TLC Determination of Marmesin, a Biologically Active Marker from *Feronia Limonia* L.

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**ABSTRACT**

*Feronia limonia* Linn. (*Rutaceae*) have gained traditional therapeutic importance owing to their high essential oil and coumarins content. Marmesin, a furanocoumarin was identified by TLC and isolated by column chromatography and further purified by Preparative TLC. Presently, there is no appropriate TLC based method available for standardization of *F. limonia*. A simple, sensitive and accurate high performance thin layer chromatographic (HPTLC) method has been developed for the estimation of marmesin in the methanolic extract of stem bark of *Feronia limonia*. HPTLC was performed on precoated silica gel 60F254 aluminium plates (20 cm × 20 cm) with Chloroform: Methanol (9.5:0.5), as mobile phase. Quantitative evaluation of the plate was performed in the absorption-reflection mode at 338 nm. The calibration curve was linear in the concentration range of 20 – 100 ng spot⁻¹. The method was validated for precision, repeatability and accuracy. The technique has been applied, for the first time, for the estimation of marmesin. The proposed method was found to be robust, precise, and accurate, it therefore holds potential for detection, monitoring and quantification of marmesin in *Feronia limonia* and its related formulation.

**Keywords**: Marmesin, *Feronia Limonia*, HPTLC

1. **Introduction**

Standardization and characterization of herbal drugs is a topic of continuous scientific interest in the herbal drug industry [1]. 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 of herbal drugs based on their phytoconstituents. This requirement is fulfilled by thin layer chromatography (TLC) [2,3]. *Feronia limonia* is (family *Rutaceae*, subfamily *Aurantioidae*), commonly known as wood-apple, belongs to the tribe *Citreae* and subtribe Balsamocitrinae[4] which is widely distributed in dry warm regions of India, Bangladesh, Barma, Ceylon, Java & Srilanka [5,6]. This plant recently gained a great therapeutically relevance owing to their high Coumarins and monoterpenoids content, which is explored for treatment of snake bite [7]. Stem bark mainly consists of furan Alkaloid; Coumarins; Flavanones; Lignan; triterpene [8]. It is useful as tonic in diarrhoea, dysentery, stomatitis, tumors, cough, asthma, leucorrhoea, wounds and ulcers. Fruits, leaves and stem bark of *F. limonia* have been studied for anti-tumor [9], larvicidal [10] and antimicrobial activity [8].

Marmesin is one of the most prevalent linear dihydro-furanocoumarin, is abundant in species belonging to the families of Umbelliferae, Apiaceae, Rutaceae, Moraceae, and Leguminoseae [11,12]. It is originally isolated from indigenous indian plants, *Aegle marmelos Correa* [13], and later from the Hawiian shrub *Pelea barbigera* [14] both of these are from rutaceae family. It has an amazing array of scientifically acknowledged benefits for key areas of health, as dermal photosensitizing activity beneficial in the treatment of leucoderma [15], antifungal activity [16], phytoalexin [17], feeding deterrence effects [18] and radical scavenging activity [19]. Currently HPTLC is often used as an alternative to HPLC for the quantification of plant products because of its simplicity, accuracy, cost-effectiveness and rapidity [20]. The present study is based on development of methods for determination of marmesin by HPTLC in *F. limonia* stem bark that may contribute in standardization of raw material of the plant and its formulation.
2. Experimental

2.1. Reagents and Chemicals

All the chemicals, including solvents, were of analytical grade from E. Merck, India. The HPTLC plates Si 60F254 (20 cm × 20 cm) were purchased from E. Merck (Darmstadt, Germany).

2.2. Plant Materials

The plant material of *Feronia limonia* was collected in the months of September–October 2008 from campus of The M.S. University, Vadodara (Gujarat). They were authenticated in the Botany Department and a voucher specimen (No.Pharmacy/FL/ 08-09/01/MJ) has been deposited in the Pharmacy Department of The M. S. University of Baroda, Vadodara, India.

