

Phytochemical Characterization and Biological Properties of *Justicia adhatoda* L. Leaves: An Investigation Using HPTLC and GC-MS Techniques

Parameswari A.**, Pavithra S.** and Suresh K.*

Abstract

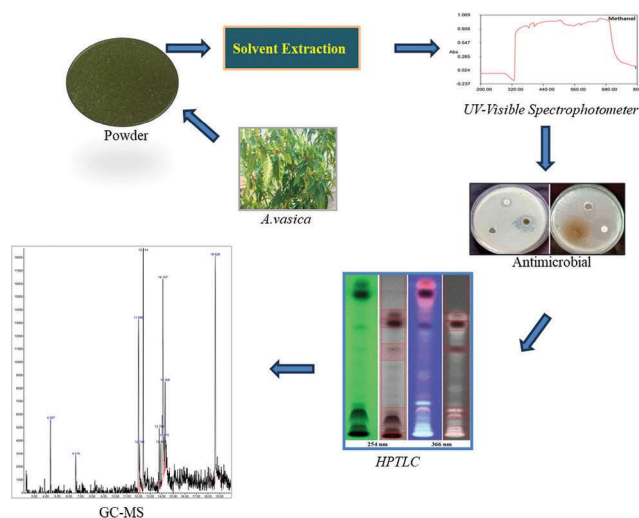
The present study was undertaken to characterize the phytochemical constituents and evaluate the biological activities of *Justicia adhatoda* L. using different analytical approaches. Qualitative phytochemical screening indicated the occurrence of alkaloids, phenolic compounds, flavonoids, saponins, steroids, and tannins, whereas anthocyanins, glycosides, and terpenoids were not detected. Among the quantified phytoconstituents, alkaloids and flavonoids were present at relatively higher levels, with concentrations of 8.46 and 8.24 µg/mL, respectively, followed by tannins at 4.12 µg/mL. The antibacterial potential of the plant extracts was examined against selected bacterial strains, and the methanolic extract exhibited the highest inhibitory effect against *Enterobacter*, showing activity comparable to that of streptomycin. The antioxidant properties of the extracts were further investigated through DPPH and ABTS radical-scavenging assays. Considerable antioxidant activity was observed, particularly with the methanol and ethyl acetate fractions. Spectroscopic and chromatographic investigations using UV-Visible spectroscopy and HPTLC demonstrated characteristic absorption patterns and several prominent chromatographic peaks, indicating the presence of diverse phytochemical constituents. GC-MS profiling subsequently revealed eleven compounds, with fatty acid derivatives and terpenoid-related compounds constituting the major identified components.

Keywords: *Justicia adhatoda* L.; Phytochemical composition; Antibacterial activity; Antioxidant activity; GC-MS analysis

** Department of Microbiology, K. R. College of Arts & Science, K. R. Nagar, Kovilpatti, 628 503 Tamil Nadu, India; parameswari_micro@krcollege.net

* Department of Biotechnology, M.G.R. College, Hosur 635130, Tamil Nadu, India; sureshbioteck@yahoo.com

Graphical abstract



1. Introduction

Medicinal plants continue to play an important role in healthcare because they contain numerous naturally occurring compounds with potential therapeutic value. One such plant is *Justicia adhatoda* L., commonly known as Malabar nut or Vasaka, which belongs to the family Acanthaceae. The plant has a long history of use in traditional medical practices, including Ayurveda and Unani, particularly for the management of respiratory and inflammatory disorders [1]. It is widely distributed across tropical and subtropical regions of Southeast Asia and has attracted considerable scientific interest because of its broad range of biological activities, including antimicrobial, anti-inflammatory, and antitussive effects [2].

The leaves are considered an important medicinal part of *J. adhatoda* because they contain several classes of secondary metabolites. Alkaloids such as vasicine and vasicinone are among the characteristic constituents, along with flavonoids and phenolic compounds. The presence and concentration of these compounds are closely associated with the biological properties attributed to the plant, making phytochemical characterization an important aspect of its scientific evaluation [3]. Modern chromatographic techniques have considerably improved the assessment of medicinal plants. High-Performance Thin-Layer Chromatography (HPTLC) is particularly useful for generating characteristic chemical fingerprints and comparing the distribution of different constituents, whereas Gas Chromatography-Mass Spectrometry (GC-MS) enables the detection and characterization of volatile and thermally stable compounds within complex plant extracts [4].

