Quantitative and Qualitative Investigation of the Main Flavonoids in Heartsease (*Viola tricolor* L.)

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Abstract

Liquid chromatography coupled to electrospray ionization tandem mass spectrometry (MS^n) is used for the analysis of flavonoids in heartsease (*Viola tricolor* L.). Our data suggested that the two main flavonoid components were violanthin (6-*C*-glucosyl-8-*C*-rhamnosyl apigenin) and rutin (3-*O*-rutinosyl quercetin). The identification of rutin was confirmed by comparing its retention time, UV spectrum, molecular mass, and fragmentation pattern with the reference standard. In this paper, we also report on the quantitative analysis of rutin by high-performance liquid chromatography. According to our results, heartsease herb contained 420 ± 1.17 µg/g rutin.

Introduction

Heartsease, also known as wild pansy (*Viola tricolor* L., Violaceae), has a long history in phytomedicine. It has been utilized to treat various skin disorders, upper-respiratory problems, and also used as a diuretic (1). Although the use of heartsease as herbal medicine goes back centuries, the biological activity of its secondary metabolites has hardly been studied. Reports deal only with heartsease extract's cyclic peptides' cytotoxicity (2) and its antimicrobial activity (3). Our knowledge about its composition and chemical structure is not complete either. Papers from as early as the 1980's report on compounds such as carotenoids (4–7), antocyanidins (8), and flavonoids (1) being present in heartsease.

Flavonoids represent an important group of secondary plant metabolites. They contain a three-ring C_{15} flavone skeleton and show great structural variety because of the plethora of possible hydroxyl, metoxyl, and glycosyl substituents. The *O*-glycosidic flavonoids have sugar moieties bound to a hydroxyl group, whereas the *C*-glycoside flavonoids have sugar substituents bound to a carbon of the aglycone. The structural characterization of flavonoid glycosides is usually performed by a combination of spectroscopic methods, including UV, nuclear magnetic resonance (NMR), and mass spectrometry (9). In contrast to offline NMR techniques, which represents one of the most precise methods for chemical structure determination (including stereochemistry) of unknown components, other methods like liquid chromatography–mass spectrometry LC–MS do not require isolation of the target analyte, and thus offer a good choice for the analysis of crude plant extracts (10–12). The usual soft ionization techniques such as electrospray ionization (ESI) and fast atom bombardment (FAB) generate mainly protonated molecules. The molecular mass alone, however, is not sufficient for structural elucidation, therefore fragment information by collision-induced dissociation tandem mass spectrometry (CID-MS–MS) is necessary. A careful study of the fragmentation patterns obtained by CID-MS–MS can be of a particular value in the determination of the nature and site of attachment of important substitution groups such as sugars moieties (13–16).

In this paper, we report on the quantitative and qualitative investigation of the two major flavonoid components of heartsease extract, namely violanthin and rutin. These compounds were identified by regular LC–MS and nanoLC–MS, and rutin was quantitated by high-performance liquid chromatography (HPLC).

Materials and Methods

Chemicals and plant material

Chloroform, HPLC-grade acetic acid, acetonitrile, methanol, and water were purchased from Sigma-Aldrich (St. Louis, MO). Heartsease herb (*Viola tricolor* L.) (SN = 28-56-05-VI/24) was purchased from Fitopharma Ltd. (Budapest, Hungary).

Qualitative analysis of heartsease extract

Sample preparation

Dried and freshly powdered plant material (5.00 g) was sonicated two times with 50 mL chloroform for 25 min in an ultrasonic bath at 30°C. The plant residue was dried at room temperature and re-extracted two times with 40 mL methanol for 15 min in an ultrasonic bath at 30°C. The methanol extract

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was evaporated to dryness under reduced pressure at 55°C. Dry extract (5.3 mg) was re-dissolved in 5.3 mL mixture of 3.7 mL MeOH, and 1.6 mL 2.5% CH_3COOH and was purified by solid-phase extraction (SPE). The SPE cartridge (Supelclean LC-18, 500mg/6mL, Sigma-Aldrich) was activated with 5 mL MeOH, then with 5 mL 2.5% CH_3COOH. After introduction, the sample was washed with a mixture of 3.7 mL MeOH and 1.6 mL 2.5% CH_3COOH. The loading and washing solvents were collected in the same vial, and filtered on an Acrodisc Nylon 0.20-µm membrane Sartorius syringe filter (Sigma-Aldrich).

