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A NEW HPLC METHOD APPROACH FOR THE QUANTIFICATION OF QUERCETIN IN SEVEN DIFFERENT ANATOLIAN PLANT EXTRACTS

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ABSTRACT. The use of natural antioxidants, especially phenolic compounds derived from foods and plants, has been extensively investigated in the context of preventive and therapeutic medicine. Quercetin, a flavonoid of the flavonol class, exhibits multiple health-promoting properties, including antioxidant, antimicrobial, and antifungal activities. The present study aimed to quantify quercetin in seven plant species native to Türkiye: Zosima absinthifolia, *Anarrhinum orientale, Fumaria asepala*, *Ferulago stellata, Salvia pseudeuphratica, Rhabdosciadium microcalycinum*, and *Diplotaenia cachrydifolia*. A novel reversed-phase HPLC-DAD method was developed for the determination of quercetin in these plant extracts and subsequently validated in accordance with ICH guidelines. The method demonstrated linearity over the concentration range of 0.4–1.2 µg/mL ($r^2 > 0.999$), with mean recovery values ranging from 96.5% to 98.3%. All validation parameters were evaluated and confirmed in accordance with ICH guidelines, demonstrating the method's reliability for quantifying quercetin in plant matrices.

Keywords: *Zosima absinthifolia, Anarrhinum orientale, Fumaria asepala, Ferulago stellata, Salvia pseudeuphratica, Rhabdosciadium microcalycinum, Diplotaenia cachrydifolia, Quercetin, HPLC, Validation*

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INTRODUCTION

Flavonoids are low molecular mass plant secondary metabolites that can serve as plant growth promoters or inhibitors by mediating plant-microbe interactions. Various external variables also influence the formation of these metabolites. Developmental factors alter the initiation and differentiation of plant parts responsible for secondary metabolite synthesis and quantity. Many factors also play a role in altering the biosynthesis of these metabolites (such as region, height, soil structure, climate, and growing conditions) [1, 2].

There are differences in the chemical structure of flavonoids depending on the degree of hydroxylation and polymerization, as well as structural classes, other conjugations, and substitutions. Flavonoids can be classified into many subclasses comprising flavonols (e.g., quercetin and kaempferol); flavonols constitute a subclass of flavonoids characterized by the presence of a ketone functional group [3], flavones (e.g., apigenin and flavones), isoflavonoids, flavanones (e.g., flavanone and hesperetin), catechins, anthocyanidins, and isoflavones. There are various studies and publications on the effectiveness of flavonoids against free radicals and their use in treatment. Flavonoids, characterized by their structural diversity and widespread occurrence in plants, confer numerous health benefits, including potent antioxidant, anti-inflammatory, antibacterial, anti-ageing, and metabolic disease–modulating properties. Emerging evidence also highlights their potential in preventing cardiovascular disorders. Diets rich in flavonoids have been linked to improved endothelial function, lower blood pressure, and a reduced risk of atherosclerosis. Specific flavonoids, such as quercetin and catechins, have been shown to regulate nitric oxide production, thereby promoting vasodilation and enhancing blood flow, which are crucial for maintaining cardiovascular health. Beyond their cardiovascular effects, flavonoids may exert neuroprotective actions by alleviating oxidative stress and inflammation, key mechanisms involved in the development of neurodegenerative conditions, including Alzheimer's and Parkinson's diseases [4-7].

Quercetin, a plant pigment, is classified as a plant secondary metabolite of flavonoids and grouped under flavonols. It is widely present in tea and onions. Additionally, its antioxidant properties were well known. Quercetin exhibits a broad spectrum of potential health-promoting effects in humans. Clinical investigations have been carried out in specific contexts, such as its role in lowering blood pressure [8]. A substantial body of in vitro and in vivo animal research has explored its antioxidant capacity [8].

Furthermore, both experimental models and cell-based studies have demonstrated its antiallergic and anti-asthmatic activities. In addition, extensive in vitro and in vivo evidence supports the anticancer properties of quercetin [9]. In addition to its antioxidant properties, it also provides numerous

medical benefits, including anticancer, antiviral, anti-diabetic, anti-inflammatory, and antitumor effects. Quercetin is a yellow, crystalline powder; despite its general insolubility, it is slightly soluble in alcohol, for instance, ethanol [10-12].

