**Isolation of Histamine Producing Bacteria (*Escherichia coli*) From *Penaeus monodon* (Tiger PRAWNS) And Its Control Measures Using Plant Extracts**

**ABSTRACT**

**Aims:** The study aims to isolate, molecularly identify, and estimate histamine-producing bacteria from *Penaeus monodon* (tiger prawns) and evaluate their antibiotic resistance and histamine degradation potential using plant extracts.

**Study Design:** Experimental study involving microbiological, molecular, and chromatographic techniques to analyze bacterial presence and histamine production.

**Methodology:** Samples were processed using specific agar media (Cetrimide, TCBS, MacConkey, etc.) followed by streaking, broth culturing, and antibiotic susceptibility testing through the disc diffusion method. Bacterial DNA was extracted, amplified using PCR, and sequenced for 16S rRNA identification. Gel electrophoresis confirmed DNA presence, and the bacterial strain was identified as *E. coli* (NCBI accession number PV156073). Histamine was extracted and quantified using Thin Layer Chromatography (TLC), UV spectrophotometry, and High-Performance Liquid Chromatography (HPLC).

**Results:** Bacterial isolates obtained from *Penaeus monodon* exhibited varying degrees of antibiotic resistance, with inhibition zones ranging from 22-26 mm for levofloxacin, 28-29 mm for ciprofloxacin, and 19-26 mm for amikacin. The presence of histamine-producing bacteria was confirmed through TLC analysis, which showed RF values of 0.6575 and 0.726. UV spectrophotometric analysis further estimated histamine levels, with optical densities of 0.640 in bacterial cultures and 0.625 in raw fish samples. HPLC and FTIR analyses validated the presence of histamine in bacterial supernatants. Additionally, plant extracts from *Annona muricata, Jatropha curcas,* and *Cestrum nocturnum* exhibited notable antimicrobial properties, with flavonoid content analysis indicating a potential role in histamine degradation.

**Conclusion:** The study successfully identified histamine-producing *E. coli* in tiger prawns, highlighting potential health risks. Antibiotic resistance patterns and histamine quantification suggest contamination risks in seafood. Plant-based extracts demonstrated potential in mitigating histamine-related allergic reactions, paving the way for natural therapeutic interventions.

*Keywords:Histamine-producing bacteria, Antibiotic resistance, Penaeus monodon, Plant extracts, Antimicrobial activity.*

1. **INTRODUCTION**

Seafood is a vital source of protein and essential nutrients, widely consumed across the globe. However, its perishable nature makes it prone to bacterial contamination, posing serious risks to food safety. One of the most concerning risks is histamine fish poisoning, which occurs due to the accumulation of histamine in seafood, particularly in scombroid fish and crustaceans such as *Penaeus monodon* (tiger prawns) (Lehane and Olley, 2000). Histamine poisoning is associated with various symptoms such as headaches, flushing, nausea, and abdominal pain, resulting from the decarboxylation of histidine by histamine-producing bacteria. Consequently, effective detection, identification, and control of these bacteria are essential for ensuring seafood safety.Histamine production in seafood is primarily attributed to bacteria belonging to species such as *Morganella morganii, Klebsiella pneumoniae, Escherichia coli, and Photobacteriumdamselae*. These bacteria produce the enzyme histidine decarboxylase, which catalyzes the conversion of histidine to histamine under improper storage conditions (Kanki *et al.,* 2004). When histamine levels exceed permissible limits, they can induce toxic effects, even after cooking or freezing, making seafood monitoring crucial. Previous studies have highlighted that bacterial contamination in seafood is closely linked to unhygienic handling, improper storage conditions, and bacterial growth during distribution and processing (Vinci & Antonelli, 2002).

In addition to histamine production, antibiotic resistance among histamine-producing bacteria presents a major concern in seafood safety. In aquaculture systems, antibiotics are widely used to control bacterial infections and enhance shrimp growth. However, excessive antibiotic usage has contributed to the emergence of multidrug-resistant (MDR) strains, complicating disease management and increasing the risk of resistance gene transfer to human pathogens (Cabello *et al.,* 2013). The presence of MDR strains such as *Escherichia coli and Klebsiella pneumoniae* in seafood emphasizes the urgent need for effective monitoring and control strategies. Natural antimicrobial agents have gained attention as promising alternatives for controlling bacterial contamination in seafood. Plant extracts are known to possess bioactive compounds with strong antibacterial properties that can effectively inhibit histamine-producing bacteria (Khullar *et al.,* 2023). Extracts from plants such as *Annona muricata, Jatropha curcas, and Cestrum nocturnum* have been widely studied for their antimicrobial, anti-inflammatory, and antioxidant properties. These plant-based treatments provide a natural, eco-friendly alternative to synthetic antibiotics and can play a significant role in improving seafood quality and safety.

Molecular techniques such as 16S rRNA sequencing and PCR methods offer high specificity and accuracy in detecting bacterial species associated with histamine production (Wongsariya *et al.,* 2013). Additionally, gel electrophoresis facilitates precise DNA profiling, ensuring accurate confirmation of bacterial identity. Such molecular tools are essential for studying bacterial contamination in seafood and formulating targeted intervention strategies. For effective detection of histamine in seafood products, various analytical techniques are employed. Thin Layer Chromatography (TLC), High-Performance Liquid Chromatography (HPLC), and UV-visible spectrophotometry are well-established methods for histamine estimation. TLC provides qualitative data for identifying histamine bands, while HPLC offers precise quantification, enabling accurate assessment of histamine levels in seafood products (Vinci &Antonelli, 2002). The combination of these techniques has proven effective in ensuring reliable histamine detection and enhancing seafood safety standards.

In addition to histamine detection, integrating remedial strategies such as the use of plant-based interventions has shown promising results. Studies have demonstrated that certain plant extracts can reduce histamine levels by inhibiting bacterial growth and promoting histamine degradation. These natural remedies provide a sustainable solution for managing bacterial contamination in seafood while minimizing the risks of antibiotic resistance (Khullar *et al.,* 2023).This study aims to investigate the presence of histamine-producing bacteria in *Penaeus monodon*, assess their antibiotic resistance profiles, and evaluate the antimicrobial potential of selected plant extracts. By combining molecular identification techniques, chromatographic analysis, and natural antimicrobial strategies, this research seeks to enhance seafood safety protocols and provide innovative solutions for reducing histamine-related health risks.

