ANTI-BIOFILM FORMING ACTIVITY OF NATURAL PRODUCTS EXTRACT PUNICA GRANATUM L. AND MAGNIFERA INDICA L. AGAINST ESCHERICHIA COLI BIOF ILM

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ABSTRACT

OBJECTIVES

To evaluate the biofilm activity of Escherichia coli and the anti-biofilm forming activity of Pomegranate peels Punica granatum L. and Mango leafs Mangifera Indica L. extracts against Escherichia coli and their combined synergistic effect using 96 well microtiter plate.

METHODOLOGY

The study design was a cross-sectional study. The sample size was 150. The samples were collected from patients at PNS Shifa Hospital Karachi. The age group of the individuals included was from 15 to 50 years. The specimens received in the lab were inoculated on CLED agar, Blood agar, and MacConkey's agar culture plates. Escherichia coli was identified by colony morphology, gram staining, TSI, and further biochemical test analysis. After identification, the samples were processed for biofilm activity on 96 well microtiter plate method and using serial dilution method to assess the anti-biofilm activity of natural product extracts. Patient's age, gender, and hospital number of patients were recorded on specially designed proforma with ERC approval no 83/2021.

RESULTS

Among 150 patients, 64% were males, and 36% were females. Overall mean age was (33.79±9.94) and (34.02±10.59) years. 90% of samples showed biofilm formation. We found a significant relationship between culture and examination (p-value 0.000), while no significant association was found between gender (p-value 0.69), age (p-value 0.44) and biofilm formation (p-value 0.57). Anti-biofilm forming activity of pomegranate peel extract against Escherichia coli was (24.46±19.09) with mean and standard deviation. Anti-biofilm forming activity of Mango leaf extract against Escherichia coli was (14.90±9.56). Significant synergistic relation was observed in both extracts, Punica granatum L. and Mango leaf extract Mangifera Indica L. used in combination.

CONCLUSION

It was concluded that a novel combination of natural product extracts had shown higher effectiveness against the rapid emergence of biofilm-forming pathogens.

KEYWORDS: *Biofilm, Escherichia Coli, Punica Granatum, Mangifera Indica, 96 Well Microtiter Plate*

INTRODUCTION

The rising spread of antibiotic-resistant bacteria poses a significant health threat to morbidity and mortality worldwide.[1] Because it's a serious public health problem with social impacts, MDR-bacteria has long been considered a global priority for investment in new drugs by the WHO.[2] Escherichia coli belongs to Enterobacteriaceae, a facultatively anaerobic, non-spore-forming, gram-negative rod.[3] Escherichia coli resides in the normal flora of the human colon and is frequently found in the large intestine.[4] These organisms are responsible for most of the clinical and subclinical infections, including urinary Tract Infection (UTI), the most frequently reported diagnosis (16.1%) throughout Pakistan, according to studies published in the last decade. E. coli was found in 28 (30.11%) of the investigations, with strong resistance to first-line medicines, followed by the bloodstream and respiratory tract infections, and frequently found in hospital-acquired infections.[5] Organisms that cause these infection has developed resistance mechanisms by forming antibiotic-resistant biofilms.[6] Biofilms, which are complex bacterial summative encased in a self-generated matrix of extracellular polymeric substances (EPS), are one of the main approaches to the survival of many species.[7] A majority of bacterial infections are related to these antibiotic-resistant biofilms. Targeting bacterial biofilms is considered a good strategy to limit microbial virulence.[8] The World Health Organization

(WHO) promotes using medicinal herbs as a natural remedy to benefit in the absence of conventional treatment options.[9] Therefore the usage of herbal medicines is quickly expanding worldwide, among many individuals increasingly turning these natural items for treatment in various health challenges in different national and international healthcare settings.[10] Several natural compounds or products isolated from multiple sources (plants, animals or microorganisms) have antibacterial and antibiofilm effects.[11] Magnifera indica L. (mango leaves) extract is used for medicinal purposes to cure diabetes, bronchitis, diarrhoea, asthma, kidney, scabies, metastasis problems, syphilis, and urinary infections.[12] Mangiferin is one of the main constituents with most of the biological activity of the Mango Leaf extract. Mango Leafs have an excellent scope of valorization as antimicrobial and antibiofilm activity.[13] Antimicrobial activity of Punica granatum L, peel, seeds and juice has been established against Escherichia coli, H.pylori, Klebsiella, Listeria monocytogenes, Pseudomonas aeruginous, and other microorganisms.[14] So our study is conducted to evaluate the biofilm formation by Escherichia coli and the anti-biofilm forming activity of pomegranate peels Punica granatum L. and Mango leave Mangifera Indica L. extracts against Escherichia coli. There combined synergistic effect using 96 well microtiter plate.

