Rutin Release of Gel Formulations Containing *Persea Americana* Mill. Extract in Vertical Diffusion System *in Vitro*

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**Abstract**

This work aims to incorporate the hydroethanol extract of *Persea americana* leaves into a hydrogel formulation and to determine the presence of rutin, the release of the active substance (using the rutin as marker), the antioxidant activity and the spreadability of the formulations developed. The extract of *P. americana* was obtained by hydroethanolic maceration and incorporated in hydrogels at doses of 1% (*P. americana* gel 1%) and 3% (*P. americana* gel 3%). Spreading ability was performed using the circular glass plate method and the rutin release in a vertical diffusion system was done using cellulose acetate membranes. Determination of the rutin in the gel and in the receptor solution was performed by high performance liquid chromatography, and the antioxidant capacity was analyzed by the 2,2-diphenyl 1-picrylhydrazyl radical method and the ferric reducing antioxidant power assay. *P. americana* gel 1% showed better propagation ability, but lower release of rutin than the gel 3%. Both formulations presented similar antioxidant effects, which can be explained by the presence of other phenolic compounds in the extract. *P. americana* gel 3% released the largest rutin amount within 1 hour and therefore produced better results than *P. americana* gel 1%.

**Keywords:** Free radical; Flavonoid; Cosmetics; Hidrogel; Avocado

**Introduction**

Hydrophilic gels or hydrogels are widely used as carriers for cosmetic products because of their easy preparation and excellent spreadability. The polymers used as raw materials in the production of these gels feature a high molecular weight and, when dispersed in water, provide viscosity to the preparation. In general, these polymers may be classified as ionic or nonionic according to their chemical properties. Gel-based formulations are non-greasy and can incorporate water-soluble active substances; thus, they are best suited for oily and acne-prone skin [1].
The type of polymer employed in a formulation directly influences the rheological properties of the end-product, as well as its physico-chemical stability, spreadability, and ability to release the active substance from the carrier. Use of the correct polymer also affects film formation on the skin and the sensory perception of the formulation, which could influence whether a consumer will accept or reject the product. As such, proper selection of the polymer is an important step in the production process [2].

Carbomer, an acrylic acid polymer is one of the most used for the formula gel because it is compatible with many active substances, presented bioadhesive properties and patient acceptance, besides not causing skin irritation [3,4].

The growing demand for stable, safe, and effective cosmetic formulations with the appeal that drives their consumption has required increasingly complex and efficient methodologies to assess the stability of these products [5,6]. The skin protects the body against the entrance of external agents and loss of water. The cutaneous surface is divided into two layers, called the epidermis (more superficial) and the dermis (deep layer). The corneal layer of the epidermis is composed of several layers of closely arranged cells, known as corneocytes, surrounded by protein and lipid materials that form a lipoprotein envelope around them. This region is the most superficial layer of the skin and responsible for protecting the body against the entry of pathogenic microorganisms and mechanical trauma; it also prevents water loss while maintaining the hydration and flexibility of the skin. This organization constitutes an efficient barrier against the entry of foreign substances into the skin [7-11].

Under normal conditions, penetration of substances through the skin is very difficult because of its organization. The entry of active substances through the skin is increased only when these substances contain constituents similar in composition to the stratum corneum and the lipophilic active will more adequately permeate this region [12-14].

The penetration of active substances into the skin depends on the release of these substances from the vehicle and their penetration through the skin layers. Thus, during the development of formulations for topical use, the physico-chemical properties of the active substances and their possible interactions with the vehicle must be taken into account [12-15].

Analysis of the release and permeation of substances through the skin constitutes a fundamental step during formulation development to ensure the effectiveness of the product. Several methods have been proposed and implemented to simulate the release of the active substance and its permeation through the skin layers. The vertical diffusion method using Frantz cells is an in vitro assay approved by the US Food and Drug Administration. In this method, Fick's law is applied to evaluate the phenomena that occur when the product is applied to the skin and its effects are observed; the method produces fast results, is very practical, and does not interfere with biological factors [11,15]. This test is based on the small amounts of substances that cross the membrane or are contained therein, which can be quantified by an appropriate analytical method [15,16].

