Analysis of Rutin in the Extract and Gel of *Viola tricolor*

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Heartsease, also known as wild pansy (*Viola tricolor* L., Violaceae), has a long history in phytomedicine (1). The therapeutic activity of pansy has been used in treating various skin conditions, such as eczema, seborrhea, impetigo and acne (2).

Wild pansy contains 0.3% of salicylic acid and its derivatives, such as the methyl ester and violutoside (the glucosidoorabino-side of salicylic acid methyl ester); phenol carboxylic acids such as trans-cafeic acid, protocatechuic acid and p-coumaric acid; 10% of mucilages [made up of glucose (35%), galactose (33%), arabinose (18%), and rhamnose (8%)]; 2.4–4.5% of tannins and flavonoids (rutin, violanthin, scoparin, saponaretin, orientin, vicenin and anthocyanidin glycosides); carotenoids (violaxanthin and four geometrical isomers and zeaxanthin); coumarins; umbelliferone; small amounts of saponins; ascorbic acid and tocopherol. The salicilates and rutin contained in the plant are anti-inflammatory (2).

In the form of plant extracts, flavonoids have been used in dermatology and cosmetics for a long time (3). The primary reason for the increasing popularity of these substances is their beneficial biochemical activities, and the primary factor affecting the activity of flavonoids in the skin is their ability to penetrate skin (4). The available data indicate that the compounds of this group permeate through the stratum corneum and can reach the viable layers of the epidermis and dermis (3).

The worldwide trends are to standardize extracts by fast and efficient techniques such as chromatography and spectrometry, with the purpose of determining the present substances that are responsible for cosmetic activity, even in small concentrations (5).

Two basic factors determine the composition of an extract: the quality of plant material and the process of production (6). The standardization of plant extracts should be performed to determine the contents of one or more constituents (7). The increasing demand for safe and effective products has required the scientific community to provide more complex studies and to use more efficient techniques to determine stability. The immense Brazilian biodiversity has led to the development of several products in different forms, which further complicates the standardization of experimental protocols to certify the stability of these preparations (8).

The development and validation of an efficient analytical method is an integral part of the quality control of the source material to guarantee the safety and effectiveness of the resulting compound (9). This study investigates the phytoconstituents and the antioxidant capacity of rutin in the crude extract of the flowers of *V. tricolor* and in the extract incorporated in gel, and validates a method for the quantification in rutin. High-performance liquid chromatography (HPLC) was used with ultraviolet (UV) detection, and the validation followed the characteristics of precision, linearity, limit of detection, limit of quantification, specificity, accuracy and robustness.

**Experimental**

**Reagents**

All reagents used in this study were of analytical grade. Ethanol, methanol, vanillin, gallic acid, salicylic acid and spectrophotometric grade methanol were purchased from Merck (Darmstadt, Germany). Folin-Ciocalteau reagent, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), rutin and catechin were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagents were of analytical grade and purchased locally. The chemicals used in the formulation were provided by the pharmacotechnic laboratory of the University Federal of Santa Maria.

**Methods**

**Collection and extraction of plant**

The flowers of *V. tricolor* were collected in the city of Gaurama (Rio Grande do Sul, south Brazil) between September and November of 2009. A dried voucher specimen is preserved in the herbarium of the Department of Biology at Federal
University of Santa Maria (SMBD 12.958). The flowers were dried in a stove (temperature above 40°C) and chopped in a knife mill. The chopped flowers were macerated at room temperature with 70% ethanol for one week, with a daily shake-up. After filtration, the hydroalcoholic extract was evaporated under reduced pressure in a rotary evaporator to remove the ethanol. The aqueous extract was dried in a stove (temperature above 40°C) to produce the crude extract.

**Analysis of polyphenols, flavonoids, condensed tannins, alkaloids and antioxidant capacity in the crude extract of V. Tricolor flowers**

The analysis was performed in a Shimadzu UV-1201 spectrophotometer (Shimadzu, Kyoto, Japan).

**Polyphenols**

The polyphenol content was measured by the Folin-Ciocalteu method described by Chandra and Mejia (10). 2N Folin-Ciocalteu reagent (0.5 mL) was added to 1 mL of the sample. This blend was allowed to stand for 5 min before the addition of 2 mL of Na2CO3 20%. The solution was left standing during 10 min before measurements at 730 nm. The data were expressed in mg of gallic acid equivalent (GAE) per g of crude extract, based on the calibration curve of gallic acid.