In this study, *Zosima absinthifolia, Anarrhinum orientale, Fumaria asepala, Ferulago stellata, Salvia pseudeuphratica, Rhabdosciadium microcalycinum*, and *Diplotaenia cachrydifolia* were studied for the determination of quercetin content. All plants were collected from different areas of Türkiye. Previous studies on *Zosima absinthifolia* and *Salvia pseudeuphratica* have demonstrated remarkable antioxidant and anticholinesterase activities [13, 14]. *Anarrhinum orientale* is recognized as a medicinal plant and exhibits antimicrobial properties [15]. Previous studies have shown that Fumaria asepala has antifungal and antibacterial properties [16]. *Diplotaenia cachrydifolia* and *Rhabdosciadium microcalycinum* are endemic species [14] and are known as medicinal plants [12].

In light of the significant health-promoting properties of quercetin, the present study aimed to quantify its content in seven plant species and to assess those with the highest levels for potential applications in dietary supplements or as sources of bioactive compounds.

To date, no studies have quantified quercetin in these plant species, highlighting a critical gap in the literature and underscoring the need to evaluate their potential as sources of this bioactive compound.

A simple and selective chromatographic method was developed for the quantification of quercetin. Validation parameters for the developed method were also studied according to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) requirements for specificity, accuracy, linearity, and precision (ICH) [17, 18].

RESULTS AND DISCUSSION

Analytical procedure

Concentrations of quercetin in the plant extract samples were measured by using high-performance liquid chromatography with a diode array detector (HPLC-DAD) system. A new and simple chromatographic method was developed for the quantitative analysis of quercetin. Each solution was injected into the HPLC system in duplicate, and the mean peak area was calculated.

Validation studies

Specificity

There was no interfering peak at the intended separation in the analytical technique.

Linearity

The new chromatographic method was developed and applied for the quantification of quercetin in plant extract. The calibration curve was drawn using concentrations of 0.4, 0.6, 0.8, 1.0, 1.1, and 1.2 µg/mL ($r^2 > 0.999$), as shown in Table 1.

Table 1. Calibration curve parameters (n=6)

	Slope	**Intercept**	**r^2**	**Slope RSD**	**Intercept RSD**	**Residual sum of squares**
y= ax+b	19251.33	-2808.6	0.99914	0.848	0.751	4.0

RSD: Relative Standard Deviation

LOD and LOQ

The LOD and LOQ values were found to be 0.1 µg/mL and 0.4 µg/mL, respectively.

System suitability test

At least two of these criteria are required for the acceptability of system suitability for the developed method. The values obtained meet the accepted criteria of a tailing factor ≤1.5 and a theoretical number of plates >2000, and the system was found to be suitable for analyzing the targeted substance [19].

Accuracy and precision

The mean recovery values were found in the range of 96.5% and 98.3%. For precision studies, the intra-day and inter-day variations were carried out for 0.6, 1.0, and 1.2 µg/mL. The results of the method throughout the linear range, the highest RSD value for six replicate sample analyses, were 1.12% for intra-day precision studies and 1.28% for inter-day precision studies, which are less than 2.0% [17, 18]. The acceptability criteria specified that accuracy should be within 85–115% of the nominal values, and precision should demonstrate a relative standard deviation (RSD) of not more than 15% (**Table 2**).

Table 2. Inter-day and intra-day accuracy and precision results (n=6)

	Inter-day			**Intra-day**		
Added (µg/mL)	**0.6**	**1.0**	**1.2**	**0.6**	**1.0**	**1.2**
Found, $\bar{x}$ µg/mL)	0.59	0.97	1.17	0.59	0.97	1.19
Precision; RSD (%)	1.28	1.01	0.69	1.12	0.94	0.47
Recovery (%)	98.3	96.5	97.8	98.6	96.8	98.2

$\bar{x}$: Mean value, RSD: Relative Standard Deviation.

The peak of quercetin was well separated from the other interfering peaks, and good resolution was achieved. Figure 1 shows the chromatogram of a quercetin-free plant extract obtained from *Salvia pseudeuphratica*. Figure 2 also shows a plant extract (obtained from *Fumaria asepala)* chromatogram that contains 0.808 mg/g of quercetin. There was no interference around the quercetin peak. The retention times for the quercetin peak were found as 6.27±0.008 min (Mean±Standard Deviation). **Table 3** gives the results of quantified quercetin concentrations from the different plant extracts.

