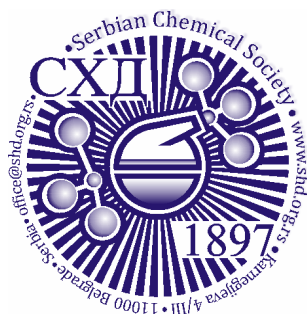




ACCEPTED MANUSCRIPT

This is an early electronic version of an as-received manuscript that has been accepted for publication in the Journal of the Serbian Chemical Society but has not yet been subjected to the editing process and publishing procedure applied by the JSCS Editorial Office.

Please cite this article as I. Davkova, Z. Zhivikj, A. Trajkovska Matevska, V. Stoilkovska Gjorgievska, I. Cvetkovikj Karanfilova, G. Stefkov, and M. Karapandzova, *J. Serb. Chem. Soc.* (2026) <https://doi.org/10.2298/JSC260422062D>

This “raw” version of the manuscript is being provided to the authors and readers for their technical service. It must be stressed that the manuscript still has to be subjected to copyediting, typesetting, English grammar and syntax corrections, professional editing and authors’ review of the galley proof before it is published in its final form. Please note that during these publishing processes, many errors may emerge which could affect the final content of the manuscript and all legal disclaimers applied according to the policies of the Journal.



J. Serb. Chem. Soc. **00(0)** 1-16 (2026)
JSCS-13925

Optimized and validated HPLC method for simultaneous quantification of rutin and verbascoside in multicomponent herbal mixture for wound healing

ISKRA DAVKOVA^{1*}, ZORAN ZHIVIKJ², ANA TRAJKOVSKA MATEVSKA¹,
VERONIKA STOILKOVSKA GJORGIEVSKA¹, IVANA CVETKOVIKJ KARANFILOVA¹,
GJOSHE STEFKOV¹, MARIJA KARAPANDZOVA¹

¹Institute of Pharmacognosy, Faculty of Pharmacy, Ss. Cyril and Methodius University in Skopje, Republic of North Macedonia, ²Institute of Applied Biochemistry, Faculty of Pharmacy, Ss. Cyril and Methodius University in Skopje, Republic of North Macedonia.

(Received 22 April; revised 5 July; accepted 19 October 2026)

Abstract: Rutin (a flavonoid glycoside) and verbascoside (a phenylethanoid glycoside) are bioactive phytochemical constituents widely recognized in traditional and modern phytotherapy for their antioxidant, anti-inflammatory and antibacterial activities. The presence of both rutin and verbascoside in a multicomponent herbal mixture (*Verbena officinalis* L., *Hypericum perforatum* L., *Achillea millefolium* L. and *Plantago major* L.) is expected to enhance its therapeutic efficacy in wound healing. In this study, an HPLC–DAD method was developed and optimized for the simultaneous determination of these two marker compounds within a single analytical run. The method was confirmed for selectivity, specificity, linearity, accuracy, precision, robustness, detection and quantification limits, as well as the stability of the standard mixture. Complete chromatographic resolution ($R_s > 1.5$) was achieved for both analytes. Excellent linearity ($R^2 \geq 0.999$) was achieved for both compounds. Accuracy was verified at 50 %, 100 %, and 150 % of the working concentration, while precision was demonstrated by relative standard deviation values below 2 %. Robustness testing showed consistent performance under minor variations in chromatographic conditions. Low detection and quantification limits were obtained for both analytes, and the standard mixture remained stable for up to 24 hours at room temperature. The validated method provides a rapid, reliable, and cost-effective tool for routine quality control and standardization of complex multicomponent herbal formulations.

Keywords: validation; flavonoids; phenylethanoids; herbal drugs.

* Corresponding author. E-mail: iskradavkova@ff.ukim.edu.mk
<https://doi.org/10.2298/JSC260422062D>

INTRODUCTION

Polyphenolic compounds are widely present in everyday life, being found in foods, beverages, and cosmetic products and are also highly valued in traditional and modern phytotherapy due to their broad spectrum of biological activities.

Among flavonoids, rutin is widely recognized for its broad biological potential and is frequently studied in various medicinal plant applications. On the other hand, verbascoside, a phenylethanoid compound, is emerging as a particularly interesting target for future research, with growing interest in its potential therapeutic applications.

Both rutin and verbascoside stimulate the antioxidant defense mechanism by increasing the production of antioxidant enzymes such as superoxide dismutase 1 (SOD1), catalase (CAT), and glutathione peroxidase (GPx), through activation of nuclear factor erythroid 2 related factor 2 (NRF2).¹⁻³ These compounds also contribute to the reduction of the inflammatory response in damaged tissue by decreasing pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF- α), IL-6, and IL-1 β , while increasing anti-inflammatory cytokines.^{1,3} Moreover, their antibacterial effect is attributed to their interaction with microbial cell membranes, modifying membrane permeability and leading to cell lysis.⁴

Based on literature reports, *Hypericum perforatum* L., Hypericaceae and *Achillea millefolium* L., Asteraceae extracts are rich sources of rutin.^{5,6} In contrast, *Verbena officinalis* L., Verbenaceae and *Plantago major* L., Plantaginaceae extracts are characterized by high levels of verbascoside.⁷⁻⁹ The presence of these two marker components in a multicomponent herbal mixture (MHM) is expected to enhance therapeutic efficacy, especially in wound healing, as both compounds exhibit complementary biological activities that may act synergistically.^{1,10,11} However, the first essential step in establishing any effective natural therapy is the accurate determination of the concentration of its active constituents. Therefore, there is a clear need to develop and optimize a reliable analytical method capable of simultaneously quantifying both rutin and verbascoside. Such a method would ensure proper standardization, quality control, and reproducibility of the herbal mixture, ultimately supporting its safe and effective use.