**Flavonoids**

The content of flavonoids was determined by the reaction with aluminum chloride using the method described by Woiisky and Salatino (11). AlCl3 2% solution (0.5 mL) was added to 1 mL of the sample. After 15 min, the absorbance was measured at 420 nm. The data were calculated based on the calibration curve of rutin and expressed in mg equivalents of rutin equivalents (RE) per g of crude extract.

**Condensed tannins**

Condensed tannins were determined by a slightly modified version of the vanillin method described by Morrison et al. (12). An extract solution (0.1 mL; 25 mg/mL of concentration) was pipetted into a test tube and 0.9 mL methanol was added. Vanillin reagent (2.5 mL equal volumes of 1 g of vanillin in 100 mL methanol and 8 mL concentrated HCl in 100 mL methanol) was added. The test tubes were placed in a water bath for 20 min. The absorbance was measured at 500 nm. The data were expressed in mg catechin equivalent (CE) per g of crude extract, based on the calibration curve of catechin.

**Alkaloids**

The alkaloids were determined by the precipitation reaction with Dragendorff’s reagent, described by Sreevidya and Mebrotra (13). Briefly, 5 mL of the solution of the extract was taken and the pH was kept between 2 and 2.5 with diluted HCl. Two milliliters of Dragendorff’s reagent was added and the resulting precipitate was centrifuged. After that, the precipitate was washed with alcohol, the filtrate was discharged and the residue was treated with 2 mL of a disodium sulfide solution, and the resulting brownish-black precipitate was then centrifuged. The residue was dissolved in 2 mL of a concentrated nitric acid; this solution was diluted in 10 mL of distilled water. To 1 mL of this solution was added 5 mL of thiourea solution. The absorbance was measured at 435 nm; the data were calculated based on the calibration curve of bismuth nitrate and expressed in mg of alkaloids per g of crude extract.

**Radical scavenging activity—DPPH assay**

The antioxidant capacity was evaluated according to a slightly modified method, previously described by Choi et al. (14). Spectrophotometric analysis was used to determine the inhibition concentration (IC50; the concentration that offers 50% inhibition) and the inhibition percentage (IP%) of the crude extract. Six different ethanol dilutions of the extract, 2.5 mL at 250, 125, 62.5, 31.25, 15.62, and 7.81 μg/mL, were mixed with 1.0 mL of a 0.3 mM DPPH ethanol solution. The absorbance was measured at 518 nm in a spectrophotometer against a blank after 30 min of reaction at room temperature. A DPPH solution (1.0 mL, 0.3 mM) with ethanol (2.5 mL) was used as a control. The same was performed with standard ascorbic acid under the same experimental conditions. Inhibition of the free radical by DPPH as a percentage (IP%) was calculated according to Equation 1.

\[
IP% = 100 - \left[ \frac{ABSSAMPLE}{\frac{ABSBLANK}{ABSCONTROL}} \right] \times 100
\]  

where **ABS SAMPLE** is the absorbance of the test compound, **ABS BLANK** is the absorbance of the blank and **ABS CONTROL** is the absorbance of the control reaction (containing all reagents except the test compound). IP% was plotted against the sample concentration, and a linear regression curve was established to calculate the IC50.

**HPLC analysis**

The HPLC method was performed on a Shimadzu Prominence system (Kyoto, Japan) equipped with an SIL-20A autosampler and reciprocating pumps connected to the DGU 20A5 degasser with CBM 20A integrator, ultraviolet-visible (UV-VIS) SPD-M20A diode-array detector (DAD) and 1.22 SP1 LC solution software. The mobile phases and all solutions and samples were filtered through a 0.45 μm Millipore membrane filter (Bedford, MA), and then degassed by an ultrasonic bath before use. The quantification was conducted by integration of the peaks using the external standard method. The chromatographic peaks were confirmed by comparing their retention times and DAD–UV spectra with those of the reference standards. All chromatographic operations were conducted at room temperature and in triplicate.

