 2204513 500C, diameter 13 mm, pore size 0.22 µm, Analitica®; and an hatcher Chocmaster® (37ºC and 65% of relative humidity) (Curitiba, Paraná, Brazil), digital microscope with camera Dino-lite® (Almere, Flevoland, Netherlands), fertilized white Leghorn chicken eggs (Irineu Salviato® Breeding – Porto Feliz, São Paulo, Brazil) were also utilized.

4.2 Sample Preparation

An aqueous gel for the incorporation of ACLOR, API, KAPF, and NAR was prepared using the Aristoflex® AVC (ARX), an emulsifying polymer (ARIEDE *et al*., 2020). The aqueous gel (Blank gel) was prepared by slowly adding, at room temperature, the ARX to the water in a mortar and stirring with a pestle until clear and transparent gel is obtained. The addition of the active ingredient, isolated, was performed slowly with the help of a pestle and stirring until a homogeneous gel was obtained (pH value = 6.5). **Table 1** describes the starting materials and proportions (% w/w) of the samples used in this study.

Table 1 - Qualitative and quantitative composition (% w/w) of samples.

Ingredients	Concentration (% w/w)				
	API	**KAPF**	**ACLOR**	**NAR**	**Blank gel**
Ammonium Acryloyldimethyltaurate/VP Copolymer	0.3	0.3	0.3	0.3	0.3
Apigenin	0.1	-	-	-	-
Kaempferol	-	0.1		-	-
Chlorogenic acid	-	-	0.1		-
Naringenin	-	-	-	0.1	-
Aqua (purified water)	99.6	99.6	99.6	99.6	99.7

4.3 *In Vitro* safety establishment by the HET-CAM method (Hen's Egg Test – Chorioallantoic Membrane

The HET-CAM assay was conducted using fertilized white Leghorn chicken eggs incubated for 10 days at 37°C and 65% relative humidity in a Chocmaster® incubator. After the incubation period, the shell around the air chamber was carefully removed with tweezers, revealing the shell membrane, which was then hydrated with 300µL of NaCl 0.9% w/v (FS) and subsequently removed. The chorioallantoic membrane of each egg used in this study was exposed, and 300µL of a solution that

contains only starting material evaluated to 0.1% (w/v) (chlorogenic acid, apigenin, kaempferol, naringenin, and Aristoflex AVC®), previously diluted in NaCl 0.9% w/v (FS), was deposited on it.

Each egg used contained only one sample per evaluation. The positive control of the test was performed with a 1.0% (w/v) sodium dodecyl sulfate (SDS) solution, and the negative control was with physiological saline solution (FS) (NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES, 2006). Each fertilized egg containing the test sample was placed in a fixed support, and the camera was positioned optimally to capture the yellow yolk or vitelline sac in the background, with blood vessels highlighted, thus recording the changes in the chorioallantoic membrane with clarity. Vascular events such as bleeding, lysis, or coagulation were recorded for five minutes by a Dino-Lite® camera (Model: AM-211). Subsequently, vascular events were assessed and analyzed.

The ocular irritation index (IS) was calculated according to **Equation 1** (KALWEIT *et al*., 1990; NATIONAL INSTITUTE OF ENVIRONMENTAL HEALTH SCIENCES, 2006). All assays were performed in triplicate. According to **Equation 1**, the samples can be classified according to **Table 2**.

Equation 1 - Calculation of the ocular irritation index.

$$\mathbf{IS} = \frac{(301 - \mathbf{H})}{300} \times 5 + \frac{(301 - \mathbf{L})}{300} \times 7 + \frac{301 - \mathbf{C}}{300} \times 9$$

Legend: IS – Ocular irritation index; H – time (s) to initiate a hemorrhagic event; L - time (s) to initiate vascular lysis; C - time (s) to initiate coagulation.

Table 2 - Classification of ocular irritation potential – HET-CAM test (KALWEIT *et al*., 1990).

Ocular irritation index (IS)	Classification
0 – 0.9	Non-irritating
1.0 – 4.9	Mildly irritating
5.0 – 8.9	Moderately irritating
9.0 - 21	Strongly irritating

4.4 *Ex vivo* and *in vivo* Efficacy Evaluation

Participants in this research were duly informed and clarified about the purpose and method used in the study, and capable of understanding and complying with the test steps. Consent was obtained through the signing of the consent form, maintaining participant anonymity, and ensuring voluntary withdrawal at any time (BRAZIL, 2012). The project was approved by the Ethics Committees of the Faculty of Pharmaceutical Sciences, University of São Paulo (protocol 4.885.215), and Lusófona University (protocol CE.ECTS/P11.21). Studies were conducted following current legislation (BRAZIL, 2012). Procedures were in line with ethical principles, norms on human experimentation, and the Declaration of Helsinki. The HPLC-TBARS-EVSC protocol was performed in 8 volunteers and the anti-inflammatory method with Laser Doppler flowmetry was conducted with 14 volunteers.

Participants were kept at a temperature of 21 ± 2 °C and relative humidity of 40-60%. They were admitted to this study if they were non-smokers, had healthy skin without any declared dermatological or circulatory pathology, aged between 18 and 60

years, with skin types II-V, not allergic to the category of products used, pregnant or breastfeeding women, immunodeficient individuals, with active atopic dermatitis, intense intense sun exposure for at least 4 weeks before the study, or use of immunosuppressive medications, corticosteroids, antihistamines, retinoids, and anti-inflammatories. It was recommended not to apply cosmetic products in the 24 hours before the experiment in the area to be assessed.

