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Isolation of Multidrug Resistant Enteric Pathogen from Poultry Fecal Matter and Application of Natural Extract Against the Bacteria

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Abstract:
This study examined multidrug-resistant (MDR) Enterobacter cloacae isolated from poultry fecal matter alongside evaluation of Justicia adhatoda leaf extract's antibacterial properties for replacing chemical preservatives in poultry feed. Morphological and molecular tests verified the presence of Enterobacter cloacae strain ARMB which demonstrated antibiotic resistance against Penicillin-G, Ampicillin, and Methicillin but retained sensitivity to Chloramphenicol. Tests of antibiotic sensitivity and Hi-Comb analysis confirmed elevated β-lactam antibiotic resistance through plasmid-based resistance pathways. The aqueous extract from Justicia adhatoda produced 19 mm zone of inhibition comparable to Chloramphenicol while exhibiting strong bactericidal activity through MIC–ELISA and viable count analysis and DNA fragmentation tests resulting in bacterial DNA destruction. The bioactive functional groups listed in FTIR results combined with total phenolic content analysis and antioxidant testing demonstrated extract bioactivity potential. Justicia adhatoda extract added to poultry feed treatment demonstrated an improved storage period as well as minimized bacterial counts and strong counteracting capabilities against infections. The study indicates that Justicia adhatoda leaf extract shows potential for acting as a natural feed preservative with antibiotic alternatives to improve feed safety against MDR bacterial infections.

Keywords: *Multidrug-resistant (MDR) Enterobacter cloacae; Justicia adhatoda; Minimum Inhibitory Concentration; DNA Fragmentation Assay; Poultry Feed*

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I. INTRODUCTION

The widespread use of antibiotics in livestock and poultry farming during veterinary operations and agricultural practices across the world increasingly creates multidrug-resistant (MDR) bacterial strains according to Davies and Davies (2010). The poultry industry stands as a major antibiotic user because farmers apply antibiotics both for disease prevention and for bacterial infection treatment and to enhance growth promotion practices. The improper antibiotic use along with excessive administration in poultry farms has enabled MDR pathogens to spread throughout the food chain which jeopardizes the health of both humans and animals [1]. *Enterobacter cloacae* represents a main antibiotic-resistant bacterial pathogen in poultry because this microorganism demonstrates successful resistance gene acquisition abilities combined with environmental survivability [2]. As a member of *Enterobacteriaceae* the *Enterobacter cloacae* bacterium functions as Gram-negative facultative anaerobic bacteria. The bacteria exist abundantly in soil along with water and human and animal gastrointestinal tissues. The pathogen *Enterobacter cloacae* normally takes advantage of opportunities but its ever-growing ability to develop antibiotic resistance makes it difficult for healthcare professionals to control. Through contaminated feed, water and faeces in poultry farms MDR *Enterobacter cloacae* causes both outbreaks of disease and lower productivity rates in poultry operations. The presence of resistant microorganisms in poultry waste affects farm animals and generates human health risks because consumers might encounter contaminated poultry products. The strong evidence shows that the poultry industry demands new measures to prevent MDR bacteria spread.

The use of plant-derived bioactive compounds represents a promising method to control antibiotic-resistant bacteria in poultry farming operations. *Justicia adhatoda* or Vasaka serves as an established medicinal

plant which traditional medicine utilizes to fight infections because of its multiple active properties that include antimicrobial activities and anti-inflammation and antioxidants [3]. The bioactive components found in *Justicia adhatoda* leaves include alkaloids as well as vasicine and vasicinone and flavonoids and tannins that show effective antibacterial properties against various Gram-positive and Gram-negative bacteria. *Justicia adhatoda* displays antibacterial properties against gastrointestinal and respiratory pathogens according to existing research although investigators have not studied its efficacy against MDR *Enterobacter cloacae* in poultry farming [4]. The growing interest in natural antimicrobial agents makes investigating *Justicia adhatoda* leaf extract as an alternative to synthetic antibiotics both vital and critical. The bioactive compounds of this plant demonstrate their antibacterial effects through bacterial cell membrane disruption combined with inhibition of biofilm formation and interference with vital metabolic pathways of resistant microorganisms [5]. The antimicrobial agents derived from natural plants show a reduced capability to generate bacterial resistance compared to traditional antibiotic interventions.

Another important aspect of this study is the application of *Justicia adhatoda* extract in poultry feed preservation. Poultry feed is often contaminated with bacteria and fungi, leading to spoilage, economic losses, and potential health risks to poultry [6]. Conventional chemical preservatives used in poultry feed can have adverse effects on animal health and may leave harmful residues in poultry products. Therefore, exploring plant-based preservatives with antimicrobial properties is a sustainable alternative. This study assessed the impact of *Justicia adhatoda* extract on microbial load reduction and the shelf-life extension of poultry feed, providing insights into its potential as a natural feed preservative. The integration of *Justicia adhatoda* leaf extract into poultry farming aligns with the global movement toward reducing antibiotic dependency and promoting sustainable livestock production. By incorporating plant-based antimicrobials into poultry feed, farmers can enhance food safety, improve animal health, and minimize the risk of antibiotic resistance transmission. This research contributes to the growing body of evidence supporting the use of medicinal plants as viable alternatives to conventional antibiotics, thereby fostering eco-friendly and antibiotic-free poultry farming practices.