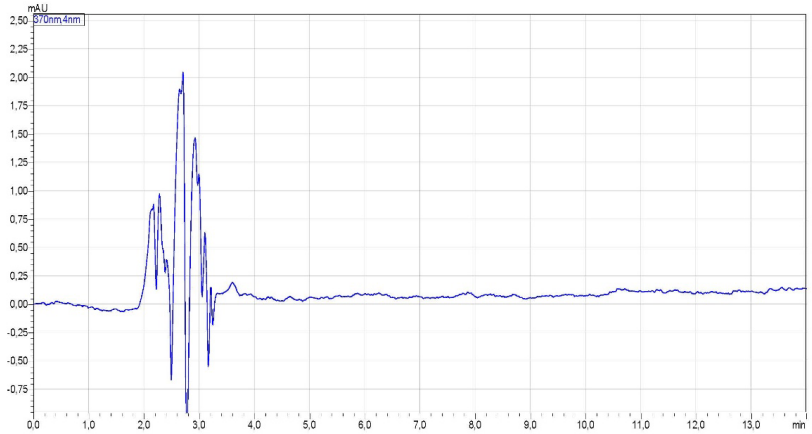

Figure 1. Chromatogram of the quercetin-free plant extract (*Salvia pseudeuphratica*)

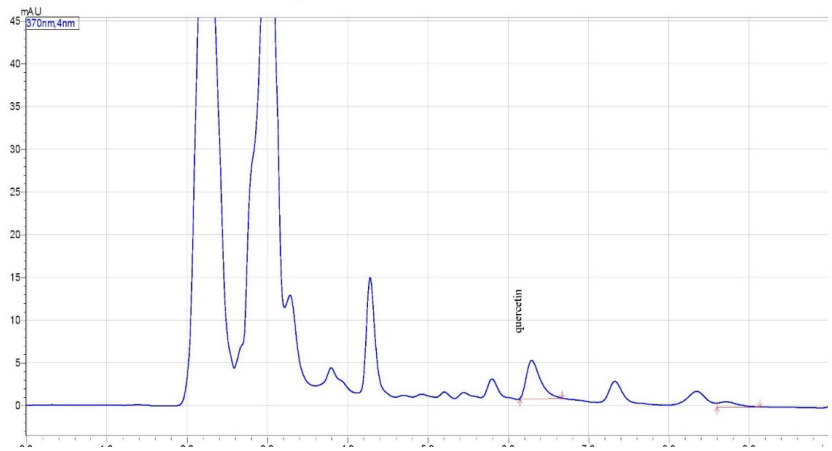

Figure 2. Chromatogram of plant extract (*Fumaria asepala)* containing 0.808 mg/g of quercetin

There are several methods for the determination of quercetin in plants. The developed method is superior according to the used mobile phases and short retention time. The chromatographic system used is an isocratic elution, and complex and gradient mobile phase mixtures were not used for the quantitation of quercetin. In the literature, studies have reported quercetin sensitivity at the mg/g level or as molarity. The developed method is more sensitive and selective compared to other methods, especially in terms of LOD and LOQ values (0.1 µg/mL and 0.4 µg/mL) [20-22]. For the plant extracts studied, quercetin content could not be determined in two of them. Among the analyzed samples, *Fumaria asepala* exhibited the highest quercetin concentration (0.807 mg/g extract), which was approximately 13 times greater than that of the most dilute sample (*Ferulago stellata*). The extracts of *Anarrhinum orientale* and *Rhabdosciadium microcalycinum* showed nearly identical quercetin concentrations (**Table 3**).

Table 3. Quercetin from the plant extract (n=6)

Plant	Calculated concentration (mg/g extract) ± SD
Zosima absinthifolia	0.197 ± 0.03
Anarrhinum orientale	0.118 ± 0.001
Fumaria asepala	0.808± 0.019
Ferulago stellata	0.063 ± 0.003
Salvia pseudeuphratica	Not determined
Rhabdosciadium microcalycinum	0.105 ± 0.002
Diplotaenia cachrydifolia	Not determined

SD: Standard Deviation

CONCLUSION

In the present study, a new, simple, accurate, and precise reversed-phase HPLC-DAD method was developed and validated for the determination of quercetin in accordance with guidelines of the International Conference on Harmonization (ICH). This method was applied easily for the quantification of quercetin in plant extracts. In this study, seven different plant extracts obtained from different areas of Türkiye were studied by using this novel chromatographic method. Studied plants have also not been analyzed for the quercetin content before. This study gives a new approach for the identification of these seven plants.

EXPERIMENTAL SECTION

Chemicals and reagents

Analytical standard of Quercetin was purchased from Merck, (Darmstadt, Germany). Acetonitrile (HPLC grade, CARLO ERBA, Italy), methanol (HPLC grade, CARLO ERBA, Italy), and NaH_2PO_4 (Sigma-Aldrich, USA) were purchased. Quercetin stock solution was prepared with ethanol at 1 mg/mL, and the solution to be used in the analysis was prepared daily by diluting the stock solutions with an ethanol: water (1:1, v/v) mixture to the desired concentration values. Stock solutions were stored at -20 °C for only 1 week.

