ANTI-BIOFILM FORMING ACTIVITY OF NATURAL PRODUCTS *PUNICA GRANATUM L.*, *MAGNIFERA INDICA L.*, AND PROBIOTIC AGAINST BIOFILM FORM BY *ESCHERICHIA COLI* AND *PSEUDOMONAS AERUGINOSA* AMONG DIFFERENT CLINICAL SPECIMENS.

DR. HADIA KHURSHEED

(06-114202-002)

This thesis is submitted in fulfillment of the requirements for the award of the degree of Master of Philosophy (pathology)

DEPARTMENT OF PATHOLOGY

BAHRIA UNIVERSITY ISLAMABAD

PAKISTAN

SEPTEMBER, 2022

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TO MY BELOVED “FATHER AND MOTHER”

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ABSTRACT

Escherichia coli and *Pseudomonas aeruginosa* are gram negative rods. The members of *Escherichia coli* and *Pseudomonas aeruginosa are* capable of causing infectious diseases in humans and common cause of community acquired infections. *Escherichia coli* is a type of bacteria normally present in your intestines and some of its strains that cause gastroenteritis in humans can be grouped into six categories: Shiga toxin-producing E. coli (STEC), enteroaggregative (EAEC), enterohemorrhagic (EHEC), enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), and diffuse adherent (DAEC) can cause serious infections like diarrhea if ingested in any contaminated food or water, or can be transmitted by person-to-person through fecal shedding and accounts for an estimated 11% of infections, most commonly *Escherichia coli* causes Gastrointestinal illness and outside intestines Urinary tract infections (UTI). An important strain of *Escherichia coli*, facultative anaerobic bacteria is Shiga toxin strain O157: H has developed immense attention in food and water borne diseases that causes diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) in humans. O157: H infects the alimentary tract and induces abdominal discomfort, cramps and hemorrhagic diarrhea. *Pseudomonas aeruginosa* classified as strict aerobes and is present on the surface of water, soil and other moist environment in hospitals cause pneumonia, urinary tract infections (UTIs), and bacteremia. In addition to biofilm formation there are various virulence factors that causes chronic infections in humans like elastase, phospholipase C, protease A, exotoxins and cytotoxins, flagella and pili, pigment production, and QS regulatory system proteins, which regulate both virulence factor transcription and biofilm formation. *Escherichia coli* and *Pseudomonas aeruginosa* display a characteristic biofilm forming activity, biofilm consist of colonies of bacteria embedded on extracellular

polymeric substances (EPS), cell to cell communications within these biofilm are major cause and it further enables bacteria to display infections and intensify its pathogenicity. The objective of this study were to identify the biofilm forming bacteria i.e. *Escherichia coli and pseudomonas aeruginosa*. To determine the anti-biofilm activity of natural product extracts i.e. pomegranate *Punica granatum L. and Mango leafs Mangifera Indica L*. To determine the anti-biofilm activity of probiotics having *Lactobacillus acidophilus*. To evaluate the synergistic activity of Natural product extracts and probiotics. The study design is cross-sectional study. The samples were collected from patients at PNS Shifa hospital Karachi. The sample size is 150. The age groups of the individuals include 15 to 50 years. Ethical permission was taken from hospital review committee informed consent for the study was taken from patients. The data collection was designed to collect the demographic data of patients. The specimens received in lab will be inoculated on Blood agar and MacConkey's agar culture plates. Culture plate will be inoculated at 37 ℃ in incubator for 24 to 48 hours. Identification of *Escherichia coli* and *Pseudomonas aeruginosa* is to be done by colony morphology, gram staining, TSI, Different biochemical test. After identification, 96 well micro titer plate will be used to detect biofilm forming activity of *Escherichia coli and Pseudomonas aeruginosa*. Then use of natural product extracts and probiotics to evaluate there, anti-biofilm forming activity using 96 well micro titer plate. Results among 150 patients 64% were males and 36% were females. Over all mean age was (33.79±9.94) and (34.02±10.59) years. Urine was the most common specimen (66.7%). (68.7%) patients were found with *Escherichia coli* and (31.3%) with *Pseudomonas aeruginosa*. While mean biofilm formation was (1.68±0.85) biofilm formation was achieved among (89.3%) of the patients. We found significant relationship between culture and examination (p-value 0.000) while no significant relationship was found between gender, age and biofilm formation (p-value 0.69) (p-value 0.44) and (p-value 0.57) respectively. Anti-bioiflm forming activity of pomegranate peels extract against *Escherichia coli* and *pseudomonas aeruginosa* is (24.46±19.09) and (29.26±19.09) respectively. Anti-biofilm forming activity of Mango leafs extract against *Escherichia coli* and *pseudomonas aeruginosa* is (14.90±9.56) and (11.45±9.52) respectively. Anti-biofilm forming activity of probiotic having *Lactobacillus acidophilus against Escherichia coli* and *pseudomonas aeruginosa is* (0.5x $10^{\wedge 6}$ CFU/ml) and (0.5x$10^{\wedge 5}$ CFU/ml) respectively. It was concluded that novel combination of natural products extract and probiotics has shown higher effectiveness against these rapid emergence of biofilm forming pathogens and has tremendously

decreased the mortality and morbidity and significantly reduced the biofilm production. Therefore, natural products and probiotics represent as an alternative treatment option in this era of multi-drug resistances gram negative bacteria. Hence, it was worth mentioning these new alternatives and providing beneficial aspects in reducing different health care associated infections.

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Keywords:

Escherichia coli, *Pseudomonas aeruginosa*, Biofilm, Natural products, Probiotics, Antibiofilm, Synergism.

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LIST OF ABBREVIATIONS

S No	Abbreviations	Stand For
1	WHO	World Health Organization
2	AMR	Antimicrobial resistances
3	ECM	Extra-Cellular matrix
4	CLSI	Clinical and laboratory standards institute
5	ESP	Exopolysaccharide
6	TSI	Triple sugar iron
7	MDR	Multi drug-resistant
8	TSB	Tryptone soya broth
9	AST	Antibiotic susceptibility test
10	ESBL	Extended-spectrum beta-lactamase
11	QS	Quorum sensing
12	PPE	Pomegranate peel extract
13	MLE	Mango leaf extract
14	CAP	Community-acquired pneumonia
15	CDC	Center of disease control
16	AK	Amikacin
17	PB	Polymixin B

18	CIP	Ciprofloxacin
19	ATM	Aztreonam
20	CAZ	Ceftazidime
21	MEM	Meropenem
22	LEV	Levofloxacin
23	CN	Gentamycin
24	TZP	Pipra-tazobactam
25	FEP	cefepime
26	TGC	tigecycline
27	P	penicillin
28	TET	tetracyline
29	LZD	linzolid
30	FOS	fosfomycin
31	NIT	Nitrazepam
32	VAN	vancomycin
33	IMP	imipenem

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CHAPTER NO 1

INTRODUCTION

1.1 BACKGROUND:

Numerous Pathogenic microorganisms are constantly emerging worldwide. Laboratory studies on microbial evolution is constantly providing a better understanding of evolutionary dynamics, identify the adaptive and environmental changes, and answer key questions that affects human health. [Alfonso Santos-Lopez et al., 2019]. However, in human pathogenic microbial infections, and the evolutionary parameters that affect the infected population can be much more complex than the ones tested in the laboratory. [Culyba, M. J., et al., 2021]. Thus, human infections can be viewed as a naturally occurring in vivo bacterial evolutionary experiment that can teach us about antibiotic resistance, mutations, environmental adaptations, etiology, and infection. [Forsyth, Valerie S., et al., 2018]

The rising spread of antibiotic-resistant bacteria poses a significant health threat to morbidity and mortality worldwide. Because it's a serious public health problem with social impacts, MDR-bacteria have long been considered as a global priority for investment in new drugs by the WHO [Lorena Varriale et al., 2022]. In 2016, WHO create a

priority list of other antibiotic-resistant bacteria to support research and development of effective drugs and other alternatives that can help in eliminate MDR-bacteria [Alessia Savoldi et al., 2018]. The predominant growth mode for most of the microbes is on surfaces, and this biofilm lifestyle is critical for AMR particularly in chronic infections. [Capozzi, C. et al., 2019]

Therefore, our knowledge of this behavior that affects the development of AMR, due to different dynamics genetically or molecular is restricted. [Ebrahim, M., et al., 2016] One of the examples, the vicinity among cells in biofilms can expedite the transfer horizontally and resistances that persist in the genes in bacterial populations. [Ciofu, O., et al., 2017]. This appearance of AMR in biofilms is important because,

1) The environmental structure of biofilms can increase cell to cell

Communication make selection less efficient and improving genetic diversity.

2) Distinct ecological conditions within the biofilm may promote functionally distinct adaptations to different niches.

3) The biofilm itself may protect its residents from exposure to external stresses such as antibiotics or host immunity host and weaken selection.

4) Steady growth within the biofilms may lessen the effectiveness of drugs that specially attack the rapidly growing cells. [Asma Ghanizadeh et al., 2021]

As we all know that rapid Antibacterial resistance (AMR) threatens the health care system for people around the world. [Al-Marri, T., et al 2021] This challenge is addressed by adequately prescribing, dispensing, and consumption of antimicrobials in clinical settings and in the daily lives of the population. [Amer, Y.,et al., 2022] Individuals need to be contacted outside the clinical setting to prepare them for the necessary chemical changes in drug regarding the treatment of infectious diseases. [Mutagonda, R. F et al., 2022]

An effective response must be invested in both the development of new drugs and also measures to slow the rapid progress in emergence of resistance. [Walters, J. H., et al 2022] The major focus is on low and middle-income countries with pluralistic health systems, where people obtain much of their antibiotics in underground markets. [Booth, A., & Wester, A. L. (2022]

There is evidence that these markets have enabled people to treat many infections and reduce the mortality. [Harant, A. (2022)] However, they also encourage the overuse of antibiotics and likely to encourage the emergence of resistance bacteria. [Bartosiński, J., et al., 2022] It concludes that effective strategies are needed to measures and ensure an easy access to antibiotics, as well as those aimed at influencing providers and users of these drugs to use them appropriately. [Murugaiyan, J., et al., 2022]

1.2 *ESCHERICHIA COLI:*

The *Escherichia coli* reside in the normal flora of human colon and frequently found in large intestine. [Mohamed, J. A. (2022)] They are the member of gram-negative rods family with some special and distinctive characteristics. [Doma Jr et al., 2018] These organisms are responsible for most of the clinical as well as subclinical infection including urinary tract infection, blood stream infection, respiratory tract infection, and found frequently in hospital acquired infections. Most frequently found in clinical specimens of urine, blood, pus, CSF and respiratory specimens of infected person. [Jang, J., Hur, H. G. et al., 2017]

Escherichia coli is a gram-negative bacteria, facultative anaerobic, non-spore-forming, and belongs to the order Enterobacter. [Horiuchi, s et al., 2022] It has a diameter of about 0.4 μm and a length of 2 to 3 μm. It can be capsular or non-capsular, and is generally motile as it has a peri-capsular flagella, grows over a vast temperature range (15-45 ° C) and can survive for a long period of time in the environment (figure no 1.1). [McDaniel, A et al., 2022]

Escherichia coli has multiple virulence factors (VFs), including toxins, adhesins, siderophores, and polysaccharide capsules. Numerous VFs are associated with *Escherichia coli* pathogenicity, with a wide range of pathogenic activities.[Meroni, G.et;al., 2022] In addition to VFs, antibiotic resistance and biofilm formation also complicate the elimination of microorganisms from the human body.[Al-Marri, T., et al., 2022]

More than 700 strains of *Escherichia coli* are present, these strains are serologically classified according to the antigenic differences between the 173 O (somatic) and 56 H (flagella) antigens based on the classification system introduced by Kaufmann in 1947 and are still internationally recognized [Furevi, A.et al., 2020] and use the fixed set of bacterial strains that feature the O antigen constitutes "serogroup", whereas a specific O antigen and H antigen combination determines these "serotype" of isolates. In the classification of Kaufman additionally analyzed antigens that are the K (capsule) F (fibrin) antigens [Krogfelt, K. A.et al., 2019]

However, numerous lines which have establish precise virulent genes that had been remain located in outside and inside of the intestine causing infections [Brubaker, J.et al; 2021]. Based at the combine pathogenicity of altered genes, pathogenic *Escherichia coli* are grouped into six pathotypes: Entero-hemorrhagic *E. coli* (EHEC), entero-pathogenic *E. coli* (EPEC), entero-invasive *E. coli*, entero-toxigenic *E. coli* (ETEC), and diffusely adherent *E. coli*. [Sanchez-Villamil, J. I., et al., 2019]

An important strain of *Escherichia coli* is Shiga toxin strain O157: H that has developed immense attention in food and water borne diseases that causes diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) in humans. O157: H infects the alimentary tract and induces abdominal discomfort, cramps and hemorrhagic diarrhea. [Halkilahti, J et al.,2020]

The assembly of Shiga toxins production may be a key issue tributary in the events of hemolytic uremic syndrome (HUS) that exhibit as hemolytic anemia, thrombocytopenia, and acute excretory organ failure. [Khalid, M., & Andreoli, S. (2019)] it may result in each acute, probably severe and lifelong, chronic illness the event of antimicrobial resistance in *Escherichia coli* is one among the difficult public health challenges. [Lusta, M. V.,et al., 2022] *Escherichia col*i exhibit resistance to variety of antibiotics, principally extended spectrum beta-lactams (ESBLs) and carbapenems. [Abdel-Rhman, S. H., et al., 2021]

ESBL producer isolates that is immune to penicillin and cephalosporin. The need has been determined as variants of TEM and SHV or the accelerator CTX-M. [Bakran-Lebl, K. et al., 2019] It is additional common in atmospheric isolates, suggesting that the environment may be a reservoir of ESBL producers. This often is a significant issue

tributary to inflated antibiotic burden, higher medical aid costs, poor health outcomes and restricted therapeutic options. [Alamneh, E., et al.,2021]

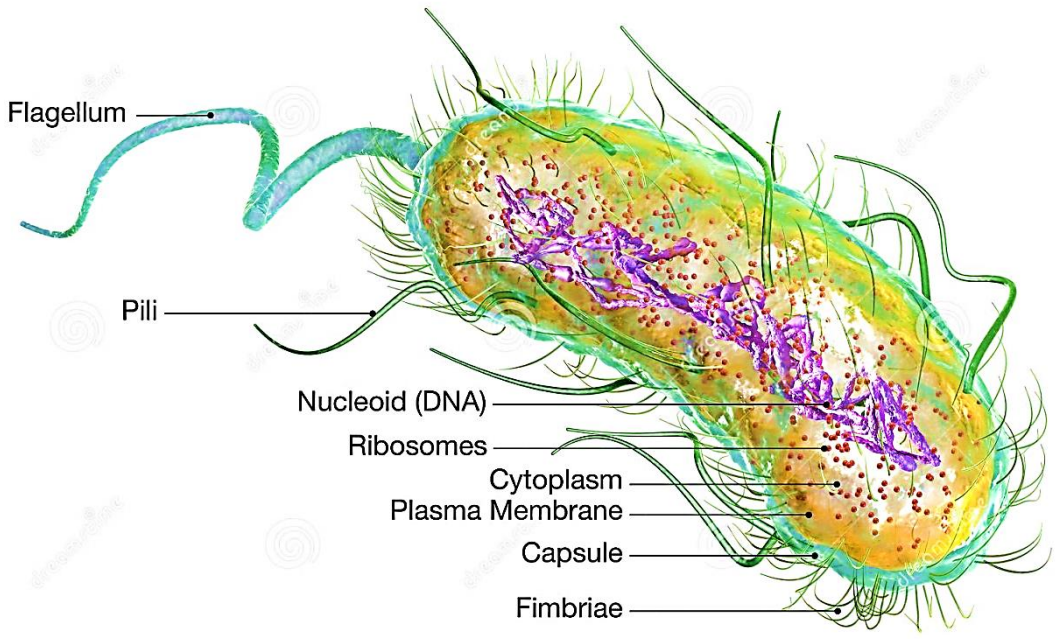


Figure 1.1: Structure of *Escherichia coli*

Ref: (Dreamstime.com)

1.3 PSEUDOMONAS AERUIGINOSA:

Pseudomonas aeruginosa is opportunistic and non-fastidious encapsulated, Gram-negative, bacillus that can cause infectious disease in humans, plants and animals. [Diggle, S. P., & Whiteley, M. (2020)] These are the species of considerable medical importance, *Pseudomonas aeruginosa* is a multidrug resistant pathogen intrinsically it is advanced in antibiotic resistance mechanisms, [El Ghali, A. et al., 2022] and is associated with serious illnesses such as hospital-acquired infections including those who are dependent on devices such as catheters (blood, I/v & urinary) ventilator-associated pneumonia and various sepsis syndromes. (Figure no 1.2) [Duszynska, W .et al., 2021]

In 2017, *Pseudomonas aeruginosa* was recognized as one of the most life-threatening bacteria and listed as top priority pathogen for Research and Development of new antibiotics by the World Health Organization (WHO). [Cheng, Z.et al., (2019).]

A growing problem worldwide is antibiotic resistance due to their limited efficacy, there adaptability and higher level of intrinsic antibiotic resistance [Botelho, J.et al., 2019] specially in gram-negative rods it is increasing at alarming levels, making it difficult to obtain treatment options for various life threatening hospital-acquired infections. [Tümmler, B.et al., 2019]

Infections caused by these organisms that have developed resistance mechanisms by forming biofilms. [Rehm, B. H. et al., 2020] In clinical settings highly notorious persistence is recognized for its ability to form multiple antibiotic-resistant biofilms [Sbhatu, D. B.et;al 2019] which unfortunately in turn leads to a poor prognosis for the relevant treatment of the patients and also overloads in the health care system, which represents a high expenditure. [A., Sorlí, L., et al., 2019]

Such capability is encouraged by means of potent cell-to-cell communications within the microbial groups of *Pseudomonas aeruginosa* called quorum sensing (QS).[Lee, J. H. et al., 2019]

Resulting, tremendously structured biofilms that are shaped in regularly organized fashion identified in continual infections in patients, including chronic pulmonary infection, chronic contaminated wound and chronic rhino sinusitis. [Rehm, B.et al., 2020]

It has been predicted that biofilms have a huge role relating more than 90% of persistent wound infections, result in terrible healing of the wound within the USA alone, [Galiano, R. D.et al., 2019] approximately 6.5 million sufferers are suffering from chronic infections of the wounds, that ended in excessive health-care burden and disturbing financial results envisioned at over billions of dollars annually. [Sen, C. K. (2021] Therefore, It is essentially helpful to identify *Pseudomonas aeruginosa* infections at initial levels before these biofilms are formed which leads into the development, or perhaps decreases the susceptibility of *Pseudomonas aeruginosa* towards antimicrobial treatment.[Al-Dahmoshi, et al., 2020]

However, the growing prevalence of acute and chronic continuing infections worldwide also highlighted to increase healing strategies as an opportunity to therapeutic antibiotics, expectedly eliminate this gram negative bacteria [Pachori, P., et al., (2019)] Depend upon its capacity to form biofilms the center of bacteria that can enhance and to cover up the mucosal surfaces or prying devices. [Grønseth, T. et al., 2020] These are the biofilms that compile the state for the tenacious bacteria more favorable, since encapsulated bacteria are constitutionally more difficult to abolish. [Poonguzhali, P. et al., 2021]

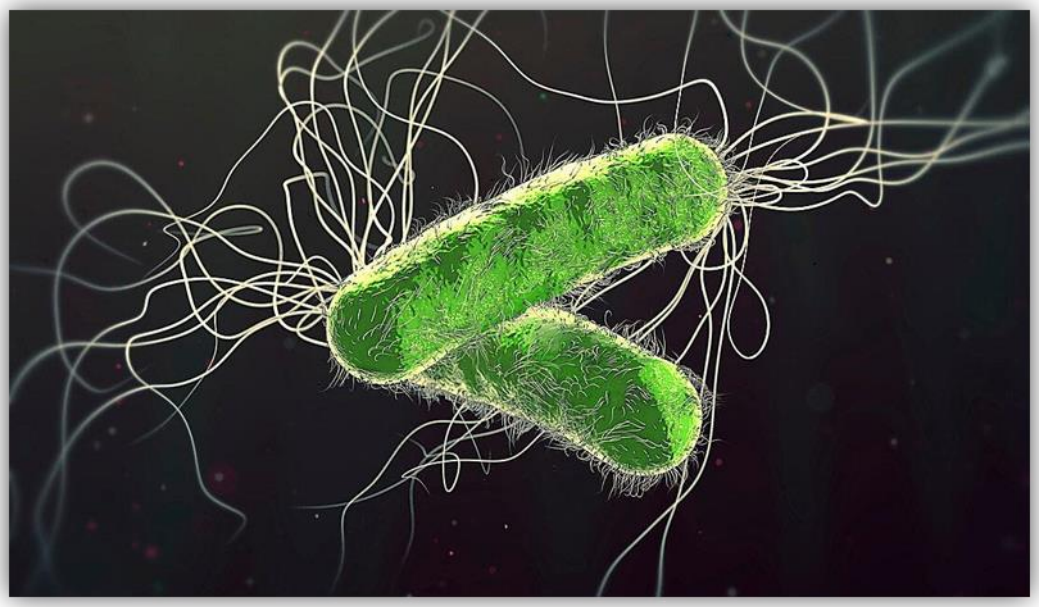


Figure 1.2: Structure of *Pseudomonas* a*eruginosa.*

Ref :(dreamstime.com)

1.4 BIOFILM DEVELOPMENT AND ITS RELATION TO SURFACES:

The biofilm is an intricate summative of bacteria encased in a self-generated environment of extracellular polymeric substances (EPS) and is one of the key approaches for the existences of many species [Muraro R. et al.,2020] against unexpected changes of living situations such as temperature and nutrient accessibility (Figure no 1.3). [Prasad, R. et al; 2020] They can ensure the automatic stability of the biofilm, mediate its bond to the surface, and form a coherent multi-dimensional polymeric network that enhance bonding and temporarily immobilize the cells of the biofilm. [Lin, Y., Zhou, X., & Li, Y. (2022)] Furthermore, the biofilm lattice also acts as an extracellular digestion mechanism a system that supports enzymes. Exogenous cells are adjacent to the cells, allow them to ingest solids, colloids and disintegrated biopolymer. [Rehman, Z. U et al., 2022]

A majority of bacterial infections are related to these biofilms. Targeting biofilms is taken into account a good strategy to limit microbial virulence whereas, to minimizing the events of multidrug antibiotic resistance (MDR). Biofilms formation cause continuous infections associated with wide span of medical implants and are well linked with diseases such as chronic wounds, chronic obstructive pulmonary disease, urinary tract infections, and cystic fibrosis. [Caldara, M., et al., 2022] In addition, the formation of biofilm lattice by pathogenic bacteria at the infection place that increased in the acerbity of the disease. [Delavar, M. A., & Wang, J. 2021] Biofilms are bacterial communities that are sheltered by an extracellular polysaccharide matrix (EPS), which guards them from dehydration or nutritional stress and facilitates the longevity of the environment. [Flemming, H et al., 2019] The capacity of microbial biofilms to resist antibiotics and the part of the host's immune system is the main reason behind the problematic continuous infections they cause. [Høiby, N.et al., 2022]

Antibiotics used can increase the amount of bacteria decrease in biofilms, but do not entirely annihilate biofilms and therefore relapses of biofilm infections are common. [Farshadzadeh, Z et al., 2022] Long-term follow-up antimicrobial therapy may be required to treat biofilm infections, immature biofilms are much more endangered to antibiotics than developed biofilms. [Cheng, H.et al., 2022] Many actions have been developed and used to hinder the formation of biofilms. Now, researchers have concentrated on natural strategies to overcome pathogenic biofilm using anti-biofilm forming products. [Chaves-López, C et al., 2022]

Regarding *Escherichia coli*, biofilm formation (BF) donates to the incidence of different pathogenic infections and makes their eradication problematic. [González pasayo, r. A. et al., 2022] Resistance of antibiotics has been identified as a global public health concern international health care organizations have now raised resistance of drugs to one of the top health apprehensions of the 21st century [Cernava, T. et al., 2022]

However there are unquestionable infectious strains of Escherichia coli that cause significant fatality. *Escherichia coli* additionally had been the explanation for numerous medical appliance mix infections in appliances akin to prosthetic grafts and joints, shunts furthermore as invasive catheters. [Zamani, H., & Salehzadeh, A. 2018] The emergence of biofilm by *Escherichia coli* on catheters makes catheter-associated tract infections (CAUTI) among the foremost significant health care associated facility infections. [Dowling, J. A. R. et al., 2019]

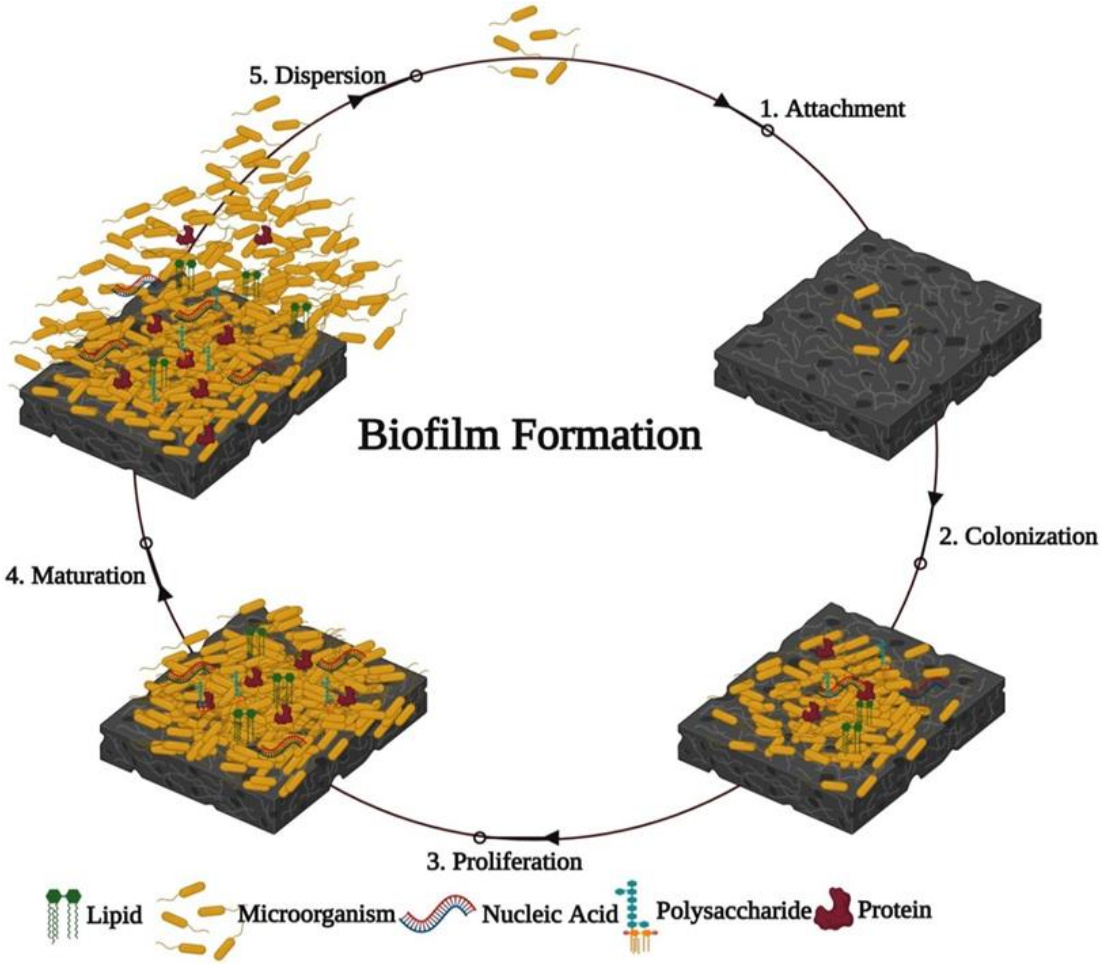


Figure 1.3: Stages Involved In Bacterial Biofilm Formation.