Application of these techniques to *J. adhatoda* leaves can provide valuable information regarding their chemical composition and assist in establishing appropriate quality control parameters [5]. In addition, the biological significance of the identified constituents can be examined through appropriate pharmacological assays. Previous investigations have reported antibacterial, antioxidant, and cytotoxic effects associated with extracts or isolated constituents of the plant, suggesting that its phytochemical composition may contribute to several of its reported medicinal properties [6,7]. Consequently, integrating phytochemical characterization with biological assessment provides a useful approach for establishing the therapeutic relevance of *J. adhatoda* leaves. Such investigations may also contribute to the identification of promising natural compounds for further pharmacological research and potential drug-development applications [8]. This work aims to conduct an in-depth examination of the phytochemical composition of *Justicia adhatoda* L. leaves using sophisticated techniques such as HPTLC and GC-MS. The study tries to find a link between the detected phytochemicals and the plant known biological functions. The study aims to discover the therapeutic potential of *Justicia adhatoda* L. leaves by combining traditional medical knowledge and current scientific approaches.

2. Materials and Methods

2.1 Plant Collection and Extract Preparation

Fresh leaves of *Justicia adhatoda* L. were collected from the K.R. College campus situated between 78°04'47"E–78°11'23"E longitude and 9°50'59"N–9°57'36"N latitude. The plant was identified and authenticated based on its diagnostic morphological characteristics using the relevant taxonomic descriptions and identification keys for the family Acanthaceae, with particular reference to *Justicia adhatoda* L. [9]. The identification was supported by characteristic features including the shrub-like habit, opposite, large elliptic to lanceolate leaves, prominent venation, and the characteristic inflorescence and floral morphology of the species. The collected material was carefully inspected, and extraneous matter was removed before processing. The leaves were subsequently spread in a well-ventilated shaded area and allowed to dry naturally for approximately three weeks. Shade drying was adopted to reduce the possible deterioration of heat-sensitive phytoconstituents. After complete drying, the leaves were pulverized into a fine, uniform powder.

2.1.1 Preparation of Solvent Extracts

The powdered leaf material was subjected to Soxhlet extraction using six different solvents, namely methanol, ethanol, ethyl acetate, acetone, chloroform, and petroleum ether. Each extraction was carried out separately at 60°C for 4 h to obtain solvent-specific extracts. Following extraction, the

resulting solutions were passed through Whatman No. 1 filter paper to remove residual plant particles. The filtrates were then concentrated under reduced pressure to remove the respective solvents. The concentrated extracts were transferred into clean, properly labelled amber coloured glass bottles and preserved until further phytochemical and biological analyses.

2.2 Qualitative and Quantitative Phytochemical Analysis

The different solvent extracts prepared from *Justicia adhatoda* L. leaves were subjected to preliminary phytochemical screening to determine the major classes of secondary metabolites and other bioactive constituents. Alkaloids were examined using Dragendorff's reagent, whereas flavonoids were detected by the alkaline reagent reaction. Phenolic compounds and tannins were evaluated using ferric chloride. The presence of terpenoids was investigated by the Salkowski reaction, while saponins were screened using the foam test. Phlobatannins were assessed by the hydrochloric acid test, and quinones and coumarins were examined using sodium hydroxide. Cardiac glycosides were identified through the Keller–Killiani reaction. Protein content was qualitatively assessed by Lowry's method. For each test, the observed reaction was recorded as either positive (+), indicating detection of the respective constituent, or negative (–), indicating its absence under the test conditions[10,11].

2.2.1 Total Phenolic Content

The concentration of phenolic constituents in the crude extracts was determined by employing the Folin–Ciocalteu colorimetric assay. Gallic acid was used as the reference standard, and a calibration plot was prepared using concentrations ranging from 0.0 to 25 µg/mL. The absorbance of the developed reaction mixtures was recorded at 760 nm with a Shimadzu UV-Visible spectrophotometer (UV Mini-1240, Japan). The amount of phenolic compounds present in each extract was calculated from the gallic acid calibration curve and expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

2.2.2 Total Flavonoid Content

2 g of the powdered *Justicia adhatoda* leaf sample was treated with 10 mL of 80% methanol. The extraction mixture was maintained at room temperature for 4 h to facilitate the release of flavonoid constituents into the solvent. Following incubation, the extract was filtered to separate the insoluble plant material. The resulting filtrate was allowed to evaporate at room temperature overnight until a dry residue was obtained. The dried residue was weighed, and the flavonoid content was calculated as a percentage of the initial sample weight using the following formula:

$$\text{Alkaloid Content (\%)} = \frac{\text{Weight of extracted material}}{\text{Weight of sample}} \times 100$$