Brazil has an enormous biodiversity and one of the richest forests in the world. However, this biodiversity has not been fully exploited and research and development of new products is necessary, especially those that use raw materials with antioxidant activity, photoprotective and preventive of skin aging [17,18]. The use of plants that make up the Brazilian flora has led to increased research and development of new cosmetic products through a series of approaches. The use of a marker to establish a standardized concentration of the chemical constituents of a plant was proposed to solve this question and to guarantee the effectiveness, safety and quality of the products obtained from the plants [6].

Persea americana (Lauracea), also known as avocado, has been described extensively in the literature because of its various biological effects (e.g., antitussive, anti-diabetic, and analgesic) [19,20] which are related to its chemical constituents (e.g., triterpenoids glycosides, coumarins, alkaloids, saponins, tannins, flavonoids, and reducing sugars) [21]. The leaves of the P. americana have been reported to exhibit anti-inflammatory [22] and antioxidant [23] effects. Phenolic compounds, especially flavonoids (rutin and quercetin), which are also chemical constituents of avocado, are related to these activities [24,25].

The objective of this work is to incorporate the hydroethanolic extract of Persea americana leaves into hydrogel and evaluate the spreadability, active substance release (using rutin as a marker) and antioxidant activity of the resulting formulations, once there are no reports in the literature about these parameters in the skin.
Materials and Methods

Materials

High performance liquid chromatography (HPLC-DAD) was performed with a Prominence Auto-Sampler (SIL-20A) HPLC system (Shimadzu, Kyoto, Japan) equipped with Shimadzu LC-20 AT reciprocating pump connected to a DGU 20A5 degasser with a CBM 20A integrator, a DAD SPD-M20A UV-VIS detector, and LC solution 1.22 SP1 software. All reagents used in this study were of analytical grade. Butylhydroxytoluene and parabens (methylparaben and propylparaben) were acquired from Galena. Disodium EDTA, imidazolidinyl urea, propyleneglycol, and Carbopol Ultrez 20 were acquired from Fagron.

Methods

Plant collection and extraction

P. americana leaves were collected in February 2014 in the city of Cruz Alta, Rio Grande do Sul, Brazil, with the following geographical coordinates: longitude: -53° 36′ 23′′ W; latitude: -28° 38′ 19′′ S; altitude: 452 m [26]. Botanical identification was performed at the Department of Biology of the University of Cruz Alta (UNICRUZ) by Professor Graciela Sonego Pretto, where a voucher specimen has been deposited in the Herbarium of Poisonous and Medicinal plants under the registration code 1109 was deposited in the Herbarium of Poisonous and Medicinal Plants.

The fresh leaves were coarsely divided and dried in an oven at 45 °C for 7 days. The dried leaves were reduced to powder in a knife mill and extracted with hydroethanol solvent (ethanol:water 70:30, v/v) by maceration at room temperature for 7 days with periodic shaking. After that period, the extraction liquid was filtered, and the maceration process described above was repeated once again. After reuniting both filtrates, they were concentrated under reduced pressure with the bath at 100 °C for ethanol removal and submitted to lyophilization until completely dryness.

Preparation of formulations

The lyophilized hydroethanolic extract of the P. americana leaves was added at doses of 1% and 3% to a gel base prepared with Carbopol Ultrez 20® carbomer; this gel was previously formulated according to the National Formulation with modifications [27] according to Table 1.

