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Development and Validation of Analytical Method for Determination of an Important Flavonoid in *Malva neglecta*

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Authors' contributions

This work was a collaborative work involving all the authors. Author NNA designed wrote the protocol and performed the experiment. Author MI analysed the data and wrote the first draft of the manuscript. Author MAM managed the literature review. The final manuscript was read, edited and approved by all the three authors.

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ABSTRACT

Development of genuine and dependable analytical methods which profile marker phytoconstituents in an extract containing a mixture of several components is a challenging task. A simple, rapid, precise, and reliable HPLC method was developed for quantification of quercetin from the methanolic extract of *Malva neglecta*. The estimation was carried out using Phenomenex Gemini-NX-5 µm C18 .The parameters considered for validation were accuracy, precision, linearity and robustness. The calibration curve was found to be linear in a concentration range of 20–100 µg/mL.The correlation coefficient was r^2=0.9996. The % average recovery of quercetin was found to be in the range of 99.82 to 100.52% which was within the acceptance criterion indicating the accuracy of the method. The results of the robustness study indicated that there is no influence of minor changes. The developed and validated method can be successfully used for the determination of quercetin in *Malva neglecta*; thereby helping in authentication and quality control of this plant.

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1. INTRODUCTION

During the past few decades, the demand for herbs as medicine is on the rise due to its vast chemical diversity and hence a need has been felt for ensuring the quality, safety and efficacy of the herbs. Standardization methods for herbs are developed to maintain the quality of the raw material used in the treatment of the various diseases and disorders. For evaluating the quality of the herbs, it is important to standardize the content of the active phytoconstituents [1]. A wide numbers of herbs are available in the market and most of the time the authentication of these herbs is difficult and hence there is a need to have analytical techniques for analysis to determine quality parameters for the safe and effective use of these herbs. Hence it becomes mandatory to identify some constituents that may serve as the important constituents for the biological activity exhibited by the herb or may serve as biomarkers [2]. Quantitative analysis of major constituents and also the bioactive marker compounds is a big challenge to scientists. This can be achieved only when the herbs are evaluated and analyzed using sophisticated analytical methods.

Various Chemical and chromatographic techniques have been successfully used in the identification of an herbal material or extract [3]. In any extract, there are a number of unknown constituents and many of them are in low amount. The complex nature and variability of the chemical constituents of the plant based drugs makes it is difficult to establish the quality control parameters. HPLC and HPTLC are some of the important modern analytical techniques that offers very powerful separation ability, such that the complex constituents present in extract can be separated into many simpler sub fractions [4,5].

Flavonoids are an important class of naturally occurring compounds consisting of a wide spectrum of biological activities, some of them being anti-oxidative, anti-inflammatory, anti-mutagenic, wound healing and other medicinal properties [6]. Quercetin is a flavonoid and review reports its various biological activities like antioxidant, anti-inflammatory, anti- cancer, anti-ulcer, antibacterial, antiviral activity and in certain types of allergies [7].

Malva neglecta is referred to as Khebaiz or Khobbeiza in Arabic and belongs to Malvaceae family and is wildly grown in the Northern Border Province, Saudi Arabia. It has been traditionally used for insect bites, bladder infection, burns, inflammation, ulcers and wounds, as astringent, demulcent, diuretic, expectorant and laxative. Some of the phytoconstituents reported in the literature are quinic acid, aconitic acid, chlorogenic acid, caffeic acid, coumaric acid, rutin, hyperoside, myricetin, fisetin, coumarin, quercetin, naringenin, luteolin, kaempferol, apigenin, rhamnetin and chysin [8-12]. WHO has published guidelines to ensure the reliability and repeatability of research on herbal medicines to specify the identity, purity, strength and manufacturing practices [13]. Literature review reports the method development and validation of quercitin in several other plants [14-16]. However there are no reports pertaining to quantification and validation of the phyto-constituents of this plant. Hence a HPLC method was used in the present work for quantification and validation of quercetin from methanolic extract of dried leaves of *Malva neglecta.*

2. METHODOLOGY

2.1 Collection and Extraction

The leaves of *Malva neglecta* were collected from Northern Border Province, Saudi Arabia. Shade dried and powdered. 250 gm of the powder was extracted with methanol. The residue was concentrated, dried and stored in the desiccator until further analysis.

2.2 Determination of Flavonoids [17]

The total flavonoids content was estimated spectrophotometrically using Aluminum chloride. The plant extracts in methanol were mixed with 1.5 mL of methanol, 1M potassium acetate, 10% aluminum chloride and distilled water. The absorbance of this mixture was measured at 415 nm. Different concentrations of the quercetin i.e. 100 µg/ml, 200 µg/ml, 300 µg/ml,400 µg/ml and 500 µg/ml were taken to obtain a calibration curve. The total flavonoid content was calculated as quercetin equivalent from the calibration curve.

