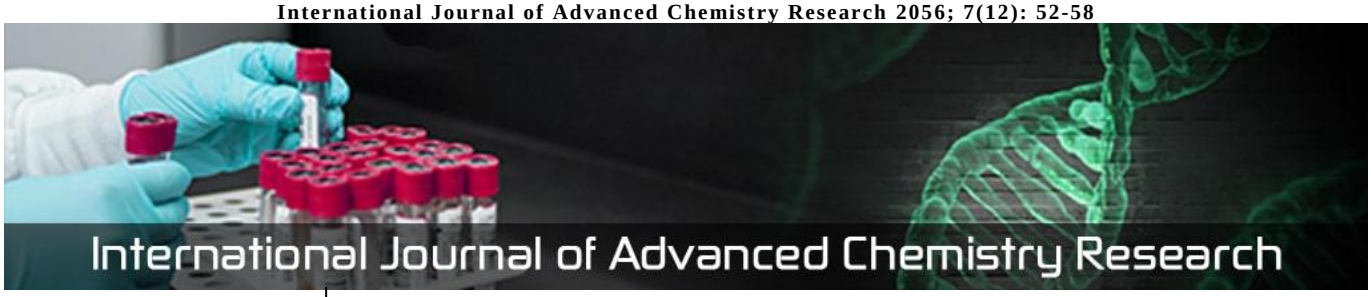

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Qualitative phytochemical profiling and estimation of vasicine using high-performance thin layer chromatography in leaf extract of *Justicia adhatoda L.* from Thanjavur region of India

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Abstract
Justicia adhatoda L. (Acanthaceae) is an important medicinal plant widely used in traditional systems of medicine for the management of respiratory and inflammatory disorders, primarily due to the presence of the bioactive alkaloid vasicine. The present study aimed to evaluate the phytochemical profile and quantify vasicine content in *J. adhatoda* leaves collected from the Thanjavur region of Tamil Nadu, India. Ethanolic extraction of the dried leaves yielded 5.83% and qualitative phytochemical screening confirmed the presence of alkaloids, flavonoids, phenols, tannins, terpenoids, proteins and glycosides. Vasicine was quantitatively estimated using a validated High Performance Thin Layer Chromatography (HPTLC) method, which showed excellent linearity (R^2 = 0.9962) over the range of 100-800 ng/spot. Vasicine was identified at an R_f value of 0.34 and quantified as 2.032% (w/w) on a dry weight basis. FTIR analysis further confirmed the presence of characteristic functional groups corresponding to vasicine. The study highlights the significance of regional evaluation for quality control and standardization of *Vasaka* based herbal formulations.

Keywords: FT-IR, HPTLC, *Justicia adhatoda* L., vasicine, herbal formulations

1. Introduction
Acanthaceae family comprises a wide range of plant species, including numerous medicinally important plants as well as ornamental varieties (Sharma and Kumar, 2016) [17]. Within the family Acanthaceae, the genus *Justicia* comprises approximately 600 species and is widely distributed across tropical and temperate regions (Carneiro *et al.*, 2023) [3]. *Justicia adhatoda* L. (Figure 1) is an important medicinal plant traditionally used by Indian tribal healers and is also a well-established herbal remedy in Ayurveda and Unani systems of medicine for the treatment of cold, cough, asthma, bronchitis, tuberculosis, as well as for managing cuts, wounds, and fever (Wangujare *et al.*, 2023; Nandhini and Ilango, 2020; Bagchi *et al.*, 2003) [23, 12, 1].

This plant, commonly known as Vasaka or Malabar nut, has been used for over 3000 years in Indian traditional medicine for the prevention, management and treatment of various diseases, particularly respiratory disorders, owing to its antibacterial, antifungal, anti-asthmatic, anti-inflammatory, and anti-ulcer activities (Chowdhury *et al.*, 2020) [4]. In addition, the Ministry of AYUSH recommended *Adathodai manapagu*, a Siddha formulation containing *J. adhatoda* as the principal ingredient, along with *Kabasura kudineer*, an Ayurvedic preparation, for prophylaxis and treatment during the COVID-19 pandemic (Banerjee and Gupta, 2021) [2], in continuation of its earlier use against swine flu and dengue fever.