EXPERIMENTAL

Plant materials

Aerial parts of *Verbena officinalis* L., Verbenaceae (VO), *Hypericum perforatum* L., Hypericaceae (HP) and *Achillea millefolium* L., Asteraceae (AM), as well as leaves of *Plantago major* L., Plantaginaceae (PM), were used in this study. HP and AM were supplied by Alkaloid AD Skopje, while VO and PM were purchased from the herbal pharmacy.

Extractive procedures for the preparation of extracts from plant materials

Extraction of the plant raw materials was done from 0.5 g of dry and crushed herbal parts with 10 ml of 50 % ethanol EtOH (v/v). The obtained ethanol extracts were placed in an ultrasonic bath (frequency of 50/60 Hz and power of 720 W) for 15 minutes, and then filtered

through filter paper and supplemented with 50 % ethanol. To obtain dry extracts, extracts were evaporated on a rotavapor (BUCHI Labortechnik AG Flawil, Switzerland) with a vacuum controller (175 mbar) at 40 °C, and then lyophilized on a lyophilizer (Freeze Dryer Labconco, FreeZone5L Freeze Dry System, USA) at 40 °C with vacuum controller (175 mbar) (VUSNI Labortechnik AG Flawil, Switzerland). The masses of the obtained dry extracts corresponding to 0.5 g of the respective plant material were 0.50 g for VO, 0.54 g for PM, 0.49 g for HP and 0.51 g for AM.

Preparation of multicomponent herbal mixture (MHM)

After determining the marker's compound content of individual extracts obtained from VO, PM, HP and AM, 4 different potential multicomponent herbal mixtures (MHM) were prepared by combining previously lyophilized plant extracts. Four different MHM were prepared at a final concentration of 10 mg/mL in a ratio of 1:1:1. The composition of each MHM is given in Table I.

Table I. Composition of MHM

MHM	<i>Verbena officinalis</i>	<i>Plantago major</i>	<i>Hypericum perforatum</i>	<i>Achillea millefolium</i>
1	+	+	+	-
2	+	+	-	+
3	+	-	+	+
4	-	+	+	+

Standards and chemicals

The following standards and chemicals were used to carry out the planned tests: rutin (Sigma Aldrich, USA), verbascoside (Sigma Aldrich, USA), 85 % *o*-phosphoric acid (HPLC grade, Carlo Erba, Germany), acetonitrile (HPLC grade, Carlo Erba, Germany), 96 % ethanol (Alkaloid, Skopje, R. Macedonia), HPLC water (Faculty of Pharmacy, UKIM, Skopje).

Development and validation of a method for quantification of components of interest in herbal extracts/MHM

Validation of the method used in this study was performed in accordance with the recommendations of the International Council for Harmonization (ICH). For this purpose, the selectivity, specificity, linearity, accuracy, precision and robustness of the method were determined, as well as the detection limit, quantification limit and stability of the standard mix.

Specificity and selectivity

The specificity and selectivity of the method were determined by comparing the chromatograms obtained after application of solvent, individual standards of rutin and verbascoside, as well as their mixture. This was determined by analyzing the retention times and spectra of each standard both individually and in combination.

Linearity

The linearity of the analytical method was determined using the Least Squares method and a regression equation from the peak areas of rutin and verbascoside individually, in the concentration range of 0.02 mg/mL to 3 mg/mL.

Accuracy and precision

The accuracy and precision of the method were evaluated by spiking the standard mixture with known amounts of rutin and verbascoside at 50 %, 100 %, and 150 % of the working

standard concentration. The precision of the method was determined by 6 consecutive injections of a mixture of rutin and verbascoside applied on two different days.

Detection limit and quantification limit

Detection limit (DL) and the quantification limit (QL) of the analytical method were assessed by the residual standard deviation (σ) and the slope (S) of the regression equation.

Robustness

The robustness of the method was determined by 3 consecutive injections of a mixture of standards at a working concentration under varying chromatographic conditions: flow (± 0.2 mL/min), column temperature (-7 °C, $+2$ °C) and the concentration of o-phosphoric acid in the aqueous mobile phase (± 0.1 %).

Stability of standard mix

The stability of the standard mix at working concentration was established at room temperature, with the solutions being injected after 12 and 24 hours.

Quantification of active/marker components of interest (HPLC-DAD method) in herbal extracts/MHM

Quantification of the active/marker components of interest was performed using high-performance liquid chromatography with a diode array detector (HPLC-DAD). HPLC 1200 Series Agilent Technologies system, consisted of a G1322A degasser, G1311A quaternary pump, G1329A autosampler, G1316A column thermostat and DAD detector G 1315 D. The separation of components was done using an Agilent Zorbax Eclipse XDB-C18 (4.6 x 150 mm, 5 μ m, Agilent Technologies, USA) column. The injection volume was 10 μ L, the flow rate 1 mL/min and the temperature was 25 °C. Mobile phase A consisted of acetonitrile, while mobile phase B consisted of a 1% (v/v) aqueous solution of o-phosphoric acid. The phase ratio during elution of the components of interest is given in Table II. Identification of the components was made by comparing the retention time (Rt) and UV spectra on the components from the analyzed extracts, with Rt and spectra on standard substances, on wavelength of 360 nm (for rutin) and 330 nm (for verbascoside).

