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Quantification of flavonoids of *Psidium guajava* L. preparations by Planar Chromatography (HPTLC)

S. M. El Sohafy, A. M. Metwalli, F. M. Harraz and A. A. Omar

Department of Pharmacognosy, Faculty of Pharmacy, Alexandria University, Alexandria, Egypt.
Samahelsohafy@yahoo.com

ABSTRACT
Guava leaves, as most medicinal plants used in folk medicine, lack any systematic methodology needed to demonstrate genuine efficacy. The method described in this study is the first step towards establishing consumer confidence in guava preparations and demonstrates the use of HPTLC-UV to quantify the quercetin content of *Psidium guajava* L. leaves (quercetin content of the leaves ranged from 0.181 – 0.393 %) The results obtained were accurate (recovery ranged from 96 – 97.20 %) and precise (% RSD 0.856 and SD 0.01).
KEY WORDS : Thin layer chromatography, Quercetin and quercetin glycosides, *Psidium guajava* L., Guava leaf HPTLC

INTRODUCTION

Guava leaf "*Psidium guajava* L." has a long history of folk medicinal uses in Egypt and worldwide as a cough sedative, an anti-diarrheic, in the management of hypertension, obesity and in the control of diabetes mellitus [1-7]. The leaf extract was found to possess anticestodal [8], analgesic, anti-inflammatory properties [9], antimicrobial [10] and hepatoprotective activities [11]. In addition the leaf extract is used in many pharmaceutical preparations as a cough sedative.

Guava leaf extract contains flavonoids, mainly quercetin derivatives, which are hydrolyzed in the body to give the aglycone quercetin which is responsible for the spasmolytic activity of the leaves [4].

Quercetin has several pharmacological actions; it inhibits the intestinal movement, reduces capillary permeability in the abdominal cavity [12] and possesses dose-dependent antioxidant properties [13], anti-inflammatory activity [14-20], antiviral and antitumor activities [21-26]. It also inhibits the aldose reductase enzyme [27]. Quercetin has a well identified yellow colored spot on TLC, futhermore most of the glycosidal constituents of guava leaf are quercetin derivatives, namely quercetin, avicularin, guaijaverin, isoquercetin, hyperin, quercitrin, quercetin 3-O-gentiobioside, quercetin 4'-glucuronoide [4, 28-32].

Therefore quercetin was selected as the principal therapeutically active marker compound for the quantitative evaluation of the leaf extracts. Several analytical techniques are available for the quantitative analysis of quercetin e.g. HPLC, spectrophotometry and several others but very few are available for quercetin and its glycosides in plant complex extracts, e.g. HPLC [33-39], HPTLC [40] and LC coupled to coulometric detection [41], furthermore few methods are available in the literature for the evaluation of the extract of *P. guajava* L.. Two examples are the microwave-assisted extraction and acid hydrolysis prior to the GC determination of quercetin [42] and the spectrophotometric method which was reported to facilitate the quantification of tannins and flavonoids of guava [43]. The present work demonstrates the use of a HPTLC method for the quantitative determination of *Psidium guajava* L. leaf, extracts and pharmaceutical preparations.

MATERIALS AND METHODS

Materials

Psidium guajava L. leaf was collected from different farms around Alexandria (location one: Agamy and location two: Maamora). Quercetin and rutin were supplied by E.Merck, Darmstadt, Germany. The HPTLC analyses were performed on precoated HPTLC plates (silica gel 60 F_{254}) with adsorbent layer thickness 0.2

mm, E-Merck, Darmstadt, Germany. Three pharmaceutical liquid dosage forms from the Egyptian market containing guava leaf extract as an active ingredient were separately analyzed by the proposed method.

Instrumentation and method

Standard preparation: 10.0 mg of quercetin was accurately weighted and dissolved in 10 mL of methanol, then 5 mL of this standard (1 mg mL^{-1}) was quantitatively diluted to 10 mL with methanol. 10.0 mg of rutin was dissolved in 10 mL methanol.

Sample preparation: Powdered guava leaves (2 g) were extracted by boiling for 5 min with 20 mL water, adjusting the volume to 20 mL and filtering. 10 mL of this filtrate was hydrolyzed by adding 2 mL 25% HCl and heating it in a boiling water bath for 25 min. The mixture was then extracted with four successive 25 mL portions of n-butanol. The combined n-butanol extracts were dried under reduced pressure and re-dissolved in 10 mL methanol (equivalent to 1 g powdered guava leaf).