2.3 Method Development and Validation [18,19]

2.3.1 Mobile phase preparation

Mobile phase was prepared by mixing 70% of HPLC grade methanol and 30% of 0.005M

phosphate buffer of pH 3. This solution was filtered using a 0.45 micron Millipore filter paper and was sonicated for 10 mins. The total volume of the mobile phase prepared was 1500 ml.

2.3.2 Standard preparation

10 mg of quercetin was taken in 10 ml volumetric flask and the volume was made to 10 ml with methanol. From this solution; 1 ml was pipetted into 10 ml volumetric flask and volume was made up to the mark with acetonitrile. This was the working solution. Further different concentration ranging from 20 µg/ml, 40 µg/ml, 60 µg/ml, 80µg/ml and 100 µg/ml was prepared by transferring required aliquots of solution to 10ml volumetric flask and made the volume up to the mark by methanol. This was sonicated for 8 mins then the solution was filtered using 0.45 micron Millipore filters.

2.3.3 Sample preparation for Assay

1 mg of the extract was weighed and dissolved in 1 ml of methanol. This solution was filtered using a 0.45 micron Millipore filter paper and was sonicated for 10 mins. 20 µl of this solution was injected.

2.4 HPLC Method Development

Quercetin in the sample was analyzed by HPLC technique using the conditions as shown in Table 1.

2.4.1 HPLC method validation

The above method was validated according to ICH guidelines. The parameters used for validation were Specificity, Precision, Robustness, Accuracy, Linearity, limit of quantitation and detection.

Accuracy: Was performed in triplicate for various concentrations of quercetin to determine the accuracy of the proposed method. Amount equivalent to 80%, 100% and 120% of the standard amount was injected into the HPLC system in accordance with the procedure. Accuracy was assessed as the percentage accuracy and mean % recovery.

Precision: Interday and intraday were studied to determine the intermediate precision of the proposed analytical method. Quercetin in the concentration of 60 µg/mL was analyzed for intraday and interday variation. The results were expressed as %RSD (Relative Standard Deviation).

Specificity: The specificity of the method was ascertained by analyzing the standard drug and extract. Quercetin in the sample was confirmed by comparing the Rf values with that of the standard.

Detection limit: The Limit of detection LOD and limit of quantitation LOQ values were calculated from the calibration curves as per the protocol.

Linearity: Linearity was established by triplicate injections of solutions containing standard quercetin. The linearity range maintained was 20 µg/ml, 40 µg/ml, 60 µg/ml, 80 µg/ml and 100 µg/ml.

Robustness: Robustness was tested by deliberately introducing small changes during the development of the analytical procedure and examining the effect in a particular aspect of its performance, normally its accuracy. The influence of independent variables on the response value was investigated using this method. The variables used in the study were temperature, pH and flow rate of mobile phase. 60 µg/ml concentration is selected for carrying out robustness. The parameters varied is as shown in Table 2. The experimental runs were performed in triplicate.

Table 1. Optimized chromatographic conditions

Parameters	Description
Detector	Shimadzu spd10A uv-vis, Japan
Pump	Shimadzu LC-10ATVP, Japan
Software	Baseline chromatography Data System N2000
Injection valve	7725i Rheodyne 20 µl, USA
Column	Phenomenex Gemini-NX-5 µm C18(2) , LC Column 250 x 4.6 mm
Elution Type	Isocratic
Elution A	Methanol
Elution B	Methanol: Phosphate buffer pH 3 (70:30)
Flow Rate	1 mL/min
Col. Temp	Ambient
Detection	UV-Vis Abs.-Variable Wave. (UV) at 280 nm

System suitability: System suitability was established by injecting six replicate injections into HPLC system as per test procedure. The system suitability parameters like retention time, peak area, peak height, resolution, tailing factor, asymmetry, theoretical plates were evaluated from standard chromatograms obtained, by calculating the % RSD.

3. RESULTS AND DISCUSSION

3.1 Collection and Extraction

The leaves of *Malva neglecta* were collected, powdered and extracted with methanol as described in the experimental section. The plant has been identified and authenticated by Dr Heba Salem, Department of Pharmacognosy, Faculty of Pharmacy, Northern Border Province, Saudi Arabia.

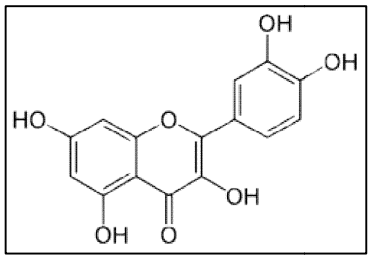

Fig. 1. Structure of quercetin

3.2 Determination of Flavonoid Content

The total flavonoid content was evaluated by the Aluminum chloride colorimetric method and was calculated as quercetin equivalent. The amount of total flavonoids in the methanolic extract was found to be 292 µg/ml.

