ANTIMICROBIAL ACTIONS OF ZANTHOXYLUM AGAINST E.COLI

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Antimicrobial actions of Zanthoxylum against E.Coli

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Abstract

Zanthoxylum armatum DC., a medicinally significant plant of the Rutaceae family, has been traditionally used for its varied therapeutic uses such as antimicrobial, anti-inflammatory, and antioxidant activity. The current study examines its phytochemical content and estimates its antimicrobial activity by the minimum inhibitory concentration (MIC) assay. Sequential solvent extraction of plant material was performed using ethanol as the major solvent followed by qualitative phytochemical screening for the identification of major bioactive classes including alkaloids, flavonoids, tannins, phenolics, terpenoids, and saponins. Antimicrobial activity of extracts was evaluated against chosen Gram-positive and Gram-negative bacterial strains through the measurement of MIC values by a broth microdilution technique. Findings established the occurrence of several secondary metabolites with notable bioactivity and dose-dependent antimicrobial growth inhibition. They identify Z. armatum as an interesting lead plant for phytochemicals of interest in the formulation of plant-based antimicrobial agents and justify the use of the species in traditional medicine on a scientific basis.

Introduction

Zanthoxylum armatum Dc. Timur is commonly known in Hindi. Tejovti in Sanskrit under family Rutaceace. Lanceolate large crown leaves. Distributed across India from Kashmir to Bhutan Altitude of up to 2,500m and in north east India. also found in China, Nepal, Japan at altitude of 1,300-1,500m. Small trees or sharp spiny leaves shrubs. Late stalk less than 2.0-7.5 x 1.0-1.7 cm [1]

Zanthoxylum armatum, or green Sichuan pepper, is not merely a pungent plant. In the hills and valleys where it is found, it performs three roles — as a food, as a medicine, and as a means of livelihood. Its sour, tongue-prickling pericarp provides a novel kick to local cuisine, so that it is a sought-after spice in kitchens. In conventional medicine, it has been highly prized over centuries to relieve stomach ailments, toothache, and colds, but its essential oils are increasingly being introduced into new-age pharmaceuticals and cosmetics. For rural people, fruit production and trade of Z. armatum fruits generates critical income, connecting biodiversity conservation with economic gain. In brief, this small tree is able to flavor food, promote health, and support livelihoods — at the same time. [2,5]

Zanthoxylum armatum, often called green Sichuan pepper or Timur in local markets, is a small but striking tree from the citrus family (Rutaceae). If you've ever brushed past its branches, you'd notice its sharp thorns and aromatic leaves — a combination of beauty and caution. Traditionally found across the foothills of the Himalayas and parts of Southeast Asia, this plant has been part of human life for centuries. Its fruits, with their characteristic tingling taste, are not only utilized in food preparation but also prized in folk medicine and even cosmetics. [7,8] Current studies indicate that Z. armatum contains a high concentration of essential oils, amides, alkaloids, flavonoids, and polyphenols — substances responsible for imparting it its taste, smell, and health benefits. From spice racks in kitchens to drug laboratories, this plant has come a long way, closing the loop between tradition and science.

Fig. 1 A pictures of plant Zanthoxylum armatum DC

Extraction method

For the isolation of bioactive compounds from the herb Zanthoxylum armatum DC, Soxhlet extraction process was employed with the use of ethanol as a solvent. Ethanol was selected because it is capable of extracting a diverse range of polar and semi-polar phytochemicals, and also because it is a safer, environmentally gentle solvent that can be applied for biological research. [9-10]

Step 1.

Plant Material Preparation

Plants of Zanthoxylum armatum DC were collected, cleaned thoroughly to eliminate dirt and impurities, and then shade-dried at room conditions for 6–10 days. After drying, the plant material was powdered into coarse form using a clean mechanical grinder and kept in dry, air-tight containers in a cool, dry place until extraction.

Step 2.

Solvent Selection

Ethanol was used as extraction solvent. Ethanol is efficient in dissolving a wide range of phytochemicals such as alkaloids, flavonoids, tannins, and phenolics—most of which occur in Z. armatum. Ethanol is also safer than

methanol and thus more appropriate for extracts that are going to be used for biological assays. Ratio of which was ethanol: water i.e. 100 ml: 100ml

Step 3.

Soxhlet Extraction Protocol

About 20 grams of dried powder plant material was accurately weighed and charged into a cellulose extraction thimble. This was charged into the main chamber of the Soxhlet extractor.

