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# Simultaneous Quantification of Umbelliferone and Quercetin in Polyherbal Formulations of *Aegle Marmelos* by HPTLC.

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## ABSTRACT

In the present investigations, methanolic extracts of four marketed *Aegle marmelos* formulations ($F_1$, $F_2$, $F_3$, and $F_4$) were prepared and subjected to simultaneous quantitative determination of two biologically active compounds; umbelliferone and quercetin. Analysis of umbelliferone and quercetin was performed on TLC aluminum plates pre-coated with silica gel 60F-254 as stationary phase. Linear ascending development was carried out in twin trough glass chamber saturated with mobile phase consisting of toluene: ethyl acetate: formic acid (6:4:1, *v/v/v*), and densitometric determination of these compounds was carried out at 300nm in reflectance/absorbance mode. The system was found to give compact spots for umbelliferone and quercetin with R*f* value of 0.66 and 0.68, respectively. The present method was validated for precision, recovery, repeatability, and accuracy in accordance with International Conference on Harmonisation (ICH Q2) guidelines. Statistical analysis of the data showed that the method is reproducible and selective for estimation of umbelliferone and quercetin. This method may be used for routine quality control and standardization of the herbal drugs and there formulations.

**Key words:** *Aegle marmelos*, Quercetin**,** Umbelliferone**,** HPTLC

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## INTRODUCTION:

*Aegle marmelos* L. belongs to the family rutaceae, an important medicinal plant being used in folk therapy. Different parts of the plant show various medicinal properties. The fruit, generally known as "*bael",* is widely consumed as '*sherbet*' (liquid fruit concentrate) and '*murabha*' (jam). Unripe fruit is highly recommended for diarrhoea and infusion of dried unripe fruits has been used as anti-diarrheal and anti-dysentery agents[1]. Sweet drink (sherbet) is prepared from the pulp of the bael fruits and produces a soothing effect in the patients who have just recovered from bacillary dysentery[2]. The fruit is widely consumed as astringent; it is also helpful in a number of other complicated intestinal disorders such as irritable bowel syndrome and ulcerative colitis[3]. Some Ayurvedic medicines (Bilvadi churna, Bilva panchaka qwath and Bilva tailam), contain bael fruit as the principle ingredient. The fruits of *A. marmelos* hold considerable amount of mucilage, pectin, marmelosin, marmelide, umbelliferone, quercetin, tannins, Flavonoid & phenolic compounds and show antioxidant activity[4,5]. Among these constituents, umbelliferone and quercetin show significant anti-inflammatory, antihistaminic, antiallergic, antioxidant and antitumor activities[6,7]. Umbelliferone and quercetin (Figure. 1) exhibit antioxidant activity and inhibit the release of autacoids and prostaglandins[8, 9].

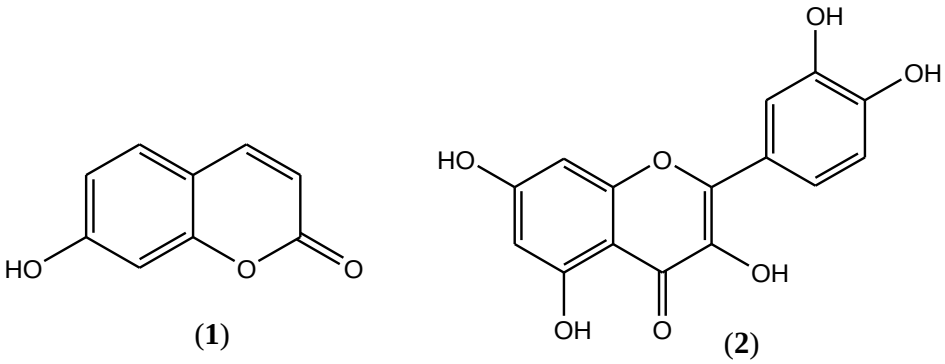


**Figure 1: Chemical structures of Umbelliferone (1) and Quercetin (2).**

A number of chromatographic techniques have been used including high performance liquid chromatography (HPLC), high speed counter-current chromatography (HSCCC) capillary zone electrophoresis and high performance thin-layer chromatography (HPTLC) in the quantification of valuable chemical compounds from medicinally important plants[10,11]. A HPTLC finger printing profile has been carried out for the standardisation of *Laghugangadhar churna*, a widely used Ayurvedic formulation for diarrhoea and dysentery, contains *A. marmelos* (fruit pulp) as one of the main ingredients[12]. Furthermore, *in vitro* antioxidant activity of umbelliferone and psoralen present in the methanol extract of *A. marmelos* fruit suggests that the fruit is a potential

source of natural antioxidants[13]. In view of these points and the therapeutic importance of *A. marmelos*, the present studies were designed to investigate the methanolic extracts of four *A. marmelos* formulations for the quantification of umbelliferone and quercetin in an efficient, simple and precise HPTLC method. Therefore, an attempt was made to develop accurate, specific, repeatable and robust HPTLC method for the determination of these biologically active compounds.