(https://www.frontiersin.org/articles/10.3389/fmicb.2021.676458/full)

1.5 Anti-biofilm Agents Based on Natural Products extracts:

Natural products have traditionally been a wealthy supply of various chemical along with several other organic activities, and feature performed a critical function in drug discovery in lots of regions together with infectious disease. Synthetic and medicinal chemistry were, to keep critical equipment to comprehend the capacity of herbal merchandise as therapeutics and as chemical probes. The formation of biofilms through microorganism in a contamination placing is a considerable element within side the recalcitrance of many bacterial infections, conferring multiplied tolerance to many antibiotics and to the host immune response, and as but there aren't any authorized therapeutics for combatting biofilm-primarily based totally bacterial infections.[Atanasov, A.etal., 2021] Small molecules that intervene with the capacity of microorganism to shape and preserve biofilms can triumph over antibiotic tolerance conferred through the biofilm phenotype, and feature the potential to shape mixture treatments with traditional antibiotics. Herbal merchandise with anti-biofilm is recognized from plants, microbes, and marine life. [L Huang, S Ahmed et al., 2021]

Due to the increasing prevalence of life-threatening bacterial, fungal and viral infections and the ability of these human pathogens to develop resistance to current treatment strategies, there is a great need to find and develop new agents to combat them. [Darwish, W. S.,et al., 2022] These molecules must have low toxicity, specific activity and high bioavailability. The compounds best suited for this task are generally derived from natural sources (animal, plant or even microbial). [Gao, Z., Du, et al., 2022] This presents and evaluates the latest and most promising natural products to combat bacteria, filamentous fungi, and viruses. These include plant extracts, essential oils, small antimicrobial peptides of animal origin, bacteriocins and various groups of plant compounds (triterpenoids, alkaloids, phenols, flavonoids) with antimicrobial and antiviral effects. [Mašković, P.et al ., 2019]

Natural products are an important source of new drugs or serve as models for the development of new synthetic drugs, ranging from cancer therapies to antibiotics.[Alhazmi, H. A. et;al 2022] A significant number of natural product drugs are actually produced by microbes or through their interaction with hosts [Newman and Cragg, et al., 2020] One of the

main reasons for research into natural products with antimicrobial activity is the constant expansion of antibiotic resistance genes carried by plasmids and the presence of diseases (mainly respiratory and neurological) that are not covered by natural or plant-derived substances.[Giles, M.,et al; 2021] The World Health Organization (WHO) promotes the use of medicinal herbs as a remedy to help in the absence of conventional treatment. [Miranda, J. J. M., 2021]

The focus is on studies of bioactive compounds, their chemical composition and the pharmacological potential of different plant species to produce compounds with lower toxicity than existing molecules. [Abert Vian, M., et al., 2019] Because of their numerous benefits, natural compounds are now used to treat a number of diseases, including microbial diseases, inflammatory processes, and cancer. [Panda, S. K., et al., 2020]

This is mainly due to the accessibility and good therapeutic potential of natural remedies [Boccolini and Boccolini, 2020]. Plants play an important role in the medical world as a source of natural products of medicinal importance and represent the largest resource for new and highly effective drugs/therapies. [Khan, M. S. A., & Ahmad, I. (2019)] BBC Research reports that the global herbal medicines market has grown from US$29.4 billion in 2017 approximately $39.6 billion in 2022 [Acosta-Torres, L. S., et al., 2018]

There are a number of natural compounds isolated from various sources (plants, animals or microorganisms) that have an antibacterial effect. [Atanasov, A. G. et al., 2021] However, due to structural differences between gram-negative and gram-positive bacteria, the effectiveness of antimicrobials can vary. [Kovanda, L. et al., 2019] The prevalence of antibiotic resistance among Gram-negative strains is of serious concern, particularly in hospital settings where immune-compromised patients are at higher risk [Morris and Cerceo, et al., 2020]. However, multidrug resistant strains (MDR) are not only found in the hospital environment, but also in our diet due to the extensive use of antibiotics in cattle to treat infections, promote growth and prophylaxis. Therefore, finding new agents to combat these MDR strains is crucial. [Beyer, P. et al., 2022]

Many plant-derived natural products possessed antimicrobial and antibiofilm functions in vitro. A variety of molecules derived from natural plant or medicinal herb extracts and the mechanisms underlying antibiofilm agent functions have been identified. [El Garah, F. et al; 2022] The antibiofilm effects of natural products are mainly based on the following aspects: inhibition of polymer matrix formation, suppression of cell adhesion

and attachment, disruption of ECM generation, and reduced production of virulence factors, thereby blocking the QS network and biofilm development. [Khadraoui, N.et al., 2022]

Plants have attracted interest from both academia and the pharmaceutical industry [Mostafa et al., 2018]. Although many herbal extracts have been shown to have antibiofilm effects, the bioactive molecules or components are still unknown and require further study. Therefore, the separation and extraction of effective antibiofilm components are important. [Chemat, F. et al., 2020] In the last decade, techniques such as chromatographic separation and structure-based virtual screening (SBVS) have been extensively studied to identify bioactive ingredients that act as plant antibiofilm agents and have laid a solid foundation for the unearthing of new molecules for biofilm control, biofilms and bacterial infections. [Li, M.et al., 2019]

The Anacardiaceae family is widely used for medicinal purposes and contains phenols and tannins that have shown antimicrobial properties [Raj, S. P., et al., 2022]. Mangifera indica L. var. "Ubá" is a member of this family, distributed across most continents, with a global production of about 40 million tons per year [Babu, R. S. H.et al., 2022]. Mango is associate evergreen tree with a great deal of ancient medicative resources aside from its very notable fruits. Its seeds have an antimicrobial effect when formulated in ointments and creams for topical use in humans and show zones of inhibition against bacteria. (figure no 1.4) [Egbuonu, A. C. C. (2022). Extracts of these mango leafs are used for antique medications to cure diabetes, bronchitis, diarrhea, asthma, kidney, scabies, metastasis problems, syphilis, and urinary disorders [Mohammad Saleem, et al., 2019]

The foremost active organic constituent of Mango Leafs is mangiferin, followed by phenolic resin acids, benzophenones, and different antioxidants akin to flavonoids, carotenoids, quercetin, isoquercetin, ascorbic acid, and tocopherols. Mangiferin is that the key donor of most of the biological activities of Mango Leafs extract. Mango Leafs have an excellent scope of valorization as they're recognized to possess varied phytochemical, biological, and pharmacologic properties, namely anti-microbial, antibiofilm [Mekhemar, M. et al., (2021)]

Figure 1.4: *(Mangifera Indica L)* Mango leafs

The food processing industry wastes a lot of fruit every year because raw fruit is processed into higher value-added products. [Ahmed, I., & Green, I. R. et al; 2022] Punica granatum L., better known as pomegranate, has developed into an interesting functional food over the past two decades, Food industries have therefore to rethink their waste management systems, mainly based on massive landfilling, and turn more sustainable strategies aimed at reducing the amount of waste to be disposed. [Li, Z., & Tu, Y. et al; 2021] 50% of the mass of the Punica granatum fruitlet consists of undigestible parts, i.e. the peel, while the residual half contains of about 20% seeds and 80% juice [Chahdi, F. O.et al., 2021].

Studies show that the fruit of P. granatum is abundant in anthocyanidins, flavonoids and phenolic combinations, but the phytochemical composition varies in diverse parts of the fruit and also depends on geographic and ecological factors that can affect the level of bioactivity of secondary metabolites [Zhao, X., & Yuan, Z. (2021)] . In addition to several micro- and macronutrients and bioactive combinations present, bases shows that P. granatum fruits possess unique antioxidant properties, as well as having anti-atherosclerotic, hypotensive, anti-inflammatory and anti-mutagenic properties and having a beneficial effect on wound healing [Ennahar, S. et al., 2021]. However, the fruit of Punica granatum L., and its parts are most commonly used due to their antimicrobial properties [Hemashekhar, B et al., 2021].

Antimicrobial activity of Punica granatum L, peel, seeds and juice has been established against different foods and marine bacteria and human pathogens together with *Escherichia coli, Bacillus subtilis, Candida albicans, Clostridium spp., Helicobacter pylori, Klebsiella pneumoniae, Listeria monocytogenes, Pseudomonas aeruginos, Salmonella typhi, Shigella spp., Staphylococcus aureus, Vibrio cholera, Yersinia enterocolitica* and numerous others [Balia, R. L.et al., 2022].

Various polyphenols have been successfully sequestered in pomegranate, namely punicalagins, punicalins and other ellagitannins [Bishayee, A. et al; 2021]. These components are known for their organic activities (antioxidant, anti-inflammatory, anti-biofilm and antimicrobial) [Aygün, A. et al; 2022], while punicalagins have been shown to be the source of the anti-biofilm activity of peels (Punica granatum L.,) [Benchagra, L., Berrougui, H. et al; 2021]. Punica granatum peels are used as food waste product in the production of pomegranate juices. The bio-active components are extracted especially those with antibiofilm activity

from Punica granatum peels can be used for antibiotics and help fight antibiotic resistance biofilms. [Demir, T. 2021] In addition, such use of waste parts in foods can subsidized to circular low cost and bearable growth & organization of bio-waste (e.g. haling the amount of residual municipal waste in the EU by 2030, as proposed in the Circular Economy Action Plan 2020). [Rashid, M. I., & Shahzad, K. 2021] (Figure no 1.5)

Therefore, biofilms with increased resistance to drugs and antimicrobials are identified, posing a problematic load on human healthcare system. Therefore, treatment of biofilm-related infections is currently a complex challenge for practitioners and microbiologists. [Bhardwaj, D. K. et al; 2021] There is a crucial need to develop new antimicrobial strategies to overcome the problems of antibiotic resistance in microbial infectious diseases. [Mpundu, M. et al; 2022] As previously mentioned, natural resources have provided an excellent source for the detection of antibiofilm agents. In recent years, research has increasingly focused on plants and natural foods for their health-promoting effects. To date, a number of studies have examined the inhibitory antibiofilm effects of natural products on the formation and development of bacterial biofilms, suggesting their potential as alternative agents for bacterial infections. It is consistent with the current evidence, most of the natural antibiofilm agents showed encouraging preclinical data on antibiofilm efficacy in different bacterial species. Its potential regulatory mechanism was mainly based on repression of any step in biofilm formation or inhibition of the QS network. [D yuan, g yi, y zhou, et al; 2019]

Figure 1.5: *(Punica granatum L)* pomegranate peels

1.5 RESEARCH GAP:

The rapid emergence of biofilm forming pathogens i.e. *Escherichia coli* and *Pseudomonas aeruginosa* has tremendously increased mortality and morbidity due to multidrug resistances and is a major public health concern, associated with a high social and economic burden. The multiple and various mechanisms employed by these organisms to survive antibiotic treatment while growing in a biofilm represent an important therapeutic challenge. The molecular mechanisms underlying tolerance of biofilms to antibiotics are targets for therapeutic interventions for potentiating the anti-biofilm effect of antibiotics. To counterbalance against these pathogens, therefore it was necessary to introduced anti-biofilm agents/products i.e. probiotics and natural products extracts and there novel combinations are providing beneficial aspects in reducing these pathogenic biofilm being form in different health care associated infections.

1.5.1 THEORETICAL GAP:

The diversity of biofilm-associated infections is increasing over time and its impact may be underestimated. This peculiar form of development endows associated bacteria with a high tolerance to conventional antimicrobial agents. A small percentage of persistent cells developing within the biofilm is known to be highly tolerant to antibiotics and has typically been involved in causing relapse of infections. Knowledge of the pivotal role played by biofilm-growing microorganisms in related infections will provide new treatment dynamics for this biofilm-related disease. Furthermore, the antibiotics available till date are ineffective for treating these biofilm related infections due to their higher values of minimum inhibitory concentration (MIC) and minimum

bactericidal concentration (MBC), which may result in in-vivo toxicity. Hence, it is critically important to design or screen anti-biofilm molecules that can effectively minimize and eradicate biofilm related infections. A major focus has been put on various anti-biofilm molecules discovered or tested till date which may include herbal active compounds. From our study, we conclude that the molecules considered here are used to treat biofilm-associated infections after significant structural modifications, thereby investigating its effective delivery in the host. It should also be ensured that minimum effective concentration of these molecules must be capable of eradicating biofilm infections with maximum potency without posing any adverse side effects on the host. Many of the compounds that exhibit anti-biofilm activity need to undergo further modifications and in-vivo tests followed by clinical trials before using them commercially. It is evident from the previous studies that bacteria underneath biofilms are more resistant to antibiotics than their planktonic counterparts. Therefore, the use of combinatorial therapy is more preferable instead of antibiotic monotherapy these anti-biofilm molecules follow different mechanisms to inhibit biofilm formation in different bacteria. These anti-biofilm polysaccharides, especially the ones that are of bacterial origin portray broad-spectrum anti-biofilm activity while only some are capable of dispersing biofilms in their initial stages before attaining maturity. Different oligosaccharides or polysaccharides also exhibiting anti-biofilm properties can be used further in industrial and clinical setting and should report these gaps, they can be used as adjuvant with available antibiotics and natural anti-biofilm agents that help in reducing their biofilm eradication. Till date, a lot has already been studied and understood we encourage further studies based on these natural product extracts to find a significant and effective alternative against biofilm forming pathogens.

1.2.2 CONTEXTUAL GAP:

We already know enough to act, but what key gaps remain to help better understand and address the problem multiple times. While actionable evidence of the importance of the environmental dimensions of AMR has been mounting, some key data and gaps, including on interlinkages and negative feedback loops that increase AMR proliferation in the environment. This hinders systematic priority-setting and the selection of cost-effective, context-specific mitigation action. Additional data on the following topics would enhance and facilitate systematic priority setting and decision making studies to assess the global burden of antimicrobial resistance, particularly improving bacterial infections reported to align with recent antibiotic-resistant mortality estimates, including improvements to understanding infection emergence and spread from environmental and hospital sources the type, quantity and dynamics of environmental releases of chemicals (e.g. with co-selection potential) and biological pollutants (e.g. rich in resistant microbes) that affect AMR development, transmission and spread in local environments settings or specific conditions. Collectively, as this information becomes available it can be used to prioritize interventions to mitigate AMR development and spread in the environment. This report demonstrates the immediate need for ambitious, collaborative action by all health care sectors and practitioners. The public sector has an important role to play, not only in regulating these pollutants and their sources, but also in putting in place enabling policies and actions. Although some private sector actors have taken first steps to control the dimensions of AMR, considerable additional work is required. Further research will provide more data and evidence to better understand the complex dynamics of AMR and will lead to improved science-policy interfaces to ensure informed decision-making. However, despite evidence being sufficient there is enough knowledge to act now. Human action is responsible for worsening AMR, but also has the potential to mitigate it.

1.2.3 METHODLOGICAL GAP:

Problems of public health caused by bacterial biofilms have emphasized the lack of information about their development. Some screening methods are available for evaluating the ability of bacterial strains to adhere and to test their susceptibility to anti-biofilm agents. Among them are observations by different microscopy techniques another one of the most convenient techniques to evaluate bacterial adhesion consists of washing, staining and de-staining of sessile cells with crystal violet, in tubes or 96 well micro titer plates, one of the most convenient techniques to evaluate bacterial adhesion consists in a 96 well micro titer plate method after washing, staining and de-staining of sessile cells with crystal violet or safranin, in tubes or micro titer plates this technique requires at least 24 to 48 hours of bacterial cultures and estimates the adhered cells stained after washing steps. These washing steps, used to remove non-adherent cells,

Similarly, use of microscopy techniques (epifluorescence, laser-scanning confocal, transmission electron and scanning electron microscopy) are also laborious methods, often requiring numerous steps for sample preparation and the observation of many fields to avoid observer error. Thus, these conventional methods are not adapted to high throughput screening of a great number of bacterial strains or to test one microorganism under different environmental factors. Today, the mainly recognized and developed technique allowing such screening consists in a 96 well micro titer plate method based on the staining of sessile cells by crystal violet dye. In conclusion, the 96 well micro titer plate may be used as a rapid, reproducible and easy-handling method to study the kinetic of bacterial biofilm formation in BHI medium. This assay can be implemented for high throughput screening of a great number of bacterial strains or mutants by using different growth parameters (pH, temperature, culture medium) or for the rapid screening of anti-biofilm molecules (soluble or grafted on surface). In this way, the output of this method will be dramatically increased by its future automatisation. Hence, national studies and laboratories should use this method to detect biofilm forming pathogenic microorganism we encourage more studies to be done in this direction and use of this method in our country.

1.3 PROBLEM STATEMENT/PROBLEM OF THE STUDY:

Enhance level of resistance and prevalent formation of biofilm by *Escherichia coli and Pseudomonas aeruginosa* pathogens leading to poor treatment outcomes and multiple drug resistances. Knowledge of the complexity of biofilm-specific antibiotic tolerance and of the biofilm-specific dynamics of antibiotic resistance evolution will ultimately provide a basis for the development of better therapeutic solutions for patients suffering from chronic biofilm infections. This study will help to evaluate and assess the antibiofilm activity of the natural products extracts and probiotics against *Escherichia coli and Pseudomonas aeruginosa* to provide alternative treatment option to health care system and avoid rapid biofilm emerging drug resistances.

1.4 RESEARCH QUESTION/HYPOTHESIS:

- **NULL HYPOTHESIS:**

  There is no antibiofilm forming activity of natural product and probiotics.

- **ALTERNATIVE HYPOTHESIS:**

There is antibiofilm forming activity of natural product and probiotics.

1.5 OBJECTIVE OF THE STUDY

1. To evaluate the biofilm forming activity of *Escherichia coli* and *pseudomonas aeruginosa.*
2. To determine the antibiofilm activity of natural products i.e. pomegranate *Punica granatum L.* and Mango leaves *Mangifera Indica L.*
3. To determine the antibiofilm activity of probiotics i.e. *Lactobacillus acidophilus.*
4. To evaluate the synergistic activity of Natural product and probiotics.

1.6 SIGNIFICANCE OF THE STUDY:

To find out the biofilm forming activity of *Escherichia coli* and *Pseudomonas aeruginos*a and antibiofilm forming activity of probiotics and natural products and there combine synergistic effect , in our setup as an alternative therapeutic option for treatment modalities. The study will highlight on the patterns and effective use of natural products and probiotics against *Escherichia coli* and *Pseudomonas aeruginosa.* The formation of biofilm by these bacteria has decreased the likelihood of antibiotic action and developed a protective defense mechanism against it. Therefore, a study like this will immensely help. In order to evaluate the antibiofilm activity of natural products and probiotics *against Escherichia coli* and *Pseudomonas aeruginosa* in this part of the world where infections against these pathogens are common, this study hold a lot of significance.

This study will build a foundation in detecting a significant solution towards large number of multi drug resistant infections being caused by *Escherichia coli* and *Pseudomonas aeruginosa* and to evaluate their synergistic anti-biofilm effects of natural product and probiotics (*Lactobacillus acidophilus*) against *Escherichia coli* and *Pseudomonas aeruginosa.*

CHAPTER NO 2

LITERATURE REVIEW

Antimicrobial resistance (AMR) is an evolutionary response of different bacteria to withstand antimicrobial drugs introduced into their environment. In 2020, the COVID-19 pandemic has clearly demonstrated how fragile our world is and how infections do not respect borders. However, we have long been living in a silent pandemic of AMR (Jasovsky et al., 2016). Each year, infections caused by resistant bacteria cause 68,000 deaths in the Europe and the United States combined (Cassini et al., 2019; CDC, 2019), and are contributing to55 billion economic loss in the United States and to 1.6 billion in the EU/EEA annually (Ahmad and Khan, 2019). Although the AMR problem is not evenly distributed across the globe (Klein et al., 2019), there is no single country on Earth that can safely state that it will not be affected by the AMR spread.

The first warning of a possible catastrophe came from Fleming (1945) in his acceptance speech for the Nobel Prize for the discovery of penicillin, the first industrially produced antibiotic. Although he did not anticipate the spread of antibiotic resistance worldwide, he was the first to recognize the threat of resistance to those who depend on

These drugs. In 2019, the Centers for Disease Control and Prevention (CDC) released a report indicating that we have entered the post-antibiotic era (CDC, 2019). [Wei Wang et al 2018] Some bacteria are inherently resistant to some antibiotics due to their cell wall structure, efflux pump activity or the presence of porins (Eichenberger and Thaden, 2019). In this case all strains within the species are insensitive to a given antibiotic. Acquired resistance, on the other hand, occurs when some of the strains within the species become resistant to the antibiotic to which they were previously sensitive. Mechanisms include mutations in existing genes, for example in intracellular targets (Musser James et al., 2020) or central metabolic genes (Lopatkin et al., 2021) or the acquisition of new antibiotic resistance genes (ARG) by horizontal gene transfer (HGT) (Eichenberger and Thaden et al., 2019).

The latter enables intra- and interspecies transmission of ARGs and is responsible for an AMR pandemic (von Winters dorff et al., 2016; Sun et al., 2019). Although ARGs existed long before the discovery and spread of antibiotics (Hall and Barlow 2017) and (Allen et al., 2016), our irresponsible use of antibiotics in animal husbandry, overuse of antibiotics in healthcare, inadequate wastewater treatment, and poor sanitation in low- and middle-income countries have led to the widespread spread of resistant bacteria around the world (Prestinaci et al., 2015; Holmes et al., 2016; Ma et al., 2020).

Escherichia coli one of the most common gram-negative bacterial pathogen present in both clinical and epidemiological environment. Over the past years, several successful high-risk and multi-drug resistant strains of these pathogens have emerged, largely due to increasing selective pressures for the use in antibiotics. [Bin Liu and Axe et al., 2022] These strains show greater suitability and pathogenicity, and are effective in transmitting and colonizing capability, global scattering due to well organized distribution, and provide resistance to various antimicrobial agents. [Yossi paitani et al., 2018]

(Amy A O'Callaghan et al., 2021) describes that *Escherichia coli* reside in the intestines of healthy individuals and animals, facultative anaerobe in colon and human fecal matter. Also the characteristics of *Escherichia coli* are ferments lactose and produce pink color colonies on MacConkey Agar. *Escherichia coli* strain O157: H7 do not ferment sorbitol, which serves as an important benchmark that distinguishes it from other strains of *Escherichia coli* on EMB agar, as it characteristically produce diagnostic metallic

green sheen. It is Indole positive, give rise to indole from tryptophan, It is motile, It decarboxylates lysine, It uses acetate as the only source of carbon. (Nicole Jackson et al., 2021)

Most strains of *Escherichia coli* are innocuous or cause diarrhea for a relatively short period of time. The prominent causes of *Escherichia coli* are enteritis, urinary tract infection, septicemia and other clinical infections, such as neonatal meningitis. However, some strains such as *Escherichia coli* O157: H7 are the most common cause of severe stomach cramps, bloody diarrhea, and vomiting. You can be exposed to *Escherichia coli* through contamination of food or water, nearly raw vegetables and half cooked ground beef. Healthy adults generally recover from *Escherichia coli* O157: H7 infection within a week. Juvenile children and older adults are at an increased risk to develop a life-threatening form of kidney failure (Zou, Y., Duan, N., et al., 2018)

During the year 2000-2010, a significant increase in the use of antibiotics was observed globally. The increase in population, increased access of individuals to antibiotics, and the socioeconomic status were found to be the primary reasons in the development of antibiotic resistance (Morrill & LaPlante, 2015) Community-acquired uncomplicated urinary tract infections (UTIs) account for a large proportion of infectious diseases in females [Gupta K. et al.,2016], and a substantial amount of oral antibiotics is prescribed on a daily basis to treat UTIs among females in community-based outpatient clinics. However, the rate at which *E. coli* strains are becoming resistant to the vast majority of antibiotics is increasing worldwide.

In addition, gram negative rods harbor gene(s) conferring resistance to almost all antibiotics [Kang C., Kim J et al .,2018] and plasmids harboring these resistance determinants can be transferred between bacteria, even between species, such that the acquisition of resistance to new antibiotics may only be a matter of time. Therefore, it is much more important to recognize practical rationales, including prescribing antibiotics when there is evidence of an infection, promoting appropriate use of antibiotics and increasing efforts for preventing UTIs. Following such strategies is essential because the abuse or misuse of antibiotics can lead to resistance via the emergence of mutant strains [M., Choe H. et al., 2016], and unresolved, relapsed UTIs tend to be resistant to previously used antibiotics [Stief C. et al., 2017]

Because of their extra ordinary ability to fine-tune their physiology and metabolism, bacteria adapt quickly in available resources and environmental variation. It can disclose new aspects of bacterial biology. [Sylvie letoffe et all, 2017] In nature, most bacteria can attach themselves to various surfaces and form biofilms [Donlan R.M. et al.,2017]. A biofilm is a complex assemblage of bacteria enclosed in a self-made matrix of extracellular macromolecules (EPS) and is one of the key strategies for species survival urging unexpected changes in living conditions such as temperature fluctuations and nutrient availability [Wozniak D.J et al.,2017]. Bacteria in biofilms can evade host immune responses and are 1,000 times more resistant to antibacterial treatments than their plankton counterparts. *P. aeruginosa* is a well-known former biofilm, making it an excellent model for studying biofilm formation [Crespo A et al., 2018]. Resilient biofilm is an essential weapon for P. aeruginosa to compete, survive and dominate in the pulmonary polymicrobial environment of cystic fibrosis [Oluyombo O. et al., 2019].

P. aeruginosa is also effective on various surfaces including medical devices (urinary catheters, implants, contact lenses, etc.) [Wilton M et al., 2016] and food industry equipment (mixing tanks, barrels and tubes). Therefore, a better understanding of the composition and structure of biofilms, as well as the molecular mechanisms underlying the antibiotic resistance of bacteria growing in biofilms, is essential to design strategies to develop biofilms effective strategy for the management, prevention and, more importantly, eradication of biofilm-associated infections.[Ahrens, C. H. et al ., 2022] Anton Von Leeuwenhoek was the first scientist that described the term Biofilm during the seventeenth century as microbial aggregates present of the tooth surfaces, collected by scraping the surface called plaque.