1. **MATERIALS AND METHODS**
   1. **Collection of Samples**

Fresh *Penaeus monodon* (tiger prawns) samples were collected from the Ukkadam fish market in Coimbatore, Tamil Nadu, India. The prawns were sourced from the Thoothukudi coastal region, known for its rich seafood availability. Upon collection, the prawns were cleaned, and the edible muscle region was separated for analysis. The sample collection followed appropriate hygienic protocols to minimize contamination

* 1. **Isolation of Histamine producing bacteria from the sample**

A selective culture medium was prepared to isolate and identify histamine-producing bacteria. Peptone broth was prepared by dissolving 0.45 g of peptone in 50 mL of distilled water in a conical flask. The solution was sterilized in an autoclave at 121°C for 15 minutes. The prepared prawn muscle tissue was crushed into a fine paste using a sterile mortar and pestle. The paste was then transferred into the prepared peptone broth. The inoculated broth was incubated in an orbital shaker at 120 rpm for 24–48 hours at 37°C to facilitate bacterial growth (Tsai *et al.,* 2005).

* 1. **Specification of Bacteria**

To identify bacterial strains, various selective agar media were prepared, including Cetrimide medium Agar (46.7g/L), TCBS Agar (86 g/L), EMB Agar (46.7g/L), MacConkey Agar (35.96 g/L), and SS Agar (63.01 g/L). The following concentrations were prepared. The prepared agar media were sterilized in an autoclave at 121°C for 15 minutes. After cooling to approximately 45°C, each medium was poured into sterile Petri plates and allowed to solidify under aseptic conditions. Following the preparation of agar plates, a small amount of bacterial culture was transferred onto each plate using a sterile inoculating loop. The streaking technique followed a zigzag pattern to ensure even distribution and clear bacterial colony formation. Plates were incubated at 37°C for 24 hours to allow bacterial growth (Andrews, 2001). Post-incubation, bacterial colonies were identified based on their characteristic growth patterns on selective media. Colonies displaying distinct growth on Cetrimide Agar, TCBS Agar, and MacConkey Agar were examined for histamine production potential which was sub cultured and further subjected to molecular identification techniques.

* 1. **Antibiotic Susceptibility Test by Disc Diffusion Method**

The disc diffusion method was employed to confirm the bacterial strains and evaluate their antibiotic resistance, particularly in relation to histamine production (Bauer *et al.,*1966). Nutrient agar media was used for the test. The prepared media was sterilized in an autoclave at 121°C for 15 minutes. After cooling to approximately 45°C, the sterilized agar medium was poured into three sterile Petri plates and allowed to solidify under aseptic conditions. Following solidification, the bacterial cultures identified as histamine producers were swabbed uniformly over the surface of each agar plate using a sterile cotton swab to ensure even bacterial distribution. Subsequently, selected antibiotic discs were carefully placed onto the agar plates to assess bacterial resistance. The antibiotics used in this study included Levofloxacin (20mcg), Penicillin-G (10 mcg), Amikacin (30mcg), Vancomycin (30mcg), and Ciprofloxacin (5mcg). The plates were subsequently incubated at 37°C for 24-48 hours to allow bacterial growth and antibiotic interaction. Upon completion of the incubation period, the zones of inhibition were measured to assess bacterial susceptibility.

* 1. **Molecular Identification of the Isolate**
     1. **DNA Isolation**

DNA extraction was performed using a modified protocol of Ramaswami *et al.,* (2018) to ensure efficient recovery of bacterial genetic material. Approximately 2 mL of bacterial culture was transferred into sterile Eppendorf tubes and centrifuged at 5,000 rpm for 5 minutes to obtain a pellet. The resulting pellet was suspended in 500 µL of extraction buffer, followed by the addition of 10 µL of lysozyme to facilitate cell wall lysis. The suspension was thoroughly mixed and incubated at room temperature for 30 minutes to enhance the lysis process. Following incubation, 150 µL of 10% sodium dodecyl sulfate (SDS) was added to the mixture, which was then incubated at 65°C for an additional 30 minutes. To extract the DNA, an equal volume of phenol-chloroform-isoamyl alcohol was introduced, and the sample was centrifuged at 10,000 rpm for 10 minutes. The aqueous phase containing DNA was carefully collected, and 0.2 volumes

of sodium acetate along with 5 volumes of isopropanol were added to precipitate the DNA. The sample was centrifuged again at 10,000 rpm for 10 minutes, resulting in a visible DNA pellet. The pellet was washed with 500 µL of absolute ethanol, followed by 70% ethanol, and centrifuged at 5,000 rpm for 5 minutes to enhance DNA purity. The obtained pellet was air-dried to remove any residual ethanol. Finally, the dried pellet was dissolved in 40 µL of 1X TE buffer for preservation. DNA integrity was assessed through agarose gel electrophoresis to confirm successful extraction

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* + 1. **PCR Amplification**

Polymerase Chain Reaction (PCR) was conducted to amplify the 16S rRNA gene for bacterial identification. The reaction was carried out using specific primers: 16SF (5'-AGAGTTTGATCMTGGCTCAG-3') as the forward primer and 16SR (5'-ACGGYTACCTTGTTACGACTT-3') as the reverse primer. The PCR conditions included an initial denaturation step at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1.5 minutes. A final extension was performed at 72°C for 5 minutes to ensure complete amplification. The amplified PCR products were separated by electrophoresis on a 1.5% agarose gel containing 0.5 µg/mL ethidium bromide and visualized under UV light using a Biotech Submarine Gel System. The purified 16S rRNA gene fragments were subsequently prepared for sequencing to enable accurate bacterial identification.

* 1. **Qualitative method of Histamine estimation**
     1. **Thin Layer Chromatography**

Thin Layer Chromatography (TLC) was employed to detect and quantify histamine content in bacterial extracts following a modified method adapted from Stahl (1969).A solvent system was prepared by combining 9 mL of acetone with 5 mL of 20% ammonium hydroxide in a clean beaker. The mixture was stirred thoroughly to ensure homogeneity. TLC plates were marked with baseline points for sample application. The extracted bacterial supernatant was carefully spotted onto the marked TLC plate 30 times using a micropipette to ensure consistent sample application. After drying, the TLC plate was placed in a developing chamber saturated with the prepared solvent system. Once the solvent front had travelled an appropriate distance, the plate was removed and allowed to air dry. To visualize the separated compounds, the dried TLC plate was sprayed with ninhydrin solution, a reagent that reacts with amines such as histamine. Distinct purple bands were observed, and the Rf (Retention factor) values were calculated.