## MATERIAL AND METHODS

### Chemicals and reagents

Umbelliferone (purity 98%, w/w) and quercetin (purity 98%, w/w) were purchased from Sigma–Aldrich (Bangalore, India). All chemicals and reagents were of AR grade. Methanol and Aluminium-backed TLC plates pre-coated with 0.2 mm layer of silica gel 60 $F_{254}$ (20 cm × 10 cm) were purchased from E. Merck (Germany); supplied by Anchrom Technologists, Mumbai. Four formulations of *A. marmelos* were purchased from different local markets in Delhi, India and marked as $F_1$, $F_2$, $F_3$, and $F_4$.

### Instrumentation and chromatographic conditions

The HPTLC densitometric scanning was performed on CAMAG TLC scanner III connected to PC running WINCATS software under MS Windows, connected with microlitre syringe and linked to a nitrogen tank. The samples were spotted on pre-coated silica gel aluminium plate 60 $F_{254}$ (20 cm × 10 cm with 0.2 mm thickness) using a CAMAG Linomat V (Switzerland) applicator. The plates were prewashed by methanol and activated at 60°C for 5 min, prior to chromatography. A constant application rate of 150 nL/sec was employed and bands of width 6 mm, space between two bands was 11.3 mm. The slit dimension was kept at 6 mm×0.45 mm. Each track was scanned thrice and baseline correction was used. The mobile phase consisted of toluene: ethyl acetate: formic acid (6:4:1, *v/v/v*) used for chromatography. Linear ascending development was carried out in 20 cm × 10 cm twin trough glass chamber (CAMAG, Switzerland) saturated for 30 min with the development solvent system allowed to start at the position 10 mm and migrate up to a height of 80 mm from the lower edge at room temperature (25±2 °C) and relative humidity of 60±5%. The chromatogram was developed for 30 min to a distance of 80 mm and dried in a current of hot air using an air dryer. Deuterium and tungsten lamps, in the absorbance mode at 190 and 400 nm emitting a continuous UV spectrum was used as the source of radiation. Each plate pre-coated silica gel accommodated 16 tracks of samples and standards applied according to volume (1-8 µL for standard and 10-20 µL for formulation

with gas flow 10s/l). A constant application rate of 150nL/sec was used. The scanner was set for maximum light optimization and with the settings of slit dimension, scanning speed 20 mm/sec and data resolution speed 100 m/step. The scan started at position 10 mm and ended at position 80 mm[6,14].

**Preparation of samples**

Each formulation ($F_1$, $F_2$, $F_3$, and $F_4$) was weighted accurately (5 gm) and sonicated for 30 min and then extracted with methanol (3 × 50 mL) under reflux on water bath at 60°C. The pooled extract of each sample was concentrated and transferred to 100 mL volumetric flask individually. It was used as working sample and applied to the plates in duplicates.

**Calibration curve for Umbelliferone and Quercetin**[6, 14]

The stock solutions of umbelliferone (1 mg/mL) and quercetin (1 mg/mL) were prepared in methanol. One millilitre of standard umbelliferone and quercetin were transferred into a 10 mL volumetric flask and made up volume with methanol. Different concentrations (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 µL) of the solution were spotted in duplicates on TLC plate to furnish 100, 200, 300, 400, 500, and 600 ngm per spot of umbelliferone and quercetin, respectively. The plate was developed in the solvent system and scanned at 300 nm for umbelliferone and quercetin. After development, peak area and amount of drug were analysed by linear least square regression. Calibration curve of umbelliferone and quercetin were prepared by plotting peak area versus concentration.

**Method validation**

Each sample (10-20 µL) was applied in duplicate on TLC plates. The plates were developed and scanned at 300 nm for umbelliferone and quercetin. The amount of umbelliferone and quercetin in formulations was calculated using the linear regression equation of calibration curve.

