Inhibition of *Acinetobacter baumannii* Biofilm Formation by Black Pepper Extract

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Declaration

It is hereby declared that

1. The thesis submitted titled **"Inhibition of *Acinetobacter baumannii* Biofilm Formation by Black Pepper Extract"** is our original work while completing our degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material that has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. We have acknowledged all main sources of help.

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Dedicated To

This research is entirely dedicated to our parents for providing us with spiritual, mental, and financial support throughout our lives.

Finally, we thank the Almighty for giving us strength, mental power, and a healthy life.

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Abstract

Acinetobacter baumannii, a Gram-negative opportunistic pathogen, is considered a significant concern in clinical aspects due to its ability to form biofilms and its resistance to multiple antibiotics. This study aims to evaluate the inhibitory effect of Black Pepper Extract on *Acinetobacter baumannii* biofilm formation. The clinical isolates of *A. baumannii* were collected from patients at the National Institute of Traumatology and Orthopedic Rehabilitation (NITOR) and identified using physical and molecular methods such as Colony morphology pattern, Gram staining technique, and PCR confirmation. An antibiotic susceptibility test (AST) was also conducted which showed that the strain was resistant to multiple antibiotics including ampicillin-sulbactam, amoxicillin, and ceftazidime. In this study, various biofilm formation tests such as: Congo Red Agar (CRA) method, tube method, and Tissue Culture Plate (TCP) method were employed to find the potential of black pepper extract in the inhibition biofilm formation. The findings indicate that black pepper extract exhibits significant anti-biofilm activity, which can be used as a non-antibiotic strategy to combat biofilm-related infections caused by *Acinetobacter baumannii*.

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List of Acronyms

A. baumannii - *Acinetobacter baumannii*

AMP - Ampicillin-Sulbactam

AST - Antibiotic Susceptibility Test

bp - Base pair

BP - Black Pepper

CAZ - Ceftazidime

CIP - Ciprofloxacin

CLSI - Clinical and Laboratory Standards Institute

CRA – Congo Red Agar

LAB - Leeds Acinetobacter Agar Base

DNA - Deoxyribonucleic Acid

EDTA - Ethylenediamine Tetra acetic Acid

EPS - Extracellular Polymeric Substances

ICU - Intensive Care Unit

MCT - Micro Centrifuge Tube

MDR - Multi Drug Resistance

MEM - Meropenem

MHA - Muller Hilton Agar

NA - Nutrient Agar

NITOR - National Institute of Traumatology and Orthopedic Rehabilitation

PBS - Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

PIT - Piperacillin-Tazobactam

QS - Quorum Sensing

TCP – Tissue Culture Plate

TE - Tris-EDTA

UV - Ultra Violet

Chapter 1: Introduction

Background:

In recent decades, *Acinetobacter baumannii* has become a major opportunistic pathogen in hospital environments. This Gram-negative, non-fermentative bacterium is known for causing severe nosocomial infections, especially in critically hospitalized patients. It is commonly associated with biofilm-related infections such as ventilator-associated pneumonia, bloodstream infections, urinary tract infections, and wound infections which is often linked to medical devices like catheters, cerebrospinal fluid shunts, and ventilators (Gedefie et al., 2020; Babapour et al., 2016). The rapid spread of *A. baumannii* biofilms on medical devices, combined with their antibiotic resistance has emerged as a major global health issue. These biofilms are often enveloped in a thick polysaccharide layer which enhances their resistance to antibiotics and makes them very difficult to eliminate (Magiorakos et al., 2012; Babapour et al.,2016).

Biofilms are complex communities of microorganisms that attach to surfaces (both biotic and abiotic) and enclose them within a self-produced extracellular polymeric substance (EPS) matrix. This EPS matrix is primarily composed of proteins (<2%), DNA (<1%), polysaccharides (1–2%), and RNA (<1%), water up to (97%) providing structural stability and protection to the microbial community within the biofilm (Guan et al., 2023). Several steps are involved in the formation of biofilm which are as follows:

Initial Attachment: Microorganisms initially adhere to the surface through weak, reversible interactions such as van der Waals forces and hydrophobic interactions. (Zhang et al., 2022).

Irreversible Attachment: In this stage, the attachment becomes stronger and irreversible. The cells begin to produce EPS, which anchors them firmly to the surface. Adhesion proteins and pili play an important role in this process. (Guan et al., 2023).

Maturation I (Microcolony Formation): Cells start to proliferate and form microcolonies. During this stage, the cells communicate through quorum sensing, a process where bacteria

communicate by releasing and detecting signaling molecules called autoinducers such as N-acylated homoserine lactone (AHL) (Rather et al., 2021)

Maturation II (Biofilm Maturation): The biofilm reaches its maximum thickness and complexity. Channels are developed within the biofilm which allows for nutrient and waste exchange. At this stage, cells exhibit increased resistance to antibiotics and the host immune response due to the protective EPS matrix (Rather et al., 2021)

Dispersion: In the final stage, cells at the edges of the biofilm become motile and disperse to colonize new surfaces. This stage is essential for the spread of biofilm-related infections into the environment. (Zhang et al., 2022).

According to the phytochemical analysis, black pepper contains various compounds such as piperine, phenols, flavonoids, alkaloids, amides, steroids, lignans, neolignans, terpenes, chalcones etc. From them, piperine is a major bioactive compound that has been shown to inhibit biofilm formation in various microbial species (Shityakov et al., 2019).

Literature Review:

Acinetobacter Baumannii and Biofilm Formation:

Acinetobacter baumannii is a Gram-negative, opportunistic pathogen that has become a significant concern in healthcare settings due to its ability to form biofilms and its resistance to multiple antibiotics (Antunes et al., 2014). Biofilm formation enhances its survival and persistence in hospital environments, making infections difficult to treat and eradicate (Harding et al., 2018). According to a study, out of all hospital-linked infections caused by Gram-negative bacteria, *A. baumannii* is responsible for 2–10% of nosocomial infections (Gedefie et al., 2020). Another study found that biofilm formation found to be the major cause of many chronic infections such as diabetic foot ulcers, cellulitis, and necrotizing fasciitis which led to the re-emergence of multidrug-resistant strains thereby resulting in treatment failure Biofilms greatly affect healthcare environments and are associated with 65% of nosocomial infections (Harika et al., 2020). In another study, it was found that the ability of *A. baumannii* to form biofilms on medical devices further complicates treatment and increases healthcare costs (Lee et al., 2008).

Antibiofilm Activity of Black Pepper Extract:

Piperine is the primary bioactive compound found in black pepper. It is an alkaloid responsible for the spice's pungency. It has shown promising antibiofilm properties, making it a subject of interest in combating biofilm-related infections.

Inhibition of Bacterial Adhesion: Initial adhesion of bacterial cells to surfaces is a significant step in biofilm formation. Piperine hinders the adhesion process by altering the expression of adhesion-related genes and proteins, thereby preventing the establishment of biofilms (Gupta et al., 2015).

Interference with Quorum Sensing: Piperine can inhibit quorum sensing by disrupting the signaling pathways mediated by Acylated Homoserine Lactone (AHL). By interfering with AHL synthesis or signal reception, piperine prevents the bacteria from communicating with each other which is crucial for biofilm formation and maintenance. Some studies have shown that piperine and other phytochemicals can degrade AHLs or block their binding to receptor proteins, thereby inhibiting QS-regulated gene expression (Khan et al., 2009; Gopu et al., 2015).

Interference with EPS: The EPS matrix is an important component of biofilms which provide structural integrity as well as protection to the bacterial community. Piperine reduces the synthesis of EPS thereby weakening the biofilm structure. (Rudrappa & Bais, 2008).