Table II. Gradient elution method using HPLC - DAD

Minutes	Mobile phase A [% v / v]	Mobile phase B [% v/v]
0-3	15	85
3-10	25	75
10-14	40	60
14-17	60	40
17-21	25	75
21-23	15	85

RESULTS AND DISCUSSION

Development and validation of a method for quantification of rutin and verbascoside in herbal extracts/MHM

Although numerous HPLC methods for individual determination of rutin or verbascoside have been reported, validated methods enabling their simultaneous quantification in complex MHM within a single chromatographic run remain

scarce. A C18 column was selected based on its widespread use, robustness, and easy accessibility across analytical laboratories, ensuring broad applicability of the proposed method. Acetonitrile, as one of the most commonly employed organic modifiers in reversed-phase HPLC, was initially chosen as the primary mobile phase. Methanol, another usually available solvent frequently reported in the literature, was first evaluated as a secondary mobile phase. However, it resulted in unsatisfactory chromatographic performance, including poor peak shape and resolution. Consequently, methanol was excluded, and a third mobile phase consisting of a 1 % *o*-phosphoric acid solution was introduced. The acidic aqueous phase was selected to improve peak symmetry and suppress ionization of phenolic groups, resulting in improved chromatographic performance without adverse effects on column stability under the applied conditions. During method optimization, an initial flow rate of 0.5 mL/min was applied. However, improved chromatographic performance was observed with increasing flow rate, so 1.0 mL/min was selected as the optimal condition. Robustness testing revealed that a reduction in flow rate did not significantly affect peak resolution, although a noticeable shift in retention times was observed, with peaks eluting approximately 4 minutes later. To further enhance method efficiency, the total analysis time was reduced from the initial 35 minutes to 25 minutes, thereby shortening a single chromatographic run without compromising chromatographic resolution. Method performance resulted in improved chromatographic behaviour, providing clear peak separation and satisfactory resolution. This optimized system enabled further method optimization and demonstrated suitability for the simultaneous determination of rutin and verbascoside.

The validation of the method confirmed its specificity, selectivity, linearity, accuracy, precision, and robustness. Additionally, the limit of detection, the limit of quantification, and the stability of the standard mix were determined.

Specificity and selectivity

Chromatograms obtained after applying the solvent, individual standard solutions of rutin and verbascoside, as well as their mixture, showed complete separation of the components of interest, indicating the specificity and selectivity of the developed method (Figure 1). The retention factor (k') was 2.06 and 2.20 for rutin and verbascoside, respectively, with a selectivity factor (α) of 1.07. The USP tailing factors were 1.12 for rutin and 1.09 for verbascoside, indicating good peak symmetry, while the column efficiencies (theoretical plates, N , half-height method) were 13054 and 12252, respectively.

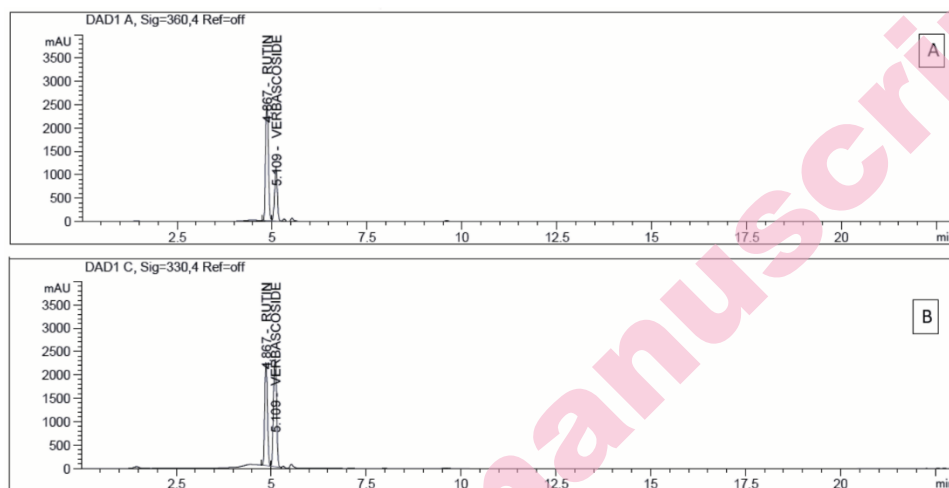


Figure 1. HPLC–DAD chromatograms of a standard mixture of rutin and verbascoside recorded at 360 nm (A) and 330 nm (B).

Representative chromatograms of the MHM(s) are provided to demonstrate that the compounds present in the mixtures do not interfere with the separation of the analytes (Figure 2 at 360 nm and Figure 3 at 330 nm). Peak purity assessment was performed using DAD spectral analysis of the standard mixture. The purity factors for rutin and verbascoside peaks were below the calculated threshold limits, confirming peak homogeneity and absence of co-eluting compounds. The purity factor/threshold values were 749.601/999.654 for rutin and 939.739/999.817 for verbascoside. The calculated resolution values between rutin and verbascoside were 1.62 at 330 nm and 1.77 at 360 nm, confirming satisfactory chromatographic separation at both detection wavelengths.

To further assess chromatographic selectivity, resolution was evaluated between the analytes and their closest-eluting neighboring peaks in all four MHMs at both detection wavelengths, in accordance with ICH Q2(R2) recommendations. The calculated values ranged from 1.08 to 2.76 (Table III). Satisfactory separation between rutin and verbascoside was observed in both the standard mixture and the investigated herbal mixtures, while some lower resolution values were associated with neighboring matrix components. Furthermore, satisfactory recovery results at three concentration levels (50%, 100%, and 150%) supported the accuracy of the method in the complex herbal mixtures.