2.2.3 Total Alkaloid Content

The alkaloid fraction was quantified by treating 5 g of the powdered plant sample with 50 mL of 10% acetic acid prepared in ethanol. The suspension was heated in a water bath for 4 h to facilitate extraction of the alkaloidal constituents. Following extraction, concentrated ammonium hydroxide (NH₄OH) was added gradually to the extract until no further precipitation was observed, indicating maximum precipitation of the alkaloids. The resulting precipitate was separated, dried to constant weight, and accurately weighed. The alkaloid content was subsequently expressed as a percentage of the original sample weight using the following formula:

$$\text{Alkaloid Content (\%)} = \frac{\text{Weight of extracted material}}{\text{Weight of sample}} \times 100$$

2.2.4 Total Tannin Content

The tannin concentration of the *Justicia adhatoda* leaf extracts was determined using a ferric chloride-potassium ferrocyanide colorimetric method. An aliquot of the extract was reacted with ferric chloride and potassium ferrocyanide in the presence of hydrochloric acid, with distilled water used as the reaction medium. The resulting colour intensity was measured at 605 nm using a UV-Visible spectrophotometer. Tannic acid was employed as the reference standard, and a calibration curve was established using concentrations ranging from 0.0 to 10 µg/mL.

2.3 Antibacterial Activity

The antibacterial properties of the different *Justicia adhatoda* L. leaf extracts were examined against five bacterial strains: *Bacillus subtilis* MTCC 1134, *Escherichia coli* MTCC 406, *Klebsiella pneumoniae* MTCC 7407, *Proteus vulgaris* MTCC 7299, and *Pseudomonas aeruginosa* MTCC 1748. The bacterial cultures were procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology (CSIR-IMTech), Chandigarh, India. The cultures were maintained as laboratory stock cultures and subcultured prior to the antibacterial assay to obtain actively growing cells. The assay was performed using the agar well-diffusion technique with Mueller-Hinton agar as the test medium. Following inoculation of the bacterial cultures, wells measuring approximately 5 mm in diameter were aseptically prepared in the agar surface. Each well was loaded with 20 µL of the respective plant extract obtained using methanol, ethanol, ethyl acetate, acetone, chloroform, or petroleum ether as extraction solvents. The

inoculated plates were maintained at 37°C for 24 h. Antibacterial activity was assessed by recording the diameter of the clear zone formed around each well, and the results were expressed in millimetres.

2.4 Antioxidant Activity

2.4.1 DPPH Assay:

The free-radical scavenging ability of the *Justicia adhatoda* L. extracts was examined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. A DPPH stock solution was prepared by dissolving 24 mg of DPPH in 100 mL of methanol. The solution was subsequently diluted with methanol to obtain a working solution with an appropriate absorbance at 517 nm. Different concentrations of the plant extracts, ranging from 10 to 500 µg/mL, were separately mixed with the DPPH working solution. The reaction mixtures were kept at room temperature in the absence of light for 15 min. After incubation, the absorbance was measured at 517 nm against a suitable blank using a UV-Visible spectrophotometer. The decrease in absorbance relative to the DPPH control was used to determine the radical-scavenging activity of the extracts.

$$\text{scavenging effect(\%)} = \frac{\text{control absorbance} - \text{sample absorbance}}{(\text{control absorbance})} \times 100$$

2.4.2 ABTS Assay:

The antioxidant potential of the *Justicia adhatoda* L. extracts was further assessed using the ABTS radical cation decolorization assay. The ABTS radical cations were generated by combining a 7 mM ABTS solution with 2.45 mM potassium persulfate. The mixture was allowed to react to facilitate radical formation and was subsequently diluted with methanol to obtain the working solution. The absorbance of the diluted ABTS solution was adjusted and recorded at 745 nm. Different concentrations of the plant extracts were then added to the ABTS working solution and allowed to react under the assay conditions. The reduction in absorbance resulting from radical scavenging was measured spectrophotometrically, and the antioxidant activity was expressed as the percentage inhibition of ABTS radical cations.

$$\text{scavenging effect(\%)} = \frac{\text{control absorbance} - \text{sample absorbance}}{(\text{control absorbance})} \times 100$$

2.5 UV-Visible Spectrophotometry

The methanolic extracts of *Justicia adhatoda* L. leaves were subjected to UV-Visible spectroscopic analysis using a double-beam UV-Visible spectrophotometer (SYSTRONICS). The absorption spectra of the extracts were recorded across the wavelength range of 200–800 nm to characterize their spectral profiles and identify the major absorption regions.

