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Original Article

# Box–Behnken Design Based Optimisation of HPTLC Method for Analysis of Jatrorrhizine from *Tinospora cordifolia* Roots with Antidiabetic Activity Assessment

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## ABSTRACT

**Introduction:** *Tinospora cordifolia* root (*T. cordifolia*) is a well-known Ayurvedic herb with immune-boosting, anti-inflammatory, and antioxidant properties. It contains Jatrorrhizine (JZ), a bioactive alkaloid with antimicrobial, hepatoprotective, and metabolic health benefits, similar to berberine. **Objectives:** The study aimed to evaluate the JZ content in *T. cordifolia root* obtained via different extraction method and to optimise the High-Performance Thin Layer Chromatography (HPTLC) method via Response Surface Methodology (RSM). The extracts were assessed for *in vitro* test evaluation. **Materials and Methods:** A Box-Behnken Design (BBD) of RSM, a mathematical and statistical method, was applied to optimize the impact of independent variable on the response. Three independent variables like the different concentration of the mobile phase were studied. The *in vitro* analysis was also done. **Results:** The content of JZ was quantified using the HPTLC densiometric method. HPTLC confirmed the presence of JZ with a prominent band at an $R_f$ value of 0.70±0.008 using a mobile phase of chloroform: toluene: methanol: formic acid (7:4:2:0.2 v/v). The *in vitro* analysis of the extract showed a significant result and was found to be α-amylase in TRM 27.71±0.70 and α-glucosidase in TRM 43.45±1.31. **Conclusion:** This work examines various extraction procedures, including Soxhlet, maceration and reflux, and introduces a novel methodology to enhance extraction efficiency, advance the phytochemical analysis of JZ. This suggests that *T. cordifolia* may have therapeutic potential in diabetes management.

**Keywords:** Jatrorrhizine, HPTLC, Response Surface Methodology, α-Amylase, α-Glucosidase.

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## INTRODUCTION

The climbing woody shrub *Tinospora cordifolia* (*T. cordifolia*), often called Guduchi, is a member of the Menispermaceae family and a tropical or subtropical plant native to Southeast Asia and India.[1] Renowned for its diverse therapeutic properties, it is widely used in Ayurveda as a tonic, energizer, and treatment for diabetes, jaundice, and metabolic disorders. The Indian Ministry of AYUSH also endorsed Guduchi as a preventive remedy during the COVID-19 pandemic.[2,3]

The medicinal properties of *T. cordifolia* from its rich chemical composition, which includes alkaloids such as Jatrorrhizine (JZ) and berberine, diterpenoid steroids, phenolics, glycosides, and lactones. These compounds exhibit antiviral, antioxidant, hepatoprotective, and anti-inflammatory effects.[4] It offers diverse therapeutic benefits through its various parts. The stem, leaves, bark, juice, root, and fruit are traditionally used to treat conditions like skin disorders, gout, ulcers, diabetes,[5] malaria, inflammation,[6] and rheumatism, as well as in emergency remedies for snakebites and scorpion stings, showcasing its medicinal versatility.[7] *T. cordifolia*, is categorized as a *Rasayana* in Ayurveda due to its renowned ability to enhance health, longevity, and overall well-being.[8] Its wide-ranging therapeutic applications have established it as a vital component of traditional medicine systems for centuries,[9] Esteemed for its safety, effectiveness, and versatility, *T. cordifolia* continues to play a significant role in holistic healing practices. To effectively utilize its medicinal potential, it is essential to optimize extraction methods to maximize the yield of its bioactive phytocompounds.[10]

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Jatrorrhizine is a plant-based compound found in traditional medicinal herbs like *Coptis chinensis, Berberis* and *Tinospora* species. It's been used for centuries and is now drawing scientific interest because of its wide range of health benefits. Research shows that jatrorrhizine may help with conditions like diabetes, high cholesterol, obesity, infections, and even some cancers.[11] It also appears to support brain health, protect the liver, and strengthen bones. Scientists have managed to synthesize it in the lab, but its natural production in plants isn't fully understood yet. While early studies show it's generally safe, more research is needed to confirm how well it works in humans and whether it interacts with other medications.[12]

India strongly promotes its traditional medicines through supportive government policies, integration into national healthcare programs, and extensive research efforts. Institutions like AYUSH, CSIR, and CDRI play crucial roles in developing innovative herbal formulations.[13] India possesses more than 10,000 units of industrialised traditional medicine. The Indian traditional medicine sector generates approximately $1 billion annually in both traditional and modern product formats, adhering to regulated production standards.[14]

The increasing global reliance on herbal medicine is driven by the drawbacks of conventional treatments such as high costs and adverse effects and the improved safety and quality of herbal alternatives.[15] Nearly 80% of the world's population depends on traditional healing systems like Ayurveda and Traditional Chinese Medicine. India, rich in biodiversity, is home to approximately 21,000 medicinal plant species, with around 150 holding notable commercial importance. Known for their therapeutic efficacy and low risk of side effects, these plants are essential to both traditional practices and modern healthcare.[16]

High-Performance Thin-Layer Chromatography (HPTLC) and HPLC/ESI-QTOF-MS/MS are complementary techniques used for comprehensive phytochemical analysis.[17] HPTLC offers a quick, cost-effective method for both qualitative and quantitative evaluation of plant metabolites, ideal for routine quality control.[18] Meanwhile, HPLC/ESI-QTOF-MS/MS provides high-resolution mass accuracy and precise structural identification, successfully detecting 36 metabolites in *T. cordifolia* and revealing variations between male and female plants.[19] Combined, these methods form a powerful platform for metabolite profiling, standardization, and quality assessment of medicinal plants.[20]

Response Surface Methodology (RSM) is a statistical technique used to study the effects of multiple variables at the same time.[21,22] One of the key benefits of this approach is that it reduces the number of experiments needed, while also improving the reliability of the results. It also helps identify whether the variables being studied influence each other. A commonly used design within RSM is the Box-Behnken Design (BBD). Unlike other designs, BBD avoids combinations of variables that fall at the extreme corners of the experimental range, which can be helpful when such conditions are difficult or unsafe to test. It's especially useful for building reliable models without the need for a large number of experimental runs.[23]

This study systematically evaluates three extraction techniques Soxhlet extraction, maceration, and reflux for isolating JZ from the root of *T. cordifolia*. The integrative approach combining multiple extraction methods is novel and has not been previously reported in the literature. The extracted JZ was quantified using a HPTLC densitometric method. To optimize the quantification process, RSM was employed for experimental design, variable screening, and method validation. In addition, *in vitro* bioactivity assays were performed to assess the biological efficacy of the extracts. The findings revealed significant biological activity when compared to standard controls. This stepwise strategy not only enhances extraction efficiency but also underscores the therapeutic potential of *T. cordifolia* root, largely attributed to its JZ content.