4.4.1 HPLC-TBARS-EVSC (high-performance liquid chromatography–thiobarbituric acid reactive substances–*ex vivo* stratum corneum) protocol

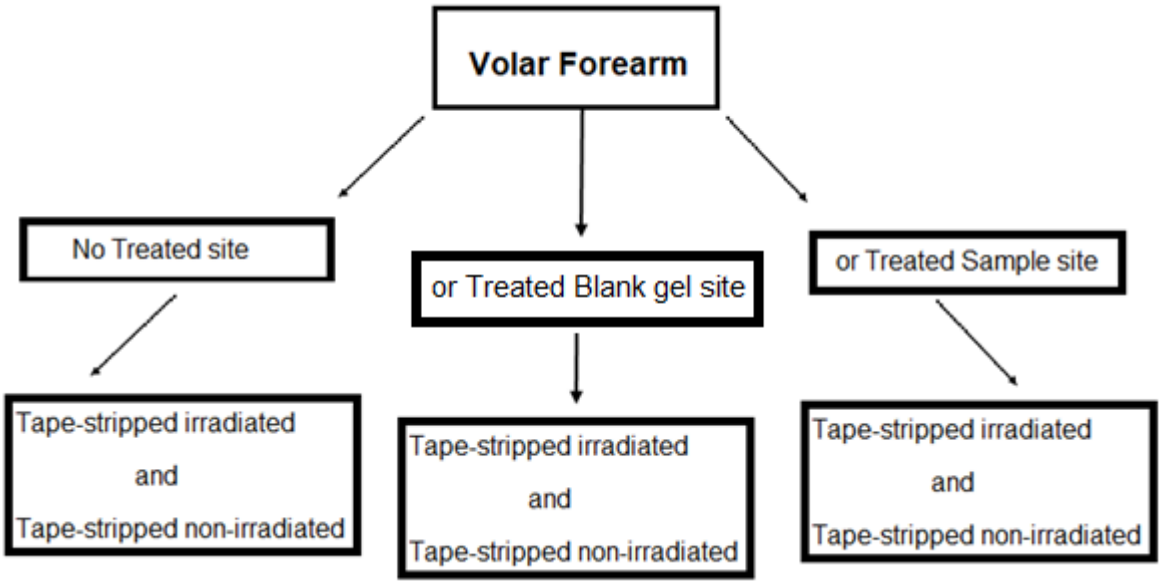
The volar forearm of eight (08) participants was cleaned once with the gentle application of dry cotton. Subsequently, aliquots of the samples (2.0 mg/cm^2), containing each of the polyphenols evaluated (incorporated into the gel, as described in Table 1), and the gel without the incorporation of actives (blank), were applied to the previously delimited areas. After 2 hours of application, the stratum corneum from the site was removed using the tape stripping technique, with six (06) strips (BENFELDT *et al*., 2007; ALONSO *et al*., 2009; OLIVEIRA *et al*., 2015; PERES, 2015).

4.4.1.1 Tape Stripping procedure

The tape stripping method for the *ex vivo* functional characterization was applied to the volunteers, and the stratum corneum was removed using the tape stripping technique (BENFELDT *et a*l., 2007; CLAUSEN *et al*., 2016; OLIVEIRA, 2015; PERES, 2015). Previously delimited areas on the volar forearm of volunteers, as illustrated in

Figure 7, were treated or not with the samples, as described above. The samples and the control were applied with the help of a disposable plastic spatula and distributed evenly on the defined area. The proportion of applications was defined according to current recommendations for sunscreens (ALONSO *et al*., 2009; OLIVEIRA, 2015; PERES, 2015).

Figure 7 - Tape stripping treatment flowchart (adapted from ALONSO *et al*., 2009).



After two hours of application, the tape stripping technique was performed using six (06) strips per area to remove the stratum corneum (ALONSO *et al*., 2009; OLIVEIRA, 2015; SAUCE *et a*l., 2021a). The sample application, control, and untreated area were randomized. The strips were applied with constant pressure at each designated location for stratum corneum extraction. During its removal, the first strip from each, and the following five (05) strips were treated according to the flowchart (**Figure 7**) (ALONSO *et a*l., 2009; SAUCE *et a*l., 2021a). For the removed strips to be irradiated (exposed to UV radiation), the photostability chamber (Atlas Suntest® CPS+)

with a xenon lamp (1500 W) and a filter responsible for simulating solar radiation, allowing the passage of wavelengths above 290 nm, was used. Irradiation was performed at 765 W/m^2, with a fixed dose and a determined period of two hours of irradiation. The temperature was controlled at 35°C to avoid sample overheating (ALONSO *et a*l., 2009; COLIPA, 2011; OLIVEIRA, 2015; PERES, 2015).

4.4.1.2 Extraction of the Stratum Corneum

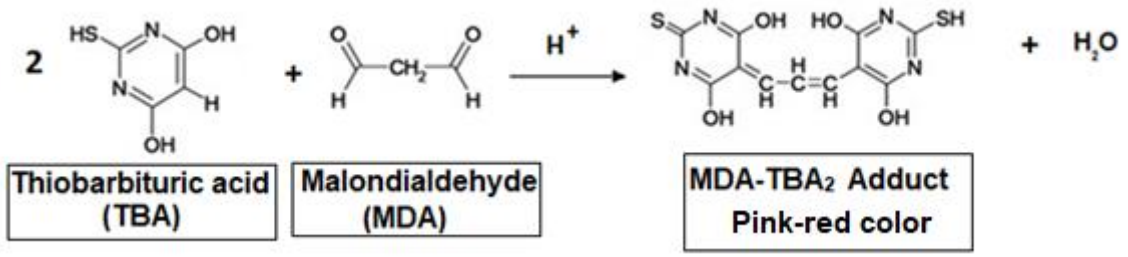
All collected adhesive strips were transferred to 50.0 mL Falcon tubes, previously identified. Next, all samples in the tubes were treated with aliquots of 10.0 mL of HPLC-grade methanol. The extraction of the stratum corneum by the solvent was performed by ultrasonic bath for 15 minutes (ALONSO *et al*., 2009; PERES, 2015). The extracted stratum corneum was evaluated by the HPLC-TBARS-EVSC protocol (SAUCE *et al*., 2021a).