2.6 HPTLC Analysis

High-Performance Thin-Layer Chromatography (HPTLC) was employed to obtain the chromatographic profile of the *Justicia adhatoda* L. extracts. The analysis was performed on precoated silica gel 60 F254 plates measuring 7.0 × 10 cm. The samples were applied onto the plate in the form of narrow bands using a CAMAG Linomat-V applicator. Chromatographic development was carried out in a suitable developing chamber using chloroform and methanol in a 9:1 (v/v) ratio as the mobile phase. After completion of the development, the plates were examined under ultraviolet illumination at 254 and 366 nm, and the separated bands were recorded based on their characteristic absorption profiles.

2.7 GC-MS Analysis

The chemical constituents present in the *Justicia adhatoda* L. extract were investigated by Gas Chromatography–Mass Spectrometry (GC–MS) using an Agilent 7820A gas chromatograph interfaced with a 5977E mass selective detector. For chromatographic separation, the oven was programmed from an initial temperature of 100°C and increased at a rate of 10°C/min until reaching 270°C, followed by an isothermal hold for 10 min. A sample volume of 2 µL was introduced into the GC system, with helium employed as the carrier gas at a constant flow rate of 1.2 mL/min. Mass spectral detection was carried out using an electron ionization energy of 70 eV. The complete GC–MS analysis was performed over a total runtime of 18.62 min.

3. Results and discussion

3.1 Phytochemical screening

The qualitative screening of *Justicia adhatoda* L. leaf extracts revealed the occurrence of several classes of phytochemicals. Alkaloids, phenolic compounds, flavonoids, saponins, steroids, and tannins gave positive reactions in the screening assays, whereas anthocyanins, glycosides, and terpenoids were not detected under the experimental conditions (Table 1). The corresponding observations are illustrated in Figure 1.



Figure 1: Leaves of *Justicia adhatoda* L. collected from the K.R. College campus.

Quantitative estimation further demonstrated the presence of appreciable amounts of selected phytoconstituents. The alkaloid concentration was 8.46 $\mu\text{g}/\text{mL}$, followed by flavonoids at 8.24 $\mu\text{g}/\text{mL}$ and tannins at 4.12 $\mu\text{g}/\text{mL}$. The relatively higher levels of alkaloids and flavonoids compared with tannins indicate that these constituents may represent important components of the chemical profile of the investigated leaf extract.

The phytochemical profile obtained in the present investigation is consistent with the recognized medicinal importance of *J. adhatoda*. The detection of alkaloids, phenolics, flavonoids, saponins, steroids, and tannins indicates that the leaves contain a chemically diverse mixture of secondary metabolites. Among these groups, alkaloids are of particular interest because *J. adhatoda* is well known for quinazoline alkaloids, especially vasicine and related compounds. These constituents have been extensively investigated in relation to the traditional use of the plant in respiratory disorders [12].

Differences between the present phytochemical profile and findings reported in earlier studies should be interpreted with consideration of the experimental conditions. In the current investigation, anthocyanins, glycosides, and terpenoids were not detected, whereas some previous reports have recorded these constituents in *J. adhatoda* or related preparations [13,14]. Such differences can arise from several factors, including geographical origin, environmental conditions, developmental stage of the plant, season of collection, drying conditions, extraction solvent, and the sensitivity or specificity of the qualitative test employed. Therefore, a negative result in a preliminary screening assay should be interpreted as non-detection under the conditions used rather than definitive evidence that the compound class is completely absent from the plant.

The present findings provide preliminary chemical evidence supporting the traditional medicinal relevance of *J. adhatoda*. The occurrence of alkaloids, flavonoids, phenolic compounds, saponins, steroids, and tannins, together with the quantified levels of alkaloids, flavonoids, and tannins, provides a basis for the subsequent evaluation of the extract's antibacterial and antioxidant properties. However, the therapeutic effects traditionally attributed to *J. adhatoda*, including its use for respiratory and inflammatory conditions, should not be ascribed solely to the phytochemical screening results. Further chromatographic characterization, isolation of individual constituents, and biological studies are required to establish specific compound-activity relationships [15-17].

Table 1: Qualitative Phytochemical Analysis of extracts *Justicia adhatoda* L.