## MATERIALS AND METHODS

### Collection of the Plant and authentication

Root of *T. cordifolia* were collected from Khari Baoli, Old Delhi, India. The root identified and authenticated by Department of Botany, SCLS, Jamia Hamdard, New Delhi, The voucher was kept for future reference.

### Chemicals

The following materials were provided by SD Fine Chemicals, located in Mumbai, India: methanol, water, gallic acid, $AlCl_3$, Potassium Acetate, 3,5-Dinitrosalicylic acid (DNS), Porcine pancreatic α-amylase solution (Sigma aldrich), sodium hydroxide, p-Nitro-α-D-Glucopyranoside (pNPG), sodium carbonate and sodium nitrite. TLC Silica gel 60F254 were purchased by Merck, located in Mumbai, India. The chemical JZ was purchased by Tokyo chemical industry Co. Ltd., (TCI) located in Tokyo, Japan.

### Preparation of Plant Material

The *T. cordifolia* roots were rinsed into in the middle of a stream after being carefully cleaned to remove any remaining dust and clinging foreign objects. They were also pulverised using a mixer (Company: SUJATA India, Model: 01W2015, 810 Watts), let to dry naturally before being passed through a 40-mesh sieve before being kept in an airtight container.[24] JZ was extracted using hydro-alcoholic solvents (methanol: water, 1:1). Reflux, Soxhlet, and maceration were the three methods used for the extraction of JZ from *T. cordifolia* root.[25]

### Extraction of Jatrrohizine

#### *Maceration*

The powdered *T. cordifolia* roots were mixed with 100 mL of solvent, maintaining a solvent-to-drug ratio of 10 mL/g, and

allowed to stand undisturbed for 2–3 days with occasional stirring. After the incubation period, the mixture was filtered, and the filtrate was concentrated using a rotary evaporator (Model: HS-2005V, Hahnshin Scientific Co., Korea. The filtrate was then dried using a rotary evaporator under vacuum (Model: HS-2005V, Manufacture by Hahnshin scientific Co. Made in Korea.[26,27]

## Soxhlet Extraction

The Soxhlet method was used for extraction, which is continuous hot solvent extraction. A ratio of 10 mL/g of solvent-to-drug was used, with temperatures ranging from 60 to 65°C for 4-5 hr. After the extraction, rotary evaporation is carried out on the filtrate with plant residue inside by the use of vacuum drying.[28]

## Reflux Extraction

The procedure was performed in a reflux device called the hot solvent extraction method.[29] 10 g of sample in 100 mL of solvent was used (ratio 10 mL/g), and the solvent was heated to 50°C for an hour. The plant residue was subsequently filtered and the filtrate was vacuum-dried in a rotary evaporator following this procedure.

## HPTLC Optimisation via BBD

### *Optimization of HPTLC parameters by Box Behnken Design*

The optimization of the JZ for the HPTLC was carried out using Design Expert software (Version 11, Stat-Ease, MN, USA) with BBD.[30] Volume of chloroform ($X_1$), volume of toluene ($X_2$), and volume of methanol ($X_3$) were treated as independent variables, while retention factor (Rf) ($R_1$) were taken as dependent variables in (Table 1). A total of 15 runs were developed, as shown in Table 2. The effect of the independent variables was further analyzed using response surface plots and polynomial equations. Significance of the factors was determined using Fisher's statistical test for Analysis of Variance (ANOVA) model that was anticipated. These elements were then employed to determine an F ratio that approximates the utility of the model. When the F-ratio possibility is low, the model is stated to be a better statistical robust for that data. All the experiments were conducted in random order to minimize the bias effects of uncontrollable factors. A BBD statistical screening approach was employed to validate the parameters and to establish the quadratic effect of varying composition of mobile phase and saturation time on Rf.[23,29]

## Standard and Sample Preparation for HP-TLC

The standard solution i.e. JZ was prepared by dissolving 1mg of the JZ in methanol (1 mg/mL). The sample solution *T. cordifolia* Root Extract soxhlet (TRS), *T. cordifolia* root extract Maceration (TRM) and *T. cordifolia* Root Extract Reflux (TRR) was taking the One gram from each of the three extracts was separately dissolved in methanol. The JZ solution, TRS, TRM and TRR were filtered by dint of a 0.22 µm Millipore (Burlington, MA, USA) syringe filter for analysis.[31]

## Development of the solvent system

Chromatography elution utilizes a wide range of mobile phases. Initially, we tested several solvent systems including ethyl acetate, chloroform, hexane, methanol, toluene and acetonitrile in various ratios and under consistent conditions using a silica gel column at room temperature. These early attempts resulted in poor separation and overlapping bands, indicating suboptimal resolution. Improved resolution was eventually obtained in a novel solvent system consisting of chloroform: toluene: methanol: formic acid (7:4:2:0.2 v/v) at room temperature.[32]

## HP-TLC instrumentation and conditions

Before chromatographic analysis, TLC Silica gel 60F254 plates were pre-heated at 110°C for 15 min. The reference samples and hydroalcoholic extract were spotted on 10 × 10 cm plates in 6-mm bands with the help of an automatic CAMAG Linomat 5 applicator, using nitrogen gas blowing at 150 nL/s. The bands were 15 mm apart and were spotted 10 mm from plate edges. Chromatographic development was carried out in a twin-trough vertical glass chamber (CAMAG) after a 30-min pre-saturation.[33]

The mobile phase was (7:4:2:0.2 v/v) chloroform: toluene: methanol: formic acid. The plate was air-dried at room temperature after the solvent migrated 90 mm in 10 min and dried at 85°C for 4 min to visualize the bands. Densitometric analysis was performed under tungsten (W) light using a CAMAG TLC Scanner 3 and winCATS software (Version 1.2.0). The slit size was 5.0 mm × 0.45 mm, with a scanning speed of 20 mm per second. The chromatographic condition required for the HPTLC determination of JZ is mentioned in Table 3.

**Table 1: Coded and actual levels of the three factors used in the Box-Behnken design for optimizing jatrorrhizine extraction from *Tinospora cordifolia*.**

| Variables | Low (-1) | High (+1) |
|---|---|---|
| **Independent variables** | | |
| A = Volume of chloroform (mL) | 6 | 8 |
| B =Volume of toluene (mL) | 3 | 5 |
| C = Volume of methanol (mL) | 1.5 | 2.5 |
| **Dependent variables** | | |
| $Y_1$ = Retention factor ($R_f$) | Maximise | |

## HP-TLC method validation

The International Council for Harmonisation (ICH) guide lines were adopted for the validation of the parameters presented below.[34]

## Linearity

Estimate of linearity was carried out with the JZ concentration range 2-10 µg/spots. The slope, intercept, and regression values of the calibration plot were determined using least squares linear regression, which was carried out on concentration vs. peak area. A linear relationship between the concentration tested and the peak area was established by the calibration plot (2-10 µg/spot). Densitometric scanning and a calibration plot were used to determine JZ in the TRS, TRM and TRR.

