Bioactive Potential of *Adhatoda vasica* Leaves: Comprehensive Phytochemical Profiling and Dual Biological Evaluation with Computational Validation

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ABSTRACT

Introduction

The advancement of pharmacognosy and analytical sciences has facilitated the identification of numerous plant-derived bioactive compounds with significant pharmacological properties. Adhatoda vasica, a medicinal plant rich in alkaloids and phenolics, has been explored for its antimicrobial and cytotoxic potential. This study aims to isolate, characterize, and evaluate the biological activity of ethanolic extract from A. vasica leaves (EE-LAV).

Objectives

This study aims to comprehensively profile the phytochemical constituents of Adhatoda vasica leaves using advanced analytical techniques and evaluate their dual biological activities. Additionally, key bioactive compounds will be validated through in silico computational approaches to support their therapeutic potential.

Materials and Methods

Leaves of A. vasica were extracted using ethanol and AR/LR-grade solvents. Preliminary phytochemical screening and spectroscopic techniques (TLC, UV-Vis, LC-MS) were used to identify active constituents. Antimicrobial activity was assessed using the disc diffusion method. In silico molecular docking (PDB ID: 3G75) evaluated the binding affinities of isolated compounds to bacterial DNA gyrase. Cytotoxicity was tested on COLO-205 colon cancer cells using cell viability and proliferation assays, with trypan blue staining for visual confirmation.

Results and Discussion

The bioactive compounds identified included tryptamine, dopamine, vasicine, vasicinone, and p-hydroxy benzoic acid. Docking results revealed strong binding affinities (–80.8423 to –257.627

kcal/mol), with dopamine and p-hydroxy benzoic acid nearing ciprofloxacin's affinity. EE-LAV showed significant antimicrobial activity, particularly against Gram-positive bacteria. In cytotoxicity assays, EE-LAV inhibited COLO-205 cell growth by up to 90.89% at 400 µg/mL and reduced viability to 50.05%, comparable to the standard drug TTN. EE-LAV demonstrated dose-dependent antimicrobial and cytotoxic effects. Visual and quantitative data confirmed reduced cell proliferation and increased cell death, indicating potent bioactivity.

Conclusion

EE-LAV exhibits promising antimicrobial and anticancer potential. Further in vivo and mechanistic studies are warranted to validate therapeutic applications.

Keywords: Antimicrobial activity, cytotoxicity, DNA gyrase, molecular docking, LC-MS, phenolic compounds, alkaloids, and COLO-205 etc.

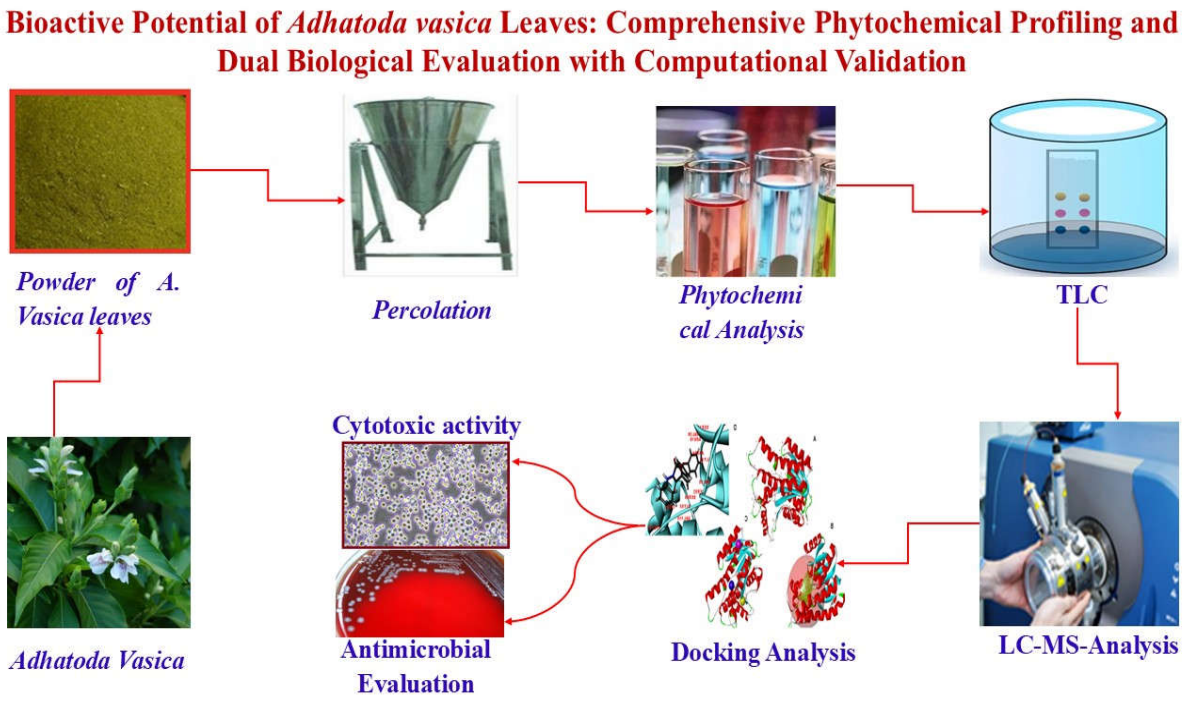


Fig-1: Graphical Abstract

1. INTRODUCTION

Vasaka (Justicia adhatoda), also known as Adhatoda or Malabar nut, is a well-known medicinal shrub belonging to the Acanthaceae family, native to the Indian subcontinent and Southeast Asia. It holds a prominent place in traditional systems of medicine like Ayurveda, Siddha, and Unani. Its therapeutic use is well-documented in ancient Ayurvedic texts such as the *Charaka Samhita* and *Sushruta Samhita*. The leaves are the most pharmacologically active part of the plant and are primarily used to treat respiratory conditions like cough, asthma, and bronchitis. The main bioactive constituent, vasicine, is a quinazoline alkaloid known for its bronchodilatory, expectorant, and anti-inflammatory effects. Other important compounds include vasicinone, essential oils, flavonoids, and tannins [1].

Traditionally, Vasaka leaves are used for their expectorant properties, helping to clear mucus from the lungs and ease breathing. Scientific studies have shown that vasicine and vasicinone stimulate bronchial secretions and reduce airway resistance, making them effective in treating chronic respiratory disorders. Beyond its respiratory benefits, Vasaka exhibits antimicrobial, antioxidant, anti-inflammatory, wound healing, and hepatoprotective properties. Ethnomedicinal uses include external applications for treating wounds and skin infections. Its antioxidant effects are attributed to polyphenolic compounds, which protect cells from oxidative stress [2, 3].

Botanically, *Justicia adhatoda* is an evergreen shrub with lance-shaped leaves arranged oppositely on short petioles. The leaves are about 8–9 cm long, dull green when dry, and bitter in taste. Microscopic examination reveals oval stomata and glandular hairs, while cystoliths are present beneath the lower epidermis. The shrub has long, yellowish branches with white flowers arranged in dense axillary spikes. Its fruit is a pubescent, club-shaped capsule [4].

Fig-2: Flowering plant of Justicia adhatoda L. (Vasaka/Basuti)

2. EXPERIMENTATION

2.1. Experimental Phytochemistry

Materials Required

The materials required for extraction include powdered plant material as the source of bioactive compounds and solvents like ethanol or water to dissolve phytochemicals. Equipment such as a percolator with a stopcock, measuring cylinders, a weighing balance, grinder, sieve, filter paper, and an evaporator or drying oven are essential for extraction, filtration, concentration, and measurement processes.

Mythology

Percolation is an effective phytochemical extraction method used to isolate soluble constituents from plant materials by passing a solvent slowly through a column of powdered drug. In this study, clean and dried *Vasaka* leaves were coarsely powdered and sieved (60–80 mesh) to ensure uniformity. The powder was moistened with 25–30% of the total ethanol and left to imbibe for 4–6 hours. The moistened material was then packed into a percolator lined with filter paper or cotton at both ends to ensure smooth solvent flow. Optionally, the material was macerated within the percolator for 24 hours to improve yield. The stopcock was opened to initiate slow percolation at a controlled rate (1–3 mL/min), and the extract was collected until it became nearly colourless. Finally, the percolate was concentrated under reduced pressure using a rotary evaporator to give final Ethanolic Extract of Leaves of *Adhatoda vasica* and stored for further analysis [5-9].

Phytochemical Screening

The phytochemical screening of *Adhatoda vasica* included a series of chemical tests to identify major bioactive constituents such as carbohydrates, amino acids, peptides, phytosterols, carotenoids, polyphenols, and alkaloids. Carbohydrate tests confirmed the presence of reducing sugars, starch, and mono/disaccharides through Benedict's, Seliwanoff's, and Osazone tests.

Amino acid identification revealed the presence of aromatic amino acids, tyrosine, tryptophan, arginine, and sulphur-containing amino acids using standard biochemical assays such as Xanthoproteic, Million's, and Ninhydrin tests [10-11]. Polyphenols were confirmed through reactions with lead acetate, ferric chloride, and Tollen's reagent, indicating strong antioxidant potential [12]. Alkaloids were identified using Dragendorff's, Mayer's, Wagner's, and Hager's reagents, all yielding characteristic precipitates. A specific confirmatory test, the Vanillin-Sulfuric acid test, confirmed the presence of quinazoline alkaloids in *A. vasica* [13-17].

Isolation of alkaloids and Phenolic compounds by TLC

Thin Layer Chromatography (TLC) was performed to identify alkaloids and phenolic compounds in the extract. For alkaloids, silica gel 60 F_{254} plates were used with a chloroform: methanol: ammonia (9:1:0.1) solvent system. After spotting and development, Dragendorff's reagent was sprayed for visualization, where alkaloids appeared as orange spots with Rf values around 0.4–0.6, compared to standard vasicine [14]. For phenolics, different solvent systems like ethyl acetate: formic acid: acetic acid: water were employed. Visualization involved UV light and reagents such as $AlCl_3$ and NaturSoft reagent, producing characteristic fluorescent spots. Rf values were calculated to aid compound identification [15].

Determination of total phenolic content

To determine total phenolic content, prepare gallic acid standard solutions (0–100 µg/mL) by diluting a 1000 µg/mL stock. For each standard and sample, mix 0.5 mL solution with 2.5 mL diluted Folin–Ciocalteu reagent, stand 5 minutes, then add 2 mL of 7.5% sodium carbonate. Incubate for 30 minutes in the dark at room temperature, then measure absorbance at 765 nm using a UV-Vis spectrophotometer.

Prepare the extract by dissolving 100 mg dry extract in 10 mL methanol, dilute 1:10, and follow the same reaction steps. Plot absorbance vs concentration to create a standard curve [18-19].

Calculation

Perform linear regression to get the equation of the line, usually in this form:

$$\boldsymbol{Y = mx + c}$$

Where:

- **y** = Absorbance of sample (A).
- **m** = Slope.
- **x** = Concentration of unknown sample.
- **c** = Intercept

Calculate TPC: The total phenolic content is determined by using the calibration curve of gallic acid, expressed as mg gallic acid equivalents per gram of dry extract (mg GAE/g).

$$\textbf{Total Phenolic content (mg GAE/ g sample)} = \frac{\boldsymbol{C \times V}}{\boldsymbol{M}}$$

Where:

- C = concentration from standard curve (µg/mL)
- V = volume of extract used in reaction (mL)
- M = mass of the dry sample extracted (g)

2. 2. Isolation of Bioactive molecules by LCMS

Preparation of 100 ppm Plant Powder Extract for LC-MS: Start by drying fresh plant material at 40–50°C until constant weight, then grind it into a fine powder. Weigh 1 g of the powder and add 10 mL of 80% methanol in a centrifuge tube. Vortex or sonicate for 15–30 minutes, then centrifuge at 10,000 rpm for 10 minutes. Filter the supernatant through a 0.22 µm syringe filter to obtain the crude extract. To prepare a 100-ppm solution, dissolve 10 mg of dried extract in 100 mL of solvent (methanol or methanol: water 50:50), vortex, and filter again if needed. Transfer an aliquot to an LC-MS vial and store at 4°C or –20°C until analysis. Recommended solvents include 80% methanol or 70% acetonitrile with 0.1% formic acid, avoiding buffers with high salts [20-25].