In conclusion, this study aims to bridge the gap between traditional medicine and modern poultry farming by investigating the antibacterial potential of *Justicia adhatoda* against MDR *Enterobacter cloacae*. The findings will provide valuable insights into the feasibility of using plant-based antimicrobial agents for pathogen control and feed preservation in poultry farming. By promoting natural alternatives, this study supports global efforts to combat antibiotic resistance and ensure sustainable poultry production.

II. MATERIALS AND METHODS

Sample Collection and Processing

Poultry faeces were collected from a local poultry farm in Eachanari, Tamil Nadu, India, and transported to the laboratory in a sterile container. A 0.5 g sample was inoculated into 10 mL of nutrient broth, incubated at 37°C for 24 hours, and used for further studies [7].

Fresh leaves of *Justicia adhatoda*and *Colocasia esculenta*were collected from the Manimala River streamside at Peringara, Thiruvalla, Kerala. A 10% leaf extract was prepared by soaking the leaves in 100 mL of water and ethanol, followed by incubation in a shaking incubator at 40°C for 24 hours at 60–70 rpm. The extract was then filtered using Whatman No.1 filter paper and used for further studies.

Morphological and Molecular Identification of the Bacteria

Bacterial identification was carried out using selective media, sub culturing, and molecular techniques. MacConkey agar (55.07 g/L), XLD agar (56.68 g/L), and CLED agar (36.25 g/L) were prepared, sterilized in an autoclave at 121°C for 15 minutes, cooled to 45–50°C, and poured into sterile Petri plates for solidification. Once solidified, a bacterial culture loop was streaked onto the media using a sterile wire loop and incubated at 37°C for 24 hours to facilitate bacterial growth and identification. The isolated bacterial colonies were further sub cultured in Nutrient Broth (13 g/L, Himedia, Mumbai, India) for further studies [8].

For molecular characterization, DNA was isolated following standard protocols [24]. The quality and quantity of the extracted DNA were assessed using 0.8% agarose gel electrophoresis (AGE) with 1X TAE buffer followed by visualization under a UV transilluminator.PCR amplification was conducted using a 20 µL reaction mixture containing 2 µL of total DNA, 2 µL of forward primer (5'- AGAGTTTGATCCTGGCTCAG-3'), 2 µL of reverse primer (5'- ACGGCTACCTTGTTACGACTT-3'), 8 µL of PCR master mix, and 6 µL of distilled water. The reaction conditions included an initial denaturation at 94ºC for 3 minutes, followed by 20 cycles of denaturation (94ºC for 15 seconds), annealing (53–55ºC for 15 seconds at 30ºC), and extension (72ºC for 2 minutes), with a final extension at 72ºC for 15 seconds. The PCR product was analysed using 1.5% agarose gel electrophoresis and subjected to partial 16S rRNA gene sequencing using a genetic analyser. The sequence was analysed using BLAST, and the closest culture sequence was retrieved from the National Centre for Biotechnology Information (NCBI) [9].

Antibiotic Susceptibility Testing and MIC Determination by Hi Comb Assay

The 24-hour-old nutrient broth culture of *Enterobacter cloacae* (80µL) was swabbed on nutrient agar (28g in 1000 mL). Antibiotic discs, including Penicillin-G (P10) (10 units/disc), Ampicillin (AMP10) (10µg/disc), Methicillin (MET5) (5µg/disc), Cefazolin (CZ30) (30µg/disc), and Chloramphenicol (C30) (30µg/disc), were placed on the plate and incubated at 37°C for 24 hours. The zone of inhibition was measured in millimetres to determine the susceptibility.

For the Hi-Comb MIC assay, a 24-hour-old *Enterobacter cloacae* culture (80µL) was swabbed on Muller-Hinton agar plates (38g in 1000 mL). The HiComb™ MIC Test MD015-1PK Cefotaxime (CTX) strips (Part A: 240-0.01 µg; Part B: 30-0.001 µg) were placed on the plates, and the plates were incubated at 37°C for 24 hours. The zone of inhibition was measured in millimetres, following the Hi-Comb assay method [10].

Characterization of the Sample by FTIR

The analysis used Fourier Transform Infrared Spectroscopy (FTIR) to identify functional groups in the extracted compounds. FTIR spectroscopy revealed the functional groups that exist within the extract sample. The absorption spectrum reveals vital information about molecular structure through this analytical method. A small amount of extract received testing within a sample holder using a Shimadzu infrared spectrometer. The spectral scan was performed from 4000 to 400 cm^{-1} over the sample using FTIR spectroscopy which yielded an IR spectrum for interpretation [11].