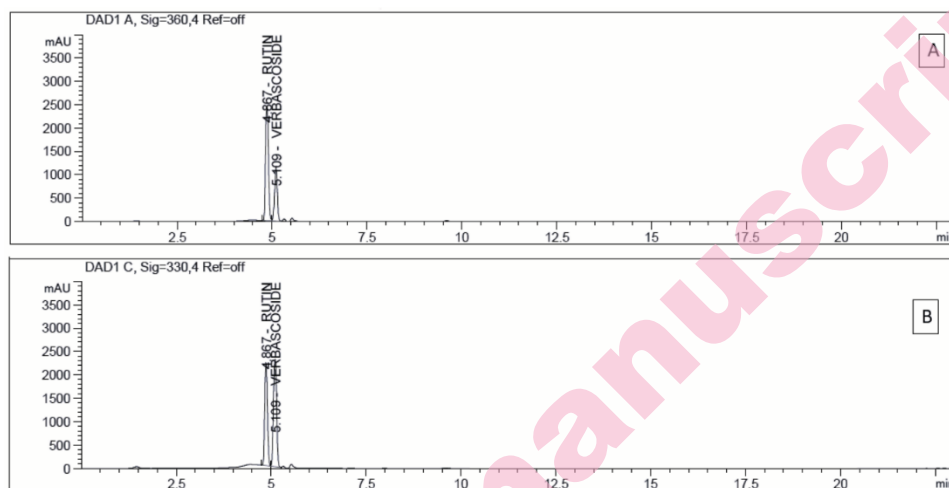


Figure 1. HPLC–DAD chromatograms of a standard mixture of rutin and verbascoside recorded at 360 nm (A) and 330 nm (B).

Representative chromatograms of the MHM(s) are provided to demonstrate that the compounds present in the mixtures do not interfere with the separation of the analytes (Figure 2 at 360 nm and Figure 3 at 330 nm). Peak purity assessment was performed using DAD spectral analysis of the standard mixture. The purity factors for rutin and verbascoside peaks were below the calculated threshold limits, confirming peak homogeneity and absence of co-eluting compounds. The purity factor/threshold values were 749.601/999.654 for rutin and 939.739/999.817 for verbascoside. The calculated resolution values between rutin and verbascoside were 1.62 at 330 nm and 1.77 at 360 nm, confirming satisfactory chromatographic separation at both detection wavelengths.

To further assess chromatographic selectivity, resolution was evaluated between the analytes and their closest-eluting neighboring peaks in all four MHMs at both detection wavelengths, in accordance with ICH Q2(R2) recommendations. The calculated values ranged from 1.08 to 2.76 (Table III). Satisfactory separation between rutin and verbascoside was observed in both the standard mixture and the investigated herbal mixtures, while some lower resolution values were associated with neighboring matrix components. Furthermore, satisfactory recovery results at three concentration levels (50%, 100%, and 150%) supported the accuracy of the method in the complex herbal mixtures.

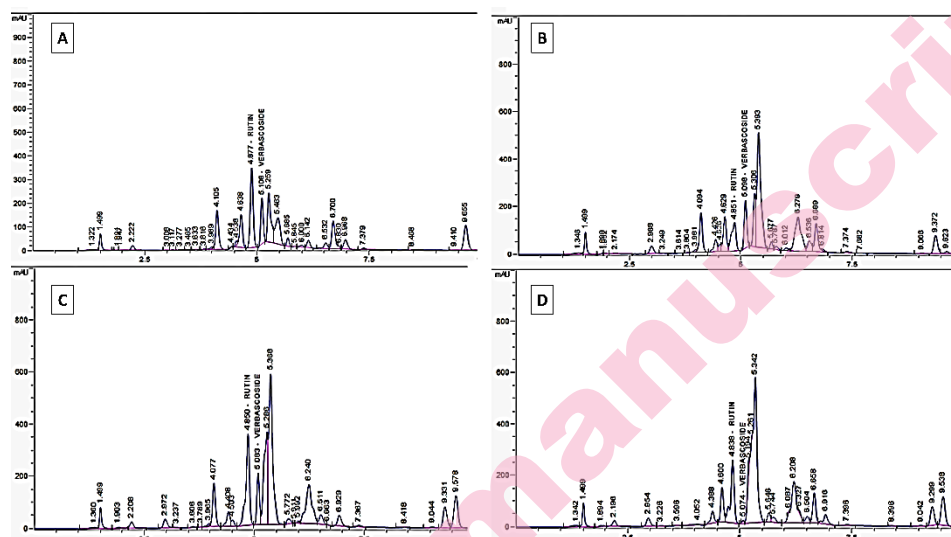


Figure 2. HPLC-DAD chromatograms of MHM1 (A), MHM2 (B), MHM3 (C), and MHM4 (D) recorded at 360 nm.