2.3. Extraction and Isolation of Reference Compound (MR-1) from *Feronia Limonia*

Air-dried and finely powdered stem barks of the plant (500 g) were exhaustively extracted at temperature (60-80°C) with methanol (3 × 1.5 L) in a soxhlet apparatus and the pooled extracts then obtained were concentrated under vacuum to give methanolic extract. Methanolic extract was made hydroalcoholic by addition of hot distilled water in 1:1 ratio partitioned with chloroform (100 mL × 4), and combined chloroform fraction was concentrated in vacuum to afford a brown residue (4.5 g). This residue was chromatographed over a Silica gel (60#120 mesh size) column eluting with toluene followed by increasing concentrations of ethyl acetate and methanol. Fraction 9-10 (toluene: ethyl acetate, 60:40) yielded yellowish crystal resulted in mixture of compounds on TLC. Further purification of MR-1 was achieved by preparative TLC (chloroform: methanol, 9.8:0.2) and confirmed by analytical HPLC. MR-1 obtained as white crystal (118 mg). The structure elucidation of MR-1 was performed with the help of 13CNMR, mass (ESI-MS) spectra and CHN analysis that confirmed as marmesin reported earlier [2,3-dihydro-2-(1-hydroxy-1 methyl ethyl)-7H-furo[3,2-g][1]benzopyran-7-one] (Figure 1) [21].

Marmesin (MR-1): C14H14O4 m.p.188-1900 (CHCl3-petrol); IR spectra: 3479,2977, 2929, 1703, 1630, 1572, 1485,1444, 1404 and 819 cm⁻¹; ¹H NMR: δ 1.23 and 1.37 (>CMe₂, 1.85(1H, br), 3.23 (2H, br d, J 8.8 Hz, H₂-1’), 4.74 (1H, t, J 8.8 Hz, H-2’), 6.21(1H, d, J 9.5 Hz, H-3), 6.74(1H, s, H-8), 7.22(1H, s, H-5), 7.59(1H, d, J 9.5 Hz, H-4); m/z (%) 246 (M +,39), 213(20), 188 (75), 187(100), 175(15), 160(30), 131(19), 59(66), 43(7) ; CHO % elements- (Oxyzen-25.915), (Carbon-67.191) and (Hydrozen-5.480).

2.4. Preparation of Crude Extract

Accurately weighed 5 g of the coarse powder of *F. limonia* stem barks were extracted with methanol (3 × 50 mL) under reflux (30 min each time) on a water bath. The combined extracts were filtered and concentrated, and transferred to a 25 mL volumetric flask and the volume was made up with methanol.

2.5. Preparation of Standard Solution

A stock solution of marmesin (100 µg mL⁻¹) was prepared by dissolving 1 mg of accurately weighed marmesin in methanol and making up the volume of the solution to 10 mL with methanol.

2.6. Chromatography

A Camag TLC system equipped with Camag Linomat V an automatic TLC sample spotter, Camag glass twin trough chamber (20 × 10 cm), Camag scanner 3 and integrated win CATS 4 Software were used for the analysis. TLC was performed on a pre-coated TLC plate silica gel60F254

![Figure 1. Mass spectroscopy and chemical structure of marmesin.](image-url)
TLC Determination of Marmesin, a Biologically Active Marker from *Feronia limonia* L.

(20 cm × 10 cm). Samples and standards were applied on the plate as 8 mm wide bands with an automatic TLC sampler (Linomat V) under a flow of N₂ gas, 10 mm from the bottom and 10 mm from the side and the space between two spots were 15 mm of the plate. The linear ascending development was carried out in a Camag twin trough chamber (20 cm × 10 cm) which was presaturated with 20 mL mobile phase chloroform: methanol (9.5:0.5 v/v) for 20 min at room temperature (25 ± 2°C and 40% relative humidity). The length of the chromatogram run was 9 cm. Subsequent to the development, TLC plates were dried under stream of hot air and then subjected to densitometric scanning using a Camag TLC scanner III (Camag, Switzerland) with win CATS software (version 1.4.1) in the absorbance- reflectance scan mode. Quantitative evaluation of the plate was performed in absorption-reflection mode at 338 nm. Quantification of marmesin in the extract of *F. Limonia* stem barks was performed by external standard method, using pure marmesin as standard.

2.7. Calibration Curve for Marmesin

Stock solution of marmesin (100 µg mL⁻¹) was prepared in methanol and different amounts (20–100 ng spot⁻¹) were applied on a TLC plate, using Linomat V for preparing five point calibration graphs of peak area versus concentration. The regression equation for marmesin was 1089.554 + 230.603x and co-relation coefficient ($r^2$) was 0.999.