Preparation of 100 ppm Liquid Plant Extract for LC-MS: For liquid extracts like juice or decoctions, centrifuge at 10,000 rpm for 10 minutes and filter through a 0.22 µm syringe filter. To estimate concentration, evaporate 1 mL of the extract to dryness and weigh the residue. For example, a 10 mg residue equals 10,000 ppm concentration. Use this information to dilute the extract to 100 ppm with methanol or acetonitrile and deionized water, then transfer to LC-MS vials for analysis.

Dilution to 100 ppm

To prepare 100 ppm the following formula is used: $\boldsymbol{C1V1 = C2V2}$

Where:

C1 = Concentration in per ml (10000 ppm).

C2 = Concentration of sample to be prepared (100 ppm).

V1 = Volume of sample to be prepared (ml or µ/L)

V2 = Desired volume (10 mg/ml).

$$V\mathbf{1} = \frac{\mathbf{C2} \times \mathbf{V2}}{\mathbf{C1}}$$

$$V\mathbf{1} = \frac{\mathbf{100} \times \mathbf{10}}{\mathbf{10,000}}$$

$$\boldsymbol{V1 = 0.1mL \ = \ 100\ \mu/L}$$

Filter the 100-ppm solution through a 0.22 µm syringe filter into LC-MS vials. Store at 4°C (short-term) or –20°C (long-term). Pipette **100 µL** of stock extract and dilute to 10 mL using methanol: water (50:50, v/v). Notes for LC-MS Compatibility

- Preferred solvents: 50:50 methanol:water or 80: 20 methanol: water.
- Optional: Add **0.1% formic acid** for better ionization in positive mode.
- Avoid sugar-rich matrices for LC-MS (they may cause ion suppression).

Methodology

The analysis was performed using an LC-MS system equipped with an Ion-Trap mass spectrometer (Agilent model). In the liquid chromatography stage, either standard solutions or plant extracts were injected into a reverse-phase (RP) column, which separates compounds based on hydrophobic interactions. As compounds eluted from the column, they were introduced into the mass spectrometer, where ionization occurred, allowing the mass-to-charge ratios (m/z) of the molecular ions to be detected. For compound identification, tandem mass spectrometry (MS/MS) was employed, wherein selected precursor ions such as [M+H] + of vasicine or vasicinone were further fragmented to generate characteristic fragment ions, enabling structural elucidation.

2.3. Computational Chemistry

Molecular docking is an optimization technique used to predict the best-fit orientation of a ligand binding to a target protein. It considers conformational changes in both the ligand and protein, a concept known as "induced fit." The goal is to minimize the system's free energy by achieving optimal protein–ligand interaction [26].

Posing and scoring function of docking

Posing the process of determining whether a given conformation and orientation of a ligand fits the active site. This is usually a fuzzy procedure that returns many alternative results. pose score is a measure of the fit of a ligand into the active site. Scoring during the posing phase usually involves simple energy calculations (Vdw + H-bond + dE = Vender walls + Hydrogen bond + Dessolve energy. EE= Electrostatic Energy. TIME = Total Intermolecular Energy) [27]. One early general-purposed empirical scoring function to describe the binding energy of ligands to receptors was developed by Böhm. This empirical scoring function took the form:

$$\Delta G_{bind} = \Delta G_0 + \Delta G_{hb} \sum \mathrm{n} - \mathrm{bonds} + \Delta G_{ionic} \sum_{ionic-int} + \Delta G \ \mathrm{lipophilic} \ |A| + \Delta G_{rot} \mathrm{NROT}$$

A more general thermodynamic "master" equation is as follows:

$$G_{bind} = -\mathrm{RTlnK_d}$$

$$K_d = \frac{[\mathrm{Ligand}][\mathrm{Receptor}]}{[\mathrm{Complex}]}$$

$$\Delta G_{bind} = \Delta G_{desolvation} + \Delta G_{motion} + \Delta G_{configuration} + \Delta G_{interaction}$$

Methodology

For computational analysis, the crystal structure of bacterial DNA gyrase (Topoisomerase-II, PDB ID: 3G75) was retrieved from the Protein Data Bank. The protein structure was prepared by cleaning and adding missing hydrogen atoms. Docking of bioactive compounds present in EE-LAV and standard drug ciprofloxacin was performed using Discovery Studio Client, and the best docking poses were selected based on interaction studies.

2.3. Experimental oncology

The in vitro cytoxic activity of EE-LAV was evaluated by SRB assay.

Principle

Sulforodamine B (SRB) is a bright pink aminoxanthine dye with two sulfonic acid group. Under mild acidic conditions SRB dye binds to basic amino acid residues in trichloro acetic acid (TCA) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two orders of magnitude [28-29]**.**

Reagents

1. PBS (Phosphate buffer saline)
2. 40-50% TCA
3. 1% acetic acid solution
4. Sulforhodamine B (0.4% in 1% TCA)
5. 10 Mm Tris (P^H = 10.5).

Cell culture

The cell culture human colon adenocarcinoma cell line COLO-205 was provided by Aakaar Biotechnologies Pvt. Ltd., Lucknow, and were grown in Eagles Minimum Essential Medium (EMEM) which contained 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 100% relative humidity, 5% CO2, 95% air and the culture medium was changed twice a week.

Preparation of stock solution of EE-LAV

Materials

EE-LAV extract (dried, powdered form)

Solvent: DMSO (for stock) and culture medium (e.g., DMEM or RPMI) for dilution

Sterile 1.5 mL microtubes or falcon tubes

0.22 µm syringe filters (for sterilization)

Pipettes and tips

96-well plate with seeded cells (usually 5,000–10,000 cells/well)

Step 1: Prepare Stock Solution

Make a concentrated stock in DMSO (e.g., 10 mg/mL).

- Weigh 10 mg of EE-LAV extract.
- Dissolve it in 10 mL of solvent (100% DMSO).
- This gives a 1 mg/mL (1000 µg/mL) stock solution.
- (Optional but recommended) Filter sterilize using a 0.22 µm syringe filter.

Step-2: Dilution Procedure

Use the formula:

$$\mathbf{C1V1 = C2V2}$$

Where:

- C1 = concentration of stock (e.g., 1000 µg/mL)
- V 1= volume of stock to use
- C2 = desired concentration (100, 200, or 300 µg/mL)
- V2 = final volume you want (e.g., 10 mL)

For 100 µg/mL in 10 mL:

- C1 = 1000 µg/mL
- C2 = 100 µg/mL
- V2 = 10 mL

$$V1 = \frac{(C2 \times V2)}{C1}$$

$$\mathrm{V1} = \frac{(100 \times 10)}{100}$$

$$\mathrm{V1} = 1\ \mathrm{mL}$$

So, mixed 1 mL of stock solution + 9 mL of solvent

For 200 µg/mL in 10 mL:

$$\mathrm{V1} = \frac{(200 \times 10)}{100}$$

$$\mathrm{V1} = 2\ \mathrm{mL}$$

Mixed 2 mL stock + 8 mL solvent

For 300 µg/mL in 10 mL:

$$\mathrm{V1} = \frac{(300 \times 10)}{100}$$

$$\mathrm{V1} = 3\ \mathrm{mL}$$

Mixed 3 mL stock + 7 mL solvent.

For 400 µg/mL in 10 mL: Mixed 4 ml stock +6 mL solvent

Assay Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x10^5 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately) 10,000 cells was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off, washed once and100 µg/ml, 200 µg/ml and 300 µg/ml of different concentration EE-LAV were added to the cell in micro titre plate. The plates were incubated at 37 ^{0}c for 72 hrs in 5% CO2 incubator, microscopic examination was carried out and observations were recorded every 24 hrs. After 72 hrs, 25µl of 50% TCA was added to wells gently such that it forms a thin layer over EE-LAV to form overall concentrations 10%. The plates were incubated at 4^{0}c for 1 hr. The plates were flicked and washed five times with tap water to remove traces of medium sample and serum and were then air dried. The air-dried plates were stained with 100 µl SRB and kept for 30 mints at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air dried. 100 µl of 10 mM Tris base was then added to the wells to solubilise the dye. The plates were shaken vigorously for 5 mints. The absorbance was measured using micro plate reader at a 540 nm. The % growth inhibition was calculated by the following formula [30]:

$$\mathbf{PCGI}(\%) = \mathbf{100} - \left(\frac{\mathbf{A_t} - \mathbf{A_b}}{\mathbf{A_c} - \mathbf{A_b}}\right) \times \mathbf{100}$$

Where:

- $\mathbf{A_t}$ = Absorbance of treated cells (with extract or drug)
- $\mathbf{A_c}$ = Absorbance of control cells (untreated, with vehicle only)
- $\mathbf{A_b}$ = Absorbance of blank (medium only, no cells).

Positive control for Cytotoxicity (Test): cells treated with a cytotoxic drug/chemical +SRB + solubilizing buffer

Negative control for cytotoxicty (control): cells left untreated + SRB + solubilizing buffer.

Blank: medium without cells + SRB + Solubilizing buffer.

Trypan blue exclusion assay for the determination of cell viability

Trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live (viable) cells do not take up certain dyes, whereas dead (non-viable) cells do. Staining facilitates the visualization of cell morphology [31].

Trypan Blue has a greater affinity for serum proteins than for cellular protein. If the background is too dark, cells should be pelleted and resuspended in protein-free medium or salt solution prior to counting.

Trypan Blue Protocol

1. Prepare a cell suspension in a balanced salt solution (e.g., Hanks' Balanced Salts [HBSS], Cat. No. H9269).

2. Transfer 0.5 ml of 0.4% Trypan Blue solution (w/v) to a test tube. Add 0.3 ml of HBSS and 0.2 ml of the cell suspension (dilution factor = 5) and mix thoroughly. Allow to stand for 5 to 15 minutes.

3. With the cover-slip in place, use a Pasteur pipette or other suitable device to transfer a small amount of Trypan Blue-cell suspension mixture to both chambers of the hemacytometer. Carefully touch the edge of the cover-slip with the pipette tip and allow each chamber to fill by capillary action. Do not overfill or underfill the chambers.

4. Starting with chamber 1 of the hemacytometer, count all the cells in the 1 mm center square and four 1 mm corner squares. Non-viable cells will stain blue. Keep a separate count of viable and non-viable cells. Count cells on top and left touching middle line of the perimeter of each square. Do not count cells touching the middle line at bottom and right sides.

5. Repeat this procedure for chamber 2.

6. Withdraw a second sample and repeat count procedure to ensure accuracy.

Calculations

Cell Counts – Each square of the haemocytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10-4 cm^3. Since 1 cm^3 is equivalent to approximately 1 ml, the subsequent cell concentration per ml (and the total number of cells) will be determined using the following calculations

Ca**lculations:**

Cells Per mL = the average count per square × dilution factor × 10^4 (count 10 squares)

The average counts per square are **43 cells × 5 × 10^4 = (2.15 ×10^2× 10^4) = 2.15×10^6 cells/ml**

Total Cells = cells per ml × the original volume of fluid from which cell sample was removed.

2.15×10^6 (cells/ml) × 10 ml (original volume) = 2.15 × 10^7 total cells.