Disruption of Biofilm Architecture: Piperine has the potential to penetrate biofilms and disrupt their architecture by breaking down the EPS matrix and detaching bacterial cells from the biofilm. This disruption accelerates the efficacy of antibiotics against biofilm-associated bacteria (Chatterjee et al., 2016).

Despite numerous studies on *A. baumannii's* resistance mechanisms and biofilm formation, there remains a significant gap in exploring alternative non-antibiotic strategies to combat these infections. Specifically, the potential use of plant extracts, such as black pepper extract in inhibiting biofilm formation and enhancing antibiotic efficacy has not been extensively studied. This research aims to fill this gap by investigating the efficacy of black pepper extract in inhibiting biofilm formation by *Acinetobacter baumannii* collected from patients at the National Institute of Traumatology and Orthopedic Rehabilitation (NITOR).

This study utilized both physical and molecular methods for identifying *Acinetobacter baumannii* such as colony morphology, Gram staining, and PCR confirmation in accordance with established literature (Babapour et al., 2016). The antibiotic susceptibility of the isolates was assessed using various antibiotics and the potential of black pepper extract as an alternative strategy to inhibit biofilm formation was explored.

Chapter 2: Materials and Methods

Study Design:

- ► Sample collection, processing, and confirmation
- ► Preparation of Black pepper extract
- ► Biofilm formation screening
- ► Effect of Black pepper extract on *A. baumannii* Biofilm

Sample collection

In this study, the tested clinical isolates of *A. baumannii* were collected from patients admitted to the National Institute of Traumatology & Orthopedic Rehabilitation (NITOR), Sher-E-Bangla Nagar, Dhaka, Bangladesh.

Confirmation of the sample

Various physical, and molecular identification techniques and antimicrobial susceptibility tests were performed to identify *Acinetobacter baumannii*.

Media for plate culture method

- ► Nutrient agar
- ► Leeds Acinetobacter Agar Base

In this method, bacteria were first sub-cultured on a Nutrient Agar (NA) medium. Following this, the white or beige colonies from the NA plate were streaked onto Leeds Acinetobacter Agar Base, which serves as the standard selective medium for isolating *Acinetobacter baumannii*.

Preparation of Nutrient agar:

- ► To prepare NA, the standard formula of 28.0g per 1000 ml of distilled water was used to make the required amount.
- ► The mixture was then boiled until fully dissolved.
- ► For sterilization, the media was autoclaved at 121°C for 15 minutes.
- ► The sterilized media was then poured into sterile Petri dishes under aseptic conditions and allowed to solidify before use.

Preparation of Leeds Acinetobacter Agar Base:

- ► To prepare it, the standard formula of 53.42g per 1000 ml of distilled water was used to make the required amount.
- ► The mixture was then boiled until fully dissolved.
- ► The media was cooled to 45-50°C
- ► The FD271 MDR selective supplement was rehydrated with the addition of 5ml distilled water per vial.
- ► The supplement solution was mixed gently to dissolve the contents completely, aseptically added to the Leeds Acinetobacter Agar Base, and mixed well.
- ► The media was then poured into sterile Petri dishes under aseptic conditions and allowed to solidify before use.

Physical characterization:

a) **Colony morphology:** This method was used to examine the characteristics of bacterial colonies as they developed on agar in Petri dishes. Different bacterial colonies exhibit diverse shapes, sizes, colors, and textures on agar media. Under sterile conditions within a laminar flow cabinet, the bacterial strain was streaked onto both Nutrient Agar and Leeds Acinetobacter Agar Base plates for evaluation. The plates were then incubated at 37°C for 24 hours. After the incubation period, the cultural properties of the colonies were observed and carefully recorded.

b) **Gram Staining:** A small amount of the bacterial isolate was smeared onto clean slides with a drop of distilled water, air-dried, and heat-fixed by passing the slides through a flame two or three times. Afterward, crystal violet was applied to the smear for one minute and then rinsed off with distilled water. Gram's iodine was added to the smear for one minute before rinsing with distilled water. The slides were decolorized with 95% ethyl alcohol for 15 seconds. The slides were then counterstained with safranin for about 30 seconds and rinsed with distilled water. After air drying, the slides were examined under a microscope. Under the microscope, cells were distinguished by color: purple for gram-positive bacteria and pink or red for gram-negative bacteria.

Molecular identification by using PCR:

a) **DNA extraction:** In this method, the genomic DNA was extracted by boiling method (Babapour et al., 2016). To extract the DNA, 3 to 4 single colonies were selected from pure culture and transferred to a microcentrifuge tube containing 1x TE buffer (Tris-EDTA). After vertexing, the microcentrifuge tube (MCT) was placed in a heat block at 95°C for 20 minutes. The MCT was then centrifuged at 4°C at 10,000 rpm for 10 minutes. The supernatant containing the DNA was carefully collected without disturbing the pellet, transferred to a new MCT, and stored at -20°C.

b) **Primer used in PCR:** A single pair of bla-OXA-51 primers was used for the molecular detection of *Acinetobacter baumannii***.** Two separate microcentrifuge tubes (MCTs) were utilized to prepare 10 µM concentrated solutions of both the forward and reverse primers from a stock solution containing 100 µM concentrated primers. To achieve a total volume of 20 µL of 10 µM working solution, 2 µL each of the forward and reverse primers from the stock solution were combined with 18 µL of nuclease-free water in their respective MCTs.

Table 1: bla-OXA-51 Primer used in PCR to detect *Acinetobacter baumannii*

Primer name	Primer sequence	Target organism	Amplicon size	Reference
bla-OXA-51	F:5'-TAATGCTTTGATCGGCCTTG-3' R:5'-TGGATTGCACTTCATCTTGG-3	*Acinetobacter baumannii*	353bp	(Falah et al., 2019)

c) **Reaction mixture and Condition of PCR:** The PCR reaction mixture was prepared in autoclaved PCR tubes, consisting of 12.5 µL of 2X Takara Bio emerald PCR master mix, 1 µL of each primer (forward and reverse) at 10 µM concentration, 5.5 µL of nuclease-free water, and 5 µL of DNA template. Each step involved gentle re-pipetting for better mixing. To minimize bubbles, all PCR tubes were placed in a rotating machine. The PCR was conducted using the Bio-Rad T100 Thermal Cycler under the following conditions: initial denaturation at 94°C for 5 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, primer annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes (Falah et al., 2019).

d) **Gel Electrophoresis:** After PCR, 6 µL of PCR product from each reaction sample was loaded onto a 1.5% agarose gel prepared with TBE buffer containing ethidium bromide dye. The gel electrophoresis was run at 110 volts for 40 minutes. A 100 bp DNA ladder was used for assessing the size of the PCR products. Afterward, the electrophoresis gel was visualized using a UV transilluminator thereby images were captured and appropriately labeled for the analysis of PCR results.

Antibiotic Susceptibility Test:

The bacterial isolates confirmed by PCR were subjected to antibiotic susceptibility testing (AST) using the Kirby-Bauer method. After subculturing on Nutrient Agar plates and overnight incubation at 37°C, pure cultures of the isolates were prepared by suspending them in 0.9% saline to achieve a turbidity equivalent to the McFarland 0.5 standard. Using a sterilized cotton swab, the bacterial suspension was evenly spread (lawned) onto Mueller-Hinton Agar (MHA) plates. Antibiotic discs were then carefully placed on the agar surface using sterilized forceps to facilitate diffusion. Following an 18–24 hour incubation at 37°C, the plates were removed, and the zones of inhibition around each disc were observed, measured in millimeters (mm), and interpreted according to CLSI guidelines.