* + 1. **UV-Visible Spectrophotometric Analysis**

The UV-Visible spectrophotometric method was employed to assess the presence and concentration of histamine in bacterial extracts, following the method described by Moniente *et al*. (2022).The analysis was performed using a Labtronics LT 291 UV-Visible Spectrophotometer The sample was scanned within the wavelength range of 200–400 nm to detect characteristic peaks corresponding to histamine absorption. The resulting spectrum was analysed to identify the peak wavelength and corresponding absorbance value, which confirmed the presence of histamine in the sample.

* + 1. **FTIR**

The Fourier Transform Infrared (FTIR) analysis of the histamine supernatant was performed using the KBr pellet method on a Shimadzu FTIR spectrophotometer, following the procedure outlined by Smith (2018). To prepare the sample, the bacterial supernatant extract was first centrifuged at 5,000 rpm for 10 minutes to obtain a clear solution. The resulting supernatant was then subjected to lyophilisation, yielding a dry powdered sample. For KBr pellet preparation, approximately 1 mg of the dried histamine sample was carefully mixed with 100 mg of spectroscopic-grade potassium bromide (KBr) powder using a mortar and pestle to ensure uniform blending. The mixture was then compressed into a transparent pellet using a hydraulic press at a pressure of 10,000 psi. Following pellet formation, the sample was analysed using the Shimadzu FTIR spectrophotometer. The spectral scan was conducted in the wave length range of 4000–400 cm⁻¹, targeting the identification of functional groups characteristic of histamine.

* 1. **Quantitative estimation of Histamine by colorimetric method**

The estimation of histidine was conducted using a modified method adapted from standard protocols for amino acid quantification (Eggerth *et al.,* 1933).A specialized broth was prepared by dissolving 0.25 g of tryptone, 0.25 g of yeast extract, 0.25 g of sodium chloride (NaCl), 0.05 g of calcium carbonate (CaCO₃), and 1.35 g of histidine in 50 mL of distilled water. The broth was thoroughly mixed and sterilized before further application. To determine histidine concentration, 5 mL of culture filtrate was collected and centrifuged at 5,000 rpm for 5 minutes to obtain a clear supernatant. From this supernatant, 1.0 mL was mixed with phosphate-buffered saline (PBS), followed by the addition of 0.5 mL of hydrogen peroxide (H₂O₂) and 0.1 mL of crystal violet reagent. The mixture was incubated at 37°C for 15 to 30 minutes to allow the color reaction to develop. The optical density (O.D.) of the resulting solution was measured at 596 nm using a UV-Visible spectrophotometer.

* 1. **HPLC Analysis of Histamine**

The High-Performance Liquid Chromatography (HPLC) analysis of histamine was conducted following the method described by Jinadasa *et al.* (2016) with slight modifications. The bacterial culture supernatant was first centrifuged at 5,000 rpm for 10 minutes to obtain a clear supernatant. Subsequently, 500 µL of the collected supernatant was mixed with an equal volume (500 µL) of dichloromethane to facilitate the extraction of histamine. The mixture was thoroughly vortexed for 2 minutes to ensure proper phase separation. The resulting solution was carefully transferred to a clean vial and subjected to HPLC analysis. The HPLC system (Shimadzu LC-20AD) was equipped with a reverse-phase column for optimal separation. The mobile phase consisted of methanol and 0.1% trifluoroacetic acid (TFA) in water (70:30 v/v), and the flow rate was maintained at 1.0 mL/min. Detection of histamine was achieved using a UV detector set at a wavelength of 220 nm, ensuring accurate identification and quantification. Injection volume for each sample was 20 µL, and the total run time was 20 minutes. Histamine was identified by comparing the retention time of the sample peak with that of a standard histamine solution.

* 1. **Remedial Measures for Allergic Resistance using Plant Extracts**

The study explored the potential remedial effects of plant extracts from *Annona muricata* (Soursop), *Jatropha curcas*, and *Cestrum nocturnum* (Night Flower Jasmine) due to their known antimicrobial, antibacterial, and anti-inflammatory properties. These plants were collected from the streets of Ondipudur, Coimbatore. Approximately 10–20 leaves from each plant were gathered and thoroughly dried to facilitate the extraction process. The dried leaves were finely ground to obtain a uniform powder for subsequent analysis.

* 1. **Total Flavonoid content**

The presence of quercetin, a bioactive compound known for its allergy-resistance properties, was assessed using a standard colorimetric method (Saeed *et al.,* 2012). To perform the test, 1 mL of plant extract was taken in a test tube, followed by the addition of 0.1 mL of AlCl₃ and 0.1 mL of potassium sodium tartrate. Next, 2.8 mL of distilled water was added to the solution. The mixture was incubated at room temperature for 30 minutes to allow the reaction to develop. After incubation, the optical density (OD) was measured at 419 nm using a UV-Visible spectrophotometer. The formation of a yellow coloration indicated the presence of quercetin, confirming the flavonoid content in the plant extracts.

* 1. **Degradation of the Component Using Plant Extracts**

The degradation study was conducted to evaluate the antimicrobial potential of selected plant extracts against bacterial growth (Snuossi *et al*., 2016). Initially, a 50 mL broth medium was prepared using 0.25 g tryptone, 0.25 g yeast extract, 0.25 g NaCl, and 0.05 g CaCO₃. The prepared broth was sterilized to ensure aseptic conditions. Following sterilization, 100 µL of bacterial culture was added to the broth, and the mixture was incubated for 24 hours to allow bacterial growth. After incubation, the culture was divided into four separate screw-capped bottles: a control containing 20 mL of the culture without plant extract, P1 containing 10 mL of the culture with 1 g of Ann*ona muricata* (Soursop) extract, P2 containing 10 mL of the culture with 1 g of *Cestrum nocturnum* (Night Flower Jasmine) extract, and P3 containing 10 mL of the culture with 1 g of *Jatropha curcas* extract. To assess the total viable count (TVC) of bacterial cells, four Petri plates were prepared by pouring 25 mL of sterilized nutrient agar into each plate. After the agar solidified, 5 µL of the corresponding plant extract mixed with bacterial culture was added onto the surface of each plate and spread plating method was carried out. The plates were left to dry under sterile conditions before incubation at 37°C for 24 hours. This incubation period allowed observation of bacterial growth patterns, enabling the evaluation of the antimicrobial efficacy of each plant extract.

