Anti-oxidant capacity, total phenolic contents and HPLC determination of rutin in *Viola tricolor* (L) flowers

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ABSTRACT

Background: Natural products with anti-oxidant properties have gained importance in recent years due to their ability to neutralize free radicals or their actions. *Viola tricolor* belong to *Violaceae* family and is known as heartsease. The aims of the study was to evaluate the phenolic contents and anti-oxidant capacity in the different flowers and leaves/roots fractions of *Viola tricolor*, as well as was identified and quantify rutin in the flowers fractions. **Materials and Methods:** Total phenolic content was determined using the Folin-Ciocalteu assay, the anti-oxidant capacity by the 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) scavenging method and thiobarbituric acid reactive species (TBARS) assay. The presence of rutin was investigated by High Performance Liquid Chromatography (HPLC). **Results:** In the flowers fractions the phenolic content varied from 12.84 ± 0.072 to 0.23 ± 0.042 mg/g; and 7.49 ± 0.002 to 0.63 ± 0.002 mg/g for leaves/roots. IC_{50} DPPH values ranged from 13.40 to 14.18 mg/ml in flowers, and 32.84 to 284.87 μg/ml for the leaves/roots. The fractions showed inhibition against TBARS, following order: ethyl acetate fractions > butanolic fractions > dichloromethane fractions. HPLC results indicate a very high content of rutin (177.46 mg/g) in this species that could explain the good anti-oxidant activities and the high phenolic contents in these fractions. **Conclusions:** These findings suggest that *V. tricolor* contains anti-oxidant principles and rutin contribute, in party, for the action.

Keywords: Anti-oxidant activity, HPLC, rutin, *Viola tricolor*.

INTRODUCTION

Viola tricolor L. (heartsease) has been traditionally utilized as an herbal medicine internally to treat upper-respiratory problems (catarrh and cough) and externally to treat skin disorders such as eczema, seborrhea, impetigo, and acne, also employed in cystitis and as a diuretic. *Viola* taxes revealed to be a rich source of cyclotides and flavone glycosides.[1] Anthocyanins, flavonoids, coumarins, tannins, saponins, carotenoids, salicylic acid and other phenolic acids are constituents of *V. tricolor* herb.[2,3]

Rutin was earlier isolated by the use of paper chromatography as the main flavonoid constituent from the plant.[2] The flavonoids contents of heartsease were reinvestigated in the methanolic extracts of a commercial dried plant material by Vukics *et al.*[3] using NMR, LC-MS and HPLC techniques. According to these authors, the main flavonoid component was identified as violanthin (6-C-glucosyl-8-C-rhamnosylapigenin) followed by rutin, both in significant quantities.[3,4] Rutin (3-O-rhamnoglucosyl quercetin) is found in many typical nutrimental plants and is an important dietary constituent of food and plant-based beverages. Several analytical techniques have been described for rutin

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determination[5,6] but there are few reports for the quantitative determination of rutin in *V. tricolor*.[4,5]

According to previous studies, *V. tricolor* contains large quantity of flavonoids, for this reason the assessment of its antioxidant properties, as well the total phenolic contents and rutin determination is of great interest to guarantee the positive effects of these compounds especially in phytomedicine. Moreover, there is an increase interest in new applications of edible flowers in culinary as a garnish or ingredient in salads, soups, entrees, desserts, and drinks besides their traditionally use in the medicinal herbs industries.[7] To the best of our knowledge, there is no report in the literature on the differences of the antioxidant activities and the phenolic contents among the different parts of *V. tricolor*. In this way, the present study aimed to evaluate the anti-oxidant activity and the total phenolic contents in plant fractions obtained from different parts of the plant (leaves/roots and flowers) in order to establish for the first time a comparative study between them and to govern future studies to prepare standard fractions. HPLC was used to carry out quantitative analysis of the plant fractions which presented the best antioxidant activities, using rutin as a phytochemical marker.