## Precision

Five replicate running's of the pre-determined JZ concentration (4 µg/spot) were carried out to estimate the instrument's accuracy. Precision, repeatability (inter-day), followed by reproducibility (intra-day) was tested at five varied concentration ranges (2-10 µg/spot) by quantifying three individual spots of JZ pipetted on different plates. These estimated values were expressed as relative Reference Deviation (RSD).

## Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOQ was calculated based on the reference deviation (σ) of the response and slope as 10 times the reference deviation of the lowest concentration-to-slope ratio (µg/spot) of the calibration curve, whereas LOD was calculated as 3 times σ for the low concentration-to-slope ratio (µg/spot) of the calibration curve. We employed the following concepts to determine the LOD and LOQ of the JZ marker: The calibration curve slope is denoted as

**Table 2: The chromatographic condition required for the HPTLC determination of JZ in *T. cordifolia* root.**

| Application mode | CAMAG Linomat 5 applicator |
|---|---|
| Stationary Phase | Silica gel 60GF$_{254}$ |
| Mobile Phase | Chloroform: Toluene: Methanol: Formic acid (7:4:2:0.2 v/v) |
| Band width | 6mm |
| Saturation time | 30 min |
| Solvent front distance | 85 mm |
| Plate activation time | 30 min |
| Detection lamp | Deuterium |
| Wavelength | 254 nm,350 nm |
| Scanning rate | 20 mm/spot |

**Table 3: 15 experimental runs generated using Box-Behnken Design (BBD), including $R_f$ values, actual responses, predicted responses, and residuals based on the Response Surface Methodology model.**

| Run | A Volume of chloroform (mL) | B Volume of toluene (Ml) | C Volume of methanol (mL) | $Y_1$ (Observed value) Retention factor ($R_f$) | Predicted value | Residual |
|---|---|---|---|---|---|---|
| 1 | 7 | 5 | 2.5 | 0.64 | 0.6463 | -0.0063 |
| 2 | 7 | 3 | 1.5 | 0.68 | 0.6738 | 0.0062 |
| 3 | 6 | 4 | 2.5 | 0.65 | 0.6463 | 0.0037 |
| 4 | 7 | 4 | 2 | 0.71 | 0.7167 | -0.0067 |
| 5 | 6 | 4 | 1.5 | 0.73 | 0.7313 | -0.0013 |
| 6 | 7 | 4 | 2 | 0.72 | 0.7167 | 0.0033 |
| 7 | 8 | 3 | 2 | 0.63 | 0.6325 | -0.0025 |
| 8 | 7 | 5 | 1.5 | 0.73 | 0.7313 | -0.0013 |
| 9 | 7 | 4 | 2 | 0.72 | 0.7167 | 0.0033 |
| 10 | 8 | 4 | 2.5 | 0.61 | 0.6088 | 0.0012 |
| 11 | 6 | 5 | 2 | 0.73 | 0.7275 | 0.0025 |
| 12 | 8 | 5 | 2 | 0.69 | 0.6850 | 0.0050 |
| 13 | 7 | 3 | 2.5 | 0.65 | 0.6488 | 0.0012 |
| 14 | 8 | 4 | 1.5 | 0.63 | 0.6338 | -0.0038 |
| 15 | 6 | 3 | 2 | 0.72 | 0.7250 | -0.0050 |

S, while the reference deviation is denoted as is. 3/S and 10/S are these values, respectively.

The LOQ and LOD were determined using Equations 1 and 2:

$$LOQ = 10*\frac{\sigma}{S} \qquad \text{equation (1)}$$

$$LOD = 3*\frac{\sigma}{S} \qquad \text{equation (2)}$$

## Accuracy

The accuracy of the procedure was determined by comparing the recovery of JZ in with reference values. The precision was evaluated by adding known amounts of to reference JZ volumes in concentrations of 60%, 80%, and 100%. The response (peak area) was computed, and the percentage recovery was calculated.

The % recovery were determined using Equation 3:

$$\%\ \text{Recovery} = \frac{\text{Amount found} - \text{Original amount}}{\text{Amount spiked}} * 100 \qquad \text{equation (3)}$$

## Robustness

The robustness of the method was assessed through testing at five levels of concentrations. The solvent system ratio of chloroform: toluene: methanol: formic acid (7:4:2:0.2 v/v) for JZ showed some fluctuation in the course of the chromatogram run. The temperature, saturation time, and quantity of the solvent system were kept within the range of less than±7%. Reference spotting times on TLC Silica gel 60F254 plates, solvent build-up times, and times between plate development and scanning were varied between 15, 30, and 45 min.[35]

## Specificity

The specificity of the method was determined by critically examining the reference, test sample, diluents, and solvent system. The JZ position was determined by the $R_f$ values of the isolated bands. The reference peak purity was determined by comparing the spectra at three different peak levels, such as the start peak, apex peak, and end of the spot peak.

## Quantification of Jatrorrhizine

By employing the suggested HP-TLC technology, JZ from the hydroalcoholic extract of *T. cordifolia* was estimated. The reference sample JZ and the both samples of TRS, TRM and TRR were placed to the TLC plate also set in the mobile phase. Five samples were employed to estimate JZ. The amount of JZ quantified is detailed in Table 7.

### *In vitro*

#### *α-amylase inhibition assay*

Inhibition activity of TRS, TRM and TRR extracts against α-amylase was evaluated by the standard *in vitro* test. Plant extracts in ethanol were used at 20-320 µg/mL concentrations preincubated with the solution of porcine pancreatic α-amylase (1 U/mL) at 37°C for 30 min. Afterward, 1% starch solution was added, and the mixture was further incubated for 20 min at 37°C. To stop the reaction and allow color development, the tubes were heated in a boiling water bath for 5 min, and DNS reagent was added.[36]

The solution was diluted with distilled water and the absorbance measured at 540 nm using a spectrophotometer. Acarbose was taken as a positive control, and the percentage inhibition of α-amylase was determined in relation to the solvent control. In this method, the TRR, TRS and TRM were assayed for inhibitory activities on α-amylase and thus potentially have a regulatory function in glucose and carbohydrate metabolism.

