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ORIGINAL ARTICLE

# HPTLC densitometric quantification of stigmasterol and lupeol from *Ficus religiosa*

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**KEYWORDS**

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**Abstract** This study presents the first report of TLC densitometric method, which has been developed and validated for simultaneous quantification of the two marker compounds (stigmasterol and lupeol) from methanolic extract using the solvent system of toluene:methanol (9:1, v/v). The method employed TLC aluminum plates precoated with silica gel 60 $F_{254}$ as the stationary phase. Densitometric analysis of stigmasterol and lupeol was carried out in the reflectance mode at 525 nm. The system was found to give compact spots for stigmasterol and lupeol ($R_f$ value of 0.37 and 0.60, respectively). The method was validated using ICH guidelines in terms of precision, repeatability and accuracy. Linearity range for stigmasterol and lupeol was 80–480 ng/spot and 150–900 ng/spot and the contents were found to be 0.06 ± 0.005% w/w and 0.12 ± 0.02% w/w, respectively. The limit of detection (LOD) value for stigmasterol and lupeol were found to be 20 and 50 ng, and limit of quantification (LOQ) value were 60 and 100 ng, respectively. This simple, precise and accurate method gave good resolution from other constituents present in the extract. The method has been successfully applied in the analysis and routine quality control of herbal material and formulations containing *Ficus religiosa*.



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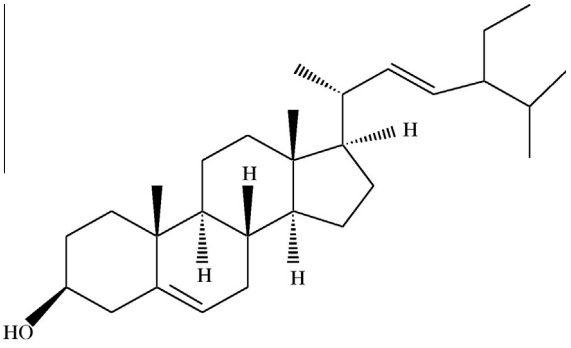
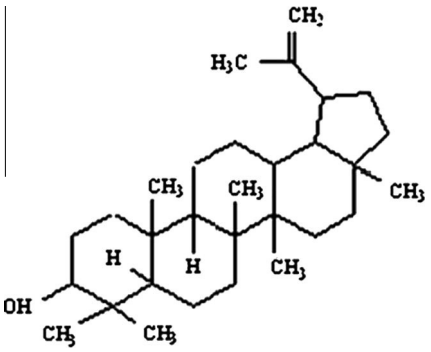
## 1. Introduction

*Ficus religiosa* Linn a perennial plant belonging to the Amaryllidaceae family grows in Sub-Himalayan tracts, commonly known as the Bodhi tree, have traditionally been used in Indian folk medicines for respiratory disorders and some skin diseases (Mousa et al., 1994). It is recommended for throat diseases, kidney stones, blindness, otitis, rheumatism, bone dislocations, sprains and fractures, mastitis, jaundice, bloody dysentery, diarrhoea, glossitis, haematuria, miscarriage, indigestion, hernia (http://www.divineremedies). The bark and leaves are taken for diarrhoea and dysentery. The powdered fruit is taken for asthma and the latex is used to treat warts. The bark is astringent, cooling, haemostatic and laxative, also used in diabetes, diarrhoea, leucorrhoea, menorrhagia and nervous disorders, for vaginal and other urinogenital disorders (Kirtikar, 1975). Medicated oil made from the root bark is applied externally to skin diseases such as eczema, leprosy and is also used in rheumatism. Recently, the methanol extract of *F. religiosa* has been reported to have neurotrophic effects and acetylcholinesterase inhibitory activity (Vinutha et al., 2007). It also exhibits antiinflammatory properties (Jung et al., 2008) and several studies have focused mainly on its antitumor, antibacterial (Nair and Chanda, 2007), anthelmintic activity (Iqbal et al., 2001), antifungal activity (Khan et al., 2007; Aqil and Ahmad, 2003), kidney and urinary disorders (Ballabh et al., 2008). The plant is reported to contain beta-sitosteryl-D-glucoside, Vitamin K, *n*-octacosanol, methyl oleanolate, lanosterol, stigmasterol, lupeol, campestrol, 28-isofucosterol, a-amyrin, β-amyrin. Bergapten and bergaptol have been isolated from the bark. Asparagine and tyrosine have been isolated from the fruit (http://www.divineremedies). Polyphenols and sterols are also reported to be present in the fruits. Stigmasterol (Scheme 1a) is reported to have antioxidant, thyroid inhibitory, antiperoxidative, hypoglycemic (Panda et al., 2009; Jamaluddin et al., 1994) hypocholesteromic (Battaab et al., 2006) and anti-inflammatory activity (Gabay et al., 2008) and lupeol (Scheme 1b) is reported to have anti-inflammatory (Geetha and Varalakshmi, 2001), hepatoprotective (Sunitha et al., 2001) and anticancer activity (Chaturvedi et al., 2008; Saleem et al., 2001; Nigam et al., 2009; Saleem, 2009).