All parts of the plant are widely used in traditional medicine in the form of decoctions, infusions, extracts, juices, and powders to treat various ailments (Claeson *et al.*, 2000) [6]. The therapeutic potential of *J. adhatoda* is attributed to key phytoconstituents, particularly pyrroloquinazoline alkaloids such as vasicine, vasicol, adhatonine, vasicinone, vasicinol, and vasicinolone.

Among these, vasicine (Figure 2) and vasicinone are the major bioactive alkaloids and are reported to possess bronchodilatory, respiratory stimulant, and uterine stimulant activities (Soni *et al.*, 2008) [19]. As the plant grows across diverse climatic and geographical regions, vasicine content shows considerable variation, highlighting the need to identify superior chemotypes with higher alkaloid levels. Vasaka leaves are in high demand in the herbal pharmaceutical industry and are largely sourced from the wild; however, such raw materials exhibit significant quality variability (Raja *et al.*, 2008) [19]. This variation is also seasonal, with alkaloid concentrations peaking during the flowering phase in spring (Vasant ritu) and declining during the vegetative stage (Sharma *et al.*, 2019) [18]. Thus, it is essential to analyze the concentration of vasicine from different region to formulate a standardized herbal formulation.

The present study focuses on qualitative phytochemical screening, quantification of vasicine using High Performance Thin Layer Chromatography (HPTLC) and its confirmation through FTIR analysis in extracts of *J. adhatoda* collected from the Thanjavur region of Tamil Nadu, India.

Fig 1: *Justicia adhatoda* L.

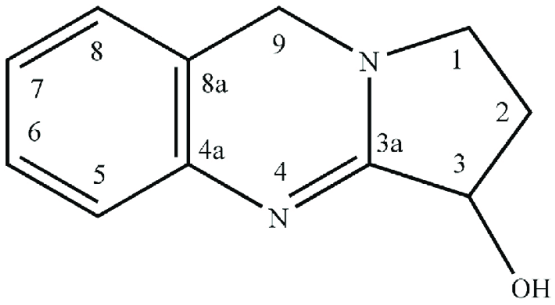

Fig 2: Structure of vasicine

2. Materials and Methods

2.1 Collection and authentication of plant materials

Fresh leaves of *J. adhatoda* L. (Syn. *Adhatoda vasica*) were collected from various locations in Orathanadu, Thanjavur district, Tamil Nadu. The plant material was authenticated by the Department of Pharmacognosy, Siddha Central Research Institute, Arumbakkam, Chennai, and the herbarium specimen (J22042319A) was prepared and deposited in the institute repository. The leaves were shade-dried, finely powdered using a pulverizer and stored in airtight containers for further analysis.

2.2 Extraction and yield percentage of plant materials

One hundred grams of dried *J. adhatoda* leaf powder were exhaustively extracted with ethanol (1000 mL) using a Soxhlet apparatus. The extraction was continued until the solvent in the siphon tube became colorless, indicating completion of the process. The obtained extract was concentrated under reduced temperature (25-30 °C) and vacuum (40 mbar) employing a rotary evaporator (Büchi Rotavapor R-300, Switzerland). The concentrated extract was preserved at 4 °C for subsequent analyses. The percentage yield of the extract was calculated using the following formula:

$$\text{Percentage yield} = \frac{\text{Final weight of the dried extract}}{\text{Initial weight of the powder}} \times 100$$

2.3 Qualitative phytochemical analysis of crude extract

Freshly prepared *J. adhatoda* leaf extracts were subjected to preliminary qualitative phytochemical screening following standard methods described by Trease and Evans (1989) [21]. The screening was performed to identify major phytoconstituents such as alkaloids, anthraquinones, flavonoids, glycosides, phenols, tannins, steroids, resins, saponins, and terpenoids. The specific tests employed for this analysis are summarized in Table 1**.**

2.4 HPTLC estimation of vasicine from leaf extracts of *J. adhatoda*

2.4.1 Chemicals and reagents

Vasicine (Product No PHL89821) was procured from Sigma-Aldrich (M/s Sigma Aldrich Chemicals Pvt. Ltd., USA). Precoated HPTLC silica gel 60 F_{254} aluminum plates (20 × 10 cm, 0.25 mm thickness) were obtained from E. Merck (M/s Merck Specialities Ltd., Worli, Mumbai, India). All other chemicals and reagents used were of analytical grade**.**

2.4.2 Standard and sample preparation

A stock solution of vasicine was prepared by dissolving 1 mg of the standard in 1 mL of methanol. This solution was subsequently diluted tenfold with methanol to obtain a working concentration of 100 µg/mL. The *J. adhatoda* leaf extract was also dissolved in methanol to yield a concentration of 10 mg/mL for spotting on the HPTLC plates.