3.3 HPLC Method Development and Validation

An HPLC method was developed and validated for the determination of quercetin in the methanolic extract of *Malva neglecta*. Review reports the development and validation methods for quercetin by various groups of researchers in various plants using different chromatographic conditions [14-16]. However there are no reports for method development for quercetin from this plant. The Chromatographic conditions were optimized to provide a good performance. The parameters used for validation of the method were accuracy, linearity, precision and robustness. The best and satisfactory results were obtained using Phenomenex Gemini-NX-5 µm C18(2) , LC Column 250 x 4.6 mm and themobile phase consisting of Methanol: Phosphate buffer pH 3 at a ratio of 70:30, flow rate was 1.0ml/min .The retention time for quercitin was 2.848. The method produced linear responses in the concentration range of 20-100%. LOD and LOQ were found to be 0.544 µg/mL and 1.3/ µg mL respectively. The amount of quercetin was found to be 0.23 µg/gm. Literature review reports several methods for simultaneous estimation of various flavonoids. The amount of quercetin varied in the range of 0.02 -0.254 µg/mL [20-22]. The HPLC method was found to be selective. The method was performed and validated for the various parameters as per ICH guidelines. The results of the Method development and validation of quercetin are as shown in the following chromatograms and tables.

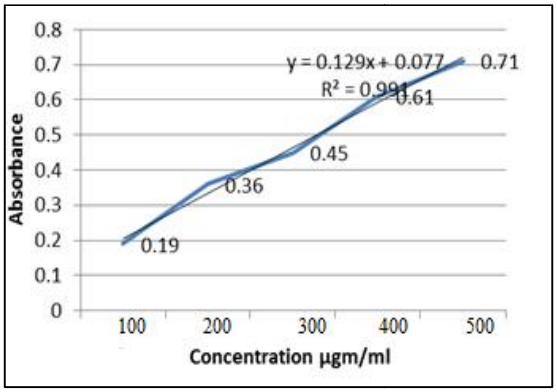

Fig. 2. Calibration curve for quercetin for estimation of total flavonoids

3.4 Linearity of the Developed Method

The concentration, peak area and retention time for linearity of quercetin and the regression line relating standard concentrations of drug using regression analysis were evaluated. The calibration curves were linear in the studied range and equations of the regression analysis were obtained i.e. R_2 =0.9996 for quercetin. Linearity concentration was in the range of 20/ µg/ml to 100/ µg/ml. Good linearity was observed over the above-mentioned range indicating that the method is linear over the concentration range studied Table 3 depicts the linearity data for quercetin.

Table 2. Parameters varied for robustness

Sl. no	Parameters varied	I	II	III
1	pH of the mobile phase	2.9	3.0	3.1
2	Temperature of the column	28°C	30°C	33°C
3	Flow rate	0.98 ml/min	1.00 ml/min	1.02 ml/min

