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A validated HPTLC Method for Simultaneous estimation of two marker compounds in *Aegle marmelos* (L.) Corr., (Rutaceae) root bark

Sweety Lanjhiyana[a], Kartik Chandra Patra[b*], Dheeraj Ahirwar[a], Avtar Chand Rana[c] and Sanjay Kumar Lanjhiyana[b]

[a]School of Pharmacy, Chouksey Engg. College, Bilaspur
[b]Institute of Pharmaceutical Sciences, Guru Ghasidas University, Bilaspur
[c]Rayat Institute of Pharmacy, Railmajra

ABSTRACT

To develop and validate a HPTLC method for the simultaneous quantification of aegelin and skimmianine in the barks of Aegle marmelos (L.) Corr. (Rutaceae) for the first time. The HPTLC system (CAMAG, Muttanz, Switzerland) with WinCATS software under window XP was used for the analysis of constituents. Separation of each constituents were obtained with the help of HPTLC plates pre-coated with silica gel 60F-254 plates (10×10 cm^2) and solvent system of toluene-ethyl acetatate (7: 3, v/v). Scanning wavelength 262 nm was used for analysis in reflection/absorption mode. The developed method was validated for linearty, LOD, LOQ, accuracy and precision. Well defined spots were obtained in the solvent system of toluene: ethyl acetate (7:3,v/v).The linearity range for aegelin and skimmianine were found to be 1-12 µg/spot with correlation coefficients of 0.996±0.0054 and 0.987±0.0046, respectively, which were indicative of good linear dependence of peak area on concentration. Limit of detection (LOD) (0.101 ng and 0.125 ng for aegelin and skimmianine), limit of quantification (LOQ) (0.639 ng and 0.736 ng for aegelin and skimmianine), accuracy (% recovery 94.45 ±0.984 for aegelin and 95.76 ± 1.103 for skimmianine), and precision (CV< 5%, for both intra-day and inter-day precisions). The method was found to be reproducible and convenient for quantitative analysis of these compounds in Aegle marmelos (L.) Corr.

Key words: *Aegle marmelos* (L.) Corr., aegelin, skimmianine, HPTLC.

INTRODUCTION

Aegle marmelos (L.) Corr., (Rutaceae) popularly known as bael is native to India found widely throughout the Indian peninsula, Pakistan, Thailand, Bangladesh, Burma, and Sri Lanka. It is widely used for the treatment of wide variety of ailments [1] in Ayurveda, Unani and Siddha systems of medicines. The leaves of bael were reported to posses several pharmacological activities such as anti-inflammatory, antipyretic, [2] analgesic [3], hepatoprotective [4] and

antifungal [5] properties. It is also useful in treatment of cancer, diarrhoea, dysentery, asthmatic complications [6], hypoglycemic and antioxidant [7-10]. Stem and root bark are used to treat ulcers [11], dyspepsia, and stomachalgia [12]. The fruit (unripe) used for diarrhea, dysentery, ulcers [12], hypoglycemic [12]. The fresh flowers allay thirst and vomiting, useful in dysentery where as seeds were reported to posses antimicrobial, anthelmintic, hypoglycemic activities. Documented report suggests that the plant consisting of numerous biologically active compounds such as aegelin, citronellal, cineole, citral, marmelosin, aurapten, cuminaldehyde (4-isopropylbenzaldehyde), eugenol, lupeol, skimmianine, marmesinin, luvangetin, fagarine, marmelide, tanin, marmin and psoralen [6]. Further aegelin and skimmianine are two important marker components of bael which has not been reported in the root bark of *Aegle marmelos* (L.) Corr., further more there is no documented report for the simultaneous estimation of these two marker compounds. So we have taken attempt to isolate these marker components from root bark of the same and to develop a validated HPTLC method for the simultaneous estimation of these two marker components in root bark.

MATERIALS AND METHODS

2.1 Plant drug

Aegel marmeolos (L.) Corr. root bark was collected from dense forest of Achanakmar foothills located at Chhattisgarh State (India) in the month of August 2008 and authenticated by Dr N Shiddamallayya of Regional Research Institute (Ay.), Bangalore (India), where Voucher Specimen (No. *RRCBI/Mus/3*) of the plant had been submitted.