* 1. **Ointment and Product Formulation**

The ointment formulation was prepared using plant extracts with potential antimicrobial and skin-protective properties (Yassine *et al.,* 2020). Initially, 1 g of powdered plant extract, obtained from three different plant species, was mixed with 30 mL of distilled water. This mixture was placed in an orbital shaker for 24 hours to ensure proper extraction of bioactive compounds. Following incubation, the extract was filtered using filter paper, yielding approximately 30 mL of filtrate. For the ointment preparation, 0.5 mL of glycerol was added to the plant extract solution, and the mixture was stirred using a magnetic stirrer under mild heat for 1 hour. Next, 1% Carbopol was introduced to the mixture and stirred under the same conditions for an additional hour. Subsequently, 0.5% gelatin was added, followed by continuous stirring under heat to ensure uniform blending. After incubation overnight, 18 mL of isopropanol and 1.5 ml of Lavender essential oil were added, and the mixture was stirred for another hour using the magnetic stirrer. Finally, 1 g of xanthan gum was added as a gelling agent to achieve the desired ointment consistency.

* 1. **Antibacterial activity of the Prepared Formulation**

To evaluate the antimicrobial effectiveness of the formulated ointment, the well diffusion technique was employed. Nutrient agar was prepared by sterilization and poured to sterile petriplates for solidification. After the agar solidified,80μl of bacterial culture was added and spread uniformly over the agar surface using a sterile swab. Wells were created in the agar plates, and the formulated ointment was carefully introduced into these wells. The plates were incubated at 37°C for 24 hours, after which the zone of inhibition was observed and recorded to determine the antibacterial potential of the ointment.

1. **results and discussion**
   1. **Collection of Prawn Sample and Processing**

The prawns used in this study were collected from the Thoothukudi district fish market located in Ukkadam, Coimbatore, India. This region is known for its extensive seafood trade, which makes it a suitable site for obtaining marine species commonly linked to histamine production. Prawns, particularly those sourced from coastal areas, are prone to microbial contamination due to their perishable nature and protein-rich composition. Studies have indicated that seafood, especially crustaceans like prawns, can rapidly accumulate biogenic amines such as histamine under improper storage conditions (Oktariani *et al.,* 2022).The selection of the muscle region for analysis was strategic, as histamine and other biogenic amines tend to concentrate in protein-dense tissues. The muscle tissue provides an ideal medium for bacterial growth and subsequent histamine formation, primarily because decarboxylase-producing bacteria thrive in such nutrient-rich environments (Kanki*et al.,* 2004). Moreover, bacterial strains such as *Morganella morganii, Klebsiella pneumoniae*, and *Photo bacterium damselae* have been commonly linked to histamine accumulation in seafood products (Visciano *et al.,* 2014).Research further indicates that histamine poisoning incidents are frequently associated with scombroid fish species, yet non-scombroid species like prawns are also susceptible, particularly when subjected to temperature abuse during storage.

A peptone-based medium was prepared to facilitate the growth of histamine-producing bacteria. Peptone is widely recognized as an effective nitrogen source that supports the proliferation of various bacterial strains, especially those responsible for histamine production (Food and Agriculture Organization [FAO], 2013). Peptone media enhances bacterial growth by providing essential amino acids, carbon sources, and growth factors that stimulate enzyme activity involved in histamine biosynthesis (Feng *et al.,* 2016).Histamine-forming bacteria such as *Morganella morganii, Proteus vulgaris, and Enterobacteraerogenes* demonstrate optimal growth in protein-enriched environments like peptone broth. Studies have confirmed that peptone-based media are effective in isolating bacteria capable of producing histamine via the decarboxylation of histidine, a common amino acid present in seafood (Lehane & Olley, 2000). Additionally, peptone media creates favorable conditions for bacterial metabolism, supporting the expression of histidine decarboxylase genes. According to Kobayashi *et al.* (2016), peptone-based media significantly enhances the detection of histamine-producing bacteria, particularly in seafood samples stored at elevated temperatures. This enrichment method is crucial for isolating target bacterial strains that actively contribute to histamine accumulation. The prepared peptone medium, therefore, ensured optimal growth conditions for histamine-producing bacteria, aligning with established methods for food microbiological analysis (Qasim*,* 2023).

* 1. **Specification of bacteria**

Bacterial identification was performed using selective agar media to isolate and confirm histamine-producing species commonly found in seafood samples. Various selective media, including Citro-medium Agar, Thiosulfate-Citrate-Bile-Sucrose (TCBS) Agar, Eosin Methylene Blue (EMB) Agar, MacConkey Agar, and Salmonella-Shigella (SS) Agar, were prepared in appropriate concentrations to enhance bacterial growth and differentiation. Each of these agars offers unique properties that target specific bacterial strains associated with histamine formation.The use of Citro-medium Agar is known for isolating *Citrobacter* species, which are opportunistic pathogens found in contaminated seafood (Ravishankar*et al.,* 2002). TCBS Agar is selective for Vibrio species, particularly *Vibrio parahaemolyticus* and *Vibrio vulnificus*, which have been linked to histamine production in marine environments (Su *et al.,* 2011). EMB Agar is commonly employed to isolate Gram-negative enteric bacteria such as *Klebsiella pneumoniae* and *Escherichia coli,* which are capable of producing histamine by decarboxylating histidine (Yoshinaga and Frank, 1982). MacConkey Agar, another effective medium for detecting enteric pathogens, was crucial in identifying *Morganella morganii*, a primary histamine producer known to thrive in protein-rich seafood samples (Koutsoumanis*et al.,*2009). SS Agar further aids in isolating Salmonella and *Shigella* species, which, although less common, can also contribute to seafood spoilage and histamine formation (Fang *et al.,* 2003).The preparation of these selective media under sterile conditions ensured effective bacterial isolation by minimizing contamination. This method is widely adopted in food microbiology for the identification of histamine-producing bacteria and their potential to cause scombroid fish poisoning (Lehane & Olley, 2000).