**Analysis of salicylic acid in extract**

The analysis of salicylic acid was performed using a slightly modified version of the method described by the British Pharmacopoeia (15). For the quantification of salicylic acid, reversed-phase chromatographic analyses were conducted in isocratic conditions using a Shimadzu C-18 column (4.6 × 150 mm) packed with 5 μm diameter particles. The mobile phase was water–methanol (60:40, v/v) containing 1% glacial acetic acid (v/v). The salicylic acid was quantified at 300 nm; injection volume was 40 μL and the flow rate was 1 mL/min.
**Method validation for quantitative analysis of rutin**

The method was validated according to the Guidelines of the International Conference on Harmonization (16).

**Preparation of standard solution**

A rutin reference standard solution was prepared in methanol–water (1:1, v/v). Standard calibration solutions at seven levels were prepared by serial dilution of a stock solution at concentrations of 10–700 μg/mL.

**Test solution of the extract**

An amount of 0.05 g of the extract was weighed in a flask, a methanol–water (1:1, v/v) solution was added to bring the solution to 10 mL, and the solution was placed in an ultrasonic bath for 15 min.

**Chromatographic conditions**

For the quantification of rutin, reversed-phase chromatographic analyses were conducted under isocratic conditions using a Shimadzu C-18 column (4.6 × 150 mm) packed with 5 μm diameter particles; the mobile phase was water–methanol (1:1, v/v) containing 0.002% (v/v) phosphoric acid (pH 2.8). The rutin was quantified at 356 nm; the injection volume was 20 μL and the flow rate was 1 mL/min.

**Specificity**

The specificity is defined as the ability of the method to accurately and specifically measure the analyte in the presence of components in the sample matrix; specificity was determined by analysis of the chromatograms of the standard and sample solutions. A photodiode-array detector (PDA) was employed to compare the sample and the reference standard profile.

**Linearity and range**

The linearity between peak area and concentration was analyzed using three calibration curves obtained with standard solutions of rutin at eight different concentrations each: 10–700 μg/mL. The data for peak area versus rutin concentration were treated by linear regression analysis. The range was obtained from the standard linear curve.

**Sensitivity**

The limit of detection (LOD) and limit of quantization (LOQ) were determined from the calibration curves of the rutin standard. LOD was calculated according to the expression 3σ/S, where σ is the standard deviation of the response and S is the slope of the calibration curve. LOQ was established by using the expression 10 σ/S (16).

**Accuracy**

The accuracy was evaluated by means of recovery tests conducted by adding known amounts (18, 25 and 53% of rutin reference standard) to the sample at three different levels; three solutions each in triplicate. The percent bias was determined by comparing the results of the analyses of the fortified samples.

**Precision**

The repeatability test was conducted using eight samples in the same concentrations on the same day. For the intermediate precision, the same experiment was conducted on another day. The relative standard deviation (RSD) was used as statistic parameter.

**Robustness**

The robustness of a method is its ability to remain unaffected by small, deliberate variations in the method, and was evaluated by changing the pH of the mobile phase (±0.3) and flow rate, in triplicate.

**Method validation for quantitative analysis of rutin in gel**

The gel was prepared by utilizing Natrosol QP 400H Farmaquimica (Porto Alegre, Rio Grande do Sul, Brazil), EDTA Proquimios (São Paulo, São Paulo, Brazil), Nipagin Belga (Santa Maria, Rio Grande do Sul, Brazil) and the extract of *V. tricolor*.

The method was validated according to the guidelines of the International Conference on Harmonization (16). The chromatographic conditions and sensitivity, precision and robustness parameters were the same as those used in the validation method for quantification of rutin in the extract.

**Preparation of standard solution**

A rutin reference standard stock solution was prepared in methanol–water (1:1; v/v). Calibration standard solutions were prepared at seven levels by serially diluting the stock solution to concentrations of 5–400 μg/mL.

**Test solution of the gel**

An amount of 1 g of the gel was weighed in a flask, methanol–water (1:1, v/v) solution was added to bring the solution to 10 mL, and the solution was placed in an ultrasonic bath for 60 min.

**Linearity and range**

The linearity between peak area and concentration was analyzed using three calibration curves obtained with standard solutions of rutin at seven different concentrations each: 5–400 μg/mL. The data for peak area versus rutin concentration were treated by linear regression analysis. The range was obtained from a standard linear curve.

**Sensitivity**

The specificity was determined by analysis of chromatograms of sample solutions of the gel without extract to verify the absence of gel interferents.