Phytochemicals / Solvent	Methanol	Ethanol	Acetone	Chloroform	Petroleum Ether	Observation
Glycosides	+	+	+	+	-	Reddish brown ring at interphase
Phenolics	+	-	+	+	-	Formation of an intense yellow color
Tannins	+	+	+	+	+	Blue green coloration
Saponins	+	-	+	+	+	Formation of foam on the top of the test tubes
Flavonoids	+	+	+	+	-	Yellow solution turned colorless
Proteins	+	+	+	+	-	Turn to blue
Quinones	+	+	-	-	+	Changing to red
Terpenoids	+	+	-	-	+	Reddish-brown coloration
Phlobatanin	+	+	-	-	+	Formation of a red precipitate
Coumarins	+	+	+	+	-	Colored complex
Alkaloids	+	+	+	+	+	Orange red precipitate
Steroids	+	-	+	+	-	Green blue formation

3.2 Antibacterial evaluation

The antibacterial activity of the different solvent extracts of *Justicia adhatoda* L. leaves was evaluated against five bacterial pathogens using the agar well-diffusion method. The inhibition zones produced by the methanol, ethanol, ethyl acetate, acetone, chloroform, and petroleum ether extracts are presented in Table 2. The extracts were tested at a concentration of 75 mg/mL, and the assay was carried out in triplicate. The inhibition zones were measured in millimetres and expressed as mean values. Appropriate solvent controls were included to determine whether the solvents themselves contributed to the observed antibacterial activity, while standard antibiotics were used as positive controls.

Among the tested extracts, methanol generally produced the largest inhibition zones against the bacterial strains examined. The methanolic extract produced inhibition zones of 13, 15, 19, 19, and 13 mm against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus vulgaris*, respectively. The ethanol extract also demonstrated appreciable antibacterial activity, with inhibition zones ranging from 14 to 17 mm. In comparison, the ethyl acetate and acetone extracts produced moderate inhibition, whereas the chloroform extract exhibited the lowest activity, with inhibition zones ranging from 10 to 11 mm. The petroleum ether extract showed slightly greater activity than chloroform against some of the tested organisms but remained less effective than the methanol and ethanol extracts.

The highest inhibition zone among the plant extracts was observed with the methanolic extract against *Escherichia coli* and *Klebsiella pneumoniae*, both producing zones of 19 mm. These values were lower than the corresponding antibiotic controls, which produced inhibition zones of 25 and 24 mm, respectively. Against *Bacillus subtilis*, the ethanol extract produced the highest inhibition zone (14 mm), while the methanol extract showed the highest activity against *Pseudomonas aeruginosa* (15 mm). For *Proteus vulgaris*, ethanol produced the largest zone (16 mm), followed by petroleum ether (14 mm). Thus, the antibacterial response varied according to both the extraction solvent and the bacterial species.

The present findings indicate that the antibacterial activity of *Justicia adhatoda* L. leaf extracts varied with the extraction solvent and the bacterial species tested. Methanol and ethanol extracts generally produced greater inhibition than the other solvent extracts, with methanol showing the strongest overall activity. The methanolic extract produced the highest inhibition zones against *Escherichia coli* and *Klebsiella pneumoniae* (19 mm each), while ethanol showed comparatively greater activity against *Bacillus subtilis* (14 mm) and *Proteus vulgaris* (16 mm). *Pseudomonas aeruginosa* exhibited the highest response to the methanolic extract (15 mm). The relatively greater activity of the methanol and ethanol extracts may be related to their ability to extract a broad range of polar and moderately polar phytoconstituents, including phenolics, flavonoids, and alkaloids. Differences in bacterial susceptibility may also reflect variations in cell-envelope structure, membrane permeability, and interactions with specific phytochemical constituents. The present observations are in agreement with previous reports describing antibacterial activity of *J. adhatoda* extracts, particularly those obtained using methanol and other polar solvents [18,19].

The comparatively low activity of the chloroform extract, together with the stronger inhibition produced by methanol and ethanol, does not support the assumption that non-polar fractions possess greater antibacterial activity.

Instead, the results suggest that antibacterial efficacy is influenced by the nature and concentration of compounds extracted by each solvent rather than by solvent polarity alone. Variations between the present findings and earlier studies may be attributable to differences in geographical origin, plant maturity, extraction conditions, extract concentration, bacterial strain, inoculum density, and assay parameters. Although the observed antibacterial activity supports the biological potential of *J. adhatoda* leaves, the activity of the crude extracts cannot be assigned to any particular phytochemical group without further isolation and characterization. Additional fractionation and compound-level investigations would therefore be useful for identifying the constituents responsible for the antibacterial effects [20].