MATERIAL AND METHODS

Chemicals

All the chemicals were of analytical grade. Solvents for the extractions, ascorbic acid, pyrogallic acid and methanol HPLC grade were purchased from Merck (Darmstadt, Germany). Rutin, hyperoside, chlorogenic and caffeic acids, Folin-Ciocalteau phenol reagent 2 *N* and DPPH radical (1, 1-diphenyl-2-picrylhydrazyl) were acquired from Sigma Chemical Co. (St. Louis, MO, USA).

Plant material

Viola tricolor L. was harvested in Santa Maria, State of Rio Grande do Sul, Brazil from November of 2005 to January of 2006 (during the flowering stage of the plant). Samples of the collected material were identified by Botanist Dr. Thais Scotti do Canto-Dorow and archived as voucher specimens in the herbarium of Department of Biology at Federal University of Santa Maria by register number SMDB 10126.

Extraction and partition of the aerial parts

Flowers from each plant were separated by hand. Leaves and roots were processed together as a unique extract. The extraction process is illustrated below [Figure 1]:

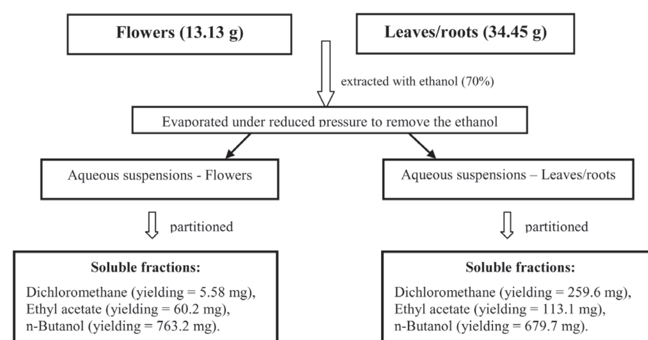

Figure 1. Diagram of the extraction process.

Thin-layer chromatography (TLC) and preliminary phytochemical screening

Analytical TLC was performed on 20 × 20 cm plates precoated with silica gel GF_{254} nm from Merck. Volumes of 10 ml of methanolic solution of standards and investigated fractions (flowers and leaves/roots) were spotted on the plates. TLC analysis was performed using two systems as mobile phase (system 1 = ethyl acetate: formic acid: glacial acetic acid: water: 100:11:11:27 v/v and system 2 = chloroform: ethanol: water 40:40:0.5 v/v).[8] Reference standards were hyperoside, rutin and chlorogenic and caffeic acids. Spots were first observed under UV light at 254 and 366 nm without any treatment. Detection was performed with anisaldehyde: H_2SO_4 reagent followed by heating (110ºC) for 10 min. Phytochemical tests were carried out as described by Boligon *et al*.[9] and each result was qualitatively expressed as negative (–) or positive (+).

Determination of total phenolic contents

Each dried fraction was diluted with water to get a final concentration of 0.15 mg/ml. The total polyphenol concentrations were determinate as described by modified Folin-Ciocalteu method as described by Boligon *et al*.[9] using pyrogallic acid as a reference standard. Briefly, 0.5 ml of 2 *N* Folin-Ciocalteu reagent was added to a 1 ml of each sample and this mixture was allowed to stand for 5 min before the addition of 2 ml of 20% Na_2CO_3. The solution was then allowed to stand for 10 min before reading at 730 nm in a Shimadzu-UV-1201 (Shimadzu, Kyoto, Japan) spectrophotometer. The estimation of phenolic compounds in the fractions was carried out in triplicate and expressed as milligram equivalents of pyrogallic acid per gram of dried fraction. Total polyphenol contents are given in Table 1. The equation obtained for standard curve of pyrogallol in the range of 0.005 – 0.030 mg/mL was $y = 34.443x - 0.0942$ ($r = 0.994$).