**Linearity**

Linearity of the proposed method was checked by analysing solutions in the range of 100-600 ng/spot (100, 200, 300, 400, 500, 600 ng/spot) for umbelliferone and quercetin, respectively. Each level was made in duplicate for each solution.

**Accuracy**

Method accuracy was performed by adding known amounts of umbelliferone and quercetin to the pre analyzed sample and then comparing the added concentration with the found concentration. Three levels of solutions were made which correspond to 50, 100 and 150% of the nominal analytical concentration. Each level was made in duplicate and spot 2.5 µL of each solution.

**LOD and LOQ**

To calculate LOD and LOQ, the LOD was expressed as (3.3 × $\sigma$) / (slope of calibration plot) and LOQ was expressed as (10 × $\sigma$) / (slope of calibration plot).

**Precision**

For evaluating the within-day precision, results of six replicates ($n$ = 6) were calculated on a single day. The between-day precision was calculated from the samples analyzed on three different days. Precision was expressed as the coefficient of variation (CV, %) of measured concentrations for each calibration level.

**Stability**

Sample solutions of the extracts were prepared and stored at room temperature for 10, 30, 60, 120 and 240 min. and then applied on the same chromoplate; after development the chromatogram was evaluated for additional spots. Similarly spot stability was analyzed by keeping the resolved plates and inspecting at intervals of 10, 30, 60, 120 and 240 min.

## RESULTS AND DISCUSSION

In present investigation quantitative analysis of umbelliferone and quercetin was studied in four *A. marmelos* formulations.

**Optimization of the mobile phase**

The standard solutions (umbelliferone and quercetin) and test solutions (methanolic extracts of formulations) were spotted on HPTLC plate and different individual solvents as well as combination of solvents have been tried to get a good separation and a stable peak. Initially toluene: ethyl acetate: methanol (6:4:2 *v/v/v*), ratio was used as the mobile phase. At this composition although both components were resolved but peak shape was not good. Reshuffling of methanol with formic acid improved the resolution between bands. With the mobile phase compose of toluene: ethyl acetate: formic acid (6:4:1, *v/v/v*) gave good resolution sharp, symmetric, and well resolved peak that belong to umbelliferone and quercetin with a retention factor of 0.66 and 0.68 respectively (Figure 2)

**Calibration Curve**

The developed chromatographic method was validated using ICH guidelines[14]. For umbelliferone densitometry data show good linear relationship with r = 0.98116 and standard deviation (S.D.) = 6.50 and 6.39 with respect to peak height area in the concentration range 100 – 600 ng/µL. The regression equation was found to be y = 111.674+0.044*x and y = 2309.424+1.628*x with respect to peak height area and for quercetin the calibration plot which

showed good linear relationship with r = 0.98103 and standard deviation (S.D.) = 18.65 and 11.50 with respect to peak height area in the concentration range 100 - 600 ng/µL. The regression equation was found to be y = 270.676+0.481*x and y = 401.636+21.217*x with respect to peak height area respectively. The slopes of the standard plots were not significantly different.