Cell Viability (%) = [total viable cells (unstained) ÷ total cells (stained and unstained)] × 100.

$$\textbf{Cell Viability}\ (\%) = \left(\frac{\textbf{Number of viable cells (unstained)}}{\textbf{Total number of cells (stained + unstained}}\right) \times \mathbf{100}$$

Where:

- Viable cells are unstained because their membranes exclude the dye.
- Non-viable cells are stained because the dye penetrates their compromised membranes.
- Total cells = viable (unstained) + non-viable (stained).

2.4. Experimental Microbiology

Requirements for *In Vitro* Antimicrobial Activity

The *in vitro* antibacterial assay was performed using Mueller-Hinton Agar (MHA) plates (SRL Chem, Cat No.-24756) as the growth medium. Bacterial strains used for the study included *Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 1133), and *Escherichia coli* (MTCC 452), all procured from the Microbial Type Culture Collection (MTCC), Chandigarh. Whatman No. 1 filter paper discs (5 mm diameter) were used to apply 10 µL of each test sample. Dimethyl sulfoxide (DMSO, SRL Chem 28580) served as the solvent control. Ciprofloxacin (2 mg/mL, SRL Chem 78079) was used as the standard antibiotic for comparison.

The antifungal assay was conducted using Sabouraud Dextrose Agar (SDA) plates (SRL Chem, Cat No.-19427) as the culture medium. The fungal strain employed was *Aspergillus niger* (MTCC 281), also obtained from MTCC, Chandigarh. Similar to the antibacterial assay, 5 mm Whatman No. 1 filter paper discs were loaded with 10 µL of each test extract. DMSO (SRL Chem 28580) was used as the vehicle control, and Amphotericin B (Amphocare, 10 mg/mL) was used as the standard antifungal agent.

Preparation of Culture Media

Nutrient agar and Sabouraud dextrose agar are widely used media for routine bacterial and fungal cultivation, respectively. The nutrient agar medium was prepared by dissolving peptone, beef extract, sodium chloride, and agar in 1000 mL of distilled water (pH 7.0), followed by boiling and sterilization at 15 lbs pressure and 121°C for 15 minutes [32–33]. Similarly, Sabouraud dextrose agar medium was prepared by dissolving glucose, peptone, and agar in 1000 mL of distilled water, adjusting the pH to 5.4, and sterilizing under the same conditions. Both media were cooled and poured into sterile Petri dishes for further use.

Table-1: Composition of Nutrient Agar Medium

Sl. No.	Ingredients	Quantity (g/L)
1.	Peptone	10 g
2.	Beef Extract	10 g
3.	Agar	20 g
4.	Sodium Chloride	5 g
5.	Distilled Water	1000 mL
6.	pH	7.0

Table-2: Composition of Sabouraud Dextrose Agar Medium

S. No.	Ingredients	Quantity (g/L)
1.	Glucose	20 g
2.	Peptone	10 g
3.	Agar	20 g
4.	Distilled Water	1000 mL
5.	pH	5.4

Method of Evaluation of In Vitro Antibacterial and Antifungal Activity

The antimicrobial activity was evaluated using the Zone of Inhibition Method (Kirby-Bauer method). For antibacterial testing, Mueller-Hinton Agar (MHA) plates were inoculated with 100 µL of *Staphylococcus aureus* culture prepared in Mueller-Hinton Broth and adjusted to 0.5 McFarland standard (≈1.5 × 10^8 CFU/mL). Sterile Whatman No.1 discs were loaded with 10 µL of test solutions

(0–100%), placed on the inoculated plates, along with one disc containing only DMSO as vehicle control and a Ciprofloxacin disc (3 µg) as positive control. Plates were incubated at 37°C for 24 hours (Basil Scientific Corp. India), and zones of inhibition were measured in millimeters. For antifungal activity, Sabouraud Dextrose Agar (SDA) plates were similarly inoculated with 100 µL of *Aspergillus niger* culture prepared in Sabouraud Dextrose Broth and adjusted to 0.5 McFarland standard (≈1.5 × 10^8 CFU/mL). Discs loaded with 10 µL of different test concentrations were placed on the surface along with DMSO (control) and Amphotericin B (100 µg) as the positive control. Plates were incubated at 37°C for 48 hours, and the zones of inhibition were measured and recorded [34-35].

3. RESULT AND DISCUSSION

3.1. Phytochemical screening and isolation phenolics and alkaloidal compounds

Table-3: Phytoconstituents present in EE-LAV

Sl. No.	Types of phytoconstituents	Visibility
1.	Carbohydrates	++
2.	Reducing sugar group	-
3.	Proteins and amino acids	++
4.	Oils and Fats	+
5.	Steroids	+++
6.	Alkaloids	+++
7.	Phenolic compounds	+++
8.	Flavanoids	+
9.	Tannins	+
10.	Saponins	+
11	Cardiac glycoside	-
12.	Anthocyanidin	-

Note: ++ = Present (Clearly Visible), + = Present (Dark), - = Absent

Table-4: R_f value of unknow constituents

Sl. No.	Standard compounds	R_f Value	Observed R_f Value	Probable bioactive molecules Observed R_f Value
1.	Vasicine	0.42–0.45	0.51	Quinazoline alkaloids
		0.50–0.55	0.59	Quinazoline alkaloids
2.	Adrenaline	0.10–0.30	0.29	Catecholamines
3.	Tryptamine	0.35–0.50	0.48	Amines
4.	Gallic acid	0.30–0.35	0.33	Phenolic Compounds
5.		0.25–0.30	0.28	Phenolic Compounds

Table-5: Absorbance of Standard Drug gallic acid

Concentration	Absorbance
0	0.00
10	0.011
20	0.019
40	0.028
60	0.039
80	0.052
100	0.069
10 (EE-LAV)	**0.003**

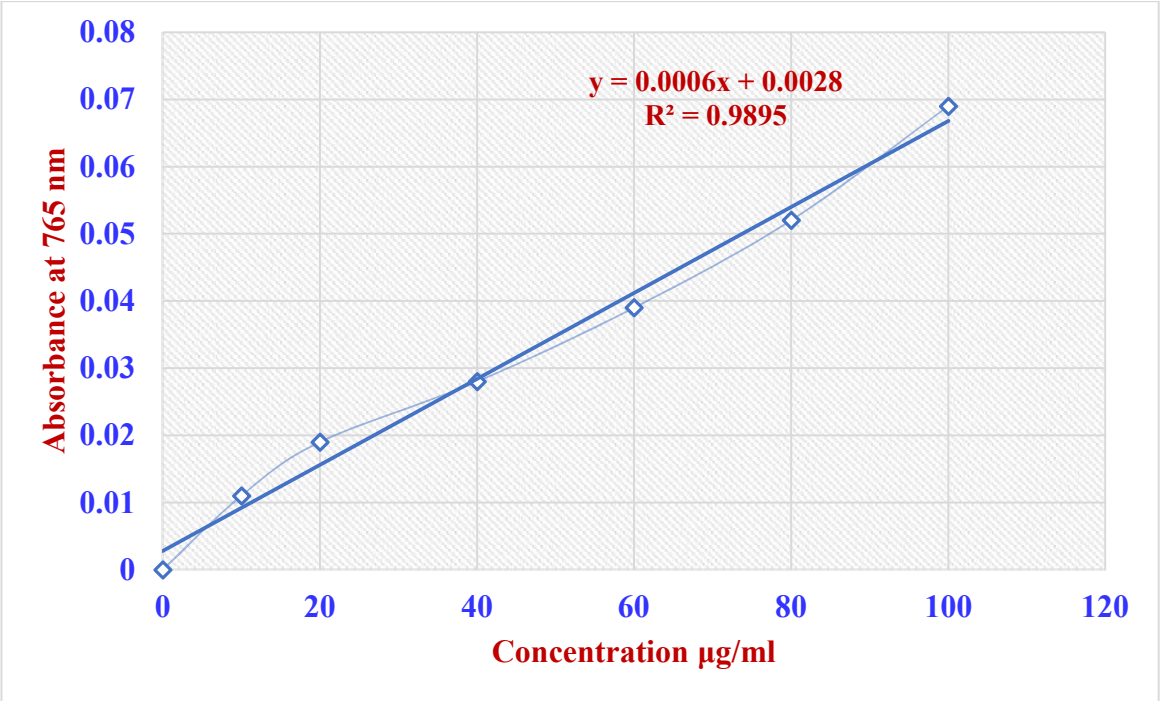


Fig-3: Standard graph of gallic acid

$$y = mx + c$$

Where:

y(A) = absorbance of the sample = **0.003**

m = slope of the standard curve

c = intercept of the standard curve = **0.0028**

$$x = \frac{(y - c)}{m}$$

$$x = \frac{(0.003 - 0.0028)}{0.0006}$$

$$\text{So}, x = 0.333\ \mu g/mL$$

$$\textbf{Total Phenolic content (mg GAE/ g sample)} = \frac{\boldsymbol{C \times V}}{\boldsymbol{M}}$$

Where:

- C = concentration from standard curve (µg/mL) = **0.333 µg/ml**
- V = volume of extract used in reaction (mL) = **0.5 mL**
- M = mass of the dry sample extracted (g) = **100 mg (0.1gm)**

$$\textbf{Total Phenolic content } \left(\textbf{mg}\frac{\textbf{GAE}}{\boldsymbol{g}}\ \textbf{sample}\right) = \frac{\mathbf{0.333 \times 0.5}}{\mathbf{0.1}} = \mathbf{1.66\ mg\ GAE/\ g\ extract}$$

Summary and Findings

The ethanolic extract of *LAV* (EE-LAV) was screened for major phytoconstituents. The phytochemical analysis (Table 3) revealed the clear presence (+++) of alkaloids, phenolic compounds, and steroids, while carbohydrates, proteins, and amino acids were also present (++) along with minor traces of flavonoids, tannins, saponins, and oils/fats (+). Reducing sugars, anthocyanidins, and cardiac glycosides were absent.

Thin Layer Chromatography (TLC) (Table-4) was employed to identify potential bioactive molecules based on Rf values. The observed Rf values (0.51 and 0.59) were consistent with quinazoline alkaloids (such as vasicine), and additional spots matched standards for phenolics (gallic acid, Rf = 0.33 and 0.28), amines (tryptamine, Rf = 0.48), and catecholamines (adrenaline, Rf = 0.29).

Total Phenolic Content (TPC) was quantified using the Folin–Ciocalteu method. Based on the absorbance of the EE-LAV extract (0.003) and the gallic acid standard curve (slope = 0.0006, intercept = 0.0028), the concentration of phenolics was calculated to be 0.333 µg/mL. Using the TPC formula. Total Phenolic Content was found to be 1.66 mg GAE/g extract.

3.2. Interpretation of LC-MS data and Characterization of alkaloids and phenolic compounds.

The characterization of isolated alkaloids and phenolic compounds were characterized by LCMS.