Approximately 200 mL of ethanol was poured into a 500 mL round-bottom flask that was attached to the Soxhlet apparatus. The setup was done, and the system was minimally heated by a controlled heating mantle. As ethanol started boiling, vapours condensed and dripped through the plant sample, gradually leaching out the bioactive components.

This was permitted to operate for 6 hours, allowing several cycles of solvent washing for a thorough and efficient extraction.

Step 4.

The final ethanol extract of Zanthoxylum armatum DC was yellowish green in appearance, odor, and rich in phytochemicals.

Fig. 2 Dried powder of plant

Fig. 3 Soxhlet apparatus

2. Material and Method

2.1 Phytochemical tests

s.no	Phytochemicals	Tests	Requirement	Procedure	Result
1.	Alkaloid	a) Mayer's test	Mayer's reagent	1 ml sample Few drops of Mayer's reagent.	+++
		b) Wagner's test	Wagner's reagent	1ml sample 1ml Wagner's reagent and shake it reddish brown ppt formed.	+++
2.	Tannin acid test	a) Ferric chloride test	0.1%Feric acid	0.5 mg dried plant extract with 20ml of water in a test tube. Then	+++

				Boil and filter it. Add 0.1% Ferric chloride.	
		b) Gelatine test	1% Gelatine 10% NaCl Solution	1ml of sample Add 1% Gelatine Add 10% NaCl solution.	++++
		c) Braymer test	10% $FeCl_3$.	1ml sample Few drops of 10% $FeCl_3$.	++
3.	Steriod test	Liebermann's test	Acetic anhydride Concentrated H_2SO_4	1ml sample with acetic anhydride boil and cool it Add conc.H_2SO_4	+++
4.	Flavonoid detection test	a) Alkaline reagent	NaOH Dilute HCl	1ml sample add few drops of NaOH solution Yellow colour will appear when disappear add dil. HCl , it showing presence of flavonoid.	+++
		b) Lead acetate test	Lead acetate	Few drops of lead acetate solution in 1ml sample.	+++
5.	Terpenoid test	Salkowski test	Chloroform H_2SO_4	1ml test solution with 2ml of chloroform and few drops of H_2SO_4. And shake it sit for while reddish brown colour will appear it indicate the presence of terpenoid.	+++
6.	Test for Saponins	Froth test	Water	1 ml sample with water and shake it well and check the stability of froth in last 2 minutes.	+++
7.	Test for Phenols	a) Ferric acid test	Gelatine	1ml extract solution Add Gelatine.	--
		b) Lead acetate test	Alcoholic solution, 20%H_2SO_4, NaOH	1 ml extract 1ml alcoholic solution Dilute with 20% H_2SO_4 then add NaOH.	--
8.	Cyanidin test	Cyanidin test	50% methanol Metal magnesium Conc. HCL	1ml aq. Extract Dissolve in 1-2 ml 50%methanol (heat) Add metal magnesium and 5-6 drops of conc. HCL	--

9.	Benedict's test- .	Benedict's test	Benedict's reagent.	0.5ml filtrate 0.5ml benedict's reagent. Boil for 2 minute.	+++
10.	Dragendroff's	Kraut's test	Dragendroff's reagent	1ml filtrate 1-2 ml reagent.	+++
11.	Carbohydrate test	Molish test	2ml filtrate 2 drops of alcoholic 1 Napthol. Add 1ml conc. H_2SO_4.	2ml filtrate 2 drops of alcoholic 1-Napthol. Add 1ml conc. H_2SO_4.	+++

Positive +++, partially positive ++, negative --

Phytochemical Screening

Phytochemical screening was carried out to identify major classes of bioactive compounds present in the plant extract using standard qualitative tests. Alkaloids were detected by Mayer's, Wagner's, Dragendorff's, and Kraut's tests, where characteristic cream, reddish-brown, or orange precipitates confirmed their presence. Tannins were analyzed using the ferric chloride test, which produced a blue, green, purple, or black coloration, the gelatine test yielding a white precipitate in the presence of sodium chloride, and the Braymer test, where decolorization of bromine water indicated hydrolysable tannins. Steroids and sterols were confirmed by the Liebermann–Burchard test, producing an emerald-green or blue-green color, and by the Salkowski test, where a red to reddish-brown interface signified the presence of steroids and terpenoids. Flavonoids were identified using the alkaline reagent test, showing an intense yellow color that disappeared with dilute hydrochloric acid, and the lead acetate test, producing a yellow or white precipitate. Phenolic compounds were verified using ferric chloride and lead acetate tests, which yielded characteristic colorations and precipitates. Saponins were confirmed by the froth test, in which vigorous shaking with water resulted in a stable foam persisting for 10–15 minutes. Cyanidin-type flavonoids were detected by heating with concentrated hydrochloric acid, producing a pink, red, or purple color. Terpenoids were further confirmed by the Salkowski test, giving a reddish-brown interface. Reducing sugars were identified using Benedict's reagent, with a color transition from blue to green, yellow, orange, or brick-red depending on sugar concentration. Collectively, these classical assays provided a preliminary phytochemical profile of the extract, enabling differentiation of compound classes for further biochemical and pharmacological analysis.