METHODOLOGY

The study was conducted between the duration of Sep 2021- May 2022. Permission was taken from Institutional Ethical Review Committee ERC approval no 83/2021. Informed consent from all 150 patients was taken for this study. Age, gender and hospital number of patients were recorded on specially designed subject evaluation proforma. Non-probability convenient sampling technique was utilized. Specimens received in the laboratory, including blood, urine, CSF, respiratory and pus, were collected from PNS Shifa Hospital Karachi. Among them, Escherichia coli was isolated. Specimens received in the lab were inoculated on CLED agar, Blood agar, and MacConkey's agar culture plates. Culture plates were inoculated at 37 ℃ in an incubator for 24 to 48 hours. Escherichia coli was identified by colony morphology, gram staining, TSI, and different biochemical test analysis. After identification, a suspension equal to 0.5 McFarland turbidity standard of all the samples was processed on a 96-well microtiter plate and serial dilution methods. Escherichia coli was isolated from the patient's clinical specimens, including Urine, Blood, Pus, CSF, and respiratory. The age group of the individuals is from 15 to 50 years of age, and both genders were included. Fifty years of age and both genders were included. Repeated samples from the same patients, HIV-infected patients, and malignancy-associated patients were excluded. All the data was entered in a specially designed Subject Evaluation proforma and assessed through SPSS version 23. All experiments were conducted at least in triplicate. The current study used SPSS 27.0 software (SPSS Inc.) for statistical analysis. Quantitative data were expressed as mean ± standard deviation. Fresh mango leaves are collected and properly cleansed with deionized water to remove dirt particles before being air-dried for a week. The air-dry leaves are then crushed into a fine powder using a domestic electric grinder. In a sterile conical flask, 250 g of leaf powder was immersed in 500 ml of methanol and kept at room temperature for 72 hours. This technique extracted the bioactive constituents. The powdered extract was then filtered using Whatman no. 1 filter paper, and the filtrate was concentrated under a vacuum at 40°C with a rotary evaporator (Heidolph equipment) to evaporate the methanol. After storing the dried extract, several dilutions of the mango extract of leaves were generated by dissolving it in 10% DMSO at different concentrations of 50 mg/ml, 100 mg/ml, and 150 mg/ml for use in various experiments.[15] To prepare pomegranate peel extract samples, the seeds were manually separated from the peel, then the separated peels were cut into small pieces, and then air-dried at room temperature until uniform volume was obtained. Air-dried peels were then homogenized with a household electric grinder until a fine powder was obtained. A constant amount of peel powder of (15g) was used to extract the natural bioactive ingredients when placed in a beaker containing (150ml) methanol and left at room temperature for 72 hrs. After preparation, the solution was filtered with 0.45 m filter paper, then concentrated and evaporated to dryness under vacuum using a mini rotary evaporator at 40°C (Soxhlet apparatus) until almost all solvent was vaporized. The dry extract obtained from the solvent was stored at -20°C for further use in various tests.[16] 96 well microtiter plate method; Isolates from newly cultivated plates were then subcultured in Tryptone soy broth (TSB) containing 1% glucose and incubated for 24 hours at 37 C before diluting (1:100) with bacterial suspension. Individual wells of sterilized, polystyrene, flat-bottom tissue cultivation plates were subsequently loaded with 0.2 ml aliquots of the diluted cultures, with broth in well no 12, serving as a control to assure sterility and non-specific medium adherence. The cell culture plates were kept for 24-72 hours in an incubator at 37°C. After incubation, the components in each well were carefully removed by tapping the surfaces of the 96-well microtiter. The wells were washed four times using 0.2 ml of phosphate buffer solution (PBS pH 7.2) to remove free-floating planktonic bacteria, and the remaining adherent

bacterial biofilms were dyed with 25 µl of 1% solution of crystal violet dye was incorporated into each well (this dye stains the cells but not the polystyrene plates). The plates were then incubated at room temperature for approximately 15 minutes before being carefully rinsed with distilled water several times. Crystal violet dye was used to stain adherent bacterial stained cells that developed biofilm across all sides of the wells. The crystal violet dye stained biofilms were solubilized in 200µl of 95% ethanol (to remove the violet dye color), of which 125µl of which was transferred into a new polystyrene96 well microtiter plate, which would then be read Using a micro ELISA auto reader, the optical densities (OD) of 570 nm stained adhering bacteria were determined (model 680, Bio-rad) The wavelength of OD 570nm estimation was analyzed to measure which microbes were adhering to the surface and forming biofilms. The experiments were done in triplicate for each strain.[17]

