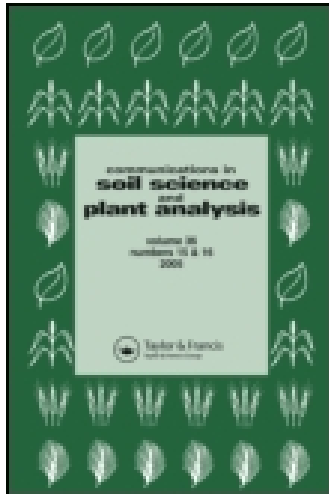


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Analysis of Quercetin and Kaempferol in an Alcoholic Extract of *Convolvulus pilosellifolius* using HPLC

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A simple, rapid, and sensitive high-performance liquid chromatography (HPLC) method was developed and validated for identification and determination of flavonoids in Convolvulus pilosellifolius. The chromatographic separation was achieved in less than 6 min using C18 column (150 × 4.6 mm, 3 μm) with isocratic mixture of methanol and water containing 0.1 percent v/v formic acid in the ration of 80:20 at 258 nm with a flow rate of 0.4 mL/min. The method was validated in the linear calibration curve ranged between 1 and 300 μg/mL with detection limits of 0.39 and 0.26 μg/mL and quantification limits of 1.20 and 0.79 μg/mL for quercetin and kaempferol, respectively. Good repeatability of the method were achieved at percent relative standard deviation (RSD < 2.18 percent) with respect to inter- and intraday repeatability. Recovery values were found to be in the range of 98.2–100.2 percent, indicating high accuracy of the method. The maximum flavonoid contents were 1.07 and 1.54 percent for quercetin and kaempferol, respectively.

Keywords Column liquid chromatography, *Convolvulus pilosellifolius*, flavonoids, plant extract

Introduction

The Convolvulaceae plant family comprises 85 genera and 2800 species. The largest genus of this family is *Convolvulus*; it comprises 250 species present as trees, shrubs, or herbs (Migahid 1989). Studies on the plants of this genus reported that they contain flavonoid, phenolic, tropane alkaloids, sterols, resin and sugars, and coumarins (Awaad et al. 2011; Nacef et al. 2010; Son et al. 1991; Sethiya and Mishra 2010). Plants of this genus possess different biological activities such as antibacterial, antiulcer, antimicrobial, antiasthmatic, anticancer, cytotoxic, and antioxidant activities (Nacef et al. 2010; Sethiya et al. 2010; Zain et al. 2012; Griffen and Lin 2000; Todd et al. 1995; Calvino 2002).

Convolvulus pilosellifolius Desr. is considered one of these genus plants. Although the biological activity as antiulcerogenic agent of this plant is still not scientifically proved,

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this plant is commonly used in folk medicine in Saudi Arabia for the treatment of gastric disorders (Migahid 1989).

The importance of many herbal drugs is coming from their flavonoids contents (Harborne 1986). Flavonoids have showed a lot of pharmacological activities including antimutagenic and anticarcinogenic (Closas et al. 1999), antioxidant properties (Jovanovic et al. 1996; Pekkarinen, Heinonen, and Hopia 1999), anti-microbial, spasmolytic, diuretic, capillary protection, and cytostatic activities (Bruneton 1995), and anti-inflammatory and antiallergic effects (Hollman, Hertog, and Katan 1996). Many of these activities are due to presence of quercetin (Harborne and Mabry 1982), followed by kaempferol and myricetin, which are widely spread in the plant kingdom (Tokusoğlu and Unal 2002).

High-performance liquid chromatography (HPLC) with spectrophotometric or electrochemical detection has been widely used for identification and quantification of flavonoids in plant extracts (Stewart et al. 2000; Annie Bligh, Ogegbo, and Wang 2013; Stefova, Stafilov, and Kulevanova 2003; Dailge and Conkerton 1988). Daigle and Conkerton first used reversed-phase high-performance liquid chromatography (HPLC) for analysis of flavonoids in 1988 (Khoddami, Wilkes, and Roberts 2013). Because of the presence of strong flavonoid chromophores, HPLC coupled with ultraviolet (UV) detection has become the most popular detection method used in flavonoid analysis (Stefova, Stafilov, and Kulevanova 2003; Daigle and Conkerton 1988; Khoddami, Wilkes, and Roberts 2013; Selvamani, Sen, and Gupta 2009).

Standardization and characterization of plant extract is a topic of continuous scientific interest in the plant drug industry. With the advent of modern chromatographic systems there is an ever-increasing intent to produce and develop easy, rapid, convenient, and cost-effective methods for standardization (Mahendra et al. 2011). For standardization of alcoholic extract of plants, HPLC is a sensitive and accurate tool that is widely used for the quality assessment of plant extract and its derived product/formulation (Awaad et al. 2013).