**Accuracy**

The accuracy was evaluated by means of recovery tests conducted by adding known amounts (4, 8 and 16% of rutin reference standard) to the sample at three different levels, three solutions each in triplicate. The percent bias was determined by comparing the results of the analyses of the fortified samples.
Results

The results of the phytochemical analysis are presented in Table I, which reveal the presence of active constituents. Much better antioxidant capacity was found for the extract of the flowers of *V. tricolor* than the standard ascorbic acid (Figure 1). The DPPH radical scavenging capacity of samples was dose-dependent, and the $IC_{50}$ values were $16.57 \pm 0.78 \mu g/mL$ for the standard ascorbic acid and $16.00 \pm 0.95 \mu g/mL$ for the extract of the flowers. Additionally, quantities of $5.73 \pm 0.45 \mu g$ of salicylic acid/100g of extract were found (Figure 2). The linearity and sensitivity of the method validation for the quantification of rutin in the extract are shown in Table II and the accuracy is shown in Table III. The result of the RSD obtained from the analyses of precision in the extract was 0.26, and the result for the intermediate precision was 0.86. No relevant change was observed when the mobile phase pH and flow rate were modified, which shows the robustness of the method. Analysis of the chromatogram of the sample by PDA indicated the specificity of the method (data not shown). The results showed higher amounts of rutin in the extract: 79.16 $\mu g/g$ (Figure 3).

The linearity and sensitivity results of the validation method for the quantification of rutin in gel are shown in Table IV. The result for the accuracy of the method is shown in Table V. The analyses of precision in gel showed an RSD of 0.75 and the analyses for the intermediate precision obtained an RSD of 0.07. The absence of interferences in the chromatograms of the samples of the gel without the extract at 356 nm confirmed the specificity of the method (data not shown). No relevant change was observed when the mobile phase pH and flow rate were modified, which shows the robustness of the method. The gel presented 233 $\mu g$ of rutin/100 g of gel (Figure 4).

Discussion

Several extracts have antioxidant capacity, and among the compounds found in extracts, flavonoids and other phenolic substances play an important role (17). The quantity of

### Table I

<table>
<thead>
<tr>
<th>Secondary metabolite</th>
<th>Amount ± RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyphenols</td>
<td>109.32 $\mu g/g$ of extract ± 1.29</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>99.40 $\mu g/g$ of extract ± 1.27</td>
</tr>
<tr>
<td>Condensed tannins</td>
<td>10.83 $\mu g/g$ of extract ± 0.49</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>0.55 $\mu g/g$ of extract ± 0.79</td>
</tr>
</tbody>
</table>

### Table II

#### Linearity and Sensitivity Results of the Method for Quantification of Rutin in Crude Extract of *V. tricolor*

<table>
<thead>
<tr>
<th>Parameter statistic</th>
<th>Rutin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range</td>
<td>10–700 $\mu g/mL$</td>
</tr>
<tr>
<td>Standard curve</td>
<td>$Y = 39481x + 98918$</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>$R = 0.9994$</td>
</tr>
<tr>
<td>LOD</td>
<td>10.06 $\mu g/mL$</td>
</tr>
<tr>
<td>LOQ</td>
<td>33.49 $\mu g/mL$</td>
</tr>
</tbody>
</table>

### Table III

#### Recovery of the Standard Solution of Rutin Added to the Samples Analyzed by the Proposed Method

<table>
<thead>
<tr>
<th>Added amount (%)</th>
<th>Recovery* (%)</th>
<th>RSD (%)</th>
<th>Bias † (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>100.8</td>
<td>0.49</td>
<td>0.8</td>
</tr>
<tr>
<td>25</td>
<td>96.76</td>
<td>0.22</td>
<td>–3.24</td>
</tr>
<tr>
<td>33</td>
<td>96.07</td>
<td>0.33</td>
<td>–3.93</td>
</tr>
</tbody>
</table>

*Mean of three measurements.

†Bias = [(measured concentration – nominal concentration) / nominal concentration] $\times$ 100.