The % inhibition of α-amylase were determined using Equation 4:

$$\%\text{Inhibition} = \frac{\text{X control} - \text{Y sample}}{\text{X control}} * 100 \qquad \text{equation (4)}$$

## α-glucosidase inhibition assay

The α-glucosidase inhibitory activity of TRS, TRM and TRR was determined using a standard assay. TRS, TRM and TRR (20-320 µg/mL in ethanol) were pre-incubated with 120 µL of 0.1 M phosphate buffer (pH 6.9) and 20 µL of enzyme solution at 37°C for 15 min. The reaction was initiated by adding 20 µL of 5 mM pNPG, and the mixture was incubated at 37°C for an additional 15 min. To terminate the reaction, 80 µL of 0.2 M sodium carbonate was added.[37]

Absorbance was measured at 409 nm, and the percentage of α-glucosidase inhibition was calculated relative to the solvent control, with acarbose as the positive control. This method enabled the evaluation of TRS, TRM and TRR as potential α-glucosidase inhibitors, suggesting their possible role in glucose regulation and carbohydrate digestion.

The % inhibition of α-glucosidase were determined using Equation 5:

$$\%\ \text{inhibition} = \frac{\text{X control} - \text{Y sample}}{\text{X control}} * 100 \qquad \text{equation (5)}$$

X control = control absorbance; Y sample =sample absorbance

# RESULTS

## HP-TLC method development and validation

The mobile phase of HP-TLC analysis is vital to accurately determining analytes. It was highly desirable that a solvent solution be one that gave dense and tight spots with appropriate and relatively different $R_f$ values. Chloroform: toluene: methanol: formic acid (7:4:2:0.2 v/v) was found to provide a good resolution of JZ from its surrounding matrix with $R_f$ value 0.70± 0.008 and the solvent travel distance of 85 mm with chamber saturation time of 30 min. Thus, we tested different mobile phases. The optimum mobile phase contained thick, compact, and well-shaped spots that enabled the separation of the components of the mixtures

under investigation. Figure 1 HPTLC plate of JZ reference and *Tinospora cordifolia* extracts visualized at 254 nm and 366 nm. Tracks T1 to T5 represent the JZ reference standard. Tracks T6 and T7 correspond to *T. cordifolia* extract obtained via Soxhlet extraction (TRS), T8 and T9 represent Maceration extracts (TRM), and T10 and T11 denote extracts obtained through the Reflux method (TRR).

## Optimization of HPTLC parameters

### *Response ($Y_1$): Effect of Independent variables on the retention factor*

A total of 15 experimental runs were performed and analyzed using the BBD methodology to evaluate the effects on $R_f$ values. A polynomial equation was developed to model the relationship between chloroform volume, methanol volume, and toluene volume on the Rf.

$$Y1=+0.7167-0.0338X1+0.0137X2+0.0275X3+0.0125X1X2+0.0150X1X3-0.0150X2X3-0.0221X_1^2-0.0021X_2^2-0.039X_3^2$$

## Effect of factors on response and analysis of response surfaces

A quadratic polynomial model was employed to assess the relationship between the independent variables and the response. This relationship was visually represented using two-dimensional (2D) and three-dimensional (3D) surface plots, as shown in Figures 2(a) and 2(b), which depict the interaction between saturation time and different volumes of the mobile phase. Statistical analysis via ANOVA confirmed the high significance of the model, with a p-value of 0.0001. The model also exhibited a strong F-value of 104.36, indicating that the likelihood of these results occurring due to random variation is only 0.01%. Furthermore, the lack of fit was non-significant, with a *p*-value

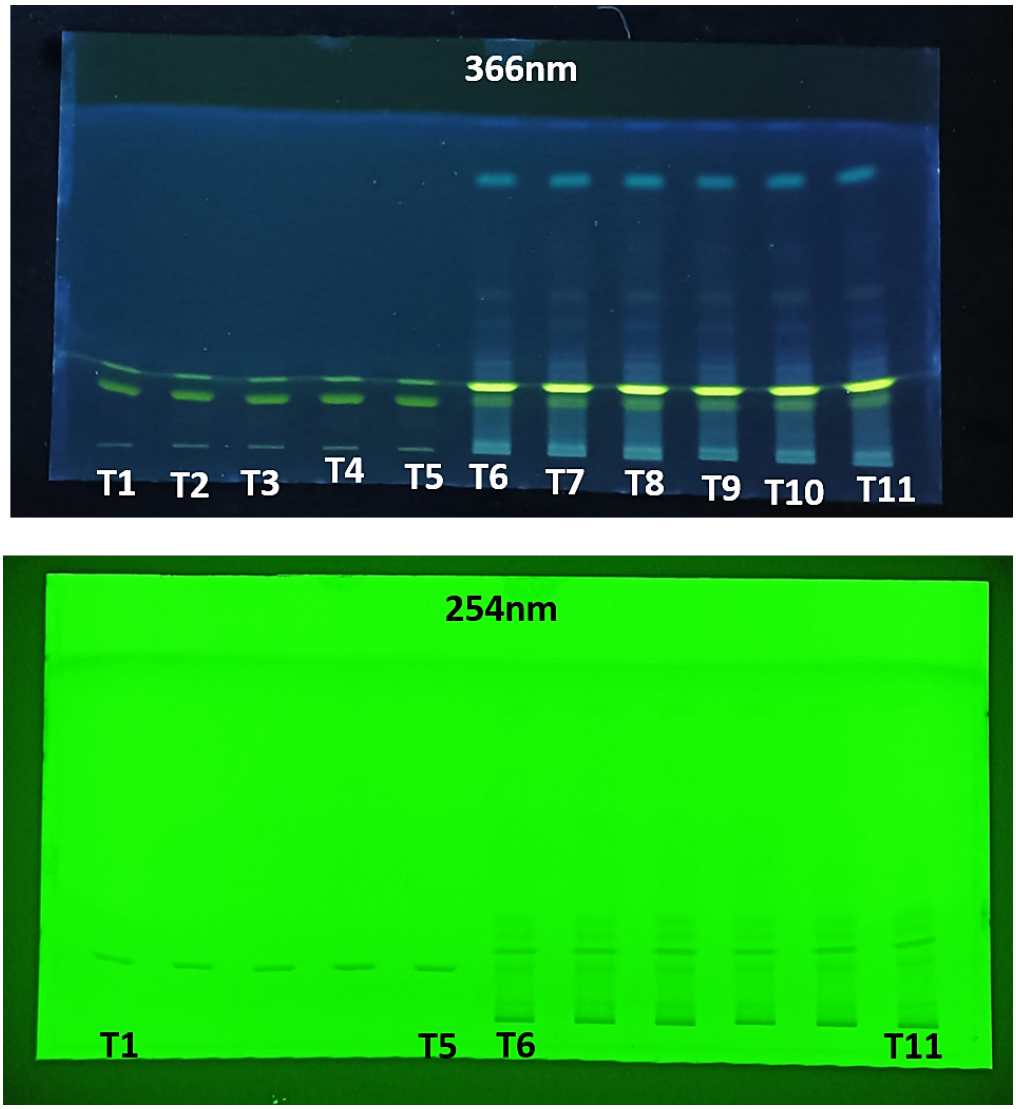


**Figure 1:** HPTLC plate of JZ reference and *Tinospora cordifolia* extracts visualized at 254 nm and 366 nm. Tracks T1 to T5 represent the JZ reference standard. Tracks T6 and T7 correspond to *T. cordifolia* extract obtained via Soxhlet extraction, T8 and T9 represent maceration extracts, and T10 and T11 denote extracts obtained through the reflux method.