2.2 Chemicals and Reagents

All chemicals were of analytical grade and obtained from Sigma Aldrich Mumbai and used with out further purification. The HPTLC plates Si 60F254 (20 cm × 20 cm) were purchased from E. Merck (Darmstadt, Germany) supplied by Anchrom Technologies, Mumbai, India).

2.3 Plant material and extraction

Bark was collected manually from the root, washed thoroughly under running tap water and shade dried at room temperature (25-30°C) for 15 days. The dried bark were subjected to pulverized using hammer mill to a coarse powder and thereafter screened through 40 # mesh sieve. Powdered mass (500 g) was extracted with methanol at 45-65°C with the help of soxhlet apparatus for 72 hours. Resulting extracts was concentrated into viscous semisolid mass (dark greenish to brown colored) in rotary evaporator at 40^0 C under reduced pressure and dried using hot air oven (45^0C) and then stored in airtight containers in refrigerator between 4-8°C for further use [Percent yield (3.6% w/w)].

2.4 Column chromatography of Aegel marmeolos (L.) Corr.

The methanolic extract was subjected to column chromatography on silica gel and eluted with n-hexane followed with a mixture of n-hexane: ethyl acetate with increasing order of their polarity (Ratio pattern: 100:00- 00:100). The flow rate through the column was adjusted to 20 drops/ min. Total 125 fractions (50 ml each) were collected and pooled according to their similarity on TLC pattern. Combined fractions 1-12 (A, 0.5 mg), 13-23 (B, 0.4 mg), 24-30 (C, 2.1 mg), 31-41 (D, 1.6 mg), 42-53 (E, 0.7 mg), 54-72 (F, 1.5 mg), 73-83 (G, 0.6 mg), 84-108 (H, 1.1 mg), 109-118 (I, 0.9 mg) and 119-125 (J, 1.2 mg) were obtained. The fraction D was applied into a preparative thin layer chromatography (PTLC) using n-hexane: chloroform (60:40). Two compounds were isolated and purified from fraction D. The isolated compounds were undergo spectral analysis, by comparing the FTR, H-NMR, COSY, and Mass spectra, spectral data (Riyanto et al., 2001) with that of standard the isolated compounds were found to be aegelin and skimmianine.

2.5 Simultaneous estimation of aegelin and skimmianine by HPTLC

2.5.1 HPTLC Instrumentation

HPTLC was performed on 10 cm × 10 cm aluminum foilbacked plates coated with 250-µm layers of Si 60F254 (E. Merck, Darmstadt, Germany; supplied by Anchrom Technologies, Mumbai, India). Samples and standards were applied to the plates as 5 mm bands, 10 mm apart, 15 mm from the bottom and left edge of the plate by use of a CAMAG (Muttenz, Switzerland) Linomat IV sample applicator equipped with a 100-µL Hamilton syringe. Plates were developed using a mobile phase consisting of toluene: ethyl acetate (7:3, v/v). Linear ascending development was carried out in 10 cm×10 cm twin trough glass chamber (Camag Muttenz, Switzerland) equilibrated with mobile phase. The optimized chamber saturation time for mobile phase was 15 min at room temperature. The length of chromatogram run was 7 cm. Approximately, 10ml of the mobile phase (5 ml in trough containing the plate and 5ml in the other trough) was used for each development, which required 8 min. It results in better apparent resolution with more convenient capability of the detecting device to perform integration of peak area. Subsequent to the development, TLC plates were dried in a current of air with the help of an air-dryer. The slit dimension settings of length 5.00 mm and width 0.45 mm, and a scanning rate of 20 mm/s was employed. The monochromator band width was set at 20 nm. Densitometric scanning was performed on Camag TLC scanner in the reflectance mode at 262 nm and operated by win CATS Planar chromatography version 1.1.5.0. The source of radiation utilized was halogen tungsten lamp. Concentrations of the compound chromatographed were determined from the intensity of diffusely reflected light. Evaluation was via peak area with linear regression.

2.5.2 Development of the optimum mobile phase

The composition of the mobile phase for development of chromatographic method was optimized by testing different solvent mixtures of varying polarity.

2.5.3 Preparation of standard curve

Standard stock solution of aegelin and skimmianine was prepared in methanol in the concentration range 1mg/ml each respectively. Different volumes of stock solution 1,5,10 and 12 µl were spotted in triplicate on TLC plate to obtain concentrations of 1,5,10 and 12µg per spot of aegelin and skimmianine respectively. The data of peak area versus drug concentration were treated by linear least-square regression.