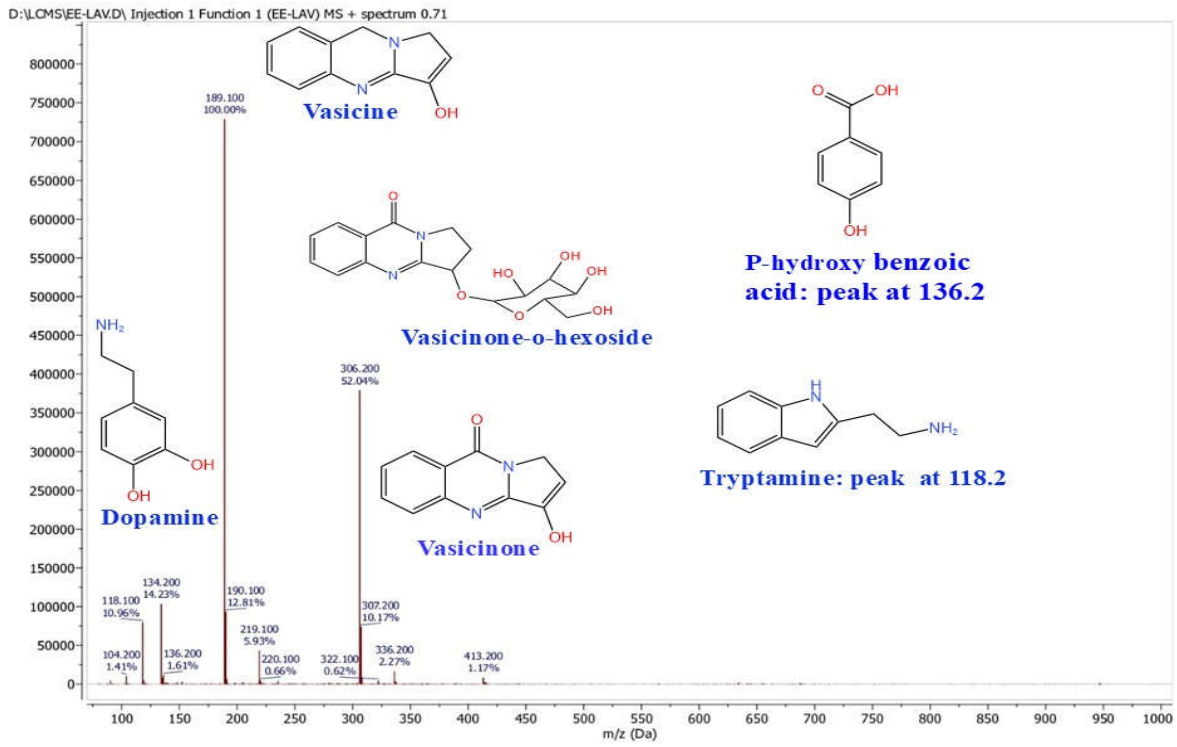


Fig-4: LCMS of EE-LAV

The LCMS data for ethanolic extracts of leaves of *Adhatoda vasica* (EE-LAV) interpreted as positive ion mode $[M+H]^+$. This includes:

- Observed m/z values
- Calculated neutral molecular weights (M)
- Possible molecular formulas
- Probable compound identities
- Fragmentation patterns

Table-6: LC-MS Data Interpretation: Positive Ion Mode ($[M+H]^+$)

m/z (Observed)	Ion Type	Neutral Mass (M)	Molecular Formula (Possible)	Probable Compound	Fragmentation
118.1	$[M+H]^+$	117.1	$C_8H_{11}N$	Tryptamine / Amine Fragment	Could be fragment of alkaloid; common in indole/phenethylamine-type structures
134.1	$[M+H]^+$	133.1	$C_8H_{11}NO$	Dopamine / Phenethylamine	May be precursor, metabolite, or breakdown product of larger molecules

189.1	$[M+H]^+$	188.1	$C_{11}H_{12}N_2O$	Vasicine	Major quinazoline alkaloid in *Adhatoda vasica*; bronchodilator
190.1	$[M+H]^+$	189.1	Possibly $C_{11}H_{13}N_2O$ or $[M+H+1]^+$	Vasicine Isotope/Adduct	May represent ^{13}C isotope peak or sodium adduct $[M+Na]^+$ if calibration slightly off
306.2	$[M+H]^+$	305.2	$C_{15}H_{14}O_7$ or $C_{13}H_{18}N_2O_6$	Flavonoid / Vasicine glucoside	Likely glycosylated flavonoid or vasicine derivative; common in plant LC-MS
307.2	$[M+H]^+$	306.2	$C_{11}H_{12}N_2O_2$	vasicinone	Structural or isobaric compound; could also be Na^+ adduct ($[M+Na]^+ = +22$ Da)
136.2	$[M-H]^-$	138.03	$C_7H_6O_3$	P-hydroxy benzoic acid	m/z 93 (loss of COOH), m/z 65, m/z 77 (aromatic ring fragments)

Final Interpretation of probable compounds present in EE-LAV.

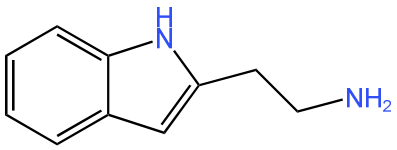
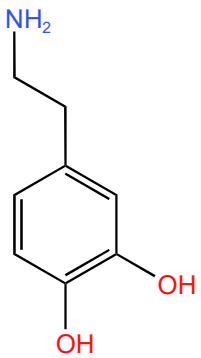
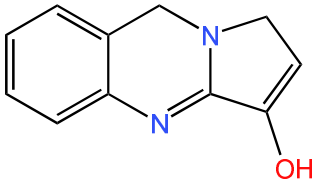
(i) 189.1 = Vasicine (major alkaloid in *Adhatoda vasica*).

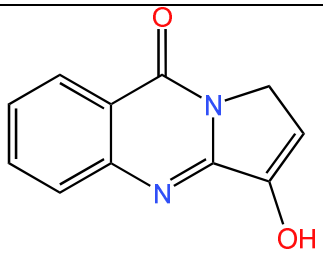
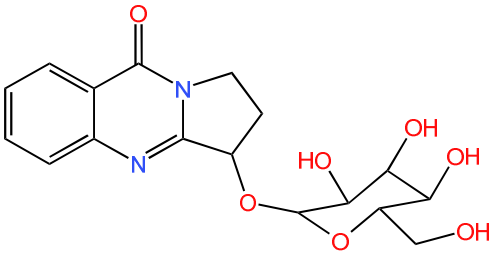
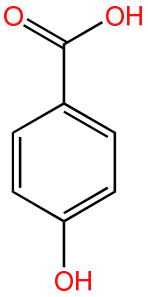
(ii) 306.2 / 307.2 = Likely glycosylated flavonoids or vasicine glucoside.

(iii) 118.1 / 134.1 = Small amine alkaloids or fragments.

(iv) 190.1 = Likely an adduct/isotope of vasicine.

Table-7: List probable compounds present in EE-LAV

Name of the compounds and Structure	
Tryptamine 	**Dopamine** 
Vasicine 	**Vasicinone**

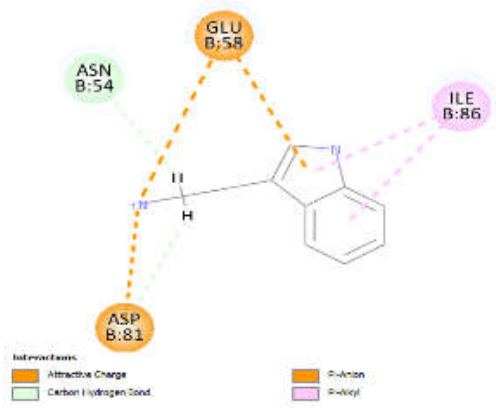
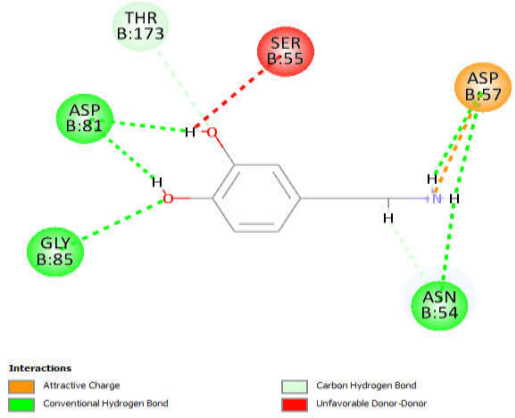
	
Vasicinone-o-hexoside 	**P-hydroxy benzoic acid** 

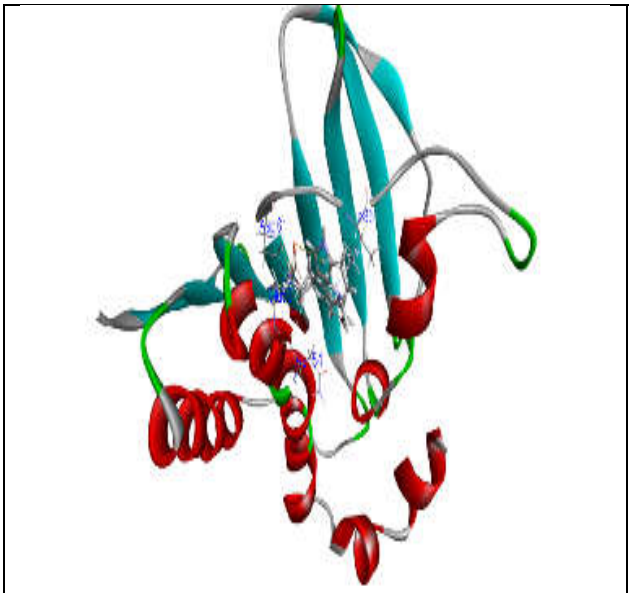
3.3. Molecular docking results interpretation

Table-8: Docking results analysis

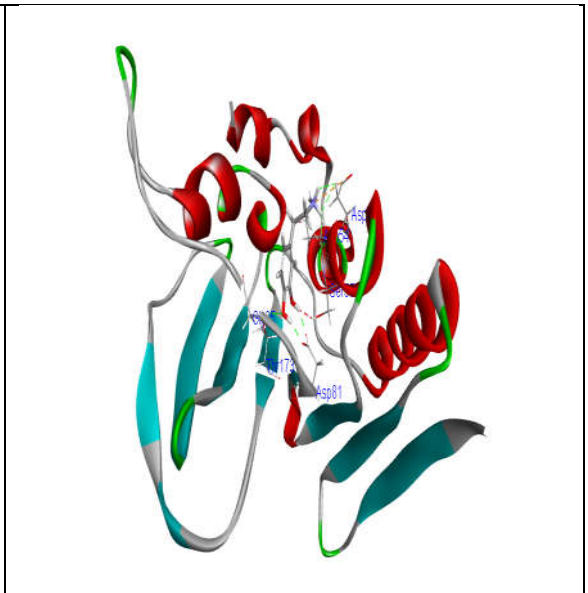
Sl. No.	Name of the compound	Cdocker Energy	Cdocker Interaction Energy	Binding Energy
1.	Tryptamine	27.4499	31.8357	-214.821
2.	Dopamine	33.7151	36.5218	-257.627
3.	Vasicine	11.2413	25.7558	-80.8423
4.	Vasicinone	12.4231	28.9939	-100.225
5.	P-hydroxy benzoic acid	33.2725	34.0812	-237.596
6.	Ciprofloxacin	23.3649	49.2324	-288.719

Table-9: 2D interaction study, 3D structures and 3D ligand-receptor complex with Bacterial DNA gyrase (3G75)

