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Original Article

High-Performance Thin Layer Chromatography (HPTLC) Analysis for the Quantification of Constituents in *Ficus religiosa*

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ABSTRACT

Background: *Ficus religiosa*, revered for its medicinal properties in traditional medicine, has drawn scientific interest due to its diverse chemical composition. This study aimed to develop a densitometric High-Performance Thin Layer Chromatography (HPTLC) technique to estimate phenolic compounds present in the hydroalcoholic extract of dried root bark of *Ficus religiosa*. **Materials and Methods:** This study utilized HPTLC to identify the constituents within *Ficus religiosa* root bark. The isolated constituent was evaluated by various analytical methods like phytochemical tests, UV, HPTLC etc. Subsequently, the HPTLC method underwent rigorous validation, confirming its reliability and accuracy in separating and quantifying the constituents within the root bark of *Ficus religiosa*. **Results:** Isolation of phytoconstituents was done by column chromatographic technique using toluene: ethyl acetate: methanol (3:3:2). The isolated component was confirmed as a flavonoid moiety by various chemical tests and by analytical evaluation. Also, HPTLC analysis of the isolate revealed the compound was Quercetin a flavonoid moiety or its derivative. **Conclusion:** These findings underscore the presence of main phytoconstituents in *Ficus religiosa* root bark which is responsible for pharmacological effects and offers insights into its application in both traditional and contemporary medicinal practices.

Keywords: HPTLC, *Ficus religiosa*, Quantification, Flavonoids, Validation.

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INTRODUCTION

Herbal medicine is the study and application of the medicinal qualities present in plants, which can generate a wide range of chemical compounds. Certain phytoconstituents included in herbal medicine have been shown to have improved systemic compatibility with humans. Medicinal plants can heal because they contain a variety of secondary metabolites and complicated compounds.[1] *Ficus religiosa* (L.), which grows in the Himalayas up to 170 meters above sea level and is widely distributed over the plains of India, is an essential herb in traditional medicine (Figure 1). *Ficus religiosa*, commonly found in Southeast Asia and the Indian subcontinent has a rich history of traditional medicinal applications for treating various ailments. Its profound cultural and religious significance in Indian mythology leads to its deliberate planting near spiritual sites within cities and villages in India.[2]

Ficus religiosa, renowned for its presence in traditional medicine and its use mainly in Ayurvedic medicine systems, has been linked to various health advantages. These encompass its potential in promoting wound healing, safeguarding the liver, reducing inflammation, countering oxidation, preventing ulcers, inhibiting tumors, aiding memory, combating certain cancers, acting as an acetylcholinesterase inhibitor, enhancing cognitive functions, and alleviating arthritis.[3] Preparations of herbal medicines use nearly every component of the plant. Numerous phytoconstituents have been identified in *Ficus religiosa* according to recent investigations. The components of the bark include phenol, alkaloids, flavonoids, bergaptol, bergapten, lanosterol, and sitosteryl-D-glucoside.[4] These substances are efficient against bacteria like *Bacillus cereus, Streptococcus facealis, Azobacterchroococcum,* and *-megaterium*. Bioactive chemicals found in the leaves help prevent gastrointestinal problems. These compounds include campesterol, isofucosterol, stigmasterol, arginine, tannins, aspartic acid, serine, threonine, glycine, proline, alanine, tyrosine, tryptophan, methionine, valine, and isoleucine. The bark leaves and fruits can all be extracted with methanol, ethanol, and water. Asparagine, undecane, tyrosine, tetradecane, tridecane, limonene, ocimene, dendrolasine, flavonoids (kaempferol, myricetin, quercetin), and the other

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Figure 1: *Ficus religiosa* plant.

phenolic components are among the beneficial substances found in the fruits.[5]

Assessing raw materials is crucial to ascertain their identification, purity, and overall quality. HPTLC stands as an invaluable tool for both qualitative and quantitative analysis, ensuring raw material identification, and purity, and maintaining quality control in final products. Recent research indicates that methods like Thin Layer Chromatography (TLC) and HPTLC can be utilized across diverse sectors such as medicine, pharmaceuticals, chemistry, biochemistry, and toxicology, addressing a wide array of qualitative and quantitative analytical challenges.[6]

Due to its simplicity and minimal sample preparation requirements, densitometric HPTLC is commonly employed to appraise the phytochemical content of herbal remedies. In this study, a densitometric HPTLC technique was devised to gauge the quantity of phenolic compounds present in the hydroalcoholic extract of dried root bark from *Ficus religiosa*. The extract was prepared using the Soxhlet extraction method.