Nowadays, HPTLC has become a routine analytical technique due to its advantages of reliability in quantitation of analytes at micro and even in nanogram levels and cost effectiveness (Rathee et al., 2010). It has proved a very useful technique because of its low operating cost, high sample throughput and need for minimum sample clean-up. The major advantage of HPTLC is in reducing analysis time and cost per analysis (Rathee et al., 2010) TLC has been known as the fast tool for the detection of compounds. Another advantage of TLC is the capability to detect more compounds than HPLC, although the resolution is poorer. In this regard, the compounds which cannot be eluted still can be detected. Moreover, the compounds having no UV absorption, e.g., sugar, still can be detected by reagent spraying. The TLC chromatogram pattern comparison seems to be promising for fingerprinting the active compounds in plant extracts. Thus, it can be used as a tool in the quality control in order to warranty that the active compounds are extracted. By means of data analysis system and optimized experimental conditions, HPTLC is also feasible for development of chromatographic fingerprint methods to determine and identify complex herbal extracts just like HPLC and GC (Chen et al., 2006). Furthermore, the colorful picture like HPTLC image provides extra intuitive parameters of visible color and or fluorescence and, unlike HPLC and GC, HPTLC can simultaneously determine different samples on the same plate. Such an approach causes the HPTLC method to maintain its innate advantage as well as get over the limitations of developing distance and plate efficiency. Some of the analytical methods reported for the qualitative and quantitative analysis of stigmasterol and lupeol are discussed herewith. Kpoviéssia et al. (2008) developed the method for determination of sterols and triterpenes using capillary gas chromatography. Haliński et al. (2009) studied the chromatographic fractionation and analysis of the main constituent's sterols and triterpenes by HPTLC. Qualitative and quantitative standardization of stigmasterol and lupeol was performed using HPTLC but not from this plant by Singh et al. (2009). Lupeol alone was also quantified by Shrishallappa et al. (2002), Anandjiwala et al. (2007), Darekar et al. (2008), Padashetty and Mishra (2007), Shrishallappa et al. (2002), and Rahul et al. from plants and polyherbal formulation. A rapid quantification of free and esterified phytosterols using APPI-LC-MS/MS was done by Lembcke et al. (2005). Determination of stigmasterol in dietary supplements by gas chromatography was performed by Sorenson and Sullivan (2006), and HPTLC determination of Stigmasterol was done by Hamrapurkar and Karishma (2007). Literature survey revealed that no method has been reported for the simultaneous quantitation of stigmasterol and lupeol from *F. religiosa* fruits extract. So, in the present study, a HPTLC method for the simultaneous quantification of stigmasterol & lupeol has been developed.