Table 1 Total phenolic contents in *Viola tricolor* flowers and leaves/roots fractions

Fraction	Flowers (mg/g ± SD)	Leaves/roots (mg/g ± SD)
Dichloromethane	0.23 ± 0.042	0.63 ± 0.002
Ethyl acetate	1.75 ± 0.028	0.28 ± 0.007
Butanolic	10.86 ± 0.002	6.58 ± 0.011
Total	12.84 ± 0.072	7.49 ± 0.002

Results were expressed in milligram equivalents of pyrogallic acid/gram of fraction (mg/g ± SD).

Radical scavenging activity on DPPH radical

The anti-oxidant activities were evaluated by monitoring its abilities in quenching the stable free radical DPPH, according to the modified method described by Schubert *et al.*[8] four different ethanol dilutions of each fraction (2.5 ml), at 125; 62.5; 31.25; and 15.62 mg/ml were mixed with 1.0 ml of a 0.3 mM DPPH ethanol solution. After 30 min, absorption was measured at 518 nm, where the radical DPPH shows maximum absorption. A solution of DPPH (1 mL; 0.3 mM) in ethanol (2.5 mL) was used as a negative control and *L*-ascorbic acid in the same concentrations used for the fractions and the crude extracts provided the positive control. Ethanol was used to calibrate the spectrophotometer. Inhibition of free radical by DPPH in percent (IP %) was calculated in following way, according to the Equation 1:

Equation 1:

$$IP\% = 100 - [(ABS_{SAMPLE} - ABS_{BLANK}) / ABS_{CONTROL}) \times 100]$$

Where: ABS_{sample} is absorbance of each fraction; ABS_{BLANK} is absorbance of fractions without adding the DPPH and $ABS_{control}$ is absorbance the solution of ethanol in DPPH. IP % was plotted against sample concentration, and a linear regression curve was established in order to calculate the IC_{50}. Tests were carried out in triplicate and correlation coefficients were optimized following Tsimogiannis *et al.*[10] procedures.

In vitro Fe (II)-induced lipid peroxidation in rat brains

Male Wistar rats (3.0–3.5 months of age and weighing 270–320 g) were maintained groups of 3–4 rats per cage. They had continuous access to food and water in a room with controlled temperature (22 ± 3°C) and on a 12 h light/dark cycle. The animals were maintained and used in accordance to the guidelines of the Brazilian Association for Laboratory Animal Science (COBEA). The rats were killed by decapitation and the brain tissue was rapidly dissected, weighed and immediately homogenized in Tris-HCl 10 mM, pH 7.5 (1/10, w/v). The homogenate was centrifuged for 10 min at 4000 rpm and the supernatant was used for inhibition of lipid peroxidation assay.[11,12] To evaluate the inhibition of lipid peroxidation, an aliquot of 100 μL of supernatant of rat brain homogenate was incubated for 1 h at 37°C with the pro-oxidant agent ferrous sulphate (10 μM of $FeSO_4$) in the presence or absence of plant fractions. The production of TBARS (thiobarbituric acid reactive substances) was determined by the colorimetric method according to Puntel *et al.*[12] Quantification was expressed in nmol of malondialdehyde (MDA)/g of tissue.

Chromatographic conditions for rutin determination

Rutin contents were determined by HPLC in the fractions which presented highest anti-oxidant activities. Liquid chromatography was performed using a Shimadzu HPLC apparatus (Shimadzu LC-10A system, Kyoto, Japan) equipped with two LC-10AD Vp pumps, an UV-VIS SPD-10A Module, an SLC-10A system controller, a Rheodyne injector 20 μL loop with RP-8 LiChroCart column (125 mm × 4.60 mm, 5 μm particle size). Chromatograms were obtained and analyzed using the software Class-VP® (Shimadzu, Tokyo, Japan). The mobile phase consisted of a binary mixture of methanol-water (50:50 v/v) adjusted to pH 2.8 with phosphoric acid[13] at isocratic flow rate of 1.0 mL min^{-1}. The absorbance was monitored at $\lambda = 360$ nm and the analytical time was 5 min. Rutin standard stock solution was prepared series of dilutions. The calibration curve was performed in triplicate and generated by linear regression of peak area ratios against their respective concentrations. Sample solutions were diluted with mobile phase getting concentration of 50 μg of each dried fraction/mL.