Estimation of Antioxidant Activity of the Extract

Total Phenolic Content

A modified Folin–Ciocalteu reagent method measured the total phenolic content present in the extract. 0.2 mL of 10% Folin–Ciocalteau's reagent solution was added to 1 mL extract quantity then the mixture was gently shaken followed by reaction time of 5 minutes at room temperature. The reaction mixture received Na_2CO_3 (20% w/v solution) at 1 mL volume followed by water addition to reach 3 mL total volume. The reaction incubated at 45 °C for 45 minutes while an UV-Vis spectrophotometer (Labtronics LT291, Microprocessor) measured the absorbance at 765 nm. A standard curve of Gallic acid measured phenolic content which was expressed as GAE/g (gallic acid equivalent) [12].

Total Antioxidant Activity by Phosphomolybdenum Method

A modification of the phosphomolybdenum method described by [13] was used to evaluate antioxidant activities. A mixture of 0.5 mL test solution and 0.5 mL reaction solution containing 0.6 M H2SO4, 28 mM sodium phosphate, and 4 mM ammonium molybdate reagent was prepared. The prepared solutions received heating at 50°C for 90 minutes in both the test tubes and blank tubes. The tubes were exposed to room temperature after incubation before measuring absorbance at 695 nm through spectrophotometer (LT 291 Labtronics Microprocessor). The evaluation of antioxidant activity in mg/g required ascorbic acid as a standard.

Remedial measures

Minimal Inhibitory Concentration (MIC) – ELISA Method

The MIC assay used a 96-well plate procedure to evaluate the minimal extract concentration needed to prevent *Enterobacter cloacae* growth. Nutrient broth served as the medium and the isolated pathogen underwent subculture procedures. A total of 0.1 mL sterile nutrient broth solution was added to each well in the plate. The experiment used increasing extract amounts (10, 20, 30, 40, and 50 µL) to treat wells with *Enterobacter cloacae* culture (5 µL) added to each well. The researcher incubated the plate for 24 hours at 37°C.The turbidity of microorganisms in the 96-well ELISA plate reader (Robonik) was measured at 600 nm as a method to assess microbial growth following incubation. The MIC value signified the extract concentration which demonstrated limited or no visual microbial growth. The cell death percentage was calculated according to [14].

Viable Count Assay

The viable count assay served to determine the antibacterial properties of *Justicia adhatoda* and *Colocasia esculenta* leaf extracts on *Enterobacter cloacae*. The preparation of nutrient broth followed autoclaving at 121^{o}C before transferring it to sterile tubes. The test tubes received 1 mL of sterile nutrient broth inoculated with 5 µL *Enterobacter cloacae* culture and 100 µL of specific leaf extract. The incubation took place at 37°C for duration of 24 hours within the culture tubes.

The microbial viability assessment through the pour plate method occurred after incubation period. For pour plating 10 µL sample aliquot from the incubated culture tubes was added to the centre point of a sterile petri dish. A small volume of sterilized warm nutrient agar was carefully poured over the initial sample with gentle mixing. Bacterial colony counting determined antibacterial leaf extract efficacy through assessment of organism viability following plate incubation at 37°C for 24 to 48 hours [15].

Antibacterial Activity

The antibacterial properties of *Justicia adhatoda* and *Colocasia esculenta* leaf extracts were evaluated through the agar well diffusion testing method. A 70 µL bacterial culture of *Enterobacter cloacae* maintained in nutrient broth added for plating onto Mueller Hinton agar plates and swabbed over the agar surface with cotton swabs. Sterile wells prepared with a cork borer which was added with plant extracts and DMSO as negative controls and chloramphenicol solution at 30 µg/disc as the reference standard. The antibacterial activity was evaluated bythe measurement of inhibition zone diameters after 24-hour incubation at 37°C. [16]

DNA Fragmentation Study

The DNA fragmentation assay measured whether *Enterobacter cloacae* experienced DNA damage when exposed to plant extracts. Bacterial cells cultivated in Luria Bertani broth containing *Justicia adhatoda* and *Colocasia esculenta* extracts which was incubated at 37°C for 24 hours. A standard DNA extraction procedure allowed obtaining DNA from the solution [17]. The DNA analysis under agarose gel electrophoresis followed by UV transilluminator examination revealed the extent of DNA fragmentation in the extracted DNA samples.

Poultry Feed Preparation

Rice bran, wheat, corn, millet, soya, broken rice, mung bean, groundnut, pearl millet, and Bengal gram were obtained from a local Eachanari farm in Tamilnadu, India. These ingredients served as bases to create poultry feed formulations. A mixture containing *Justicia adhatoda* powder was added to the feed at percentages ranging from 0.1% to 0.5% of its total weight. The extract preparation involved dissolving the powders with water followed by overnight cultivation at 37°C while maintaining rotation within the shaking incubator. The extract passed through a Whatman No.1 filter paper after the incubation period. The finished feed contained an extracted powder concentration that ranged between 25-35%.