**Table 4: ANOVA and fit statistics for the HPTLC method. Presents the method's accuracy, precision, and reliability.**

| Source | Sum of Squares | $d_f$ | Mean Square | F-value | p-value | |
|---|---|---|---|---|---|---|
| Model | 0.0263 | 9 | 0.0029 | 60.35 | 0.0001 | significant |
| $X_1$-Volume of chloroform | 0.0091 | 1 | 0.0091 | 188.53 | <0.0001 | |
| $X_2$-Volume of Toluene | 0.0015 | 1 | 0.0015 | 31.29 | 0.0025 | |
| $X_3$-Volume of methanol | 0.0060 | 1 | 0.0060 | 125.17 | <0.0001 | |
| $X_1X_2$ | 0.0006 | 1 | 0.0006 | 12.93 | 0.0156 | |
| $X_1X_3$ | 0.0009 | 1 | 0.0009 | 18.62 | 0.0076 | |
| $X_2X_3$ | 0.0009 | 1 | 0.0009 | 18.62 | 0.0076 | |
| $X_1^2$ | 0.0018 | 1 | 0.0018 | 37.25 | 0.0017 | |
| $X_2^2$ | 0.0000 | 1 | 0.0000 | 0.3316 | 0.5897 | |
| $X_3^2$ | 0.0058 | 1 | 0.0058 | 119.69 | 0.0001 | |
| Residual | 0.0002 | 5 | 0.0000 | | | |
| Lack of Fit | 0.0002 | 3 | 0.0001 | 1.75 | 0.3838 | not significant |
| Pure Error | 0.0001 | 2 | 0.0000 | | | |
| Cor Total | 0.0265 | 14 | | | | |
| Fit statistics | | | | | | |
| Std. Dev. | | | | 0.0070 | | |
| Mean | | | | 0.6827 | | |
| C.V. % | | | | 1.02 | | |
| $R^2$ | | | | 0.9909 | | |
| Adjusted $R^2$ | | | | 0.9745 | | |
| Predicted $R^2$ | | | | 0.8887 | | |
| Adequate Precision | | | | 21.5803 | | |

of 0.3838 and an F-value of 1.75, suggesting a good fit of the model to the experimental data. This non-significant lack of fit is desirable, as it indicates that the model reliably represents the observed data. Additionally, the predicted $R^2$ value of 0.8887 is in close agreement with the adjusted $R^2$ value of 0.9745, with a difference of less than 0.2, further confirming the robustness and reliability of the model, as summarized in Table 4.

The prediction versus actual plot in the RSM serves as a key tool for validating the model's accuracy by comparing predicted outcomes with observed results. In this study, the plot shown in Figure 3 demonstrates a strong correlation, with the predicted and actual values closely aligning along a single trajectory.

## HPTLC method validation

The mobile phase of HP-TLC analysis is vital to accurately determining analytes. It was highly desirable that a solvent solution be one that gave dense and tight spots with appropriate and relatively different $R_f$ values. Chloroform: toluene: methanol: formic acid (7:4:2:0.2 v/v) was found to provide a good resolution of JZ from its surrounding matrix with $R_f$ value 0.70± 0.008 and the solvent travel distance of 85 mm with chamber saturation time of 30 min. As shown in Figure 5, the chromatogram was recorded after the run. Thus, we tested different mobile phases. The optimum mobile phase contained thick, compact, and well-shaped spots that enabled the separation of the components of the mixtures under investigation (Figure 4).

## Calibration Curve

In Table 3, the optimal HP-TLC conditions for JZ analysis are indicated. Through the plotting of peak area versus JZ concentration, a calibration curve was established, and the findings of the regression analysis are shown in Table 5. These results validate the linearity of the reference curves across the range of investigation. The linear regression of the reference curve is R2=0.98702. Linear regression analysis showed that

Y=2281.342+598.876*X±6.06%. The calibration plot is shown in Figure 5 along with the 3D HPTLC chromatogram and spectra also.

HP-TLC was quantitatively measured at the same time to determine and evaluate JZ in both TRS, TRM and TRR. ICH guidelines were used to validate the process. Optimum conditions were opted for after substantial learning regarding experimental parameters like chamber saturation time, solvent front travel, slit width, and band width.

## Linearity and range

Corresponding to a correlation coefficient ($R^2$) of 0.98702 for JZ, good linear relationship between peak area (response) in addition to amount was found for JZ at 350 nm for a series of 2-10 µg/spot. Linearity has the requirement that there is a linear relationship between the signals of the analytes and their concentrations within the test sample range. The concentration of study (2-10 µg/mL) was highly linearly correlated in the data, which meant that it was amenable to analysis (Figure 5). The content of JZ in TRS, TRM and TRR was determined to be close to 98.99%.

## Limit of detection and limit of quantification

LOD is the lowest concentration that can be measured by an instrument but not quantified, having a noise-to-signal ratio of 1:3, whereas LOQ is the measured and detected lowest concentration, having a noise: signal ratio (1:10). LOD for JZ was estimated to be 1.09 µg/band whereas LOQ was 3.65 µg/band shown in Table 5.