2.4.3 Calibration curve of vasicine

Different volumes (1-8 µL) of the working standard solution (100 µg/mL) were applied to the HPTLC plates to obtain vasicine concentrations ranging from 100 to 800 ng per band. The plates were then developed under optimized chromatographic conditions. Quantification was achieved by plotting peak area versus corresponding standard concentrations and the calibration curve was constructed using regression analysis

2.4.4 Instrumentation and chromatographic conditions

Chromatographic separation was carried out in a CAMAG glass twin-trough HPTLC chamber (20 × 10 × 4 cm) using linear ascending development. Samples were applied with a Linomat V automatic applicator (CAMAG, Muttenz, Switzerland) fitted with a 100 µL Hamilton syringe

(Bonaduz, Switzerland). Precoated silica gel 60 F_{254} HPTLC plates (E-Merck; 20 × 10 cm, 0.25 mm thickness) served as the stationary phase, with bands applied at a width of 8 mm. Densitometric evaluation was performed using an HPTLC Scanner 4 integrated with visionCATS software (CAMAG), employing a slit dimension of 6.00 × 0.45 mm and a scanning speed of 20 mm/s. Detection was carried out at 254 nm using deuterium and tungsten lamps. The mobile phase comprised toluene, ethyl acetate, methanol, and ammonia in the ratio of 30:30:15:1 (v/v/v/v). All analyses were conducted under controlled laboratory conditions at 25±2 °C and 55% relative humidity

Table 1: Procedure for qualitative phytochemical screening

S. No.	Test	Procedure	Result	Interpretation
Test for alkaloids				
1.	Mayer's test	1 ml of plant extract + 2-3 drops of Mayer's reagent	Appearance of pale-yellow colour precipitate	Presence of alkaloids
2.	Dragendroff test	1 ml of plant extract + 2-3 drops of Dragendroff's reagent	Appearance of reddish-brown colour precipitate	Presence of alkaloids
3.	Wagner's test	1 ml of plant extract + 2-3 drops of Wagner's reagent	Appearance of reddish-brown colour precipitate	Presence of alkaloids
4.	Hager's test	1 ml of plant extract + 2-3 drops of Hager's reagent	Appearance of orange or yellow colour precipitate	Presence of alkaloids
Test for terpenoids				
5.	Salkowski test	1 mL of plant extract + 2 mL of chloroform + conc. sulfuric acid along the sides.	Formation of yellow coloured lower layer	Presence of terpenoids
Test for flavonoids				
6.	Shinoda test	1 mL of plant extract + few fragments of Magnesium ribbon + conc. hydrochloric acid	Appearance of pink, scarlet, crimson red or occasionally green to blue colour after few minutes	Presence of flavonoids
7.	Alkaline reagent test	1% sodium hydroxide solution + 1 mL of test solution + few drops of dil. hydrochloric acid	Turns to colourless	Presence of flavonoids
Test for tannins				
8.	Braymer's test	1 mL of plant extract + 1 mL of water + 2-3 drops of 5% ferric chloride solution	Formation of green colour precipitate	Presence of tannins
Test for saponins				
9.	Foam test	2 ml of plant extract + 10 ml of distilled water (shaken well for 15 min)	Formation of stable foam	Presence of saponins
Test for carbohydrates				
10.	Benedict's test	1 ml of plant extract + few drops of Benedict's reagent, then boiled on water bath.	Formation of reddish-brown precipitate	Presence of carbohydrates
11.	Molisch test	0.5 ml of plant extract + few drops of alcoholic α-napthol + 0.2 ml of conc. sulfuric acid along the sides	Appearance of purple to violet colour ring	Presence of carbohydrates
Test for glycosides				
12.	Borntrager's test	1 ml of plant extract + 2 ml of dil. sulfuric acid (boiled and then filtered) + equal volume of benzene or chloroform + ammonia	Appearance of pink colour in the ammoniacal layer	Presence of glycosides
Test for cardiac glycosides				
13.	Keller-killani test	1 ml of plant extract + 0.4 ml of glacial acetic acid (containing a trace amount of ferric chloride) + conc. sulphuric acid along the sides	Appearance of blue colour appears in the acetic acid layer	Presence of cardiac glycosides
Test for phenols				
14.	Ferric chloride test	1 ml of plant extract+ dil. Ferric chloride	Formation of blue colour	Presence of phenols
Test for Proteins				
15.	Millon's test	0.5 ml of plant extract + 2 ml of Millon's reagent. Then gently heated.	Appearance of white precipitate	Presence of proteins