The present work describes a simultaneous method for identification and quantification of two relevant flavonoids compounds, quercetin and kaempferol (their chemical structures are shown in Figure 1), in an alcoholic extract of *Convolvulus pilosellifolius* plant using the HPLC method. The analytical method was validated in terms of linearity range, accuracy, precision, sensitivity, detection, and quantification limits.

Experimental

Plant Material and Chemicals

Fresh aerial parts of *Convolvulus pilosellifolius* Desr. was collected from the Alrawdā (Riyadh, Saudi Arabia) during summer 2012 and identified by our taxonomist in addition

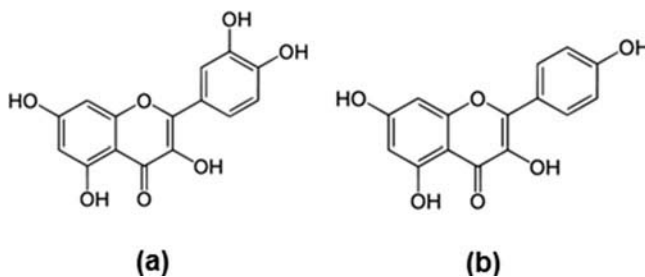


Figure 1. Structure of (a) quercetin and (b) kaempferol.

to comparison with the published data (Migahid 1989). A voucher specimen has been maintained in the herbarium of Chemistry Department. All other reagents were of HPLC grade or analytical grade as per requirement. The active compounds (quercetin and kaempferol) were purchased from Sigma-Aldrich.

Apparatus and Chromatographic Conditions

The HPLC analysis was performed on a Shimadzu UFLC system, equipped with LC 20A quaternary gradient pump using SPD-M20A PDA diode array detector (DAD). The data was acquired on the liquid chromatography solution administrator data system (Japan) using BETASIL C18 column (150 × 4.6 mm, 3 μm) (Thermo Scientific, USA) and an isocratic mixture of methanol and water containing 0.1 percent v/v formic acid in the ratio of (80:20) (Vipul et al. 2013). Always before used, the mobile phase was filtered through a 0.22-μm Millipore filter and degassed by sonication for 30 min. The flow rate was adjusted to 0.4 mL/min. Injection volume was adjusted to 5 μL and detection was made at wavelength 258 nm (Tripathi et al. 2012).

Extraction Procedure

First, 100 g of air-dried powder of *Convolvulus pilosellifolius* Desr. was extracted with methanol (500 mL × 3) in a Soxhlet apparatus until complete exhaustion (Awaad et al. 2013). The total ethanol extract was concentrated under vacuum at 35 °C to yield 5 g of the dry extract.

Preparation of Sample Solution

Then 20 mg of extracted sample was dissolved in 25 mL HPLC-grade methanol. The sample was sonicated for 10 min. After sonication, the volume was made up to 50 mL with HPLC-grade methanol and filtered through a 0.22-μm membrane filter.

Preparation of Standard Solution

Standard stock solutions of quercetin and kaempferol (10 mg) were prepared in 10 mL methanol. Working standard solutions from 1 to 300 μg/mL were freshly prepared before analysis of the samples using HPLC-grade methanol by appropriate dilutions of the stock solution. The HPLC-grade methanol was injected as the blank.

Method Validation

For establishing calibration curves, nine points ranged from 1 to 300 μg/mL of each flavonoid under study were prepared by dilution of the stock solution with HPLC-grade methanol. The solutions were always filtered using a 0.22-μm filter and injected in triplicates. Regression equations and coefficients of correlation (R^2) were derived as shown in Table 1.

The method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), and precision. Peak areas were plotted versus the respective concentrations of quercetin and kaempferol to study the method linearity. The LOD and LOQ for each detected analyte were calculated with corresponding standard solution on the basis of a signal-to-noise ratio (S/N) of 3 and 10, respectively.