Statistical analysis

IC_{50} was calculated from the linear regression lines of inhibition percentage versus concentration. One-way analysis of variance (ANOVA) was used for comparison of the experimental data. Post-hoc multiple comparisons were done by Tukey test for significance at P values ≤0.05.

RESULTS AND DISCUSSION

The flowers and leaves/roots fractions demonstrated that the plant contains tannins, amino groups, phenols, flavonoids, terpenoids, oses, steroids, and anthocyanins. Flavonoids and phenols were the main group of compounds in both extracts, suggesting their responsibility for the

good anti-oxidant activities observed.[7,9] Caffeic acid (Rf = 0.64), chlorogenic acid (Rf = 0.45) and rutin (Rf = 0.35) were also detected in the investigated extracts by TLC analysis. These results are in agreement with previous studies.[3,4,14]

Total polyphenol contents are given in Table 1. The highest contents of phenolics were obtained in the butanolic fractions from the flower and leaves/roots fractions, some investigations of plants compositions have demonstrated that polar fractions usually concentrates high amounts of phenolic compounds.[9,11,15,16] Considering the sum of these compounds, flowers fractions showed higher content (almost two times) when compared to the leaves/root fractions. It is difficult to choose suitable standards for total phenolic determination in plant extracts due to the chemical heterogeneity of plants products and the low specificity of phenolic reagents. Thus, it is only possible to get relative equivalents with the standard used and this procedure difficulty the comparisons between different plants.[7] The phenolic contents determined in the flowers of *Viola* were similar to those quantified by Ćetković *et al.*[17] in *Calendula arvensis* flowers using the same method, although these authors expressed their results as chlorogenic acid equivalents. It is difficult to choose suitable standards for total phenolic determination in plant extracts due to the chemical heterogeneity of plants, products and the low specificity of phenolic reagents. Thus, it is only possible to get relative equivalents with the standard used and this procedure difficult the comparisons between different plants.

In this study, the flowers fractions of *V. tricolor* exhibited a strong DPPH free radical scavenging activity and the IC_{50} was achieved at the smallest concentration used in the assay (IC_{50} = 13.40; 13.25 and 14.18 mg/ml, for dichloromethane, ethyl acetate, butanolic fractions, respectively) this results are similar to ascorbic acid activity (IC_{50} = 12.66 mg/ml) [Figure 2, Table 2]. The leaves/roots fractions showed a lower anti-oxidant activity, IC_{50} value of 284.87 mg/ml, 23.65 μg/ml and 32.84 μg/ml for dichloromethane, ethyl acetate and butanolic fractions, respectively. Previous work also has found similar results for leaves methanol extract of *V. tricolor* (IC_{50} = 60.64 ± 16.87 μg/mL) using a DPPH assay.[18] The IC_{50} value for rutin is 10.26 ± 0.47 μg/mL,[3] this compound may be responsible in part for the good activity found in the flowers fractions.

Positive relation between anti-oxidant capacity and phenolic content is described by several authors using similar assay systems,[16] however, in the case of *V. tricolor*, this kind of relation could not be well established because the fractions that presented higher polyphenolic contents not necessarily presented the best anti-oxidant activities. As an example, the total phenolic contents in dichloromethane, ethyl acetate and butanolic fractions of flowers ranged from 0.23 to 10.86 mg/g [Table 1], while the anti-oxidant capacity was almost the same for these fractions [Figure 2]. One explanation could be the presence of other reducing compounds that probably interfere with the Folin-Ciocalteu assay and/or the presence of other non-phenolic compounds with anti-oxidant effects.[9,11]