Chromatographic parameters and conditions: Standard zones were applied as bands on 20 x 10 cm aluminum silica gel 60 F_{254} HPTLC plates by means of (Wilmington, NC, USA) Linomat IV automated spray-on band applicator equipped with a 100-µL syringe and operated with the following settings: band length 6 mm, application rate 15 s μL^{-1}, distance between bands 4 mm, distance from the plate side edge 1.2 cm, and distance from the bottom of the plate 1.5 cm.

Plates were developed to a distance of 6.5 cm beyond the origin with toluene-acetone-methanol-formic acid (46: 8: 5: 1) in a vapour equilibrated Camag HPTLC twin trough chamber. (The development time was about 17 min). After development, the plates were air-dried for 15 min.

Densitometric evaluation of the chromatograms: Camag TLC scanner 3 was used to densitometrically quantify the bands using WINCATS software (version 4 X).and the scanner operating parameters were: (mode: absorption / reflection; slit dimension: 5 x 0.1 mm; scanning rate: 20 mm s^{-1} and monochromator bandwidth: 20 nm at an optimized wavelength 254 nm).

Calibration curve construction: As recommended by the International Committee on Harmonization (ICH) guidelines [44, 45], a calibration curve was established using six analyte concentrations of the quercetin (0.5 mg mL^{-1}) standard (1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 µL $zone^{-1}$). Each concentration was applied in triplicate and the average of each reading was calculated, representing 0.5 – 3 µg $spot^{-1}$ of quercetin.

Choice of the solvent used for extraction of quercetin from the hydrolyzed samples and determination of the number of solvent portions sufficient for extraction: In order to select the best solvent that yields the maximum recovery of quercetin from the hydrolyzed samples, three solvents (ether, ethyl acetate and n-butanol) were tested.

Three 5 mL samples (quercetin 1mg mL^{-1}) were individually treated according to the conditions of the previously described method i.e. heated to 100 °C on a water bath with 2 mL 25% HCl, seperately extracted with four successive portions (25 mL each) of either ether or ethyl acetate or n-butanol. Each combined solvent was dried, redissolved in 10 mL methanol (i.e. the concentration of the resulting solution is 0.5 mg mL^{-1}), then these extracts were quantitatively estimated against standard quercetin solution (1 mg mL^{-1}). For ether and ethyl acetate recoveries of 81.40% and 76.04% were obtained, respectively, while the best recovery was obtained using n-butanol (95.50%). Butanol was therefore used as the extraction solvent.

To determine the number of n-butanol portions sufficient for extraction, 25 mL of standard quercetin (1 mg mL^{-1}, equivalent to 25 mg quercetin) was treated under the same conditions as described before and extracted with four successive quantities of butanol (25 mL each). Every portion was collected separately, dried, redissolved in 5 mL methanol resulting in four samples that were analyzed as before. The results are given in table 1.

Table 1. Quercetin concentrations in successive n-butanol extract fractions.

Sample	Concentration	Recovered amount
	mg $5mL^{-1}$	(% from total)
First extract	15.76	63.03
Second extract	5.00	20.02
Third extract	2.21	8.84
Fourth extract	1.08	4.32
Total	24.05	96.20

The extraction efficiency of n-butanol is between 50-63% each time indicating that four repetitive extractions are enough to extract most of the quercetin from the sample with a recovery of about 96%.

RESULTS AND DISCUSSION

Development with the mobile phase described above on the HPTLC silica gel layers produced compact, flat, dark yellow bands of quercetin (R_F 0.38) detected as quenching band when viewed under a 254 nm UV light.

Analytical performance

The linearity of the method was tested by analyzing six quercetin standard solutions in triplicate in the concentration range 0.5-3.0 µg spot^{-1}. The Linear regression correlation coefficient values (r-value) were 0.9996 when using peak area and 0.9906 when using peak height. The Linear regression equations were found to be y_1 = 2565.4 x – 639.11 and y_2= 86.522 x – 19.302 where y_1 is the peak area, y_2 is the peak height and x is quercetin concentration in µg/spot. Based on the r-value results peak areas were used for the quantitative calculations in this study.

The minimum detected concentration was found to be 0.0625 µg spot^{-1} using different dilutions of standard quercetin, the minimum concentration that gave linear results was found to be 0.5 ug spot^{-1} and the maximum concentration was 3 ug spot^{-1}.