2.5.4 Determination of aegelin and skimmianine in Aegle marmelos (L.) Corr.root bark

The extracted solution was applied on TLC plate followed by development and scanning. Analysis was repeated in triplicate. The content of aegelin and skimmianine were determined by extrapolation from standard curve.

2.5.5 Purity of Spot in chromatogram

The spot obtained on the chromatogram were analysed at wavelength 262 nm which is the average of maximum wavelength for aegelin (250nm) and skimmianine (275nm) respectively at three points i.e. point start to middle, middle and finally middle to end in the standard as well as in sample.

2.5.6 Validation of the method

2.5.6.1 Sensitivity

The sensitivity of the method was determined with respect to LOD, LOQ, linearity range and correlation coefficient. Solutions containing 1-12µg of aegelin and skimmianine respectively

were spotted on TLC plate. The LOD was calculated as 3 times the noise level and LOQ was calculated as 10 times the noise level.

2.5.6.2 Accuracy (Recovery study)
Recovery of aegelin and skimmianine was determined by spiking aegelin and skimmianine in three different concentrations covering the low, medium and high ranges of the calibration curve. The samples were then extracted and analyzed. The recovery was calculated by comparing the resultant peak areas with those obtained from pure standards in methanol at the same concentrations.

2.5.6.3 Precision
Different amount of aegelin and skimmianine covering low, medium and higher ranges of the calibration curve were spotted on the TLC plate. These spots were analyzed by using above described HPTLC method. Precision was expressed as the percent relative standard deviation (% R.S.D.). Five micro litre aliquots of sample containing 5µg of aegelin and skimmianine were analyzed according to the proposed method. In order to control the scanner parameters, one spot was analyzed several times. By spotting and analyzing the same amount several times (n=3) the precision of the automatic spotting device and the derivatization technique, was evaluated.

2.5.6.4 Robustness of the method
By introducing small changes in the mobile phase composition, the effects on the results were examined. Mobile phases having different composition of toluene: ethyl acetate (7:3 and 7:2 v/v) was tried at two different concentration levels of 5 and 10 µg per spot for aegelin and skimmianine.

RESULTS

By comparing the spectral data of isolated compounds it was confirmed that the isolated compounds were aegelin and skimmianine. So a validated HPTLC method was developed to standardize the root bark for the content of aegelin and skimmianine. The composition of the mobile phase used in this method was optimized by testing solvent mixtures of different polarity. It was found that toluene-ethyl acetate (7: 1, v/v) enabled good resolution with Rf values of 0.56 and 0.65, for aegelin and skimmianine, but typical peak nature was missing. The mobile phase toluene-ethyl acetate (7: 3, v/v) gave sharp and well-defined peaks at Rf values of 0.74 and 0.85 respectively **(Fig. 1&2),** when the chamber was saturated with the mobile phase for 15 min at room temperature. This enabled more accurate integration of peak area. Aegelin and skimmianine showed a good linear relationship over the concentration range of 1-12 µg per spot with respect to peak area. The linearity was observed with the regression coefficient being 0.996 with standard error of the mean (SEM) at 0.0054 for aegelin and 0.987 with SEM of 0.0046 for skimmianine. No significant differences were observed in the slopes of the standard curve. The purity of the spots scanned at the wavelength of 250 and 275 nm had a value of r (S, M) within the range 0.998-0.999 and r (M, E) was within the range 0.997-0.998 **(Fig. 3&4).** LOD, LOQ, accuracy and precision were evaluated for quantitative purposes. LOD (0.101 ng & 0.125ng for aegelin and skimmianine) and LOQ (0.639 ng & 0.736 ng for aegelin and skimmianine) values suggest the high efficiency of the process. Accuracy in terms of percentage of recovery (94.45 ± 0.984 % for aegelin and 95.76 ± 1.103 % for skimmianine) shows a high extraction efficiency of aegelin and skimmianine.