Fe (II) induced stimulation in brain TBARS levels, which were partially reduced by *V. tricolor* fractions in a concentration-dependent manner. The inhibitory potency was in the following order: ethyl acetate fractions (IC_{50} = 19.48 and 33.62 mg/ml, for flowers and leaves/roots,

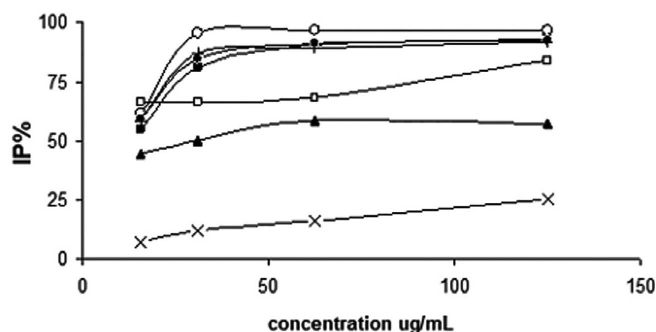

Figure 2. Inhibition Percentage (IP %) of *V. tricolor* fractions. (■) = n-butanol, (•) = dichloromethane and (+) = ethyl acetate fractions from flowers. (▫) = Ethyl Acetate, (▲) = n-butanol and (X) = dichloromethane fractions from the leaves/roots. (o) = *L*-Ascorbic acid.

Table 2 IC_{50} (μg/mL) values of the DPPH and TBARS assay in *Viola tricolor* flowers and leaves/roots fractions

Fraction	IC_{50} DPPH (μg/mL)		IC_{50} TBARS (μg/mL)	
	Flowers	Leaves/roots	Flowers	Leaves/roots
Dichloromethane	13.40	284.87	28.13	56.01
Ethyl acetate	13.25	23.65	19.48	33.62
Butanolic	14.18	32.84	22.70	29.95
Ascorbic acid	12.66			

IC_{50}: concentration required to inhibit 50% of the capacity. SD: standard deviation.

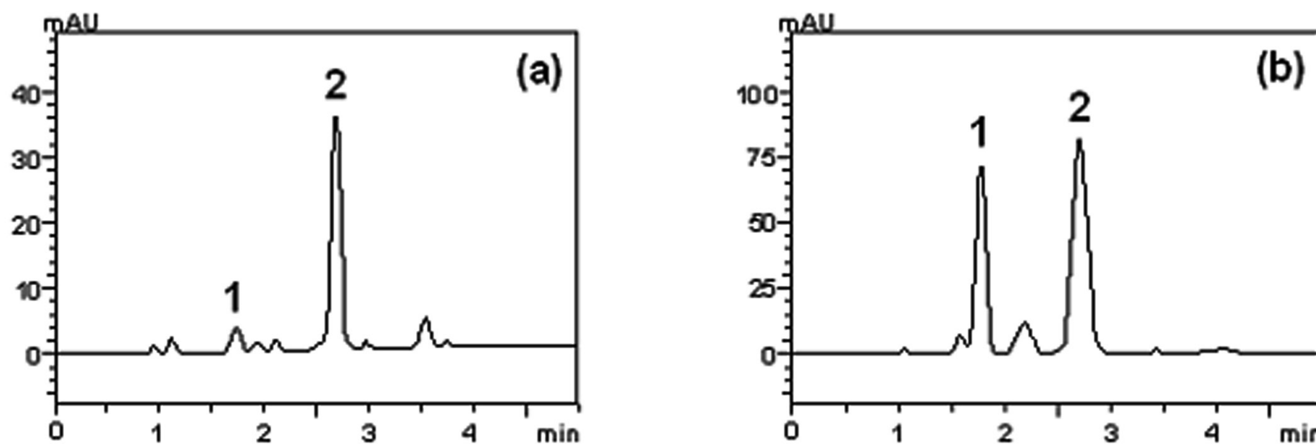

Figure 3. Representative high performance liquid chromatography profile (a) ethyl acetate and (b) butanolic fractions of *V. tricolor* flowers, detection UV was at 360 nm. 1 Represents an unknown peak and 2 correspond to rutin peak. Chromatographic conditions are described in the Methods section.