<table>
<thead>
<tr>
<th>Components</th>
<th>Amounts (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraben solution</td>
<td>3.30</td>
</tr>
<tr>
<td>Propyleneglycol</td>
<td>5.00</td>
</tr>
<tr>
<td>Disodium EDTA</td>
<td>0.10</td>
</tr>
<tr>
<td>Carbopol Ultrez 20®</td>
<td>2.00</td>
</tr>
<tr>
<td>Water</td>
<td>qs 100 mL</td>
</tr>
<tr>
<td>50% Imidazolidinyl urea solution</td>
<td>0.60 mL</td>
</tr>
</tbody>
</table>

Table 1: Composition of Carbopol Ultrez 20® Carbomer Gel Formulation (g)

Disodium EDTA and the paraben solution were added to water at room temperature. Carbopol Ultrez 20® carbomer was added to the solution, and the resulting mixture was homogenized until complete dispersion was achieved. Thereafter, imidazolidinyl urea was added to the solution.

Determination of rutin in gel

Chromatographic analyzes were performed reverse-phase HPLC under isocratic conditions using a C18 column (4.6 mm x 150 mm) loaded with 5 μm-diameter particles according to the method described by Boligon, et al. [28]. The mobile phase consisted of water: acetonitrile containing 0.5% phosphoric acid (pH 2.8; 70:30 v/v), and the rutin was quantified at 254 nm. The injection volume was 40 μL, and the flow rate was 0.8 mL/min. The mobile phase and all sample solutions were filtered through a 0.45 μm membrane filter (Millipore) and then degassed in an ultrasonic bath prior to use. The reference solution was prepared in the mobile phase for CLAE-UV/DAD at concentrations of 0.050–250 mg/mL rutin. Gels containing 1% or 3% P. americana hydroethanolic extract were diluted as follows: 1 g of gel and 9 mL of mobile phase were left in an ultrasonic bath for 60 minutes.

Chromatographic peaks were confirmed by comparison of their retention times with those of reference standards and comparison with the DAD spectra. All chromatographic operations were performed at room temperature and in duplicate.

Analysis of rutin release in a vertical diffusion system

Rutin release analyses were performed on the formulations using a Franz-type vertical diffusion cell with phosphate buffer (pH 6.0). The cell was covered with a cellulose acetate membrane (0.45 μm porosity; 7.06 cm² area) to separate the donor compartment from the receptor compartment, i.e., to form a non-interfering
barrier to a fluid called a receptor. The diffusion cell was introduced to an ultrathermostated bath at 37 °C, simulating body temperature. The coupling agents (1% and 3% formulations) were added to the membrane. During release evaluation, aliquots were collected after 1, 2, and 3 hours to verify the presence of rutin in the receptor solution. The assays were performed in duplicate [29,11]. The determination of rutin in the aliquots was analysed by HPLC/DAD [28].

**Determination of the in vitro antioxidant capacity of the formulations**

**2,2-Diphenyl 1-picrylhydrazyl radical method:** The antioxidant capacity was evaluated according method previously describe by Casagrande et al [30]. Samples were diluted in methanol in the following concentrations: 20, 10, 5, 2.5, and 1.25 mg/mL. A volume of 2.5 mL of each sample was added to 1 mL of DPPH solution (0.3 mM in methanol). After 30 minutes, readings were made in a spectrophotometer at 517 nm. A solution of 1 mL of 0.3 mM DPPH dissolved in methanol (2.5 mL) was used as a negative control, and gel of rutin were prepared at the concentrations identical to those of the samples and used as standards (positive control). Ethanol was used to zero the spectrophotometer and solutions of each sample without addition of DPPH were used as blanks. The assay was performed in triplicate, and antioxidant activity was calculated according the equation:

\[
\%\text{Inhibition} = 100 - \left(\frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs control}}\right) \times 100
\]

Where: Abs sample is the test absorbance; Abs blank is the blank absorbance, and Abs control is the DPPH solution in methanol absorbance.