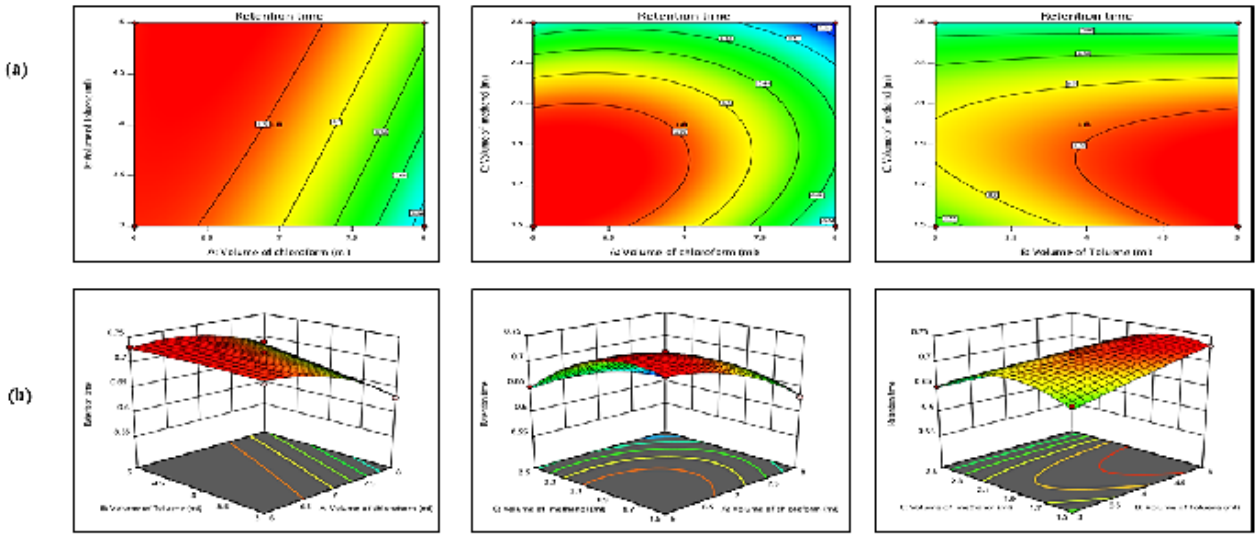


**Figure 2:** a) 2D plot (b) 3D plot showing the influence of independent factors as volume of chloroform (X1), volume of toluene (X2) and volume of methanol (X3) on the retention time.

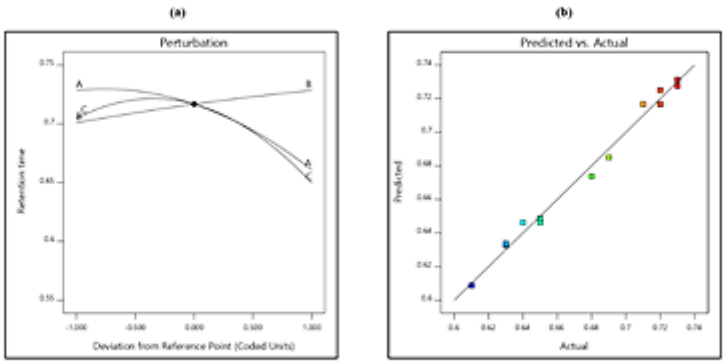


**Figure 3:** The Effect of Each Factor, X1, X2, and X3, on the Rf Value of Jatrorrhizine, is Illustrated in Two Graphs: (a) Perturbation Graph and (b) Prediction Versus Actual Graph.

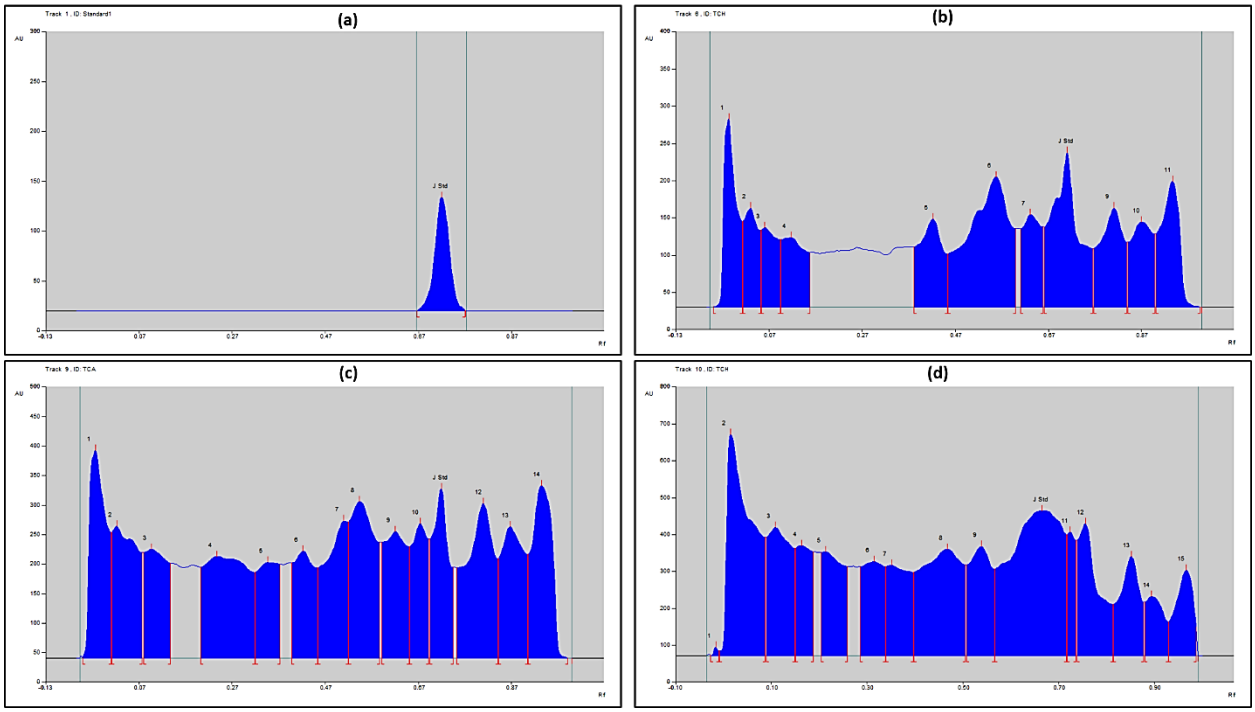


**Figure 4:** The chromatograms of the (a) Jatrorrhizine (b) *T. cordifolia* root soxhlet (c) T. cordifolia root maceration (d) *T. cordifolia* root reflux.