1) Alkaloid Test

The alkaline test is a chemical method in which a plant extract is treated with sodium hydroxide (NaOH); the appearance of an intense yellow color indicates the presence of flavonoids, which disappears on adding dilute acid (HCl), confirming the result.

Mayer's Test: Mayer's test detects alkaloids by adding Mayer's reagent (potassium mercuric iodide solution) to the sample. The appearance of a cream or white precipitate indicates alkaloids.

Wagner's Test: Wagner's test detects alkaloids by adding Wagner's reagent (iodine in potassium iodide). A reddish-brown precipitate confirms alkaloids.

2) Tannin Acid Test

This test identifies tannins using reagents such as ferric chloride ($FeCl_3$), lead acetate, or gelatine solution, which give characteristic color changes or precipitates.

Ferric Chloride Test: Adding neutral $FeCl_3$ solution to the extract produces blue, green, purple, or black coloration depending on the type of phenol or tannin present.

Gelatine Test: Adding 1% gelatine solution with sodium chloride forms a white precipitate in the presence of tannins.

Braymer Test: In this test, bromine water is added to the extract. Decolorization of bromine water confirms hydrolysable tannins.

3) Steroid Test

This test detects steroids or sterols, which react with concentrated acids to give color changes (green, blue, or red).

Liebermann–Burchard Test: The extract is treated with acetic anhydride followed by sulfuric acid. A blue-green or emerald-green color confirms steroids or sterols.

4) Flavonoid Test
Flavonoids are detected by qualitative tests producing color changes or precipitates.
Alkaline Reagent Test: Adding NaOH gives an intense yellow color, which disappears upon adding dilute HCl.
Lead Acetate Test: Adding 10% lead acetate forms a yellow or white precipitate confirming flavonoids or tannins.

5) Terpenoid Test
Terpenoids are detected by the Salkowski test: mixing the extract with chloroform and carefully adding sulfuric acid produces a reddish-brown interface, indicating terpenoids.
Salkowski Test: Steroids or terpenoids dissolve in chloroform and react with sulfuric acid to give a red, reddish-brown, or golden-yellow color at the interface.

6) Saponin Test
Saponins are confirmed by the foam (froth) test: vigorous shaking of the extract with water forms a persistent froth lasting 10–15 minutes.

7) Test for Phenols
Phenols are detected by color or precipitation reactions.
Ferric Chloride Test: Adding neutral $FeCl_3$ produces blue, green, purple, or black coloration depending on phenol type.
Lead Acetate Test: Adding 10% lead acetate solution forms a yellow or white precipitate confirming phenols, flavonoids, or tannins.

8) Cyanidin Test
This test detects cyanidin-type flavonoids. Treating the extract with concentrated HCl and heating develops a pink, red, or purple color.

9) Benedict's Test
This test detects reducing sugars using Benedict's reagent ($CuSO_4$ + Na_2CO_3 + sodium citrate). On heating, blue Cu^{2+} is reduced to red Cu_2O, causing a color change from blue to green, yellow, orange, or brick-red depending on sugar concentration.

10) Dragendorff's Test
Dragendorff's test detects alkaloids using potassium bismuth iodide reagent. Adding the reagent forms an orange, reddish-brown, or yellow precipitate confirming alkaloids.
Kraut's Test: Kraut's test uses potassium mercuric iodide reagent. The formation of a reddish-brown precipitate indicates alkaloids.

11) Carbohydrate Test
Carbohydrates are detected using color reactions or precipitations.
Molisch's Test: A general test for all carbohydrates where a purple/violet ring forms at the interface, confirming their presence.