respectively) > butanolic fractions (IC_{50} = 22.70 and 29.95 mg/ml, for flowers and leaves/roots, respectively) > dichloromethane fractions (IC_{50} = 28.13 and 56.01 mg/ml, for flowers and leaves/roots, respectively) [Table 2]. The brain is particularly susceptible to free radical damage because of its high consumption of oxygen and its relatively low concentration of anti-oxidants enzymes and free radicals scavengers. Several studies have focused in the use of natural therapeutic anti-oxidant compounds that can afford protection in a variety of *in vitro* and *in vivo* models of human pathologies, including neurotoxicity models.[11,12] Phenolics acids, such as: caffeic acid and chlorogenic acid and flavonoid rutin are known to have anti-oxidative action *in vitro* and *in vivo*,[7,11] the presence of these substances in the *V. tricolor,*[3,4] can explain the high anti-oxidant effect of ethyl acetate fraction of the plant. The IC_{50} values of quercetin, gallic acid and rutin against the pro-oxidant Fe (II) were 1.4 μg/ml, 16.3 μg/ml and 25.8 μg/ml, respectively. [7] They can act directly by entering the redox reactions, and indirectly by chelation of Fe (II).

Fang *et al*,[5] developed an HPLC method to assay wine flavonoids in a single run using mobile phase gradient elution (acetonitrile: methanol: water, mixture 1% tetrahydrofuran), Merck LiChrospher 100RP-18e column, detection wavelength at 360 nm with a retention time for rutin of 9.8 min. In our study, this method was adapted to obtain an isocratic reversed-phase HPLC system in order to reduce the experimental time, using rutin as an external standard. Calibration curves were obtained by injecting standard solutions of rutin in the chromatographic system. The mathematical expressions of the calibration curves were determined. In order to establish the linear ranges the correlation coefficients (*r*).

Identification of rutin was achieved by comparing the retention times (sample × reference standard), as well as by the addition of standard rutin to the samples. Rutin determination according to our conditions (described in methodology) gave a retention time of about 2.7 min [Figure 3], a regression linear equation $y = 26621x - 37744$ and a good correlation coefficient ($r = 0.9958$). The modifications in the method allowed an improvement in chromatographic conditions reducing the run time, as well as the volume of solvent residues. Butanolic and ethyl acetate fractions of *V. tricolor* flower presented 143.57 ± 8.48 and 33.70 ± 0.81 mg of rutin/g dry fraction, respectively [Table 3]. The content of rutin may contribute, in part, to the antioxidant capacity described, although the different fractions present the same profile of anti-oxidant activity, rutin concentrations varied in the fractions, suggesting that it is not exclusively responsible for this activity.[7]

In addition, due to the high concentration of rutin in the herb, it may be employed to prevent bruising and broken capillaries, to check the buildup of fluid in the tissues and to help to reduce blood pressure.[19]

Table 3 Rutin concentration in *Viola tricolor* flowers fractions

Fraction	Area (± SD)	Concentration (mg/g* ± SD)
Dichloromethane	8849 ± 682.95	ND
Ethyl acetate	707507 ± 17858.02	33.70 ± 0.81
Butanol	2218338 ± 15373.32	143.57± 8.48

*Concentration = mg of rutin/g dry fraction; SD = Standard deviation; ND = Not determined.

CONCLUSION

Our results suggest that *V. tricolor*, especially its flower fractions, are a promising source of natural anti-oxidants. These effects can be related mainly to their phenolic and flavonoids contents, especially the presence of rutin that was identified and quantified in the fractions of the flowers. However, more detailed *in vivo* studies are required to establish the safety and bioavailability of *V. tricolor*.

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