Precision (repeatability) of the sample application step: The content of a single sample was estimated using the previous method .The sample (3 µL) was applied on a plate six times and quantitatively scanned in order to determine the precision of the application step. The SD of the experiment was found to be 0.027 and the % RSD was 1.078.

Precision (repeatability) of the method: In accordance with the ICH guidelines [44, 45], precision was determined by repeated analysis of a homogeneous sample using the same equipment, same analytical procedures, same laboratory and on the same plate. Six aliquots of the previously prepared sample were subjected to the same method, spotted on the same plate. The SD of the experiment was found to be 0.01 and the % RSD was 0.856. The results shown meet the acceptance criterion for % RSD specified by the ICH [44, 45] which is a precision of less than 2-3 % RSD.

Accuracy: The accuracy of the method, which gives information about the recovery of the analyte from the sample, was validated by standard addition analysis. The sample solutions were spiked with two different, known, concentrations of rutin. A 10 mL aliquot of guava leaf extract; sample solution of known previously determined concentration (0.179 mg mL^{-1}); was mixed with 5 mL of standard rutin solution (1 mg mL^{-1}) to give a mixture of concentration 0.427 mg mL^{-1} and another 10 mL aliquot of the same sample solution was mixed with 2 mL of standard rutin (1 mg mL^{-1}) to give mixture of concentration 0.271 mg mL^{-1}. Another 10 mL standard rutin (1 mg mL^{-1}) was also analyzed. The unfortified guava leaf extract, standard rutin and the two fortified sample solutions were separately hydrolysed, extracted, dried, redissolved in methanol and analyzed on the same plate. The difference between the expected concentrations and the determined ones was calculated to determine the accuracy of the method. The recovery of quercetin from standard rutin was found to be 96.20% while those of quercetin from the two mixtures were 96.00% and 97.20% respectively.

Pharmaceutical Preparations and guava leaves:

The proposed method was applied to some pharmaceutical preparations (liquid dosage forms) containing guava leaf extract that are available in the Egyptian market to quantify their total quercetin content. Three samples (20 mL each) from the market were analyzed in order to quantify their total quercetin content. Sample 1 contained guava leaf extract, tilia extract, fennel oil and purified honey. Sample 2 contained thyme extract, guava leaf extract, anise oil and purified honey while sample 3 contained guava leaf extract, tilia extract and fennel oil. Quercetin concentration was found to be 6.95, 4.52 and 16.17 mg 100mL^{-1} for samples 1, 2 and 3, respectively. The accuracy of the method upon its application to the liquid preparations was assessed by fortifying 20 mL of one sample with 5 - 10 mg of standard quercetin in order to prove that the recovery of the method is unaffected by co-existing ingredients and that the method could be applied to pharmaceutical preparations. The analysis was conducted in triplicate.

The recoveries of the extraction of pharmaceutical preparations ranged from 95.30 – 97.90%. Therefore the method is applicable to the pharmaceutical preparations without the interference of co-existing compounds. Quercetin content of *P. guajava* L. leaves collected from trees at different stages of growth from the determined best two different areas (locations one and two) from trees at different stages of growth as shown in table 2 were analyzed for their quercetin

Table 2. Samples of P. guajava L. leaves collected from trees at different stages of growth and their quercetin content.

Sample	Concentration of quercetin mg 100 g^{-1}	
	Location one (Agamy)	Location two (Maamora)
Flowering stage (May)	211.25	375.00
Premature fruiting i.e. while the fruits were small and green (July – August)	***215.45***	***392.70***
Mature fruiting i.e. while the fruits were mature and yellow (October)	181.05	326.98
Post harvesting (December)	199.34	336.86
Post harvesting and preflowering (March)	206.10	374.60

content according to the proposed method in order to reveal the best stage of growth during which guava trees yield the highest quercetin content. The results are shown in table 2. The highest quercetin content is during the premature fruiting stage. Nevertheless, and from the economic point of view, samples taken post harvesting of the fruits contain good amount of quercetin glycosides, as well.

CONCLUSION

The applied HPTLC method has been shown to be selective, linear, precise and accurate. The results meet the guidelines of the International Conference on Harmonization (ICH) [44, 45] for validation of pharmaceutical assays of drug products and are comparable with those published. The method will be useful for quality control of the raw material, extracts and pharmaceutical preparations.

The proposed HPTLC method designed for the analysis of guava products is accurate, precise, sensitive, simple, easy to perform, rapid, versatile. It is easily applicable to a large number of samples in a short time and with a low cost in terms of reagents, solvents and equipment. In addition the sample treatment is very simple.

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