2.4.5 Quantification of vasicine

The *J. adhatoda* leaf extract (10 mg/mL) was applied onto clean HPTLC plates in seven different volumes (1-7 µL) along with the working standard solution. The plates were then developed (Figure 5.) and scanned following the previously described standardized conditions. Vasicine was identified by comparing the R_f values of the sample bands with those of the reference standard. Quantification was performed using the calibration data derived from standard peak areas and concentrations, and the vasicine content was expressed as a percentage of the dry weight of the powdered sample (HPTLC Association, 2021) [8].

2.5 Fourier Transform Infra-Red analysis

FT-IR analysis of the crude *J. adhatoda* leaf extract was performed to identify functional groups corresponding to different classes of phytoconstituents and to assess their compatibility using a Thermo Nicolet 6700 Fourier Transform Infrared (FT-IR) spectrometer. The liquid sample was placed in the sample cell and scanned over the wavenumber range of 4000-400 cm^{-1}.

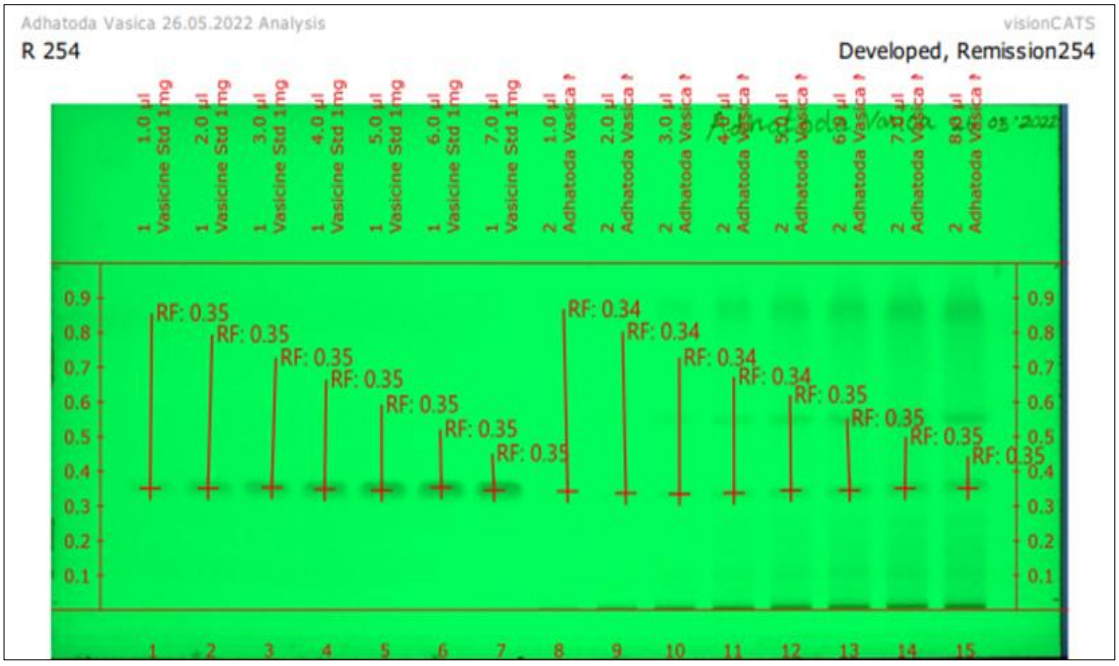

Fig 3: Developed HPTLC plate showing vasicine at R_f value 0.34

3. Results and Discussion

The percentage extraction yield of *J. adhatoda* leaves was 5.83%, which is comparable to the 7% yield reported by Vinothapooshan and Sundar (2010) [22]. In contrast, Srinivasan and Kumaravel (2015) [20] reported a higher yield of 16.30%, likely due to variations in geographical origin, solvent selection, extraction time, temperature, harvesting season, and extraction methodology (Kaneria *et al.*, 2012) [10]. The results of the preliminary phytochemical screening of the *J. adhatoda* leaf extract (Table 2) indicated the presence of alkaloids, phenols, tannins, terpenoids, flavonoids, proteins, and glycosides, in agreement with the observations of Selvarani and Jeyasimha (2020) [16].

Table 2: Qualitative phytochemical analysis of *J. adhatoda* leaf extract

S. No.	Phytochemicals examined	Name of the test	Inference
1.	Carbohydrates	Benedict's test	Negative
		Molisch's test	Negative
2.	Saponins	Foam test	Negative
3.	Alkaloids	Mayer's test	Positive
		Dragendroff"s test	Positive
		Wagner's test	Positive
		Hager's test	Positive
4.	Phenols	Ferric chloride test	Positive
5.	Tannins	Braymer's test	Positive
6.	Terpenoids	Salkowski's test	Positive
7.	Flavonoids	Shinoda's test	Positive
		Alkaline reagent test	Positive