Table 2: Antibiotics list used in AST and their zone interpreting criteria according to CLSI

Antibiotic name	Antibiotic Class	Zone interpretation criteria
Imipenem (IMP)	Carbapenem	S>=22, I=19-21, R<=18
Meropenem (MRP)		S>=18, I=15-17, R<=14
Ampicillin-sulbactam (AMP)	β lactam combination agents	S>15, I=12-14, R<=11
Amoxicillin-clavulanate (AML)		S>=18, I=14-17, R<=13
Ciprofloxacin (CIP)	Fluoroquinolones	S>=21, I=16-20, R<=15
Levofloxacin (LE)		S>=17, I=14-16, R<=13
Ceftazidime (CAZ)	Cephalosporins	S>=18, I=15-17, R<=14
Cefepime (CPM)		S>=18, I=15-17, R<=14
Tetracycline (TE)	Tetracyclines	S>=15, I=12-14, R<=11
Doxycycline (DO)		S>=13, I=10-12, R<=9
Piperacillin-Tazobactam (TZP)	β lactam combination	S>=21, I=18-20, R<=17

Preparation of Black Pepper Extract:

a) Collection of Black Pepper:

- ► Fresh black pepper was collected
- ► Dried thoroughly at room temperature for 7 days.
- ► Then the dried black pepper was ground into coarse powder using a grinding machine and stored in an air-tight jar.

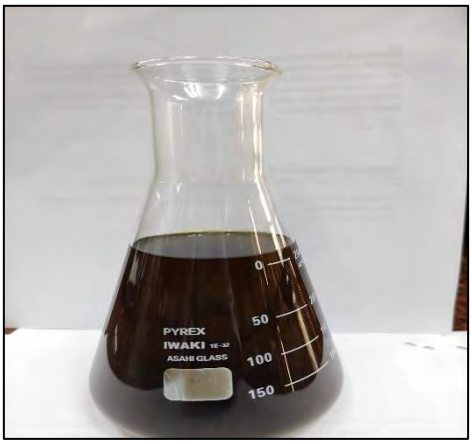
b) Extraction and Filtration Process:

- ► 670 grams of the prepared black pepper powder were weighted by using a digital balance.
- ► The powder was soaked in a 1:1 ratio mixture of dichloromethane and methanol in a flask.
- ► The flask and its contents were kept in an amber jar to protect them from light.
- ► The flask was kept at room temperature for 14 days. During this soaking period, the flask was shaken and stirred to ensure thorough mixing and to ensure the sample's compounds were dissolved in the solvent.
- ► The solution was first filtered through a piece of clean, white, 100% cotton fabric.
- ► The filtrate was collected, and the residual extract was squeezed from the residue on the cloth.
- ► The collected filtrate was then filtered using absorbent cotton.
- ► The previous step was repeated with fresh absorbent cotton.
- ► Finally, the filtrate was filtered using Whatman filter paper.
- ► The final filtrate was concentrated using a rotary evaporator set at a temperature of 40-55º C and a rotation speed of 70-80 rpm.

c) Solvent-solvent partitioning:

- ► All beakers, flasks, and vials were weighed before use.
- ► According to the Kupchan scheme method, in a measuring cylinder, 45 ml MeOH and 5 ml distilled water were taken. (Total 50 ml 90:10) (Hettiarachchi, 2022).
- ► The crude extract was transferred into a separatory funnel using a small amount of the MeOH/H_2O mixture.
- ► The rest of the prepared MeOH/ H_2O mixture was added to the funnel and mixed well.
- ► Next, 50 mL of hexane was added to the funnel, mixed well, and set aside for layer separation.
- ► The hexane layer was collected for dryness.
- ► Following this, 50 mL of chloroform was added to the funnel, mixed well, and set aside for layer separation. The chloroform layer was collected into another flask and left it to dry.
- ► The remaining MeOH/ H_2O mixture was poured into a new flask, and the MeOH was evaporated.
- ► Finally, 50 mL of ethyl acetate was added to the water layer, mixed well in the separatory funnel, and left aside for layer separation.
- ► The ethyl acetate layer was then collected into a flask and left to dry.
- ► In the process, all layers were collected and evaporated to dryness separately (Hettiarachchi, 2022).

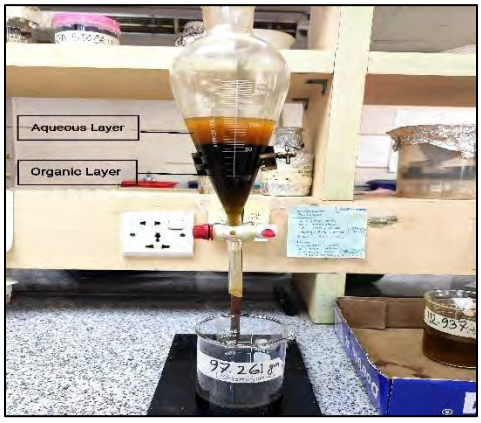
Filtration



Collection of extract

Evaporation at 42ºC

Drying the collected extract



Partitioning of black pepper crude

Partitioning layers

Figure 1: Preparation of Black pepper extract

Biofilm Formation Screening

Qualitative techniques

a) **Congo red agar (CRA) method:** Congo Red Agar (CRA) is considered a simple qualitative assay to detect biofilm production in bacterial isolates. The medium was made up of 37 g/L brain heart infusion (BHI) agar, 50 g/L sucrose, and 0.8 g/L Congo Red (Freeman et al., 1989). The solution was heated until all components were completely dissolved. The Congo Red stain was prepared as a concentrated aqueous solution and autoclaved separately at 121°C for 15 minutes. After autoclaving, the agar mixture was allowed to cool at approximately 55°C. Once cooled, the sterilized Congo Red solution was added to the agar mixture and mixed thoroughly. The media was then poured into sterile Petri dishes under aseptic conditions and allowed to solidify before use. Using a sterile inoculating loop, a single colony of *Acinetobacter baumannii* was picked and streaked onto the prepared Congo Red Agar plate. The plates were then incubated at 37°C for 24 hours. After incubation, a positive result was indicated by the presence of black colonies with a dry crystalline consistency, signifying biofilm production. In contrast, non-biofilm-forming strains were characterized by red or pink colonies with a smooth appearance This method was performed in triplicates (Freeman et al., 1989).

b) **Tube Method:** This qualitative method was used by observing biofilm that had adhered to the vial's walls and bottom. Both tubes and vials were used to perform this process.

- ► Using a sterile loop, one or two colonies of *Acinetobacter baumannii* were taken from the NA subculture plate and inoculated in 9 ml of fresh sterile LB or Luria-Bertani broth with 1% glucose in each tube.
- ► Then the tubes were placed in the rotary shaker for 3.5 hours.
- ► After shaking, 500 µL of the bacterial suspension was transferred to each vial and incubated at 37°C for 72 hours.

- After incubation, the bacterial suspensions were removed and each vial was washed with 0.9% sterile saline solution and dried.
- The vials were stained with 0.1% crystal violet for 10 minutes.
- Then, the vials were washed with distilled water to remove the crystal violet that did not specifically stain the adherent bacteria.
- After that, the vials were dried in an inverted position at room temperature.
- The results were observed, and the formation of a visible ring at the wall of the bottom of the vial was considered positive for biofilm formation (Harika et al., 2020).