MATERIALS AND METHODS

Collection of plant material

The root bark of the *Ficus religiosa* plant (Figure 2) was sourced from Kozhikode, Kerala, India. Dr. A.K. Pradeep, an Associate Professor in the Department of Botany at Calicut University, confirmed the plant's authenticity through specimen number 88488. After a meticulous cleansing with tap water, the root bark underwent shade drying and was finely pulverized. Currently, it is stored in an airtight receptacle for future research purposes.

Figure 2: Root bark of *F. religiosa*.

Physiochemical evaluation of plant material

To ascertain the quantities of extraneous organic substances, moisture loss, overall ash content, acid-insoluble residue, water-insoluble residue, and sulphated ash, a range of physicochemical parameters were gauged.

Foreign organic matter

Accurately weigh 50 g of the initial sample and distribute it as a thin layer. Examined the sample without assistance or with the aid of a 6 X lens and made every effort to remove any alien biological matter physically.

Loss on drying

Around 1 g of the coarsely powdered medication was transferred into a porcelain dish, which was weighed and dried for 30 min at 100-105°C in the oven. Weighed and cooled the contents. Retried the drying process for 30 min at 100°C to105°C while keeping the contents inside a hot air oven.

Determination total ash

The crucible was removed from the desiccator and weighed after 30 min of red-hot heating. Evenly distribute 2 g of the material being studied into the crucible. Let it air dry at 100°C to 105°C for an hour. After that, the weight was raised to a constant temperature by burning in a muffle furnace at 600 ± 25°C.

Determination of acid-insoluble ash

The ash-less filter paper was used to filter the ash that was obtained after 5 min of boiling 25 mL of 2M hydrochloric acid. The filter paper in the silica crucible came into contact with fire. After cooling, ash that remained insoluble in acid is weighed.

Determination of water-soluble ash

Boil 25 mL of water with the ash for five minutes. A gooch crucible or filter paper that does not contain ash may be used to gather the insoluble material. Light it for 15 min at a temperature below 450°C followed by thorough washing with hot water. The overall weight of insoluble stuff must be reduced by the ash's weight. This weight differential represents the water-soluble ash. It is required to use the medication that has been air-dried to calculate the proportion of water-soluble ash.

Determination of sulfated ash

To get rid of the fumes, the ash was boiled in a milliliter of sulfuric acid for 5 min. Once all the black particles had been removed, the mixture was ignited at 600 ± 25°C. After the crucible had cooled, a few drops of sulfuric acid were added and brought to a boil. Weigh and continue this process until there is not more than a 0.05 mg difference between two subsequent weights.[7]

Preparation of Extract

The extraction process involved 50 g of root bark, which was gathered, cleaned, dried, and coarsely ground before being securely sealed in a thimble. Water and ethanol were chosen as solvents for their ability to effectively extract compounds from plant materials due to their polarity and solvent strength.

Using a Soxhlet apparatus maintained at 40°C. The extraction was conducted via hot continuous percolation. A mixture of 30% water and 70% ethanol was employed as the solvent, with the extraction process running for 72 hr or until the solvent in the siphon tube became colorless. [8] Once the extraction was complete, the mixture was filtered, and the filtrate was concentrated to remove solvents. The resulting dried extract was then collected and stored in desiccators for preservation.

Phytochemical screening of the extract

The obtained extract has been subjected to several phytochemical tests like tests for phenols, flavonoids, glycosides, tannins, proteins, carbohydrates etc., to identify phytoconstituents.[9]

Isolation of Phytoconstituents

A column of silica gel (100 mesh size) was used for column chromatography of an aqueous ethanol extract. The column was prepared using a wet packing technique and a slurry of silica gel and solvent. A 30x2 cm column was loaded with approximately 5 g of crude *Ficus religiosa* root bark extract and 8 g of silica gel, and the mixture was eluted with water: ethanol in a 3:7 ratio. 5 mL of fractions were collected in each time interval until only solvents were eluted. Using a developing solvent system of hexane, chloroform, and water (3:3:4), all of the obtained fractions were subjected to TLC. Next, using a rotatory evaporator, the organic solvent was extracted from the separated fractions. The dehydrated separated fractions were once more subjected to column chromatography in silica gel; the column was gradually eluted using a mobile phase at a regulated temperature and flow rate. To isolate the phenolic component, mix toluene, ethyl acetate, and methanol at a ratio of 3:3: 2. Each fraction was examined using a rotary evaporator to remove the organic solvent after the mobile phase was removed. Fractions that were similar in terms of R_f values were combined and subjected to TLC analysis and chemical tests. UV spectrophotometry was used to measure the total phenolic content of the obtained pooled fractions at 400 nm.