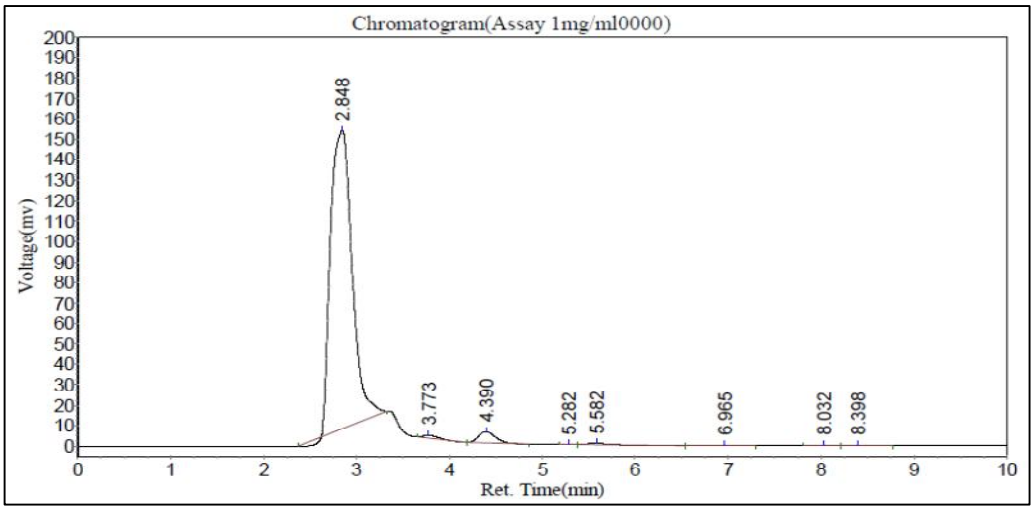

Fig. 3. HPLC chromatogram for quercetin in the sample

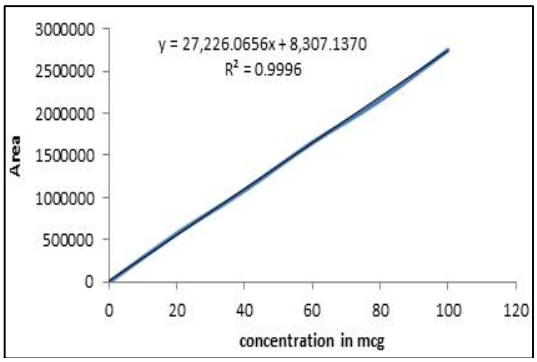

Fig. 4. Linearity graph for quercetin

3.5 Accuracy

Accuracy study was performed by adding known amounts of quercetin in three replicates. The recovery range for quercetin was found to be 99.82 to 100.52%. The accuracy data is as depicted in Table 5.

Precision of the developed method: Repeatability (Intra-day and intermediate precision data) is as shown in Tables 6 and 7. The quantity used was 60 µg/ml. The RSD values obtained for intraday & intermediate precision were 0.5597 & 0.3959 respectively.

The robustness of the proposed method was evaluated by deliberately changing the chromatographic conditions such as flow rate, temperature and pH. The results showed that varying the chromatographic conditions had no appreciable effects on the chromatographic parameters.

Table 3. Linearity data for quercetin

Concen(µg/ml)	Conc as % of analyte target	Peak are (mean of three injections)	Peak Area % RSD
20	20	572676.9587	1.96386303
40	40	1088280.208	0.229418599
60	60	1652236.333	1.72731281
80	80	2155012.167	1.21857
100	100	2749456.833	1.725641898
Y=27,226.0656x + 8,307.1370 Equation for regression line		Correlation coefficient (r^2) =0.9996	

Table 4. Data for system suitability for quercetin

System suitability parameter	Acceptance criteria	Result	Criteria met/not met
Injection precision for retention time(min)	RSD ≤ 2%	0.559734318	Met
Injection precision for peak area (n = 6)	RSD ≤ 2%	1.085541137	Met
Injection precision for peak height	RSD ≤ 2%	0.915565447	Met
Resolution (Rs)	Rs = ≥2.0	4.406	Met
USP tailing factor (T)	T = ≤ 2.0	1.165	Met
Asymmetry	K = ≤ 2.0	1.336	Met
Theoretical Plates (N)	N = ≥ 2000	2443.14	Met

Table 5. Data showing % recovery

Sample	Percentage nominal (mean of 3 inj)	Amount of standard(µg)		Recovery (%)
		Spike	Found	
1	80	48	47.9179	99.828901
2	100	60	59.4443	99.073856
3	120	72	72.3801	100.52795

Table 6. Intraday precision–data sheet

Sl. No.	Peak area	Retention time
1	1600036.125	4.648
2	1587093.375	4.632
3	1609695.5	4.623
4	1585969.5	4.598
5	1633015.5	4.582
6	1598850.625	4.642
Mean	1602443.438	4.620833333
StDev	17395.1827	0.02586439
%RSD	1.085541137	0.559734318

Table 7. Intermediate precision – data sheet showing the peak areas

Sl. No	Day-1	Day-2	Day-3
1	1571014.25	1615860	1607930.25
2	1569665.75	1613946.25	1596367
3	1568828.625	1615205.5	1606692.5
Mean	1569836.208	1615003.917	1603663.25
StDev	1102.738044	972.6698546	6348.972697
%RSD	0.07024542	0.06022709	0.395904358

Table 8. Robustness study of the proposed HPLC method

Parameter	Conditions	Retention time
Temp	28	4.40
	31	4.43
	33	4.42
pH	2.9	4.40
	3.0	4.42
	3.1	4.41
Flow rate (mL/min)	0.98	4.97
	1.0	4.50
	1.02	4.06

4. CONCLUSION

ICH guidelines were used for the development and validation of a HPLC method for determination of quercetin in *Malva neglecta*. The method was found to be simple, specific, accurate and precise .The linearity of the method was determined from the correlation coefficient and the method was found to be linear and within the range of 20/ µg/ml to 100/ µg/ml. The accuracy of the method was calculated by recovery study the results of the proposed method was found to be accurate as all the parameters were in compliance with the acceptance criteria This method can be adopted for the routine quantification and quality control of quercetin in *Malva neglecta* and traditional drugs and formulations containing quercetin.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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