Quantitative technique

Tissue Culture Plate (TCP) Method:

- NA subculture was prepared.
- Bacteria from a single colony were inoculated into 10 ml of Luria-Bertani (LB) broth and incubated at 37°C for 24 hours.
- The cultures were diluted 1:100 with fresh medium.
- Clean and dry 96-well TCP plates were prepared while drying was done in a 44°C incubator.
- The wells were filled with 0.2 ml aliquots of the diluted culture.
- Uninoculated broth was added as the negative control.
- The plates were incubated at 37°C for 48 hours.
- The contents were removed from the plates by gently tapping.
- The plates were washed twice with 0.2 ml of phosphate-buffered saline (pH 7.2).
- The plates were incubated at 37°C for an hour.
- The wells were stained with 0.2 ml of 0.1% crystal violet for 10 minutes.
- Excess stain was removed by washing twice with distilled water, and the plates were left to dry.
- 200 µl of 33% glacial acetic acid was added to the wells.
- The optical density (OD) of the isolates was determined using an ELISA machine at a wavelength of 630 nm (OD 630 nm).
- The results were recorded in triplicate.

Effect of Black pepper extract on Biofilm

The effect of black pepper extract on the biofilm-producing isolates of *Acinetobacter baumannii* was measured by the tube method as well as the tissue culture plate (TCP) method which was previously mentioned. Different concentrations of black pepper extract were used in this test. The concentrations of black pepper extract are given below:

- 5 mg/mL
- 10 mg/mL
- 20 mg/mL

Tube Method: Using a sterile loop, one or two colonies of *Acinetobacter baumannii* were taken from the NA subculture plate and inoculated into 9 ml of fresh sterile LB broth with 1% glucose. The tubes were then placed in a rotary shaker for 3.5 hours. After shaking, 500 µL of the bacterial suspension was transferred to each vial and incubated at 37°C for 72 hours. Following incubation, the required amount of black pepper extract was added to the vial and incubated at 37°C for 24-48 hours. After incubation, the solutions were removed, and each vial was washed with 0.9% sterile saline solution and dried. The vials were then stained with 0.1% crystal violet for 10 minutes, washed with distilled water to remove any non-adherent stain, and dried in an inverted position at room temperature. Afterwards, the results were observed to see the presence/absence of ring formation in the vial. By adding different concentrations of black pepper extract, it was possible to determine the most effective concentration that could reduce or prevent biofilm formation.

Tissue Culture Plate (TCP) Method:

- NA subculture was prepared.
- Bacteria from a single colony were inoculated into 10 ml of Luria-Bertani (LB) broth and incubated at 37°C for 24 hours.
- The cultures were diluted 1:100 with fresh medium.
- Clean and dry 96-well TCP plates were prepared while drying was done in a 44°C incubator.

- The wells of the first 5 rows (A, B, C, D, E) were filled with 0.2 ml aliquots of the diluted culture.
- The positive control was added in the 6^{th} row (F) and the uninoculated broth was added as the negative control in the 7^{th} row (H).
- The plates were incubated at 37°C for 48 hours.
- 10-50 µl of black pepper extract was added in first 6 row (A, B, C, D, E, F) and incubated at 37°C for 24 hours.
- The contents were then removed from the plates by gently tapping.
- The plates were washed twice with 0.2 ml of phosphate-buffered saline (pH 7.2).
- The plates were incubated at 37°C for an hour.
- The wells were stained with 0.2 ml of 0.1% crystal violet for 10 minutes.
- Excess stain was removed by washing twice with distilled water, and the plates were left to dry.
- 200 µl of 33% glacial acetic acid was added to the wells.
- The optical density (OD) of the isolates was determined using an ELISA machine at a wavelength of 630 nm (OD 630 nm) and the effectiveness of black pepper extract.
- The results were recorded in triplicate.

Chapter 3: Observation and Result

Confirmation of *Acinetobacter baumannii* by physical characterization:

The clinical isolate of *Acinetobacter baumannii* was preliminarily identified by their colony morphologies upon streaking on nutrient agar media as well as selective media. The bacterial isolate suspected to be *Acinetobacter baumannii* was cultured on nutrient agar and incubated for 24 hours at 37°C. The resulting colonies were appeared smooth, opaque, and shiny, with a white

Figure 2: Growth of *Acinetobacter baumannii* in both Nutrient Agar and Leeds Acinetobacter Agar Base

to beige coloration. Following this, the colonies from the NA plate were streaked onto Leeds Acinetobacter Agar Base which served as the standard selective medium for isolating *Acinetobacter baumannii*. After incubation, pink mucoid colonies were observed, with pink color diffused into the surrounding medium thereby indicating the utilization of nitrogenous material and production of ammonia ions.

Gram Staining: *Acinetobacter baumannii* was examined under a light compound microscope using oil immersion at 100X magnification. The bacterial cells were stained pink, almost round and rod-shaped. Therefore, it appeared as Gram-negative.

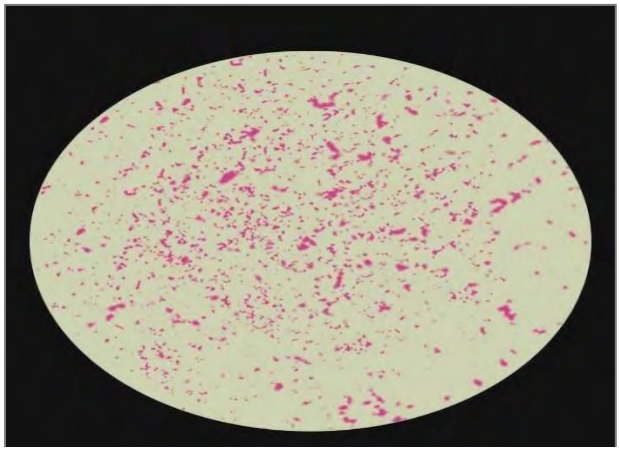


Figure 3: Microscopic Examination of *Acinetobacter baumannii*. The pink color indicated a gram-negative result.

Confirmation of A. baumannii using PCR and gel electrophoresis: To identify *A. baumannii*, PCR and agarose gel electrophoresis were used. Following agarose gel electrophoresis and visualization under a UV transilluminator, specific bands were identified at the 353 bp region (using a 100 bp DNA ladder) which indicated a positive result for *A. baumannii*. The presence of this band at the 353 bp region confirms that DNA fragments specific to Acinetobacter baumannii were amplified, thus validating the species identification. The picture of the positive PCR result is given below:

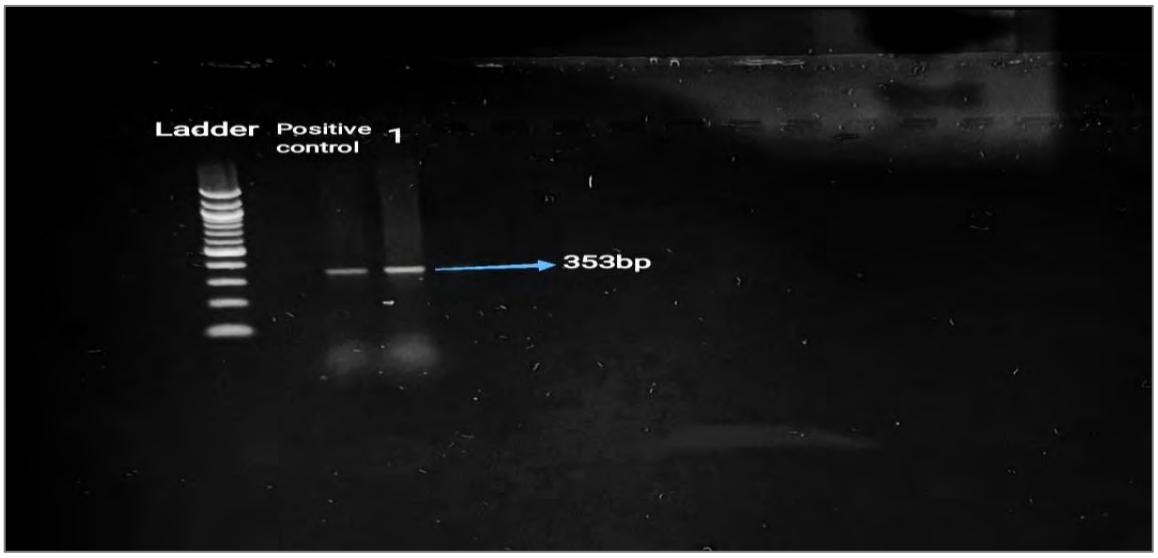


Figure 4: PCR Confirmation of *Acinetobacter baumannii*

Antibiotic Susceptibility Test Analysis:

In this study, a total of 8 antibiotics were employed to assess their effectiveness against *Acinetobacter baumannii* by measuring the zone of inhibition. The interpretation of these measurements was done as per CLSI guidelines using the ranges specified in Table 2 to categorize the antibiotics as "sensitive," "intermediate," or "resistant". By measuring the zone of inhibition, it was found that *A. baumannii* showed resistance to multiple antibiotics including ampicillin-sulbactam, amoxicillin, ceftazidime and ciprofloxacin while showed susceptible to meropenem, piperacillin, cefepime and doxycycline. During the analysis, satellite colonies were observed within several inhibition zones. These satellite colonies were carefully identified and documented, as their presence could indicate variations in antibiotic susceptibility.

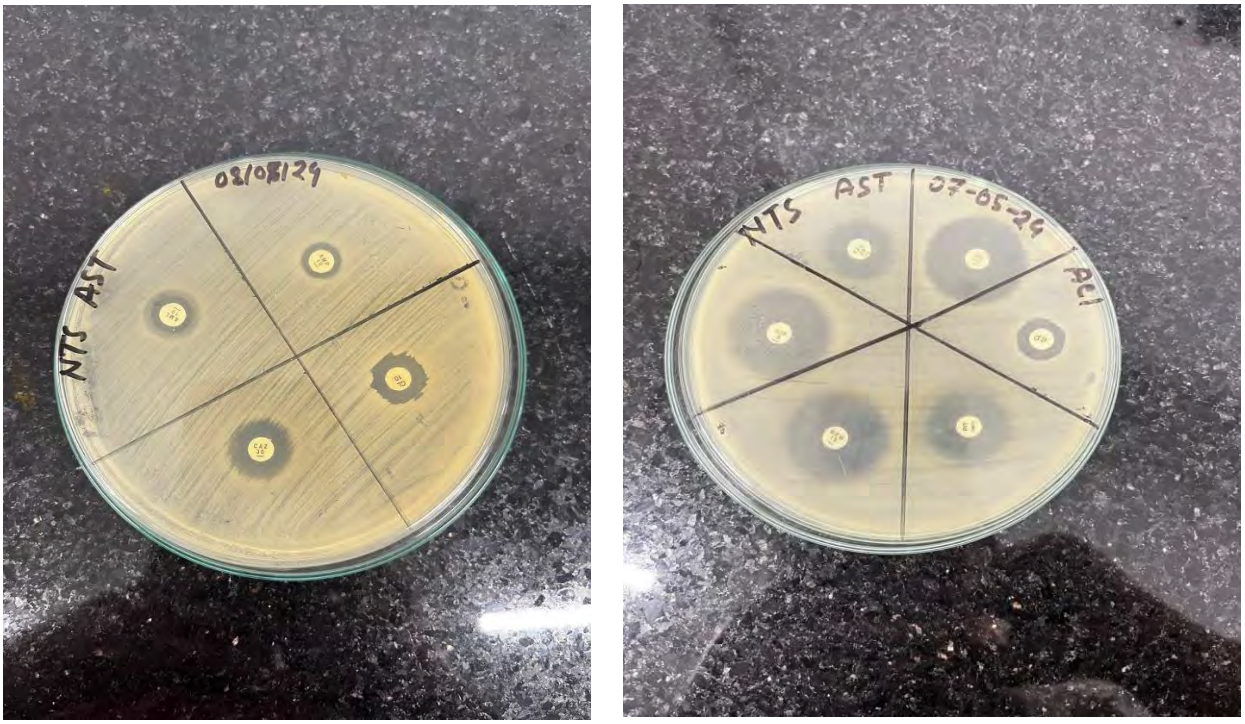


Figure 5: Antibiotic susceptibility testing of *Acinetobacter baumannii* against a list of antibiotics

Biofilm formation Result

Qualitative technique

a) **Using Congo Red Agar:** The Congo Red Agar was employed to detect biofilm production by *Acinetobacter baumannii*. After incubation, the presence of black colonies with a dry crystalline consistency, signifies biofilm production. Therefore, it indicates a positive biofilm formation result. This observation confirmed the presence of biofilm production by *Acinetobacter baumannii.*

Figure 6: Biofilm formation test using Congo Red Agar

b) Using Tube: As a qualitative assessment, the tube method was employed to observe biofilm formation adhering to the walls of tubes and vials. Ring formations were observed on the walls of the tubes and vials used for staining. These rings indicated the presence of biofilm adhering to the surfaces, confirming a positive result for biofilm formation.

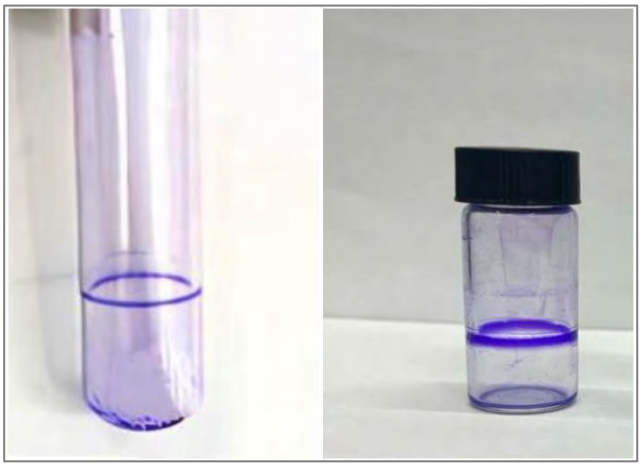


Figure 7: Ring formation in both tube and vial indicating biofilm formation

Quantitative technique

Using microtiter plate: Biofilm formation in the microtiter plate wells was evaluated using the tissue culture plate technique. The presence of stained biofilms confirmed the effective adherence and growth of microorganisms. After 48 hours of incubation, the optical density at 630 nm was measured using an ELISA reader to quantify biofilm formation.