Microbial Load and Shelf-Life Analysis

The assessment of microbial contamination in poultry feed was conducted under sterile conditions through nutrient broth (Himedia, Mumbai, India; 13 g in 1000 mL). 100 µL of prepared poultry feed was inoculated onto the prepared sterile nutrient broth and was incubated at 37°C for 24 hours. The spectrophotometer measured optical density at 600 nm to evaluate microbial growth after incubation. Shelf-life was evaluated through analysing microbial amounts present in the poultry feed throughout the period [18].

III. RESULT AND DISCUSSION

The present study suggested that the *Justicia adhatoda* leaf extract product could have a great potential to be used as natural preservatives in poultry feed replacing chemical preservative as it has less microbial load extending the shelf life of the feed and has antibacterial effect similar to antibiotics which prevent the growth of MDR bacteria *Enterobacter cloacae*.

Sample Collection and Plant Extract Preparation

Microbial contamination was detected in tests analysing poultry feces obtained from Eachanari area in Tamil Nadu which triggered the need for effective control measures. The nutrient broth used for enriching 0.5 g fecal samples allowed bacterial growth which enabled the isolation of multidrug-resistant *Enterobacter cloacae* that historically exists within poultry environments [19]. Aquatic bacterial presence in poultry waste proves hazardous because it carries potential risks of infecting humans when ingested through food or when they come into contact with the environment [20]. Research indicates that intensive antibiotic usage in poultry feeds makes farms serve as MDR pathogen reservoirs and warrants immediate development of non-antimicrobial disease prevention methods [1].

Bioactivity studies conducted on extractable compounds from *Justicia adhatoda* and *Colocasia esculenta* leaves from the Manimala River region of Kerala showed strong natural antibacterial properties. The water and ethanol extraction process maintained phytochemicals with antimicrobial properties intact and these

compounds later underwent tests for bacterial inhibition. Previous studies show that *Justicia adhatoda* contains antimicrobial compounds such as alkaloids and flavonoids and phenolic compounds [21]. Studies reveal that *C. esculenta* contains bioactive compounds which demonstrate antimicrobial action toward MDR pathogens according to [22]. The identified characteristics demonstrate plants can function as effective antibiotic substitutes within poultry feed to reduce microbial contamination and create safer feed systems while controlling antibiotic resistance development [23].

Morphological Identification of Bacteria and its Phylogenetic Analysis

Microbiological characterization involved analyzing bacterial isolates through morphology testing across different selective culture media. The colonies on MacConkey agar showed signs of lactose fermentation with their pink appearance and circular shape along with convex texture and irregular outline and medium size and opaque and mucoid characteristics. Lactose fermentation produces acidic end products which accumulate in the medium and change the pH along with transforming neutral red indicator into its pink-red color as shown by *Enterobacter sp.* [24]. On Xylose Lysine Deoxycholate (XLD) agar the microorganism produced small circular mucoid convex smooth-edged yellow colonies with fair growth. The acidic fermentation of lactose as well as xylose and sucrose leads to phenol red indicator changes from red alkalinity to yellow acidity which is typical for *Enterobacter spp*. At the same time lysine decarboxylation causes alkaline end products that generate small regions of deepened red coloration in the medium independent of hydrogen sulfide production according to [25]. On the Cysteine-Lactose-Electrolyte-Deficient (CLED) agar medium the isolated bacteria formed moderate-to-large sized round colonies that had pink to pinkish-white coloration and irregular borders along with a slightly mucoid texture. CLED medium adopts its different shades from lactose transformation which causes pH-related spectral color changes ranging from deep blue (pH 7.4) through bluish-grey (pH 7.0) to pale grey (pH 6.8), pinkish-grey (pH 6.6) up to bright red (pH 6.4–6.0) [26]. The identification process for *Enterobacter cloacae* colonies follows established research which demonstrates their typical growth behavior on these specific media types.