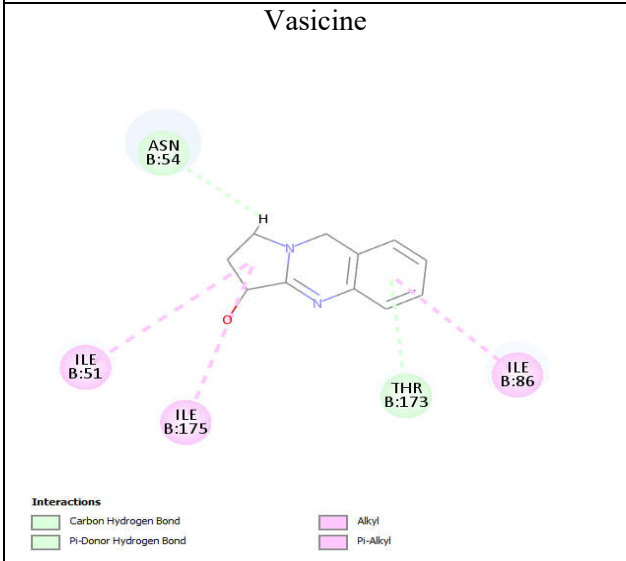
Tryptamine	Dopamine
	

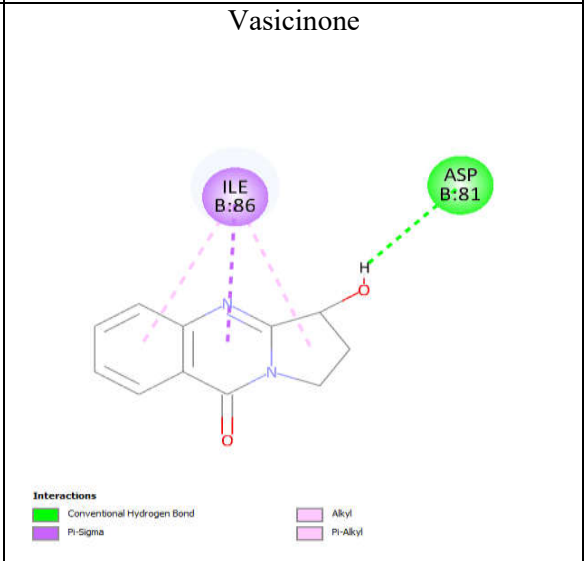


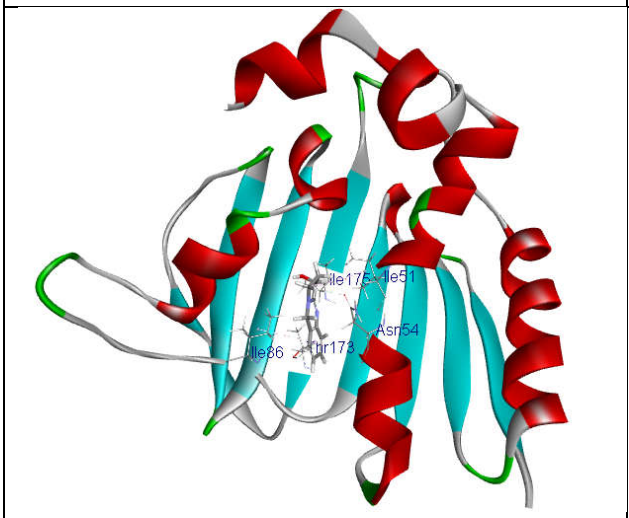
Vasicine



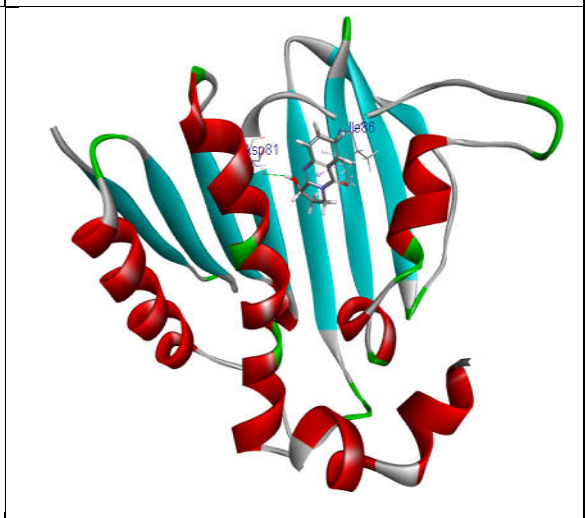
Vasicinone



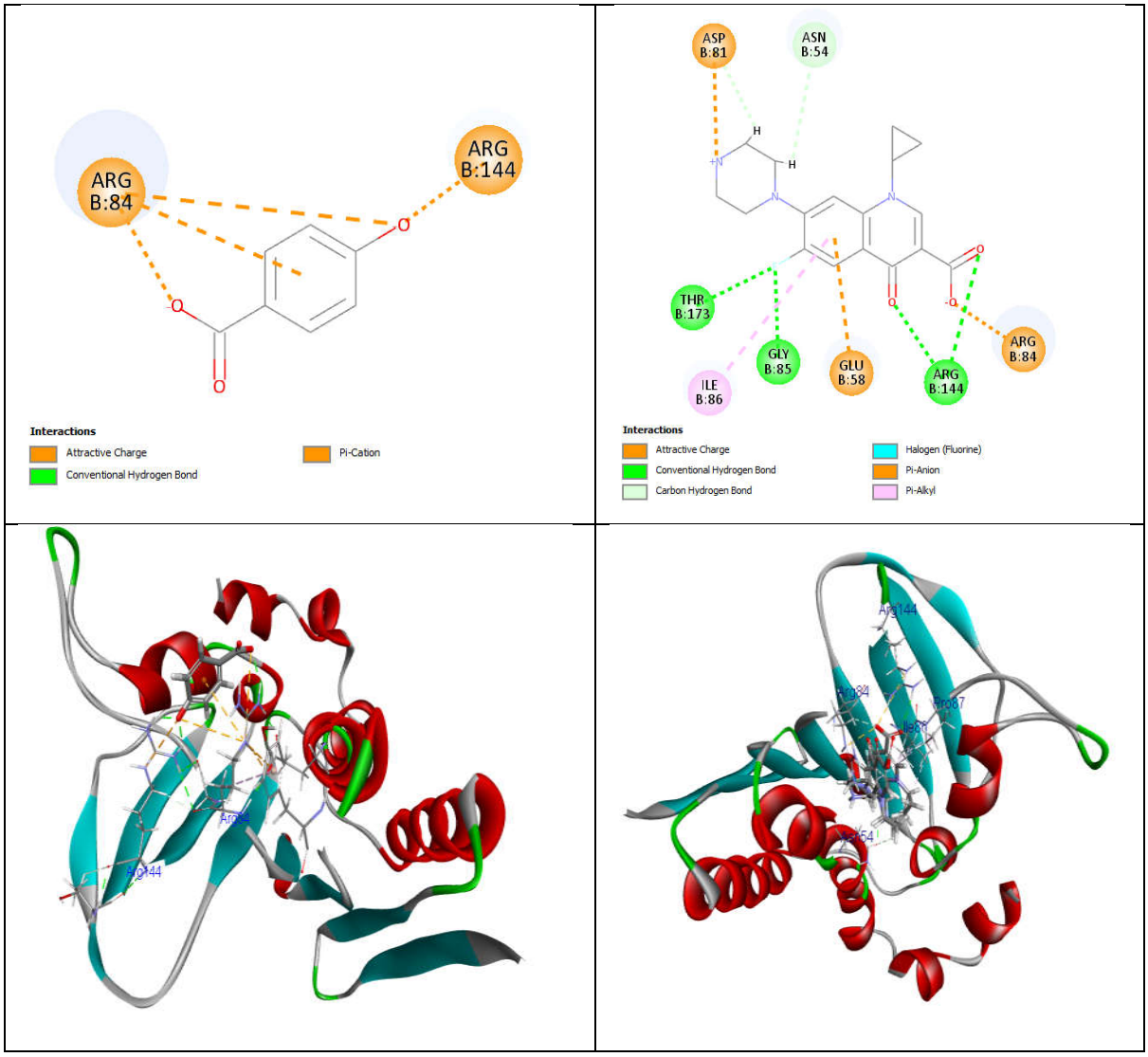




Para hydroxy benzoic acid



Ciprofloxacin



Summary and Findings

Molecular docking and interaction studies were conducted to evaluate the binding affinity of bioactive compounds isolated from EE-LAV—namely tryptamine, dopamine, vasicine, vasicinone, and p-hydroxy benzoic acid—against bacterial DNA gyrase (topoisomerase II), a validated antibacterial drug target. The crystal structure of the enzyme (PDB ID: 3G75) was retrieved and prepared using Discovery Studio, including protein clean-up and hydrogen addition. The docking analysis was guided by molecular dynamics (MD)-based scoring functions, where negative or low energy values indicate a more stable and favorable ligand-receptor interaction. Among all tested compounds, dopamine (–257.627 kcal/mol) and p-hydroxy benzoic acid (–237.596 kcal/mol) demonstrated strong binding affinities, closely approaching that of ciprofloxacin (–288.719 kcal/mol), the reference standard.

The strong binding affinities suggest these ligands effectively interact with key residues in the ATP- or DNA-binding regions of topoisomerase II through hydrogen bonding, π-π stacking, and hydrophobic interactions. Functional groups such as hydroxyl and amine moieties enhance these molecular interactions. Tryptamine also showed significant binding (–214.821 kcal/mol), while vasicine and

vasicinone displayed moderate affinities. These in silico findings align with in vitro antimicrobial activity, indicating that the inhibitory effect of EE-LAV may result from interference with DNA gyrase function. This highlights the potential of EE-LAV compounds as promising leads for developing novel antibacterial agents targeting bacterial topoisomerase II.

3.4. Evaluation cytotoxic activity of EE-LAV against Colo-205 (Human colon adenocarcinoma cell line)

Table 10: Percentage (%) of cell growth inhibition by EE-LAV

Concentrations	Absorbance (MEAN±SD)	PCGI (MEAN±SD) %
100 µg/ml	0.092±0.001	69.38±0.4504
200 µg/ml	0.062±0.001	79.55±0.026
300 µg/ml	0.033±0.0005*	88.9±0.015*
400 µg/ml	0.027±0.001**	90.89±0.045*
TTN 75 µg/ml (Positive Control)	0.015±0.0005***	94.64±0.0208***
Control	0.297±0.0005	0

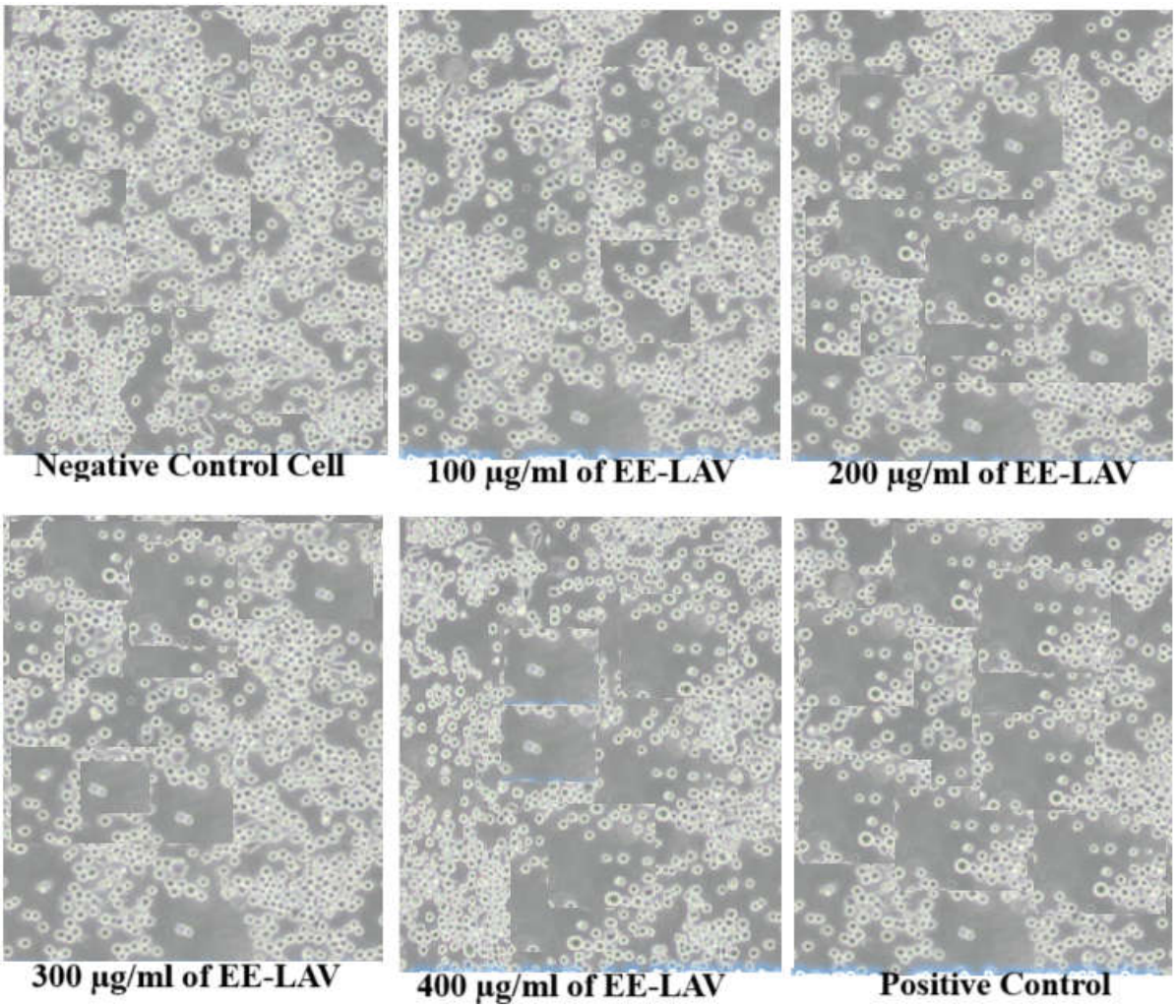


Fig-5: The growth of COLO-205 cells was inhibited by EE-LAV

Table 11: Effect of EE-LAV on Cell viability assay

Concentration (µg/ml	Cell viability (%) (MEAN±SD)
100	72.79±0.00577
200	68.6±0.0049
300	61.34±0.0058
4000	50.05±0.01000
TTN***	34.88±0.0057