LC–MS and LC–MS^n conditions

For system 1, experiments were performed on an Agilent Technologies (Waldbronn, Germany) 1100 HPLC/MSD SL system which consisted of a binary pump, a degasser, an automatic injector, a diode array detector, a thermostat, and a mass selective detector. For the LC separation, gradient elution from 10% to 40% ACN in 30 min (A = 2.5 % CH_3COOH) was performed at a flow rate of 0.5 mL/min on a Hypersil ODS (250 × 4.6 mm, 5 µm) (Sigma-Aldrich) column. The eluate was monitored with both the diode array (at 340 nm) and the mass selective detector. Scanning was performed from *m/z* 100 to 1000 in 0.2 min intervals. The mass selective detector was equipped with a normal-flow electrospray ionization (ESI) source. The electrospray conditions were as follows: drying gas flow, 13 L/min; drying gas temperature, 350°C; nebulizer pressure, 35 psi; capillary voltage, 3000 V. The Chemstation software (Agilent Technologies) was used for data acquisition and evaluation.

For system 2, experiments were also performed on a Thermo Fisher LTQ (San Jose, CA) mass spectrometer, equipped with a nanoflow electrospray ionization source (Proxeon, Odense, Denmark). The sample was introduced directly to the ionization source. The electrospray conditions were as follows: spray potential, Ues = 2.02 kV; spray current, Ies = 0.67 µA; capillary temperature, 250°C; capillary voltage, 36.93 V. Scanning was performed from *m/z* 100 to 1000 in intervals. The Excalibur software (Thermo Fisher) was used for data acquisition and evaluation.