4.4.1.3 Determination of Lipid Peroxidation of the stratum corneum

Lipid peroxides from the tape-stripped stratum were quantified by HPLC according to Marques and colleagues, 2023. Chromatography conditions were performed isocratically with a mobile phase composed of 35% methanol and 65% phosphate buffer (50nM, pH 7.0). The flow rate was standardized at 1.0 mL per minute for 10 minutes at a temperature of 30°C, with a sample injection volume of 40.0 µL. The diode detector was set at 532 nm to quantify the MDA-TBA$_2$ adduct (BASTOS,

2012; SAUCE *et al*., 2021a). Using an automatic pipette, 2.0 mL of the samples of the SC extraction obtained in section 4.5.2 were transferred to glass tubes with lids, identified according to the corresponding sample. Subsequently, 800.0 µL of H_3PO_4 (0.44M) and 288.0 µL of BHT (0.2% in methanol) were added to the tubes and they were vortexed for one minute, then allowed to stand for 10 minutes at room temperature in the dark. Next, 1200.0 µL of TBA (0.6% in H_3PO_4 - 0.44M) were added, and the tubes were vortexed for 30 seconds. The tubes were then placed in a thermostatic bath and heated to 90°C, remaining for 45 minutes. After cooling to room temperature, 1200.0 µL of n-butanol were added to the tubes, and they were vortexed for one minute. Following this process, an aliquot of the tube contents was filtered through a 0.22 µm filter, attached to a syringe with a needle, directly into the identified vial for the determination of the lipid peroxidation profile of the stratum corneum in function of the treatment through the quantification of the MDA-TBA_2 adduct (ALONSO *et al*., 2009; BASTOS *et* al., 2012; PESCIA *et al*., 2012; FRANTZEN *et al*., 2016). **Figure 8** illustrates the reaction with TBA and MDA to form the MDA-TBA_2 adduct. The results were evaluated by the ratio of the irradiated sample and the non-irradiated stratum corneum.

Figure 8 - Reaction between thiobarbituric acid (TBA) and malondialdehyde (MDA) forming the MDA-TBA_2 adduct (ALONSO *et al*., 2009; BASTOS *et al*., 2012; PESCIA *et al*., 2012; FRANTZEN *et al*., 2016).



4.4.2 *In Vivo* Anti-Inflammatory Activity Assay

Six areas of 2.0 cm^2 were randomly designated on the volar forearm of 14 participants. Five areas were defined for sample application, and one served as an untreated control. The experiment was conducted in a temperature-controlled environment (21 ± 2°C and relative humidity of 40-60%). Five areas were gently cleaned, and the samples were applied using a spatula. In each five areas, amount 10.0 mg containing 0.1mg of one the samples was applied. The sixth area was evaluated as control. The sample remained in contact with the evaluated site for two hours, also randomly assigned to either the left or right forearm (MOROCHO-JÁCOME *et al*., 2022).

After the designated time, erythema was induced in each area by applying a filter paper (4.0 cm^2) saturated with 85.0 µL of 0.5% methyl nicotinate aqueous solution for 60 seconds. Blood flow measurements were immediately taken at individual test sites using a Laser Doppler Flowmetry system (PeriFlux System 5000, Perimed,

Stockholm, Sweden) for 15 minutes. The results were statistically evaluated based on the ratio between the values obtained at each sample site and the control values for each participant, thus, minimizing the interindividual variability effect (OLIVEIRA *et al*., 2016).

4.5 Statistical Analysis

Statistical evaluation of the results was performed using one-way ANOVA, non-parametric statistics (Wilcoxon test) with a significance level of 0.05. GraphPad Prism® version 5.0 software (GraphPad® Software, Inc) was used (ROSADO *et al*., 2009; SAUCE *et al*., 2021b).

Results and Discussion

5 Results and Discussion

5.1 *In vitro* safety establishment by the HET-CAM method (Hen's Egg Test – Chorioallantoic Membrane)

The HET-CAM (Hen's Egg Test – Chorioallantoic Membrane) assay is an alternative method to animal experimentation, based on the similarity between the chorioallantoic membrane (CAM) of a developing chicken egg and the mucosa of the human or rabbit eye, due to the similar vascularized tissue (KALWEIT *et al*., 1990; STEILING *et al*., 1999). Establishing the in vitro safety profile of polyphenols – chlorogenic acid, apigenin, kaempferol, and naringenin – was significant as a preliminary assay for the future application of active ingredients in a suitable semi-solid vehicle or, considering the market, a finished cosmetic product. It is worth noting that products applied to the facial area may encounter the ocular region, either through sweat or even contact during the application of the cosmetic product in the area around the eyes, thus expanding the rationale for the HET-CAM test. The *in vitro* method was chosen as an alternative to other biochemical assays, such as 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, due to its convenient conduct, availability, and generation of results comparable to the Draize Test, which is performed on rabbits and is currently not recommended for testing raw materials or cosmetic products (ALLTOX, 2016). Furthermore, the assay is less costly, requires less complex infrastructure, and does not involve the participation of research participants (STEILING *et al*., 1999; WANG; YU; WICKLIFFE, 2011).