8.	Proteins	Millon's test	Positive
9.	Glycosides	Borntrager's test	Positive
10.	Cardiac glycosides	Keller-killani test	Negative

High Performance Thin Layer Chromatography (HPTLC) has emerged as a preferred analytical technique over conventional methods due to its simplicity, speed, accuracy, robustness, and cost-effectiveness (Dhandhukia and Thakker, 2011) [7]. In the present study, HPTLC was utilized to determine the vasicine content in *J. adhatoda* leaf extracts.

The method's linearity was evaluated using vasicine working standards over a concentration range of 100-800 ng per spot, corresponding to sample volumes of 1-8 µL. The regression analysis yielded the equation $Y = 1.192 \times 10^{-9} X + 9.399 \times 10^{-4}$, with a coefficient of variation of 3.61%. A correlation coefficient of 0.9962 confirmed excellent linearity for the vasicine standard. The calibration curve for the working standard vasicine is presented in Figure 4.

The presence of vasicine in the sample was confirmed by matching the R_f values of the standard and the extract, with vasicine observed at an R_f of 0.34 on the HPTLC plate. Representative densitograms of both the standard and sample are shown in Figure 5. HPTLC analysis revealed that the vasicine content in *J. adhatoda* leaf extract was 2.032% on a dry weight basis. This value is consistent with the findings of Priya *et al.* (2021) [14], who reported a vasicine content of 1.5%.

The FT-IR absorption spectra of *J. adhatoda* leaf extract showed the peaks at 3391.08, 2973.65 & 2901.10, 2255.36 & 2133.88, 1634.97, 1453.07, 1382.85, 1337.33, 1273.75, 1082.83, 1049.12 and 647.99 cm^{-1} indicates the presence of alcohol, alkane, alkyne, alkene, aromatic compound, primary alcohol, amine and disubstituted cis attributed to

functional groups *viz.* O-H stretching, C-H stretching, C≡C stretching, C-H bending, O-H bending, C-N stretching, C=O stretching, C-N stretching, C-H out of plane bending and C=C bending. The detail is exhibited in Figure 6 and shown in Table 3. These findings are consistent with the observations made by Islam *et al.* (2021) [9]. Furthermore, the IR spectrum of vasicine, as analyzed by Narasimhaji *et al.* (2023) [13], exhibited peaks at 3071 (hydroxyl), 2847, 1636 (>C=N), 1481, 1308, 1184, 1110, 834 and 760 cm^{-1}, aligns with current observations, indicating the presence of vasicine in the *J. adhatoda* sample under investigation.