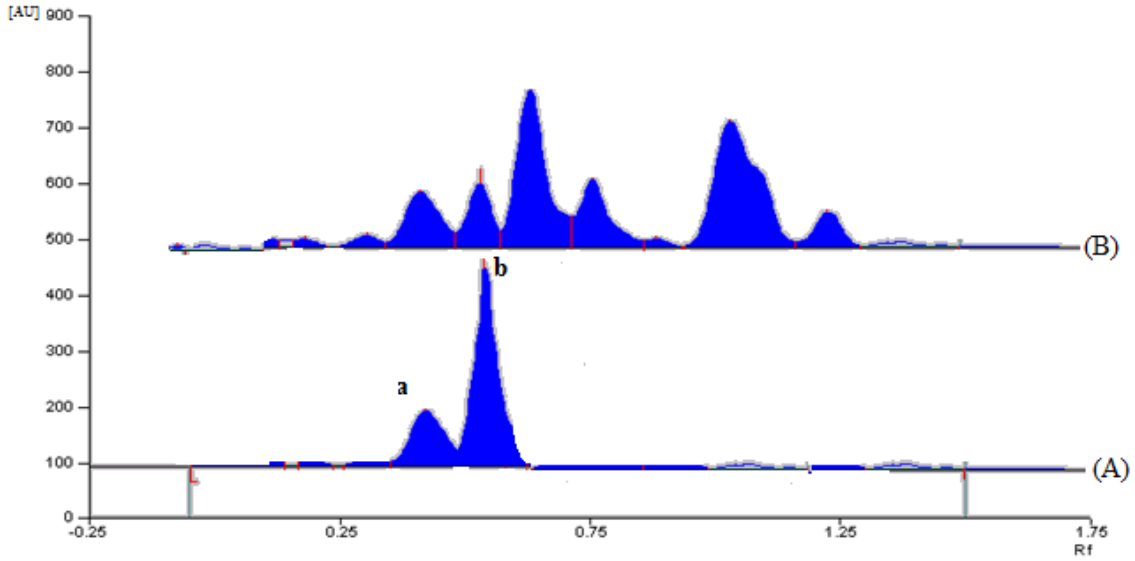


**Figure 2: Chromatogram of umbelliferone and quercetin (300 nm). AU: area under curve; R*f* = 0.66 and 0.68, mobile phase: toluene:ethyl acetate:formic acid (6:4:1, *v/v/v*).**

A: chromatogram of standard umbelliferone and quercetin;
B: chromatogram of umbelliferone and quercetin containing *A. marmelos* formulations. Peaks a and b represents umbelliferone and quercetin, respectively.

**Quantification of umbelliferone and quercetin in *A. marmelos* formulations**

A significant variation in umbelliferone and quercetin content were analysed in different *A. marmelos* formulations. Table1 &2 showed a comparative account of the umbelliferone and quercetin yield in the 4 studied formulations. The maximum amount of umbelliferone (0.094% w/v) and quercetin (0.038% w/v) was quantified in $F_4$ formulation. A good HPTLC chromatogram was obtained for standard umbelliferone and quercetin at 300nm. The recovery and the relative standard deviation for each of the analysts are given in Table 3&4.Umbelliferone and quercetin quantified and validated by the proposed HPTLC method. The validation data for both constituents meet the acceptance criteria for accuracy, precision, linearity, detection and quantification limits set by the International Conference on Harmonization(ICH) for assay of pharmaceutical products[14]. The relationship between the concentration of umbelliferone & quercetin to the peak response was linear within the concentration range of 100-600 ng/µL with correlation coefficients of 0.9811 & 0.9810, respectively.

**Table 1: Quantification of Umbelliferone in different formulations of *A. marmelos.***

| Formulation code | Umbelliferone | | | |
|---|---|---|---|---|
| | Amount µg/100mL (mean height) | Amount µg/100mL (mean area) | Mean of a and b (µg/100 mL) | % w/v |
| **$F_1$** | $5.862 \pm 0.6^b$ | $0.924 \pm 1.1^b$ | $3.393 \pm 0.6^b$ | $0.0339^b$ |
| **$F_2$** | $0.240 \pm 0.3^c$ | $1.468 \pm 0.7^a$ | $0.854 \pm 0.4^b$ | $0.0085^b$ |
| **$F_3$** | $0.061 \pm 0.9^d$ | $1.736 \pm 0.8^c$ | $0.898 \pm 0.2^c$ | $0.0081^c$ |
| **$F_4$** | $0.823 \pm 0.5^a$ | $6.003 \pm 1.4^b$ | $3.413 \pm 0.3^a$ | $0.0941^a$ |

Mean±SD of 3 replicates from two experiments. Within each column, values are followed by the same superscript letter are not significantly different at $P = 0.05$ level according to LSD test.

**Table 2: Quantification of Quercetin in different formulation of *A. marmelos.***

| Formulation code | Quercetin | | | |
|---|---|---|---|---|
| | Amount µg /100ml (mean height) | Amount µg /100ml (mean area) | Mean of a and b (µg /100 ml) | % w/v |
| **$F_1$** | $2.162 \pm 0.7^b$ | $1.373 \pm 0.9^b$ | $1.767 \pm 0.7^b$ | $0.017^b$ |
| **$F_2$** | $0.314 \pm 0.8^c$ | $0.981 \pm 0.3^a$ | $0.647 \pm 0.6^b$ | $0.016^b$ |
| **$F_3$** | $0.612 \pm 0.6^d$ | $0.608 \pm 0.9^c$ | $0.61 \pm 0.2^c$ | $0.006^c$ |
| **$F_4$** | $6.241 \pm 0.5^a$ | $1.468 \pm 0.1^b$ | $3.854 \pm 0.4^a$ | $0.038^a$ |

Mean±SD of 3 replicates from two experiments. Within each column, values are followed by the same superscript letter are not significantly different at $P = 0.05$ level according to LSD test.