ICCVAM (Interagency Coordinating Committee on Validation of Alternative Methods) (2010) advocates that HET-CAM assay results can be accepted only if the

positive control, 1.0% w/v sodium dodecyl sulfate dispersion (SDS), exhibits an ocular irritation score (IS) that classifies it as strongly irritating. The positive control showed an acceptable result, with hemorrhage and lysis observed within a few seconds (mean time = 2 s). The negative control (0.9% w/v NaCl - FS) did not trigger vascular alteration processes, as can be seen in **Table 3**. Thus, considering that the controls confirmed the adequacy of the assay, the results for chlorogenic acid, apigenin, kaempferol, naringenin, and the polymer (BLANK GEL) were acceptable.

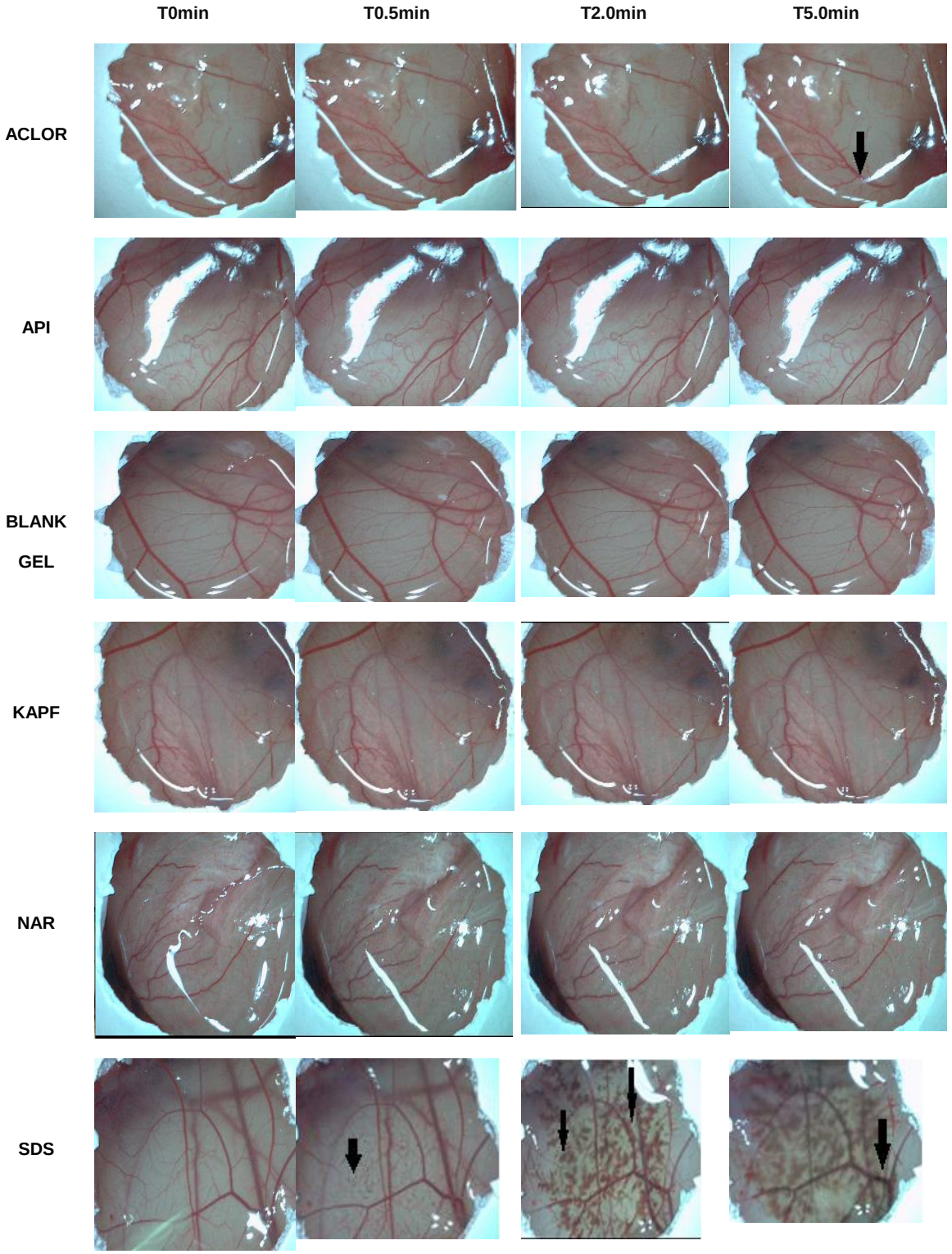
Table 3 - Classification of ocular irritation potencial – HET-CAM test of samples ACLOR, API, KAPF, NAR, SDS, FS and Blank gel.

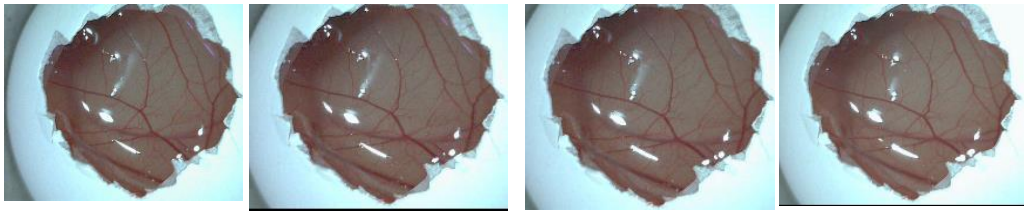
Sample	**Concentration (%w/v)**	**At (S) Hemorrhagic event**	**At (S) Vascular lysis**	**IS**	**Classification**
ACLOR	**0.1**	**0.73**	No events	**0.73**	NI
API	**0.1**	No events	No events	0.1	NI
BLANK GEL	**0.1**	No events	No events	0.1	NI
KAPF	**0.1**	No events	No events	0.1	NI
NAR	**0.1**	No events	No events	0.1	NI
FS (0.9% w/v NaCl)	0.1	No events	No events	0.1	NI
SDS (1.0% w/v sodium dodecyl sulfate)	1.0	2.0	2.0	11.96	SI

Legend: IS – Ocular irritation index; At (s) - Average time in seconds); NI – Non irritating; SI - Strongly irritating.