Following media preparation, bacterial cultures were streaked onto the agar plates using a sterile inoculating loop in a zigzag pattern to promote distinct colony formation. The streaking method is highly effective in isolating bacterial strains from mixed microbial populations, enabling the identification of individual colonies for further analysis (Rawles*et al.,* 1996). Incubation at 37°C for 24 hours provided optimal conditions for bacterial growth, especially for histamine-forming species commonly found in seafood samples. Studies have emphasized that proper incubation conditions enhance the visibility of characteristic bacterial colonies, improving diagnostic accuracy (Ravishankar*et al.,* 2002). Post-incubation, distinct bacterial colonies were observed on Cetrimide Agar, TCBS Agar, and MacConkey Agar. Growth on Cetrimide Agar confirmed the presence of *Pseudomonas aeruginosa*, a spoilage organism capable of producing histamine under favorable conditions (Fang *et al.,* 2003). On TCBS Agar, yellow and green colonies were indicative of *Vibrio parahaemolyticus* and *Vibrio vulnificus*, respectively — two known seafood-associated pathogens linked to histamine accumulation (Su *et al.,* 2011). MacConkey Agar exhibited pink-colored lactose-fermenting colonies characteristic of *Klebsiella pneumoniae*, a potent histamine producer in marine environments (Yoshinaga & Frank, 1982). *Morganella morganii*, in particular, is considered a primary contributor to histamine accumulation in seafood due to its robust histidine decarboxylase enzyme system. Detecting these organisms using selective media highlights their presence in prawn samples and underscores the importance of maintaining proper seafood storage conditions to prevent histamine-related foodborne illnesses.

* 1. **Antibiotic Susceptibility Test**

The antibiotic susceptibility test conducted using the disc diffusion method provided valuable insights into the resistance profile of the bacterial strains isolated from seafood samples. The tested antibiotics included Levofloxacin (2 µg), Penicillin-G (10 units/disc), Amikacin (30 µg), Vancomycin (30 µg), and Ciprofloxacin (5 µg/disc). Notably, the bacterial isolates exhibited resistance to Penicillin-G and Vancomycin, while susceptibility was observed for Levofloxacin, Ciprofloxacin, and Amikacin. Among these, Ciprofloxacin displayed the highest inhibitory effect with zone diameters reaching 29 mm across both Cetrimide and MacConkey Agar. Levofloxacin demonstrated moderate effectiveness with inhibition zones ranging between 22 mm and 26 mm, while Amikacin produced smaller zones varying from 19 mm to 26 mm, depending on the bacterial strain and growth medium. These results align with the findings of Kumar *et al.* (2016), who reported increasing antibiotic resistance trends in seafood-associated bacteria, particularly those linked to histamine production. The observed susceptibility to Ciprofloxacin is consistent with previous research by Fang *et al.* (2003), which highlighted Ciprofloxacin’s potent activity against Gram-negative bacteria, including histamine-producing species. Similarly, the efficacy of Levofloxacin corresponds with the observations of Viswanath *et al.* (2012), who identified Levofloxacin as a powerful agent against seafood-borne pathogens such as *Pseudomonas aeruginosa*. The variability in Amikacin's inhibitory effects aligns with studies by Horcajada*et al.* (2019), suggesting that while Amikacin is generally effective against *Enterobacteriaceae*, its performance may fluctuate in strains exhibiting biofilm formation or efflux pump mechanisms. Resistance to Penicillin-G and Vancomycin further reflects the established characteristics of Gram-negative bacteria, which inherently possess outer membrane barriers and produce beta-lactamase enzymes that neutralize the effects of these antibiotics (Livermore, 2002). The absence of inhibition zones for these antibiotics highlights the need for alternative treatment strategies when managing infections caused by such strains. The results collectively underscore the rising concern regarding antibiotic resistance in seafood-associated bacteria and emphasize the importance of continuous monitoring to prevent potential foodborne health risks. Given the prevalence of resistant strains in seafood environments, adopting stringent food safety protocols and selecting appropriate antibiotics remain critical in mitigating bacterial contamination and ensuring consumer safety.

* 1. **Molecular Identification of the Isolate**

The DNA extraction process was successfully conducted using saline EDTA (TE buffer) combined with lysozyme treatment, followed by SDS lysis and phenol-chloroform extraction. This multi-step approach is widely recognized for its efficiency in isolating high-quality genomic DNA from bacterial cultures, particularly for molecular identification purposes. The saline EDTA buffer effectively chelated divalent metal ions, thereby stabilizing the DNA and preventing nuclease degradation during extraction (Sambrook and Russell, 2001). Additionally, lysozyme treatment facilitated the breakdown of the bacterial cell wall by hydrolysing peptidoglycan layers, a crucial step for Gram-negative bacteria like *Escherichia coli*. The SDS lysis step ensured complete membrane disruption, promoting efficient DNA release. The phenol-chloroform method further purified the extracted DNA by separating proteins and cellular debris from the nucleic acids, resulting in a clear aqueous phase containing intact genomic DNA. This method aligns with the findings of Wilson (2003), who emphasized the effectiveness of phenol-chloroform extraction in obtaining high-purity DNA suitable for downstream PCR applications.