**Scheme 1a** Structure of stigmasterol.




**Scheme 1b** Structure of lupeol.

## 2. Experimental

### 2.1. Plant material

The fruits of *F. religiosa* were collected in the month of Nov–Dec 2008 from Chandigarh and were authenticated from Department of Botany, Punjab University, Chandigarh. The plant material was dried under shade, stored in airtight glass bottle at 30 °C and powdered to 40 mesh when required.

### 2.2. Chemicals

All chemicals used were of analytical grade and purchased from Qualigens Fine Chemicals, Mumbai, India. Stigmasterol (Purity: 97% w/w) and lupeol (purity: 99% w/w) were purchased from Natural Remedies Pvt. Ltd, Bangalore, India.

### 2.3. TLC conditions

TLC plates consisted of 20 × 10 cm, precoated with silica gel 60 $F_{254}$ TLC plates (E. Merck) (0.2 mm thickness) with aluminum sheet support. The spotting device was a CAMAG Linomat V Automatic Sample Spotter (Camag Muttenz, Switzerland); the syringe, 100 μL (from Hamilton); the developing chamber was a CAMAG glass twin trough chamber (20 × 10 cm); the densitometer consisted of a CAMAG TLC Scanner 3 linked to winCATS software; the experimental condition temperature 25 ± 2 °C, relative humidity 40%.

### 2.4. TLC fingerprinting profile

Though a TLC densitometric method was reported for the quantification of lupeol ( Anonymous. et al., 2005), we modified the method so that both the compounds (stigmasterol and lupeol) could be quantified simultaneously. For the quantification of Stigmasterol, we developed our own method.

### 2.5. Sample solution

Preparation of sample solutions were optimized to achieve good fingerprinting and also to extract the marker compounds efficiently. The preparation of selected sample solutions is given below:

Since the marker compounds were soluble in methanol, methanolic extract was prepared by accurately weighing 1 g of the powdered drug and extracted with methanol (25 ml × 4) under reflux on a water bath. The methanolic extract was filtered through Whatman I filter paper, filtrates were combined, concentrated under vacuum and the volume was made upto 25 ml in a volumetric flask. This extract was used for the quantification of stigmasterol and lupeol.

### 2.6. Standard solution of stigmasterol and lupeol

2 mg each of stigmasterol and lupeol were dissolved separately in methanol and the volume was made upto 25 ml with methanol in volumetric flask.

### 2.7. Solvent system

Toluene:methanol (9:1, v/v) for co-chromatography with stigmasterol and lupeol.

### 2.8. Procedure

For co-chromatography with stigmasterol and lupeol, 10 μl of sample solutions of methanolic extract along with the standard was applied on a TLC plate and the plate was developed in toluene:methanol (9:1, v/v) solvent system to a distance of 8 cm. The plates were dried at room temperature in air and derivatized with anisaldehyde–sulphuric acid reagent and heated at 105 °C for 5 min. The RF values and color of the resolved bands were noted.

### 2.9. Simultaneous quantification of stigmasterol and lupeol using HPTLC

#### 2.9.1. Sample solution

Sample solution (methanolic extract) described under the previous section was used for quantification of stigmasterol and lupeol.

#### 2.9.2. Preparation of standard solution of stigmasterol

A stock solution of stigmasterol (50 μg/ml each) was prepared by dissolving 5 mg of accurately weighed Stigmasterol in methanol and making up the volume of the solutions to 100 ml with methanol in a volumetric flask. The aliquots (1.6–9.6 ml) of each of the stock solutions was transferred to 10 ml volumetric flasks and the volume of each was adjusted to 10 ml with methanol to obtain standard solutions containing 8, 16, 24, 3, 40 and 48 μg/ml of stigmasterol.

#### 2.9.3. Preparation of standard solution of lupeol

A stock solution of lupeol (150 μg $ml^{-1}$) was prepared by dissolving 7.5 mg of accurately weighed lupeol in methanol and making up the volume of the solution to 50 ml with methanol in a volumetric flask. The aliquots (1–6 ml) of the stock solutions were transferred to 10 ml volumetric flasks and the volume of each was adjusted to 10 ml with methanol, to obtain standard solutions containing 15, 30, 45, 60, 75 and 90 μg $ml^{-1}$ of lupeol.