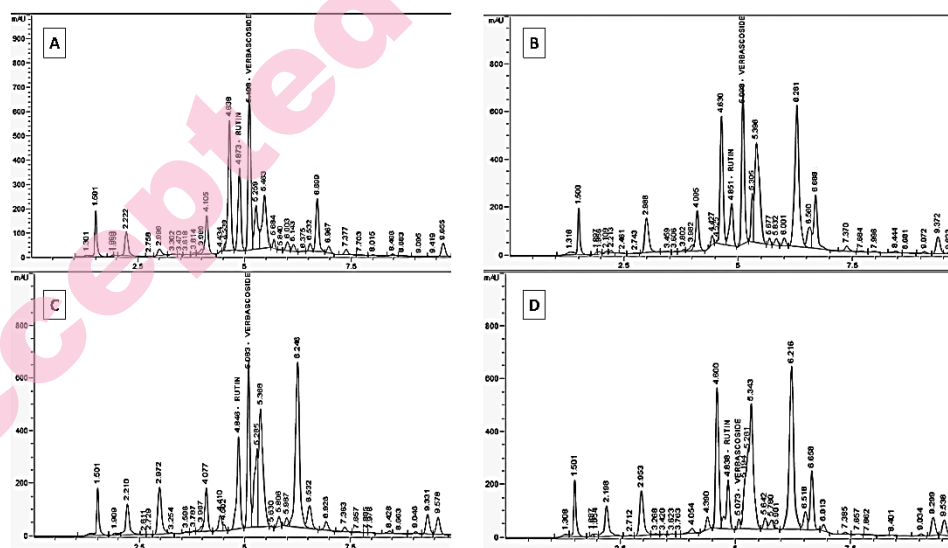


Figure 3. HPLC-DAD chromatograms of MHM1 (A), MHM2 (B), MHM3 (C), and MHM4 (D) recorded at 330 nm.

Table III. Chromatographic resolution (R_s) between rutin, verbascoside, and their closest-eluting neighboring peaks in the investigated multicomponent herbal mixtures.

Sample	Preceding peak– rutin (360 nm)	Rutin– verbascoside (360 nm)	Rutin– verbascoside (330 nm)	Verbascoside– following peak (330 nm)
MHM 1	1.92	1.91	1.86	1.08
MHM 2	1.29	1.48	1.84	1.79
MHM 3	2.76	1.86	1.85	1.29
MHM 4	1.96	2.34	1.96	1.32

Linearity

The linearity of the method was demonstrated by the correlation coefficient ($R^2 \geq 0.999$), which indicated a linear relationship between peak area and concentration of standard solutions within the concentration range of 0.02 mg/mL to 3 mg/mL for both components of interest (Figure 4).

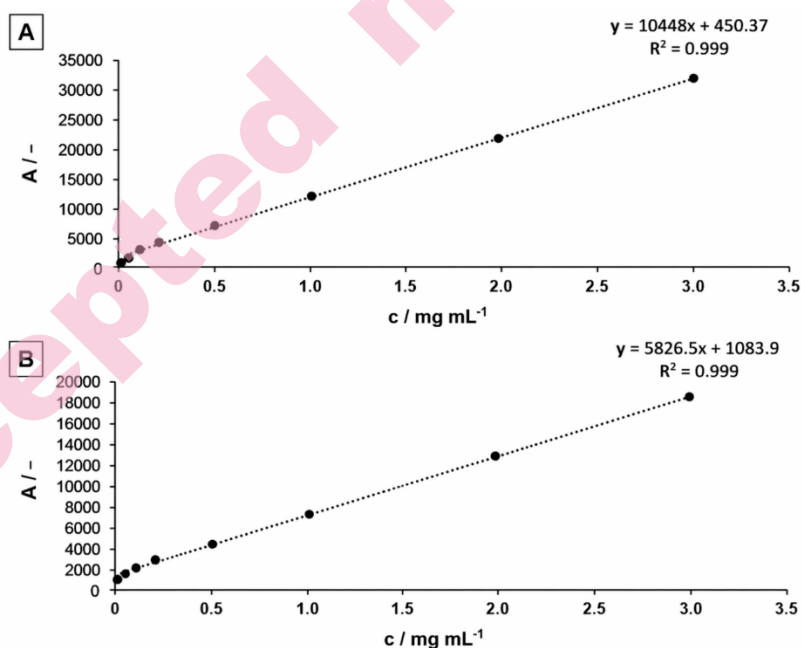


Figure 4. Calibration curves for (A) rutin and (B) verbascoside obtained by HPLC–DAD analysis.

Accuracy and precision

The obtained values for analytical yield ranged from 99.54 % to 100.58 % for rutin and from 98.25 % to 99.22 % for verbascoside, confirming the accuracy of the method within the range of 50 %, 100 %, and 150 % of the working

concentration. The precision of the method was confirmed by the relative standard deviation (RSD) value, which was less than 2 % (Table IV).

Table IV. Accuracy and precision of the method for rutin and verbascoside

Component	Level (%)	Recovery (%)	RSD (%)
Verbascoside	50	98.25	1.24
	100	99.66	0.58
	150	99.22	0.84
Rutin	50	99.54	1.65
	100	99.85	0.73
	150	100.58	1.74

Robustness

The robustness of the analytical method was tested by varying chromatographic conditions in terms of flow rate, column temperature, and salt concentration. The results obtained from these changes indicated that the method was robust under these conditions.

Detection limit and quantification limit

Detection and quantification limits were calculated using the relative standard deviation of rutin and verbascoside at the lowest concentration point and the slope from the regression equation obtained from the calibration curve for each standard individually. DL for verbascoside was 0.0011 mg/mL, while the QL was 0.0034 mg/mL. DL for rutin was 0.0008 mg/mL, while QL was 0.0024 mg/mL (Table V).

Table V. Detection and quantification limit for rutin and verbascoside

Component	Detection limit (DL)	Quantification limit (QL)
Verbascoside	0.0011 mg/mL	0.0034 mg/mL
Rutin	0.0008 mg/mL	0.0024 mg/mL

Stability of standard mix

The stability of the standard mix was tested at room temperature over multiple time intervals, and it was determined that the standard mix remained stable for up to 24 hours at room temperature.