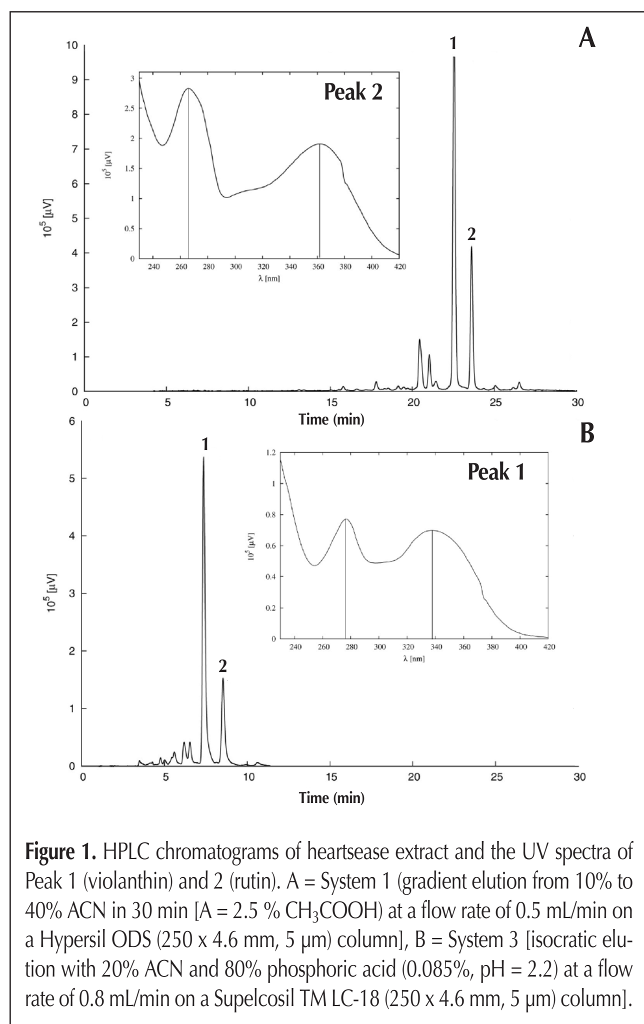

Figure 1. HPLC chromatograms of heartsease extract and the UV spectra of Peak 1 (violanthin) and 2 (rutin). A = System 1 (gradient elution from 10% to 40% ACN in 30 min [A = 2.5 % CH_3COOH) at a flow rate of 0.5 mL/min on a Hypersil ODS (250 x 4.6 mm, 5 µm) column], B = System 3 [isocratic elution with 20% ACN and 80% phosphoric acid (0.085%, pH = 2.2) at a flow rate of 0.8 mL/min on a Supelcosil TM LC-18 (250 x 4.6 mm, 5 µm) column].

Quantitative analysis of rutin

Sample preparation

Dried and freshly powdered heartsease plant material (1.25 g) was subject of continuous Soxhlet extraction (home-made Soxhlet apparatus) with 100 mL methanol for 9 h (exhaustive extraction). The extract was evaporated to dryness under reduced pressure at 55°C, weighed, and re-dissolved in 25 mL methanol (stock solution). Phosphoric acid (1.5 mL 0.085%) was added to 3.5 mL of the extract (diluted stock solution) and filtered on a MINIsart RC-15, 0.20-µm membrane syringe filter (Sigma-Aldrich). The filtered solution was ready to be analyzed.

HPLC conditions (system 3)

The ABLE-E & Jasco HPLC (Tokyo, Japan) apparatus consisted of an ERC-3113 degasser, an LG-980-02 solvent mixer, a PU-980 pump, and a 20 µL Rheodyne 7725 injector. The instrument was equipped with a UV-975 UV–vis detector. UV spectra were recorded during the HPLC separation by manually setting the recording time. A SUPELCOSIL TM LC-18 (250 × 4.6 mm, 5 µm, Sigma-Aldrich) column was applied during the experiments. Isocratic elution with 20% ACN and 80% phosphoric acid (0.085%, pH = 2.2) was performed at a flow rate of 0.8 mL/min. The eluate was monitored at 340 nm.

Fortified sample recovery test

1.2 mL of diluted stock solution (see the sample preparation section) was further diluted with 0.8 mL 70% methanol. This aliquot served as a blank solution in the fortified sample recovery test. Another 1.2 mL aliquot of the stock solution was diluted with 0.8 mL rutin solution (267 µg/mL, 70% MeOH), corresponding to the addition of 213.6 µg rutin. The recovery (*R*) was calculated as $R = 100(m_{found} - m_{initial})/m_{added}$; ($m_{found}$: rutin content in the fortified sample; $m_{initial}$: rutin content in the blank sample; m_{added}: added rutin amount).

Results and Discussion

Qualitative analysis of heartsease extract

The purified flavonoid fraction of heartsease methanol extract was first analyzed by normal-flow LC–MS (System 1). Good separation was obtained by applying gradient as elution shown in Figure 1A. Peaks A and B represent the two main flavonoid com-

ponents. By comparing retention time, UV spectrum, molecular mass and fragmentation pattern with the reference standard molecule (Table I), we suggest Peak B as rutin (Figure 2). The main component (Peak A) could not be identified under these particular MS conditions due to poor fragmentation; however, its molecular mass was determined as M = 578.2. Because the usual molecular mass of flavonoid aglycones ranges from 200 to 350, an apparent molecular mass larger than 500 indicates that Peak A is probably a conjugate.

As higher stage MS analysis provides insights about the chemical structure of the molecules of interest, Peak A was analyzed with MS–MS (System 2). Fragment ions with relative intensity higher than 1% are listed in Table II. For MS^2, *m/z* 579.14 was selected as precursor ion. The characteristic fragments might have derived from water losses and/or the cleavage of sugar units. This latter, together with the absence of Y_G^+,Y_R^+, and $Y_{R,G}^+$ (depicted in Figure 3, *m/z* 411.37, 433.37, and 271.24, respectively,) suggested that Peak A was not a di-*O*- but a di-*C*-glycosylflavonoid. In case of flavonoid-*C*-glycosides, the most usual connection site of the sugar unit is at position 6 or 8 (17) (Figure 2).