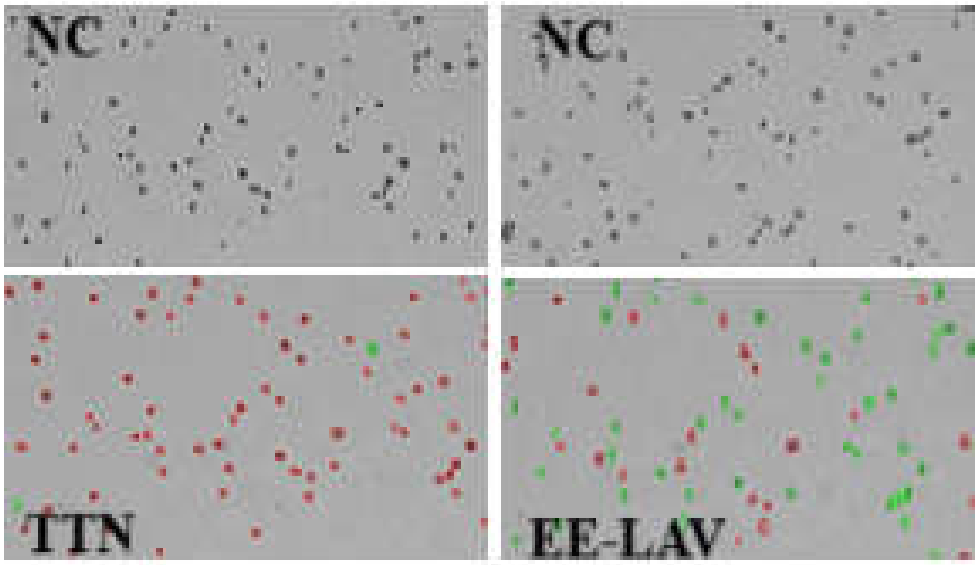


Fig-6: Shown, a stained trypan blue image and a Cello meter counted image. Counted live cells are outlined in green while the dead trypan blue positive cells are outlined in red caused by the EE-LAV at 400 µg.

Summary and Findings

The ethanolic extract of *Adhatoda vasica* (EE-LAV) demonstrated significant dose-dependent inhibition of cell growth and viability in the tested cell line. EE-LAV displayed the percentage of cell growth inhibition (PCGI) at various concentrations of EE-LAV (Table-1). At 100 µg/mL, the extract inhibited the colo-205 cell growth by 69.38%, increasing to 90.89% at 400 µg/mL. These results were comparable to the positive control (TTN 75 µg/mL), which showed 94.64% inhibition. Absorbance values decreased significantly with increasing EE-LAV concentration, confirming reduced cell proliferation. The statistically significant decreases at 300 and 400 µg/mL ($p < 0.05$ and $p < 0.01$ respectively) highlight the potent cytotoxic effect of EE-LAV.

EE-LAV shown the effect of EE-LAV on cell viability (Table-2). The percentage of viable cells decreased from 72.79% at 100 µg/mL to 50.05% at 400 µg/mL, indicating dose-dependent cytotoxicity. The positive control TTN showed the lowest viability at 34.88%, affirming the strong effect of the reference drug. This data corroborates the PCGI results, suggesting that EE-LAV reduces cell viability

significantly at higher concentrations. Figure 6 illustrates stained trypan blue images with cell counts. Live cells were outlined in green, while dead cells (trypan blue-positive) were outlined in red, particularly visible at 400 µg/mL concentration of EE-LAV. This visual evidence confirms the cytotoxic effect seen in the quantitative assays.

Discussion Points

- EE-LAV exhibits a strong inhibitory effect on cell proliferation and viability in a concentration-dependent manner.
- The decrease in absorbance values with increasing extract concentration correlates with higher cell death and lower proliferation.
- The viability assay confirms that EE-LAV induces cytotoxicity, reducing the number of live cells as concentration increases.
- Trypan blue staining provides visual confirmation of increased cell death caused by EE-LAV.
- The extract's cytotoxic effect approaches that of the standard chemotherapeutic agent TTN, suggesting potential anticancer properties.
- Future studies should explore the molecular mechanisms behind this cytotoxicity and assess in vivo efficacy.

3.5. Preliminary screening of antimicrobial activity

Table-12: Antibacterial Activity of EE-LAV against *S. aureus*

Test Organism: *S. aureus*						
X Axis: Amount (%/disc)						
Y Axis: *Zone Size (in mm)*						
Sample code: EE-LAV						
Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
CF (PC:0.03)	23	23	23	23	0	0
6.25	27	27	27	27	0	0
12.50	28	28	28	28	0	0
25	31	31	30	30.666	0.577	0.333
50	32	32	32	32	0	0
100	33	33	33	33	0	0

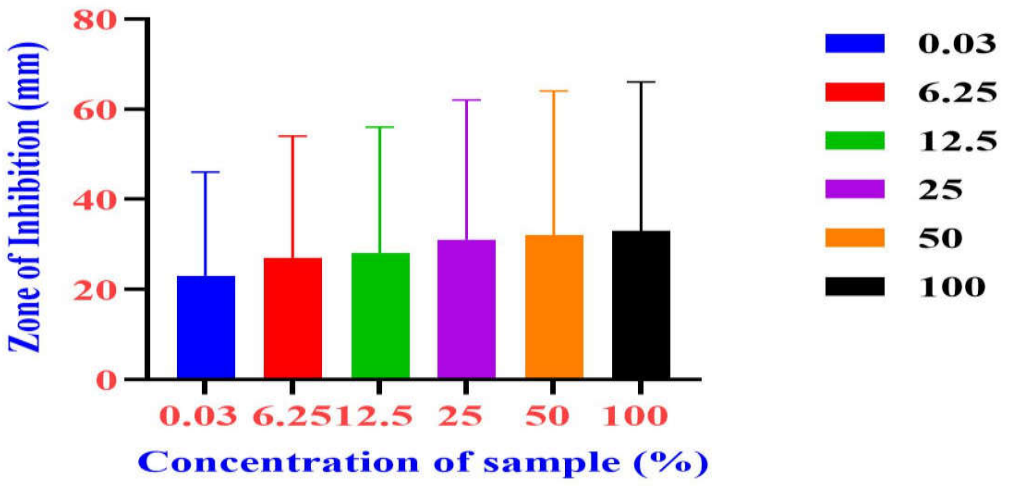


Fig-7: Antibacterial Activity of EE-LAV against *S. aureus*

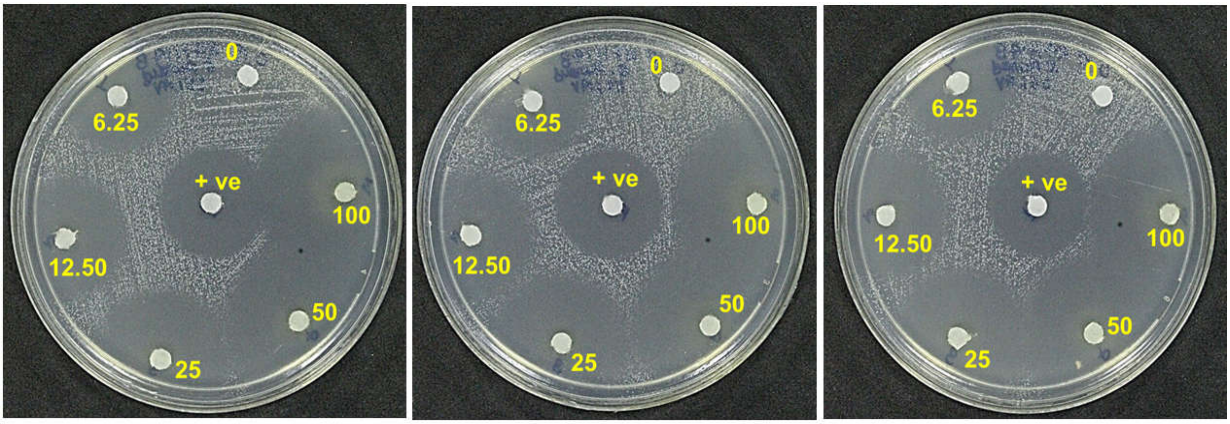


Fig-8: Zone of Inhibition of EE-LAV against *S. aureus* at concentration present per disc in percentage (%).

Table-13: Antibacterial Activity of EE-LAV against *B. subtilis*

Test Organism: *B. subtilis*						
X Axis: Amount (%/disc)						
Y Axis: *Zone Size (in mm)*						
Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
CF (PC:0.03)	23	23	22	22.666	0.577	0.333
6.25	25	25	25	25	0	0
12.5	25	25	25	25	0	0
25	27	28	27	27.3333	0.577	0.333
50	28	29	28	28.3333	0.577	0.333
100	33	33	33	33	0	0