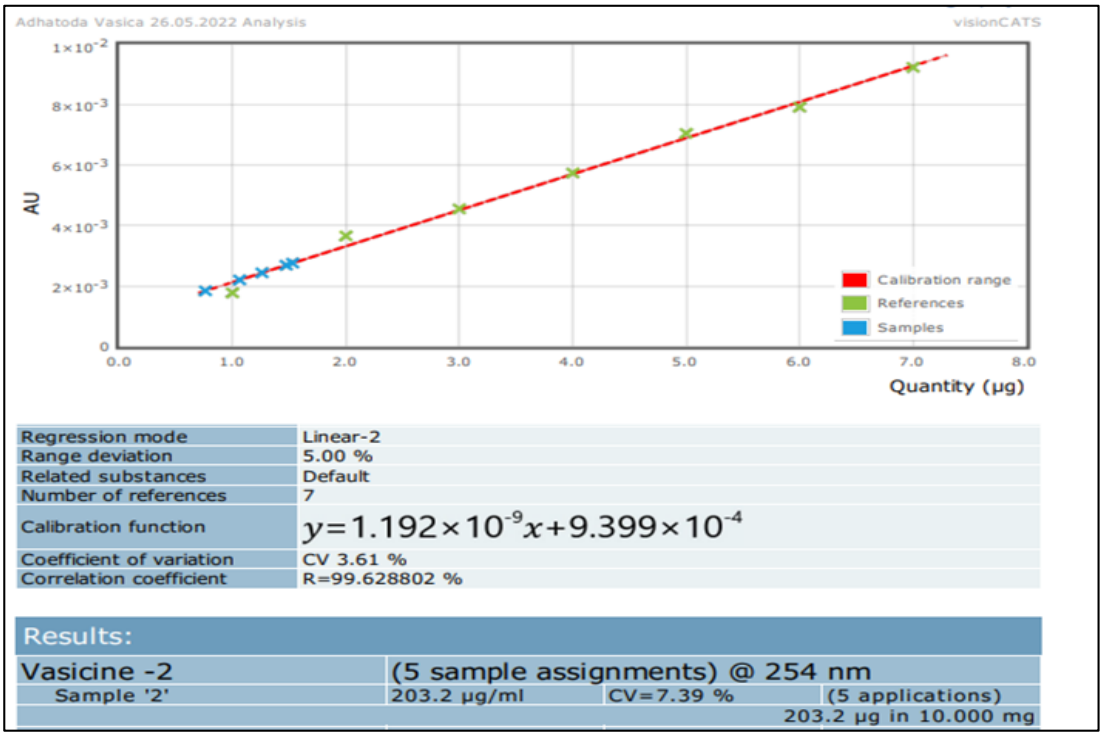

Fig 4: Calibration curve of vasicine (Standard) in *J. adhatoda* leaf extract

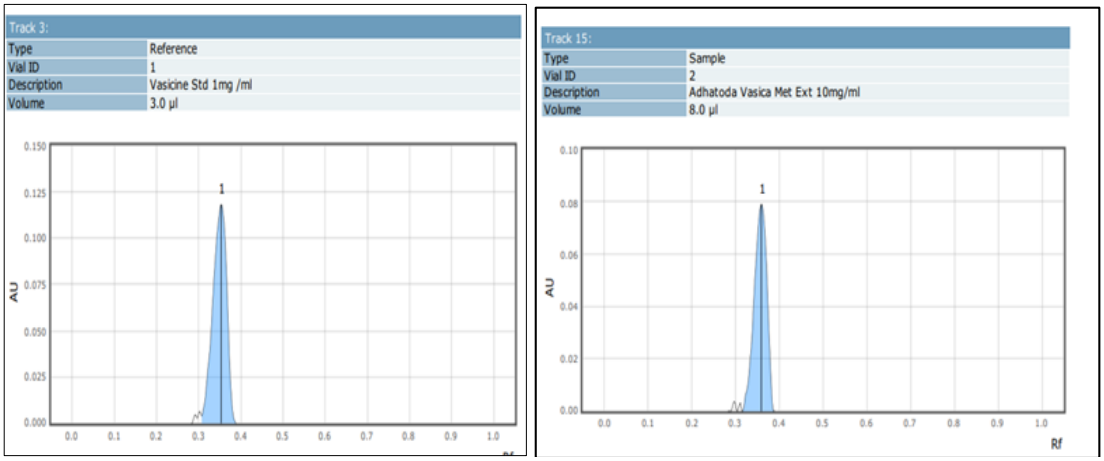

Fig 5: Representative densitograms of vasicine standard (a) and vasicine in sample (b)

Cimpoiu (2011) [15] stated that IR spectroscopy has a high potential for the elucidation of molecular structures, and the characteristic absorption bands can be used for compound-specific detection. In future, HPTLC-FTIR coupled method can be used in the laboratories for the qualitative and quantitative analysis of herbal medicines. In the present study also the vasicine content was estimated using HPTLC and its presence was qualitatively confirmed by IR spectroscopy.