**Ferric reducing antioxidant power assay:** The samples were diluted in distilled water at the following concentrations: 1.25, 2.5, 5, 10, and 20 mg/mL. 30 μL of each sample solution was added to 90 μL of DPPH reagent (acetate buffer 300 mM/L, 2,4,6-tripyridyl-S-triazine (TPTZ) 10 mM/L and ferric chloride 20 mM/L) and were mixed and kept at 37 °C for 30 minutes. The solution was read with the aid of the graphing paper, and the average diameter was calculated. This procedure was repeated successively by adding more boards at 1-minute intervals. The results were expressed as the spreadability of the sample due to the applied weight and computed according to the equation below:

\[
E_i = \frac{d^2 \times \pi}{4}
\]

Where:
- \(E_i\) = spreadability of the sample weight for a given \(i\) (mm²);
- \(d\) = diameter (mm);

The mean of three determinations was considered the final spreadability. The spreadability factor \((S_f)\) was calculating using the following equation (Fontana et al., 2011):

\[
S_f = \frac{A}{W}
\]

Where:
- \(S_f\) = spreadability factor; \(A\) = total area (mm²); \(W\) = total weight (g)

**Statistical analysis**

The results are expressed as the means ± standard error of the mean (SEM). All data were analyzed by analysis of variance (ANOVA) followed by Tukey's test. \(P<0.05\) was considered as statistically significant.

**Results and Discussion**

The skin forms an external barrier and is in continuous contact with solar radiation and pro-oxidant agents. UVA and UVB radiation induces DNA damage directly and indirectly through oxidative stress, causing photoaging, immunosuppression, cancer, and various inflammatory skin disorders [33].

The application of topical antioxidant products has proved to be an ally against oxidative stress in the skin and as a way to protect against the damages caused by solar radiation the long term. It is known that phenolic compounds are secondary metabolites present in plants with antioxidant activity. This activity is related to the content and profile of the phenolic compound found in the plant, determining the effectiveness of the extract, since the individual activity of the substances varies considerably in relation to their chemical structure [34-36].

Rutin, a polyphenol of the class of flavonoids, is known for its anti-inflammatory, antioxidant, antiallergic, antitumor, anti-bacterial, antiviral and photoprotective activities [37-39]. Rutin has a pronounced antioxidant action, which is attributed to its ability to donate electrons and hydrogen, eliminating oxidant species and preventing oxidative processes and is effective against damage induced by ultraviolet radiation [14,18,40,41].

The presence of rutin in the *P. americana* gel 1% and 3% were analyzed by HPLC / DAD and the chromatograms as well as the result of the quantification are in Figure 1.

![Figure 1: High performance liquid chromatograms from Persea americana gel: A) P. americana gel 1%; B) P. americana gel 3%; R = rutin. *Results are expressed as mean ± Standard error (SE) of three determinations.](image)

After chromatographic analysis of the gels, rutin release assays were performed. In this study, the release of rutin through a cellulose acetate membrane in a vertical diffusion model was analyzed. Rutin was used as a marker and analyzed by HPLC after the release assay. Samples were collected after 1, 2, and 3 hours, and the results are shown in Table 2.

<table>
<thead>
<tr>
<th>Formulation/Time</th>
<th><em>P. americana</em> gel 1%</th>
<th><em>P. americana</em> gel 3%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h (mg/100 g ± SD)*</td>
<td>5.4 ± 0.7&lt;a,a&gt;</td>
<td>12.6 ± 0.07&lt;a,b&gt;</td>
</tr>
<tr>
<td>2 h (mg/100 g ± SD)*</td>
<td>8.5 ± 0.35&lt;b,a&gt;</td>
<td>12.75 ± 0.14&lt;b&gt;</td>
</tr>
<tr>
<td>3 h (mg/100 g ± SD)*</td>
<td>10.75 ± 4.6&lt;ca&gt;</td>
<td>13.05 ± 0.77&lt;ab&gt;</td>
</tr>
</tbody>
</table>

*Results are expressed as the mean ± standard derivation (SD) of two determinations. Different letters represent significant differences (*p* < 0.05).

Table 2: Rutin release in a vertical diffusion system

According to table II it is observed that there was significant difference between the two formulations in the different times analyzed. However, there was no significant difference between the results displayed by *P. americana* gel 3% in the three analyzed times. Compared with *P. americana* gel 1%, more rutin release was achieved by *P. americana* gel 3% within 1 hour. *P.
**P. americana** gel 1% achieved rutin release only after 3 hours of contact.