As Becchi et al. (15) described, the presence of $^{0,2}X^+$ and the absence of $^{0,3}X^+$ indicates that the component of interest is a di-*C*-hexosylflavonoid. Because in the MS–MS spectrum of Peak A $^{0,2}X^+_{hexose}$ (neutral loss = 120 Da) is a significant fragment, and $^{0,2}X^+_{pentose}$ (neutral loss = 90) is not detectable, we suggest that the component in Peak A is a di-*C*-hexosylflavonoid. As a first approximation, we can propose the presence of glucosyl and rhamnosyl moieties from the neutral losses of $[M+H–120]^+$ and $[M+H–104–H_2O]^+$, respectively. The early work of Li et al. (14) described how isomers whose structure differ only in the binding position of the sugar units (e.g., violanthin and isoviolanthin, Figure 2) can be differentiated with the help of characteristic fragment ions. Following their detailed analysis scheme of similar *C*-glycoside isomers the fragmentation of the 6-*C*-carbohydrate substituent generally results in a set of typical product ions: $^{0,4}X^+$-2H_2O, $^{0,2}X^+$, $^{1,5}X^+$, $^{0,1}X^+$, and Y^+. Similarly, the fragmentation of the 8-*C*-carbohydrate substituent gives rise to another group of characteristic product ions: $^{0,2}X^+$-H_2O, $^{0,2}X^+$-2H_2O, and $^{0,3}X^+$-3H_2O (Table III). In case of Peak A, the fragment ions distinctive to glucose and rhamnose are $^{0,4}X^+$-2H_2O, $^{0,4}X^+$-3H_2O, $^{0,2}X^+$, $^{1,5}X^+$-2H_2O, $^{0,1}X^+$, and $^{0,2}X^+$-H_2O, respectively. Based on the analogy in the fragmentation patterns, we consider that one glucose unit is connected at position 6, and one rhamnose unit is connected at position 8 to ring C, respectively.

As demonstrated, water losses are explained by the elimination of water molecules from the 2′′-hydroxyl groups of the sugar moieties and the 5- or 7-hydroxyl groups of the aglycone part (16). The high intensity of the E_1^+, E_2^+, and E_3^+ fragments, therefore, indicate the presence of hydroxyl groups either at position 5 or 7 or both. For further examinations we recorded the MS^3 spectra of *m/z* 337. This fragment ion bears special importance because it has lost both sugar moieties, and is therefore similar to flavonoid aglycones. Ma et al. (13) suggested three main fragmentation pathways for flavone and flavonol aglycones. According to their descriptions, the characteristic fragment ions for flavones and flavonols, which derive from the cleavage of ring C, are $^{1,3}B^+$, $^{0,2}B^+$, $^{0,4}B^+$, and $^{1,3}B^+$-2H, $^{0,2}B^+$, respectively (Figure 4). One can calculate from the molecular mass of $^{0,2}B^+$ that the presence of *m/z* 153, 137, 121, and 105 suggests that ring B has 3, 2, 1, or no hydroxyl substituents, respectively. Similarly, *m/z*

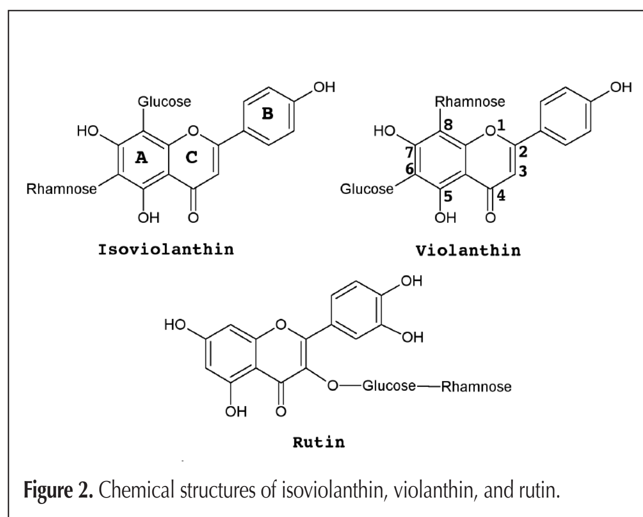

Figure 2. Chemical structures of isoviolanthin, violanthin, and rutin.