These bacterial exopolysaccharides (EPS) was initially discovered by Louis Pasture in the year 1861, in wines as microbial products and named as dextran. Later on in 1878, Van Tieghem found about dextran producing strains Leuconostoc mesenteriodes. Characklis, in 1973 define biofilm as persistent and resistant to disinfect. The term “Biofilm” was introduced by a Canadian microbiologist, Bill Costerton in 1978. He defined it as "a structured community of bacterial cells wrapped in a self-made polymer matrix and attached to an inert or living surface." (J Genet Eng Biotechnology. 2021)

The biofilm is a complex aggregate of bacteria encased in a self-generated matrix of extracellular polymeric substances (EPS) and is one of the key strategies for the

survival of species against unexpected changes of living conditions such as temperature and nutrient availability [Philip S. Stewart, et al.,2019]. Numerous studies has been shown that *P. aeruginosa* biofilm matrix primarily encompasses polysaccharides, extracellular DNA (eDNA), proteins and lipids. The matrix, which is responsible for 90% of biofilm biomass, acts as a scaffold for adhesion to biotic and abiotic surfaces and shelter for encased bacteria in harsh environmental conditions (antibiotics and host immune responses). It also provides a repertoire of public goods including essential nutrients, enzymes and cytosolic proteins for the biofilm community. The matrix also facilitates cell-to-cell communication [Doiron, A.,et al., 2019]

Biofilm formation is a natural phenomenon, which occurs when the density of the population of bacterial cells reaches a certain threshold and so as a result leads to the formation of multiple micro-colonies. This process is further classified into four different phases, i.e., surface attachment, sessile growth phase which is controlled by intercellular interaction or colonization, biofilm maturation, and detachment [Schild, S.etal(2021)] Studies conducted over the last 30 years show that bacteria live in biofilm growth mode in most environments, but the floating single-cell state is considered to be in transition. The migration of bacteria from the plankton growth mode to the biofilm state relies on the production of adhesins and extracellular matrix components that act as scaffolds and trap the bacteria in the biofilm and also on the growth conditions and age of the biofilm. Many *Pseudomonas aeruginosa* strains can synthesize three exopolysaccharides Pel, Psl, and alginate that serve as matrix components for biofilm formation. [Oana Ciofu et al and Tim Tolker-Nielsen et al 2019] Antibiotic treatment cannot eradicate these biofilm infections due to the development of their inherent antibiotic resistance and mutant antibiotic resistance. Biofilm resistance to antibiotics is multifactorial and includes physical, physiological and genetic determinants, but bacterial antibiotic resistance in biofilms is caused by mutations, resulting in high levels of bacterial antibiotics. Caused by repeated exposure to substances in 2019 eradicate of these biofilm infections with antibiotics are not completely possible due to their intrinsic tolerance to antibiotics and the development of mutational hindrances to antibiotics and assertive to repeatedly expose to high levels of antibiotics. [Castaneda, P et al., 2016] Studies showed that reduced penetration of antibiotics from the polysaccharide matrix into biofilms evacuation to extracellular space antibiotics via pump or pollins Increased outflow of change in phenotype of cells that are involved in biofilm formation.Presence

of endless cells in the biofilm changes in the inside environment due to regulatory QS Biofilm due to the gradient of oxygen and nutrients throughout the biofilm. Antibiotic inactivation by modification such as enzymatic phosphorylation post-translational modification or gene mutation [K. Brindhadevi, et all , 2019] This diversity of the structural components of the biofilm is reinforced by the development of resistance to antibiotics, which makes their eradication more difficult. Antimicrobial agents in conventional use limit the range of targets and limited effectiveness in it, underscoring the need for research into alternative therapies such as anti-adherent compounds, phytochemicals, natural products, probiotics and Nanomaterials for effective drug delivery to limit biofilm growth. [Černáková, L et al., 2019]

The anti-biofilm activity of plants derived compound has been studied. In order to obtain new anti-biofilm agents, no of researches have turned to natural products to contribute to drug discovery. Recently, as verified by many researchers, that plants extracted compound found to have anti-biofilm properties. Therefore, inhibition of biofilm is envisioned as a new target for the disease and preventive therapies. Blocking biofilm QS reduces the toxicity of harmful pathogens, makes them more susceptible to applicable treatments, and facilitates removal by the host's defense mechanisms. However, the discovery of therapeutically effective and safe anti-biofilm compounds is still in its infancy and requires further investigation using natural products. A screening program has identified several potential anti-biofilm plants, including Mangifera indica L. demonstrated by [Mishra, R. et al., 2022] In the past, natural products have a source of numerous chemicals with different biological and chemical activities and have played a role in drug discovery in many areas, including infectious diseases. Numerous studies include the potential use of natural things as therapeutic and as a chemical probes. The formation of Bacterial films in an infectious environment is a major factor in the resistance of many bacterial infections, which confer greater resistances to many drugs and the host's immune system. There is still no approved strategy for combating bacterial infection. [Roberta J Melander et al 2020]

Many of the natural products an there anti-biofilm activity has been identified from different plants, microbes, and marine life. Previous study also shows that natural products from plants, bacteria, fungi and marine organisms can be effectively used as anti-biofilm agents, inhibit the formation of polymer matrix, inhibit cell adhesion and binding, and reduce the production of biofilm factors. Virulence prevents the quorum

sensing network. [farncesca Guzzo et all; 2020] Much attention is paid to medicinal plants, which highlighted the need to introduce traditional medicinal products in policies of drug, plants serve as the great sources for wide range of plants. Study related to plants to better understand the properties, and effectiveness for benefits. The presence of components in herbal plants made them useful in curing human diseases. [Akhtar, J. (2020)]

The pomegranate Punica granatum L. an ancient fruit, mainly grows in western Asia, although it is also grown in the Mediterranean and other parts of the world. Its use has been linked to numerous health benefits since ancient times. In recent years, several different researches have demonstrated its beneficial physiological activities, particularly its antioxidative, antimicrobial, antibiofilm and analgesic properties. [P kandylis, et al, 2020] Currently, national and international studies and that focus primarily on peels and seeds of pomegranate, with little research on its flowers. Pomegranate flower contain polyphenols which has an important antioxidant effects and functions in lowering blood pressure and blood lipid contant, preventing atherosclerosis, and suppressing the growth of cancer cells. Ellagic acid is a polyphenol that is widely distributed in various berries and nuts, along with various form that dervie free ellagic acid. Therefore, this experiment was conducted to analyze the polyphenols and ellagic acid contained in pomegranate flowers. The extraction methods include leaching, infiltration and reflux which usually have longer extraction time and reduced extraction yields.Also the phenolic compounds most frequently found in the Punica Granatum L. and its derivatives. These anthocyanins are phenolic compounds in commercial red fruit juices are also good sources for these compounds for consuming. [S.Benslimane et al 2020] The ellagitannins in pomegranate are disolved by the gut microbes to form phenolic compounds. Then ellagic acid when absorbed into the blood while ellagitannins are not able absorbed and metabolized to urolithines. Reported as a by-products of pomegranate and inhibit the growth of Escherichia coli, Pseudomonas aeruginosa, Clostridia and Staphylococcus aureus as well as the growth of beneficial bacteria such as Bifidobacterium spp and Lactobacillus spp. [valeria sorrenti et al , 2019] This fruit has also been used in traditional medicine to treat dysentery, diarrhea, and respiratory diseases. [Gianmaria fabrizio ferrazzano et al., 2017]

This study shows that pomegranate extract and its main ingredient, ellagic acid, have antibiotic activity. The various bioactivities of pomegranate are attributed to the presence of tannins, especially ellagitannins which is the main component of pomegranate

extract. The two main members of the ellagitannins (viz. punicalagin and ellagic acid) have antibacterial, antioxidant, anticancer and anti-inflammatory properties (Adams et al and Glazer et al 2016). Since it is already used as a food, pomegranate has an added benefit when used medicinally as it is non-toxic; Reports have confirmed this situation for bioactive compounds from pomegranate.

For the first time, pomegranate extract has been shown to inhibit biofilm formation and disrupt preformed biofilms. Several reports indicate that biofilms are resistant to inhibitors under nutrient-restrictive or nutrient-depleted conditions, in contrast to their susceptibility to similar inhibitors under nutrient-depleted conditions. Nutrient rich conditions (Maeda et al. 2017). Pomegranate peel extract has been shown to be effective in the gastrointestinal tract under both nutrient-rich and nutrient-deficient conditions, which is an advantage for pomegranate extract as a bioactive agent.

Pomegranate peel extract or ellagic acid may be utilized in a medical putting to deal with topical infections and wounds generating stepped forward healing outcomes. They can also have the capability to be evolved as sanitizing marketers to sterilize surgical units and different clinical devices. However, some studies show that synergistic interest of pomegranate extract with antibiotics has proven stepped forward efficacies towards pathogens that are immune to antibiotics (Endo et al. 2018). Hence, the combinatorial use of Pomegranate peel extract with antibiotics may want to assist to fight drug-resistant pathogens and tool related infections in humans.

Another antibioiflm activity of plant product/extract is Mangifera indica leaves has been extensively studied. In order to and new anti-biofilms, many people are considering natural products as a contribution to drug. It has been found that medicinal and nutritional plants possess anti-biofilm properties. Resistances to the biofilm was envisaged as a target for the establishment of anti-biofilm therapies, as clogging the biofilm effect weakens its pathogenicity. [FM hussain et al, 2017] The Anacardiaceae family is widely used medicinally that have antibiofilm and antimicrobial properties. Mangifera is the common to most of the region and has a huge world production. Its seeds have an antimicrobial effect when formulated for topical use in humans and have inhibitory action against bacteria. [andressa GB Manzur et al] Mangiferin has a wide range of uses and has no reports of its side effects. It is abundant in the leaves and stems of the mango tree Mangifera Indica L. [Ngampuk tayana1 et;all , 2017]

[Manzur, A. et al 2020] In his research, we used the leaves of *M. indica* that presents great bioavailability in tropical and sub-tropical regions and produced simple extracts of easy preparation. We observed the inhibition of *S. aureus* strains and reduction of mature biofilms and, using a plant extract, is the first study recording reduction of adhered biofilms in teacup rubber, which represent an initial and relevant point of contamination in the processing of dairy products. The extracts of *M. indica* leaves show antibacterial effects that could be attributed especially to the tannins, because after removal of these compounds, the extracts reduced its effectiveness.

Mango Leafs exhibit exceptional biological, medicinal, and metabolic properties. Mango Leafs are otherwise considered as a waste material generated mainly through the pruning of mango plants; in reality, they are a most significant resource containing a wide variety of bioactive compounds (phenolics and essential oils), crude protein, dietary fiber, minerals, and vitamins. The various bioactive compounds present in the Mango Leafs include phenolic compounds, flavonoids, benzophenones, sesquiterpenes, saponins, xanthones, tannins, terpenoids, and alkaloids. Mango leafs extract is found useful for the treatment of common diseases like diarrhea, chronic ailments like diabetes, and fatty liver disease. Mango Leafs extract possessed strong anti-proliferative activity against pancreatic, breast, human colon carcinoma, and other types of cancers. The extracts of mango leafs are also crucial in as an anti-obesity agent and demonstrate hepatoprotective action. There are numerous vital chemical compounds present in mango leaves, which are instrumental to performing various metabolic, bacteriostatic, and antimicrobial activities. Some of these include gurjunene, trans-caryophyllene, humulene, selinene, and so on. Mangiferin present in mango leafs was also found to mitigate the oxidative stress involved in the treatment of numerous pathologies. Mango Leafs are thus a potential source of cost-effective food supplements and nutritive ingredients for improving human health and curing acute and chronic diseases.

These studies concluded that the plant extracts exhibiting antibacterial property coupled with antibiofilm activity. Therefore, these extracts might serve as potential candidates for developing biofilm inhibitors and may act as potent drugs against antibiotic-resistance biofilm-producing bacteria.

Probiotics have beneficial bacteria which when administered provide a health benefits to the host. Probiotics that are used in the preparing of numerous functional foods, including dairy products (milk, yogurt, cheese,) and non-dairy products (meat and

grain fiber snacks) Juices and other products. Probiotics have a positive effect on health by preventing various actions such as preventing the adhesion of pathogens, the metabolites produced and the modulation of the immune system through the production of immunoglobulin antibodies. The most common probiotics belong to the genera *Lactobacillus* spp. Probiotics provide numerous health benefits through a variety of mechanisms that involve a variety of active components. The probiotic effects include inhibit the reproduction of pathogenic bacteria by altering the pH and decreasing oxygen caring ability, which leads to less intestinal conditions and inhibitions. Probiotics synthesize important nutrients such as vitamins, amino acids and enzymes and improve the availability of nutritional components, stimulating the body's immune system and improving the carbohydrates activity. [Plaza-Diaz, J., Ruiz-Ojeda, F. J., Gil-Campos, M., & Gil, A. (2019)] Many GIT diseases, like watery diarrhea, irritable bowel syndrome and inflammatory bowel disease causes imbalance of intestinal flora, an important factor in bacterial infection.

Recent researches highlight the antibiofilm feature of Lactobacillus genus. Osama et al. (2017) demonstrated antimicrobial and antibiofilm action of *Lactobacillus rhamnosus* and *Lactobacillus gasseri* strains against *Pseudomonas aeruginosa, E. coli* and *Staphylococcus aureus*, three pathogens commonly involved in persistent infections associated with biofilm formation. As also, [Abdelhamid et al. (2018)] identified effective results in the biofilm eradication from multidrug resistant (MDR) *E. coli* isolates through bioactive compounds acting as antimicrobials. Fernandes (2019) demonstrated that cell free supernatant produced by standard and commercial probiotic strains *(L. acidpohillus LA14, L. acidophillus ATCC 4356 and L. rhamnosus ATCC 9595)* has potent bactericidal and antibacterial activity against *E. coli* MDR isolated from fish fillet samples. However, the results obtained are due to several mechanisms of action that require further investigation. For example, an effective strategy to avoid the first step of biofilm formation until biosurfactant application, alters microbial adhesion by modifying the properties of biosurfactants, physicochemical properties of the cell surface (Gmez et al., 2016 ; Sharman and Saharan, 2016 ; Kaur et al., 2018). The activity of Antibiofilm is also involved in the production of bacteriocins, which are antibacterial peptides produced by some bacteria, which work by preventing biofilm formation and have great application potential food biological preservatives, as they have a broad spectrum against many food spoilage microorganisms, including *E. . coli* (Mathur et al., 2017; Novik and Savich,

2019). In human beings, probiotic *Lactobacillus* strains can live to tell the tale after oral management and effectively colonize one-of-a-kind components of gastrointestinal tract, however the upkeep of the pressure into intestine surroundings appears to relies upon on consumption frequency of probiotic preparation (Saxelin et al., 2010; Balgir et al., 2013; Arioli et al., 2018; Taverniti et al., 2019). That might also additionally provide an explanation for while a number of those probiotic lines come to randomized scientific trials, the consequences are every so often inconclusive, and those fascinating consequences placed on doubt the scientific cost of this remedy method (Chau et al., 2018; Ten Bruggencate et al., 2015; Hegar et al., 2015; Piescik-Lech et al., 2013).

However, it ought to be taken into consideration that the anti-infectious motion of *Lactobacillus* isn't similarly powerful for all sickness prevention or remedy indication. Several elements are concerned on efficacy of probiotic lines, amongst them ok doses for suitable periods, except illnesses kind and mechanisms of motion of lines (Liu et al., 2019; Islam, 2016; Floch et al., 2015). In different words, the precise pick of probiotic ought to be pressure-specific, and ought to now no longer be generalized even amongst lines into the identical species (Sniffen et al., 2018; McFarland et al., 2018; Liu et al., 2018).
Antibiotics are currently used to treat imbalances in the intestinal flora. However, the abuse or abuse of antibiotics can lead to the development of drug resistance, which is a health related problem worldwide. Other problem that include by the formation of infectious bacteria, antibiotics are less effective in treating human and animal infections. Highly protected to not prone to changes, low activity, and resistant to drugs. Therefore, probiotic strains that have both antibacterial and antibiotic properties may be more effective in treatment as a clinical benefit of treatment. This will help in future studies by synergistic effects of both in treating these pathogenic biofilms. Research approves the effectiveness of combine synergistic use of natural products and probiotics is an effective and therapeutic option available now a days, that help overcome these biofilm formation by pathogenic bacteria which decrease the antibiotic efficacy.

OPERATIONAL DEFINITIONS:

Biofilm, Antibiofilm, natural products, probiotics, 96 well micro titer plate, *Escherchia coli, Pseudomonas aeruginosa.*

BIOFILM:

Biofilm are sessile, free floating microbial communities that are formed when come in contact with numerous medical appliances (abiotic) and human organs (biotic) surfaces and forms extracellular polymeric substances (EPS) due to which it provides a huge chance to different number of microorganism to colonize the surface and inhibit most of the antibiotics that help overcome these pathogenic microorganisms. [Bipasa Bose et;al 2022]

ANTIBIOFILM:

The anti-biofilm effects of natural products and probiotics that rely on inhibiting the formation of extracellular polymer matrix (ECM), as it suppress cell adhesion and attachment, interrupting extracellular polymeric substances (ECM) generation and decreased the virulence factor that are produced as a result, thereby it blocks the biofilm development and its duplication. [Lee, J. et;al 2021]

NATURAL PRODUCTS:

Natural products are herbal remedies that act as an effective cure and provides protection for most of the disease and are used largely as an alternative for of antibiotics. Natural plants contain flavonoids and ellagic aicds that are very much similar in properties of synthetic antibiotics. Most of the pharmacological drugs are derived directly or indirectly from plants and leaves. Many of these synthetics drug has same chemical characteristics that are also found in plants.[., Hu, W. et;al 2019]

- **Mangifera indica (Anacardiaceae)** is an important tropical fruit can considered as king of fruits, it has been found out that *mangifera indica* contain various medicinal effects including its antibiofilm activity has been determined. In order to evaluate *mangifera indica* leaf extract as effective antibiofilm forming activity in relation to various organism. And it had proved significant inhibitory effect against the biofilm formation. [Abulreesh, H. et;al 2017]

- **Pomegranate (Punica granatum L.)** has interesting sources of phenolic content and its presences in different fruits. Pomegranate has increased benefits and is a potential source in providing health promoting benefits. Pomegranate exhibits a broad range of bioactivities including its antibiofilm effects, which helps to overcome numerous antibiotic resistances. [Pramadita, J.et;al 2019]

PROBIOTICS:

Probiotics are live microorganism referred to as beneficial effects on human health when they are administered in adequate amount. Probiotics can release bioactive substances that can inhibit the growth of many biofilm producing pathogenic organisms. Some strains of probiotics like *lactobacillus* and *bifidobacterium* are widely used as a probiotic in several food and dietary supplements to improve the health. They are helpful in treating many pathogenic disease and conditions that affect humans and animals. Probiotics also help in antibiotic resistant organisms and it's the leading benefit for use of probiotics as an alternative to combat such resistance. [Frassinetti, S. et;al 2020]

96 WELL MICRO TITER PLATE:

A microtiter plate or microtiter plate a multiple well is a flat plate with several "wells" that are used as small test tubes. The microtiter plate has become a standard tool in laboratories for analytical research and clinical diagnostic tests. A very common application is the ELISA (Enzyme-Linked Immunosorbent Assay), the basis of the most modern medical diagnostic tests in humans and animals. A micro titter plate typically has 6, 12, 24, 48, 96, 384 or 1536 sample wells arranged in a 2:3 rectangular matrix.

[Goarant, C. et;al (2020).]

ESCHERICHIA COLI:

Escherichia coli and other facultative anaerobes make up about 0.1% of the gut microbiota and fecal-oral transmission is the main pathway through which strains of pathogenic bacteria cause disease. Cells can survive outside the body for a limited period of time. This makes them potential indicator organisms for testing environmental samples for faecal contamination. However, *Escherichia coli* in a particular environment can survive for many days and grow outside of a host. Generates ATP through aerobic respiration when oxygen is present, but it is able to switch to fermentation or anaerobic respiration when oxygen is not present. Strains that have flagella are mobile. The flagella have a peritric arrangement it also binds to the microvilli in the gut and clears them up through an adhesion molecule known as intimin. [Strachan, N., & Bono, J. L. et;al (2022)]

PSEUDOMONAS AERUGINOSA:

Pseudomonas aeruginosa is a frequently encapsulated, aerobic, gram-negative rod-shaped bacteria that can cause diseases in animals and plants (including humans). *Pseudomonas aeruginosa* is a species with important medical significance, with its ubiquitous existence, internal antibiotic resistance mechanism and its interaction with such as hospital acquired infections (such as concomitant pneumonia and sepsis). Known for its association linked with other serious diseases and syndromes. It is found in soil, water, skin flora, and most man-made environments around the world. [Pang, Z., Raudonis, R.et;al 2019]

CHAPTER NO 3

METHODOLOGY

3.1 STUDY DESIGN:

This observational cross-sectional in-vitro study was carried out to evaluate the biofilm forming activity against the isolated clinical specimens of gram negative rods i.e. *Escherichia coli* and *Pseudomonas aeruginosa* and to determine anti-biofilm forming activity of natural product extracts and probiotic i.e. *Lactobacillus acidophilus* and to evaluate their synergistic activity of natural product extracts and probiotics against above mention organism.

ETHICAL APPROVAL:

Since the current study involved human participants, therefore ethical approval is prime consideration of this research. Approval for the study was taken from faculty of research committee, Ethical Review Committee (ERC) of Bahria University Medical and Dental College and PNS Shifa Hospital (PNS) to project subjects' welfare and dignity. Next to this inform consent was also taken from patients.

3.2 SUBJECTS/ANIMALS:

In order to carry out the study, the samples were collected from amongst indoor and outdoor patients at PNS SHIFA Hospital, Karachi. The age group of individuals in this study includes 10-50 years. Informed consent was obtained from the patients.

3.3 SETTING:

Different samples of *Escherichia coli* and *Pseudomonas aeruginosa* were collected from different wards (Surgery, Medicine, Gynecology, ENT and Pediatrics) and ICU (intensive care unit) at PNS Shifa hospital. Next to this, the specimens received in lab will be inoculated on blood agar, MacConkey's agar culture plates. Culture plate will be inoculated at 37 ℃ in incubator for 24 to 48 hours. Identification of *Escherichia coli* and *Pseudomonas aeruginosa* will be done by colony morphology, gram staining, TSI and different biochemical test analysis. After identification the samples were processed on 96 well micro titer plate method and serial dilution method.

3.4 INCLUSION CRITERIA:

Escherichia coli and *Pseudomonas aeruginosa* were isolated from different clinical specimens of patient. Clinical specimens include Urine, Blood, Pus, CSF and respiratory specimens, Endo-bronchia wash. The specimens of age group from 10 to 50 years of age and both genders were received from different wards at PNS Shifa hospital, Karachi.

3.5 EXCLUSION CRITERIA:

- Repeated samples from same patient.
- HIV infected patients
- Any malignancy associated patients.

3.6 DURATION OF STUDY

September 2021 to May 2022.

3.7 SAMPLE SIZE ESTIMATION:

The approximated/estimated sample size is determined by using the method of sample size (WHO World Health Organization) by taking 95 % confidence interval, 5% margin of error.

FORMULA

Sample size (***n)*** **= [DEFF*Np (1-p)]/ [(d^2/$Z^2_{1-\alpha/2}$*(N-1) + p*(1-p)]**

n=0.97574/0.0065

n=150

Calculated sample size is (150) patients.

(Ref: Varriale, L., Dipineto, L., Russo, T. P., Borrelli, L., Romano, V., D'Orazio, S., .. & Santaniello, A. (2020). Antimicrobial resistance of Escherichia coli and Pseudomonas aeruginosa from companion birds. *Antibiotics*, *9*(11), 780.

3.7 SAMPLING TECHNIQUE:

Non Probability Convenient Sampling technique.

3.8 HUMAN SUBJECTS AND CONSENTS:

Samples from outdoor and indoor hospitalized patients in PNS SHIFA Hospital Karachi were included. Data was obtained about demographic characteristics like age groups and gender. Consent for participation was taken from thumb impression and signature after verbally explaining the project. In case of children, consent was taken from parents (as given in appendices c)

3.9 MATERIALS USED:

- **CLINICAL SAMPLES**

The clinical samples of patients included urine, blood, CSF, pus and respiratory specimens of patients admitted in PNS SHIFA HOSPITAL, KARACHI.

- **CULTURE MEDIA**

Blood agar, MacConkey's agar, TSI agar, different biochemical test.

- **EQUIPMENTS**

Autoclave, weighing machine, vortex, Hot air oven, Incubator, ELISA reader.

- **PERFORMA**

As mentioned in appendices.

3.11 PARAMETER OF STUDY

The study evaluated the biofilm activity of *Escherichia coli* and *Pseudomonas aeruginosa* and anti-biofilm forming activity of natural product extracts and probiotic *Lactobacillus Acidophilus* against *Escherichia coli* and *Pseudomonas aeruginosa* and there combine synergistic effect would be done using 96 well plate. Clinical data of the patients includes age, gender and clinical diagnosis.

3.12 PROTOCOL OF STUDY

All the data was entered in a specially designed Subject Evaluation Form. Permission will be taken from Institutional Ethical Review Committee. Informed consent from all the 150 patients was taken for this study.

Age, gender and hospital number of patients will be recorded on specially designed proforma. The specimens like urine pus, wound swab, sputum, blood, respiratory specimen and cerebrospinal fluid that were collected in microbiology lab PNS SHIFA Hospital Karachi.

The gram stain of the samples was performed to identify the gram negative rods by pink color of the colonies. All specimens like urine, blood, pus, CSF and respiratory specimens were inoculated on Blood agar, MacConkey's agar and TSI. Culture plates will be incubated at 37 ºC in ambient air for 24 to 48 hours. On blood agar, circular grey and moist colonies were observed, while on MacConkey's Agar circular, pink and lactose fermenting colonies were found for *Escherichia coli* and colorless colonies were found for *Pseudomonas aeruginosa*.

Further identification of organisms were done using TSI and different biochemical tests. A suspension equivalent to 0.5 McFarland of all isolates *Escherichia coli* and *Pseudomonas aeruginosa* were inoculated.

3.12.1 SAMPLE COLLECTION PROTOCOL:

- **URINE**:

The sterile container was given to the patient with brief instructions about the mid-stream urine collection.

Boric acid powder (0.1g/10ml of urine) was added in the container to preserve the sample.

The sample container was labeled and forwarded to the microbiology laboratory of PNS SHIFA HOSPITAL within 48 hours.

The sample (10ml) midstream was transferred aseptically into conical tube and centrifuged at the speed of 500-1000 g for approximately 5-mintues. The supernatant was separated and used for biochemical analysis.

The sediment in the conical tube was remixed by tapping at the bottom of the tube and transferred single drop to a sterile glass slide and spread it to make a thin smear and put coverslip on it.

The smear was allowed to air dry and protected from dust and insects and stained it through the gram technique. The smear was examined through microscope.

- **BLOOD**:

The vein was located in the upper arm and disinfected the site about 50 mm in diameter by 70% ethanol the top of the bottle was wiped by ethanol-ether swab.

About 20ml of blood was withdrawn and dispensed about into the culture medium bottle the contained about 25ml of broth.

The blood was mixed with the broth in EDTA container. The media was incubated and the culture was protected from sunlight until it was in incubation.

- **PUS**:

The pus specimen was collected by using a sterile swab and inserted the swab into the container that had amines transport medium.

Next, the stick of the swab was broken to cap the bottle tightly. The swab was made on a sterile slide for gram staining and allowed it to air dry by heat fix the smear.

- **SPUTUM:**

About 2mL purulent sputum was collected with a sterile wide-mouth container after the patients were momentarily instructed to rinse their mouths with water. Microbiological methods were not used on sputum specimens that had a lot of watery saliva. The container was labeled the sputum was smeared and analyzed right away to see

if it was suitable for culture. Specimens with more than 25 polymorphonuclear leukocytes and less than 10 epithelial cells were inoculated into MAC agar and Blood agar plates and incubated for 24 hours at 37°C.

- **Isolation and Preservation of *Escherichia Coli* and *Pseudomonas Aeruginosa* from Clinical Samples.**

Different clinical samples of subjects were taken and isolation of *Escherichia coli* and *Pseudomonas* colonies were done, these organism were kept preserved in nutrient agar with glycerol in a volume of 2ml in screw capped bottles at -20c as shown in the figure.

- **Identification of *Escherichia coli* and *Pseudomonas Aeruginosa*:**

Clinical samples of pus, urine, nasal swab, throat swab, blood and respiratory aspirate were taken and identification of gram negative rods were done by conventional phenotypic methods which include gram staining, culture media, triple sugar iron agar and different biochemical test.