Rojas, et al. [42] described that the release rate of a given substance is related to its respective concentration in the formulation, polymer properties and temperature. Thus, it was observed that the formulation with the highest concentration of active substance (*P. americana* gel 3%) was the one with the highest release of this substance (rutin). The diffusion of molecules from a more concentrated region (donor system) to a less concentrated region (receptor system) occurs during the release of semi-solid formulations and the membrane functions as a limiting medium. As the release of the molecules occurs over a given period of time, the concentration in the donor medium decreases as this concentration increases in the recipient medium. This is explained by Fick's second law, in which there is a linear correlation in relation to the concentration of molecules and their release [43]. Valandro, et al. [44] evaluated the rutin damages and protect the skin in the long term [49].

In terms of their ability to scavenge DPPH radicals (Table 3), *P. americana* gel 1% and Rutin gel 1% (standard) presented significant differences only at the concentration of 1.25 mg/mL. *P. americana* gel 1% and *P. americana* gel 3% demonstrated significant differences at concentrations between 1.25 and 10 mg/mL but no significant difference at 20 mg/mL. Compared with its standard (Rutin gel 3%), *P. americana* gel 3% showed significant differences at all concentrations studied.

In general, both formulations presented similar behaviors toward DPHH. As *P. americana* gel 1% and *P. americana* gel 3% could scavenge DPHH radicals, they may be used in formulations with antioxidant activity to prevent skin aging.

<table>
<thead>
<tr>
<th>Formulation/ Concentration (mg/mL)</th>
<th><em>P. americana</em> gel 1% (%± SD)*</th>
<th>Rutin gel 1% (%± SD)*</th>
<th><em>P. americana</em> gel 3% (%± SD)*</th>
<th>Rutin gel 3% (%± SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>0.5 ± 0.20&lt;sub&gt;a&lt;/sub&gt;</td>
<td>5.6 ± 0.10&lt;sub&gt;b&lt;/sub&gt;</td>
<td>1.36 ± 0.10&lt;sub&gt;ab&lt;/sub&gt;</td>
<td>38.3 ± 0.68&lt;sub&gt;b&lt;/sub&gt;</td>
</tr>
<tr>
<td>2.5</td>
<td>20.4 ± 2.53&lt;sub&gt;a,b&lt;/sub&gt;</td>
<td>22.7 ± 2.95&lt;sub&gt;a&lt;/sub&gt;</td>
<td>13.9 ± 2.98&lt;sub&gt;ab&lt;/sub&gt;</td>
<td>88.6 ± 0.29&lt;sub&gt;b&lt;/sub&gt;</td>
</tr>
<tr>
<td>5</td>
<td>47.1 ± 1.40&lt;sub&gt;a,a&lt;/sub&gt;</td>
<td>43.0 ± 1.90&lt;sub&gt;a&lt;/sub&gt;</td>
<td>56.5 ± 0.82&lt;sub&gt;ab&lt;/sub&gt;</td>
<td>90.5 ± 0.38&lt;sub&gt;b&lt;/sub&gt;</td>
</tr>
<tr>
<td>10</td>
<td>67.9 ± 0.86&lt;sub&gt;a,a&lt;/sub&gt;</td>
<td>69.6 ± 1.57&lt;sub&gt;a&lt;/sub&gt;</td>
<td>61.0 ± 0.20&lt;sub&gt;ab&lt;/sub&gt;</td>
<td>92.5 ± 0.28&lt;sub&gt;b&lt;/sub&gt;</td>
</tr>
<tr>
<td>20</td>
<td>68.4 ± 1.06&lt;sub&gt;a,a&lt;/sub&gt;</td>
<td>72.1 ± 1.07&lt;sub&gt;a&lt;/sub&gt;</td>
<td>71.6 ± 0.83&lt;sub&gt;a,a&lt;/sub&gt;</td>
<td>93.1 ± 0.21&lt;sub&gt;b&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

*Results are expressed as the mean ± standard derivation (SD) of three determinations. Different letters represent significant differences (p < 0.05).