2.8. Quantification of Marmesin in Test Sample

Ten microlitres of sample solution were applied in triplicate on a TLC plate and developed, scanned as above. Peak areas were recorded and the amount of marmesin was calculated using the calibration plot.

2.9. Specificity

The specificity of the method was ascertained by co-analyzing standard and sample. The band for marmesin in sample was confirmed by comparing the $R_f$ (0.49) and absorption spectra of the spot to that of reference compound. The peak purity of marmesin peak in sample track was assessed by comparing the spectra at peak start, peak apex and peak end positions of the band. Good correlation was also obtained between standards and sample overlay spectra ($r^2 > 0.99$).

2.10. Method Validation

The method was validated for precision, accuracy and repeatability [22]. Instrumental precision was checked by repeated scanning of the same spot 20 and 100 ng five times and was expressed as coefficient of variance (% RSD). Method precision was studied by analyzing the standards 20 and 100 ng per spot under the same analytical procedure and lab conditions on the same day and on different days (inter-day precision) and the results were expressed as % RSD. Accuracy of the method was tested by performing the recovery studies of the pre-analysed sample with standard at three levels (55.02, 68.78 and 82.53 µg mL⁻¹), % recovery and average % recovery were calculated.

3. Result and Discussion

Chloroform: methanol (9.5:0.5 v/v) gave the best resolution and satisfactory separation of the components in the extracts with well resolved peaks. A total of nine peaks were observed methanol extracts of samples. A comparative chromatographic display is shown in Figures 2(a) & 2(b). The densitometric scanning was therefore performed at a wavelength of 338 nm. The identities of the bands of marmesin ($R_f = 0.49$), in the sample extract were confirmed by overlaying their absorption spectra with those of the standard compounds using the TLC Scanner 3. The peak purity of the separated marmesin was confirmed by recording the absorption spectra at start to middle and middle to the end of the peak.

![Figure 2](a) TLC chromatogram, for a standard marmesin in methanol, Calibration curve and Three-dimensional overlaid chromatogram of standard track and sample track for marmesin; (b) TLC chromatogram, for stem barks methanolic extract of *Feronia Limonia*.}

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3.1. System Suitability Test

3.1.1. Linearity and Detection Limit
Linearity was checked by applying standard solutions of marmesin at five different concentration levels. The calibration curve was drawn in the concentration range of 20–100 ng spot⁻¹ (Figures 2(a) and 2(b)). The equation for the calibration curve of marmesin is $Y = 1089.554 + 230.603x$ and the correlation coefficient of the calibration plot was 0.999 indicating good linearity. Results of regression analysis on the calibration curve and detection limits are presented in Table 1(a).

3.1.2. Precision Studies
Instrumental precision was checked by repeated scanning of the same spots (20 and 100 ng spot⁻¹) of standard marmesin five times and the RSD values were 1.56 and 1.82 for 20 and 100 ng spot⁻¹, respectively. To determine the precision of the developed assay method 20 and 100 ng spot⁻¹ of the marmesin standard was analysed five times within the same day to determine the intra-day variability. The RSD values were 3.41 and 6.29 for 20 and 100 ng spot⁻¹, respectively. Similarly the inter-day precision was tested on the same concentration levels on 2 days and the RSD values were 2.68 and 2.83, respectively (Table 1(b)).

3.1.3. Sample Analysis and Recovery Studies
This developed TLC method was subsequently applied for the analysis of marmesin in the methanolic extract of Feronia limonia stem barks. The marmesin content of the stem barks by this proposed method was found to be 0.03412%. For the examination of recovery rates, 80, 100 and 120% of pure marmesin were added to preanalyzed sample and quantitative analysis was performed. The average recovery was 98.83 (Table 1(c)).

4. Conclusions
Thin layer chromatography is a globally accepted, rational and practical solution to characterize the crude plant drug along with pharmacologically active constituent enriched standardized extracts and their formulations. TLC method on silica gel 60F₂₅₄ with chloroform–methanol (9.5:0.5, v/v) was developed and densitometric evaluation was performed at 338 nm. This method is simple, specific, precise, accurate and robust for the determination of marmesin [2,3-dihydro-2-(1-hydroxy-1-methylethyl)-7H-furo[3,2-g][1]benzopyran-7-one]. This standardized TLC procedure may be used effectively for the screening analysis as well as quality evaluation of the plant or its derived herbal products.

REFERENCES