Quantification of rutin and verbascoside in herbal extracts/MHM

Quantification of the components of interest was done based on the constructed calibration curves of the standard solutions of rutin and verbascoside, using the equation of the line $y = 10448x + 450.3$ and $y = 5826.5x + 1083.9$, respectively. The validated HPLC-DAD method showed that verbascoside was detected and quantified only in the VO and PM extracts, whereas rutin was detected and quantified only in the HP and AM extracts (Table VI). The values for the content of the active component (verbascoside and rutin) in MHM are given in

Table VII. Based on the obtained results, the selected medical plants represent a rich and suitable source of rutin and verbascoside.

Table VI. Content of rutin and verbascoside in individual plant extracts

Plant material	Verbascoside (mg g ⁻¹)	Rutin (mg g ⁻¹)
<i>Verbena officinalis</i>	122.2	-
<i>Plantago major</i>	129.2	-
<i>Hypericum perforatum</i>	-	27.7
<i>Achillea millefolium</i>	-	6.2

		Rutin (mg g ⁻¹)
1	76.4	11.2
2	73.4	4.1
3	29.4	14.9
4	24.8	6.6

The validation of this method confirmed its specificity, linearity, accuracy, precision, and robustness for the simultaneous quantification of both rutin and verbascoside in a single analytical run. Importantly, the confirmed robustness of the method ensures its easy reproducibility across various laboratory settings. The use of a C18 column, one of the most commonly used columns in analytical laboratories,¹²⁻¹⁷ provides optimal resolution without increasing backpressure or requiring UHPLC systems. In contrast to many methods that rely on a single detection wavelength,¹²⁻¹⁸ this approach employs dual-wavelength detection (360 nm for rutin and 330 nm for verbascoside), which enhances both selectivity and sensitivity for each compound. Moreover, the method uses ambient temperature conditions (25 °C), avoiding overheating or potential degradation of thermolabile compounds, offering an energy-efficient alternative to methods in the literature that operate at higher temperatures up to 40 °C.^{12,13,16} The selected injection volume of 10 µL, compared to the used volume injection in literature,¹¹ minimizes the risk of system overload or peak distortion, which is especially important when analyzing complex herbal samples. Additionally, while some published methods, particularly for verbascoside report run times of up to 60 minutes,¹⁶ this method maintains a balanced runtime of 23 minutes, avoiding unnecessary delays without compromising separation quality.

While most published analytical methods focus on the individual determination of either rutin or verbascoside, validated approaches enabling their simultaneous quantification in complex herbal matrices remain limited. The absence of a unified method represents a significant analytical challenge, particularly when dealing with complex, multicomponent herbal mixtures. In the present study, the developed HPLC–DAD method was successfully applied for the simultaneous quantification of rutin and verbascoside in multicomponent herbal

mixtures, demonstrating its practical applicability and suitability for the quantitative assessment of complex herbal formulations. This approach considerably simplifies the analytical workflow, reduces analysis time and solvent consumption, and enhances overall efficiency in routine analysis.

The quantification of verbascoside and rutin in the analyzed plant extracts revealed values that are generally consistent with literature data, with some notable differences. In VO herb, verbascoside was quantified at 122.2 mg/g, which is higher than the typical literature range of 1.5–4.5 mg/g for aerial parts.¹⁹ Similarly, PM leaves contained 129.2 mg/g of verbascoside, falling within the higher end of reported ranges (6–66 mg/g).^{20,21} Rutin was measured at 27.7 mg/g and 6.2 mg/g in HP herb and AM herb, respectively, which is also higher than previously reported values (0.2–1.5 mg/g for HP and 0.19–0.44 mg/g for AM).⁶ These differences may primarily be explained by the fact that the present study evaluated lyophilized extracts rather than untreated plant material, resulting in a concentration of bioactive constituents after removal of non-extractable plant components. In addition, variations in extraction procedures, solvent composition, plant origin, environmental conditions, and harvesting period may significantly influence the accumulation and recovery of phenolic compounds. The use of a validated HPLC-DAD method with compound-specific detection wavelengths may have further contributed to reliable quantification of verbascoside and rutin.²² Therefore, the higher values observed in this study likely reflect differences in sample preparation and analytical approach rather than a discrepancy with previously reported phytochemical profiles. It should also be noted that the reported concentrations in this study are expressed as mg/g of lyophilized extract, whereas many literature values refer to mg/g of raw plant material, making direct comparison between datasets less straightforward.

When the individual extracts were combined to prepare the multicomponent herbal mixture (MHM) at a 1:1:1 ratio, the concentrations of rutin and verbascoside were observed to be lower than in the respective single extracts (Figure 5).

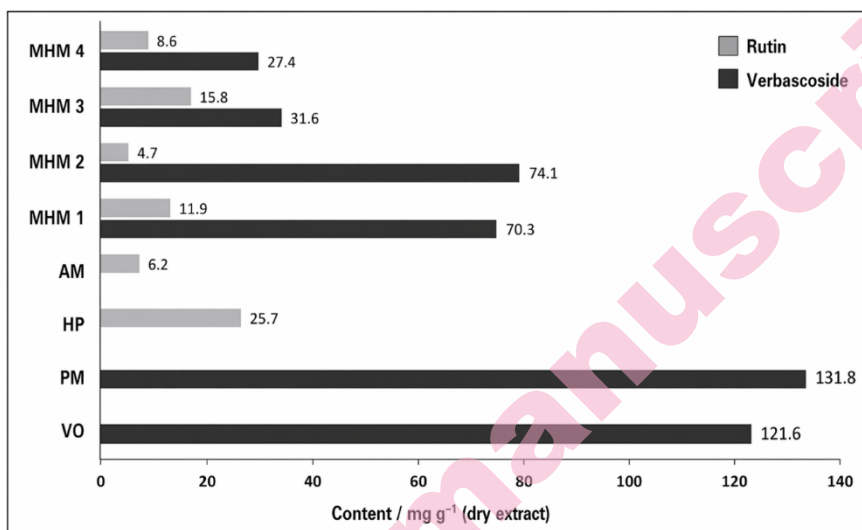


Figure 5. Content of rutin and verbascoside in individual plant extracts and multicomponent herbal mixtures (MHM), expressed as mg g⁻¹ of dry extract.