The video image records at 0, 0.5, 2.0, and 5.0 minutes are illustrated in **Figure 9**. It was observed that only chlorogenic acid 0.1% (w/v) showed the hemorrhage event at 2.5 minutes of contact with the chorioallantoic membrane. Despite causing this event, this sample was classified as non-irritating. The other polyphenols and the polymer did not show evidence of coagulation, lysis, and/or hemorrhage in the membrane vessels, as can be seen in the images in **Figure 9**, there is no evidence of these events. The polyphenols ACLOR, API, KAPF, NAR, and the polymer ARX evaluated in this study demonstrated ocular irritation index (IS) below 0.9, therefore, they were also classified as non-irritating.

Figure 9 - Effects of ACLOR, API, BLANK GEL, KAPF, NAR, SDS diluted in FS, and FS on the chorioallantoic membrane - image records at 0, 0.5, 2.0, and 5.0 minutes.



FS 

Legend: T0min – time zero minutes; T0.5min – time 0.5 minutes; T2.0min – time 2.0 minutes; T5.0min – time 5.0 minutes.

According to the results of the HET-CAM assay, all samples were suitable for further *ex vivo* and *in vivo* experiments, specifically; HPLC-TBARS-EVSC and anti-inflammatory activity assessed by laser Doppler flowmetry.

5.2 Determination of Lipid Peroxidation of the stratum corneum established by HPLC-TBARS-EVSC protocol

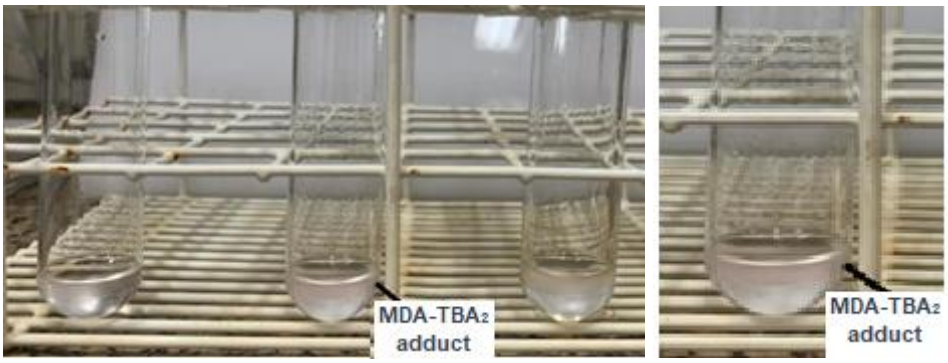
The thiobarbituric acid reactive substances (TBARS) method allows the evaluation of lipid peroxides in the outer layers of the stratum corneum, formed through UV irradiation in an artificial UV simulator. This method quantifies lipid peroxidation by measuring substances reacting with 2-thiobarbituric acid (TBA) in the medium, resulting in a pink-colored product determined by spectrophotometry at 532nm (BASTOS *et al*., 2012; SAUCE *et al*., 2021a)

Lipid peroxidation involves a chain reaction that affects the polyunsaturated fatty acids in cell membranes. One characteristic distinguishing these fatty acid structures is the presence of multiple double carbon-carbon bonds and reactive hydrogen atoms. Such structures are more susceptible to oxidative processes, exhibiting a direct relationship between the degree of unsaturation and the tendency to undergo oxidation

(MUSAKHANIAN; RODIER; DAVE, 2022). This condition of instability facilitates the generation of free radicals, and as the cell membrane is the structure affected, it results in the process of lipid peroxidation. Consequently, significant degenerative changes are triggered, compromising cell integrity and altering permeability, fluidity, electrical resistance, i.e., modifying the cell barrier and, consequently, weakening the intracellular environment and cell metabolism (TORRES *et al*., 2021).

These disruptions in balance promote the formation of certain components, such as malondialdehyde (MDA), a biomarker of lipid peroxidation. MDA is a product of the breakdown of polyunsaturated fatty acids and is the final product studied to assess cellular damage (PILZ *et al*., 2000; DEL RIO; STEWART; PELLEGRINI, 2005). This component, quantified through its detection by analytical tools, indicates an increase in oxidative stress levels resulting from lipid peroxidation in a specific tissue or sample (DEL RIO; STEWART; PELLEGRINI, 2005). The detection and analysis of these MDA levels in the system can be performed through the thiobarbituric acid reactive substances (TBARS) method (LIMA; ABDALLA, 2001; YAGI *et al*., 1994). This method assesses the final products of lipid peroxidation (lipid peroxides, malondialdehyde, and various low molecular weight aldehydes). When MDA reacts with two molecules of 2-thiobarbituric acid (TBA) in an acidic medium, it forms the MDA-TBA_2 adduct, a fluorescent pink chromogen detectable through spectrophotometric assay. **Figure 10** illustrates the formation of the pink supernatant, representing the MDA-TBA_2 adduct.