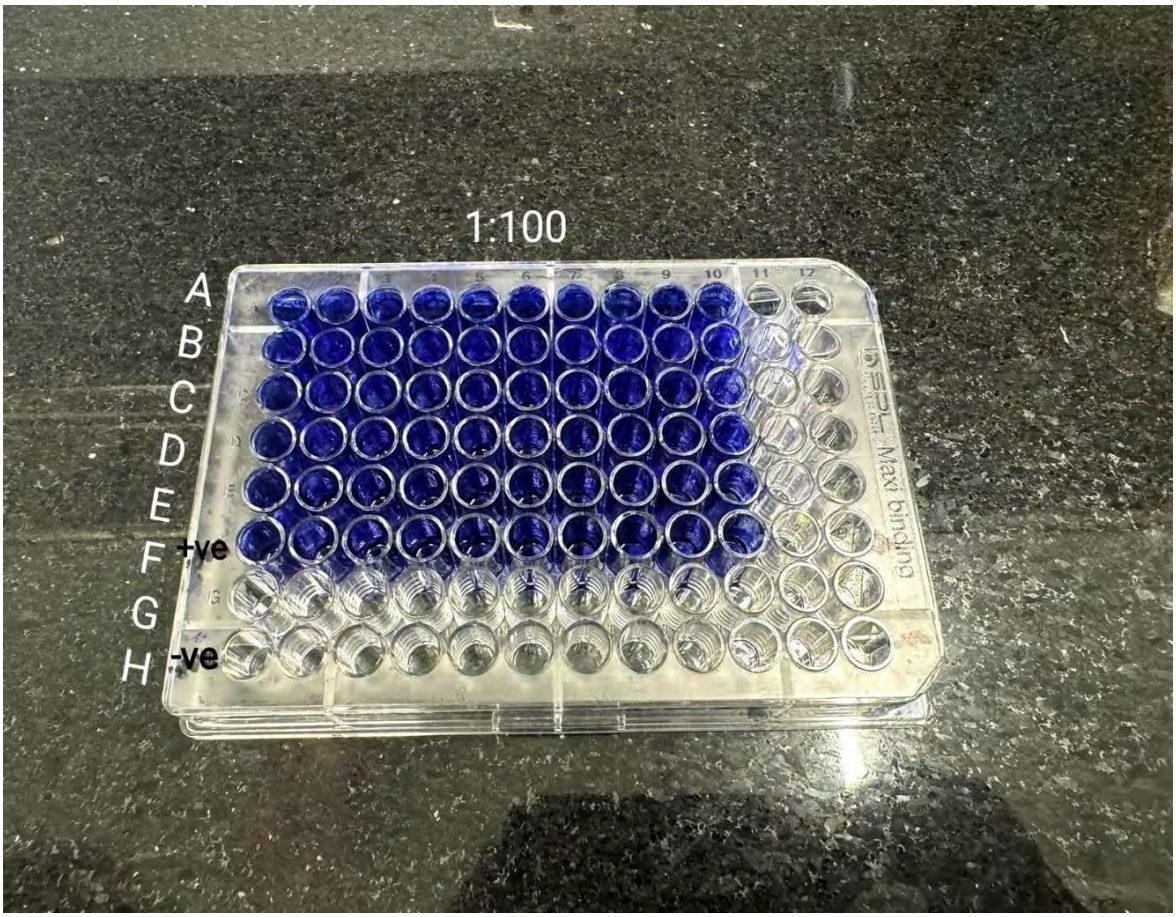


Figure 8: By using tissue culture plate (TCP) method, biofilm formation was detected and optical density (OD) was measured in a microtiter plate.

Result of the effectiveness of black pepper extract on biofilm

Tube Method: After the addition of different concentrations of black pepper extract, a noticeable reduction in biofilm formation was observed. When concentrations of 5 mg/ml and 10 mg/ml were added, the biofilm ring was visibly diminished compared to the control sample. At a concentration of 20 mg/ml, the biofilm was completely inhibited with no biofilm ring visible at all. This indicates higher concentration of black pepper extract are more effective in inhibiting biofilm formation.

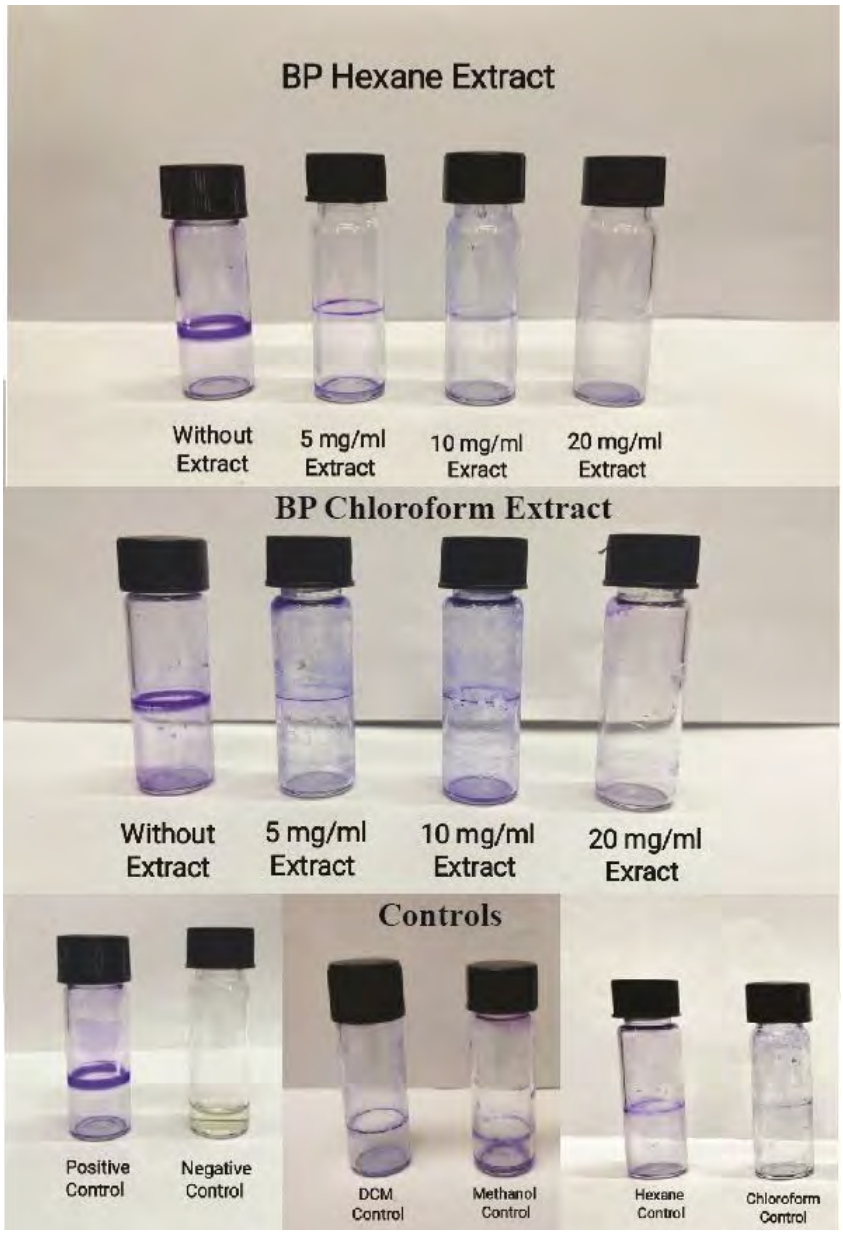


Figure 9: Both Hexane and Chloroform BP extract of different concentrations such as, 5 mg/ml, 10 mg/ml and 20 mg/ml were used. Afterwards, a significant reduction of in biofilm formation was observed. Different controls are also used for this experiment.