Plant Materials

Murat Kürşat collected and identified every plant from various parts of Türkiye in 2020. In September, *Diplotaenia cachrydifolia Boiss*. was collected at a height of 2250 meters on Karz Mountain in Bitlis. In September, *Rhabdosciadium microcalycinum Hand.-Mazz.* and *Ferulago stellata Boiss*. were collected at a height of 1750 meters on Kambos Mountain in Bitlis, in August, *Anarrhinum orientale Benth*. was collected at a height of 1600 meters on Kambos Mountain in Bitlis. In May, *Zosima absinthifolia* (Vent.) Link and *Fumaria asepala* were collected from the Baskil region of Elazığ. The plant *Salvia pseudeuphratica Rech.f.* was collected from Keban, Elazığ, in July. Bitlis Eren University has voucher specimens for every plant.

Plant extraction

50 g of dried plant material was obtained from each plant. Coarsely ground part of each plant was extracted with methanol at room temperature. The extraction procedure was carried out four times for each plant, and all plants were extracted with 1 L of freshly added methanol for 12 hours using a magnetic stirrer. At the end of the extraction studies, four extracts of solvent content were pooled. The pooled methanol phase evaporated with a rotary evaporator, and the dry crude extracts were obtained. Extracts of *Zosima absinthifolia, Anarrhinum orientale, Fumaria asepala, Ferulago stellata, Salvia pseudeuphratica, Rhabdosciadium microcalycinum*, and *Diplotaenia cachrydifolia* were studied for the application of a new chromatographic method.

Extract analysis procedure

Samples were accurately weighed using an analytical balance (Ohaus, USA), approximately 0.05 g, into the 15 mL Falcon tube and dissolved

with 10 mL of HPLC-grade methanol. The mixtures were sonicated in an ultrasonic bath for 30 min. Then the mixtures were stirred for 30 min with a magnetic stirrer. The mixtures were diluted with HPLC-grade methanol ten times or not according to the sample content. After the dissolving procedure, solutions were filtered by a 0.22 µm PTFE syringe filter, and then 10 µL of an aliquot was injected into the HPLC system. This procedure was repeated twice for each sample.

Instrumentation and chromatographic conditions

The chromatographic system consisted of a Shimadzu liquid chromatograph system (LC-2030 2D Plus Prominence-I), 10 µL injection loop, an autosampler, a Diode Array Detector, and a system controller using CLASS-VP 5.0 (Shimadzu, Kyoto, Japan). The separation was achieved by XBridge, C18 (250 x 4.6 mm (I.D.), particle size 5 µm) analytical column (The column temperature was 20 °C) (Waters, Milford, MA, USA). The mobile phase consisted of 20 mM NaH_2PO_4 (pH=4.16) and acetonitrile (65:35; (v/v)) in isocratic mode, and the detector wavelength was set to 370 nm. A 10 µL aliquot of the sample solution was injected into the HPLC system at a flow rate of 1.1 mL/min. For preparing all solutions, type 1 water (Simplicity 185 Water System, Millipore Corp., Bedford, MA, USA) was used. The mobile phase was filtered through a membrane filter (pore diameter of 0.45 µm) and kept in an ultrasonic bath for 15 min to remove the soluble gases.

Validation studies

Specificity

Selectivity was tested after the injection of drug-free plant extract and the mobile phase injection.

Linearity

Linearity studies were conducted using standard solutions prepared by quantitative dilution of the stock solution. In this method, a calibration curve was constructed by plotting the concentration values against the peak area of quercetin. The slope, intercept, and correlation coefficient of the calibration curve (y= ax+b) were calculated.

LOD and LOQ

The limit of detection (LOD) was calculated as the signal-to-noise (S/N) ratio of 3:1 (n=6), and the limit of quantification (LOQ) value was calculated as the signal-to-noise (S/N) ratio of 10:1 (n=6) for standard solution analyses.

System suitability test

System suitability tests were performed using 1 µg/mL quercetin, and the results were evaluated based on retention time, injection repeatability, capacity factor, tailing factor, and the theoretical number of plates. The values obtained must meet the accepted criteria for good separations: capacity factor (k') values between 1 and 10, tailing factor≤1.5, and theoretical number of plates>2000.

Accuracy and Precision

For accuracy analyses, recovery values were calculated for six repeated analyses (for 1 µg/mL of quercetin) by using spiking of a drug-free plant extract sample. For the reproducibility of the method, three concentration values in the linear range were analyzed at six replicates. For accuracy and precision values, relative standard deviation (RSD) values were calculated and evaluated [17, 18].

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