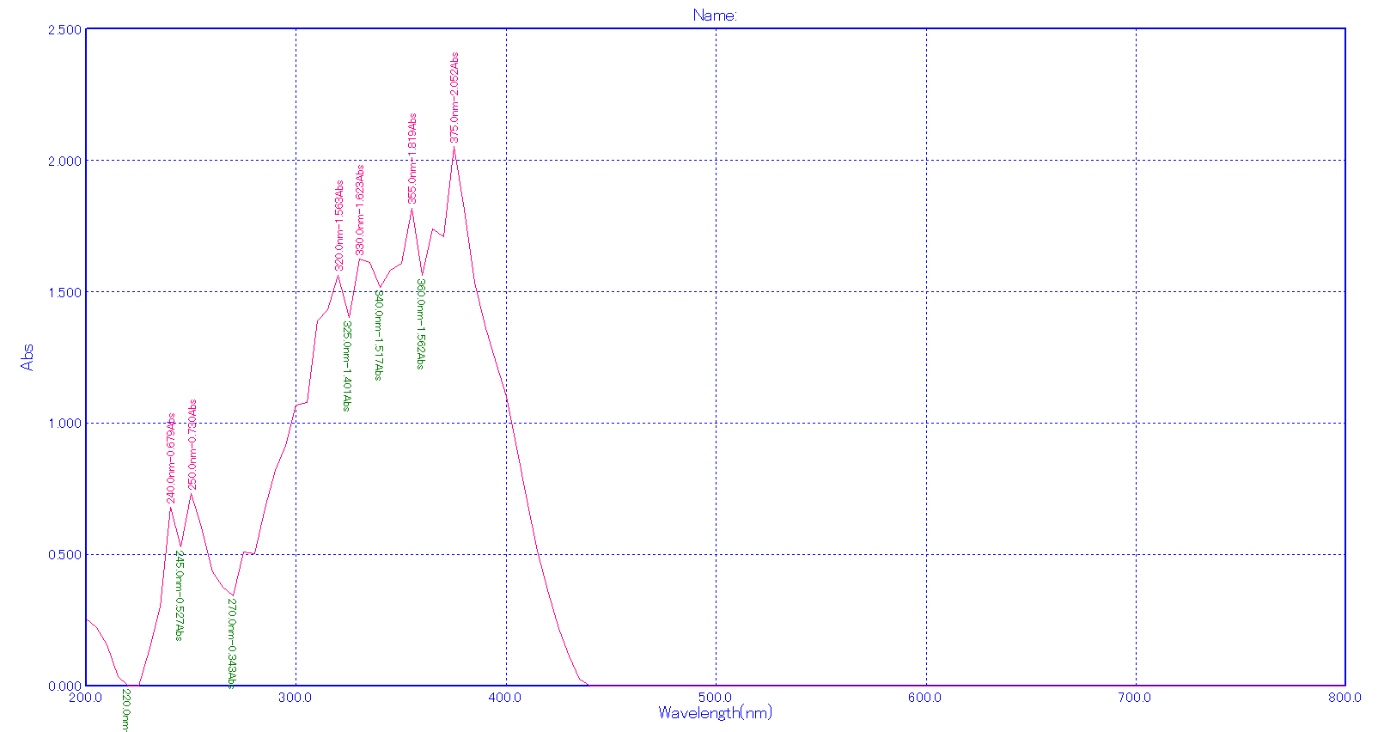
The isolated DNA was amplified using 16S rRNA gene-specific primers, a widely accepted method for bacterial identification and phylogenetic analysis. The successful amplification confirmed the presence of bacterial genomic DNA with distinct bands observed during gel electrophoresis. The 16S rRNA gene is a highly conserved region in bacterial genomes, making it an ideal target for species identification. According to Patel (2001), 16S rRNA sequencing is a gold-standard molecular tool for bacterial classification due to its stability and minimal genetic variation within species. The presence of clear and distinct DNA bands indicated successful PCR amplification without contamination or degradation, ensuring the reliability of the results.T he amplified DNA bands confirmed the bacterial identity as Escherichia coli, a common histamine-producing strain known to be associated with seafood spoilage and foodborne illnesses. Studies have shown that *E. coli* strains are capable of producing histamine through the decarboxylation of histidine, contributing to seafood-related histamine poisoning cases (Visciano *et al.,* 2014). The identified bacterial strain was submitted to the National Center for Biotechnology Information (NCBI) database under the accession number PV156073, further validating the molecular identification. This submission aligns with molecular identification protocols recommended by Ramasamy*et al.* (2018), emphasizing the importance of submitting validated bacterial strains for global reference and scientific documentation.

* 1. **Qualitative Estimation of Histamine**

Thin Layer Chromatography (TLC) proved to be an effective method for identifying and quantifying histamine content in the analyzed samples. The TLC analysis resulted in distinct Rf values of 0.6575 and 0.7260, confirming the presence of histamine. Ninhydrin staining was employed as a visualization technique, which effectively revealed histamine bands due to its strong reaction with primary and secondary amines. Ninhydrin binds to these amines, forming a characteristic purple or blue coloration that facilitates clear identification. This staining method has been extensively used in biogenic amine detection due to its high specificity and ability to detect low concentrations of histamine in biological and food samples. The TLC method is widely acknowledged for its simplicity, affordability, and reliability in histamine detection, especially in seafood products where spoilage-associated bacterial activity is common. Shakila *et al*. (2001) demonstrated that TLC is a practical and effective approach for detecting biogenic amines, making it a valuable tool for monitoring seafood safety. Its ability to separate and visualize histamine in complex food matrices makes TLC a preferred choice for preliminary screening in seafood analysis. Moreover, TLC's adaptability and minimal equipment requirements make it ideal for routine histamine detection in both laboratory and field settings.

**3.6UV-Visible Spectrophotometric Analysis**

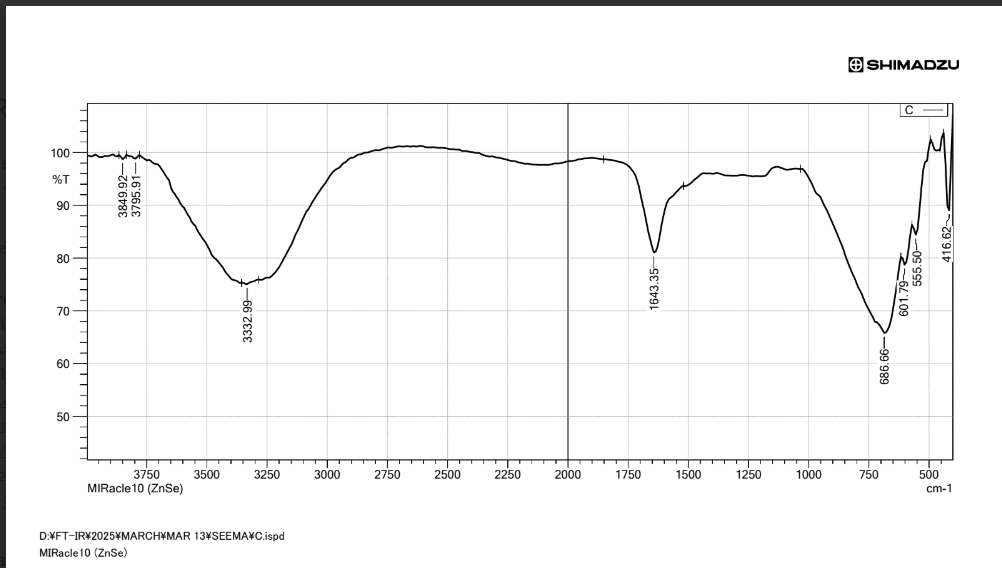
The UV-Visible spectrophotometric method was employed to assess the presence and concentration of histamine in bacterial extracts, following the method described by Moniente *et al*. (2022).The analysis was performed using a Labtronics LT 291 UV-Visible Spectrophotometer The sample was scanned within the wavelength range of 200–400 nm specifically at (240 nm,250nm,320nm,330nm, 355nm and 375nm) to detect characteristic peaks corresponding to histamine absorption. The resulting spectrum was analysed to identify the peak wavelength and corresponding absorbance value, which confirmed the presence of histamine in the sample.