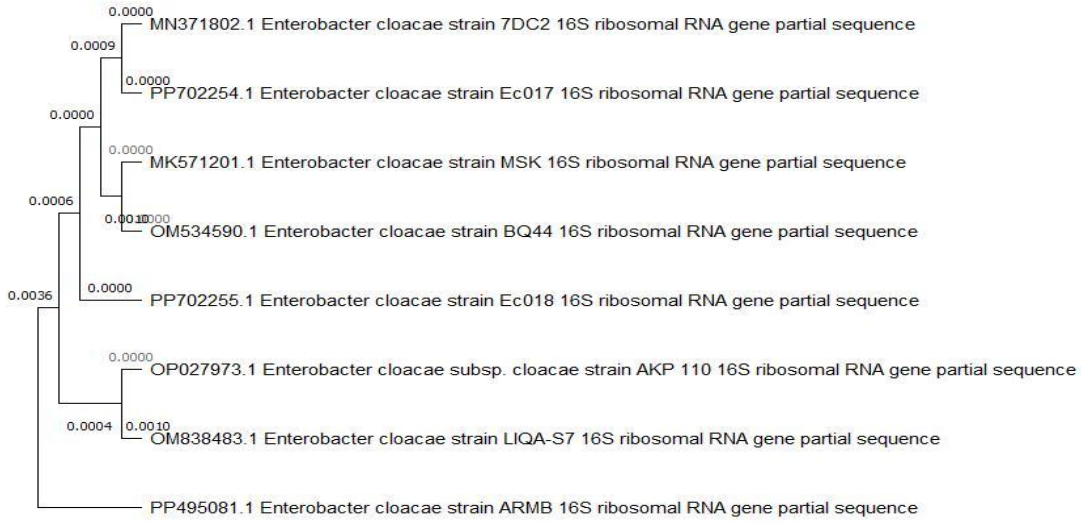

Figure 1: Phylogenetic Analysis of the Bacteria isolated from Poultry Farm

The molecular identification process employed 16S rRNA sequencing for isolates while the obtained sequences underwent BLAST searches in the National Center for Biotechnology Information (NCBI) database. The lineage BLAST results identified isolates as the *Enterobacter cloacae* strain ARMB which belongs to the *Enterobacteriaceae* family. The phylogenetic tree (Figure 1) shows how identified *Enterobacter cloacae* sequences maintain direct evolutionary ties with other *Enterobacter cloacae* sequences. The assigned accession number for this strain is PP495081.The phylogenetic correlation analysis showed that *Enterobacter sp*. ES1 matched 99% with the previously identified *Enterobacter sp*. strains which are present in GenBank: MW131452. *E. cloacae* pose serious risks to poultry environments since it displays two critical traits which make it a matter of urgent concern: multidrug resistance and disease-causing potential (Gupta et al., 2020). Previous research confirms that *Enterobacter cloacae* succeed in poultry environments which emphasize the necessity for new antibacterial measures to control its dissemination [27].

Antibiotic Susceptibility and Resistance Pattern of *Enterobacter cloacae*

Enterobacter cloacae demonstrated resistance to Penicillin-G (P10), Ampicillin (AMP10), and Methicillin (MET5) antibiotics but showed sensitivity to Chloramphenicol (C30) and intermediate resistance to Cefazolin (CZ30) according to the results of the antibiotic susceptibility test. *Enterobacter* species have become more resistant to β-lactam antibiotics because they produce extended-spectrum β-lactamases (ESBLs) and carbapenemases [28] according to research studies. The existence of antibiotic resistance in β-lactam antibiotics creates substantial concern about standard antimicrobial treatments in poultry environments necessitating immediate intervention through alternative antimicrobial approaches (Kim et al., 2021). The excessive and improper use of antibiotics in poultry operations creates resistant bacteria that traverse through food systems thus endangering community health according to [29].

Enterobacter cloacae showed susceptibility to Cefotaxime (CTX) in the Hi-Comb assay as tests with Strip A demonstrated resistance at 30 µg and below but susceptibility at 240, 120 and 60 µg whereas Strip B revealed complete resistance to the entire 30–0.001 µg concentration range. ESBL production is likely responsible for resisting lower Cefotaxime concentrations since it break down β-lactam antibiotics to make them unusable [30]. The prevailing conditions of MDR pathogens present a major challenge for poultry farms which demonstrates the need for both strict antibiotic surveillance programs and research-based developments of plant antibiotic substitutes including *Justicia adhatoda* extracts [31].

Estimation of Total Phenol and Total Antioxidant Activity

Plant extracts demonstrated diverse Total Phenolic Content (TPC) levels throughout the testing samples. Aqueous extract of *Justicia adhatoda* achieved 77.57 mg/g GAE Gallic Acid Equivalent which was greater than the aqueous extract of *Colocasia esculenta's* 63.71 mg/g GAE while ethanolic extract of *Justicia adhatoda* measured 49.54 mg/g GAE and ethanolic extract of *Colocasia esculenta* measured 46.79 mg/g GAE. The phenolic levels were found to be higher in aqueous extracts than ethanolic extracts based on the analysis results. Phenolic compounds show powerful antioxidant functions because they can donate hydrogens to cancel free radical reactions [32]. The aqueous extract of *Justicia adhatoda* and the aqueous extract of *Colocasia esculenta* highlight their pharmaceutical and nutraceutical applications because their antioxidant and antimicrobial properties make them suitable candidates for food preservation.