Table 2: Antibacterial activity of *Justicia adhatoda* L. leaves extract against pathogens

Organisms	Antibiotics mm	Metha- nol mm	Ethanol mm	Ethyl Acetate	Acetone mm	Chloro- form mm	Petroleum Ether
<i>Bacillus subtilis</i>	Tetracycline (20)	13	14	11	13	10	11
<i>Pseudo- monas aeruginosa</i>	Ampicillin (23)	15	16	13	14	11	12
<i>Escherichia coli</i>	Streptomycin (25)	19	17	15	15	11	12
<i>Klebsiella pneumoniae</i>	Amoxicillin (24)	19	17	16	14	10	12
<i>Proteus vulgaris</i>	Streptomycin (25)	13	16	12	13	10	14

3.3 Antioxidant potential

The antioxidant potential of *Justicia adhatoda* L. leaf extracts was evaluated using DPPH and ABTS radical-scavenging assays. The results obtained for the acetone, chloroform, petroleum ether, ethanol, and methanol extracts are presented in Table 3. Each experiment was performed in triplicate, and the results are expressed as mean $\pm$ standard deviation (SD). Statistical significance was evaluated by one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison post hoc test, with differences considered significant at $p < 0.05$. Different superscript letters within the same column indicate statistically significant differences.

In the DPPH assay, the methanol extract showed a value of 143 ± 3 , followed by ethyl acetate (131 ± 1), acetone (125 ± 3), ethanol (123 ± 1), and chloroform (116.0 ± 0.7). For the ABTS assay, the values recorded were 285 ± 3 for chloroform, 173 ± 2 for acetone, 165 ± 3 for methanol, 121 ± 1 for ethanol, and 64.0 ± 1 for petroleum ether. The superscript letters indicated significant differences among the respective treatments ($p < 0.05$). The variation between the DPPH and ABTS responses indicates that the extracts

did not behave uniformly in the two radical-scavenging systems, which may be related to differences in the chemical nature and reaction mechanisms of the radicals used in the assays.

The comparatively distinct responses of the solvent extracts may be associated with differences in the phytochemical constituents extracted by each solvent. Methanol is capable of extracting a wide range of polar and moderately polar compounds, including phenolics and flavonoids, which may contribute to antioxidant activity. However, the present results demonstrate that solvent-dependent antioxidant activity cannot be predicted solely on the basis of solvent polarity. The differences observed between DPPH and ABTS assays further indicate that the antioxidant response is influenced by the composition of the extract and its interaction with the particular radical system. Previous investigations have also reported antioxidant activity in *J. adhatoda* extracts and have associated this activity with its diverse phytochemical composition [21–23].

Table 3: Antioxidant potential of *Nyctanthes arbor-tristis* methanolic extract

Plant extracts	ABTS radical	DPPH radical
Acetone	173±2 ^d	125±3 ^e
Chloroform	285±3 ^e	116.0±0.7 ^a
Petroleum Ether	64.0±1 ^a	123±1 ^e
Ethanol	121.0±1 ^b	131±1 ^e
Methanol	165.0±3 ^d	143±3 ^e

Each value in the table is represented as mean ± SD (n = 3). Values in the same column followed by a different letter (a-f) are significantly different (p < 0.05).

For comparative evaluation, vitamin C was used as the natural antioxidant standard and TBHQ as the synthetic antioxidant standard. The DPPH and ABTS responses of the five plant extracts were compared with those of both reference antioxidants under identical assay conditions, and the results are presented graphically in Fig. 3. This comparison provides a clearer assessment of the relative antioxidant capacity of the plant extracts. However, the observed in-vitro activity should not be interpreted as evidence of therapeutic efficacy. Further studies involving purified compounds, concentration-dependent assays, and complementary antioxidant models are required to identify the constituents responsible for the observed activity.

3.4 UV-Visible spectrum

The UV-Visible absorption characteristics of *Justicia adhatoda* L. leaf extracts prepared using acetone, chloroform, ethanol, ethyl acetate, and methanol were recorded over the wavelength range of 200–800 nm. Distinct absorption bands were observed among the extracts, with differences in absorbance intensity and peak position depending on the solvent used (Figure 2). The

recorded absorbance values ranged from 0.027 to 0.872 OD. The variation in spectral profiles indicates differences in the composition and concentration of UV-absorbing constituents extracted by the respective solvents.

