# HPTLC METHOD DEVELOPMENT AND VALIDATION OF FLAVONOIDS IN ALCOHOLIC EXTRACT OF *CLITORIA TERNATEA PLANT: AN ANALYTICAL APPROACH* .

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***"Abstract"***

The Current research elucidate the development of an HPTLC technique for the quantification of Quercetin, Myricetin, and Kaempferol in the alcoholic extract of Clitoria ternatea flowers. The analysis utilized precoated silica gel 60 F254 plates as the stationary phase, with a mobile phase comprising toluene, ethyl acetate, and formic acid in a 5:4:1 ratio. Compounds were detected at 254 nm, with Rf values recorded at 0.63 for Quercetin, 0.58 for Myricetin, and 0.75 for Kaempferol. The detector showed linearity between 100 and 500 ng per band. The limit of detection (LOD) was 17 ng/band for Quercetin, 20 ng/band for Myricetin, and 19 ng/band for Kaempferol, while the limit of quantification (LOQ) was 53, 61, and 57 ng/band respectively. Validation parameters such as linearity, LOD, LOQ, accuracy, precision, specificity, robustness, and ruggedness confirmed the reliability of the method. The attempt was found to be accurate, specific, simple, and robust, making it suitable for the estimation of these flavonoids in Clitoria ternatea flower extracts.

**Keywords: HPTLC, TLC, Quercetin, Myricetin, Kaempferol, Method validation.**

## Introduction

Depression is a prevalent and disabling mental illness that affects over 280 million people worldwide, accounting for 5% of the adult population, and ranks higher in health burden than diabetes and cancer [1][2]. It disrupts mood, sleep, appetite, cognition, and general functioning. The COVID-19 pandemic caused a 25% global surge, with India reporting a 35% increase in severe depression and anxiety, especially among women, youth, and those with chronic illnesses [5].

Clitoria ternatea, known as Butterfly Pea, is a traditional Ayurvedic herb with multiple pharmacological properties including anxiolytic, antidepressant, antioxidant, and anti-inflammatory effects [3]. It contains flavonoids (quercetin, myricetin, kaempferol), anthocyanins, and phenolics, which modulate serotonin and dopamine, neurotransmitters crucial for mood regulation [12]. It also acts on GABA receptors, contributing to its calming effects and neuroprotective actions [12]. Though promising, more human trials are needed for conclusive evidence.

Botanically, Clitoria ternatea belongs to the Fabaceae family and grows in tropical Asia, including India [15][17]. Morphologically, it is a climbing herb with blue flowers and dark seeds [18]. It has vernacular names like Gokarna (Marathi), Aprajita (Bengali), and Shankhapushpa (Kannada) [16].

In pharmaceutical analysis, High Performance Thin Layer Chromatography (HPTLC) is vital for the standardization and quantification of herbal medicines [21]. It separates plant constituents based on affinity to the mobile and stationary phases, with the Rf value indicating the degree of movement [21]. HPTLC offers high throughput, sensitivity, and reproducibility, making it suitable for analyzing bioactives in Clitoria ternatea.

Method development includes plate preparation, sample application, mobile phase optimization, and UV or densitometric detection. Method validation ensures specificity, precision, accuracy, LOD/LOQ, robustness, and linearity, as recommended by ICH guidelines [22].

**Flower's of *Clitoria ternatea***

**8.1 Characterization of Standard Compounds**

**8.1.1 Organoleptic Properties**

The standard drugs were analyzed for color, odor, and physical appearance. Results were compared with specifications and tabulated. [23]

**8.1.2 Physical Properties**

- **Solubility:** Quercetin, Myricetin, and Kaempferol were tested in solvents such as 1 M HCl, 0.1 M HCl, methanol, water, chloroform, ethanol, and ether . [24]
- **Melting Point:** Determined by the capillary tube method. [37–39]

**8.1.3 Chemical Tests** [40]

Standard flavonoid chemical tests confirmed the presence of Quercetin, Myricetin, and Kaempferol:

- **Test 1:** Ethanol + Mg + HCl → pink/red/orange (Quercetin).
- **Test 2:** Ferric chloride → green/blue/purple complex.
- **Test 3:** Lead acetate → yellow/black precipitate.
- **Test 4:** NaOH/KOH → yellow/orange color. [24]

**8.1.4 UV Spectroscopic Analysis**

Each compound was dissolved (1–100 µg/mL) and scanned using a UV-Vis spectrophotometer (200–800 nm) to determine λmax. [25]

**8.1.5 FTIR Spectroscopy**

FTIR spectra of the three standards were recorded using Bruker Alpha IR Spectrometer and interpreted (Figure 9.2, Table 9.4). [26]

**8.2 Extraction of Plant Material**

10 g of *Clitoria ternatea* flower powder was extracted using 250 mL methanol via microwave-assisted extraction. The extract was filtered post-cooling. [27]

**8.3 TLC Profile of the Drug**

**8.3.1 TLC Plate Preparation:** Silica gel-G slurry was spread on glass plates and activated at 110°C.

**8.3.2 Sample Application:** 1 mg extract in 1 mL methanol was spotted using a capillary tube.

**8.3.3 Mobile Phase Selection:** Various solvents were tested; detection via iodine chamber and UV cabinet. [41]

**8.4 HPTLC Method Development**

**8.4.1 Standard Stock Solution:** 50 mg of each standard dissolved in methanol to make 1 mg/mL solutions.

**8.4.2 Mobile Phase Selection:** Optimal solvent system selected based on Rf values:

- Toluene: Ethyl acetate: Formic acid (5:4:1)

**8.4.3–8.4.4 Optimization:**

- **Chamber Saturation Time:** 20 min
- **Plate:** Silica gel 60 F254
- **Activation:** 110°C for 30 min
- **Sample Application:** CAMAG ATS 4 used for 8 mm bands

**8.4.5 Chromatogram Development:** Spots were developed and observed under UV for clarity and tailing.

**8.4.6 Rf Value Determination:** Plates derivatized with ninhydrin and visualized under visible light.

**8.5 Calibration Curve Preparation**

**8.5.1 Standard Solutions:** 1000 µg/mL stock diluted to 100 µg/mL.

**8.5.2 Sample Solutions:** Extract prepared at 1000 µg/mL, then diluted to 200 µg/mL.

**8.5.3 Chromatogram Development:**

- Spots applied in increasing volumes (1–7 µL for standards, 3 µL & 5 µL for samples).
- Plates scanned at 520 nm post-derivatization.

**8.6 Method Validation**

**8.6.1 Linearity:**

Spots (100–600 ng/spot) applied and scanned. Peak areas plotted.

**8.6.2 Precision:**

- **Repeatability:** 6× 1 µL standard solution (50 µg/mL) spotted; peak areas calculated.
- **Intermediate Precision:** Performed over 3 days using 100 ng, 300 ng, and 500 ng.

**8.6.3 Accuracy (Recovery):**

3µL extract (0.2 mg/mL) spotted and spiked with standard at 0.8, 1.0, 1.2 µL (0.05 mg/mL). %Recovery calculated.

**8.6.4 Specificity:**

Compared standard, extract, blank, and diluent. Scanned post-derivatization.

**8.6.5 LOD & LOQ:**

Calculated using:

- **LOD = 3.3 × (σ/S)**
- **LOQ = 10 × (σ/S)**

**8.6.6 Robustness:**

Tested at 300 ng/spot with:

- Slight changes in mobile phase (4.5:1 and 6.4:1)
- Chamber saturation time ±10%
- **1.1 Evaluation of Standard Compounds and Clitoria ternatea**
- **1.1.1 Organoleptic Properties** The organoleptic evaluation of Clitoria ternatea extract and standard flavonoids (Quercetin, Myricetin, and Kaempferol) indicated acceptable identity and purity. Clitoria ternatea showed a light blue color, odorless character, neutral taste, and crystalline powder form (Table 9.1). Quercetin exhibited a yellow crystalline solid with a rubber-like odor and bitter taste (Table 9.2). Myricetin also appeared as a yellow crystalline powder with a slightly fragrant odor and a mildly bitter taste (Table 9.3). Kaempferol had a yellow crystalline appearance with a distinct wild lemon odor and bitter taste.
- **1.1.2 Physical Properties**
- **Solubility:** All standard compounds were freely soluble in methanol. Myricetin showed additional solubility in acetone, while Kaempferol dissolved well in methanol, ethanol, and acetone.
- **Melting Point:** Quercetin melted at 316°C, Myricetin at 358°C, and Kaempferol at 278°C, conforming to standard references.