Table 1

Validation parameters of the HPLC method quantification of quercetin and kaempferol and their contents in alcoholic extract of *Convolvulus pilosellifolius*

Validation parameter	Result	
	Quercetin	Kaempferol
Linear range ($\mu\text{g/mL}$)	1–300	1–300
Correlation coefficient (R^2)	0.9993	0.9995
Regression equation	$y = 60466 \times -168479$	$y = 52065 \times -44039$
LOD ($\mu\text{g/mL}$)	0.33	0.26
LOQ ($\mu\text{g/mL}$)	1.06	0.83
Intermediate precision (RSD %)		
Intraday (n = 3)	0.68	1.72
Interday (n = 3)	1.23	2.18
Recovery (%)		
Low level (10 $\mu\text{g/mL}$)	98.2	100.2
Medium level (100 $\mu\text{g/mL}$)	98.5	99.8
High level (200 $\mu\text{g/mL}$)	99.1	98.4
Content $\mu\text{g/mL}$ extract	4.27	6.14
Content (%)	1.07%	1.54%

To study the efficiency of the extraction at the optimal point, a recovery test was performed by spiking 3 mL of the preanalyzed extract with a known amount of standard solution at three concentration levels (10, 100, and 200 $\mu\text{g/mL}$). The average percentage of recovery was estimated by applying values of peak area to the regression equations of the calibration graph. Three replicate samples of each concentration level were prepared using the proposed method.

To utilize the repeatability and reproducibility of the developed method, intraday and interday precision levels of the method were determined by analyzing standard solution for each concentration on the same day three times and on three different days, respectively. The results are reported in terms of percent relative standard deviation (RSD).

Results and Discussion

To achieve better resolution in a short period for the two flavonoids, HPLC conditions were standardized after several trials with mobile phase proportions, flow rate, and detector wavelength. Under the optimized conditions, BETASIL C18 column and UV detector at 258 nm using isocratic mixture of methanol and water with 0.1 percent formic acid as mobile phase gave well-resolved symmetric peaks and high sensitivity for quercetin and kaempferol. Figure 2 shows that the total run time was found to be less than 6 min, while the retention times of both compounds were observed at 4.43 and 5.17 min for quercetin and kaempferol, respectively. This indicates that the present HPLC method is rapid, easy, and convenient.

The calibration curves were prepared by plotting the peak area against standard concentration as shown in Figure 3; it was found linear in the range of 1–300 $\mu\text{g/mL}$ for both quercetin and kaempferol. The regression equations were found as

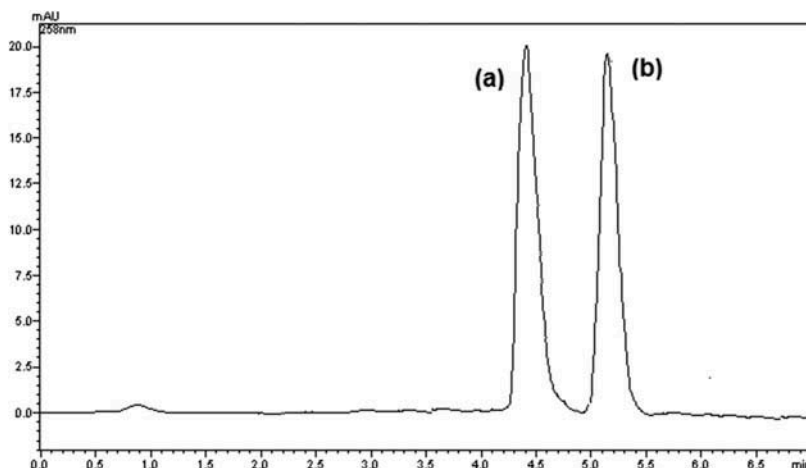


Figure 2. HPLC chromatogram of 10 $\mu\text{g/mL}$ standard flavonoids, where: (a) quercetin and (b) kaempferol.

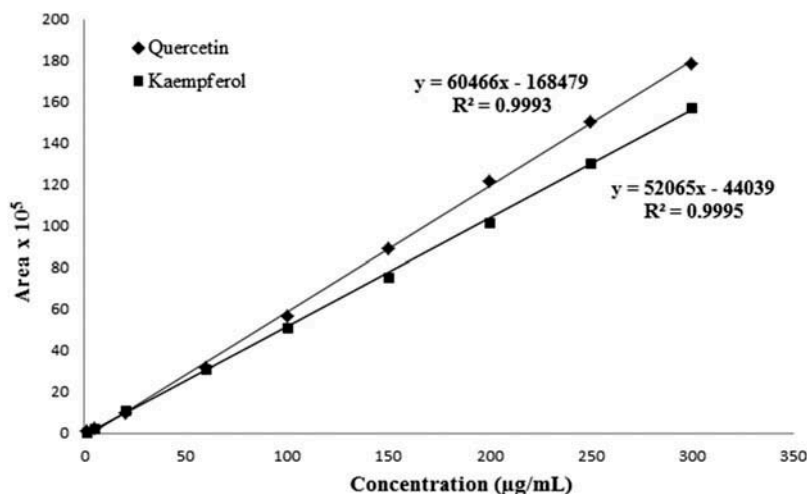


Figure 3. Calibration curves of the standard flavonoids.