Table 3: DPPH radical scavenging ability of the formulations.

The ability of the formulations to reduce Fe<sup>3+</sup> to Fe<sup>2+</sup> is shown in Figure 2. In this assay, *P. americana* gel 1% and *P. americana* gel 3% showed no significant difference at concentrations between 1.25 and 5 mg/mL. Above these concentrations (i.e., at 10 and 20 mg/mL), a significant difference between the formulations was observed. *P. americana* gel 1% and Rutin gel 1% showed no significant difference at concentrations of 1.25 and 5 mg/mL. Above these concentrations (i.e., at 10 and 20 mg/mL), a significant difference was observed. No significant difference between *P. americana* gel 3% and Rutin gel 3% was observed at all concentrations studied. Both formulations could reduce ferric ions, which suggests their potential use in anti-aging products.

As skin is exposed daily to environmental factors, especially ultraviolet radiation, it is necessary to use topical formulations that have a protective effect. It is known that solar radiation induces oxidative stress and the production of oxidant species that can damage different cellular components. In this way, formulations containing active substances with antioxidant action can prevent these damages and protect the skin in the long term [18,46-49].
In general, the formulations presented similar antioxidant activities against DPPH radicals and too in reduce ferric ions and these effects is attributed not only to the presence of rutin, but also to the presence of other phenolic compounds, such as quercetin [24], found in the extract.

The evaluation of parameters related to formulations for topical use are extremely important to predict some practical situations that may occur during their use. Among these factors, spreadability, which is defined as the expansion of a topical formulation on a surface after a certain is applied to check the behavior of the formulation on the surface of the skin time [50,51]. This feature is also considered when selecting a product package because formulations with low spreadability are difficult to obtain from a tube. In addition, a formulation should provide easy spreadability on the skin surface to ensure its effect [32,52,53].

The spreadability of the developed formulations is shown in Figure 3. Both formulations initially showed similar behavior, but P. americana gel 1% demonstrated greater spreadability than P. americana gel 3% as the weight increased (305.68, 376.40 and 447.6). The spreading factors listed in Table 4 reveal significant differences between the formulations: P. americana gel 1% presented a higher spreadability factor than P. americana gel 3%, which confirms the spreadability results presented in Figure 3. This higher scattering capacity is shown to be due to the lower concentration of the extract in the P. americana 1% gel. Fontana et al. [52] also found different spreadability values for different formulations.

Table 4: Spreadability factors (Sf) of the formulations as a function of total area (mm²) and total weight (g).

<table>
<thead>
<tr>
<th>Formulations</th>
<th>Area (mm²)*</th>
<th>Weight (g)</th>
<th>Sf (mm²g⁻¹)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. americana gel 1%</td>
<td>6622 ± 0.91</td>
<td>447.6</td>
<td>14.79 ± 2.04</td>
</tr>
<tr>
<td>P. americana gel 3%</td>
<td>3975 ± 1.76</td>
<td>447.6</td>
<td>8.87 ± 0.40</td>
</tr>
</tbody>
</table>

*Results are expressed as the mean ± standard derivation (SD) of three determinations (n = 3). Different letters represent significant differences (p < 0.05).

Taking the results together, while P. americana gel 3% presents lower spreadability than P. americana gel 1% and both formulations do not show great differences in antioxidant activity, the latter appears to be the more suitable formulation for practical applications because it releases rutin faster (within 1 hour) to the skin. More studies should be performed to confirm the antioxidants and anti-aging effects of P. americana extract and ensure an effective and safe preparation for topical use.

**Conclusion**

Persea americana gel 3%, which allowed the release of rutin to the skin within 1 hour, presented better results than Persea americana gel 1%. The formulation 1% howed better spreadability but poorer rutin release. Both formulations presented similar antioxidant effects, likely
because of the presence of other phenolic compounds in the extract.

References