- **1.1.3 Chemical Tests for Clitoria Ternatea** Phytochemical screening using magnesium and HCl, ferric chloride, lead acetate, and sodium hydroxide confirmed the presence of flavonoids in Clitoria ternatea through color changes and precipitate formation.
- **1.1.4 UV Spectroscopic Analysis** UV-Visible spectroscopy revealed the λmax of Quercetin, Myricetin, and Kaempferol at 370-380 nm, 375-385 nm, and 365-375 nm respectively. Methanol was used as a solvent and blank, and quartz cuvettes ensured precise readings
- **FTIR Spectroscopic Analysis**
- **Quercetin:** Displayed major peaks at 3333 $cm^{-1}$ (O-H), 2359 $cm^{-1}$ (C=O), and 1652 $cm^{-1}$ (C=C)
- **Myricetin:** IR spectra showed 3263 $cm^{-1}$ (O-H), 1442 $cm^{-1}$ (C=C), and 1003 $cm^{-1}$ (C-O)
- **Kaempferol:** Characteristic peaks appeared at 3305 $cm^{-1}$ (O-H), 1655 $cm^{-1}$ (C=O), and 1224 $cm^{-1}$ (C-H), consistent with phenolic compounds

**1.1.4 HPTLC Method Development**

Developed method used Silica gel 60F254 plates with mobile phase of Toluene:Ethyl Acetate:Formic acid (5:4:1). Rf values: Myricetin (0.58), Quercetin (0.63), Kaempferol (0.75). The method was validated through calibration curves using concentrations 100-500 ng/band.

**1.1.5 Linearity, LOD and LOQ**

Linearity for all compounds ranged between 10-50 µg/ml with excellent correlation ($R2 > 0.98$).

- Myricetin: slope = 0.007, R2 = 0.9893, LOD = 113.68 ng/band, LOQ = 344.46 ng/band
- Quercetin: slope = 0.0085, R2 = 0.9833, LOD = 100.21 ng/band, LOQ = 303.65 ng/band
- Kaempferol: slope = 0.0103, R2 = 0.9881, LOD = 17.75 ng/band, LOQ = 53.78 ng/band

**1.1.6 Precision**

Repeatability showed %RSD < 2% for all analytes.

- Myricetin: Mean = 0.00391, SD = 1.5E-05, %RSD = 0.38
- Quercetin: Mean = 0.00434, SD = 2.8E-05, %RSD = 0.66
- Kaempferol: Mean = 0.00351, SD = 1.9E-05, %RSD = 0.55

Interday %RSD for all analytes also remained under 0.5%, confirming excellent precision.

**1.1.7 Accuracy**

Recovery studies performed at 80%, 100%, and 120% spiking levels showed recovery within acceptable limits:

- Myricetin = 91.50%
- Quercetin = 91.37%
- Kaempferol = 82.54%

**1.1.8 Specificity**

Specificity test confirmed distinct peaks for Quercetin, Myricetin, and Kaempferol at their respective Rf values, while no interference from diluent or mobile phase was observed.

**1.1.9 Robustness**

Robustness was confirmed by small variations in saturation time (18, 20, and 22 min). %RSD values remained < 1%:

- Myricetin: %RSD = 0.39 to 0.53
- Quercetin: %RSD = 0.35 to 0.48
- Kaempferol: %RSD = 0.43 to 0.71

**Conclusion**

The developed HPTLC method for simultaneous estimation of Quercetin, Myricetin, and Kaempferol in Clitoria ternatea extract was found to be accurate, precise, specific, robust, and linear over a wide concentration range. This method can be effectively utilized for routine analysis and standardization of herbal formulations containing Clitoria ternatea.