Tissue culture plate method:

Optical Density Reading (Without BP Extract):

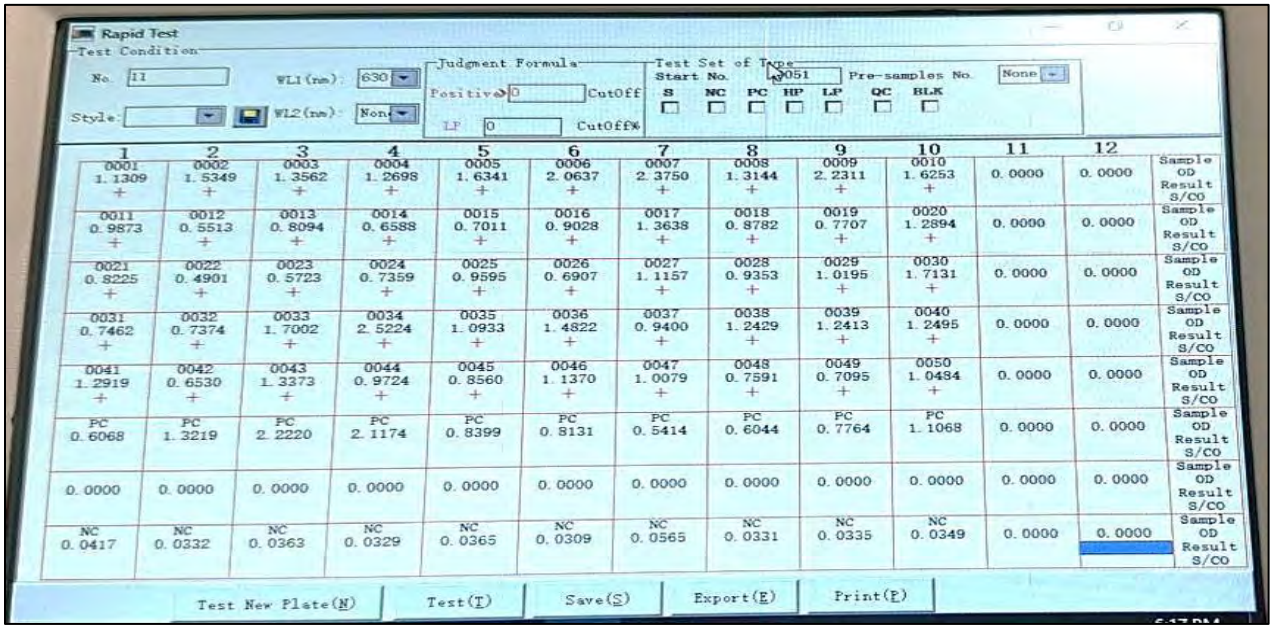


Figure 10: After biofilm formation in microtiter plate, their OD value was measured at 630nm. High OD values, indicating high biofilm formation.

Optical Density Reading (With BP Extract):

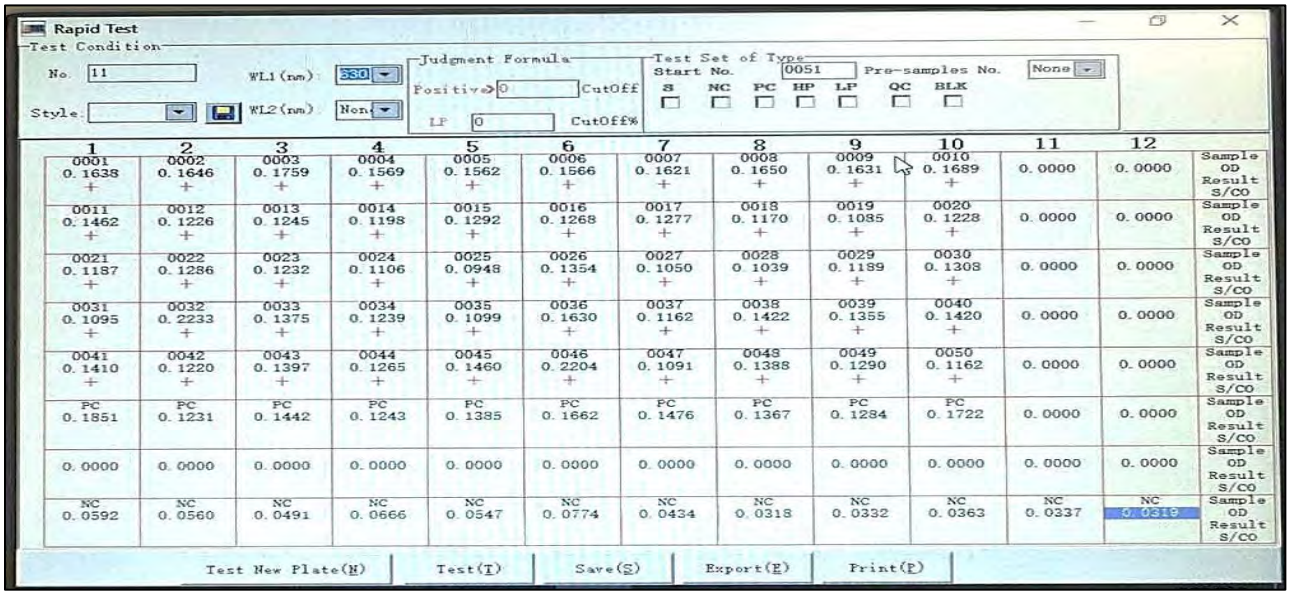


Figure 11: After adding 10 mg/ml Black Pepper extract in microtiter plate, their OD value was measured at 630nm. Lower OD values, suggesting reduced biofilm formation due to the inhibitory effect of the extract.

Determination of Biofilm Formation:

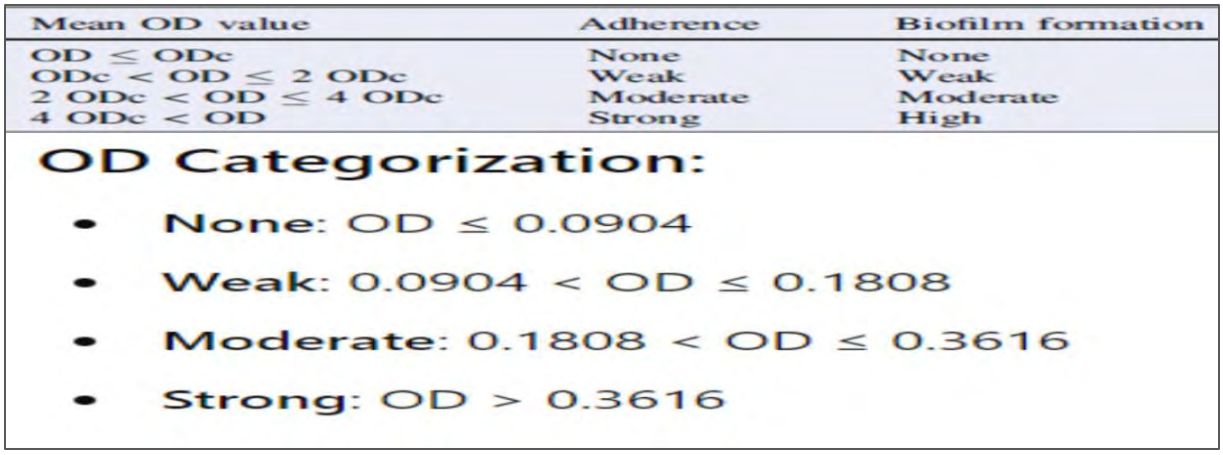
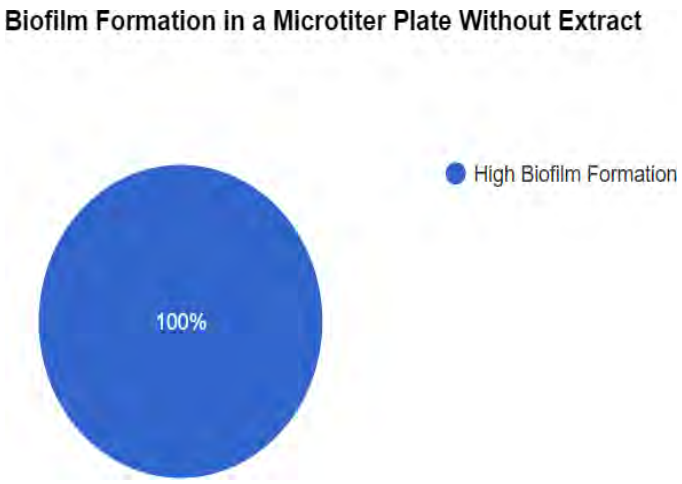