The Total Antioxidant Activity assessment revealed that *Justicia adhatoda* aqueous extract maintained the highest antioxidant potential when measured as 861.09 µg/mL Ascorbic Acid Equivalent (AAE) whereas *Colocasia esculenta* aqueous extract scored second at 742.91 µg/mL AAE followed by *Colocasia esculenta*ethanolic extract at 670.18 µg/mL AAE and the lowest scores were from *Justicia adhatoda*ethanolic extract at 624.73 µg/mL AAE. The positive relationship between TPC and TAA indicates that phenolic compounds play a major role in the antioxidant properties of plant extracts [33]. The aqueous extraction solutions achieved stronger antioxidant performance than ethanolic extracts since the phenolic compounds displayed better solubility in water. These findings demonstrate *Justicia adhatoda* and *Colocasia esculenta* extracts contain significant potential as natural antioxidant resources which might help prevent diseases from oxidative stress while providing opportunities for functional food development.

Characterization by Fourier Transform Infrared Spectroscopy

The FTIR spectrum of *Justicia adhatoda* extract presented characteristic peaks that signify its bioactive functional groups. The broad peak at 3271.96 cm^{-1} represents O-H stretching bands which is a signal commonly linked to the phenolic and flavonoid compounds [34]. The absorption spectra of C-H stretching aliphatic groups exists at 2924.89 cm^{-1} and C=O stretching vibrations related to ketones and carboxylic acids and aldehydes appear at 1635.84 cm^{-1} (Singh et al., 2019). The spectrum indicates the existence of carbohydrate and alkaloid compounds through the C-O and C-H bending vibrations detected at 1070.23 cm^{-1} and 601.79 cm^{-1} [35].

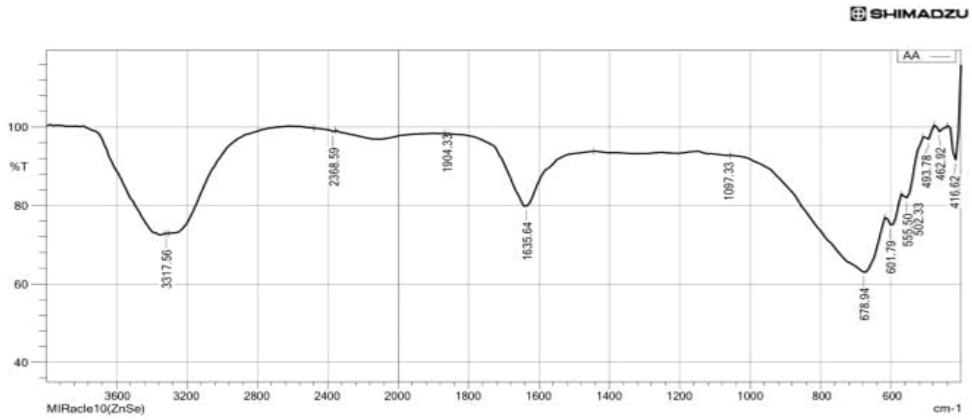

Figure 2: FTIR Spectrum of *Justicia adhatoda*Plant extract

Among documented studies regarding *Justicia adhatoda* extracts researchers have found identical functional groups that confirm its potential as a natural antimicrobial agent. Based on its hydroxyl and carbonyl groups the extract shows potential for bacterial cell wall interaction which results in antibacterial action against multidrug-resistant (MDR) pathogens including *Enterobacter cloacae*. Research verifies that Flavonoids alongside Tannins found in *Justicia adhatoda* block microbial growth by both membrane destruction and enzyme hindrance [36]. This research reveals that *Justicia adhatoda* extract demonstrates promise as a safe alternative to synthetic antibiotics in poultry feed which combines preservation benefits with longer shelf stability.

Minimal inhibitory concentration –ELISA method

Bacterial cell mortality increased in a concentration-dependent manner according to ELISA MIC tests of both *Colocasia esculenta* and *Justicia adhatoda* extracts. The ethanolic extract of *Colocasia esculenta* achieved 35.57% cell mortality when exposed to 10 µl after which the aqueous extract recorded 36.01% bacterial cell death. At 50 µl of concentration both the ethanolic extract from *Colocasia esculenta* achieved 69.54% bacterial mortality while the aqueous extract reached 71.74% mortality. Higher dosage levels of *Colocasia esculenta*'s aqueous and ethanolic extracts increased their potency against bacteria yet the aqueous extract displayed marginal superior bacterial killing potential. The bacterial cell death rate measured by MIC tests increased significantly among *Justicia adhatoda* extracts as the concentrations were elevated. Both the ethanolic solution (L:E) and aqueous solution reached 28.25% and 31.91% cell death when tested at 10 µl concentrations. The ethanolic extract achieved 69.54% cell death at its maximum concentration of 50 µl but the aqueous extract reached 75.10% cell death at this same value. *Justicia adhatoda* has demonstrated superior bacterial viability reduction abilities than *Colocasia esculenta* thus demonstrating better antimicrobial properties. The findings from this study support past research that showed *Justicia adhatoda* contains bioactive phytochemicals such as alkaloids and flavonoids which demonstrate strong antibacterial properties [37].