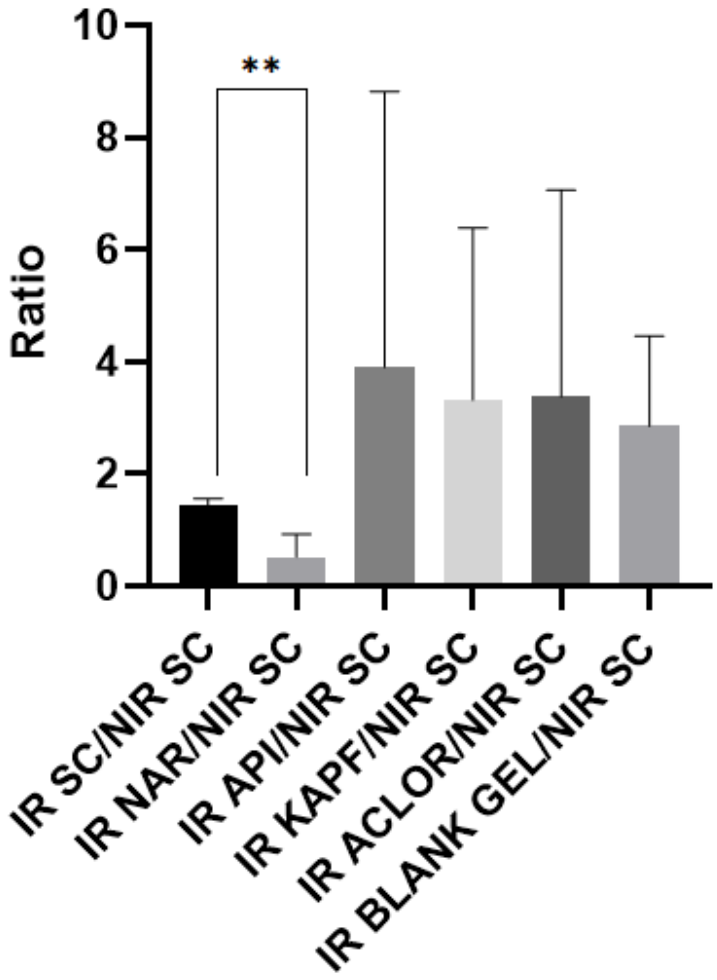
Figure 10 - Formation of the MDA-TBA_2 adduct - pink supernatant.



In this research, the HPLC-TBARS-EVSC method, developed by the Research Group (SAUCE *et al*., 2021a) was employed. This assay allows for improved specificity in the detection and quantification of MDA-TBA_2, aiming to measure the profile of lipid peroxidation (SAUCE *et al*., 2021b) in the stratum corneum of participants obtained through tape-stripping (ALONSO *et al*., 2009; OLIVEIRA, 2015; PERES, 2015). The HPLC-TBARS-EVSC method was utilized to assess the *ex vivo* antioxidant activity of chlorogenic acid, apigenin, kaempferol, and naringenin by evaluating their protection against lipid peroxidation in the stratum corneum, whether exposed to artificial UV radiation.

The results of the HPLC-TBARS-EVSC were analyzed as the ratio between the values obtained from the treatment site of each sample with the additional irradiation and the value of the control, the non-irradiated stratum corneum (NI SC) (OLIVEIRA *et al*., 2016; PERES *et al*., 2016). Eight volunteers participated in the assay, and the determined values of the adduct in the irradiated SC were higher than its corresponding non-irradiated counterpart, validating the method. This was evidenced by a significant increase in lipid peroxidation in the sample under radiation stress conditions. The ratios for the participants are illustrated in **Figure 11**.

Figure 11 - HPLC-TBARS-EVSC results for the samples as the ratio between the irradiated samples/SC and the non-irradiated (basal) SC.



Legend: NI SC – non-irradiated stratum corneum; IR SC - irradiated stratum corneum; IR ACLOR - irradiated chlorogenic acid; IR API - irradiated apigenin; IR BLANK GEL – irradiated blank gel; IR KAPF - irradiated kaempferol; **p=0.0078.

According to the scientific literature, the use of antioxidant agents enhances the overall defense capacity of the body against deleterious effects generated by free radicals, thus preventing the initiation and continuation of the lipid peroxidation

cascade (Bedard; Krause, 2007). The substances ACLOR, API, and KAPF, based on the results of the HPLC-TBARS-EVSC, did not exhibit sufficient protection for the stratum corneum (SC) against lipid peroxidation when exposed to artificial radiation, as the ratios demonstrated increases in these values.

The ratio of the SC sample treated with NAR (irradiated) to the non-irradiated SC (control) showed a statistically significant difference ($p<0.05$) from the ratio of the irradiated SC to its non-irradiated counterpart. This result profile suggested that there was protection from NAR, preventing lipid peroxidation. This finding is supported by Martinez and colleagues, 2016, who, when applying a formulation containing naringenin on dorsal skin of mice and exposing it to UVB radiation, and then observed decreased glutathione level, inhibition of superoxide radical ($O2^{\bullet-}$) production and reduced levels of highly reactive and toxic lipid hydroperoxides, indicating Increase in the antioxidant capacity of the skin and suggesting potential protective activity of naringenin against UVB radiation (KIM *et al*., 2012; MARTINEZ *et al*., 2016).