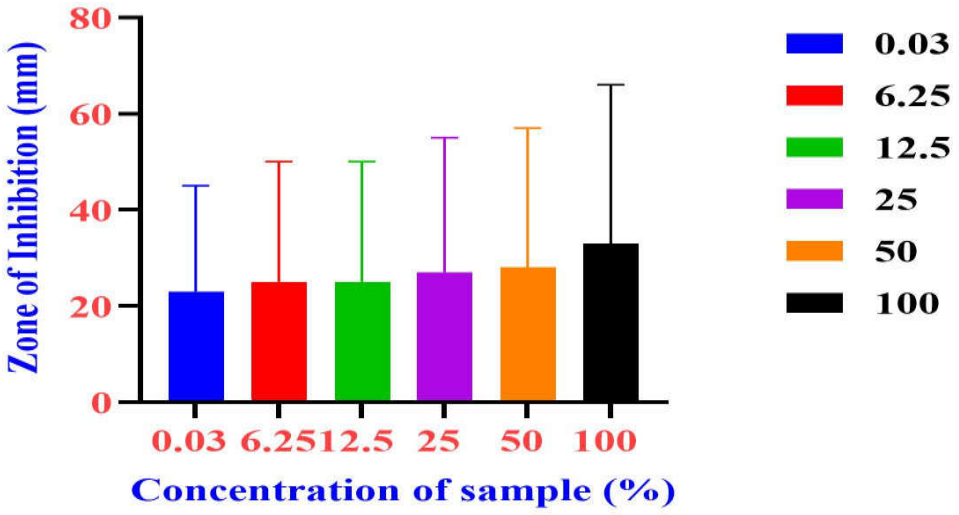


Fig-9: Antibacterial Activity of EE-LAV against *B. subtilis*

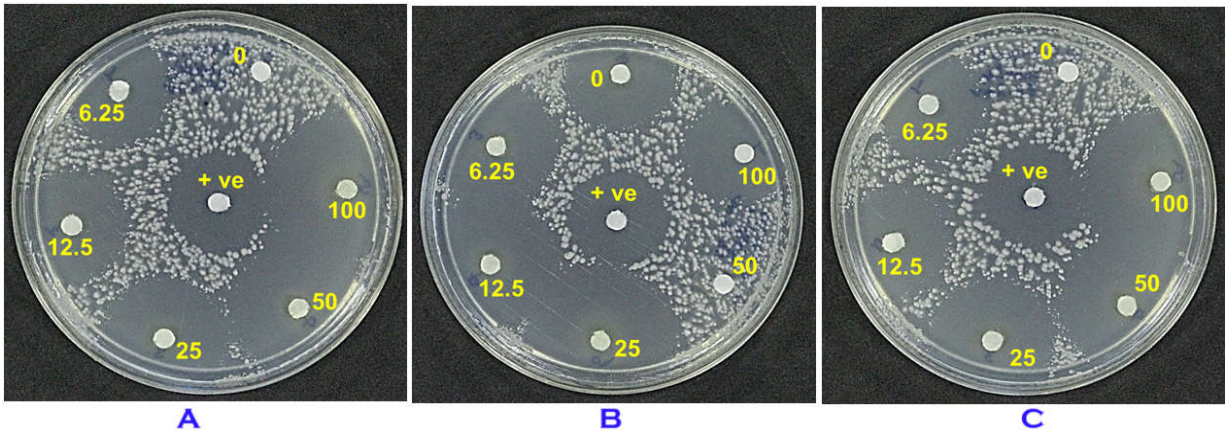


Fig-10: Zone of Inhibition of EE-LAV against *B. subtilis* at concentration present per disc in percentage (%).

Table-14: Antibacterial Activity of EE-LAV against *E. coli*

Test Organism: *E. coli*						
X Axis: Amount (%/disc)						
Y Axis: *Zone Size (in mm)*						
Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
CF (PC:0.03)	23	25	25	24.666	0.5773	0.3333
6.25	8	8	7	7.666	0.5773	0.333
12.5	8	8	8	8	0	0
25	10	9	9	9.333	0.577	0.333
50	14	14	14	14	0	0
100	17	17	17	17	0	0

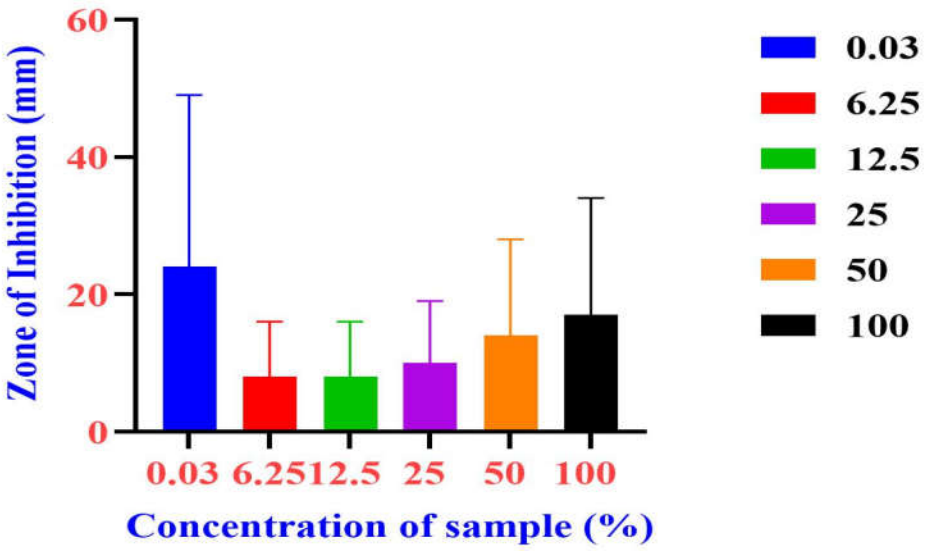


Fig-11: Antibacterial Activity of EE-LAV against *E. coli*

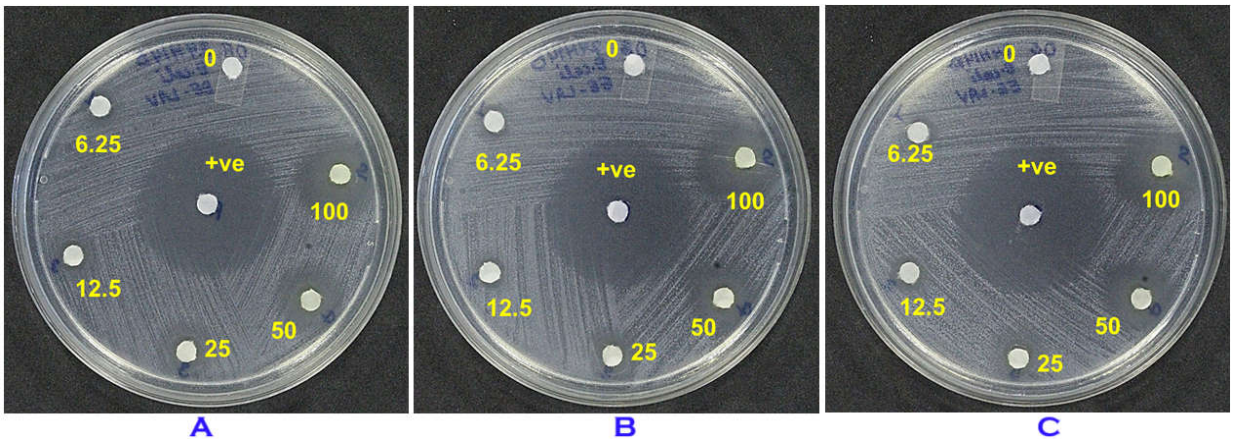


Fig-12: Zone of Inhibition of EE-LAV against *E. coli* at concentration present per disc in percentage (%).

Table-15: Antibacterial Activity of EE-LAV against *P. aeruginosa*

Test Organism: *P. aeruginosa*						
X Axis: Amount (%/disc)						
Y Axis: *Zone Size (in mm)*						
Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
CF (PC:0.03)	23	24	25	24.666	0.577	0.333
6.25	8	8	9	8.333	0.577	0.333
12.50	11	11	12	11.333	0.577	0.333
25	13	14	13	13.333	0.577	0.333
50	15	15	15	15	0	0
100	17	17	17	17	0	0

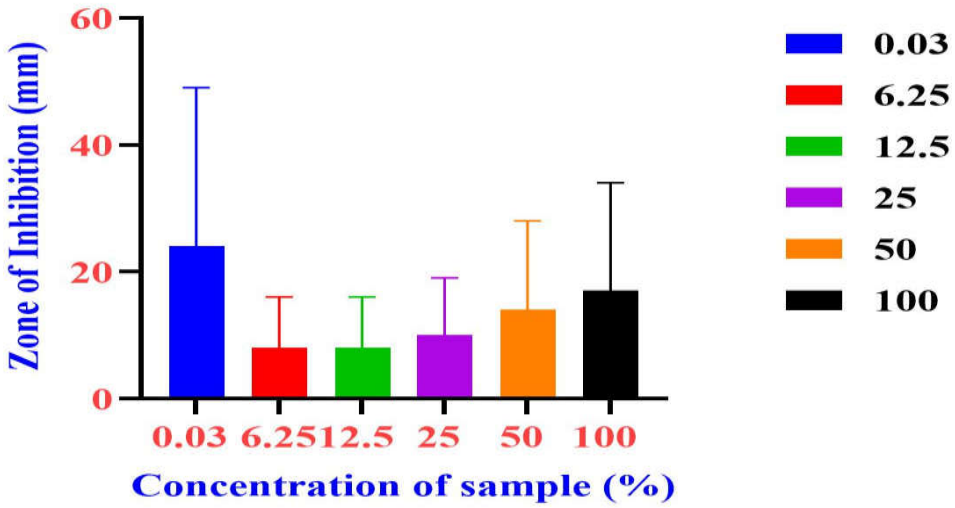


Fig-13: Antibacterial Activity of EE-LAV against *P. aeruginosa*

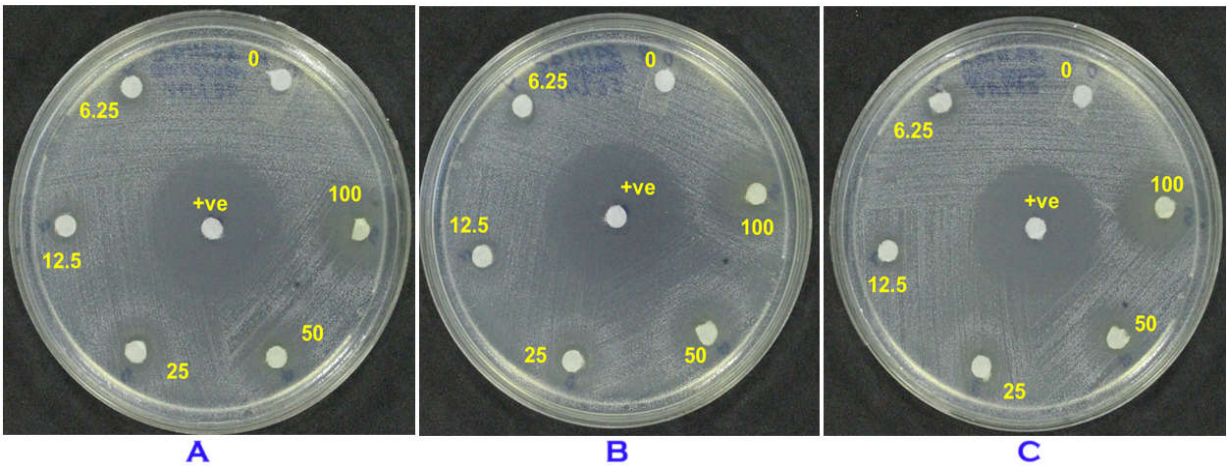


Fig-14: Zone of Inhibition of EE-LAV against *P. aeruginosa at* concentration present per disc in percentage (%).