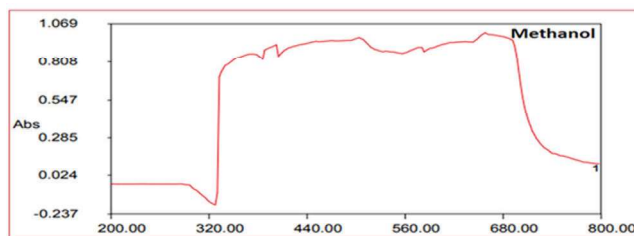


Figure 2: UV spectrum of Methanolic extract

The observed absorption maxima were interpreted by comparison with previously reported spectral characteristics of major phytochemical groups. Absorption bands occurring in the lower UV region may be associated with aromatic and other conjugated constituents, whereas bands in the approximately 250–400 nm region may correspond to chromophoric groups commonly present in phenolic and flavonoid compounds. However, the presence of an absorption peak alone cannot be considered definitive evidence for the presence of a particular bioactive compound. Therefore, the tentative assignment of the observed wavelengths was made only where the corresponding absorption range was supported by published literature [24–25]. Confirmation of individual compounds requires complementary analytical techniques such as HPTLC and GC-MS.

The differences observed between the solvent extracts may reflect the selective extraction of compounds with different chemical and spectral characteristics. The methanolic extract showed a distinct absorption profile, while the other solvent extracts also exhibited characteristic spectral patterns. These observations support the use of UV-Visible spectroscopy as a preliminary fingerprinting technique for comparing plant extracts, although it should be considered complementary rather than a definitive method for compound identification. The spectral profiles obtained in the present study provide a basis for further chromatographic and spectrometric characterization of the phytochemical constituents of *J. adhatoda* L. [26–28].

3.4 HPTLC Spot

The methanolic leaf extract of was subjected to HPTLC analysis to establish its chromatographic fingerprint. Six distinct bands were observed at 254 nm, with R_f values ranging from 0.01 to 0.98, while six bands were detected at 366 nm, with R_f values ranging from 0.06 to 0.88 (Fig. 3). The corresponding peak volume and area measurements are presented in Table 4. At 254 nm, band 4 exhibited the highest peak volume (592.8), whereas the largest peak

area was observed for band 4 (6478) at an Rf value of 0.12. At 366 nm, band 2 showed the highest peak volume (419.1), while bands 2 and 6 exhibited the largest peak area (3388), with Rf values of 0.79 and 0.06, respectively.

The HPTLC profile demonstrates the presence of multiple chromatographically separable constituents in the methanolic extract. However, the observed Rf values were not assigned to individual phytochemicals because authenticated reference standards were not included in the analysis. Therefore, the present Rf values should be considered characteristic fingerprint parameters rather than compound-specific identification markers.

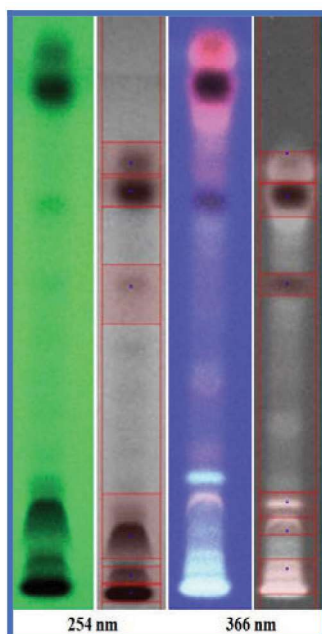


Figure 3: HPTLC Chromatogram Spot of methanolic extract at 254 and 366nm
Justicia adhatoda L.

Comparison of these values with authentic standards under identical chromatographic conditions would be necessary for assigning individual compounds. Nevertheless, the banding pattern, peak intensity, peak area, and Rf distribution provide a reproducible chromatographic profile that may be useful for preliminary comparison and quality assessment of *J. adhatoda* extracts. Further confirmation using reference standards and complementary chromatographic or spectrometric techniques would strengthen the chemical characterization of the extract [29-32].

Table 4: HPTLC pattern of *Justicia adhatoda* L. methanol extract

254 nm				366 nm		
Band	Volume	Area	R _f Value	Volume	Area	R _f Value
1	229.8	3160	0.84	6.79	2926	0.88
2	464.4	2844	0.98	419.1	3388	0.79
3	75.6	5767	0.60	159.5	2156	0.62
4	592.8	6478	0.12	53.5	2464	0.19
5	202.4	1580	0.05	158.2	1771	0.13
6	447.0	1817	0.01	98.6	3388	0.06