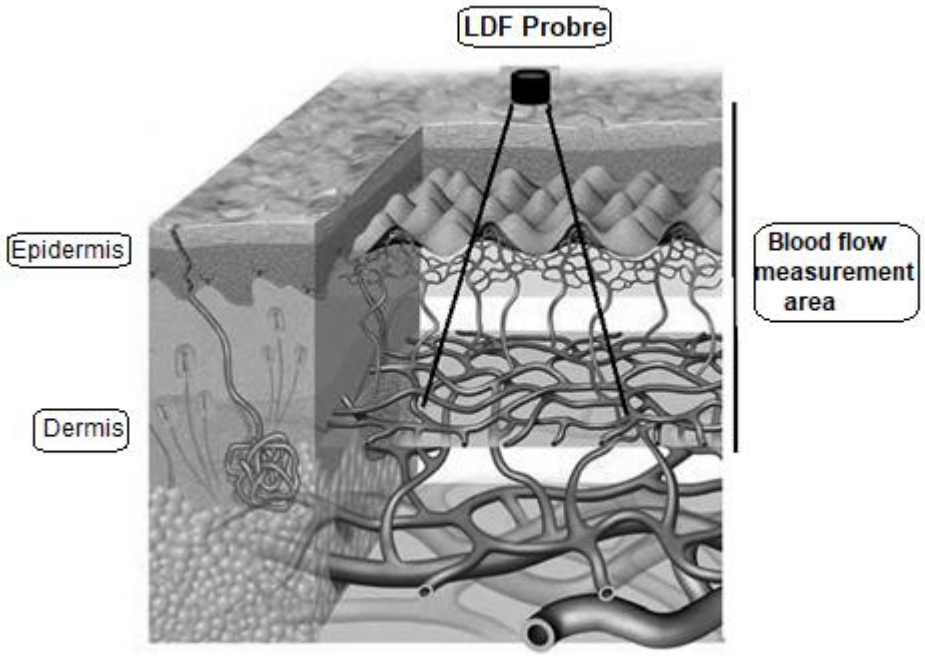
5.3 *In Vivo* Anti-Inflammatory Activity established by laser Doppler flowmetry

The non-invasive laser Doppler flowmetry (LDF) method utilizes a flowmeter based on optical principles to evaluate microvascular cutaneous perfusion (SARNIK, *et al*., 2007). This assay can be employed in clinical studies and allows for the indirect and continuous assessment of changes in blood flow the measurement of cutâneos microvascular perfusion at a specific location in contact with the probe (PETERSEN,

2013). This method evaluates the *in vivo* anti-inflammatory activity of samples on the skin surface (VANDERSEE *et al*., 2016).

The **Figure 12** illustrates the blood flow measurement region assessed by Laser Doppler Flowmetry.

Figure 12 - Diagram showing the location of the epidermis, dermis, cutaneous microcirculation, and regions assessed by Laser Doppler Flowmetry (Adapted from PINTO, 2010).



The technique employs a monochromatic laser sent through an optical fiber, which, upon penetrating the tissue, collides and illuminates red blood cells in motion in the blood vessels. The laser beam undergoes a shift and is reflected at a wavelength different from the original at the time of emission, a phenomenon known as Doppler shift (SIKUROVA; BALIS; ZVARIK, 2011). This change is related in real-time to the speed and volume of blood cells. In addition to being non-invasive, LDF is an *in vivo*

and painless technique, applied in studies to evaluate topical inflammatory reactions, for example (SIKUROVA; BALIS; ZVARIK, 2011).

In the current study, LDF allowed for the evaluation of the potential topical anti-inflammatory activity of chlorogenic acid, apigenin, kaempferol, naringenin, and the vehicle (Aristoflex® AVC), which lacks an active ingredient. Parameters such as onset time of action (time required for an increase in perfusion units, t_{onset}), area under the curve, and the slope of the tangent line (angular coefficient) during the hyperemia phase were assessed (VERTUANI *et al*., 2003; OLIVEIRA *et al*., 2016). The stimulus for cutaneous microcirculation (vasodilation) was induced by applying methyl nicotinate solution to the participants' forearms, and the samples under analysis were challenged to mitigate this response (ZHU *et al*., 2022). **Table 4** presents the values of the area under the curve (AUC) and the angular coefficient in the form of the ratio between the values obtained in each of the evaluated areas (with the presence of one of the samples) and those obtained in the control area.

Table 4 - Ratios of the area under the curve (AUC) and angular coefficient during the hyperemia phase of the investigated samples.

Parameter	Sample	Ratio (mean ± Standard Deviation	*p*-value
Area under the curve	ACLOR	0.855 ± 0.572	0.1189
	API	0.811 ± 0.305	0.3910
	KAPF	0.694 ± 0.337	0.0353 *
	NAR	0.559 ± 0.290	0.0085 **
	BLANK GEL	0.853 ± 0.357	0.2820
Angular coefficient	ACLOR	0.357 ± 0.242	0.6257
	API	0.264 ± 0.148	0.0906
	KAPF	0.260 ± 0.189	0.0785
	NAR	0.222 ± 0.178	0.0031 **
	BLANK GEL	0.686 ± 0.250	0.3808

Legend: ** $p \leq 0.01$ and * $p \leq 0.05$.

ACLOR and API did not generate responses significantly different from the control (vehicle without active ingredient) for the parameters area under the curve and slope of the tangent line (angular coefficient) ($p > 0.05$). Regarding KAPF, it did not show a different result from the control for the angular coefficient; thus, it was able to reduce the intensity of the inflammatory response compared to the control. This contrasts with Shin and colleagues (2017), who observed significant anti-inflammatory activity of API in an animal model (mice) due to the inhibition of prostaglandin E2 production. However, such a response was not identified in this study (SHIN *et al*., 2017). The results for API in terms of the area under the curve and the slope of the tangent line (angular coefficient) may be due to the concentration of the active

substance being insufficient to show efficacy under the assay conditions. It is worth noting that API, ACLOR, and KAPF also did not yield significant results in protecting the stratum corneum against lipid peroxidation through the HPLC-TBARS-EVSC protocol.