#### 2.9.4. Preparation of calibration curve of stigmasterol

10 μl each of the standard solutions of stigmasterol (80–480 ng/spot) were applied (band width: 6 mm, distance between the tracks: 12 mm) in triplicates on a TLC plate using automatic sample spotter. The plates were developed in a twin trough chamber (20 × 10 cm) up to a distance of 8 cm using a solvent system of toluene:methanol (10 ml) (9:1, v/v) at 25 ± 2 °C and 40% relative humidity. After development, the plates were dried at room temperature in air, derivatized with anisaldehyde–sulphuric acid reagent, heated at 105 °C for 5 min. and scanned densitometrically at 525 nm in absorbance mode using tungsten lamp. The area of the resolved peaks was recorded. Calibration curve of stigmasterol was obtained by plotting peak areas *vs* concentrations of stigmasterol applied.

*2.9.5. Preparation of calibration curve of lupeol*
10 µl each of the standard solutions of lupeol (150–900 ng/spot) were applied (band width: 5 mm, distance between the tracks: 10 mm) in triplicate on a TLC plate using automatic sample spotter. The plates were developed in a twin trough chamber (20 × 10 cm) up to a distance of 8 cm using a mobile phase of toluene:methanol (9:1 v/v) (10 ml) at 25 ± 2 °C temperature and 40% relative humidity. After development, the plates were dried at room temperature in air, derivatized with anisaldehyde–sulphuric acid reagent, heated at 105 °C for 5 min and scanned densitometrically at 525 nm in the absorbance mode using a tungsten lamp. The peak areas were recorded. Calibration curve of lupeol was obtained by plotting peak areas vs concentrations of lupeol applied.

*2.9.6. Simultaneous quantification of stigmasterol and lupeol in the sample*
10 µl of suitably diluted sample solution of methanolic extract was applied in triplicates on a TLC plate. The plates were developed and scanned as mentioned above. The peak areas were recorded and the amount of stigmasterol and lupeol was calculated using the calibration curve.

*2.10. Validation of the method*

ICH guidelines were followed for the validation of the analytical method developed (CPMP/ICH/281/95 and CPMP/ICH/381/95) for precision, repeatability and accuracy.

*2.10.1. Instrumental precision*
Instrumental precision was checked by repeated scanning ($n = 7$) of the same spot of stigmasterol and lupeol (160 ng/spot) and expressed as relative standard deviation (% R.S.D.).

*2.10.2. Repeatability*
The repeatability of the method was affirmed by analyzing 160 ng/spot of stigmasterol and lupeol individually on TLC plate ($n = 5$) and expressed as % R.S.D.

*2.10.3. Inter-day and intra-day variation*
Variability of the method was studied by analyzing aliquots of standard solution containing 80, 160, 240 ng/spot of stigmasterol and lupeol on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as % R.S.D.

*2.10.4. Limit of detection and limit of quantification*
For the evaluation of limit of detection and limit of quantification different concentrations of the standard solutions of stigmasterol and lupeol were applied along with methanol as blank and determined on the basis of signal to noise ratio. LOD was determined at an S/N of 3:1 and LOQ at an S/N of 10:1.

*2.10.5. Recovery*
The accuracy of the method was assessed by performing recovery study at three different levels (50%, 100% and 125% addition of stigmasterol and lupeol). The percent recoveries and the average percent recoveries were calculated.

*2.10.6. Specificity*
Specificity was ascertained by analyzing standard compounds and samples. The bands for stigmasterol and lupeol from sample solutions were confirmed by comparing the RF and spectra of the bands to those of the standards. The peak purity of all the compounds (Data not shown) was analysed by comparing the spectra at three different levels, i.e. start, middle, and end positions of the bands.