HPTLC study

The instrumental setup employed for this analysis involved the Camag HPTLC system comprising Linomat V spotting apparatus and a scanner-III operated with Win Cats 4 software. TLC aluminium sheets precoated with silica gel $60F_{254}$ were the stationary phase contained within a Twin Tough Chamber (20X10). The mobile phase consisted of a blend of toluene, ethyl acetate, methanol, and formic acid (in ratios of 3:3:0.8:0.2). A scanning wavelength of 366 nm was utilized for the process.

Procedure

Using a semi-automated Linomat V sample applicator, we applied duplicate 1, 2, 3, 4, and 5 µL portions of the standard solution onto a TLC plate, representing the 5 standard levels (1, 2, 3, 4, and 5 µg) of quercetin. After allowing the chromatograph to develop for 15 min, it was left to air dry at room temperature before being scanned at 366 nm. We then calculated the average peak areas of the two standards. Employing the Win Cats software, we established a regression equation by plotting the standard drug concentration calibration curve (X-axis) against the average peak height/area (Y-axis). This equation was subsequently utilized to estimate the quantity of isolated quercetin from *Ficus religiosa*.[10]

Estimation of quercetin

The average peak area/height of the duplicate samples had been computed, enabling the quantification of quercetin content through the utilization of the regression equation derived through the standard curve.[11]

RESULTS

Due to the intricate secondary metabolites found in various segments of these plants, *Ficus religiosa* stands out as a valuable traditional medicinal resource employed in treating diverse ailments and diseases. While the standardization and isolation of the active compound from the *Ficus religiosa* root bark have yet to be thoroughly explored, this study seeks to pinpoint the active phenolic constituent within this plant's root bark. The results of physiochemical parameter studies and phytochemical screening of hydroalcoholic extract are given in Tables 1 and 2 respectively.

Isolation of phytoconstituents

The isocratic elution method was employed in column chromatography. Initially, a high polarity mobile phase consisting of water and ethanol (3:7) was used to isolate the crude extract from the root bark. The eluted compounds were subsequently concentrated, packed into a new column, and eluted using a mobile phase composed of Toluene, ethyl acetate, and methanol (3:3:2).

Following this, the resulting fraction was concentrated, and a single spot with an R_f value of 0.86 was observed upon analysis by TLC in the same mobile phase. Subsequently, the compound underwent screening using a chemical test, with the ferric chloride test yielding a positive result.

Estimation of Quercetin by HPTLC

The mobile phase composed of Ethyl Acetate, Toluene, Methanol, and Formic Acid in a ratio of 3:3:0.8:0.2, exhibited an R_f value of 0.88, indicating the presence of a bioactive flavonoid molecule

(Figure 3). The validation of quercetin bands in the sample chromatograms was achieved by comparing them to those obtained from the reference standard solution (quercetin R_f 0.87) (Figure 4).

After allowing the chamber to saturate with the mobile phase for 20 min at room temperature, distinct spots emerged. High-Performance Thin-Layer Chromatography (HPTLC) investigations revealed well-defined peaks of isolated quercetin. Observation of the chromatogram spot under UV 280 nm (refer to Figure 5) unveiled that the *Ficus religiosa* isolate contained 3.1% (w/w) of quercetin with an R_f value of 0.88. The HPTLC chromatograms of the isolate and standard Quercetin were provided in Figures 3 and 4, respectively.

Method validation

Following ICH guidelines, all validation parameters were executed during the procedure's development. The proposed method underwent validation encompassing Limits of Detection (LOD) and Quantification (LOQ), linearity, accuracy, precision, ruggedness, and specificity, which are depicted in Table 3.

DISCUSSION

The isocratic elution method in column chromatography effectively separated bioactive compounds from *Ficus religiosa* root bark. Using the specified mobile phase yielded distinct spots on TLC plates, facilitating the identification of the flavonoid compound with an R_f value of 0.88. The validation of quercetin presence was accomplished through comparison with a reference standard solution, demonstrating the reliability of the method.

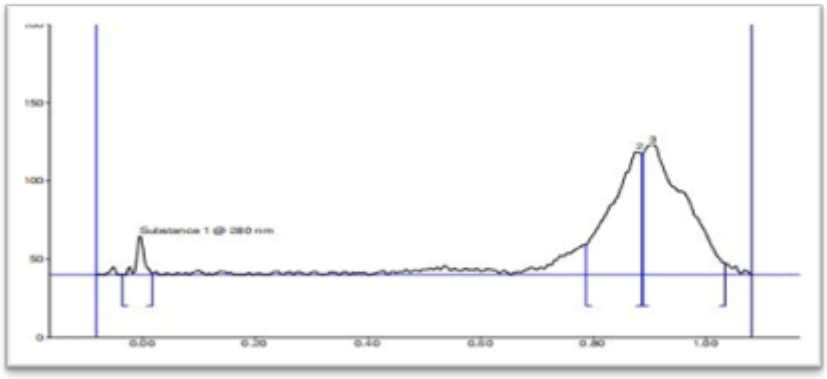

Figure 3: HPTLC chromatogram of isolate.