Table I. Fragment Ions of Peak A (Violanthin), Peak B (Rutin), and Rutin Reference Molecule (System 1)

Peak A *m/z* (RI* %)	Peak B *m/z* (RI %)	Rutin standard *m/z* (RI %)
581.2 (7)	633.2 (14)	633.2 (21)
580.2 (38)	–	613.2 (23)
579.2 (100)	–	612.2 (31)
449.0 (6)	611.2 (100)	611.2 (100)
121.0 (7)	593.4 (8)	593.4 (4)
–	465.2 (27)	465.2 (29)
–	–	316.4 (24)
–	303.0 (22)	303.0 (37)
–	–	288.2 (39)
–	–	202.2 (7)

* RI = relative intensity.

Table II. Fragment Ions Obtained by MS–MS analysis of *m/z* 579.14 (System 2)

Label	Structure	MS^2 579.14
E_1^+	$[M+H-H_2O]^+$	561.17 (100)
E_2^+	$[M+H-2H_2O]^+$	543.25 (29)
E_3^+	$[M+H-3H_2O]^+$	525.17 (12)
$^{2,3}X_{G,R}^+$-2H_2O	$[M+H-30-2H_2O]^+$	513.17 (28)
	$[M+H-2C_2H_2O]^+$	495.17 (12)
$^{0,4}X_G^+$-2H_2O	$[M+H-60-2H_2O]^+$	483.25 (11)
$^{0,4}X_G^+$-3H_2O	$[M+H-60-3H_2O]^+$	465.2 (5)
$^{0,2}X_G^+$	$[M+H-120]^+$	459.25 (14)
$^{0,2}X_R^+$-H_2O	$[M+H-104-H_2O]^+$	457.17 (24)
$^{0,2}X_G^+$-H_2O	$[M+H-120-H_2O]^+$	441.1 (4)
$^{0,1}X_G^+$	$[M+H-150]^+$	429.0 (1)
$^{1,5}X_G^+$-2H_2O	$[M+H-134-2H_2O]^+$	409.1 (1)

151, 135, 119, and 103 ($^{1,3}B^+$) indicates the presence of 3, 2, 1 and no hydroxyl substituents on ring B. The *m/z* 149, 133, and 117 ($^{1,3}B^+$-2H) demonstrates that ring B has 2, 1 or no hydroxyl substituents and that the molecule is a flavonol (3-hydroxyl substituent). In the MS^3 spectrum of *m/z* 337, the fragment ion of *m/z* 121 is significant (relative intensity = 14%), whereas the relative intensity of the fragment ion *m/z* 117 is lower than 1%. Accordingly, we propose that the component in Peak A has only one hydroxyl substituent on ring B and has no hydroxyl substituent on ring C at the position 3. As a conclusion, the component in Peak A is apparently violanthin (Figure 2).

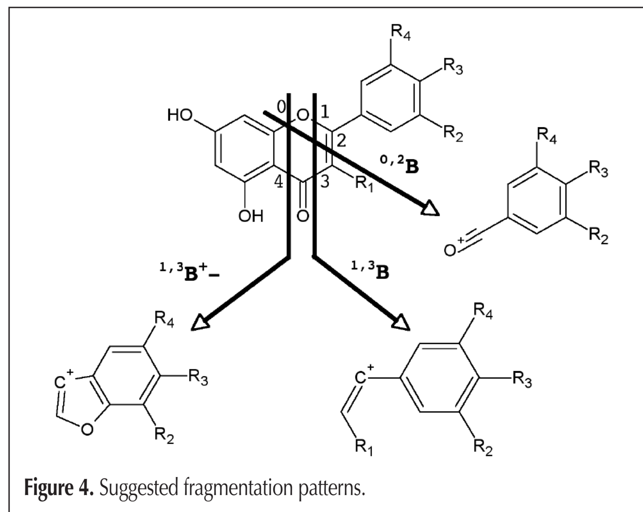

Figure 4. Suggested fragmentation patterns.