3.12.2 GRAM STAINING:

- **PROCEDURE:**

The gram stain was performed by applying the samples from infected site onto a glass slide. To fixed smear of bacterium, the slide is then treated with crystal violet dye (primary stain) followed by the addition of a mordant (Gram's Iodine), rapid de-colorization with alcohol, acetone, or a mixture of alcohol and acetone and lastly, counterstaining with safranin.

For gram negative bacteria pink color rod-shaped colonies were observed on microscope.

(FIGURE NO 3.1)

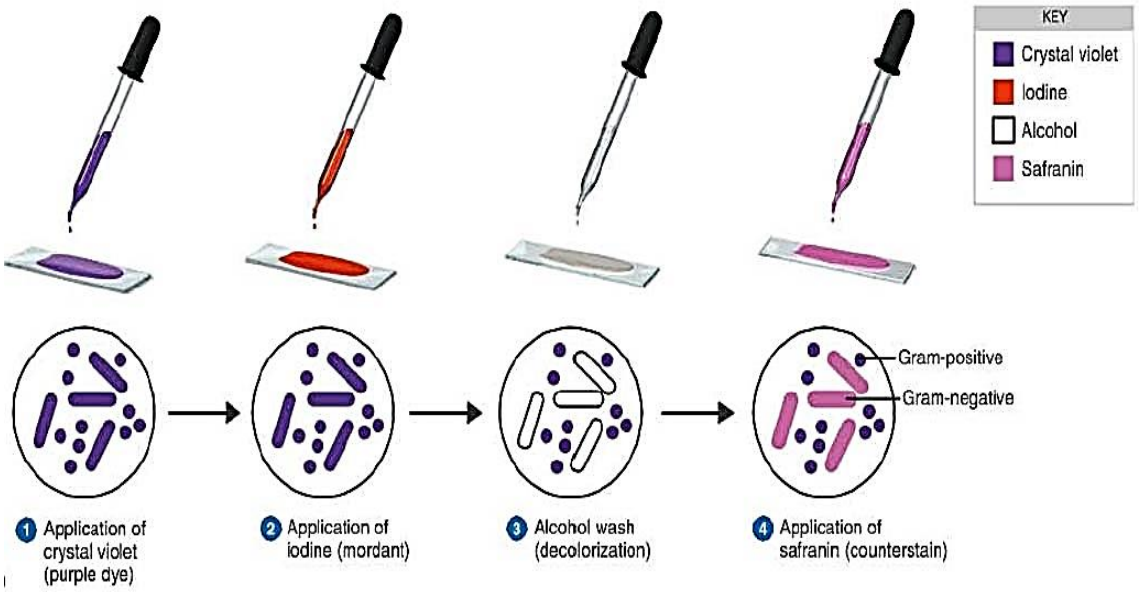


Figure No 3.1: Procedure of gram staining (Sapra A et al. Gram Staining. (Updated 2021 Aug 11)

3.12.3 CULTURE MEDIA:

- **MACCONKEY'S AGAR:**

MacConkey agar is used for the isolation of gram-negative enteric bacteria and the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria. Pancreatic digest of gelatin and peptones (meat and casein) provide the essential nutrients, vitamins and nitrogenous factors required for growth of microorganisms. Lactose monohydrate is the fermentable source of carbohydrate. The selective action of this medium is attributed to crystal violet and bile salts, which are inhibitory to most species of gram-positive bacteria. Sodium chloride maintains the osmotic balance in the medium. Neutral red is a pH indicator that turns red at a pH below 6.8 and is colorless at any pH greater than 6.8 agar is the solidifying agent.

Uses of MacConkey Agar

1. MacConkey agar is used for the isolation of gram-negative enteric bacteria.

2. It is used in the differentiation of lactose fermenting from lactose non-fermenting gram-negative bacteria. (Figure no 3.2)

Figure no 3.2: *Escherichia coli* (Lactose fermenting) Pink color colonies and *Pseudomonas aeruginosa* (Non-Lactose fermenting) pale yellow, Color-less colonies.

- **BLOOD AGAR:**

Escherichia coli a gram negative bacillus. That grows well on commonly used medium. Colonies are big, circular, gray, moist and beta hemolytic. Most Escherichia coli strains are non-pigmented. (Figure no 3.3)

Pseudomonas aeruginosa shows large, mucoid and metallic sheen colonies of pseudomonas beta hemolysis were observed when cultivation on blood agar in anaerobic conditions at the temperature of 37 C and duration of 24 hours. (Figure no 3.4)

Figure no 3.3: *Escherichia coli* colonies on Blood agar.

Figure no 3.4: *Pseudomonas aeruginosa* colonies on Blood agar.

3.12.4 BIOCHEMICAL TEST:

- **TRIPLE SUGAR IRON (TSI)**

In the context with positive test for organism, the triple sugar iron test (TSI) was assessed to evaluate ability of *Escherichia Coli* and *Pseudomonas Aeruginosa* to ferment sugar or not ferment sugar molecules and production of gas (hydrogen sulphide). Principally, the phenol red reagent and ferrous sulfate solution indicate the acidification of agar medium and hydrogen sulfide production by organism, respectively.

Initially, the bacteria that ferment sugar i.e. glucose which turns the medium acidic 8-12 hours by showing yellow appearances, whereas, butt of the tube in anaerobic condition, sustain the acidic condition due to presence of organic acid that are led by glucose fermentation. The slant turn the acidic medium into alkaline in aerobic state by oxidizing the fermentation products into CO_2 AND H_2O and peptone, the slant subsequently oxidized and produced alkaline amines.

In the present study, the 24 hour bacterial culture in Tryptic soya broth tube was used to continue TSI test assay. Initially the inoculation wire loop was sterile by heated in blue flame in vertical direction till it became red hot. Then allowed the wire loop to cool for 20-sec. the culture was taken in the needle, stabbed into medium of triple sugar iron tube and streaked slant-surface in back and forth direction of 24-hours at the temperature of 37C.

Followed by incubation period, the fermented dextrose was observed by red slant or yellow butt due to alkaline-acid reaction, fermented dextrose, lactose and sucrose were identified by formation of yellow slant and yellow butt due to acid-acid reaction, whereas, red slant and red butt (alkaline-alkaline reaction) was shown non-fermented carbohydrate.

The formation of gas molecules (CO_2) was showed by bubbles or cracks that formed in the agar, while, presences of (H_2S) turns the media black. (Figure no 3.5 and 3.6)

The 24-hour incubation time was strictly followed to prevent any false-positive outcomes because of oxidation of fermented product in aerobic conditions by sugar especially lactose and sucrose and the turning of acidic medium to alkaline state by slant.

Figure no 3.5: Triple sugar iron test on *Escherichia coli*

Figure no 3.6: Triple sugar iron test on *Pseudomonas aeruginosa*

- **OXIDASE TEST:**

The oxidase test is a biochemical reaction that assays for the presence of cytochrome oxidase, an enzyme sometimes called indophenol oxidase. In the presence of an organism that contains the cytochrome oxidase enzyme, the reduced colorless reagent becomes an oxidized into colored product. The dye is reduced to deep purple color. This test is used to assist in the identification of oxidase positive and negative oxidase organisms.

There are many method variations to the oxidase test. These include, but are not limited to, the filter paper test, direct plate method, swab method, impregnated oxidase test strip method and test tube method. (Figure no 3.7)

Figure no 3.7: Positive oxidase test on *Pseudomonas aeruginosa*

- **INDOLE TEST:**

The indole test is used to identify the capability of the specific pathogen to undergo the decomposition of tryptophan into indole. Bacteria that express tryptophanase enzyme, undergoes in the deamination and hydrolysis process of tryptophan amino acid. Indole generates by tryptophan deamination.

In indole kovac's reagent combination, the solution is turned from pale-yellow coloration to cherry red coloration on oily-layer tubes top. Escherichia coli is Indole positive while pseudomonas is indole negative.

- **CATALSE TEST:**

Catalase test can be used as an aid to the identification of Enterobacteriaceae. This test shows the presence of catalase, an enzyme that catalyzes the release of oxygen from hydrogen peroxide (H_2O_2). It is used to differentiate organism that produces an enzyme catalase, such as staphylococci, from non-catalase producing bacteria such as streptococci. Normally 3% H_2O_2 is used for the routine culture while 15% H_2O_2 is used for detection of catalase in anaerobes.

The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen

bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old.

3.12.5 METHODS OF NATURAL PRODUCT EXTRACT:

- **MANGO LEAF EXTRACT :**

Fresh mango leaves were obtained these leaves were properly washed with distilled water to remove dirt and air-dried for a week.

Then dried leaves were blended using a household electrical grinder. The leaf powder (250 g) was soaked in 500ml of methanol in a sterile conical flask and allowed to stay at ambient temperature for 72 h.

The bioactive components were extracted using the method above, the extracts were then filtered using What man no. 1 filter paper and the filtrate was concentrated in vacuo at 40ºC using a rotary evaporator (Heidolph apparatus) to evaporate methanol.

A series of dilutions of MLE were prepared by dissolving in 10% DMSO at different concentrations of 50 mg/ml, 100 mg/ml, and 150 mg/ml to be used in different experiments. ((Figure no 3.8, 3.9, 3.10, 3.11, 3.12 and 3.13)

[Refences : Hannan, A., Asghar, S., Naeem, T., Ullah, M. I., Ahmed, I., Aneela, S., & Hussain, S. (2016). Antibacterial effect of mango (Mangifera indica Linn.) leaf extract against antibiotic sensitive and multi-drug resistant Salmonella typhi. *Pakistan Journal of Pharmaceutical Sciences*, *26*(4), 715-719.]

Figure no 3.8: dried Mango leafs.

Figure no 3.9: powdered Mango leafs.

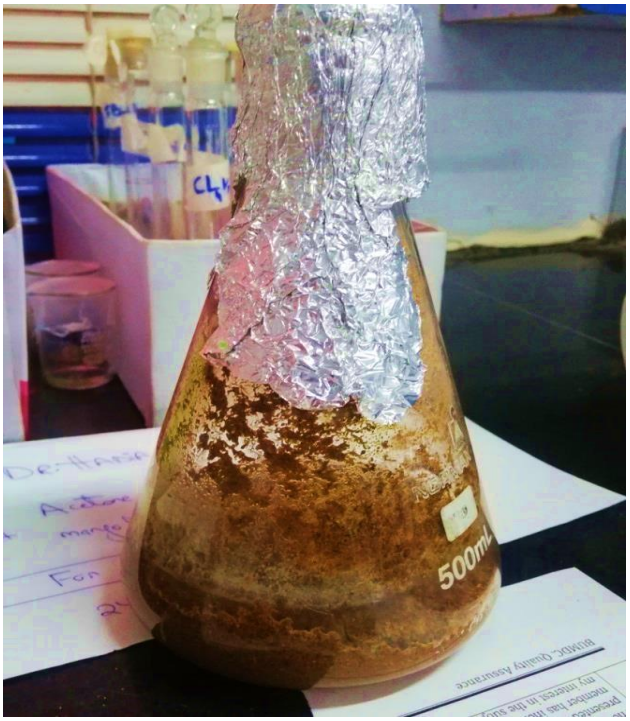


Figure no 3.10: leaf powder socked in methanol.

Figure no 3.11: extract using watman no 1 filter paper.

Figure no 3.12: evaporation of methanol from extract using Heidolph apparatus.

Figure no 3.13: Mango leaf extract dissolved in 10% DMSO.

➢ METHODFOR POMEGRANATE PEEL EXTRACT (PPE)

For sample preparation of pomegranate peel extracts, seeds were separated manually from the peel, then the separated peels were cut into small pieces, and then allowed to air-dry in a room temperature until constant weight was achieved.

Air-dried peel was then homogenized using a grinder until a fine powder was obtained a constant amount of powder peels (15 g) was used in order to extract natural bio active components when placed in beaker containing (150ml) methanol.

After the preparation of the macerated peels, the solution was filtered using 0.45 µm filter paper and then concentrated and evaporated to dryness under vacuum using a mini-rotary evaporator at 40°C using Soxhlet apparatus, until almost all the solvent was removed. The obtained dried extract from the solvent was stored at −20°C for further use in different test. (Figure no 3.14, 3.15, 3.16, 3.17, 3.18, 3.19 and3.20)

[ref: Salam Nasreddine, Amale Mcheik, Mohammad Abdalla Al-Seblani, Hawraa Shahrour, Mariam Hammoud, Ali Chokr, Influence of the Extractive Method of *Punica granatum* Peels in its Antibacterial and Antibiofilm Activities Against Gram-Negative and Gram-Positive Bacteria, *Journal of Diseases and Medicinal Plants*. Volume 4, Issue 6, December 2018]

Figure no 3.14: Dried Pomegranate Peels.

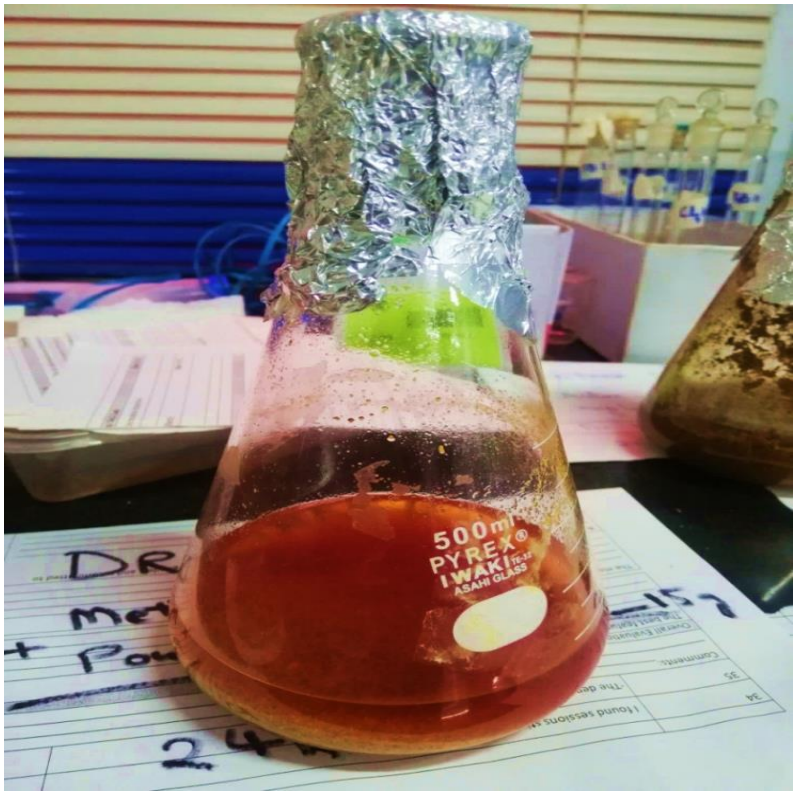


Figure no 3.15: Powdered pomegranate peels socked in methanol.

Figure no 3.16: extracted solution using 0.45µm filter paper.

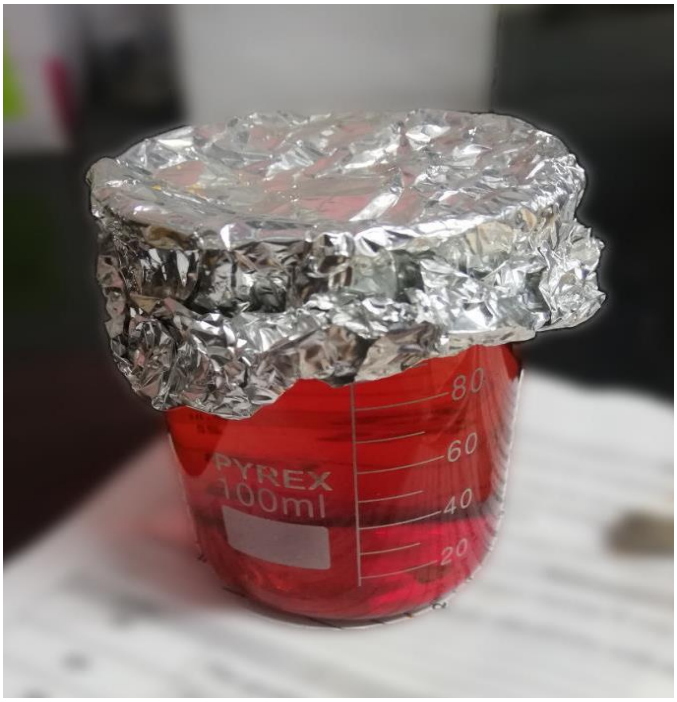


Figure no 3.17: filtered solution of dried peels.

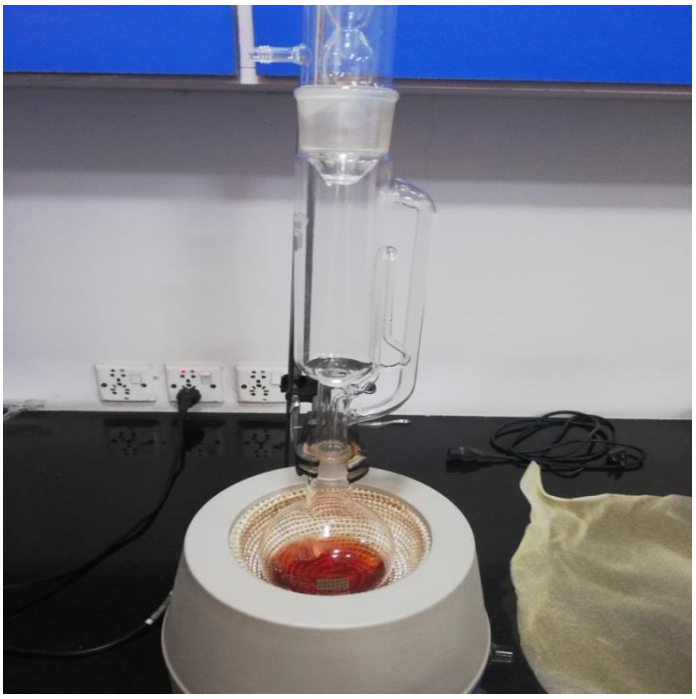


Figure no 3.18: evaporation of methanol using Socklet apparatus.

Figure no 3.19: evaporation of methanol.

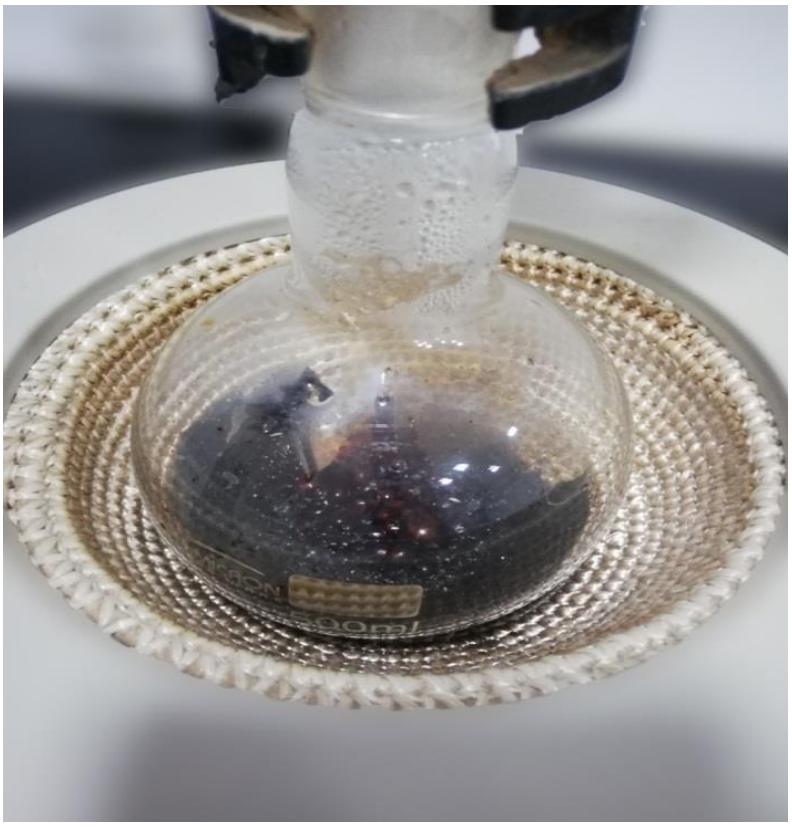


Figure no 3.20: complete evaporation of methanol (Soxhlet apparatus).

METHOD FOR BIOFILM DETECTION:

- **96 WELL MICRO TITER PLATE METHOD:**

The 96 well micro titer plate assay is most widely used and is considered as standard test for detection of biofilm formation all isolates were screened for their ability to form biofilm.

Isolates from fresh agar plates were inoculated in Tryptone soy broth (TSB) with 1% glucose and incubated for 24 hours at 37 C in stationary condition and diluted (1 in 100) with fresh medium.

Individual wells of sterile, polystyrene, flat-bottom tissue culture plates were filled with 0.2 ml aliquots of the diluted cultures, and only broth served as control to check sterility and non-specific binding of media.

The 96 well micro titer plates were incubated for 24 hours at 37°C. After incubation, the content of each well was gently removed by tapping the plates.

The wells were washed four times with 0.2 ml of phosphate buffer saline (PBS pH 7.2) to remove free floating planktonic bacteria; then 25 µl of 1% solution of crystal violet was added to each well (this dye stains the cells but not the polystyrene plates.

The plates were incubated at room temperature for 15 minutes, rinsed thoroughly and repeatedly with distill water. Adherent cells, which usually formed biofilm on all side wells, were uniformly stained with crystal violet.

Crystal violet-stained biofilm was solubilized in 200µl of 95 % ethanol (to extract the violet color), of which 125µl were transferred to a new polystyrene micro titer dish, which was then read.

Optical densities (OD) of 570 nm stained adherent bacteria were determined with a micro ELISA auto reader (model 680, Bio rad)

The wavelength of values was considered as an index of bacteria adhering to surface and forming biofilms. Experiments for each strain were performed in triplicate and repeated three times. (Figure no 3.21 and 3.22)

[REF: Gad, G. F., El-Feky, M. A., El-Rehewy, M. S., Hassan, M. A., Abolella, H., & El-Baky, R. M. (2018). Detection of icaA, icaD genes and biofilm production by Staphylococcus aureus and Staphylococcus epidermidis isolated from urinary tract catheterized patients. *Journal of infection in developing countries*, *3*(5), 342–351]

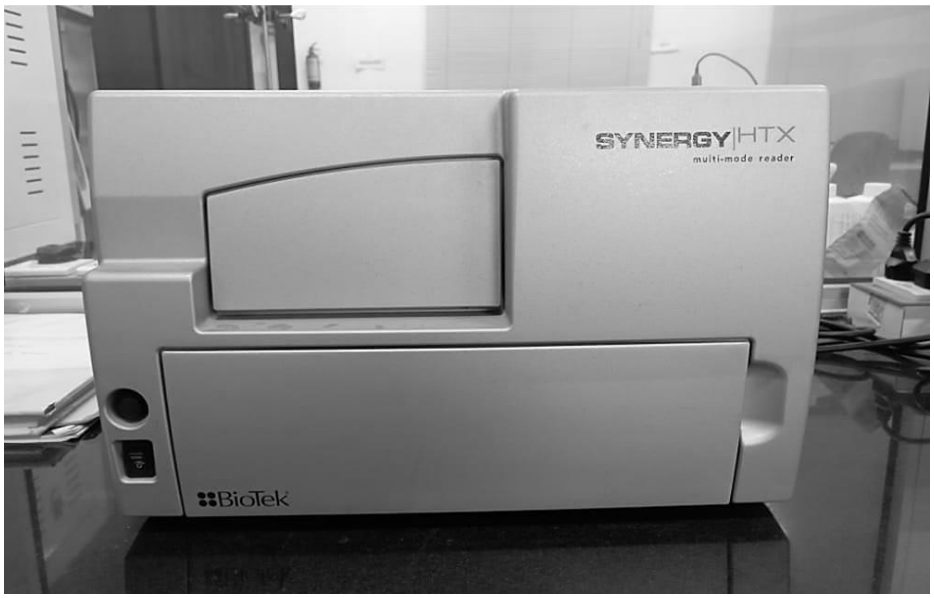


Figure no 3.21: ELISA Reader.

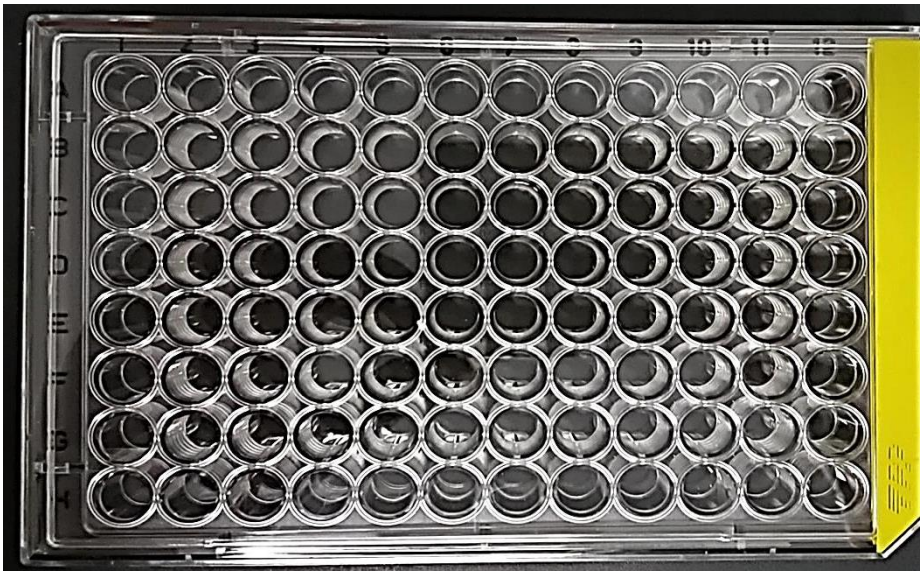


Figure no 3.22: 96 well micro titer plate.

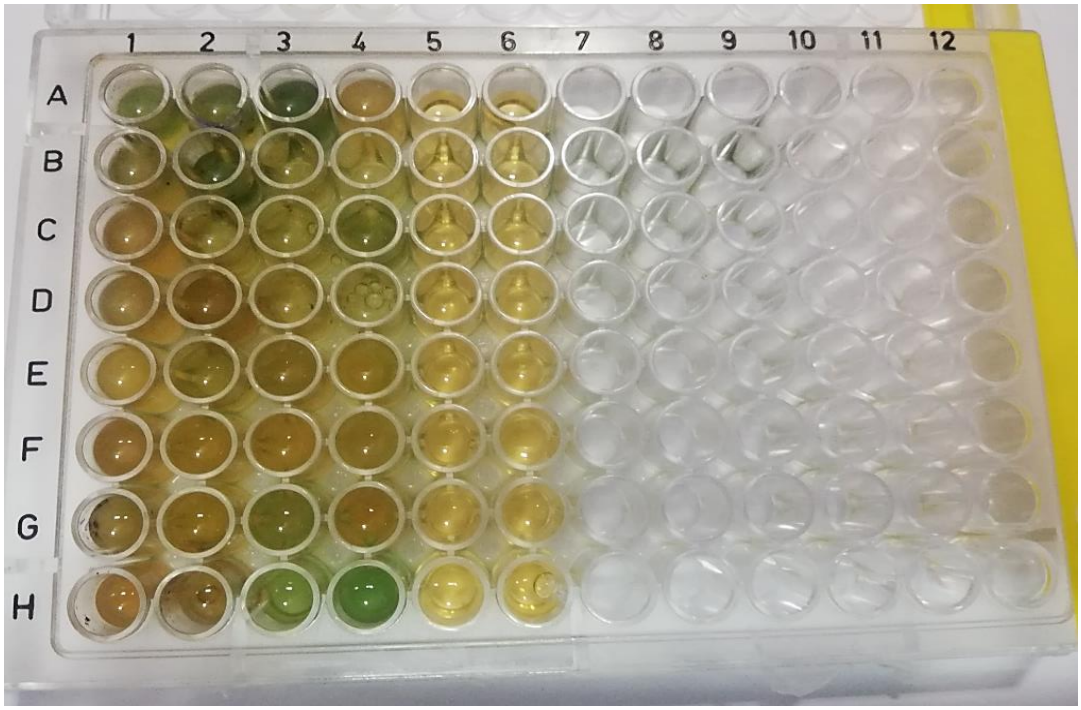


FIGURE NO 3.23: Biofilm formation by *Pseudomonas aeruginosa* isolates, 5th and 6th shows control.