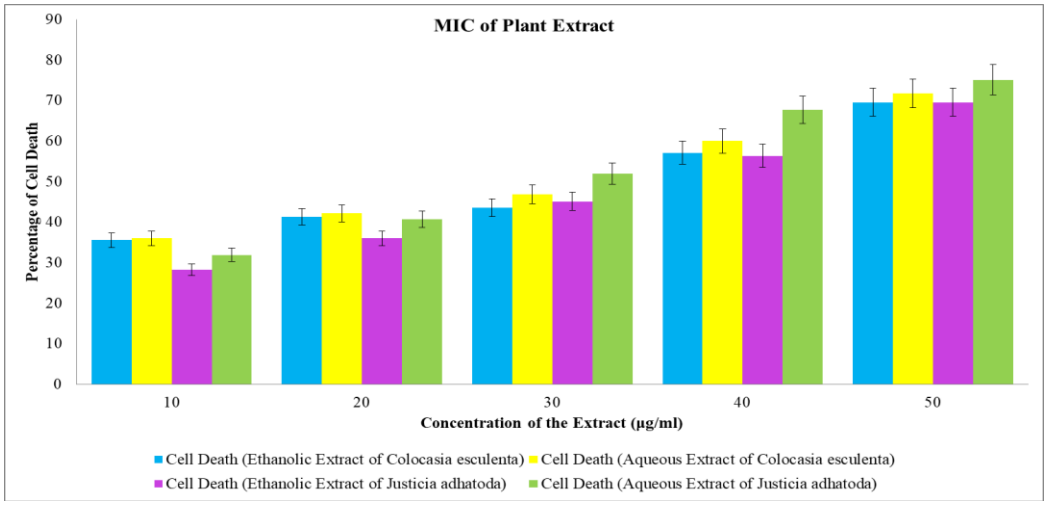

Figure 3: Minimum inhibitory Concentrations of the Plant Extracts

The examination demonstrates that *Justicia adhatoda* together with *Colocasia esculenta* exhibit substantial antibacterial activity that directly correlates with bacterial killing results. The antibacterial effect of *Justicia adhatoda* exceeded that of other tested plants making it more suitable for potential antimicrobial product development. Water-based plant extracts exhibited superior antibacterial effects than plant extracts prepared using ethanol because water aids in better solubility and extraction of active compounds.

Viable count assay

The viable count assay proved that *Justicia adhatoda* and *Colocasia esculenta* extracts effectively inhibited *Enterobacter cloacae* growth. The tube method showed bacterial growth through turbidity in the control tube but no turbidity could be detected in the leaf extract containing tubes which demonstrated bacterial inhibition. The pour plate control obtained numerous bacterial colonies that were too many to count (TNTC) but the plate containing *Justicia adhatoda* extract had no visible bacterial colonies. The antibacterial action of *Colocasia esculenta* extract was only partial so it yielded two colonies that qualified as too low to count (TLTC). The antibacterial strength of *Justicia adhatoda* exceeds that of *Colocasia esculenta* which makes it a superior natural antimicrobial substance. The antimicrobial potential of plant extracts continues to be demonstrated through research outcomes because their bioactive compounds including flavonoids, alkaloids and tannins disrupt bacterial cell wall structure while interfering with cellular metabolic activities [15]. The antibacterial results from this research demonstrate how natural plant-derived compounds work by breaking bacterial membranes and interrupting key enzymatic processes. The pour plate method produces reliable bacterial inhibition assessments based on agar dilution results according to comparative research which demonstrates better accuracy and reproducibility than broth micro dilution [16]. The tested application demonstrates how *Justicia adhatoda* extract demonstrates potential power as a natural preservative which can prevent bacterial contamination in poultry feed and decrease usage of synthetic agents.

Antibacterial activity

Among all evaluated extracts *Justicia adhatoda*'s aqueous extract achieved the best inhibition results against *Enterobacter cloacae* generating a 19 mm zone that matches the standard antibiotic Chloramphenicol's (19 mm) performance. Both extracts from *Justicia adhatoda* and *Colocasia esculenta* showed minimal antibacterial properties through their corresponding 10 to 11 mm zones of inhibition. Results indicate that the bioactive compounds in *Justicia adhatoda* aqueous extract are highly effective at inhibiting bacterial growth and thus can become a viable natural substitute to synthetic antibiotics according to [21]. Bacterial cell wall synthesis and metabolic processes suffer interference from alkaloids and flavonoids and other secondary metabolites found in *Justicia adhatoda* which leads to its substantial antibacterial effect (Rani et al., 2020).Scientific evidence shows that *Justicia adhatoda* exhibits antibacterial behavior because bioactive compounds found in plants effectively prevent drug-resistant microbes [36]. Current challenges in antimicrobial resistance against *Enterobacter cloacae* demand the evaluation of plant-based solutions to address medical and environmental AMR issues. The tested plant extracts demonstrated antimicrobial properties because the DMSO control showed no bacterial inhibition activity thus eliminating solvent effects on the bacterial growth rates. The antibacterial properties of *Justicia adhatoda* reinforce research evidence that shows medicinal plants may represent a solution to fight bacterial infections and scientists must study more to identify and define these active antibacterial compounds for pharmaceutical development.