2.2 Isolation of bacteria

The microbial world begins with one crucial step—isolating bacteria to obtain pure cultures. This process allows scientists to study a single species without interference, making accurate identification, research, and experimentation possible. The idea is simple yet powerful: separate individual bacterial cells on a solid medium so that each cell multiplies into a visible colony, representing a pure population. To achieve this, microbiologists use techniques like the streak plate method, where an inoculating loop spreads bacteria across an agar plate in progressively diluted streaks, producing well-defined colonies after incubation at 37°C for 24–48 hours. The spread plate method involves evenly spreading a small volume of diluted bacterial suspension on the agar surface to produce isolated, countable colonies, while the pour plate method mixes serially diluted bacteria with molten agar (~45°C), allowing colonies to grow both inside and on the surface of the plate. In addition, special

approaches—such as selective media like MacConkey agar for Gram-negative bacteria or enrichment cultures like selenite broth for Salmonella—help target and grow specific bacterial species with precision.

2.2.1 Biochemical test

1. Gram staining

Gram staining is one of the most powerful and widely used techniques in microbiology, helping researchers quickly distinguish between two major bacterial groups—Gram-positive and Gram-negative—based on fundamental differences in their cell wall structure. The method uses a series of stains and reagents: crystal violet as the primary stain, Gram's iodine as the mordant, alcohol or acetone as the decolorizer, and safranin as the counterstain. Gram-positive bacteria, with their thick peptidoglycan layer, retain the deep violet color of the crystal violet–iodine complex even after decolorization, appearing purple under the microscope. In contrast, Gram-negative bacteria, with a thinner peptidoglycan layer and lipid-rich outer membrane, lose the primary stain during decolorization and readily take up the red counterstain, appearing pink. The procedure involves preparing and heat-fixing a thin bacterial smear, sequentially applying the stains, decolorizing, counterstaining, and finally observing under an oil immersion lens at 100x magnification. In my test, the bacteria were identified as Gram-negative, rod-shaped organisms, which appeared pink under the microscope—similar to species like *Escherichia coli* or *Salmonella typhi*. This simple yet highly informative technique not only classifies bacteria but also guides further identification, antibiotic selection, and diagnostic decisions. Test were gram negative bacteria with rod shaped looking.

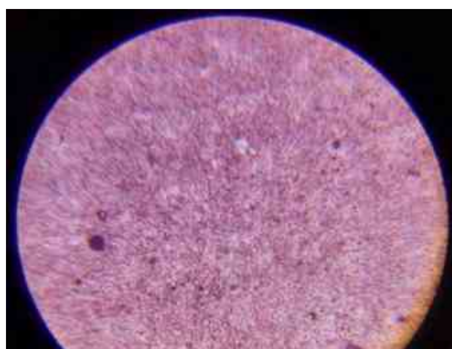

Fig.4 : Gram staining- gram's negative bacteria

2. Catalase test

The catalase test is a quick and simple way to determine whether bacteria produce the enzyme catalase, which protects cells by breaking down harmful hydrogen peroxide (H_2O_2) into harmless water and oxygen. When hydrogen peroxide is added to catalase-producing bacteria, it instantly fizzes as oxygen bubbles are released—almost like a microscopic soda reaction. This test is usually performed by placing a drop of 3% hydrogen peroxide on a clean glass slide and mixing in a fresh bacterial colony with a sterile loop or wooden stick (metal needles are avoided to prevent false bubbling). If bubbles appear immediately, the test is positive, as seen in organisms like *Staphylococcus aureus* or *Bacillus subtilis*. If no bubbling occurs, the test is negative, as in *Streptococcus* and *Enterococcus* species. The catalase test is particularly useful in clinical microbiology to quickly distinguish staphylococci (catalase-positive) from streptococci (catalase-negative), and to help classify bacteria as aerobic or anaerobic. For accurate results, it's important to use fresh hydrogen peroxide—old solutions may decompose and lead to false negatives. Here the test was positive there were bubbles as the culture came in contact with hydrogen peroxide.

Catalase test: positive test

3. Amylase test

The amylase test, also known as the starch hydrolysis test, is used to determine whether bacteria can produce amylase—an enzyme that breaks down starch into simpler sugars like maltose and glucose. In this method, bacteria are grown on starch agar plates (nutrient agar enriched with 1% soluble starch) and incubated at 30–37 °C for 24–48 hours. After incubation, the plate is flooded with Gram's iodine solution, which normally reacts with starch to form a blue-black complex. If the bacteria have hydrolyzed the starch, a clear, transparent halo appears around their growth because no starch is left to bind iodine—this indicates a positive result, as seen in *Bacillus subtilis*. In contrast, if the bacteria do not produce amylase, the entire plate turns blue-black with no clear zone around colonies, as with *Escherichia coli*. This test is widely applied in microbiology to identify amylase-producing bacteria, which are significant in industries such as brewing, food processing, and detergent manufacturing, as well as in diagnostic labs to differentiate bacterial species. The test over here were positive their the clear zone of starch hydrolysis could be seen.