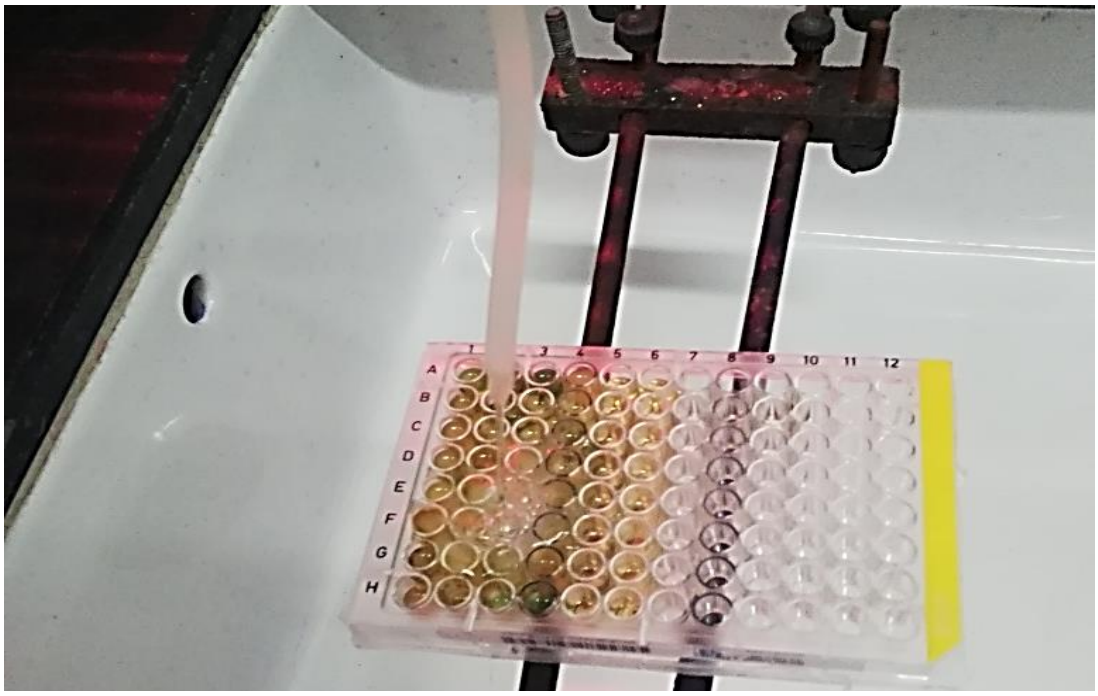


FIGURE NO 3.24: Washing with PBS (Ph. 7.2)

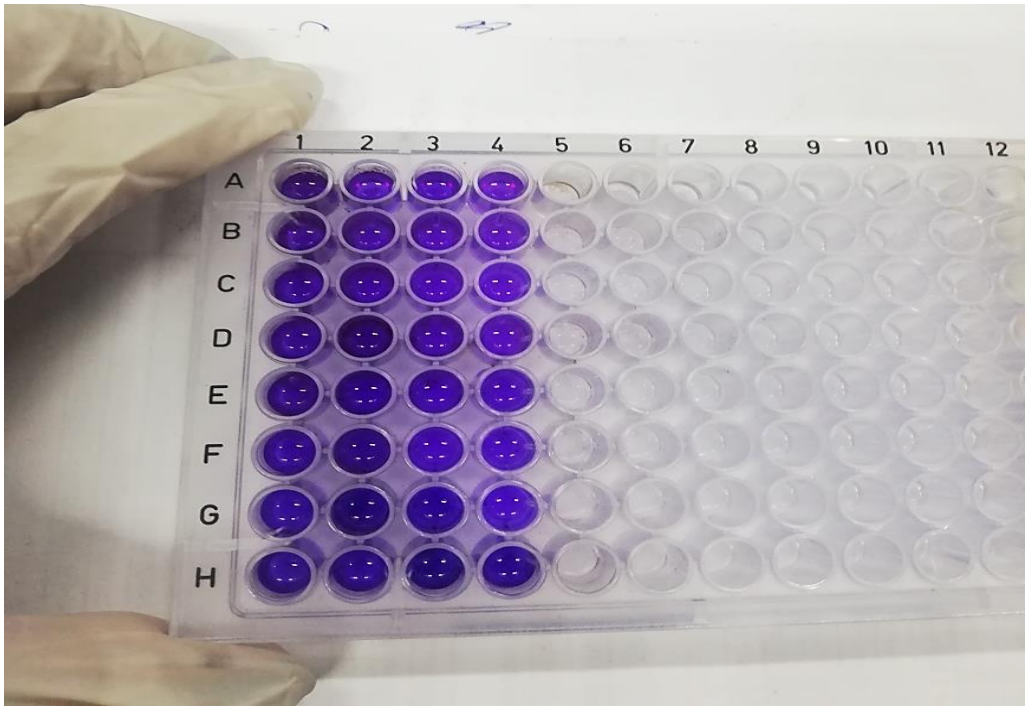


FIGURE NO 3.25: Crystal violet dye staining

METHOD OF SERIAL DILUTION:

Serial dilution is a process through which the concentration of an organism, bacteria is systematically reduced through successive resuspension of an initial solution (solution$_0$) in fixed volumes of 10 ml in liquid diluent/broth. Usually the volume of the diluent/broth is a multiple of 10 to facilitate logarithmic reduction of the sample organism.Tryptic soya broth culture broth is appropriate for cultivation of a desired organism. Prepare ten test tubes capable of storing 10 mL broth solution in a rack and label them T1-T10. Each tube number is consistent with the dilution factor it corresponds to, inoculate "solution$_0$" with a single colony from a previously streaked plate. As with serial dilutions, a logarithmic scale is employed to express bacterial concentration to Mc Farland's standard concentration that is $0.5x10^8$**.** McFarland standards is used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range to standardize microbial testing.

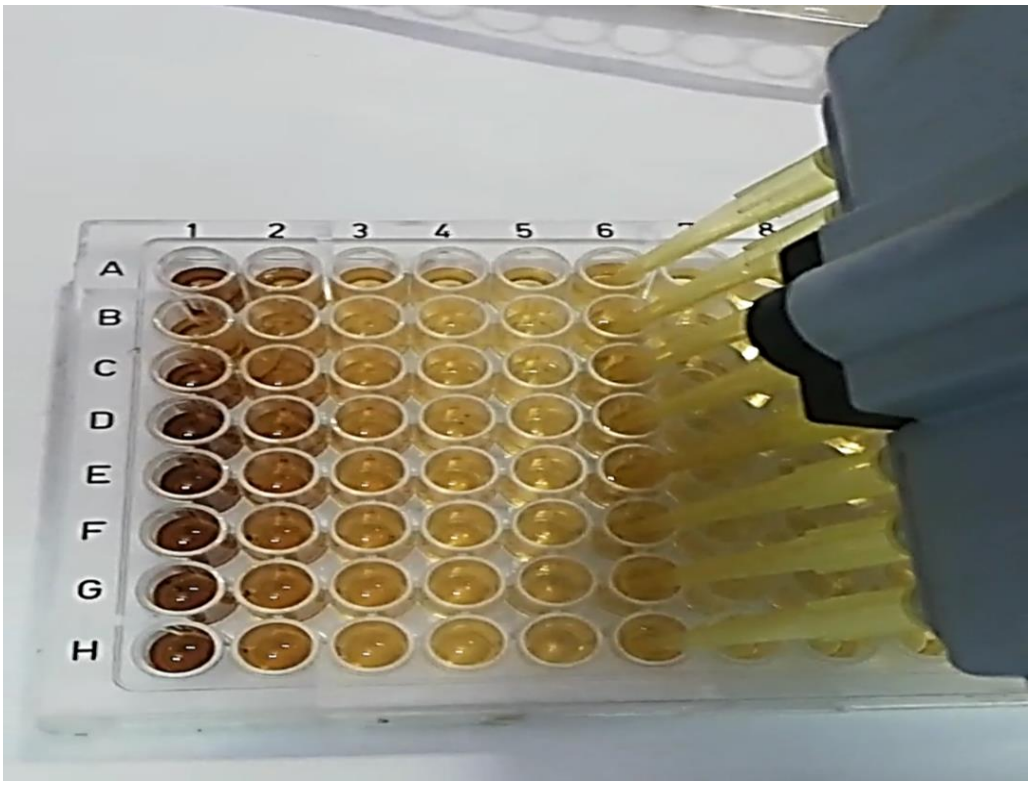


Figure no 3.26: Serial dilution of mango leaf extract using 96 well plate method.

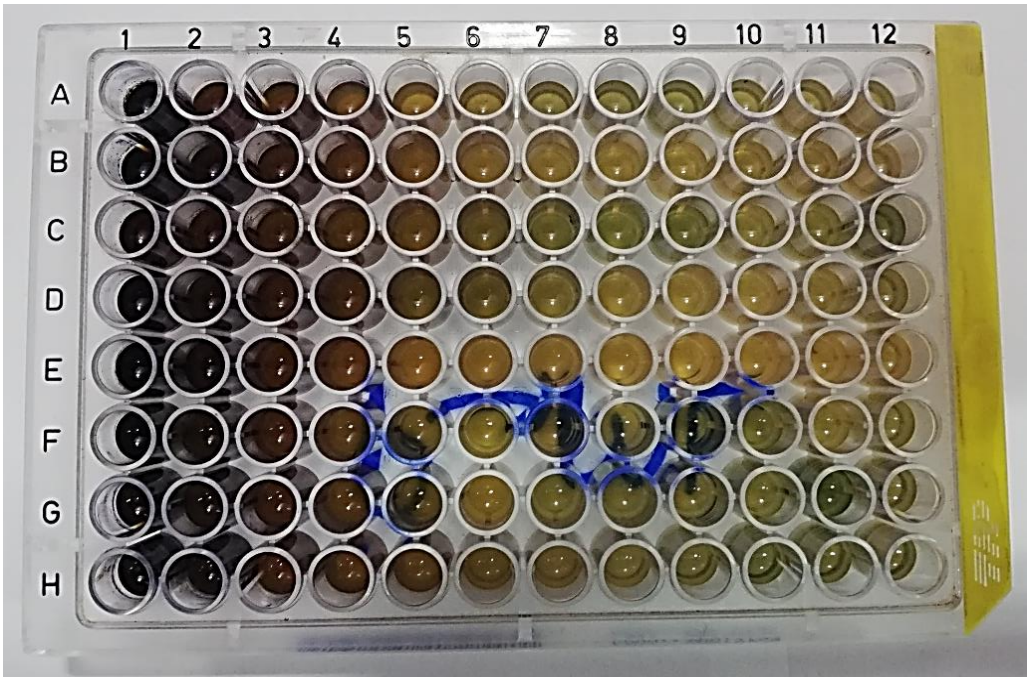


Figure no 3.27: Serial dilution of extract (pomegranate peel extract)

➢ CRYSTAL VIOLET DYE PREPARATION:

Preparing the solutions for staining 1% solution of ammonium oxalate. Dissolve 1 g of ammonium oxalate in 100 ml of distilled/demineralized water.

Crystal Violet staining solution: Dissolve 2 g of Crystal Violet powder dye in 20 ml of 95% ethanol and mix with 80 ml of 1% aqueous solution of ammonium oxalate. (Figure no 3.28)

[Ref: https://www.biognost.com/wp-content/uploads/2020/02/Crystal-Violet-powder-dye-IFU-V1-EN1.pdf]

Figure no 3.28: Crystal violet dye

Figure no 3.29: ATCC Culture *Lactobacillus acidophilus*

Ref: Liofilchem brand

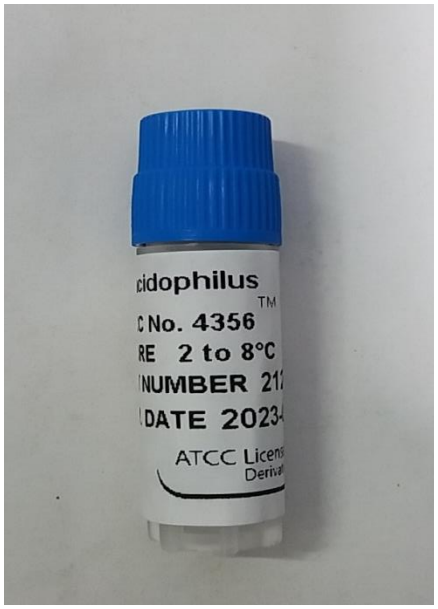
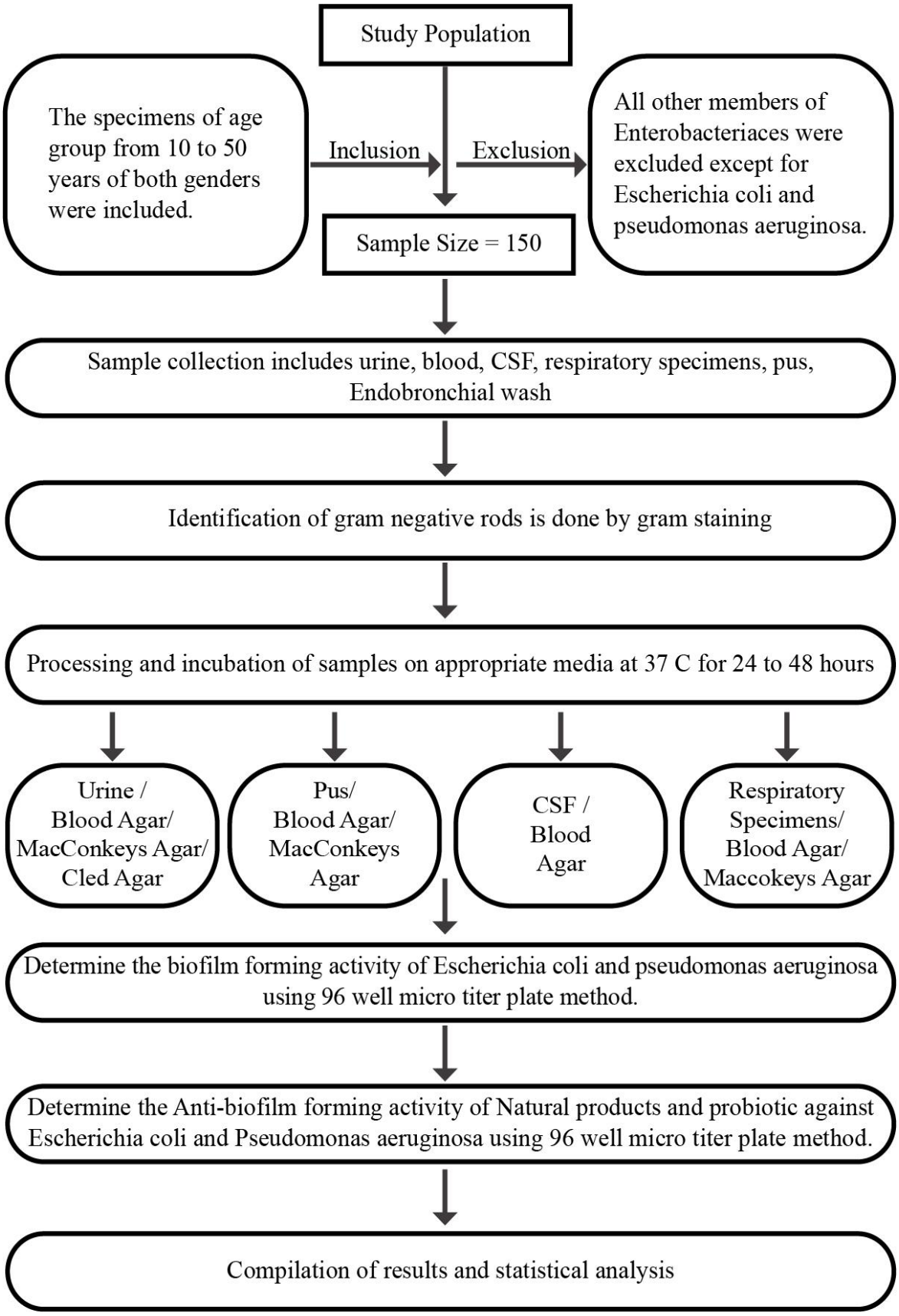


Figure no 3.30: Pallets of ATCC Culture *Lactobacillus acidophilus*

3.13 FLOW CHART OF THE STUDY:



3.14 STATISTICAL ANALYSIS:

All experiments were conducted at least in triplicate. SPSS 27.0 software (SPSS Inc.) was used for statistical analysis in the current study. Quantitative data were expressed as mean ± standard deviation. The Student's *t*-test was utilized for statistical contrast. P-value (less than 0.05) was considered significant.

CHAPTER NO 4

RESULTS

Total 150 patients with age 15 years to 50 years of either gender meeting inclusion criteria of study were evaluated to evaluate biofilm forming activity of *Pseudomonas aeruginosa* and *Escherichia Coli* as well as to determine anti biofilm activity of natural products and probiotic *Lactobacillus acidophilus*

Among 150 patients, there was 64% male and 36% female patients as presented in Table-1. The overall mean age was 33.79±9.94 years. The detailed descriptive statistics of age are presented in Table-3. The age was further stratified in groups. The frequency of patients among these groups is presented in figure no 3.
Urine was the most common examination method (66.7%) while mean biofilm formation was 1.68±0.85. Biofilm formation was achieved among 89.3% of patients as presented from Table-10

In our study, 31.3% of patients were found with *Pseudomonas aeruginosa* and 68.7% with *Escherichia Coli* as presented in Table-6. Mean age was 34.02±10.59 years and 33.69±9.69 years among *Pseudomonas aeruginosa* and *Escherichia Coli* cases as presented in Table-5. Antimicrobial susceptibility of antibiotics (*Pseudomonas aeruginosa and Escherichia Coli)* and anti-biofilm forming activity of *Pseudomonas*

Aeruginosa and Escherichia Coli are presented from Table-14 to Table-15 respectively. And synergistic activity of pomegranate peel extract, mango leaf and *lactobacillus acidophilus* detailed results are presented in table no- 22, 23, 24, 25, 26 and 27.

We found insignificant mean difference according to organism (p=0.850) as presented in Table-23 while we found significant association of organism with samples (p=0.000) while no significant association was found for gender (p=0.692), age group (p=0.446) and bio-film formation (p=0.574). Detailed results of association are presented from Table-6, 7, 9, 11, 12 and Table-13 respectively. We also determine association of organism with biofilm formation for stratified groups of gender, age and samples. Detailed results of association are presented from Table12, 13 figure no 32, 33 and Table-10 respectively.

TABLE NO– 4.1

FREQUENCY DISTRIBUTION OF GENDER IN OUR STUDY

(n=150)

GENDER	Frequency (%)
Male	96(64%)
Female	54(36%)
TOTAL	150

TABLE NO – 4.2

FREQUENCY DISTRIBUTION OF ORGANISMS

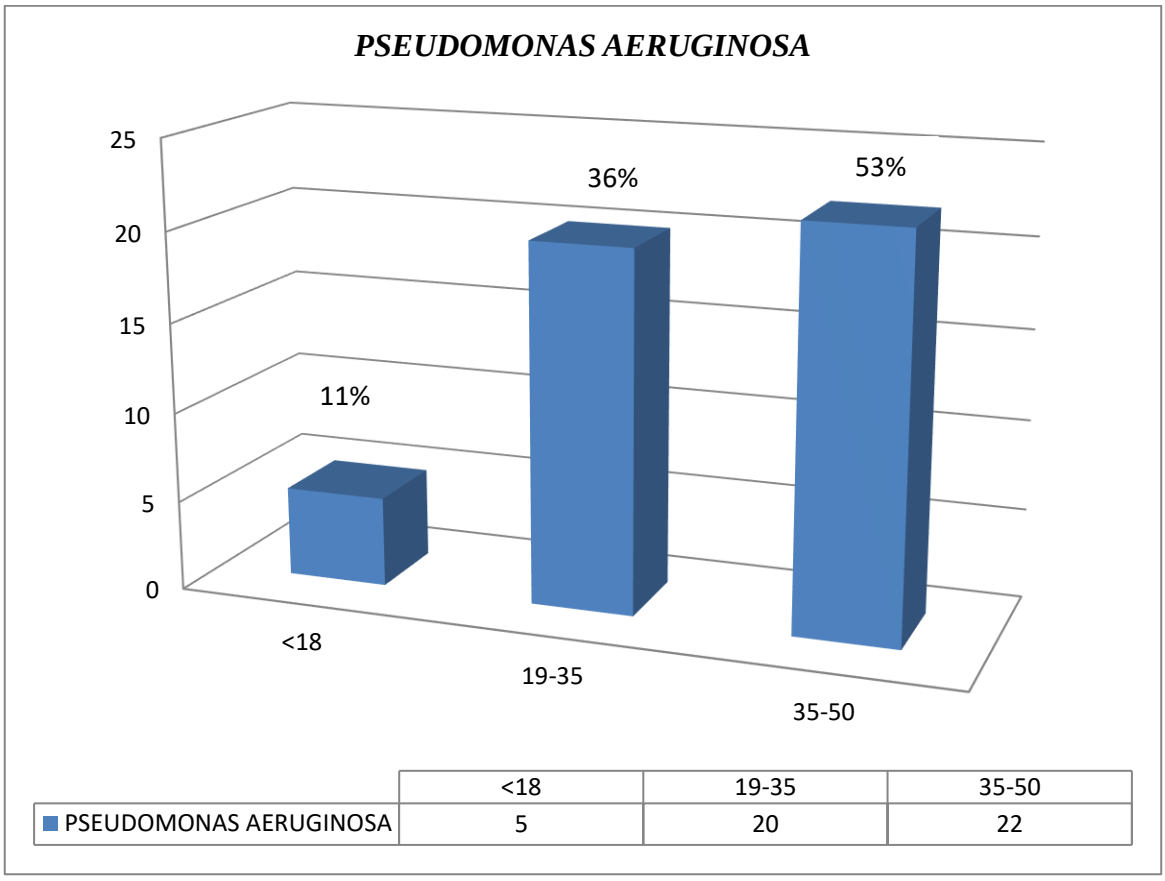
(n=150)

CULTURE	Frequency (%)
Pseudomonas aeruginosa	**47(31.3)**
Escherichia Coli	**103(68.7)**
TOTAL	**150**

FIGURE NO 3.31

FREQUENCY DISTRIBUTION OF *PSEUDOMONAS AERUGINOSA* ACCORDING TO AGE GROUPS

(n=47)

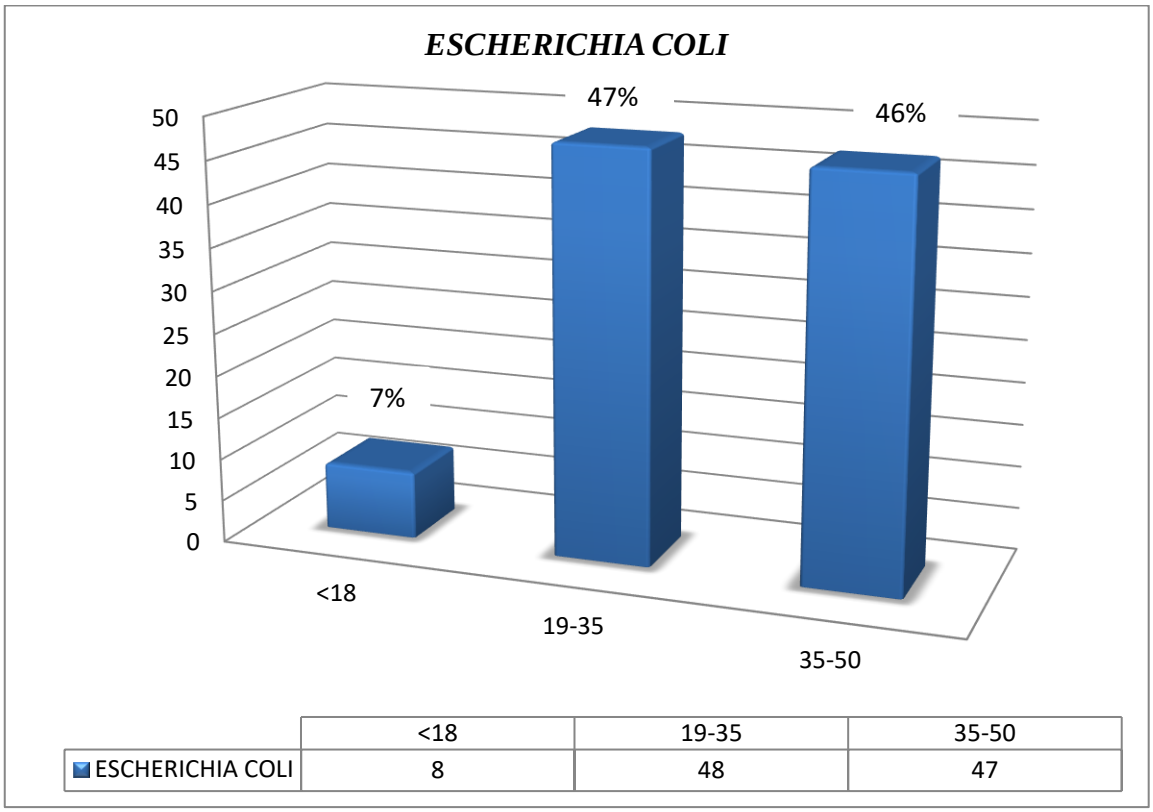


	<18	19-35	35-50
PSEUDOMONAS AERUGINOSA	5	20	22

FIGURE NO-3.32

FREQUENCY DISTRIBUTION OF *ESCHERICHIA COLI* ACCORDING TO AGE GROUP

(n=103)



	<18	19-35	35-50
ESCHERICHIA COLI	8	48	47

TABLE NO – 4.3

DESCRIPTIVE STATISTICS OF AGE

ACCORDING TO *PSEUDOMONAS AERUGINOSA* (YEARS)

(n=47)

Mean	**34.02**
SD	**±10.59**
Median	**32.00**
Inter Quartile Range	**16.00**
Range	**35**
Minimum	**15**
Maximum	**50**

TABLE NO 4.4

DESCRIPTIVE STATISTICS OF AGE

ACCORDING TO *ESCHERICHIA COLI*

(n=103)

Mean	**33.69**
SD	**±9.69**
Median	**33.00**
Inter Quartile Range	**17.00**
Range	**35**
Minimum	**15**
Maximum	**50**

TABLE NO-4.5

MEAN COMPARISON OF AGE

ACCORDING TO ORGANISM

(n=150)

	CULTURE n (%)		**P-VALUE**
	Mean	**Std. Dev**	
Pseudomonas	**34.02**	**±10.59**	**0.850****
E-Coli	**33.68**	**±9.69**	

Independent t-test was applied.

P-value <0.05 considered as significant.

****In-Significant at 0.05 level.**

TABLE NO– 4.6

FREQUENCY AND ASSOCIATION OF ORGANISM

ACCORDING TO AGE GROUP

(n=150)

	CULTURE n (%)			
	Pseudomonas	***E-Coli***	**Total**	**P-VALUE**
≤18 years	**5(38.5)**	**8(61.5)**	**13**	**0.446****
19-35 years	**20(29.4)**	**48(70.6)**	**68**	
>35 years	**22(31.9)**	**47(68.1)**	**69**	
Total	**47**	**103**	**150**	

Chi-square/Fisher exact test was applied.