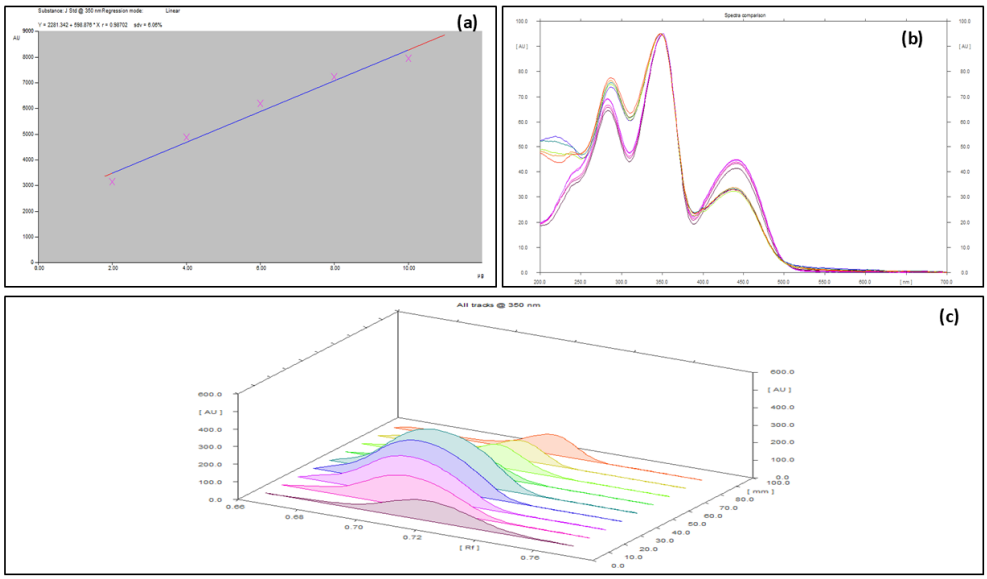


**Figure 5:** (a) Calibration plot of Jatrorrhizine at 350 nm (b) Spectra of standard Jatrorrhizine ranges between 200-700 nm (c) Overlaid 3D HPTLC chromatogram of the *T. cordifolia* extract at 350 nm.

TRS, TRM and TRR and JZ *in situ* UV spectra were overlayed, and they showed a good correlation (Figure 5). The peak clarity of JZ was compared with *in situ* UV band at different peaks of the band and peak clarity was determined.

## Precision

Repetitive scanning ($n$=3) of the three JZ spots (2.4, 2.6, and 2.8 µg/spot) was applied to check precision and track scanner parameters including the ability of repetition of the peak areas and equipment. They were reported as %RSD, and Table 5 shows that less than 2% was found, which was within the acceptable range based on ICH guidelines.

Intraday accuracy is the near-term usage of the analytical method within the lab, and interday accuracy is the comparison of study variation when a process is performed in the lab over a series of days. The interday and intraday accuracy testing study outcomes indicated that the method was indeed precise.

**Table 5: HPTLC calculation details including $R_f$ value, LOD, LOQ, recovery percentage, regression data, and both intraday and interday precision for the method developed specifically for *Tinospora cordifolia* root.**

**(a)**

| Parameter | Value of Jatrorrhizine |
|---|---|
| Linearity range | 2-10 µg/spot |
| Regression equation | Y=2281.342+598.876*X±6.06% |
| Correlation coefficient ($r^2$) | 0.98702 |
| Slope (µg/spot) | 2281 |
| Intercept | 598.9 |
| SE | 373.15 |
| SD | 834.39 |
| $R_f$ value | 0.70±0.008 |
| LOD (µg/spot) | 1.09 |
| LOQ (µg/spot) | 3.65 |
| % Recovery | 98.99 |

**(b)**

| Jatrorrhizine | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Inter-day | | | | | Inter-day | | | | |
| Conc.(µg/spot) | Area | Mean | ±SD | %RSD | Conc.(µg/spot) | Area | Mean | ±SD | %RSD |
| 2 | 3140.22 | 3143.25 | 2.68 | 0.08 | 2 | 3137.33 | 3140 | 2.56 | 0.08 |
| 4 | 4866.03 | 4862.96 | 2.84 | 0.05 | 4 | 4865.56 | 4868.04 | 3.90 | 0.08 |
| 6 | 6188.88 | 6185.70 | 2.78 | 0.04 | 6 | 6187.83 | 6187.38 | 1.75 | 0.02 |
| 8 | 7231.73 | 7233.65 | 1.97 | 0.02 | 8 | 7232.51 | 7232.45 | 0.69 | 0.00 |
| 10 | 7946.14 | 7944.63 | 1.37 | 0.01 | 10 | 7945.56 | 7945.31 | 0.97 | 0.01 |
| Mean % RSD | | | | 0.04 | Mean %RSD | | | | 0.04 |

## Accuracy (Recovery studies)

As evident from Tables 6 and 7 the mean % recovery of JZ at 3 different levels (50%, 100%, and 150%) was 98.99%, showing good method accuracy.

## Quantification of jatrorrhizine by HPTLC

The validated HP-TLC method was employed to quantify the JZ content in various extracts. The TRS, TRM and TRR extract samples were applied as spots on TLC Silica gel 60 F254. The sheets were then placed in a twin-trough chamber for development. The amount of JZ was determined using win CATS software with the help of the calibration plot.

## *In vitro* assay

### *α-amylase*

*In vitro* α-amylase inhibitory investigation was conducted to assess the percentage of α-amylase inhibition as a function of extract concentrations. Additionally, the $IC_{50}$ values were determined for acarbose, TRS, TRM and TRR. On the basis of the TRS, TRM, and TRR the $IC_{50}$ of acarbose was determined to be 59.07±0.61, 24.56±2.42, 27.71±0.70 and 25.97±1.16 respectively. As seen in Figure 6, the activity of α-amylase *in vitro* is displayed.

## α-glucosidase

An *in vitro* investigation on α-glucosidase inhibition assessed the percentage of inhibition relative to extract concentrations, and the $IC_{50}$ values were determined for acarbose, TRS, TRM and TRR. The $IC_{50}$ values of acarbose were determined to be 62.21±0.81, 36.71±1.78, 43.45±1.31 and 31.93±1.03 for the TRS, TRM and TRR, respectively. Figure 6 illustrates the *in vitro* α-amylase and α-glucosidase.

## Application

*T. cordifolia* along with JZ, are known for their numerous medicinal applications. *T. cordifolia* enhances immune capabilities, reduces inflammation, and scavenges free radicals, thus showing efficiency in infection, arthritis, and oxidative stress. This herb detoxifies the liver, increases digestive processes, normalizes diabetes, and promotes respiration in diseases like asthma and bronchitis.[12] It also acts as a febrifuge and an adaptogen to treat stress.