Table-16: Antifungal Activity of EE-LAV against *A. niger*

Test Organism: *A. niger*						
X Axis: Amount (%/disc)						
Y Axis: *Zone Size (in mm)*						
Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
Ampho-B (PC: 0.03)	17	17	17	17	0	0
6.25	7	7	6	6.666	0.577	0.408
12.5	7	7	7	7	0	0
25	8	8	7	7.666	0.577	0.408
50	8	8	8	8	0	0

100	8	8	8	8	0	0

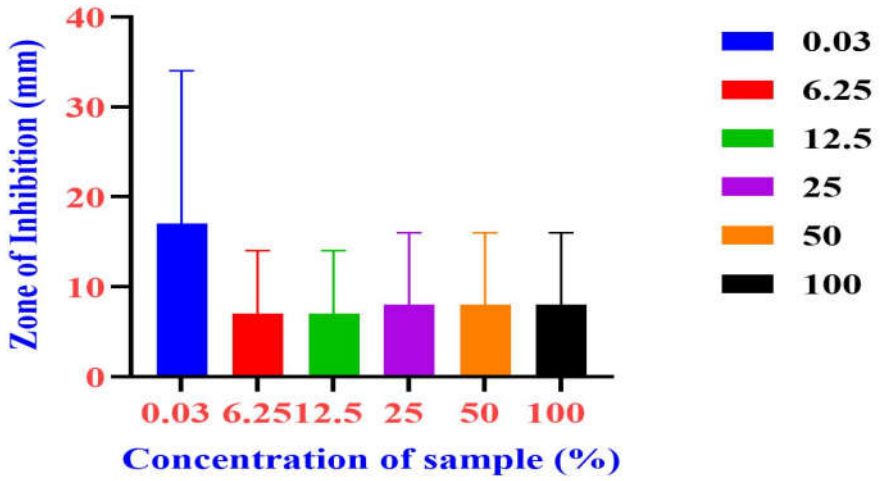


Fig-15: Antifungal Activity of EE-LAV against *A. niger*

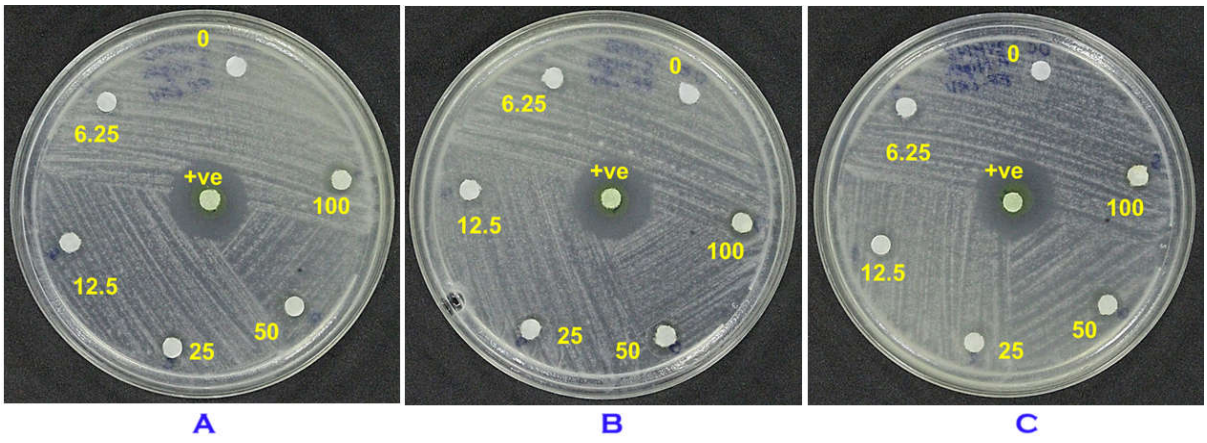


Fig-16: Zone of Inhibition of EE-LAV against *A. niger at* concentration present per disc in percentage (%).

Table-17: Interpretation of Average Zone of Inhibition at effective concentration

Sample code	Test organisms	Effective concentration	Average Zone of Inhibition at effective amount (mm)
CF (Std.)	*S. aureus*	0.03%	23.00
EE-LAV	*S. aureus*	6.25%	27.00
CF (Std.)	*B. subtilis*	0.03%	23.00
EE-LAV	*B. subtilis*	6.25%	25.00
CF (Std.)	*E. coli*	0.03%	24.66
EE-LAV	*E. coli*	6.25%	7.66

CF (Std.)	*P. aeruginosa*	0.03%	24.66
EE-LAV	*P. aeruginosa*	6.25%	8.33
Amphotericin B	*A. niger*	0.03%	17.00
EE-LAV	*A. niger*	6.25%	6.66

Discussion and summary

The ethanolic extract of *Adhatoda vasica* (EE-LAV) demonstrated strong antibacterial activity against Gram-positive bacteria, particularly *Staphylococcus aureus* (27.00 mm) and *Bacillus subtilis* (25.00 mm), exceeding the efficacy of the standard antibiotic ciprofloxacin (CF). These findings suggest that EE-LAV contains potent bioactive compounds capable of effectively disrupting the cell walls of Gram-positive bacteria. The observed antimicrobial effects are likely due to the presence of phytochemicals such as alkaloids and phenolic compounds, which were identified through TLC, UV, and LC-MS analyses.

In contrast, EE-LAV exhibited limited activity against Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and weak antifungal activity against *Aspergillus niger*. The reduced efficacy against Gram-negative strains may be attributed to their outer membrane barrier, which impedes the penetration of active compounds. Although used at a higher concentration (6.25%) than the standard drugs, EE-LAV was only effective against Gram-positive bacteria. Overall, these results suggest that EE-LAV could serve as a promising natural antimicrobial agent for Gram-positive infections but may require formulation enhancement for broader-spectrum use.

4. CONCLUSION

Based on the experimental findings, the ethanolic extract of *Adhatoda vasica* (EE-LAV) showed dose-dependent cytotoxicity against colo-205 cancer cells, significantly reducing cell growth and viability. At 400 µg/mL, EE-LAV achieved 90.89% inhibition, closely matching the standard drug TTN. Trypan blue staining confirmed increased cell death, suggesting the extract contains potent anticancer compounds. Further in vivo and mechanistic studies are needed to explore its therapeutic potential.

EE-LAV also demonstrated notable antimicrobial activity. Phytochemical and molecular docking studies identified compounds like dopamine, tryptamine, vasicine, vasicinone, and p-hydroxy benzoic acid with strong binding affinity to bacterial DNA gyrase. The extract was especially effective against Gram-positive bacteria such as Staphylococcus aureus and Bacillus subtilis, outperforming ciprofloxacin, while showing moderate activity against Gram-negative bacteria and fungi.

DNA gyrase in bacteria has human counterparts' topoisomerase IIα and IIβ involved in DNA replication and cell division. Compounds in EE-LAV may target both enzymes, inhibiting bacterial DNA metabolism and inducing cancer cell death. This dual action may explain EE-LAV's combined antibacterial and anticancer effects, warranting further investigation.

Acknowledgement

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Conflict of Interest

We declare that there is no conflict of interest regarding the publication of this research. All experiments were conducted independently without any financial or personal relationships that could influence the outcomes.

5. REFERENCES

1. Atal, C. K., Sharma, M. L., Kaul, A., & Khajuria, A. (1980). Immunomodulating agents of plant origin. Journal of Ethnopharmacology, 2(4), 333–348. https://doi.org/10.1016/0378-8741(80)90018-6
2. Dhuley, J. N. (1999). Antitussive effect of Adhatoda vasica extract on mechanical or chemical stimulation-induced coughing in animals. Journal of Ethnopharmacology, 67(3), 361–365. https://doi.org/10.1016/S0378-8741(99)00030-4
3. Singh, S., Yadav, C. M., & Kalia, A. N. (2011). Pharmacognostical and phytochemical investigation of Adhatoda vasica leaves. Asian Pacific Journal of Tropical Biomedicine, 1(2), 162–165. https://doi.org/10.1016/S2221-1691(11)60023-3.
4. Kumar, M., Dandapat, S., Kumar, A. and Sinha, M.P. Anti-typhoid activity of Adhatoda vasica and Vitex negundo Persian Gulf Crop Protection, 2013; 2(3): 64-75 http://corpprotection.ir/files_site/paperlist/Journal2-3-130906213336.pdf Archived 2015-05-29 at the Wayback Machine.
5. Kokate, C. K., Purohit, A. P., & Gokhale, S. B. (2010). Pharmacognosy. Nirali Prakashan.
6. Khandelwal, K. R. (2008). Practical Pharmacognosy: Techniques and Experiments. Nirali Prakashan.
7. Harborne, J. B. (1998). Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. Springer.
8. Azwanida, N. N. (2015). A Review on the Extraction Methods Use in Medicinal Plants. Asian Journal of Pharmaceutical and Clinical Research, 8(1), 1–6.
9. World Health Organization. (1998). Quality Control Methods for Medicinal Plant Materials. Geneva: WHO.
10. P. C Dandiya, P. K. Sharma, Bio-chemistry and clinical pathology, second edition, PP- 17-18, 24, 47-48.
11. Dr. G. Devala Rao, A Manual of Practical Biochemistry, pp 17.

12. Jaswant Kaur, PV Chemistry of Natural Products, 2010 edition, PP-113-114, 116, 344-346, 381.
13. Kokate, C. K., Purohit, A.P., Gokhale, S.B. (2015). *Pharmacognosy* (50th ed.).
14. Harborne, J. B. (1998). *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*. Springer Science & Business Media.
15. Wagner, H. & Bladt, S. (1996). *Plant Drug Analysis: A Thin Layer Chromatography Atlas*, Springer.
16. Evans, W.C. (2009). Trease and Evans Pharmacognosy.
17. A.K. Chakravarty et al., *Journal of Natural Products*.
18. Singleton, V.L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. Methods in Enzymology, 299, 152–178. https://doi.org/10.1016/S0076-6879(99)99017-1.
19. Waterhouse, A. L. (2002). Determination of total phenolics. In Current Protocols in Food Analytical Chemistry. John Wiley & Sons. https://doi.org/10.1002/0471142913.faa0101s06.
20. Pawar, H. A., and Deshmukh, V.N. (2013). Phytochemical analysis of plant extracts for LC-MS studies. Asian Journal of Pharmaceutical and Clinical Research, 6 (Suppl 2), 68-71.
21. Wolfender, J.-L., Marti, G., Thomas, A., and Bertrand, S. (2015). Current approaches and challenges for the metabolite profiling of complex natural extracts. Journal of Chromatography A, 1382, 136–164.
22. Dey, A., and De, J.N. (2012). LC-MS phytochemical screening of ethnomedicinal plant extracts. International Journal of Pharmacy and Pharmaceutical Sciences, 4(4), 38-42.
23. Toward an Integrative Strategy." *Journal of Natural Products*, 76(4), 588–603.
24. Want, E.J., et al. (2010). "Global metabolic profiling of animal and human tissues via UPLC-MS." *Nature Protocols*, 5, 1005–1018.
25. Stobiecki, M., and Buszewski, B. (2009). "Metabolomics in plant studies trends and challenges." *Bioanalysis*, 1(10), 1749–1760.
26. Pelletier JC et al. Preparation of highly substituted gamma lactam FSH receptor agonists, Bioorg. Med. Chem 2005; 13 : 5986-5 995.
27. (a) Gregory L. Warren, Stephen D. *"Chapter 16: Scoring Drug-Receptor Interactions".* WarrenPublished 2011 doi: 10.1039/9781849733410-00440.
28. "Sulforhodamine B sodium salt (CAS 3520-42-1)". Santa Cruz Biotechnology.
29. 2. Coppeta, J.; Rogers, C. (1998). "Dual Emission Laser Induced Fluorescence for Direct Planar Scalar Behavior Measurements". Experiments in Fluids. 25: 1–15. doi:10.1007/s003480050202.
30. 3. Viricel W; Mbarek A; Leblond J (2015). "Switchable Lipids: Conformational Change for Fast pH-Triggered Cytoplasmic Delivery". Angewandte Chemie International Edition. 54: 12743–12747. doi:10.1002/anie.201504661.

31. 5. Skehan P, Storeng R, Scudiero D, Monks A, McMahan J Vistica D et al. Evaluation of colorimetric protein and Biomass stains for analyzing Drug Effects upon Human Tumour Cell lines. Proceedings of the American Association for Cancer Research, 1989; 30:612.
32. Vibhor K Jain, Sudeep Mandal, Dibyajyoti Saha, Bindu Jain."*Synthesis, characterization and evaluation of antibacterial and antifungal activity of triazole derivatives of gallic acid"* Ijabpt 2010; 1 (3): 1300-1311.
33. Chandrakant R. Kokare. Pharmaceutical *Microbiology Experiments and Technique,* Second Edition, pp 63, 138-139.
34. John C. Christenson, E. Kent Korgenski, Ryan F. Relich, 286-Laboratory Diagnosis of Infection Due to Bacteria, Fungi, Parasites, and Rickettsiae, Editor(s): Sarah S. Long, Charles G. Prober, Marc Fischer, Principles and Practice of Pediatric Infectious Diseases (Fifth Edition), Elsevier, 2018, Pages 1422 1434.e3, ISBN 9780323401814,
35. R. S. Gaud, G. D. Gupta "*Practical microbiology"*. Nirali prakashan. Mumbai 3rd ed. 2004: 41.