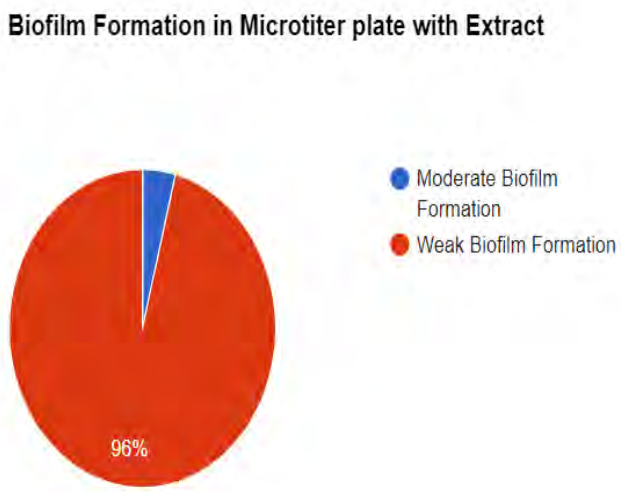
Figure 12: Different level of biofilm formation can be determined by using microtiter plate method. The cut-off absorbance value (ODc) was considered as three standard deviations above the mean OD of the negative control (Babapour et al., 2016).

Table 3: The percentage of biofilm formation in *Acinetobacter baumannii* after measuring OD and ODc values in both microtiter plates,

Test	**Biofilm Formation**			
	None	**Weak**	**Moderate**	**High**
Microtiter Plate method (**Without Extract**)	0 (0%)	0 (0%)	0 (0%)	50 (100%)
Microtiter Plate method **(With 10 mg/ml Extract)**	0 (0%)	48 (96%)	2 (4%)	0 (0%)

Pie Chart for effectiveness of BP Extract on *A. baumannii* Biofilm:





According to the pie chart, the initial measurement of biofilm formation showed 100% high biofilm formation without the addition of black pepper extract. The optical density (OD) values indicated high biofilm presence across all samples.

After the addition of 10 mg/ml black pepper extract, significant changes in biofilm formation were observed. The OD value was again measured and according to the ODc cut-off = 0.0904, the evaluation intensity of biofilm formation was categorized as: 4% showed moderate biofilm formation, and 96% displayed weak biofilm formation. These results indicate that black pepper extract has a substantial inhibitory effect on biofilm formation.

Chapter 4: Discussion

Acinetobacter baumannii has been recognized as an opportunistic pathogen and one of the main causes of nosocomial infections over recent decades. This bacterium, particularly its biofilm-producing strains and its multidrug-resistant (MDR) were found to pose significant threats to hospitalized patients by causing severe infections. The treatment of such infections was rendered challenging due to the widespread resistance of *A. baumannii* to antibiotics (Babapour et al., 2016). Given these challenges, it was important to explore a non-antibiotic strategy by using plant extracts that can effectively inhibit biofilm formation. There have been very limited studies investigating the use of black pepper extract to inhibit the biofilm formation of Acinetobacter baumannii. To fill this knowledge gap, clinical isolates of *A. baumannii* were collected from patients admitted to the National Institute of Traumatology and Orthopedic Rehabilitation (NITOR), and black pepper extracts were used in different concentrations to test their efficacy in inhibiting biofilm formation.

The identification of *Acinetobacter baumannii* was successfully achieved through both physical and molecular methods. The colony morphology on nutrient agar, Leeds Acinetobacter Agar Base, Gram staining, and PCR confirmation matched the characteristics described in the literature (Babapour et al., 2016).

The ability of *Acinetobacter baumannii* to form biofilms was confirmed using qualitative methods such as Congo Red Agar (CRA) and the tube method. In our study, a clear reduction in biofilm formation was observed with the addition of black pepper extract in a concentration-dependent manner using the tube method (Harika et al., 2020). At concentrations of 5 mg/ml and 10 mg/ml, there was a noticeable decrease in the biofilm ring adhering to the walls of the vials. At a concentration of 20 mg/ml, biofilm formation was completely inhibited, as evidenced by the absence of any biofilm ring. The observed reduction in biofilm formation was supported by the literature, which indicated that various natural extracts including black pepper, possess antibiofilm properties that can inhibit bacterial growth and biofilm formation (Lee et al., 2014).

The quantitative analysis using the tissue culture plate (TCP) method further supported these findings. The optical density (OD) measurements showed a significant decrease in biofilm formation in the presence of black pepper extract. Before the addition of the extract, 100% of the samples exhibited high biofilm formation. Similar results were reported by (Mohammad and Ahmad 2019) in Iraq, who demonstrated that 100% of samples of *A. baumannii* had a strong ability to form biofilms. Here, The OD values were directly proportional to biofilm formation, indicating that higher OD values correspond to more substantial biofilm production. After the addition of 10 mg/ml black pepper extract, The OD value was again measured, and found that 4% showed moderate biofilm formation, and the rest 96% displayed weak biofilm formation. These results indicate that black pepper extract has a substantial inhibitory effect on biofilm formation.

In our study, biofilm formers showed greater resistance to ampicillin-sulbactam, amoxicillin, and ceftazidime compared to meropenem and piperacillin. These results align with findings from a study by Badave and Kulkarni (2015). Nahar et al. reported that resistance to gentamicin, amikacin, netilmicin, ciprofloxacin, and meropenem was higher among biofilm-forming *Acinetobacter baumannii*, particularly from ICU patients. In contrast, our study found that *A. baumannii* biofilm formers were susceptible to meropenem and piperacillin. This could be due to the ability of these antibiotics to penetrate the biofilm and inhibit bacterial growth. This finding is supported by research on antibiotic permeation through biofilms, which demonstrates that piperacillin and meropenem have relatively high permeation and penetration through biofilms (Shigeta et al., 1997).

Limitations of our Study:

Despite the positive outcomes, several limitations need to be addressed. The specific mechanisms (mode of action) by which black pepper extract inhibits biofilm formation were not explored and need further investigation on it. Additionally, comparative studies with other natural extracts and commercial biofilm inhibitors were not conducted. In our study, the lack of phytochemical analysis makes it difficult to identify the specific compounds responsible for the anti-biofilm effects. Without conducting whole genome sequencing, the study may lack detailed information into the

complete genetic makeup of *Acinetobacter baumannii* thereby limiting the identification of specific genetic factors influencing biofilm formation and antibiotic resistance mechanisms.

Chapter 5: Conclusion

In conclusion, this study confirms the biofilm-forming ability of *Acinetobacter baumannii* and demonstrates the potential of black pepper extract to inhibit biofilm formation in a concentration-dependent manner. The significant reduction in biofilm formation with increasing concentrations of black pepper extract highlights its potential as a natural agent. These findings pave the way for further research into the application of black pepper extract in preventing and treating biofilm-associated infections. Further research is recommended to isolate active compounds in black pepper extract and explore their mechanisms of action in biofilm inhibition.

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