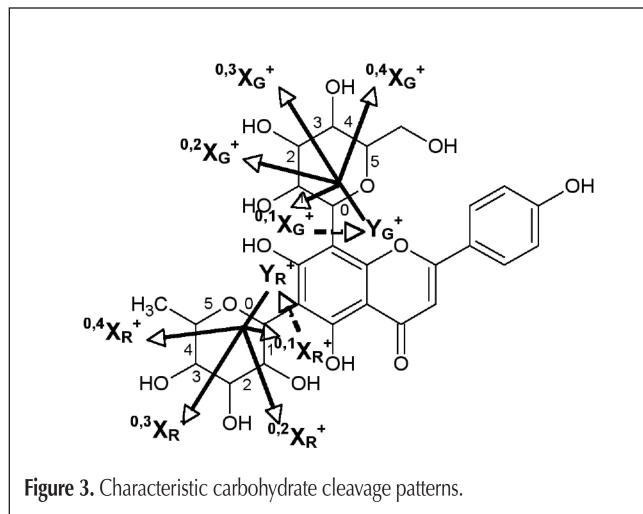

Figure 3. Characteristic carbohydrate cleavage patterns.

Quantitative analysis of rutin

For the quantitative analysis of rutin, an isocratic HPLC separation method was developed, referred to as System 3 (Figure 1B). This rapid method still featured proper resolution of Peaks A and B (R_s = 1.95). The validation process of the method is comprised of linearity determination, in addition to accuracy and precision measurements. Quantitative determination of rutin was carried out using the external standard calibration technique (18), that is, standard solution samples in a concentration range of 50–300 µg/mL were injected and measured. Good correlation [$y = (30378 \pm 251)x - 15422$, $R^2 = 0.9993$] was obtained between the sample concentration (x) and the uncorrected peak area (y). Accuracy was verified by the so called fortified sample recovery test (R = 99.36 ± 0.06 %, RSD = 0.07 %, n = 3) (19). Precision was tested by HPLC peak area reproducibility. Mean relative standard deviation values for the reference peak areas (n = 10) and the sample peak areas (n = 9) were 0.99% and 1.58%, respectively. According to our results, our heartsease herb sample contained 420 ± 1.17 µg/g rutin (RSD = 2.78 %, n = 6).

Conclusion

In this work, the two main flavonoid components of heartsease's crude methanol extract were analyzed by regular LC–MS and nanoLC–MS^n. Multistage mass spectral data analysis suggested one of the main components as violanthin. Identification of the second main flavonoid component as rutin was attempted by direct comparison of its retention time, UV, and multistage mass spectral data with a conventionally available standard. Our quantitative HPLC analysis revealed that the heartsease herb sample contained 420 ± 1.17 µg/g rutin.

Table III. Characteristic Carbohydrate Fragments (System 2)*

A* Fragments characteristic of:		B† Fragments characteristic of:	
6-*C*-carbohydrate substituent	**8-*C*-carbohydrate substituent**	**glucose**	**rhamnose**
$^{0,4}X^+$-2H_2O	–	$^{0,4}X^+$-2H_2O, $^{0,4}X^+$-3H_2O	–
$^{0,2}X^+$	–	$^{0,2}X^+$	–
$^{1,5}X^+$	–	$^{1,5}X^+$-2H_2O	–
$^{0,1}X^+$	–	$^{0,1}X^+$	–
Y^+	–	–	–
	$^{0,2}X^+$-H_2O	–	$^{0,2}X$+-H_2O
	$^{0,2}X$+-2H_2O	–	–
	$^{0,2}X$+-3H_2O	–	–

* A = described in the literature (14).
† B = obtained by the MS–MS analysis of *m/z* 579.14.

Acknowledgment

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