Fig.4 Starch hydrolysis test- clear zone of breakdown of starch.

6. Urease test

The urease test is a reliable way to check if bacteria produce the enzyme urease, which breaks down urea into ammonia and carbon dioxide. As ammonia is released, it makes the medium alkaline, and this change is detected using phenol red, a pH indicator that shifts from yellow or orange at acidic or neutral pH to a bright pink or fuchsia in alkaline conditions. In practice, the test is performed using Christensen's Urea Agar slants, which contain both urea and phenol red. The bacterial culture is heavily inoculated onto the agar and incubated at 37 °C for up to 48 hours—though some rapid urease producers can show results within a few hours. A pink color indicates a positive test, as seen with *Proteus vulgaris*, *Klebsiella pneumoniae*, or *Helicobacter pylori*, while a yellow or orange color indicates a negative result, as in *Escherichia coli*. This test is particularly useful for differentiating urease-positive *Proteus* species from other Enterobacteriaceae, identifying *H. pylori* in clinical settings (through related urease breath tests), and forming part of the biochemical profile used to classify bacteria. For accurate results, freshly

prepared media should be used, and readings should be taken within 48 hours to avoid false positives from spontaneous urea breakdown.

Urease test: positive test

5. Casein hydrolysis test

The protease test, also known as the casein hydrolysis test, is a simple yet insightful way to check whether bacteria can produce enzymes that break down proteins—specifically casein, the major protein in milk. When bacteria capable of producing proteases (like caseinase) grow on milk agar, they secrete enzymes that digest the opaque casein, leaving a clear halo around their colonies. To perform this test, bacteria are streaked or spot-inoculated on milk agar plates (nutrient agar mixed with 10% skim milk) and incubated at 30–37°C for 24–48 hours. After incubation, the plates are examined: a clear zone around bacterial growth indicates a positive result, showing the presence of protease activity (as seen with *Bacillus subtilis* or *Pseudomonas aeruginosa*), while no change in the medium indicates a negative result (as with *Escherichia coli*). This test is widely used in food, dairy, and soil microbiology to detect proteolytic bacteria, in industrial microbiology to identify organisms that produce commercially valuable proteases (used in detergents, leather processing, and more), and in diagnostic labs to help differentiate bacterial species. Test was positive an enzyme caseinase was present that hydrolysed or break down the protein present in the culture sample.

Fig.5 Casein test – hydrolysis of protein present in milk

2.3 ANTIMICROBIAL TEST OF ZANTHOXYLUM ARMATUM DC. AGAINST E. COLI.

Experiments with Zanthoxylum armatum DC. have proved to have high antibacterial activity against Escherichia coli, with varying intensity depending on extract type and purity. Isolated phytoconstituents like ombuin showed MIC values of 0.125 to 0.5 mg/mL, which indicates potent inhibitory activity,

while semi-purified hexane and ethyl acetate fractions manifested moderate activity with MIC values of 0.25 to 1 mg/mL. By contrast, crude methanolic and ethanolic extracts tend to provide minimum bactericidal concentration (MBC) values in most cases, ranging from 0.78 to 50 mg/mL, indicating lower activity than purified compounds. These results demonstrate that the antibacterial activity of Z. armatum against E. coli is primarily due to certain bioactive phytochemicals and not the crude extract, emphasizing the necessity for active compound targeting of isolation and characterization to construct plant-derived antimicrobial agent.

3.Conclusion and discussion

The study of Zanthoxylum armatum highlights its importance as a plant with strong traditional and scientific value. The presence of different phytochemicals such as alkaloids, flavonoids, tannins, saponins, terpenoids, and phenols explains why the plant has long been used in folk medicine for digestive disorders, toothaches, fevers, and as a natural spice. These bioactive compounds are also responsible for its antimicrobial, antioxidant, and anti-inflammatory effects, making it a promising candidate for pharmaceutical research. Beyond medicine, the plant has social and economic importance too — its fruits and bark are traded in local markets, and its essential oils are used in cosmetics and perfumery. This shows how one plant connects culture, health, and livelihood.

In conclusion, Zanthoxylum armatum is much more than a wild Himalayan shrub. It is a reservoir of healing compounds with applications in traditional healthcare as well as modern industries. The findings from phytochemical screening support its wide range of medicinal properties and justify its continued use in both rural traditions and scientific research. Preserving and promoting this plant can therefore benefit both community health and sustainable development.

Fig.6: Antimicrobial property against e coli.

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