**Table 3: Validation parameters for Umbelliferone.**

| Validation Parameter | Value | | | | |
|---|---|---|---|---|---|
| Regression equation | Y = 2309.424 + 1.628x | | | | |
| Linearity range | 100-600 ng/spot | | | | |
| Slope | 1.628 | | | | |
| Intercept | 2309.424 | | | | |
| Correlation coefficient($r^2$) | 0.98116 | | | | |
| LOD | 11.169 ng/spot | | | | |
| LOQ | 33.846 ng/spot | | | | |
| System Suitability (n= 6 %CV) | 0.09 | | | | |
| Instrument Precision (n= 6 %CV) | 0.515 | | | | |
| Intraday (precision) (n= 6 %CV) | 0.498 | | | | |
| Interday (precision) (n= 6 %CV) | 0.925 | | | | |
| **% Recovery of Umbelliferone** | | | | | |
| **% Spiked** | **Added quantity** | **Actual quantity** | **Quantity recovered** | **% Recovery** | **% RSD** |
| 50 % | 0.462 | 1.386 | 1.358 | 97.9 % | 0.8 % |
| 100 % | 0.924 | 1.848 | 1.839 | 99.5 % | 1.1 % |
| 150 % | 1.386 | 2.31 | 2.319 | 100.3 % | 1.4 % |

CV: coefficient variation; LOD: limit of detection; LOD: limit of quantification; ng: nanogram: RSD: relative standard deviation.

**Table 4: Validation parameters for Quercetin.**

| Validation Parameter | Value |
|---|---|
| Regression equation | Y = 401.636 + 21.217x |
| Linearity range | 100-600 ng/spot |
| Slope | 21.217 |
| Intercept | 401.636 |
| Correlation coefficient ($r^2$) | 0.98103 |
| LOD | 1.78 ng/spot |
| LOQ | 5.42 ng/spot |
| System Suitability (n= 6 %CV) | 0.09 |
| Instrument Precision (n= 6 %CV) | 0.515 |
| Intraday (precision) (n= 6 %CV) | 0.563 |
| Interday (precision) (n= 6 %CV) | 0.873 |

**% Recovery of Quercetin**

| % spiked | Added quantity | Actual quantity | Quantity recovered | % Recovery | % RSD |
|---|---|---|---|---|---|
| 50 % | 0.686 | 2.059 | 2.026 | 98.3% | 0.9 % |
| 100 % | 1.373 | 2.746 | 2.713 | 98.8% | 1.9 % |
| 150 % | 2.059 | 3.432 | 3.437 | 100.1% | 1.7 % |

CV: coefficient variation; LOD: limit of detection; LOD: limit of quantification; ng: nanogram: RSD: relative standard deviation.

The percentage recoveries at three different levels were found to be (97.9%, 99.5% and 100.3%) for umbelliferone and (98.3%, 98.8% and1001%) for quercetin, respectively. The signal to noise ratio of 3.3 and 10 were considered as LOD and LOQ, respectively. The LOD and LOQ were found to be 11.169 and 33.846 ng per spot for umbelliferone and 1.78 and 5.42 for quercetin, respectively (Table 3 and Table 4).

Umbelliferone and quercetin are gaining immense importance by virtue of their critical role in disease prevention. In this context, *A. marmelos* can rightly be mentioned as a plant of considerable interest. These constituents present in the plant extract are reported to inhibit release of autacoids and prostaglandins, thereby inhibit motility and secretion[15].

The study on medicinal plants to reveal the mechanism of action and to justify their claims by traditional healers being in continuation over the years. In present investigation four *A. marmelos* formulations were screened for quantification and estimation of umbelliferone and quercetin.

The developed method could be used for the quantification in the commercial formulations. Also it will find wide applications in standardization and quality control of formulations, herbal raw materials as well as formulation having these constituents. The present study has verified that remedial plants could be good source of antioxidant substances.

## CONCLUSION

In conclusion, the maximum amount of umbelliferone and quercetin was found in $F_4$ formulation among all the tested 4 market formulations ($F_1$, $F_2$, $F_3$ & $F_4$). The developed HPTLC method is an attractive alternative for the simultaneous quantitative determination of umbelliferone and quercetin in polyherbal formulations of *A. marmelos* with regard to its simplicity, accuracy and selectivity. This method could be widely applied directly for routine analysis and quality assurance of related extracts and drugs. The developed HPTLC method for this estimation of umbelliferone and quercetin is accurate, linear, rugged, simple and rapid. Statistical analysis indicates that the method is reproducible and selective for the analysis.

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