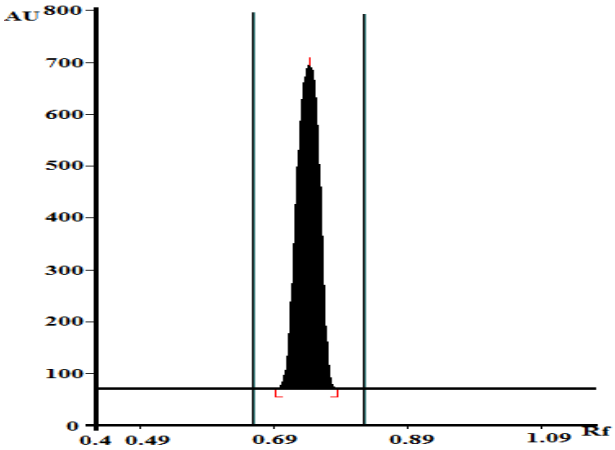

Fig 1(A) Typical HPTLC Chromatogram of aegelin (Rf=0.74).

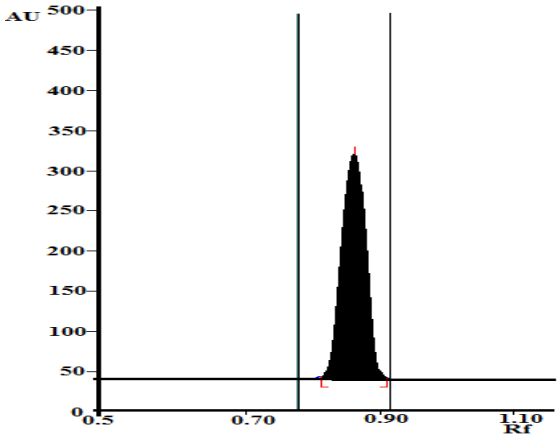

Fig 1(B)Typical HPTLC Chromatogram of skimmianine (Rf=0.85).

The percentage of coefficient of variance for intra-day and inter-day precisions was found to be 4.2 and 4.8 for aegelin and 4.7 and 4.3 for skimmianine, respectively, which is comparable and within the limits.

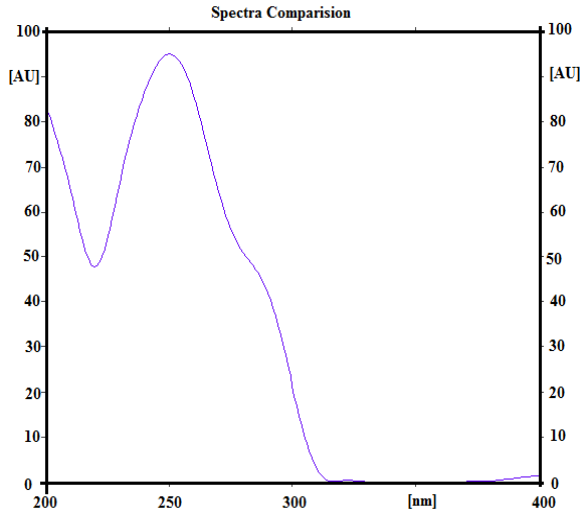

Figure 3. Peak purity spectra of aegelin at 250nm.

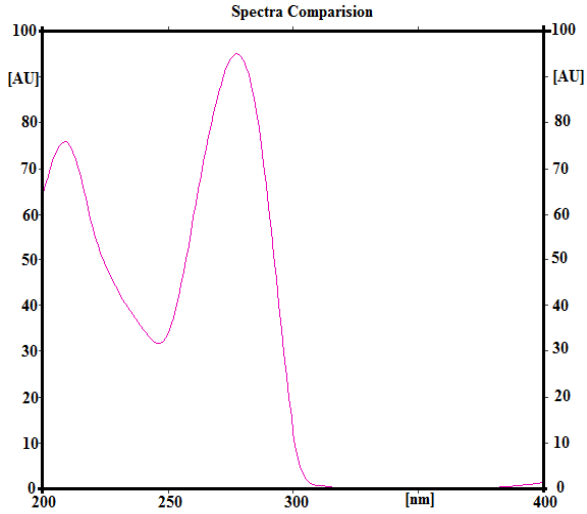

Figure 4. Peak purity spectra of skimmianine at 275nm.

DISCUSSION

Hence, the proposed method can be used for the estimation of aegelin and skimmianine in root bark. The proposed HPTLC technique is found to be precise and accurate. Further, the method is sensitive enough for the analysis of aegelin and skimmianine in crude drugs as well as pharmaceutical formulations containing *Aegel marmeolos* (L.) Correa root bark. With the growing demand of herbal drugs in the herbal drug market and with the increased belief in the usage of 'green' medicine (herbal drugs), this standardisation tool will help in maintaining the quality of this important crude drug.

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