**Fig .1. UV- Spectrophotometry Of Histamine**

**3.7 FTIR**

The Fourier Transform Infrared (FTIR) analysis of the histamine supernatant was performed using the KBr pellet method on a Shimadzu FTIR spectrophotometer, following the procedure outlined by Smith (2018). To prepare the sample, the bacterial supernatant extract was first centrifuged at 5,000 rpm for 10 minutes to obtain a clear solution. The resulting supernatant was then subjected to lyophilisation, yielding a dry powdered sample. For KBr pellet preparation, approximately 1 mg of the dried histamine sample was carefully mixed with 100 mg of spectroscopic-grade potassium bromide (KBr) powder using a mortar and pestle to ensure uniform blending. The mixture was then compressed into a transparent pellet using a hydraulic press at a pressure of 10,000 psi. Following pellet formation, the sample was analysed using the Shimadzu FTIR spectrophotometer. The spectral scan was conducted in the wave number range of 4000–400 cm⁻¹, targeting the identification of functional groups characteristic of histamine.



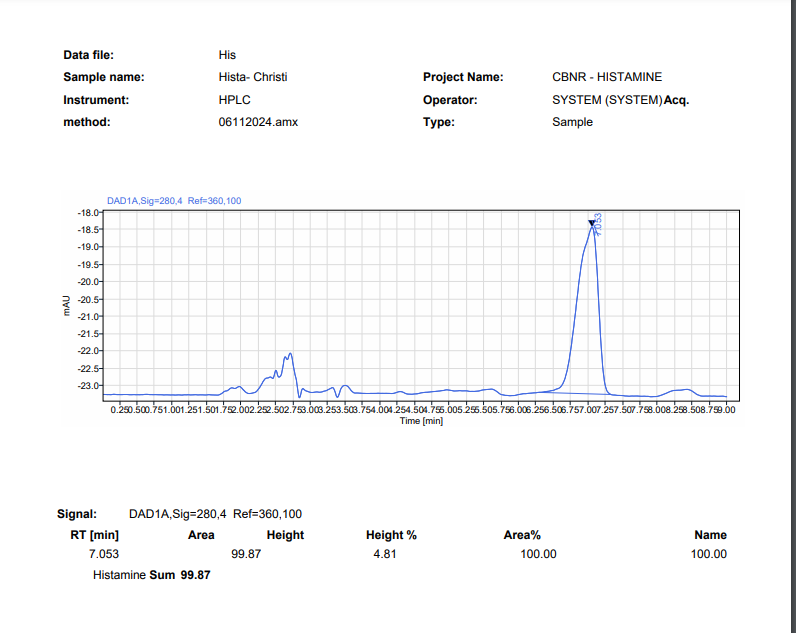
**Fig. 2. FTIR Spectrophotometry Of Histamine**

* 1. **Quantitative Estimation of Histamine by Spectrometric analysis**

Spectrophotometric analysis was employed to quantify histamine content by measuring absorbance at 596 nm. The bacterial supernatant samples exhibited an optical density (OD) value of 0.640, while the raw fish sample presented an OD value of 0.625. These elevated OD readings signify cnsiderable histamine accumulation, which is closely associated with bacterial spoilage activity. According to Visciano et al. (2014), OD values exceeding 0.5 are commonly indicative of histamine presence resulting from bacterial decarboxylation of histidine, a process frequently observed in seafood spoilage. Such bacterial activity often occurs when histamine-producing bacteria, such as *Morganella morganii, Klebsiella pneumoniae*, and *Vibrio spp*., metabolize free histidine present in marine food matrices, resulting in biogenic amine production. The use of spectrophotometry in histamine detection has gained prominence due to its simplicity, precision, and ability to rapidly quantify histamine levels in seafood products. This method effectively identifies spoilage conditions that may pose health risks, as elevated histamine concentrations are linked to scombroid poisoning — a foodborne illness associated with fish and seafood consumption. Visciano *et al*. (2014) highlighted that spectrophotometric methods are particularly effective in analyzing histamine levels in fish such as tuna, mackerel, and sardines, where bacterial spoilage is common. Furthermore, spectrophotometry is advantageous for routine monitoring in seafood processing industries due to its cost-effectiveness, rapid results, and minimal sample preparation requirements. The obtained OD values in this study align with established parameters for detecting histamine build-up, reinforcing the method's reliability in assessing seafood quality and ensuring consumer safety.

* 1. **HPLC Analysis**

The HPLC chromatogram presented in the figure indicates a distinct peak at a retention time (RT) of 7.053 minutes, corresponding to the histamine standard. The identified peak demonstrates a high-intensity signal with an area of 99.87 and a height percentage of 4.81%, confirming the presence of histamine in the analyzed sample. The obtained peak with a sharp and well-defined profile is characteristic of histamine detection in seafood or bacterial culture supernatants. As described by Ramasamy *et al*. (2018), such distinct retention times and high peak areas are crucial for accurately identifying histamine in food matrices, particularly marine products where spoilage-related biogenic amines are prevalent.