## 3. Results and discussion

### *3.1. TLC fingerprint and co-chromatography*

Chromatographic fingerprint analysis has shown to be a rational and feasible approach for the quality assessment and species authentication of traditional medicine (Xie et al., 2006; Qiao et al., 2007; Lu et al., 2006; Li and Wang, 2005). It utilizes chromatographic techniques to construct specific patterns of recognition for herbal drugs. The developed fingerprint pattern of components can then be used to determine not only the absence or presence of markers of interest but the ratio of all detectable analytes as well. Although high performance thin layer chromatography (HPTLC) has a few limitations, such as the limited developing distance and lower

**Table 1** TLC fingerprinting profile of *Ficus religiosa* fruits (sample solution and standard solution; solvent system) under UV 525 nm.

| S. No. | $R_f$ value | Color of the band |
|---|---|---|
| 1 | 0.14 | Purple |
| 2 | 0.17 | Green |
| 3 | 0.22 | Green |
| 4 | 0.26 | Green |
| 5 | 0.35 | Purple |
| 6 | 0.37 (stigmasterol) | Purple pink |
| 7 | 0.60 (lupeol) | Purple |

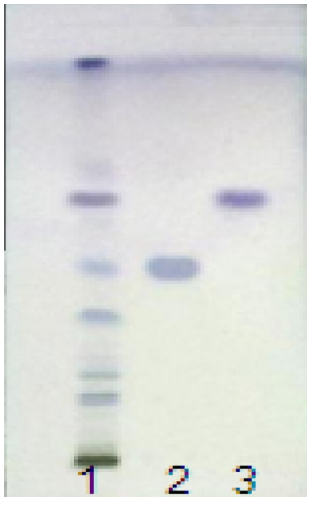



**Figure 1** TLC profile of *Ficus religiosa* fruits after derivatization at 525 nm; Spot 1 indicates sample solution and Spots 2 and 3 indicates stigmasterol and lupeol, respectively.

plate efficiency by comparison with HPLC and GC, it is still an effective tool for quality evaluation of herbal drugs due to its simplicity, low cost, and requirement, and it has been successfully utilized to develop the chromatographic fingerprint for botanical drugs (Chen et al., 2006; Anandjiwala et al., 2007; Qian et al., 2007). Moreover, the above mentioned shortcomings can be overcome by separately developing fractions of different polarity on two or several thin layer plates. Thus, the unique feature of the picture like image of HPTLC coupled with the digital scanning profile is gradually attractive to the herbal analysts to construct the herbal chromatographic fingerprint. This HPTLC could provide adequate information and parameters for comprehensive identification and differentiation of the two closely related herbal medicines.

**Table 2** Method validation parameters for the quantification of stigmasterol and lupeol by the proposed TLC densitometric method.

| S. No. | Parameter | Stigmasterol | Lupeol |
|---|---|---|---|
| 1 | Instrumental precision (% CV, $n = 7$) | 0.85 | 0.65 |
| 2 | Repeatability (% CV, $n = 5$) | 1.51 | 1.61 |
| 3 | Accuracy (average % recovery) | 99.92 | 99.79 |
| 4 | Limit of detection (ng) | 20 | 50 |
| 5 | Limit of quantification (ng) | 60 | 100 |
| 6 | Specificity | Specific | Specific |
| 7 | Linearity (correlation coefficient) | 0.996 | 0.997 |
| 8 | Range (ng/spot) | 80–480 | 150–900 |

**Table 3** Intra-day and inter-day precision of stigmasterol and lupeol.

| Marker | Concentration (ng/spot) | Intra-day precision* | Inter-day precision* |
|---|---|---|---|
| Stigmasterol | 80 | 0.13 | 0.26 |
| | 160 | 0.51 | 0.43 |
| | 240 | 0.47 | 0.61 |
| Lupeol | 80 | 0.19 | 0.21 |
| | 160 | 0.55 | 0.51 |
| | 240 | 0.43 | 0.56 |

* % R.S.D; mean ($n = 3$).