P-value <0.05 considered as significant.

****In-Significant at 0.05 level**.

TABLE NO-4.7

FREQUENCY AND ASSOCIATION OF ORGANISM

ACCORDING TO GENDER

(n=150)

	CULTURE n (%)			
	Pseudomonas	***E-Coli***	**Total**	**P-VALUE**
Male	**29(30.2)**	**67(69.8)**	**96**	**0.692****
Female	**18(33.3)**	**36(66.7)**	**54**	
Total	**47**	**103**	**150**	

Chi-square/Fisher exact test was applied.

P-value <0.05 considered as significant.

****In-Significant at 0.05 level.**

TABLE NO – 4.8

FREQUENCY DISTRIBUTION OF SAMPLE

(n=150)

Samples	Frequency (%)
Blood	4(2.7)
EB Wash	4(2.7)
PUS	23(15.3)
Sputum	15(10)
Urine	100(66.7)
Tissue	4(2.7)
TOTAL	150

TABLE NO- 4.9

FREQUENCY AND ASSOCIATION OF ORGANISM

ACCORDING TO SAMPLE

(n=150)

Samples	CULTURE n (%)			P-VALUE
	Pseudomonas	E-Coli	Total	
Blood	1(25)	3(75)	4	0.0000*
EB Wash	4(100)	0(0)	4	
PUS	14(60.9)	9(39.1)	23	
Sputum	15(100)	0(0)	15	
Urine	10(10)	90(90)	100	
Tissue	3(75)	1(25)	4	
Total	47	103	150	

Chi-square/Fisher exact test was applied.

P-value <0.05 considered as significant.

***Significant at 0.05 lev**

Figure No- 3.33

FREQUENCY DISTRIBUTION OF BIOFILM FORMER AND NON-BIOFILM FORMER AMONG *ESCHERICHIA COLI.*

(n-103)

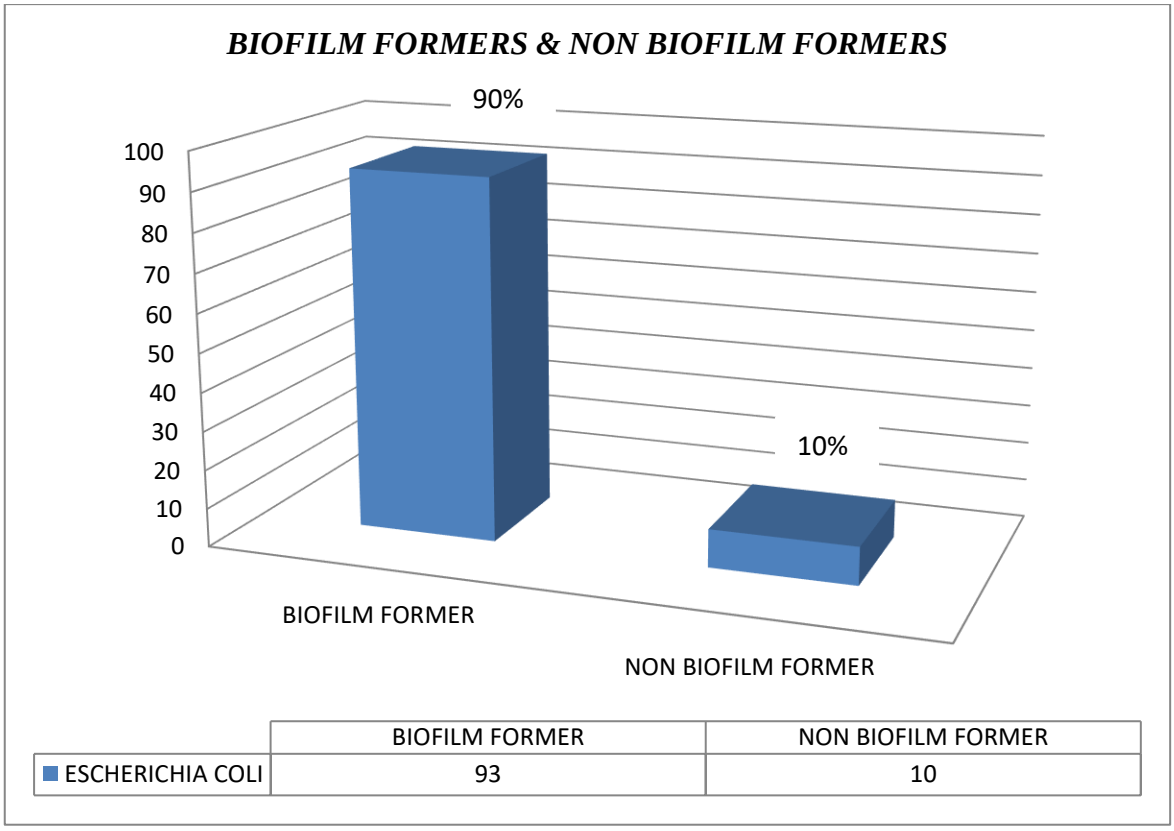
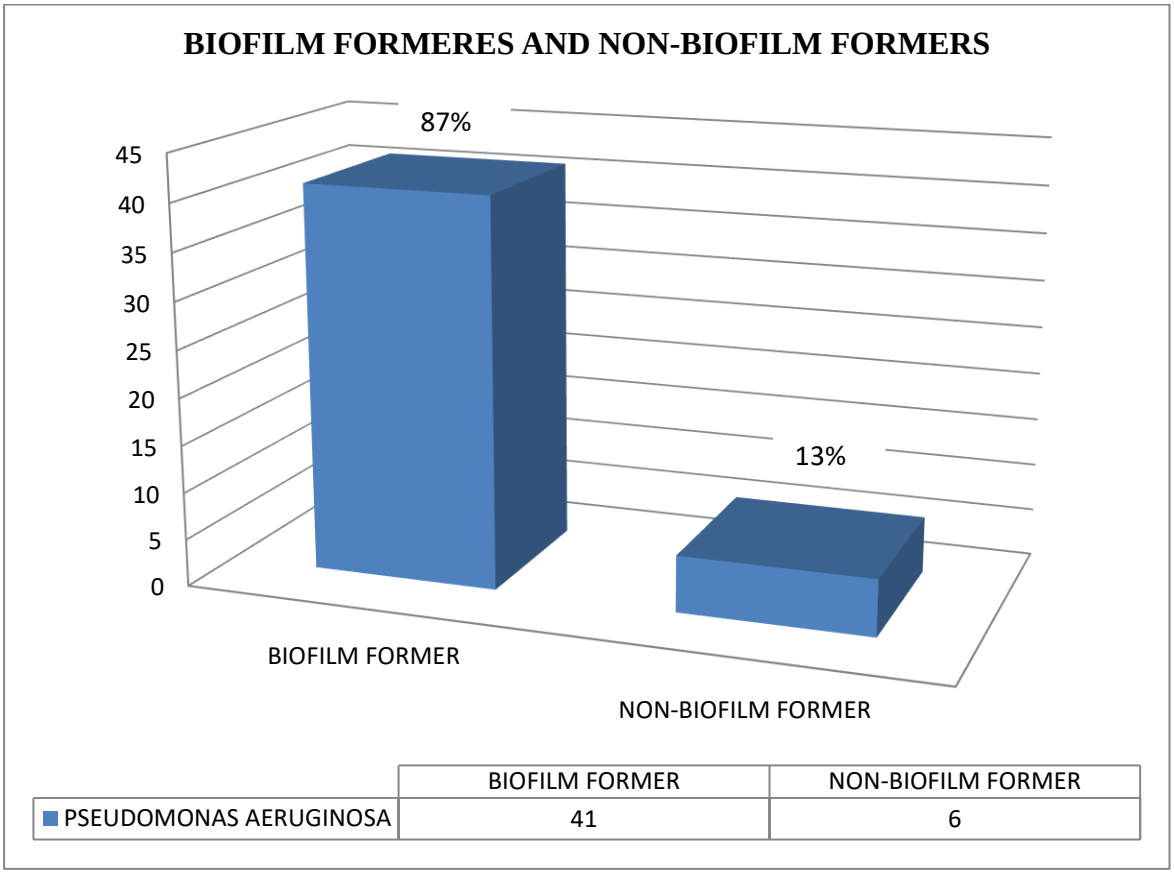


Figure No-3.34

FREQUENCY DISTRIBUTION OF BIOFILM FORMER AND NON-BIOFILM FORMER AMONG PSEUDOMONAS AERUGINOSA

(n-47)



	BIOFILM FORMER	NON-BIOFILM FORMER
PSEUDOMONAS AERUGINOSA	41	6

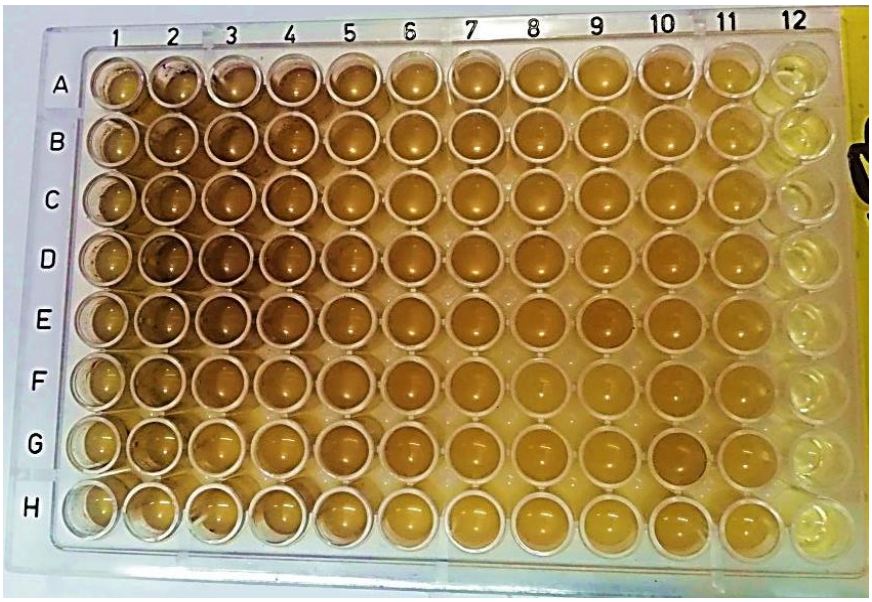


Figure no 3.35: Biofilm formers *Escherichia coli 12th* well shows control

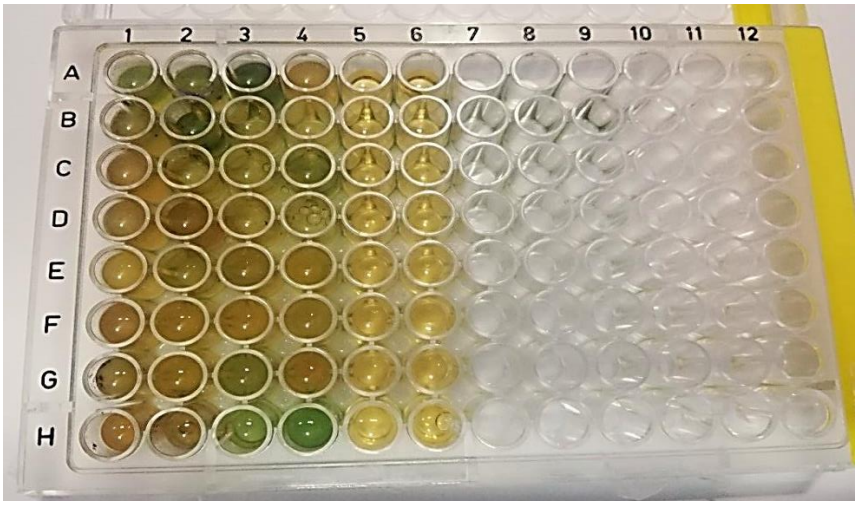


Figure no 3.36: Biofilm former *Pseudomonas aeruginosa 5 and 6th wells show control*

TABLE NO – 4.10

FREQUENCY DISTRIBUTION OF BIO-FILM FORMATION IN ALL SAMPLES (n=150)

	Frequency (%)
Yes	**134(89.3)**
No	**16(10.7)**
TOTAL	**150**

TABLE NO- 4.11

FREQUENCY AND ASSOCIATION OF ORGANISM ACCORDING TO BIO-FILM FORMATION

(n=150)

	CULTURE n (%)			**P-VALUE**
	Pseudomonas	***E-Coli***	**Total**	
Yes	**41(30.6)**	**93(69.4)**	**134**	**0.574****
No	**6(37.5)**	**10(62.5)**	**16**	
Total	**47**	**103**	**150**	

Chi-square/Fisher exact test was applied.

P<0.05 considered as significant.

****In-Significant at 0.05 level**.

TABLE NO-4.12

FREQUENCY AND ASSOCIATION OF ORGANISM

ACCORDING TO BIO-FILM FORMATION

FOR MALE

(n=96)

Bio-Film Formation	ORGANISM n (%)			P-VALUE
	Pseudomonas	*E-Coli*	Total	
Yes	25(28.7)	62(71.3)	87	0.329**
No	4(44.4)	5(55.6)	9	
Total	29	67	96	

Chi-square/Fisher exact test was applied.

P<0.05 considered as significant.

****In-Significant at 0.05 level.**

TABLE NO-4.13

FREQUENCY AND ASSOCIATION OF ORGANISM

ACCORDING TO BIO-FILM FORMATION

FOR FEMALE

(n=54)

Bio-Film Formation	CULTURE n (%)			P-VALUE
	Pseudomonas	E-Coli	Total	
Yes	16(34)	31(66)	47	1.000**
No	2(28.6)	5(71.4)	7	
Total	18	36	54	

Chi-square/Fisher exact test was applied.

P<0.05 considered as significant.

****In-Significant at 0.05 level**

FIGURE NO- 3.37

FREQUENCY DISTRIBUTION OF BIOFILM FORMER *PSEUDOMONAS AERUGINOSA* AMONG DIFFERENT AGE GROUPS

(n-41)

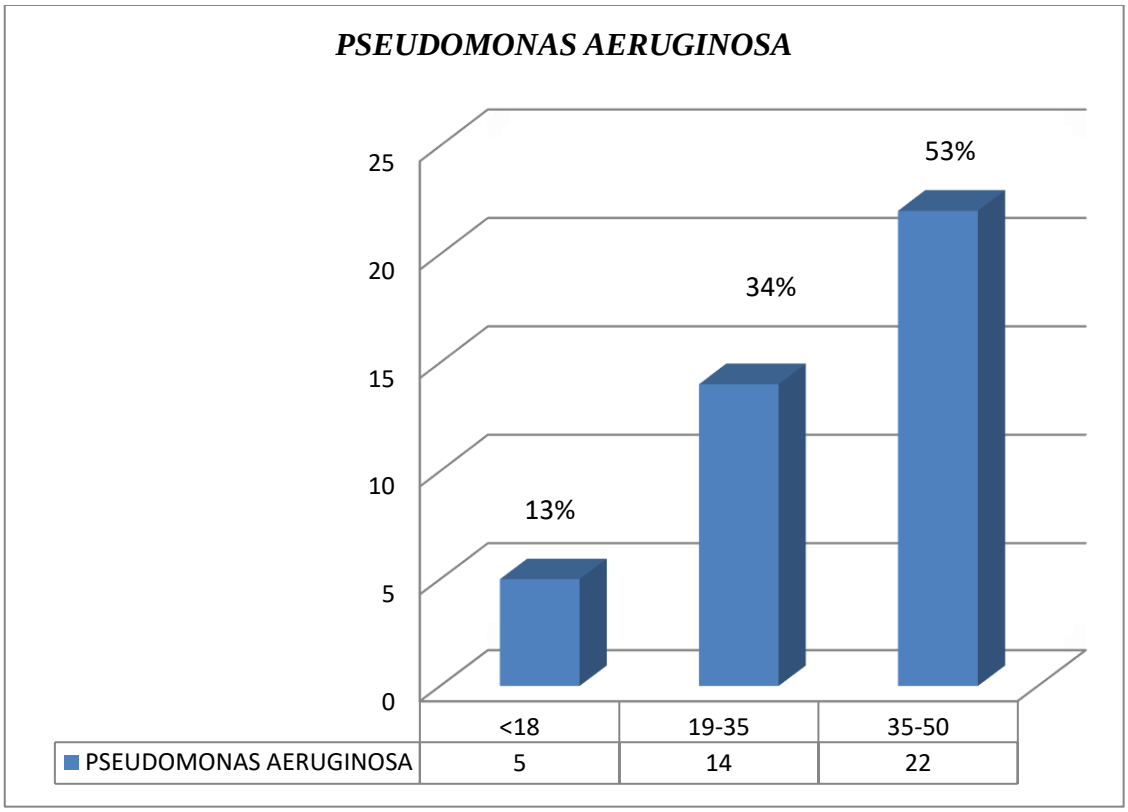


FIGURE NO- 3.38

FREQUENCY DISTRIBUTION OF BIOFILM FORMER *ESCHERICHIA COLI* AMONG DIFFERENT AGE GROUPS

(n-93)

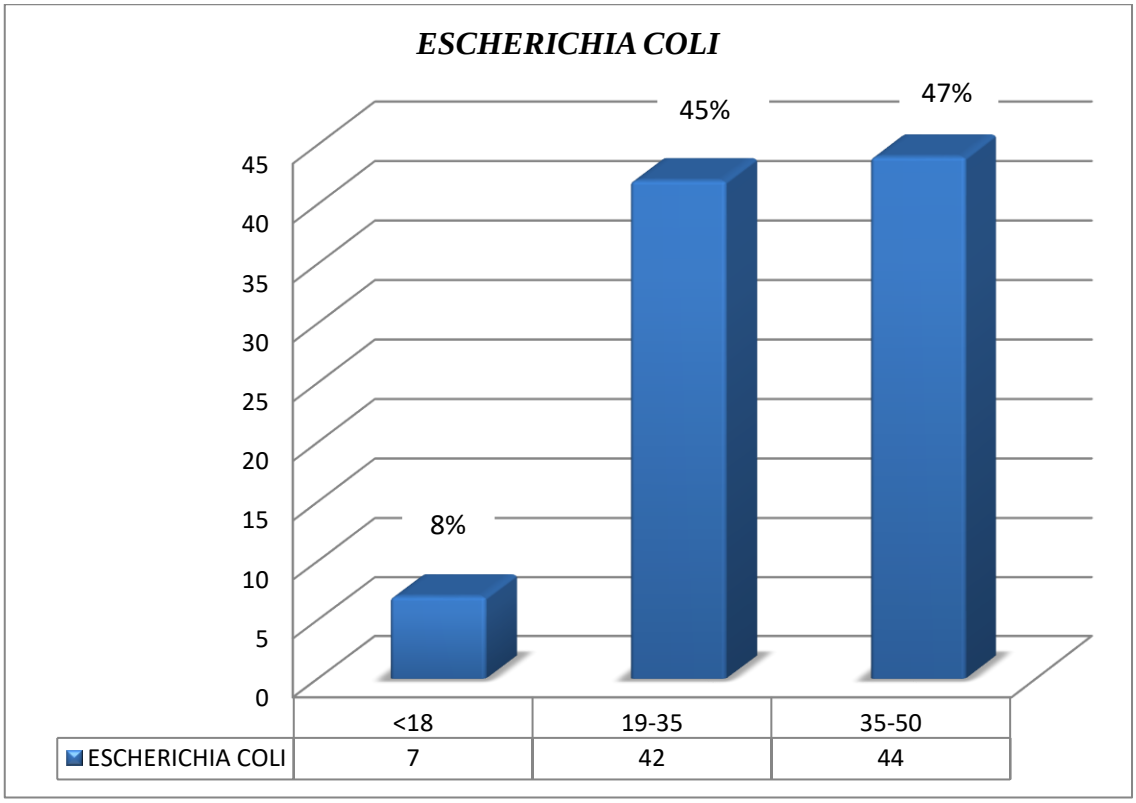


TABLE NO– 4.14

ANTIMICROBIAL SUSCEPTIBILITY OF *PSEUDOMONAS AERUGINOSA*

(n=47)

S.NO	ANTIBIOTICS	DICS (Conc.) µg	Sensitive	Resistant
1	CIPROFLOXACIN	6	13	34
2	AZITHROMYCIN	30	20	27
3	CEFTAZIDIME/AVIBACTAM	30	12	35
4	CEFEPIME	30	18	29
5	LEVOFLOXACIN	5	17	30
6	GENTAMYCIN	10	14	33
7	PIPERACILLIN/TAZOBACTAM	110	25	22
8	AMIKACIN	30	19	28
9	POLYMIXIN B	300	28	19

TABLE NO – 4.15

ANTIMICROBIAL SUSCEPTIBILITY OF ESCHERICHIA COLI

(n=103)

S.NO	ANTIBIOTICS	DICS (Conc.) µg	Sensitive	Resistant
1	**AMOX-CLAVULANATE**	30	44	59
2	**COTRIMOXAZOLE**	25	21	82
3	**AMPICILLIN**	10	38	65
4	**CIPROFLOXACIN**	5	29	74
5	**TETRACYCLINE**	30	20	83
6	**AMIKACIN**	30	31	72
7	**CEFTRIAXONE**	30	89	14
8	**SULBACTAM/CEFOPERAZONE**	105	79	24
9	**PIPRA-TAZOBACTAM**	110	39	64
10	**FOSFOMYCINE**	50	80	23
11	**NITROFURANTOIN**	300	32	71
12	**MEROPENEM**	10	82	21
13	**TIGECYCLINE**	15	38	65

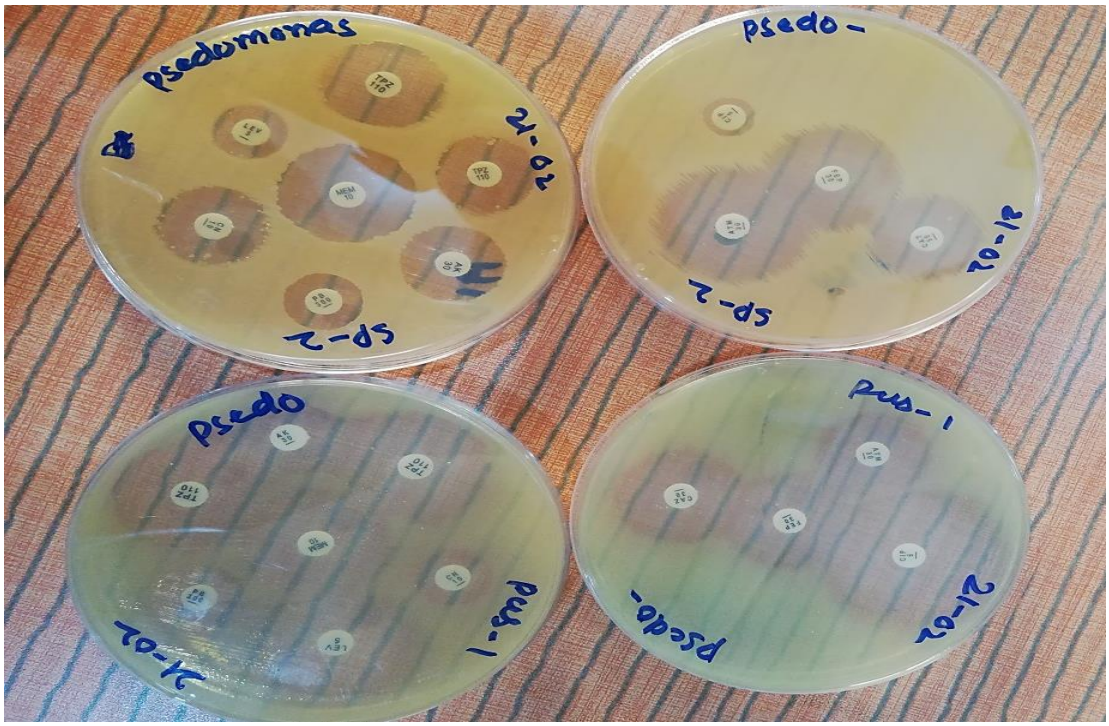


Figure no 3.39: AST PATTERN OF *PSEUDOMONAS AERUGINOSA*

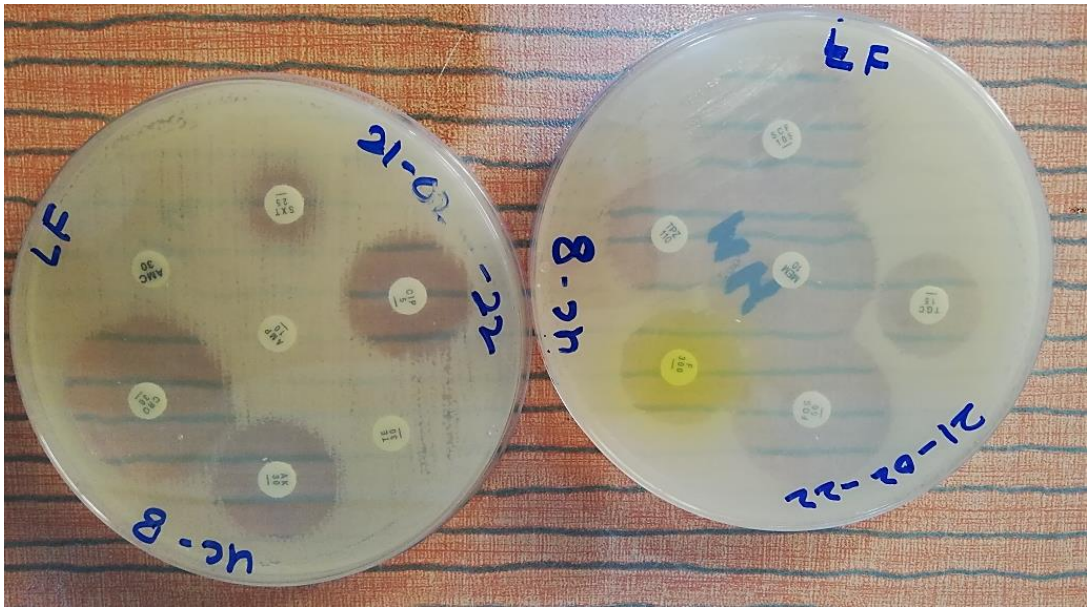


Figure no 3.40: AST PATTERN OF *ESCHERICHIA COLI*

TABLE NO -4.16

ANTI-BIOFILM FORMING ACTIVITY OF POMEGRANATE PEEL EXTRACT (PPE) AGAINST ESCHERICHIA COLI

CULTURE	COMPOUND	Anti-biofilm concentration (PPE) mg/ml
Escherichia coli	Pomegranate peel extract (PPE)	24.46±19.09

TABLE NO-4.17

ANTI-BIOFILM FORMING ACTIVITY OF POMEGRANATE PEEL EXTRACT (PPE) AGAINST PSEUDOMONAS AERUGINOSA.