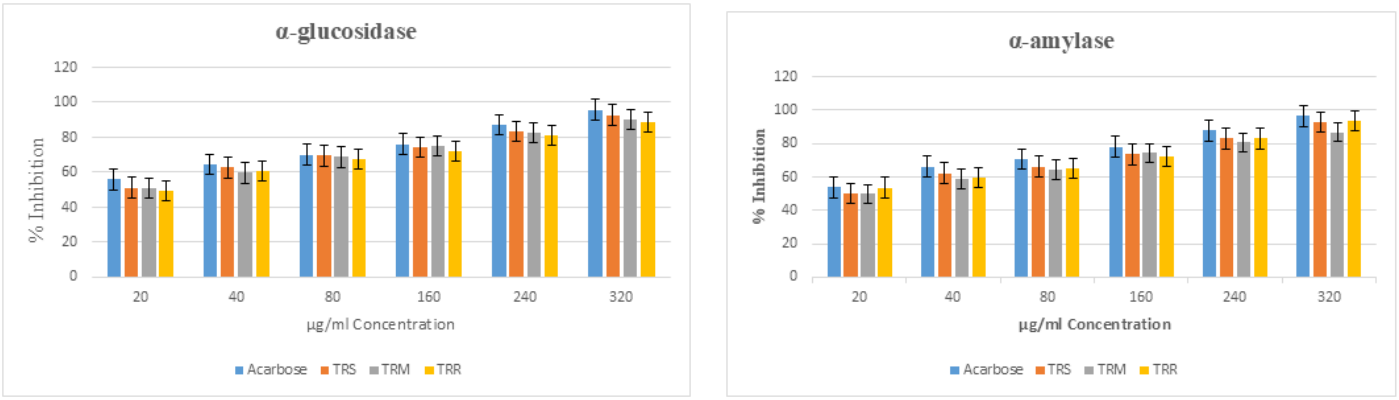


**Figure 6:** α-amylase and α-glucosidase of the *T. cordifolia* root extracts in different methods

**Table 6: Recovery investigations and content estimation of Jatrrohizine from *Tinospora cordifolia* root.**

| Standard | Amount added | Amount recovered (%mean) | SD | %RSD |
|---|---|---|---|---|
| Jatrorrhizine | 2.4 | 98.36 | 3.15 | 0.08 |
| | 2.6 | 98.94 | 3.73 | 0.09 |
| | 2.8 | 99.68 | 11.92 | 0.30 |
| Mean | | 98.99 | | |

**Table 7: Jatrorrhizine content determined by the developed HPTLC–densitometric method in the sample.**

| Sample | Jatrorrhizine (mg/g) |
|---|---|
| T6 | 1.06 |
| T7 | 2.30 |
| T8 | 1.15 |
| T9 | 2.60 |
| T10 | 1.05 |
| T11 | 1.72 |

JZ is an isoquinoline alkaloid recognised for its antimicrobial, antioxidant, and anti-inflammatory properties. This compound safeguards the liver from harmful substances, manages lipid metabolism, and supports the management of metabolic conditions like diabetes.

Antioxidant activity may prevent oxidative damage, and cardioprotective potential promotes cardiovascular health. JZ contributes to gut health and has a promising aspect in the study of cancer because of its ability to induce apoptosis in cancer cells. *T. cordifolia* and JZ have been of great research interest as both traditional medicine and modern medicine.[38]

## DISCUSSION

The study effectively extracted and quantified JZ from *T. cordifolia* roots using various methods and optimized the HPTLC technique through Response Surface Methodology. The mobile phase of chloroform: toluene: methanol: formic acid produced a distinct JZ band. Extraction method influenced JZ yield, with Soxhlet, maceration, and reflux showing different efficiencies. *In vitro* results showed significant α-amylase and α-glucosidase inhibitory activity, indicating the antidiabetic potential of the extracts. Overall, the study presents a reliable extraction and analytical approach, supporting the therapeutic promise of *T. cordifolia* in diabetes management.

## CONCLUSION

The identification of *T. cordifolia* root as a new and potent source of JZ marks a significant advancement in natural product research and herbal medicine. Traditionally derived from various Berberidaceae plants, JZ was found in high concentrations in *T. cordifolia* root.

In the study the JZ was extracted from *T. cordifolia* root using Soxhlet, maceration and reflux methods. HPTLC quantification further validated the presence and concentration of JZ in the root extracts, with the $R_f$ value of jatrorrhizine found to be 0.70±0.008, confirming the reliability of the extraction methods in the mobile phase chloroform: toluene: methanol: formic acid (7:4:2:0.2 v/v). This discovery not only broadens the phytochemical profile of *T. cordifolia* root but also enhances its therapeutic potential, especially for antidiabetic applications. The efficient extraction using eco-friendly solvents supports its viability for large-scale production. The present study highlights *T. cordifolia* root as a promising natural therapeutic for diabetes management due to its ability to improve glucose metabolism, enhance insulin

sensitivity, and reduce oxidative stress. The BBD model used to optimize HPTLC conditions demonstrated excellent predictive reliability ($R^2 = 0.9909$) and in HPTLC correlation coefficient was found to be 0.98702.

Furthermore, the extracts showed significant strong inhibitory activity against α-amylase and α-glucosidase enzymes involved in carbohydrate metabolism. These results underscore the potential of *T. cordifolia* root as a sustainable, cost-effective source of JZ and a valuable candidate for plant-based antidiabetic therapies.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**T. cordifolia:** *Tinospora cordifolia;* **JZ:** Jatrorrhizine; **TRS:** *T. cordifolia* root extract soxhlet; **TRM:** *T. cordifolia* root extract maceration; **TRR:** *T. cordifolia* root extract reflux; **DCM:** Dichloromethane; **RSM:** Response-Surface-Methodology; **DNS:** 3,5-Dinitrosalicylic acid; **UAE:** Ultrasound-Assisted Extraction; **pNPG:** p-nitro-α-D-glucopyranoside; **$AlCl_3$:** Aluminium Chloride.

## SUMMARY

JZ is a pharmacologically active isoquinoline alkaloid predominantly found in various Berberis species. It is known for its wide range of therapeutic effects, including antimicrobial, antioxidant, anti-inflammatory, and antidiabetic activities. In the present study, an attempt was made to explore a new natural source of JZ - specifically, the roots of *Tinospora cordifolia*. The extraction was conducted using three different techniques-Soxhlet, maceration, and reflux-with a hydroalcoholic solvent system.

Quantitative estimation of JZ in the various extracts was performed using HPTLC. For chromatographic optimization, RSM was employed to evaluate critical variables, including the composition of the mobile phase and development conditions. The study focused on how variations in the mobile phase affected the $R_f$ value of JZ to ensure precise separation and improved resolution.

The optimized mobile phase composition-chloroform: toluene: methanol: formic acid (7:4:2:0.2 v/v)-produced a well-defined peak for JZ with an $R_f$ value of 0.70±0.008, a solvent front migration distance of 85 mm, and a chamber saturation time of 30 min. The application of RSM significantly enhanced the method's peak resolution, symmetry, and overall analytical efficiency. Furthermore, *in vitro* analysis of the extracts demonstrated notable results in comparison to the standards, reinforcing the potential of *Tinospora cordifolia* root as a promising source of JZ.

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