### 3.2. TLC densitometric quantification of stigmasterol and lupeol using HPTLC

There is no report of simultaneous quantification of stigmasterol and lupeol in *F. religiosa* fruits. Hence we developed a simple and precise method for quantification of these marker compounds. Stigmasterol and lupeol were resolved well at $R_f$ 0.37 and 0.60, respectively (Table 1, Fig. 1) from sample solutions of methanolic extract when the plate was developed in solvent system and derivatized as mentioned above.

The simplicity of the sample preparation, and the possibility of analyzing several sample of herbal products simultaneously in a short time, make HPTLC the method of choice. In the present method stigmasterol and lupeol were quantified from *F. religiosa* fruits by TLC densitometric method using HPTLC. The TLC densitometric method was validated in terms of precision, repeatability, and accuracy (Tables 2–4). The linearity range for stigmasterol and lupeol were 80–480 ng/spot and 150–900 ng/spot with correlation coefficient ($r$ values) of 0.996 and 0.997, respectively. The TLC densitometric method was found to be precise with R.S.D for intraday in the range of 0.13–0.51 and 0.19–0.55 and for interday in the range of 0.26–0.61 and 0.21–0.56 for different concentrations of stigmasterol and lupeol (Table 3). This indicates that the proposed method was precise and reproducible. The limit of detection (LOD) value for stigmasterol and lupeol were found to be 20 and 50 ng, and limit of quantification (LOQ) value were 60 and 100 ng, respectively (Table 2). The average percent recoveries at 3 different levels of stigmasterol and lupeol were found to be 99.92% and 99.79%, respectively (Table 4). The

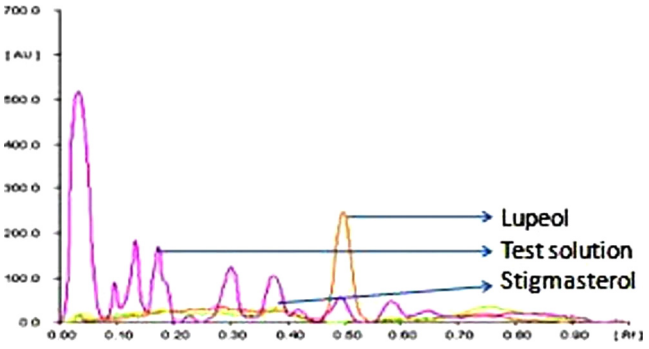



**Figure 2** TLC densitometric scan at 525 nm of test solution of *Ficus religiosa* fruits; stigmasterol and lupeol standard solution (green line and brown line); test solution (pink line).

**Table 4** Recovery studies of stigmasterol and lupeol at 50%, 100% and 125% addition by the proposed TLC densitometric method.

| Marker | Amount of marker present (μg) | Amount of marker added (μg) | Amount of marker found (μg) | Recovery* (%) | Average recovery (%) |
|---|---|---|---|---|---|
| Stigmasterol | 45 | 22 | 67.54 ± 4.71 | 100.81 ± 1.05 | 99.92 |
| | 45 | 45 | 89.75 ± 7.14 | 99.72 ± 1.19 | |
| | 45 | 56 | 99.23 ± 3.13 | 99.22 ± 0.87 | |
| Lupeol | 50 | 25 | 74.73 ± 2.51 | 99.42 ± 0.64 | 99.79 |
| | 50 | 50 | 100.19 ± 2.11 | 100.11 ± 0.40 | |
| | 50 | 62 | 110.69 ± 2.01 | 99.86 ± 0.34 | |

* Mean ± SD ($n = 3$).

contents of stigmasterol and lupeol quantified using TLC densitometric methods were found to be 0.06 ± 0.005 and 0.12 ± 0.02% w/w, respectively (Fig. 2).

## 4. Conclusion

A TLC densitometric method for the quantification of stigmasterol and lupeol from *F. religiosa* fruits using HPTLC was established. The method was found to be simple, precise, specific sensitive and accurate and can also be used for the quantification of stigmasterol and lupeol in the herbal raw materials. It can also be used in routine quality control of herbal materials as well as formulations containing any or both of these compounds.

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