DNA Fragmentation Analysis

The DNA fragmentation results showed that *Enterobacter cloacae* suffered DNA damage when subjected to treatment with_both*Justicia adhatoda* and *Colocasia esculenta* leaf extracts. The detection of DNA fragmentation confirms bacterial cell death because such genomic disruption makes it impossible for cells to survive. The antimicrobial properties with antibacterial qualities found in these plant extracts seemed to contribute to DNA fragmentation by inhibiting cellular DNA replication and affecting metabolic functions. Foreign DNA damage in bacterial cells occurs through plant-derived bioactive compounds such as alkaloids and flavonoids and tannins through ROS production and essential DNA synthase inhibition [38]. Independent evidence of DNA fragmentation confirms the antibacterial mechanism through which these extracts specifically recognize and damage bacterial genomic material. The assay technique for detecting bacterial cell death through natural antimicrobial agents corresponds to established research reports [39]. DNA fragmentation analysis with selective PEG precipitation along with Hoechst fluorescence assays demonstrated that DNA damage directly corresponds to apoptotic cell death thus confirming the basis for plant extract bactericidal activities. *Justicia adhatoda* and *Colocasia esculenta* show potential as natural antimicrobial agents because they trigger DNA fragmentation in *Enterobacter cloacae* solutions thus they offer an alternative to conventional antibiotics to reduce antimicrobial resistance (AMR). Studies should identify active antibacterial compounds that cause DNA damage to develop novel antibacterial treatment methods.

Poultry Feed Preparation and Evaluation of its Microbial Load and Shelf Life Analysis

The feed preparation for poultry included rice bran together with wheat along with corn and millet and legumes to deliver balanced nutrition to poultry. Experimental feed received *Justicia adhatoda* powder with increasing amounts of 0.1% to 0.5% during preparation. Aqueous extraction followed by incubation and filtration procedures led to the development of feed formulation with the incorporation of the targeted substances. Plant-derived bioactive chemicals such as alkaloids and flavonoids found in *Justicia adhatoda* have shown antimicrobial properties at key concentrations that qualify it for use as a natural poultry feed additive according to [40]. The researchers have integrated plant-derived additives into this research to create higher quality feed that requires diminished synthetic antibiotics and preservatives.

Results from microbial load analysis demonstrated that *Justicia adhatoda* extract reduced bacterial contamination in poultry feed better than the control without any treatment. During a 30-day test period the microbial levels stayed lower across the treated feed whereas untreated feed did not show similar reductions thus confirming its function as a food preservative. The inhibitory effect of *Justicia adhatoda* on microorganisms stems from its phytochemical content that causes bacterial membrane destruction and interferes with their metabolic functions [41]. The prevention of foodborne diseases and better poultry health depends on decreased microbial pollution within feed thus leading to improved meat and egg production efficiency. This study confirms previous research which demonstrates plant extracts as poultry feed additives improve both productivity and nutrient absorption and immune function while maintaining animal wellness according to [41]. Using *Justicia adhatoda* as an ingredient extends the food quality duration of poultry feed supply while providing farmers with a natural replacement for chemical protectants. The investigation shows how natural plant-based additives from *Justicia adhatoda* can contribute both to safer foods and better poultry production despite rising antibiotic resistance and chemical preservative worries in poultry farming.

IV. CONCLUSION

The research reveals that extracts from *Justicia adhatoda* and *Colocasia esculenta* possess notable antimicrobial and antioxidant properties suitable for medical and poultry nutrition applications. Results from total phenolic content testing and antioxidant activity evaluation proved bioactive compounds existed within samples mostly found in aqueous extracts. The antibacterial effect against *Enterobacter* cloacae was proven through the MIC and DNA fragmentation tests indicating both structural cellular disruption and growth inhibition. The usage of plant-derived antimicrobials shows promise as natural antibiotic alternatives to tackle antimicrobial resistance concerns. *Justicia adhatoda* extract added to poultry feed reduced microbial growth which improved dietary shelf stability and followed safety requirements. The investigation validates the potential of plant compounds to fulfil sustainable agricultural requirements through reduction of synthetic preservative usage. Future research should aim to determine optimal dosages while examining extended effects and investigating the complementary actions of natural compounds that enhance their benefaction for food preservation along with animal health maintenance.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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