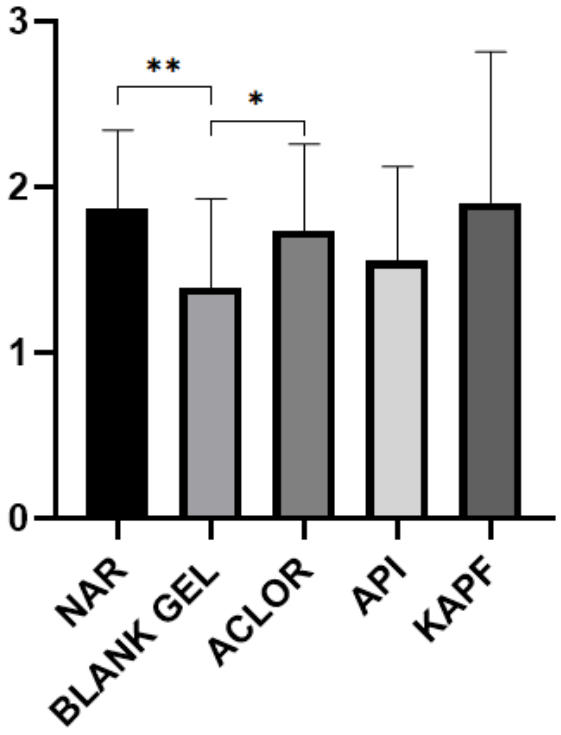
In **Table 4**, differences in the area under the curve parameter between NAR and the control ($p < 0.05$) can be observed, as well as for the slope of the tangent line (angular coefficient) during the hyperemia phase ($p < 0.05$). This response profile is supported by Frade, 2020, who observed that stimulating inflammation using polysaccharides in RAW macrophages 264.7 and treating them with naringenin 50 and 100nM, was reducing secretation of IL-6 and tumor necrosis factor-alpha (TNF-α), such fact suggested the phenolic compounds exert anti-inflammatory potential by inhibiting the cytokines release

According to Katzman and colleagues, 2003, the contact of methyl nicotinate with the cutaneous surface induces local hyperemia due to the release of prostaglandins D2 and E2, causing vasodilation in the peripheral dermal capillaries (KATZMAN *et al*., 2003). The release of these prostaglandins may also be associated with the skin's interaction with UV radiation, a stressor that generates reactive oxygen species (ROS) (SAUCE *et al*, 2021b). Polyphenols have the potential to modulate pro-inflammatory cytokines, such as interleukin-6, inhibiting their release and preventing exacerbated inflammatory processes (ABAD et al., 2011). Among the compounds mentioned in the literature that act to prevent inflammation from becoming more intense are naringenin and kaempferol (SHIN *et al*., 2017), supporting the results obtained in this investigation regarding the area under the curve parameters for both samples and the angular coefficient during the hyperemia phase for NAR. However, according to the literature, KAPF exhibited satisfactory anti-inflammatory activity when

associated with quercetin, a flavonoid. The mixture modified the inflammatory process by modulating the production of interleukins and TNF-α (TODOROVA; TRENDAFOLOVA, 2014).

Regarding the t_{onset} (time of increased perfusion units), the ratio was determined between the time required for the sample to show an increase in blood perfusion and the time for the control region to exhibit the same behavior. This result is illustrated in **Figure 13**.

Figure 13 - Time to increase perfusion units (t_{onset}) of the samples as the ratio of chlorogenic acid (ACLOR), apigenin (API), blank gel, kaempferol (KAPF) and naringenin (NAR).



Legend: **p=0.005 and *p=0.0168.

According to t_{onset}, ACLOR and NAR showed a significant response in delaying the onset of increased perfusion units ($p < 0.05$). Topical treatments with ACLOR and NAR delayed the onset of methyl nicotinate's action on the skin, i.e., the noticeable beginning of vasodilation in the blood capillaries by the equipment. However, only NAR demonstrated the ability to decrease the intensity of the inflammatory response based on the results obtained for the area under the curve and angular coefficient parameters.

According to the specialized literature, this assay had not been previously explored for the investigation of the topical anti-inflammatory behavior of chlorogenic acid, apigenin, kaempferol, and naringenin in humans. This study utilized methyl nicotinate, which has the ability to stimulate peripheral vasodilation and blood perfusion. Considering that exposure to UV radiation can promote an inflammatory response, a strategy to prevent damage caused by sun exposure would be the use of active ingredients that inhibit the inflammatory response (ZHU *et al*., 2022). Naringenin has anti-inflammatory action by inhibiting the activity of the enzyme COX-2, which triggers the pro-inflammatory effect (MANCHOPE *et al*., 2016). In light of the results obtained by the applied protocols, NAR, in addition to protecting the stratum corneum from lipid peroxidation, was able to reduce the effect of methyl nicotinate on the study participants.

Conclusion

6 Conclusion

Through the utilization of the *in vitro* safety assessment, specifically the Hen's Egg Test-Chorioallantoic Membrane (HET-CAM) assay, it was discerned that ACLOR, API, KAPF, and NAR were categorized as non-irritating active ingredients. This classification was attributed to their absence of adverse reactions within the vascularization of the chorioallantoic membrane, thus establishing their safety profile.

To explore the protective capabilities of the compounds ACLOR, API, KAPF, and NAR against lipid peroxidation in the stratum corneum, a comprehensive analysis was undertaken utilizing the HPLC-TBARS-EVSC protocol. Employing this protocol on samples obtained through tape-stripping and subsequent artificial irradiation (UV radiation) of the skin, it was observed that only NAR exhibited a notable reduction in epidermal lipoperoxidation, indicating a superior anti-radical potential. Conversely, ACLOR, API, and KAPF displayed a pro-oxidant profile under the specified test conditions.

Laser Doppler flowmetry demonstrated the anti-inflammatory potential of NAR and KAPF, with NAR demonstrating superior efficacy. Notably, NAR emerged as the sole polyphenol capable of mitigating the intensity of the inflammatory response induced by the methyl nicotinate solution in the study participants, as compared to both the control group and the gel lacking an active ingredient. This reduction in inflammation was quantified through parameters such as the area under the curve, angular coefficient, and t_{onset}.

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