CULTURE	COMPOUND	ANTIBIOFILM CONCENTRACTION (PPE) mg/ml
Pseudomonas Aeruginosa	Pomegranate peel extract (PPE)	29.26±19.09

TABLE NO-4.18

ANTIBIOFILM FORMING ACTIVITY OF MANGO LEAF AGAINST ESCHERICHIA COLI

CULTURE	COMPOUND	ANTIBIOIFILM FORMING ACTIVITY OF (MLE) mg/ml
ESCHERICHIA COLI	MANGO LEAF EXTRACT (MLE)	14.90±9.56

TABLE NO-4.19

ANTIBIOFILM FORMINGACTIVITY OF MANGO LEAF AGAINST PSEUDOMONAS AERUGINOSA

CULTURE	COMPOUND	ANTIBIOFILM ACTIVITY OF (MLE) mg/ml
PSEUDOMONAS AERUGINOSA	MANGO LEAF EXTRACT (MLE)	11.45±9.52

TABLE NO-4.20

ANTIBIOFILM FORMING ACTIVITY OF LACTOBACILLUS AGAINST *ESCHERICHIA COLI*

CULTURE	**CULTURE**	**ANTIBIOFILM ACTIVITY OF LACTOBACILLUS ACIDOPHILLUS**
ESCHERICHIA COLI	***LACTOBACILLUS ACIDOPHILUS***	**0.5x 10^6 (CFU/ml)**

TABLE NO-4.21

ANTIBIOFILM FORMING ACTIVITY OF LACTOBACILLUS AGAINST PSEUDOMONAS AERUGINOSA

CULTURE	**CULTURE**	**ANTIBIOFILM ACTIVITY OF LACTOBACILLUS ACIDOPHILUS**
PESUDOMONAS AERUGINOSA	***LACTOBACILLUS ACIDOPHILUS***	**0.5x10^6 (CFU/ml)**

TABLE NO-4.22

COMBINE EFFECT OF POMEGRANATE PEEL EXTRACT (PPE) AND MANGO LEAF EXTRACT (MLE) AGAINST *ESCHERICHIA COLI.*

CULTURE	PPE INDIVIDUAL (mg/ml)	PPE COMBINE (mg/ml)	MLE INDIVIDUAL (mg/ml)	MLE COMBINE (mg/ml)	FIC INDEX	RELATION
ESCHERICHIA COLI	24.46±19.09	3.46±2.38	14.90±9.56	3.56±2.41	0.3	SYNERGISM

TABLE NO -4.23

COMBINE EFFECT OF POMEGRANATE PEEL EXTRACT (PPE) AND MANGO LEAF EXTRACT (MLE) AGAINST *PSEUDOMONAS AERUGINOSA*

CULTURE	PPE INDIVIDUAL (mg/ml)	PPE COMBINE (mg/ml)	MLE INDIVIDAL (mg/ml)	MLE COMBINE (mg/ml)	FIC INDEX	RELATION
PSEUDOMONSA AERUGINOSA	29.26±19.09	3.55±2.38	11.45±9.52	1.68±1.19	0.24	SYNERGISM

TABLE NO-4.24

COMBINE EFFECT OF POMEGRANATE PEEL EXTRACT (PPE) AND LACTOBACILLUS ACIDOPHILUS AGAINST *ESCHERICHIA COLI.*

CULTURE	PPE INDIVIDUAL (mg/ml)	PPE COMBINE (mg/ml)	LACTOBACILLUS INDIVIDUAL (CFU/ml)	LACTOBACILLUS COMBINE (CFU/ml)	RELATION
ESCHERICHIA COLI	24.46±19.09	3.11±2.37	0.5x10^6 (CFU/ml)	0.5X10^4 (CFU/ml)	SYNERGISM

TABLE NO-4.25

COMBINE EFFECT OF POMEGRANATE PEEL EXTRACT AND LACTOBACILLUS ACIDOPHILUS AGAINST *PSEUDOMONAS AERUGINOSA.*

CULTURE	PPE INDIVIDUAL (mg/ml)	PPE COMBINE (mg/ml)	LACTOBACILLUS INDIVIDUAL (CFU/ml)	LACTOBACILLUS COMBINE (CFU/ml)	RELATION
PSEUDOMONAS AERUGINOSA	29.26±19.09	3.60±2.27	0.5x10^6 (CFU/ml)	0.5x10^5 (CFU/ml)	SYNERGISM

TABLE NO-4.26

COMBINE EFFECT OF MANGO LEAF EXTRACT AND LACTOBACILLUS AGAINST *PSEUDOMONAS AERUGINOSA*.

CULTURE	MLE INDIVIDUAL (mg/ml)	MLE COMBINE (mg/ml)	LACTOBACILLUS INDIVIDUAL (CFU/ml)	LACTOBACILLUS COMBINE (CFU/ml)	RELATION
PSEUDOMONSA AERUGINOSA	**11.45±9.52**	**1.70±1.19**	**0.5x10^6 CFU/ml**	**0.5x10^3 CFU/ml**	**SYNERGISM**

TABLE NO-4.27

COMBINE EFFECT OF MANGO LEAF EXTRACT AND LACTOBACILLUS AGAINST *ESCHERICHIA COLI*.

CULTURE	MLE INDIVIDUAL (mg/ml)	MLE COMBINE (mg/ml)	LACTOBACILLUS INDIVIDUAL (CFU)	LACTOBACILLUS COMBINE (CFU)	RELATION
ESCHERICHIA COLI	**14.90±9.56**	**3.61±2.38**	**0.5 x 10^6 CFU/ml**	**0.5x10^4 CFU/ml**	**SYNERGISM**

- Synergistic calculations are frequently seen using the following formula to obtain Fractional Inhibitory Concentration (FIC) index: FIC = (Ac/A) + (Bc/B), where Ac and Bc are the inhibitory biofilm concentration of compounds in combination, and A and B are the individual biofilm inhibition concentration of compound A and B alone.

  Synergism by the checkerboard method is defined as a Fractional Inhibitory Concentration (FIC) index of ≤ 0.5, represents synergistic effect is defined as an FIC index of > 0.5 and ≤ 1, Indifference effect is defined as an FIC index of > 1 and ≤ 2 and antagonism effect is defined as an FIC index of > 2 or > 4. Concentrations within the FIC panel were such that the inhibitory concentration of each compound and lactobacillus acidophilus is in the middle of the range of concentrations tested.

  Ref: (Sophia Hawas et al 2022)

CHAPTER NO 5

DISCUSSION

5.1 SEQUENCE OF DISCUSSION EXPERIMENT/ HYPOTHESIS WISE.

Pathogenic microorganisms and their chronicity are major concerns in biomedical and pharmaceutical research. Traditional antimicrobial treatments consist of drugs which target different essential functions in pathogens. [Abdus-Salam, I.et;al 2020] Nevertheless, bacteria continue to evolve new mechanisms to evade this drug-mediated killing with surprising speed on the deployment of each new drug and antibiotics worldwide, a phenomenon called antimicrobial resistance (AMR). Nowadays, AMR represents a critical health threat, for which new medical interventions are urgently needed. By 2050, it is estimated that the leading cause of death will be through untreatable AMR pathogens. [MacDonald, J.,et;al 2018]

Currently, AMR pathogens already cause 700,000 deaths/year worldwide, with a project 10 million deaths/year in 2050 a figure even higher than that caused by cancer today [Rotello, V. M. et;al(2019)]. In 1946, Alexander Fleming predicted the global threat of AMR with the phrase "There is probably no chemotherapeutic drug to which bacteria, under appropriate circumstances, will not respond in some way by acquiring ‘fastness’ (resistance)" [Grad, Y. H. et;al 2018].

A plethora of mechanisms have led to antimicrobial resistance in microorganisms. The classification includes intrinsic, acquired and adaptive resistance mechanisms. By contrast, the acquired resistance relies on the acquisition of new functions. When bacteria become resistant to one or more antibiotics that were initially effective, they are referred to as multi-drug resistant and often called “superbugs” [El Aidy, S. et;al (2022)]

Adaptive resistance is the ability of the bacteria to cope with antibiotics by transient changes in gene expression in response to specific stimuli. The acquired phenotype is reversible and when the stimulus is removed, the inherent bacterial susceptibility is restored [M., Sulaiman, A. M et;al 2021]. One of the mechanisms of this type of resistance is the formation of biofilms.

A biofilm is a collection of bacteria present on biotic or abiotic surfaces, entrapped in a self-produced matrix of extracellular polymeric substances, including proteins, exopolysaccharides, metabolites and extracellular DNA. [Musa Hassan Muhammad et al., 2020] Microbial cells growing in biofilms are less sensitive to antimicrobial agents and host immune responses than free-floating planktonic cells. [Christine Murphy et;al 2020]

This is due to the fact that the biofilm protects bacteria by preventing antibiotic penetration, alters the microenvironment to induce slow bacterial growth, determines an adaptive response to stress, and differentiates bacterial cells towards the persistent phenotype.

The alarming rate of antibiotic resistance cases, the World Health Organization (WHO) published in 2017 a priority list of pathogens (classified as critical, high and medium priority) to guide the discovery of appropriate prophylactic and therapeutic strategies. As part of the critical category of the WHO priority list, multidrug resistant Gram-negative bacteria, highlighted by Pseudomonas aeruginosa and Escherichia coli are

becoming a public health concern due to high levels of resistance to many commercially available drugs [Bartaula, B. et;al,2019]

Today, the time it takes for bacteria to become resistant to newly introduced antibiotics is becoming shorter and shorter. Indeed, the misuse or overuse of antibiotics continually leads to evolutionary pressures for the generation and transmission of resistant pathogens.

Persistent biofilm-associated infections are not easy to treat due to the antimicrobial tolerance of biofilms has become a major challenge for medical scientists in various health care areas. Synthetic drugs, combination therapies and antibiotic hybrids have not been able to achieve and deliver the desired and effective results during the treatment. The low efficacy of various treatments and the in vivo toxicity of available antibiotics led researchers to the discovery of many potent natural antibiofilm agents. Natural extracts and natural product-based antibiofilm agents are more efficient than chemically synthesized counterparts with fewer side effects.

The study mainly focuses on several natural antibiofilm agents known for its medicinal values such as antimicrobial, anti-inflammatory and antibiofilm properties contain phytochemicals, antimicrobial peptides, and microbial enzymes along with their sources, mechanism of action by interfering with quorum-sensing pathways, disruption of extracellular polymeric substance, mechanism of adhesion, and their existing inhibitory concentrations in the previous literature.

Our study provides a better understanding of antibiofilm activity of natural product against *Escherichia coli and Pseudomonas aeruginosa*. This information can be further used to improve therapeutic strategy by combining more than one natural antibiofilm compounds from different sources.

As discussed previously *Escherichia coli and Pseudomonas aeruginosa* are able to survive on abiotic surfaces and form biofilms. In our study, Table no 1 shows the frequency distribution among gender were 94 males and 54 were females out of 150 samples which reveal a high incidence of *Escherichia coli* among males than females in all samples collected. Similar results are in accordance with the research done by [Haley J Appaneal et al., 2021] identified 21,938 residents with UTI treated in 120 VA and CLCs, between year (2013-2018) of whom 96% were male patients with UTI. Another study

which is not in accordance with our study, it is conducted by [Maryam Montazeri et;al in 2021 & Yawei Zhang et;al 2018] which reveal higher infections rate of *Escherichia coli* in urinary tract infection among females probably due to the shortness of their urethra and its proximity to the anal orifice which provides easy access of bacteria to the bladder.

While in our study, In Table no 2 the frequency distribution of organism, *Pseudomonas aeruginosa* were 47 out of 150, that is (31.3%) while in case of *Escherichia coli* it is 103 out of 150 that is (68.7%). The results of our study are consistent with previous investigations done by [Ali Reza Nateghian et al., in 2021] which showed *Escherichia coli is* (77.6%) & *Pseudomonas aeruginosa* is (2.4%) another study conducted by [Alexey Glazunov et al., 2020] results shows *Escherichia coli* is most common isolated organism (49.1%) followed by *Pseudomonas aeruginosa* (1.7%). Hence, that shows *Escherichia coli are* among one of the most well-adapted and pathogenically versatile bacteria. As it cause variety of human infections both intestinal and extra intestinal most commonly known for UTIs which concludes its prevalence more among infected population.

According to figure no 1&5 and table no 3&5 of our study the frequency distribution of *pseudomonas aeruginosa* into the 3 different age groups which are selected shows maximum prevalence of *pseudomonas aeruginosa* in age group of 35-50 years that is a total of (22) patients out of (47) were among this group mean age (34.02±10.59) and its p- value is statistically insignificant in our study. [Farooq ali et al., 2021] conduct a study in Manshera, findings were mostly similar to our study that higher-frequency rate was detected in the age group more than (≥31 years) 43%. Similarly, [momna tariq et al., 2020] carried out a study in Gujranwala also illustrate higher infectious rate of (64%) in age group 45-66 years of patients. With reference to our study and other researchers data shows that we observed a significantly higher overall incidence of infection in elderly patients as it is attributed to the higher rates of comorbidities decreased immunity and special medical procedures in the elderly group.

While in figure 2&6 and table no 4&5 the prevalence of *Escherichia coli* is most in an age group of 19-35 (33.69±9.69) out of 150 samples. Amongst 48 of them showed the presences of *Escherichia coli* in middle age groups while *Pseudomonas aeruginosa* in older age groups also its p- value is in significant in our study. Similar finding from a study conducted by [M., Adedoja et;al 2021] were participants ranged from age group15

to 27 years; their average age was 19.57 ± 2.44 years. Another study conducted in north-western provenance, Libya by [Samia Tayeb Hawisa et;al 2021] Both, male & female patients were from age group 19 to 35 years. The most prominent bacteria isolated from the samples studied were *Escherichia coli*, accounting for 37.5% of the total positive isolates. This observation concludes the relationship between organism and age may have several implications. As it may influence the behavior of prescriptions concerning different age groups, as UTIs with resistant bacteria are more prevalent in girls, especially above 19 years of age. Factors of causing UTI are accelerated by low socioeconomic status, sexual behavior, and the use of unhygienic food.

While in table no 6 the frequency and association of both the organism according to age groups, *Pseudomonas* and *Escherichia coli* is 68% in 19-35 years of age, which concludes more vulnerable situation among both bacterial infection in this age group and its p-value is statistically insignificant in our study.

Moreover, in table no 7 the frequency and association of both microorganism according to gender, shows majority infectious rate in male patients (96) out of (150) samples. [Waleed Al-Momani et; al 2022] conducted a study in Northern Jordan that includes 24 total samples from which15 positive culture cases, 9 (60%) belong to male patients and 6 (40%) belong to female patients. Therefore, in this study infectious rate was higher in male patients than in female patients, and this could be due to males being more active outdoors and exposed more to infectious agents. Another cross-section study conducted by [ashfaq nasir khan et al., 2022] duration of the study was from 2019-2020. In his study 36 (36%) patients were male whereas 64 (64%) patients were females *Escherichia coli* was found in 13(43.33%) patients followed by *Pseudomonas aeruginosa* 7(23.33%) his study results showed that among male patients, the positive culture was found in 20(55.56%) patients whereas among female patients, the positive culture was found in 10(15.62%) patients. Observing our data and pervious observations we conclude that men and women are different and this fundamental observation extend to their susceptibility and response to different infectious diseases. Apart from cultural and behavior differences between the sexes that play a prominent role in the exposure to pathogens, also biological differences in the immunity against infections exist between women and men. Our study showed that men are more porn to infections.

In table no 8 when we evaluate the distribution frequency among positive samples in our study we see that organism isolated from urine shows the maximum frequency which is (66.7%). However, in table no 9, Escherichia coli is more prominent isolate from urine among different samples we see 90 positive isolate out of 103 samples. *Escherichia coli* as it accounts for 70–90% of uncomplicated and 50–60% of recurrent or complicated infections. Appropriate empirical antibiotic treatment of UTIs is important for successful outcome and preventing complications. Therefore, antimicrobial resistance to antibiotics commonly used against *E. coli* has emerged worldwide and has converted UTIs into infectious diseases that are challenging to treat. Similar results were found in national and international studies, a study conducted by [Jahanzeb Malik et;al 2020] a prospective, observational study conducted at Benazir Bhutto Hospital, Rawalpindi, Pakistan. Out of 440 urine samples, 144 culture-positive samples had been obtained from male participants and 296 culture-positive samples had been obtained from female participants. The most common organism on analysis was *Escherichia coli*.

Another study by [Sameer S. Kadri et;al 2020],This cohort study included 17430 adults admitted to 104 US hospitals between January 2009 and December 2015, data analysis took place from January 2018 to December 2019. The most common culture-positive sites were urine (9077 [52.1%]), the most common pathogens were *Escherichia coli* (5873 [33.7%]) Hence, Urinary tract infections are the most common type of bacterial infection and *Escherichia coli* is the most common cause of urinary tract infection in both sex.

Pseudomonas aeruginosa is an important opportunistic pathogen with strong virulence and an invasive nature. In our study, table no 9 exclusively shows the presences of pseudomonas aeruginosa that 15 out of 47 samples pseudomonas positive isolates are from sputum. In cystic fibrosis (CF) cases, *Pseudomonas aeruginosa* forms cellular aggregates called biofilms that are thought to contribute to chronic infections they form aggregates and use different mechanisms, each with its own pathogenic implications. These findings are consistent with a similar study conducted by [Abdul Samad et;al 2017] A cross sectional study was conducted from January to December 2014 in Northwest General Hospital and Research Centre, Peshawar. Total of 615 sputum samples were collected from both in and out-patients. Out of 615 sputum samples, 354 (57.56%) were culture positive. Out of these a total of 71 (20.05%) strains of *Pseudomonas* were

isolated. It is also co related with the fact that main organism for UTI is *Escherichia coli* and the association between these two organisms in different sample is significant. *Pseudomonas aeruginosa* thrives in cystic fibrosis lung infections and is the leading cause of mortality in cystic fibrosis patients.

In figure no 3&4 and table no 10-11 the presences of biofilm formers among *Escherichia coli* is approximately 90% that is 93 out of 103 isolates. A biofilm is a community of microbes that typically inhabit on surfaces and are encased in an extracellular matrix. Biofilms display very dissimilar characteristics to their planktonic counterparts. Biofilm forming uropathogenic *Escherichia coli* are associated with persistent and chronic inflammation leading to complicated and or recurrent UTIs. Therefore, biofilms provide an environment for poor antibiotic penetration and favors the development of Multidrug-resistant organisms (MDRO). Similar findings were in accordance with the study conducted by [Zara Rafaque et;al 2020] A total of 155 UPEC strains were scrutinized samples were collected from a tertiary care hospital located in Islamabad out of which113 (73%) were strong biofilm formers.

While in case of *Pseudomonas aeruginosa* 41are biofilm former which is 87% and 6 are non-biofilm formers among 47 samples as shown in figure no 3&4 and table no 10-11. *Pseudomonas aeruginosa* is a bacterium known to produce robust biofilms. *Pseudomonas aeruginosa* biofilms cause severe problems in immunocompromised patients, including those with cystic fibrosis or wound infection. Moreover, the unique biofilm properties further complicate the eradication of infections, leading to chronic infections. The finding of our research was very close to the previous observations done by [Abu Baker Siddique et; al 2019] a total of 200 clinical samples were collected from different hospitals of Punjab, Pakistan. The results showed that from 200 samples 52 (26 %) were *Pseudomonas aeruginosa*. MPA detected 49 (94.23 %) isolates as biofilm producers.

However, in table no 10-11-12&13 the frequency distribution of biofilm formation and its association with organism and gender show that among (134) positive biofilm former (93) were *Escherichia coli* and (41) were *Pseudomonas aeruginosa*. Among 134 positive biofilm formers 87 were males and 47 were females therefore, this correlation is insignificant in our study. Biofilm formation among males in our study is

prominent finding as biofilms are associated with persistent infection and patient risk factors for colonization or infection with biofilm forming isolates.

Increasing multidrug resistance among pathogens is an alarming situation in a hospital setting and requires prompt management of these cases. In our study table no 14 the antimicrobial susceptibility of *pseudomonas aeruginosa* shows maximum sensitivity against polymixin B while they have shown resistance against ceftazidime and ciprofloxacin. The findings are in accordance with the study done by [Lubna Farooq et;al 2019] it's an-vitro Clinical study, conducted in department of Pharmacology, Ziauddin University shows highest susceptibility to Colistin (100%) followed by Ceftazidime (78%) & Ciprofloxacin (80.4%). Colistin is Polymyxin B antibiotic which is used for gram negative bacteria (MDR Bacteria).[Zaheer Ali et;al 2015] study comprised clinical isolates of four strains of Ps. aeruginosa that were collected from patients admitted in different hospitals of Karachi between July 2012 and June 2013. From 204 samples the most effective drug against *Pseudomonas aeruginosa* with 100% susceptibility was Colistin followed by Polymyxin B (98.5%) and meropenem 175(85.8%) highest resistance with Ciprofloxacin (60%) ,Ceftazidime (53.9%). Multidrug-resistant *Pseudomonas* continue to challenge the healthcare community and complicate antimicrobial decisions. *Pseudomonas aeruginosa* remains a public health threat despite ongoing antibiotic stewardship and drug development efforts. This is due in large part to *Pseudomonas aeruginosa* ability to evade host defenses and render multiple antibiotic classes ineffective via rapid development of complex biofilm resistance mechanisms.

Microbial infections of urinary tract are among the most frequently occurring bacterial infections across the globe. Empirical treatment based on global susceptibility data is the preferred method for treatment of urinary tract infections. In table 15, antibiotic susceptibility of *Escherichia coli* is shown that *Escherichia coli* has maximum sensitivity against meropemem, while that maximum resistances tetracycline, Ceftriaxone and trimethoprim/sulfamethoxazole. Similar findings were from [Nazia Khursheed et;al 2020] her retrospective study was conducted at the Indus Hospital Network, Karachi, Of the 10,667 isolates analyzed, 6380(60%) were *Escherichia coli* isolates showed high resistance towards co-amoxiclav, ampicillin, ceftriaxone and ciprofloxacin, leaving little empirical options for treating patients. [Muhammad Ali Shah et;al 2020] conducted a study was carried out in the microbiology laboratory of Abbas Institute of Medical Sciences (AIMS) and Combined Military Hospital (CMH), district Muzaffarabad, AJK,

Pakistan. Urine samples were collected all the 100 *E. coli* isolates, 50 each from AIMS and CMH were susceptible to Imipenem, nitrofurantoin. Resistance was observed in 5 (10%) and 4(8%) isolates from AIMS and CMH, respectively. Resistance against Cefixime 47, Co-trimoxazole 30(60%), Co-amoxiclave 32(64%), Ciprofloxacin 27(54%) was observed in *E. coli* isolates from AIMS. Similarly pattern of resistance against Cefixime 34(68%), Cotrimoxazole 47(94%), and Co-amoxiclave 37(74%), Ciprofloxacin 18(36%) was found in *E. coli* isolates from CMH. Hence concluded, as MDR phenomenon among the microorganism is an evolving rapidly, regular antimicrobial surveillance and monitoring studies should be conducted on local levels to provide physicians with knowledge about the most successful empirical treatment of UTIs.

The failure of conventional antibiotic therapies indicates that biofilm treatments need auxiliary up-gradation (Zhang et al., 2020). Natural anti-biofilm agents selectively exterminate the persistent biofilms and allow the diffusion of bioactive constituents into the biofilm matrix. These natural extract target various phases of biofilm cycle to degrade the biofilm matrix and finally inhibit the cell growth. In our study table no 16-17 shows the anti-biofilm forming activity of pomegranate peel extract (PPE) against *Escherichia coli* and *Pseudomonas aeruginosa* which is 24.46±19.09 mg/ml and 29.26±19.09mg/ml respectively. Pomegranate peels contain Punicalagins and ellagic acid can be extracted on large scales as it shows a significant inhibitory effect on in vitro biofilms produced by these pathogenic organism *Escherichia coli* and *Pseudomonas aeruginosa*. [Salam Nasreddine1 et;al 2018] conducted a study that shows pomegranate peels extracts against *E. coli* (ATCC35218), with an anti-biofilm concentration of 25 mg/ml this shows that minimum biofilm inhibitory concentration of Pomegranate peel extract shows prominent anti-biofilm activity. This finding is insistent with our study as well. Hence, it is proven that these bioactive molecules punicalagin and ellagic acid derived from Pomegranate peel extract play an important role in disruption of biofilms

Although, our study explore mango leafs extract as anti-biofilm activity against *Escherichia coli* and *pseudomonas aeruginosa.* Our research data support that Mango Leaf extracts and its active constituents act as a potential candidate for exploiting anti-biofilm agent against pathogenic and strong biofilm producing *Escherichia coli and Pseudomonas aeruginosa*. As shown in Table no 18-19 antibiofilm forming activity of Mango Leaf Extract against *Escherichia coli* and *pseudomonas aeruginosa*. This shows

14 mg/ml and 11 mg/ml which is slower than the concentration required for Pomegranate Peel Extract against both of these organisms. However, a study conducted by [O.C.Nwiniyi et al 2015] in his study he determined the antibiofilm activity of Mangifera indica leaf extracts against biofilm form on catheters. In another study conducted by [Fahad M. Husain et al 2017] used the leafs of *Mango in* his study the broad spectrum anti-biofilm properties of different fractions from Mango Leaf extract were tester against different bacteria including *Pseudomonas aeruginosa* . Our study and studies conducted by other researchers on leafs of *Mango,* its *extract* suggest that it has a potential anti-biofilm activity and this could serve as a source that has a therapeutic potential for the treatment of infections related with above mentioned bacterial biofilm.

Probiotic bacteria are microbial food supplements with beneficial properties on human health. Lactobacillus is the part of normal flora of the intestine. It plays an important role in human health and stimulating the immune system. In our study table no 20-21 it is shown that anti-biofilm forming activity of probiotic (*Lactobacillus acidophilus*) against *Escherichia coli* and *pseudomonas aeruginosa* which shows 0.5x10^6 and 0.5x10^6 CFU/ml respectively. These finding are consistent with our finding, a study done by [Khaled M. Hassanein, et;al 2021] were he used *Lactobacillus acidophilus,* and its anti-biofilm effects were evaluated against *Pseudomonas aeruginosa* it was shown that *Lactobacillus acidophilus* was able to remove 91.8% of biofilm formed by *Pseudomonas aeruginosa* strains. Another study conducted by [Boris et al. 2016] identified that *Lactobacillus* isolated from dairy inhibit growing of *Pseudomonas aeruginosa,* and *Escherichia coli*. The inhibitoriest effect was for *Escherichia coli*. Similar finding is from a study conducted by [Coconnier et al 2016] study demonstrated that *L. acidophilus* possesses an inhibition effect on *P. aeruginosa, L. acidophilus* reduces these organisms' attachment to epithelial cells and decreases the colony count of these bacteria. Therefore, bacterial-mediated therapy could be considered as an effective treatment. Our research study aimed at developing a new anti-biofilm agent using *lactobacillus acidophilus* bacteria that can be used as anti-biofilm agent for antibiotic-resistant *P. aeruginosa and Escherichia coli* strains.

The formation and development of biofilms is a complicated procedure involving different stages and can be a target of natural anti-biofilm agents for the prevention of biofilm development. The natural anti-biofilm agents either act solely or synergistically

by diverse mechanisms. In our study table no 22-23 further extends the potential of synergistic effect of pomegranate peel extract and mango leaf extract as a source for anti-biofilm agents against *pseudomonas aeruginosa* and *Escherichia coli*. Both of these extract combination display shows a significant synergistic effects. In case of *Escherichia coli* table no 22 the pomegranate peel extract and mango leaf extract the individual antibiofilm concentration is 24mg/ml and 15mg/ml respectively. When used in combination changes to 3.5mg/ml and 3.5mg/ml respectively. While in table no 23 in case of *pseudomonas aeruginosa* the pomegranate peel extract and mango leaf extract shows an individual anti-biofilm activity of 29mg/ml and 11mg/ml respectively. When combine changes to 3.5mg/ml and 1.7mg/ml respectively. It shows there is a drastic change in the concentration which when combine both of these extracts shows synergistic antibiofilm activity. Similar findings are in accordance with a study conducted by [Dhamodharan Bakkiyaraj et al 2013] shows a prominent antibiofilm activity based on the data obtained in his study, Apart from inhibiting the formation of biofilm of entero-pathogenic *Escherichia coli*, pomegranate peel extract disrupted pre-formed biofilms at various concentrations. Thus, it is proven in our study that the inhibition of biofilm formation by pomegranate peel extract and mango leaf extract is observed at different concentrations that suppressed bacterial growth with synergistic combination identified to play an important role in the treatment and prevention of infections.