This can be explained primarily by the dilution effect resulting from combining the individual extracts, which contained different concentrations of the investigated compounds. Therefore, the concentration of a given compound in the final MHM depends on its contribution from each individual extract and its proportion in the mixture.^{23,24} Importantly, the lower quantified levels of rutin and verbascoside in the multicomponent mixtures do not indicate degradation or absence of these compounds. The reliability of the quantification was confirmed by the good chromatographic resolution of both analytes and by spiking/recovery experiments, which demonstrated that the matrix did not significantly affect their determination.

CONCLUSION

Rutin and verbascoside play an important role in the treatment of chronic wounds due to their antioxidant, anti-inflammatory and antibacterial properties. Accurate quantitative determination of these components in the following plant extracts: *Hypericum perforatum* L., *Achillea millefolium* L., *Verbena officinalis* L., *Plantago major* L. as well as in their multicomponent herbal mixtures (MHM) may provide valuable insights into their therapeutic potential, support evidence-based optimization of extract combinations, and contribute to the development of standardized and effective herbal formulations for wound healing. Unlike most published methods, which are typically designed for the individual determination either of rutin or verbascoside, the proposed method offers a cost-effective and time-efficient solution by eliminating the need for separate analyses, making it

particularly suitable for the analysis of complex herbal mixtures. Finally, the method relies on commonly available reagents and chromatographic conditions and does not require specialized additives, solvents, or more sophisticated equipment, making it accessible and practical for routine analysis in standard laboratory environments.

ИЗВОД

ОПТИМИЗОВАНА И ВАЛИДИРАНА HPLC МЕТОДА ЗА ИСТОВРЕМЕНУ КВАНТИФИКАЦИЈУ РУТИНА И ВЕРБАСКОЗИДА У ВИШЕКОМПОНЕНТНОЈ БИЉНОЈ МЕШАВИНИ ЗА ЗАРАСТАЊЕ РАНА

ISKRA DAVKOVA¹, ZORAN ZHIVIKJ², ANA TRAJKOVSKA MATEVSKA¹, VERONIKA STOILKOVSKA GJORGIEVSKA¹, IVANA CVETKOVIKJ KARANFILOVA¹, GJOSHE STEFKOV¹, MARIJA KARAPANDZOVA¹

¹Institute of Pharmacognosy, Faculty of Pharmacy, Ss. Cyril and Methodius University in Skopje, Republic of North Macedonia, ²Institute of Applied Biochemistry, Faculty of Pharmacy, Ss. Cyril and Methodius University in Skopje, Republic of North Macedonia.

Рутин (флавоноидни гликозид) и вербаскозид (фенилетаноидни гликозид) су биоактивни фитохемијски састојци широко прихваћени у традиционалној и модерној фитотерапији због својих антиоксидативних, антиинфламаторних и антибактеријских дејстава. Очекује се да ће присуство рутина и вербаскозида у вишекомпонентној биљној мешавини (*Verbena officinalis* L., *Hypericum perforatum* L., *Achillea millefolium* L. и *Plantago major* L.) побољшати њену терапеутску ефикасност у зарастању рана. У овој студији, развијена је и оптимизирана HPLC–DAD метода за истовремено одређивање ова два једињења маркера у оквиру једног аналитичког циклуса. Метода је потврђена у погледу селективности, специфичности, линеарности, тачности, прецизности, робустности, границе детекције и квантификације, као и стабилности стандардне смеше. Потпуна хроматографска резолуција ($R_s > 1,5$) постигнута је за оба анализита, као и одлична линеарност ($R^2 \geq 0,999$). Тачност је верификована на 50%, 100% и 150% радне концентрације, док је прецизност демонстрирана вредностима релативне стандардне девијације испод 2%. Тестирање робустности показало је конзистентне перформансе под мањим варијацијама хроматографских услова. Ниске границе детекције и квантификације добијене су за оба анализита, а стандардна смеша је остала стабилна до 24 сата на собној температури. Валидирана метода пружа брз, поуздан и ефективан алат за рутинску контролу квалитета и стандардизацију сложених вишекомпонентних биљних формулација.

(Примљено 22. априла; ревидирано 5. јула; прихваћено 19. октобра 2026.)

REFERENCES

1. F. Irfan, F. Jameel, I. Khan, R. Aslam, S. Faizi, A. Salim, *Regener. Ther.* **21** (2022) 225–238 (<https://dx.doi.org/10.1016/j.reth.2022.07.011>)
2. L. Y. Chen, C. N. Huang, C. K. Liao, H. M. Chang, Y. H. Kuan, T. J. Tseng, K. J. Yen, K. L. Yang, H. C. Lin, *Antioxidants* **9** (2020) 1122 (<https://dx.doi.org/10.3390/antiox9111122>)
3. N. D. De Moura Sperotto, L. Steffens, R. M. Veríssimo, J. G. Henn, V. F. Péres, P. Vianna, J. A. B. Chies, A. Roehle, J. Saffi, D. J. Moura, *J. Ethnopharmacol.* **225** (2018) 178–188 (<https://dx.doi.org/10.1016/j.jep.2018.07.012>)