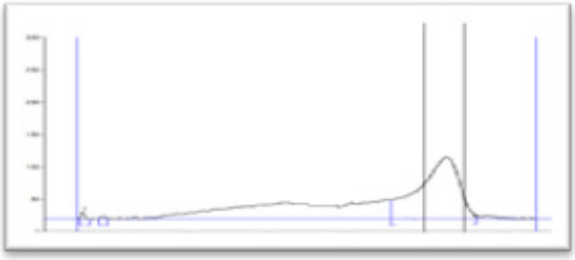

Figure 4: Quercetin HPTLC chromatogram.

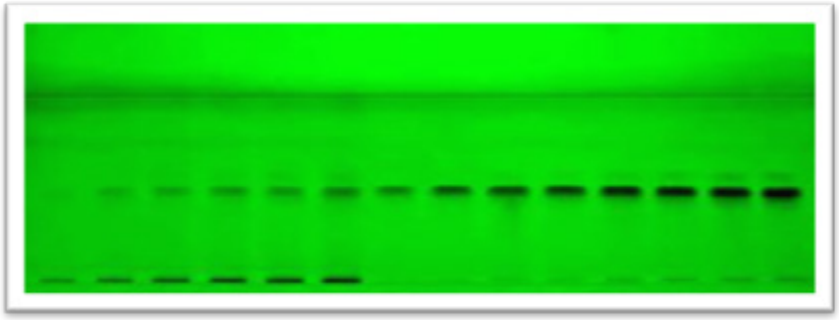

Figure 5: Photography of HPTLC.

Table 1: Physiochemical parameters.

Parameters	Values	Limit
Foreign organic matters	1.12%	NMT 2%
Loss on drying	0.11%	
Total ash	4.53%	NMT 7%
Acid insoluble ash	0.16 mg	NMT 0.3 mg
Water insoluble ash	0.15 mg	NMT 50 mg
Alcohol soluble extractives	6.21%	NMT 8%
Water soluble extractives	5.8%	NMT 9%

Table 2: Preliminary phytochemical analysis of hydro alcoholic extract of *Ficus religiosa*.

Phytochemical constituents	Extract
Carbohydrate	+ve
Tannins	+ve
Phenolic compounds	+ve
Flavonoids	-ve
Alkaloids	+ve
Saponins	+ve
Terpenoids	-ve
Steroids	+ve

Table 3: Validation parameters.

Method property	Value
Linearity range (ng/mL)	500-2000
Standard Deviation (SD)	2.12±0.02
Coefficient of Correlation (r)	0.99±0.003
LOD (ng/mL)	150
LOQ (ng/mL)	50
Intraday precision %RSD *n*=3	0.88±0.002
Precision %RSD	1.81±0.004

HPTLC analysis further confirmed the presence of quercetin, with the isolated sample containing 3.1% (w/w) of the compound. The chromatograms of the isolated compound and standard quercetin exhibited similar profiles, validating the identity of the isolated compound as quercetin. These findings highlight the potential of *Ficus religiosa* as a natural source of quercetin, which possesses various pharmacological properties.

CONCLUSION

In conclusion, this study successfully isolated and identified quercetin from *Ficus religiosa* root bark using column chromatography coupled with TLC and HPTLC techniques. The utilization of an appropriate mobile phase enabled the effective separation of bioactive compounds, leading to the isolation of quercetin with a purity of 3.1% (w/w). The validation of quercetin presence through comparison with a reference standard solution reinforces the reliability of the isolation method. The results underscore the potential of *Ficus religiosa* as a valuable source of quercetin, which holds promise for further pharmacological exploration and therapeutic applications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

CM: Centimeter; **g:** Grams; **HPTLC:** High-performance thin layer chromatography; **R_f:** Retention factor: **mL:** Milliliter; **nm:** Nanometer.

AUTHOR'S CONTRIBUTION

Jyothisree G. prepared the manuscript which is guided by S. Umadevi.

SUMMARY

This study aims to identify the active phenolic constituent in *Ficus religiosa* root bark, a traditional medicinal resource used for treating various ailments. The hydroalcoholic extract was isolated using the isocratic elution method in column chromatography. The compound was then screened using a chemical test, yielding a positive result. The mobile phase, composed of Ethyl Acetate, Toluene, Methanol, and Formic Acid, exhibited an R_f value of 0.88, indicating the presence of a bioactive flavonoid molecule. High-Performance Thin-Layer chromatography (HPTLC) investigations revealed well-defined peaks of isolated quercetin, revealing that the *Ficus religiosa* isolate contained 3.1% (w/w) of quercetin with an R_f value of 0.88.

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