$y = 60466x - 168479$ with R^2 of 0.9993 for quercetin and $y = 52065x - 44039$ with R^2 of 0.9995 for kaempferol. Regression equations and coefficient of correlations revealed a good linearity response for the developed method.

The method was also validated in terms of precision, accuracy, and other validation method parameters. Signal-to-noise ratios (S/N) of 3 and 10 were considered as LOD and LOQ, respectively. The LODs for quercetin and kaempferol were 0.33 and 0.26 $\mu\text{g/mL}$, and the LOQs for the same analytes were 1.06 and 0.83 $\mu\text{g/mL}$, respectively. This indicated that the proposed method exhibits good sensitivity for the quantification of flavonoids.

The values of intraday precisions (expressed in terms of %RSD) were less than 0.68 and 1.72 percent for quercetin and kaempferol, respectively, while the interday values were less than 1.23 and 2.18 percent for quercetin and kaempferol, respectively. The results

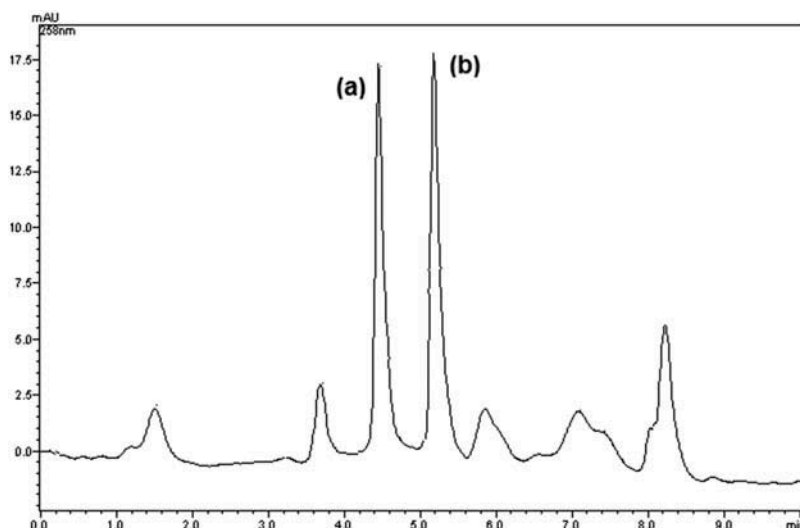


Figure 4. HPLC chromatogram for alcoholic extract of *Convolvulus pilosellifolius* Desr., where: (a) quercetin and (b) kaempferol.

demonstrated good intra- and interday precision for the proposed method. The accuracy of the proposed method was expressed as the recovery of standard compounds added to the preanalyzed sample. The recovery in all cases was found to be in the range of 98.2–100.2 percent. These values demonstrate good extraction efficiency. All of the quantitative estimation of quercetin and kaempferol in *Convolvulus pilosellifolius* are summarized in Table 1.

The presence of flavonoids in the plant extract was confirmed by comparison of their retention times and overlaying of ultraviolet (UV) spectra with those of standard compounds. A typical chromatogram of the alcoholic extract is illustrated in Figure 4. The alcoholic extract of the air-dried plant showed the presence of flavonoids, quercetin, and kaempferol, among which kaempferol (6.14 $\mu\text{g}/\text{mL}$ extract, 1.54 percent) was present in greater amounts than quercetin (4.27 $\mu\text{g}/\text{mL}$ extract, 1.07 percent). The contents and the percentages of quercetin and kaempferol found in the plant extract are given in Table 1. According to our finding results, kaempferol was found in greater percentage than quercetin in the *Convolvulus pilosellifolius* plant under investigation.

Conclusion

Phytochemical analysis is a very important laboratory process and scientific process. This process identified essential components of any plant part such as bark, leaves, stem, and root. No one knows exactly how many different plants are used in the world today, but we do know that medicinal plants are enormously important in both traditional and modern medicine. In this work, simultaneous determination of two flavonoids (kaempferol and quercetin) in total alcoholic extract of *Convolvulus pilosellifolius* was developed. The proposed HPLC method for identification and determination of quercetin and kaempferol was found to be simple, precise, specific, sensitive, and accurate. It can be used for routine quality control analysis of kaempferol and quercetin in herbal formulations containing flavonoids such as the *Convolvulus pilosellifolius* plant. Based upon the HPLC results, it

can be concluded that kaempferol is more abundant than quercetin in *Convolvulus pilosellifolius* plant. The percentage content of quercetin was 1.07 percent compared with 1.54 percent for kaempferol content.

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