4. T. P. T. Cushnie, A. J. Lamb, *Int. J. Antimicrob. Agents* **26** (2005) 343–356 (<https://doi.org/10.1016/j.ijantimicag.2005.09.002>)
5. S. Samadi, T. Khadivzadeh, A. Emami, N. S. Moosavi, M. Tafaghodi, H. R. Behnam, *J. Altern. Complement. Med.* **16** (2010) 113–117 (<https://dx.doi.org/10.1089/acm.2009.0317>)
6. S. Vitalini, G. Beretta, M. Iriti, S. Orsenigo, N. Basilico, S. Dall'Acqua, M. Iorizzi, G. Fico, *Acta Biochim. Pol.* **58** (2011) 203–209 (<https://hdl.handle.net/2434/161403>)
7. M. Deepak, S. S. Handa, *Phytother. Res.* **14** (2000) 463–465 ([https://doi.org/10.1002/1099-1573\(200009\)14:6%3C463::aid-ptr611%3E3.0.co;2-g](https://doi.org/10.1002/1099-1573(200009)14:6%3C463::aid-ptr611%3E3.0.co;2-g))
8. A. R. Bilia, M. Giomi, M. Innocenti, S. Gallori, F. F. Vincieri, *J. Pharm. Biomed. Anal.* **46** (2008) 463–470 (<https://dx.doi.org/10.1016/j.jpba.2007.11.007>)
9. S. Jarić, O. Kostić, Z. Mataruga, D. Pavlović, M. Pavlović, M. Mitrović, P. Pavlović, *J. Ethnopharmacol.* **211** (2018) 311–328 (<https://dx.doi.org/10.1016/j.jep.2017.09.018>)
10. L. Y. Chen, C. N. Huang, C. K. Liao, H. M. Chang, Y. H. Kuan, T. J. Tseng, K. J. Yen, K. L. Yang, H. C. Lin, *Antioxidants* **9** (2020) 1122 (<https://dx.doi.org/10.3390/antiox9111122>)
11. N. D. De Moura Sperotto, L. Steffens, R. M. Veríssimo, J. G. Henn, V. F. Péres, P. Vianna, J. A. B. Chies, A. Roehle, J. Saffi, D. J. Moura, *J. Ethnopharmacol.* **225** (2018) 178–188 (<https://dx.doi.org/10.1016/j.jep.2018.07.012>)
12. P. Sampaio, R. Grasielly, P. Sá, N. Souza, J. Alencar Filho, J. Pedro, R. Jackson, L. Rolim, *Afr. J. Biotechnol.* **17** (2018) 1048–1056 (<https://dx.doi.org/10.5897/AJB2018.16588>)
13. A. O. J. Alvarenga, S. Duarte Ozelin, L. Fernandes Magalhães, A. C. B. B. Candido, V. M. M. Gimenez, M. L. A. Silva, W. R. Cunha, A. H. Januário, D. C. Tavares, L. G. Magalhães, P. M. Pauletti, *Front. Plant Sci.* **14** (2023) 1324680 (<https://dx.doi.org/10.3389/fpls.2023.1324680>)
14. R. Fu, *Agilent Technologies* (2011) (<https://www.agilent.com/cs/library/applications/5990-9633EN.pdf>)
15. L. Fernández, J. de Santos, A. M. Díaz-Lanza, E. Pérez, L. Villaescusa, *Pharm. Biol.* **43** (2005) 226–229 (<https://dx.doi.org/10.1080/13880200590928799>)
16. D. Šatinský, K. Jägerová, L. Havlíková, P. Solich, *Food Anal. Methods* **6** (2013) 1353–1360 (<https://dx.doi.org/10.1007/s12161-012-9551-y>)
17. Q. Liu, W. Cai, X. Shao, *Talanta* **77** (2008) 679–683 (<https://dx.doi.org/10.1016/j.talanta.2008.07.011>)
18. N. Vachirapatama, B. Chamna, *Sci. Technol. Asia* (2012) 27–33 (<https://ph02.tci-thaijo.org/index.php/SciTechAsia/article/view/41173>)
19. P. Kubica, A. Szopa, A. Kokotkiewicz, N. Miceli, M. F. Taviano, A. Maugeri, S. Cirmi, A. Synowiec, M. Gniewosz, H. O. Elansary, E. A. Mahmoud, D. O. El-Ansary, O. Nasif, M. Luczkiewicz, H. Ekiert, *Molecules* **25** (2020) 5609 (<https://dx.doi.org/10.3390/molecules25235609>)
20. T. Janković, G. Zdunić, I. Beara, K. Balog, D. Pljevljakušić, D. Stešević, K. Šavikin, *Biochem. Syst. Ecol.* **42** (2012) 69–74 (<https://dx.doi.org/10.1016/j.bse.2012.02.013>)
21. N. Öztürk, M. Tunçel, İ. Potoğlu-Erkara, *Pharm. Biol.* **47** (2009) 120–127 (<https://dx.doi.org/10.1080/13880200802437073>)
22. S. Kulevanova, G. Stefkov, M. Karapandzova, I. Cvetkovikj Karanfilova, *Farmakognozija* (2023) 38–49 (<https://ukim.edu.mk/e-book/00068/>)

23. A. Ćirić, H. Prosen, M. Jelikić-Stankov, P. Đurđević, *Talanta* **99** (2012) 780–790 (<https://dx.doi.org/10.1016/j.talanta.2012.07.025>)
24. G. P. Domínguez Moré, M. I. Cardona, P. M. Sepúlveda, S. M. Echeverry, C. M. Oliveira Simões, D. M. Aragón, *Pharmaceutics* **13** (2021) 535 (<https://dx.doi.org/10.3390/pharmaceutics13040535>).