4. Conclusion

The present study systematically evaluated *Justicia adhatoda* L. leaves collected from the Thanjavur region of Tamil Nadu, confirming their phytochemical richness and suitability for standardization. HPTLC analysis demonstrated excellent linearity and precision for vasicine estimation, with the compound clearly identified at an R_f value of 0.34 and quantified at 2.032% (w/w) on a dry weight basis, indicating that the regional plant material is a good source of this therapeutically important alkaloid. FTIR spectral data further supported the presence of vasicine and related functional groups, corroborating the chromatographic findings. Collectively, these results highlight the importance of regional and chemotypic evaluation of *J. adhatoda* due to natural variability in alkaloid content and establish HPTLC, supported by FTIR, as a reliable approach for quality control and standardization of *Vasaka* based herbal formulations.

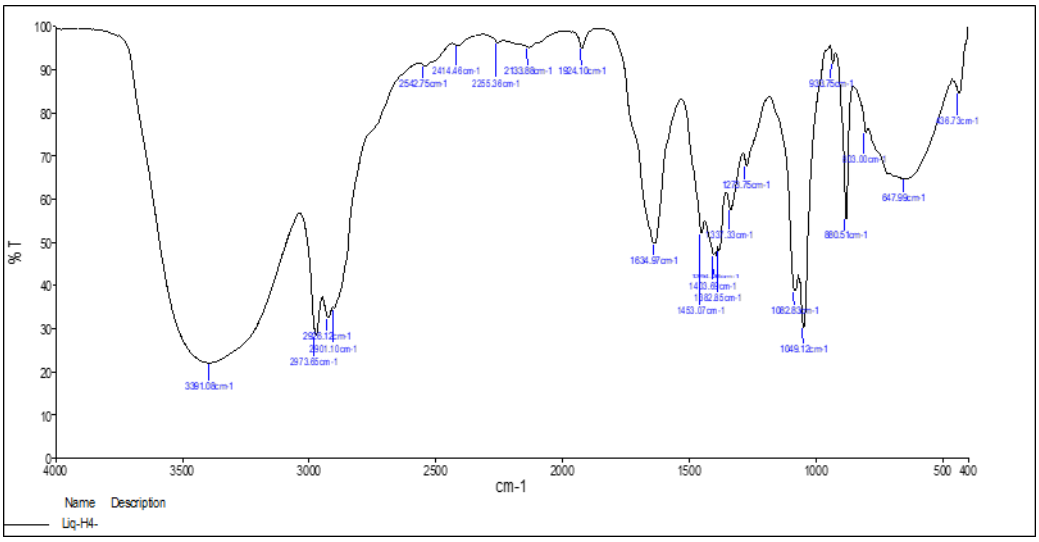

Fig 6: FTIR spectrum of *J. adhatoda* leaf extract

Table 3: FTIR analysis report of *J. adhatoda* leaf extract

S. No.	Wave number (cm^{-1})	Functional groups	Compound class	Bonding Pattern
1.	3391.08	O-H stretching	Alcohol	Strong, broad
2.	2973.65, 2901.10	C-H Stretching	Alkane	Strong
3.	2255.36, 2133.88	C≡C stretching	Alkyne	Weak
4.	1634.97	C=C stretching	Alkene	Medium
5.	1453.07, 1382.85	C-H bending	Alkane	Medium
6.	1337.33	O-H bending	Alcohol	Medium
7.	1273.75	C-O stretching	Aromatic compound	Medium
8.	1082.83	C-O stretching	Primary alcohol	Strong
9.	1049.12	C-N stretching	Amine	Strong
10.	880.51	C-H Out of plane bending	Aromatic group	Medium
11.	647.99	C=C bending	Disubstituted cis	Medium broad

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