### Table IV

#### Linearity and Sensitivity Results of the Method for Quantification of Rutin in the Gel of *V. tricolor*

<table>
<thead>
<tr>
<th>Parameter statistic</th>
<th>Rutin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range</td>
<td>5–400 $\mu g/mL$</td>
</tr>
<tr>
<td>Standard curve</td>
<td>$Y = 32890x + 21599$</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>$R = 0.9994$</td>
</tr>
<tr>
<td>LOD</td>
<td>1.99 $\mu g/mL$</td>
</tr>
<tr>
<td>LOQ</td>
<td>6.64 $\mu g/mL$</td>
</tr>
</tbody>
</table>

### Table V

#### Recovery of the Standard Solution of Rutin Added to the Samples, Analyzed by the Proposed Method

<table>
<thead>
<tr>
<th>Added amount (%)</th>
<th>Recovery* (%)</th>
<th>RSD (%)</th>
<th>Bias † (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>100.37</td>
<td>0.67</td>
<td>0.37</td>
</tr>
<tr>
<td>8</td>
<td>99.32</td>
<td>0.23</td>
<td>–0.68</td>
</tr>
<tr>
<td>16</td>
<td>98.79</td>
<td>0.68</td>
<td>–1.21</td>
</tr>
</tbody>
</table>

*Mean of three measurements.

†Bias = [(measured concentration – nominal concentration) / nominal concentration] $\times$ 100.
polyphenols was 109.32 mg of GAE/g and for flavonoids, 99.40 mg of RE/g was found, showing that most of the polyphenols existent in the extract are flavonoids. Vukics et al. (18) also found considerable quantities of flavonoids in this species by using the method of the European Pharmacopoeia. The excellent antioxidant capacity may be due to the amounts of polyphenols and flavonoids found in the extract. Vukics et al. (18) found a positive correlation between the amounts of flavonoids and the antioxidant capacity of this species. Results from a study by Mustafa et al. (19) suggested that phenolic compounds, particularly the flavonoids, are the major contributor to the antioxidant capacity of the tested plants, and consequently can be utilized in the development of functional ingredients with potent antioxidant properties for commercial exploration.

Considerable quantities of condensed tannins were found, 10.83 mg of CE/g, which is superior to the results of Rimkiene et al. (2), which found amounts ranging from 1.3 to 4.5% tannin between 1995 and 2002. In vitro tests of extracts rich in tannins showed bactericidal and fungicidal activity, inhibition of lipid peroxidation and free radical scavenging. They also can help in the healing process of wounds, burns and inflammation, by forming a protective layer (tannin-protein complex) in the damaged skin (20). Studies by Toiu et al. (22) encountered higher quantities of salicylic acid in the aerial parts of this plant. Salicylic acid also has anti-inflammatory and antibacterial properties (23).

Until now, the literature had only qualitative data about the existence of alkaloids in V. tricolor (21), but this study found small amounts (0.55 mg) of alkaloids/g; therefore, it is probable that this metabolite has little influence on the therapeutic effect and antioxidant capacity of this species.

The various phytochemicals present in plants have been reported to possess great potential in the treatment of various diseases. These constituents act directly in antioxidant, anti-inflammatory and anti-microbial activities (24). The compounds found, such as polyphenols, flavonoids, tannins and salicylic acid, make this a promising species for developing formulations and ensuring the quality of the extract and the gel; however, appropriate analytical methods must be established. Rutin is one of the constituents of the flowers of this species, so it was used as a marker to evaluate the quality of the extract and the gel.

The method validation for the quantification of rutin in the extract showed linearity and sensitivity between the concentrations of 10–700 µg/mL. The results of the precision were satisfactory because the RSD was less than 2. The accuracy was determined by analyzing a sample of known concentration and comparing the measured value with the true value, using the standard addition method. The results were expressed as percentage bias, and the method showed adequate accuracy.

Vuckis et al. (18) found flavonoid rutin to be the primary constituent of the flowers of V. tricolor at a concentration of 420 µg/g rutin; this difference may be due to differences in soil, climate, time of collection and parts of the plant that were analyzed.

The linearity and sensitivity were adequate in the concentration range of 5–400 µg/mL. The results of the precision were satisfactory and the accuracy of the method was considered to be adequate. Additionally, it was observed that the sonication time of 45 min was ineffective for the complete extraction of rutin from the gel, because the method indicated quantities of 209.94 ± 0.11 mg of rutin/100 g of gel (below 90%).

Conclusion

V. tricolor presents a considerable amount of polyphenols and flavonoids: large amounts of rutin contribute to the antioxidant capacity of this species, and constituents such as condensed tannins, alkaloids and salicylic acid may also help in this capacity. The proposed method for the quantification of rutin in the extract and gel is linear, sensitive, precise, specific, accurate and robust. This validated method can be used to the quality control of the extract and the gel.

Acknowledgments

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