3.4 GC-MS Spectrum

The methanolic leaf extract of *Justicia adhatoda* L. was subjected to GC-MS analysis to obtain a preliminary profile of its volatile and semi-volatile constituents. Based on chromatographic separation and comparison of the obtained mass spectra with entries in the NIST mass spectral library (National Institute of Standards and Technology), eleven compounds were tentatively identified in the extract (Fig. 4). The major constituents included 9,17-octadecadienal, diisooctyl phthalate, 1H-cyclopentaquinoline, 2,3,5,6,7,8-hexahydro-9-amino-, and pentadecanoic acid. Other compounds detected at comparatively lower abundance were octadecanoic acid, 3[2H]-pyridazinone, 6-methyl, chrysene, 1,3,5-heptatriene, 3-quinolinecarboxamide, 2-amino-, oxirane, decyl, and 3-buten-1-ol, 3-methyl. The differences in peak abundance indicate variation in the relative representation of these tentatively identified constituents within the methanolic extract. Since the compound assignments were based on mass spectral library matching, the identities should be considered tentative and require confirmation using authentic reference standards and/or complementary analytical techniques.

Several of the compounds tentatively identified in the present analysis have previously been reported in studies of medicinal plants and have been associated with diverse biological activities. For example, octadecanoic acid has been investigated for antimicrobial and other biological properties, while fatty acids and related lipid-derived compounds have been reported to exhibit antioxidant and antimicrobial effects [33–36]. The detection of such constituents may contribute to the biological properties observed for the plant extract; however, their presence in the GC-MS profile should not be interpreted as direct evidence of biological activity in the present extract. The pharmacological effects of individual compounds depend on their concentration, purity, bioavailability, and interaction with other constituents present in the extract.

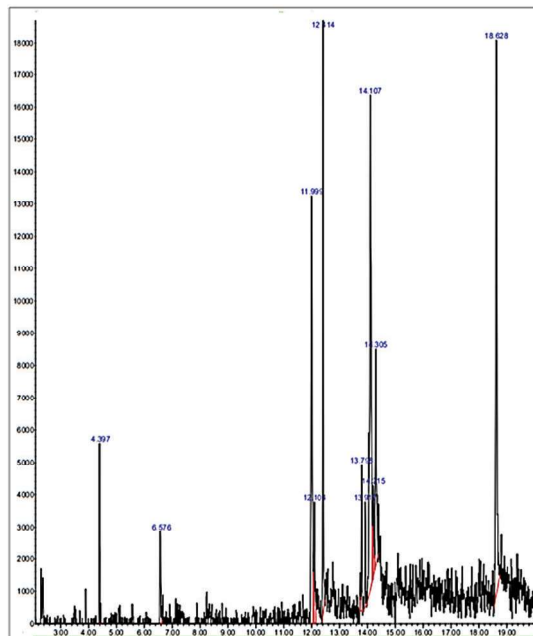


Figure 4: GC-MS Chromatogram of methanolic extract

The GC-MS profile therefore provides useful preliminary information regarding the chemical composition of *J. adhatoda* leaves and supports the presence of a chemically diverse mixture containing fatty-acid derivatives and other organic compounds. Nevertheless, the compounds identified through spectral library matching should be regarded as tentative assignments unless confirmed using authentic reference standards and/or complementary analytical techniques. Further isolation and structural characterization would be required to establish the identity and biological contribution of individual constituents. The GC-MS chromatogram and corresponding compound profile are presented in Figure 4.

4. Conclusion

In conclusion, the phytochemical profile of *Justicia adhatoda* L. is rich, confirming both its traditional medicinal use and its potential for therapeutic uses. Significant amounts of bioactive substances, including alkaloids, flavonoids, tannins, and phenolic compounds all of which support its antibacterial, antioxidant, and anti-inflammatory properties were found during the qualitative study. Twelve primary chemicals were found by GC-MS analysis; carbamic acid, piperazine acid, and N-hexadecanoic acid were particularly prominent and each contributed to the plant's medicinal potential. The evaluation of antibacterial activity further supported the

effectiveness of methanol extracts, demonstrating similar inhibition to that of conventional antibiotics and emphasizing the usefulness of plants in antimicrobial treatment. Significant radical scavenging activity was shown by antioxidant assays, confirming the use of *N. arbor-tristis* in the treatment of disorders linked to oxidative stress.

Declarations

Ethical Approval: Not applicable.

Competing Interests: Declared that no competing interests

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Authors Contribution

Parameswari A. designed the study, supervised the GC-MS and HPTLC analyses, and contributed to data interpretation. S. Pavithra conducted the laboratory experiments and analyzed the data. Suresh K. assisted in analyzing the biological activity assays and supported result interpretation. All authors contributed to manuscript drafting and discussion of findings.

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Conflict of Interest

“The authors declare no competing financial interest.”

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