In our study in table no 24-25 we evaluate the synergistic effect of pomegranate peel extract and *lactobacillus acidophilus* against *Escherichia coli* and *pseudomonas aeruginosa* by using 96 well micro titer plate. Both of these combination shows synergistic effect because in case of *Escherichia coli* (in table no 24) shows the pomegranate peel extract and *lactobacillus acidophilus* there individual antibiofilm concentration is 24mg/ml and 0.5x10^6CFU/ml respectively. When used in combination change to 3.1mg/ml and 0.5x10^4 CFU/ml respectively, which shows there is a drastic change in the concentration combine. While in table no 25 individual anti-biofilm activity of pomegranate peel extract and *lactobacillus acidophilus* against *pseudomonas aeruginosa* there individual anti-biofilm concentrations are 29mg/ml and 0.5x10^6 CFU/ml respectively, but when used in combination there synergistic effect shows 3.6mg/ml and 0.5x10^5 CFU/ml respectively. Similar findings are in accordance with the research conducted by [Philip M. et al 2016] his findings demonstrate that probiotics *lactobacillus acidophilus* prevent preform biofilm cells colonization induced by these

pathogenic *Escherichia coli* strains O157:H7 and O127:H6. Thus, probiotics have also been employed with success in vivo and in vitro antibiofilm agents. Regarding the anti-biofilm activity of probiotics *lactobacillus acidophilus*, in our study they significantly inhibited the biofilm cells proliferation of *Escherichia coli* and *pseudomonas aeruginosa* thereby reducing their cell count is significant.

Now in our study table no 26-27 evaluated the combine synergistic effect of mango leaf extract and *lactobacillus acidophilus* against *pseudomonas aeruginosa* and *Escherichia coli* using 96 well micro titer plate. Both of these combination shows synergistic effect because in case of *pseudomonas aeruginosa* in table no 26 Mango leaf extract and *lactobacillus acidophilus* shows the individual antibiofilm concentration is 11.5mg/ml and 0.5X10^6 CFU/ml respectively. After used in combination change to 1.7mg/ml and 0.5x10^3CFU /ml respectively. This reveals an extreme change in biofilm inhibitory concentration synergistically. While in table no 27 individual concentration of mango leaf extract and *lactobacillus acidophilus* is 14mg/ml and 0.5x10^6 but when used synergistically concentration is reduced to 3.61mg/ml and 0.5x10^4 respectively. Similar results from a study conducted by [Rachael M. Wilson et al 2021] his study shows that *L. acidophilus* reduced biofilm formation as well as destroyed established *Pseudomonas aeruginosa* biofilm. Another study in accordance with our findings related to mango leaf extract [FA.Awe, A.M. Hammed et al 2019] findings reveal that mango leaf extract at a concentration of 10mg/ml contain sufficient phytochemicals to inhibit biofilm formation by pseudomonas aeruginosa. It is therefore suffice to say that plant extract are a good source of natural products and can serve as an alternative to antibiotics also the anti-biofilm activity of these probiotic strains may be primarily linked to the release of self-produced substances, which reduced the number of cultural pathogens in biofilms. Additionally, the integration of probiotic cells into the biofilm may have contributed to the destabilization of the biofilm organization. This proof-of-principle study supports the potential of *Lactobacillus acidophilus* strain to be used as biofilm control agent against pathogenic biofilms development.

5.2 IMPLICATIONS OF THE STUDY.

5.2.1 THEORETICAL IMPLICATIONS:

The antimicrobial tolerance of biofilms has emerged as a significant challenge to medical scientists across diverse healthcare sectors. Synthetic drugs, combinational therapy, and antibiotic hybrids could not achieve and deliver the desired results during the treatment. A panel of studies suggested that conventional antibiotic resistance mechanisms are unable to explain the various cases of antibiotic-resistant biofilm infections. On the basis of limited data it has been reported earlier that repeated exposure of antibiotics in biofilm-growing *Pseudomonas aeruginosa* and *Escherichia coli* has developed a conventional type of intrinsic antibiotic resistance in biofilms infections. In biofilm communities, antibiotics resistance appears due to various strategies such as slow or incomplete penetration of the antibiotics into the biofilm, an altered chemical microenvironment within the biofilm and a subpopulation of micro-organisms in a biofilm. The hunt for anti-biofilm in drug resistance emergency insists on the scientific society to search innovative natural anti-biofilm agents. The focus of the our study and its implication represent that it is to assess various natural products to overcome the biofilm-forming microorganisms and provide concise information on existing confines and recent developments in the modification of different natural anti-biofilm agents to make them effective drug candidates for clinical use and to avoid drug misuse.

5.2.2 PRACTICAL IMPLICATIONS:

Till date, a lot has already been studied and understood about the bacterial biofilm formation. Emergence of severe biofilm infections and its resistance to antimicrobial treatment, has posed a great challenge in the medical field. To find a significant and effective alternative against biofilm formation, and focus on the discovery of different natural anti-biofilm compounds/agents along with modifications, our study provides the information regarding natural extract provides a better understanding about the nature of biofilms, which can be further used to develop new and successful anti-biofilm agents which targets the main cells. This can also bring improvement in the efficacy of the previously known drugs that are becoming resistant. The naturally derived anti-biofilms extracts and probiotics have more biochemical and structural diversity as compared with synthetic drugs, thus could be very useful in developing various alternative therapies allowing enhanced binding to the target and has inhibitory effects. In contrary, synthetic drugs are cost effective and consume less time but many of them show adverse side effects and rapidly gaining resistances. Therefore, the use of natural remedies has also been widely embraced in many developed countries with complementary and alternative medicines so remodeling or incorporating the previously used drugs with these natural alternatives as it is easy to extract in limited time and there abundant availability in nature prove to be a more appropriate and constructive idea and we encourage practitioners to limit the antibiotic usage and provide alternative natural options to treat bacterial infections and minimize resistances.

5.2.3 POLICY IMPLICATIIONS

The agents of natural origin are structurally and functionally more diverse in comparison with conventional antibiotics. The structure and function of natural anti-biofilm agents from various sources have been exploited to develop/study numerous advanced therapeutic strategies showing increased activity, stability, and reliability. Our study use natural product extract, mainly pomegranate peels, mango leaves and probiotic *lactobacillus acidophilus* that act as anti-biofilm agents contain bioactive compounds punicalagins, anthocyanins and flavonoids. We encourage national authorities to take action and incorporate such bioactive molecules that help inhibit biofilm forming pathogens and help prevent the development of chronic infections.

5.3 STRENGTH AND LIMITATIONS OF THE STUDY

A) Strengths of the study:

- Pomegranate peel extract, mango leaf extract and probiotic lactobacillus acidophilus combinations synergistic use is promising therapy for treatment of *Escherichia coli and Pseudomonas aeruginosa* infection.

- Addition of natural product extracts and probiotics facilitates the physicians to treat multi drug resistant microorganisms. It is a valuable treatment option against emerging antimicrobial drug resistant producing gram-negative rods.

- Antibiofilm forming activity of natural product extracts and probiotic at different concentration calculated by 96 well micro titer plate method, that is easy and cost effective method.

- The next future antibiofilm technologies would be focused in the development of in vivo evaluation methods with the end to determine the action of new antimicrobial drugs against biofilm formation which can predict the clinical outcome in persistent infections of drug combinations or new treatment strategies, through application of specific parameters, that can be useful in establishing a successful anti-biofilm activity/therapy.

- Our study demonstrated solid evidences that plants are an excellent source to provide abundant natural compounds for the development of preventative and therapeutic agents against biofilm-based infections.

B) Limitations of the study:

- The study is being conducted at only one setting, it should be multi centered keeping with the rampant level of infections by *Escherichia coli and pseudomonas aeruginosa* in the general population and all age groups.

- Sample size is small.

- Biofilm formation and anti-biofilm forming activity of natural product extract and probiotic (*lactobacillus acidophilus*) against only two members among gram negative rods that is *Escherichia coli* and *pseudomonas aeruginosa* are being studied.

- Identification of biofilm formation among both micro-organisms is done only by 96 well micro titer plate methods.

5.4 FUTURE RESEARCH DIRECTIONS/RECOMMENDATIONS:

- Clinical trials should be done further to analyses safety and efficacy of natural product extract and lactobacillus acidophilus on local population in Pakistan.

- More studies should be directed in this regard for converting the novel anti-biofilm compounds into drugs. As a result, more research in this direction can enhance success rate in clinical trials at the final stage. Further novel natural anti-biofilm agents/compounds in therapeutics may be possible if rigorous studies will be done in our country or needed to test the efficiency of natural anti-biofilm agents in the future.

- Future clinical trials should be done further to analyses Safety and efficacy of natural product extract and *lactobacillus acidophilus* on local population in Pakistan.

- Here, we continue to analyze the efficacy of specially targeted drug-tolerant pathogenic biofilms produced by *Escherichia coli* and *pseudomonas aeruginosa* without disturbing the natural micro-flora. Incorrectly prescribed antibiotics also contribute to the promotion of resistant bacteria. Thus, the possible reason for this failure to treat multiple infections may be the availability of the antibiotics which are unregulated and available over the counter without a prescription.

- This lack of regulation results in antibiotics that are easily accessible, plentiful, and cheap, which promotes overuse. One possible solution to this problem is a combination of strategies like antibiotics, along with natural anti-biofilm agents should be used for better results combination therapy of natural agents with commercial antibiotics needs urgent exploitation in the future to advance anti-biofilm activity of natural origins along with antibiotics can be a novel lead for species-specific biofilm destruction, as it has a promising utilization aspect in health care industries.

- Detailed analysis/further experiments can be conducted that compare the anti-biofilm activity of the synergistic use of pomegranate peel extract with mango leaf extract and probiotics (*Lactobacillus acidophilus*). Most of the previous studies show that these natural extracts and *lactobacillus acidophilus* is very effective drug against biofilm forming gram-negative rods.

- The future studies should be conducted on *Escherichia coli* and *Pseudomonas aeruginosa* infected patients having other health related complications.

- Awareness programmers should be conducted about antimicrobial-resistant and proper use of antibiotics for patients on government level and local levels.

5.5 CONCLUSION:

A major setback in the treatment of biofilm-related infections is the ineffectiveness of existing antibiotics due to the protective layers built by cells in the biofilm. There is therefore limited antibiotic penetration, so the community of sedentary cells persists even in the presence of antibiotics effective against their motile counterparts. The dispersion of matured biofilms may primarily require the disruption of the EPS matrix. Our research study aimed at developing a new anti-biofilm agent from Pomegranate peel extract and mango leaf extract that is less expensive, uses waste resources, produces higher yields, and can be used as biofilm inhibitory agents. Hence, plants based extract represent a virtually inexhaustible and sustainable source of free anti-biofilm agents with novel targets, unique modes of action and proprieties with potential for utilization in a plethora of medical, agricultural, and industrial fields. On the one hand, realization of this possibility has so far been hindered by insufficient fundamental research done in this way to comprehensively understand the ecologically relevant functions of plant-derived compounds in the real natural environments and there properties that inhibit pathogenic biofilms. We also discovered that including probiotic bacteria into treatment techniques can be extremely beneficial in preventing and/or treating hospital-acquired illnesses as well as reduce the possibilities of opportunistic infections. Possibly, for the first time we have utilized Pomegranate peel extract and mango leaf extract and lactobacillus acidophilus to demonstrate synergizing anti-biofilm effect their extensive biofilm inhibition/removal activity suggests, that they could be used as a biofilm control agent for antibiotic-resistant or multi drug resistant *Pseudomonas aeruginosa* and *Escherichia coli* strains. Therefore, natural products have provided considerable value to the health care system over the past half century. In particular, the therapeutic areas of infectious diseases have been benefited from numerous drug classes derived from natural product sources. Despite many new and interesting molecules with biological activity have been published in the past few years. If natural product materials/extracts continue to be tested for desirable therapeutic activities, we believe that significant progress in identifying new antibiotics, therapeutics and other useful medicines will be made.

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Valerie S. \Forsyth, Chelsie E. Armbruster https://orcid.org/0000-0002-2165-2759, Sara N. Smith, Ali Pirani, A. Cody Springman, Matthew S. Walters Greta R. Nielubowicz

Varriale, L., Dipineto, L., Russo, T. P., Borrelli, L., Romano, V., D'Orazio, S., Pace, A., Menna, L. F., Fioretti, A., & Santaniello, A. (2020). Antimicrobial Resistance of *Escherichia coli* and *Pseudomonas aeruginosa* from Companion Birds. *Antibiotics (Basel, Switzerland)*, *9*(11), 780.

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APPENDICES

A) FRC APPROVAL LETTER

Bahria University
Discovering Knowledge
Medical and Dental College, Karachi

Ref no: FRC/BUMDC -14/2021-Path-104 **MS-11**

Approval of Research Proposal

Mr/Miss/Ms/Mrs/ **Dr. Hadia Khursheed**

Registration No:

Dear MS/MPhil Student,

I am pleased to inform you that your research proposal on **"Antibiofilm forming activity of natural products Punica granatum L., Magnifera indica L. and Probiotic against biofilm form by Escherichia coli and Pseudomonas aeruginosa among different clinical specimens"** has been approved. You may, therefore, continue your research on this theme and produce a quality thesis, as per the HEC requirements.

I take this opportunity to remind you that you must complete your thesis, and defend it successfully, by **SPRING 2023**; this is the date which marks the end of the Extended Duration of your programme. However, to remain eligible for honours and awards, you must complete the thesis, and successfully defend it, by the end of 10 week of final semester.

I wish you every success.

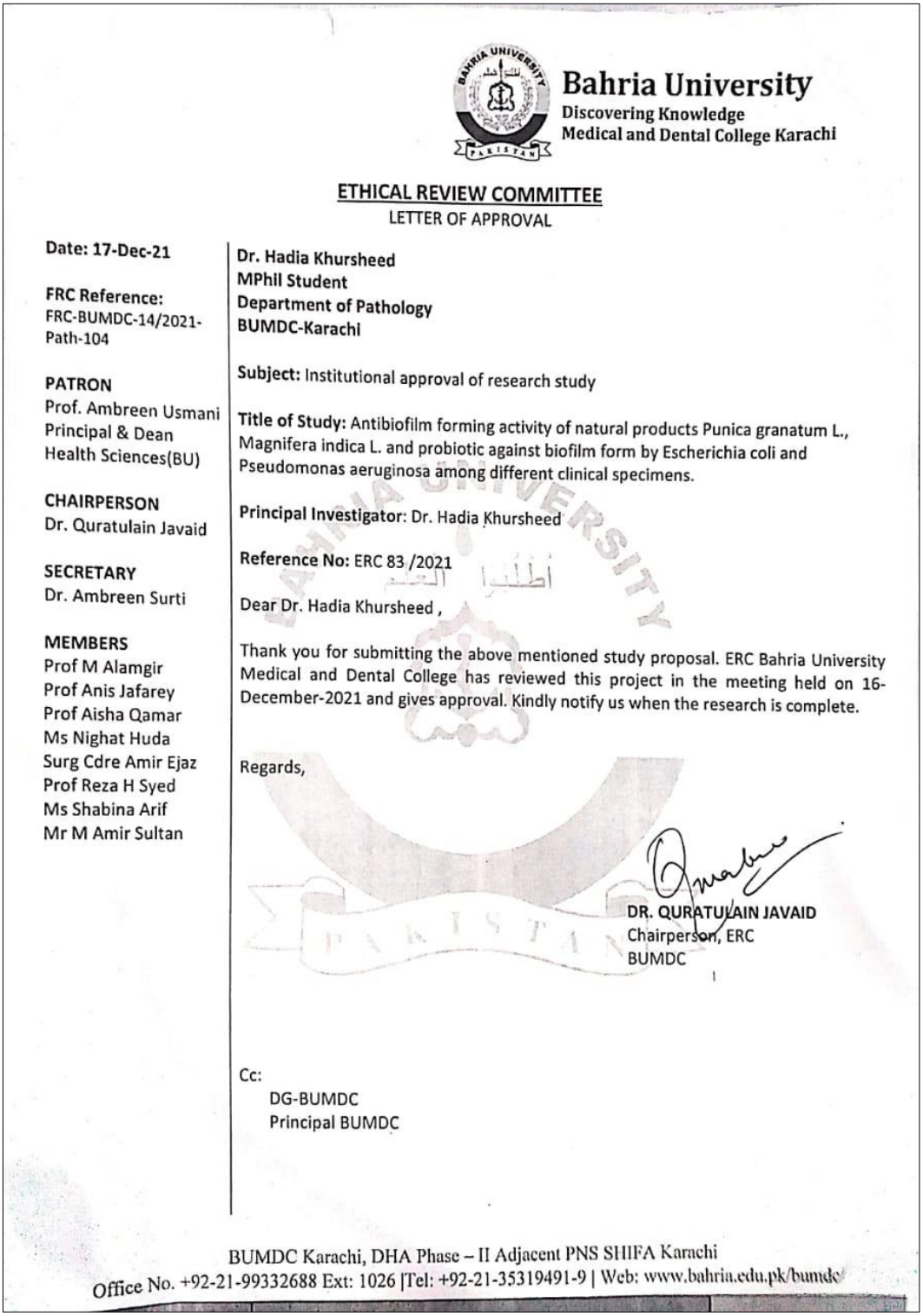
Dated: 16/9/2021

CHAIRPERSON FRC, BUMDC

Distribution:

- DG
- Principal
- Student's File (with the HOD/PGP Coordinator)
- Student

[B] ERC APPROVAL LETTER



Bahria University
Discovering Knowledge
Medical and Dental College Karachi

ETHICAL REVIEW COMMITTEE
LETTER OF APPROVAL

Date: 17-Dec-21

FRC Reference:
FRC-BUMDC-14/2021-Path-104

PATRON
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Principal & Dean
Health Sciences(BU)

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Prof Reza H Syed
Ms Shabina Arif
Mr M Amir Sultan

Dr. Hadia Khursheed
MPhil Student
Department of Pathology
BUMDC-Karachi

Subject: Institutional approval of research study

Title of Study: Antibiofilm forming activity of natural products Punica granatum L., Magnifera indica L. and probiotic against biofilm form by Escherichia coli and Pseudomonas aeruginosa among different clinical specimens.

Principal Investigator: Dr. Hadia Khursheed

Reference No: ERC 83 /2021

Dear Dr. Hadia Khursheed ,

Thank you for submitting the above mentioned study proposal. ERC Bahria University Medical and Dental College has reviewed this project in the meeting held on 16-December-2021 and gives approval. Kindly notify us when the research is complete.

Regards,

DR. QURATULAIN JAVAID
Chairperson, ERC
BUMDC

Cc:
DG-BUMDC
Principal BUMDC

BUMDC Karachi, DHA Phase – II Adjacent PNS SHIFA Karachi
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[C] INFORMED CONSENT FORM (ENGLISH)

INFORMED CONSENT FORM FOR THE PATIENTS

I am giving my consent to participate voluntarily and at my own will in this research project which aims to determine effectiveness of new strategies that is the use of probiotics and natural products and its antibiofilm forming activity against *Escherichia coli* and *Pseudomonas aeruginosa* for the purpose of research.

I have been told that finding of my disease and my data will be kept strictly confidential and will be used only for the benefit of the community, through publications and paper presentations.

I fully agree to give my samples at the beginning and end of the study and when required in between. Also, i have been told that there are no risk involve in this study as it is in-vitro study, a benefical treatment option will be available to eradicate the disease.

I also agree to give all relevant information when needed, in full and to the best of my knowledge to the researcher. It is clarified to me that no incentive will be provided to me for participating in the study, whereas I do have the right to with draw from the study at any time.

I am advised to contact DR.HADIA KHURSHEED on mobile no: 03480266935/03242072499 or visit PNS SHIFA Hospital in case of any query/ emergency related to my disease.

NAME OF THE PATIENT: _____

SEX: _____

S/O, D/O, W/O, F/O: _____

AGE: _____

SIGNATURE/ THUMB IMPRESSION OF THE PATIENTS AND GUARDIANS OF THE MINORS:

_____

NAME OF THE RESEARCHER: _____

SIGNATURE OF THE RESEARCHER:

_____

DATE: _____

[C] INFORMED CONSENT FORM IN URDU

(آگاہ شدہ رضاکارانہ فارم برائے مریض)

میں رضاکارانہ طور پر اجازت دیتی / دیتا ہوں کہ میں اپنی مرضی سے اس تحقیقی منصوبے میں حصہ لے رہی / رہا ہوں جس کا مقصد پیٹ کی بیماریوں کی ایک نئی دوا کی تاثیر کا تعین کرنا ہے۔

مجھے بتایا گیا ہے کہ اس سلسلے میں میرے جسم سے ایک نمونہ (خون، پیشاب اور پاخانہ) لیا جائے گا۔ مجھے یہ بھی بتادیا گیا ہے کہ میری بیماری کے نتائج اور اعداد و شمار کو خفیہ رکھا جائے گا اور صرف برادری، اشاعت اور مطالعہ کی پیشکش کے فائدے کے لیے استعمال کیا جائے گا۔

میں مکمل طور پر اپنے نمونے دینے کے لیے اتفاق کرتی / کرتا ہوں چاہے مطالعہ کے شروع، آخر یا بیچ میں کبھی ضرورت پڑے، ضرورت کے تحت میں اپنی تمام تر معلومات فراہم کرنے پر بھی اتفاق کرتی / کرتا ہوں۔

مجھے بتایا گیا ہے کہ اس تحقیق کے لیے مجھے کوئی حوصلہ افزائی نہیں دی جائے گی اور مجھے اپنی خواہش کے مطابق اس مطالعہ سے نکلنے کا حق حاصل ہے۔ اس کے علاوہ، مجھے بتایا گیا ہے کہ اس تحقیق میں کوئی خطرہ نہیں ہے کیونکہ یہ ایک ان وٹرو مطالعہ ہے، بیماری کو ختم کرنے کے لیے ایک فائدہ مند علاج کا آپشن دستیاب ہوگا۔

مجھے مشورہ دیا گیا ہے کہ میں ڈاکٹر سے انکے موبائل نمبر03242072499 / 03480266935 یا پھر PNS SHIFA HOSPITAL KARACHI ہسپتال میں کسی بھی سوال یا ہنگامی صورتحال میں رابطے میں رہوں۔

مریض کا نام:_____

جنس:_____(مرد / عورت)

والد / شوہر کا نام:_____

عمر:_____

دستخط / اور نابالغوں کے سرپرست:_____

نشان انگوٹھا:_____

[D] SUBJECT EVALUATION PERFORMA

PROFORMA

Name of the patient: _____

Father/ Husband name: _____

Signature/thumb impression of the patient: _____

Name of the researcher: _____

Signature of the researcher: _____

Date: _____

SUBJECT EVALUATION PERFORMA

PRELIMINARY DATA

- **Date: _____**
- **Ward no: _____**
- **ID no: _____**
- **Patient name:**

  _____

- **S/O D/O W/O F/O :**

  _____

- **Age: _____ Years**

- **Gender: Male / Female**

- **Occupation:**

  _____

- **Address:**

- **Presenting complains:**

  _____

- **Past medical history:**

  _____

- **Laboratory test:**

  _____

- **Samples: _____**

- **Gram staining: _____**

- **Bacterial growth of blood agar:**

  _____

- **Bacterial growth on MacConkey agar:**

  _____

- **Triple sugar iron TSI agar:**

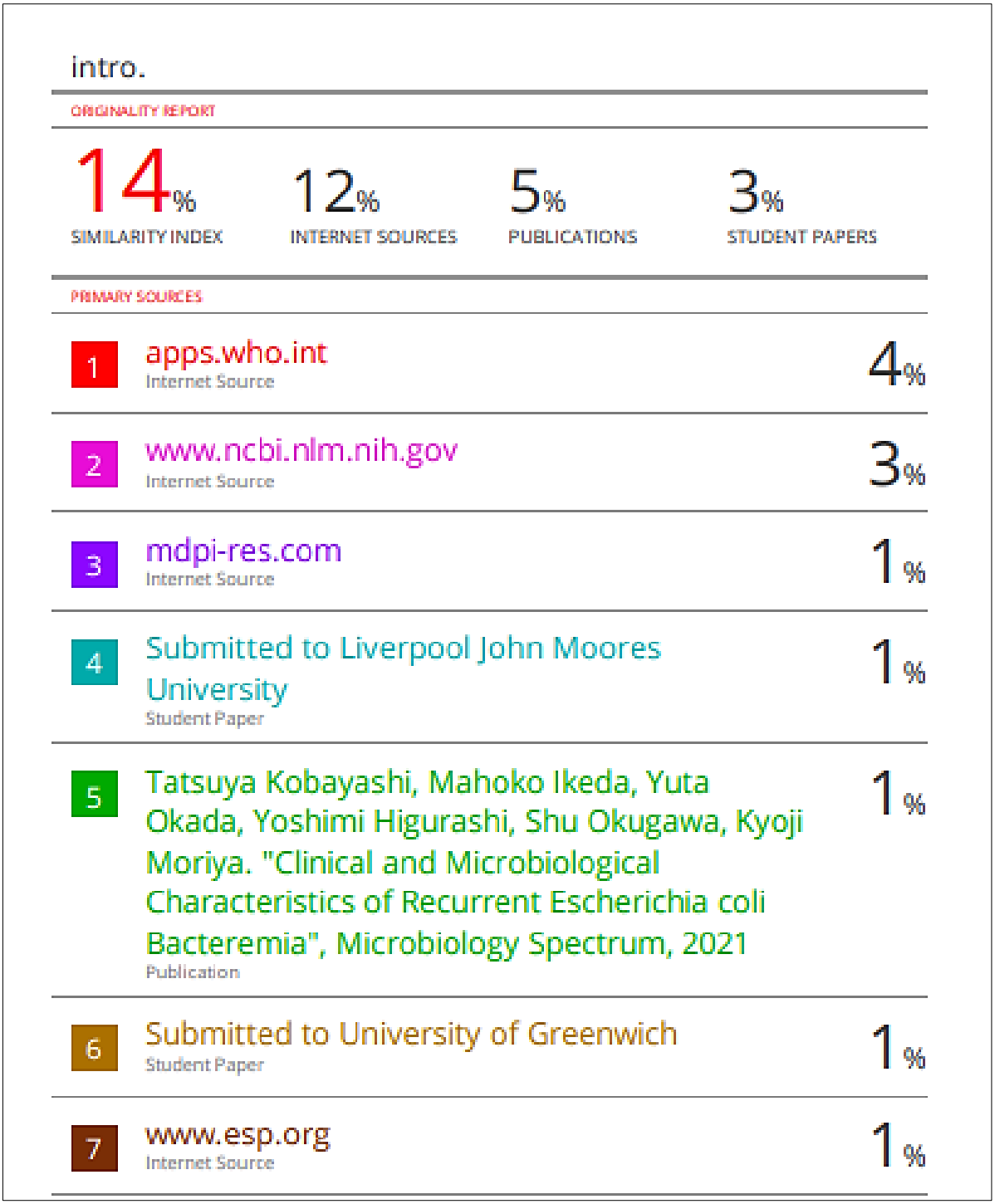
  _____

- **Biochemical test: _____**

- **Adverse effect if any : _____**

[E] HOSPITAL CARD:

[F] TURNITIY PLAGIRISM REPORT



intro.

ORIGINALITY REPORT

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