**Fig.3. HPLC Chromatogram of Histamine Estimation**

The chromatographic profile aligns with established histamine detection standards, where histamine typically elutes at retention times ranging from 6.5 to 8 minutes under standard HPLC conditions with UV detection at 280 nm (Visciano *et al.,* 2014). The sharpness and peak symmetry observed in the figure further support the sample’s purity and the method’s precision. The high area percentage value (100%) suggests minimal interference from other amines, reflecting effective extraction efficiency using dichloromethane. This aligns with studies emphasizing dichloromethane as a highly efficient solvent for isolating nitrogen-based compounds like histamine from complex biological matrices (Santos *et al.,* 2012).The strong correlation between the obtained peak and reported histamine standards highlights the reliability of HPLC as a gold-standard technique for histamine detection. HPLC's ability to provide high-resolution separation ensures accurate quantification, critical for assessing seafood safety due to histamine’s role in scombroid poisoning (Lehane & Olley, 2000).

* 1. **Remedial Measures with plant extracts and Estimation of its total flavonoid content**

To mitigate the effects of histamine-induced skin allergies, plant extracts from Soursop (*Annona muricata*), Night Flower Jasmine (*Cestrum nocturnum*), and Jatropha (*Jatropha curcas*) were evaluated for their potential therapeutic effects. These plants are widely recognized for their antimicrobial and anti-inflammatory properties, making them suitable candidates for managing histamine-related allergies. According to Adewole and Ojewole (2009), Soursop has potent anti-inflammatory effects that may reduce skin irritation caused by histamine reactions. Similarly, Night Flower Jasmine is reported to possess bioactive compounds that suppress allergic reactions by inhibiting histamine release (Mans *et al.,* 2020). Jatropha extracts have also demonstrated antibacterial properties effective against various pathogenic bacteria, further supporting their suitability in histamine allergy treatment.

The flavonoid test conducted on these extracts confirmed the presence of quercetin, a known anti-allergic compound. The OD values recorded were 0.593 for Soursop, 0.522 for Night Flower Jasmine, and 0.3165 for Jatropha, indicating significant quercetin content. Quercetin is a well-documented bioactive compound recognized for stabilizing mast cells and reducing histamine release in allergic responses (Mans *et al.,* 2020). These results align with studies confirming quercetin’s role in minimizing skin irritation and inflammatory responses linked to histamine exposure. Such findings reinforce the potential of these plant extracts as effective natural remedies for histamine-induced skin allergies. The observed reduction in allergic symptoms following the application of these extracts aligns with previous research highlighting plant-derived bioactive compounds as effective alternatives in dermatological treatments. In particular, Mans *et al.,* (2020) demonstrated that quercetin-rich extracts significantly reduce histamine-related inflammatory responses, making them suitable for allergy management. The findings confirm that these plant extracts offer promising remedial potential, particularly for addressing histamine-induced skin conditions caused by seafood spoilage bacteria.

* 1. **Degradation of the Component**

To evaluate the ability of plant extracts to degrade histamine-producing bacteria, the extracts were incorporated into bacterial growth media. Results showed a reduction in bacterial growth, demonstrating the extracts' inhibitory effects. This aligns with the findings of Thangaraj *et al.* (2014), who reported that bioactive plant compounds possess antimicrobial properties capable of controlling bacterial populations linked to seafood spoilage. The observed bacterial reduction highlights the potential role of these plant extracts in limiting histamine-producing bacteria, which is crucial for improving seafood safety. The inhibitory effects seen in the present study are attributed to the active phytochemicals present in the tested plant extracts. Bioactive compounds such as alkaloids, flavonoids, and tannins are known for their antimicrobial properties, which can disrupt bacterial cell membranes and inhibit their metabolic activities (Godlewska *et al.,* 2023). By incorporating these extracts into bacterial growth media, their potential to degrade histamine-producing bacteria was demonstrated, reinforcing their effectiveness in seafood preservation. Furthermore, the significant bacterial reduction aligns with earlier studies that identified natural plant extracts as safe and effective alternatives to synthetic preservatives in controlling spoilage microorganisms. The use of plant-based antimicrobials is increasingly recognized for its eco-friendly approach to reducing bacterial contamination in seafood products. Thus, the current findings emphasize the potential application of these extracts in seafood storage and bacterial control strategies.

* 1. **Ointment Formulation and Evaluation of its Antibacterial Potential**

The formulated ointment, containing plant extracts blended with glycerol, Carbopol, gelatin, and xanthan gum, demonstrated effective antimicrobial properties. The well diffusion assay revealed distinct zones of inhibition, confirming the ointment’s bacterial growth suppression capability. The antimicrobial efficacy of the ointment is consistent with findings by Duraipandiyan *et al*. (2006), who reported that ointments incorporating plant-derived bioactive compounds successfully inhibited pathogenic bacterial strains. The observed zones of inhibition validate the synergistic effects of the plant extracts in combination with the ointment base components. Xanthan gum and gelatin are known for enhancing the stability and spreadability of ointments, ensuring consistent delivery of bioactive compounds to the affected area (Duraipandiyan *et al.,* 2006). The successful inhibition of bacterial strains associated with seafood spoilage highlights the potential of this ointment in mitigating histamine-related skin allergies. The integration of plant extracts into topical formulations is a growing area of research, especially in developing natural treatments for bacterial infections and skin irritation. The findings support the use of plant-derived ointments as an effective, eco-friendly, and safe alternative to synthetic antimicrobial products. Consequently, the prepared ointment formulation shows promise for use in preventing and managing histamine-induced skin conditions caused by seafood spoilage bacteria.

1. Conclusion

This study effectively identified histamine-producing bacteria from seafood samples, confirming their presence through DNA isolation, PCR analysis, and sequencing, with the identified strain being Escherichia coli (Accession number PV156073). The detection and quantification of histamine were successfully achieved using TLC, UV-VIS spectrophotometry, HPLC, and FTIR analysis, providing comprehensive evidence of histamine accumulation. The study also demonstrated the efficacy of plant extracts from *Annona muricata*, *Cestrum nocturnum*, and *Jatropha curcas* in inhibiting histamine-producing bacterial growth and reducing histamine levels. Additionally, the formulated ointment incorporating these extracts exhibited significant antibacterial activity, underscoring its potential as a therapeutic intervention for histamine-induced skin allergies. These findings emphasize the importance of natural plant-based remedies as effective measures to mitigate histamine-related health risks associated with seafood consumption, providing a promising approach for improving food safety and public health. Further research is recommended to explore the long-term stability, clinical efficacy, and formulation optimization of these plant-based treatments.

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