RESULTS

Table 1: Frequency Distribution of Genders (n=150)

Gender	N(F)
Males	96(64%)
Females	54(36%)

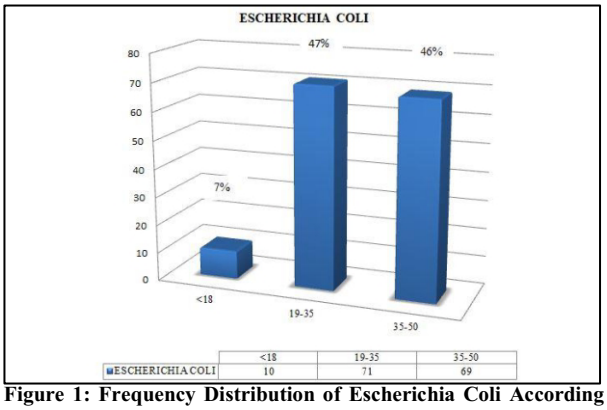

Figure 1: Frequency Distribution of Escherichia Coli According to Age Groups (n=150)

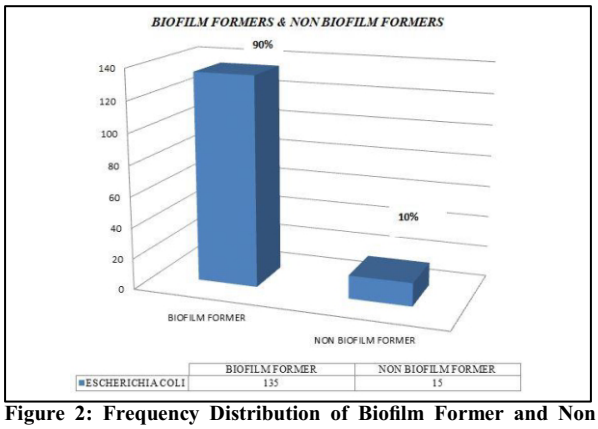

Figure 2: Frequency Distribution of Biofilm Former and Non-Biofilm Former among Escherichia Coli (n=150)

Table 2: Anti-Biofilm Forming Activity of Pomegranate Peel Extract against Escherichia Coli

Organism	Compund	Antibioifilm Forming Activity of (MLE) Mg/Ml
Escherichia Coli	Pomegranate Peel Extract (PPE)	24.46±19.09

Table 3: Antibiofilm-Forming Activity of Mango Leaf against Escherichia Coli

Culture	Compund	Antibioifilm Forming Activity of (MLE) mg/ml
Escherichia Coli	Mango Leaf Extract (MLE)	14.90±9.56

Table 4: Combine the 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

Synergistic calculations are seen using the 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 compounds A and B alone. FIC index of ≤ 0.5 represents a synergistic effect. FIC index of > 0.5 and ≤ 1, Indifference effect. FIC index of > 1 and ≤ 2 and antagonism effect.[18]

DISCUSSION

Numerous Pathogenic microorganisms are constantly emerging worldwide. Laboratory studies on microbial evolution continuously provide a better understanding of evolutionary dynamics, identify adaptive and environmental changes, and answer key questions affecting human health. 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 has long been considered a global priority for investment in new drugs by the WHO.[19] One the examples, the vicinity among cells in biofilms can expedite the transfer horizontally and resistances that persist in the genes in bacterial populations. Therefore biofilm itself may protect its residents from exposure to external stresses such as antibiotics or host immunity host and weaken selection. Steady growth within the biofilms may lessen the effectiveness of drugs that especially attack the rapidly growing cells.[20]

In Table 1, based on our study findings, the frequency and association of both microorganisms according to gender showed a majority infectious rate in male patients, 64% (96) and 36% (54) out of 150 samples. These findings are consistent with Waleed Al-Momani et., al 2022 study that includes 24 total samples from which positive 15 cultured case, from which 9 (60%) belong to male patients and 6 (40%) to female patients.[21] Our data showed that men are more commonly infected with Escherichia coli infections. While our findings in Figure 2 show that Escherichia coli is most prevalent in the age group of 19-35 years, 47% (71) out of 150 samples. Another study showed that male & female patients were from the age group 19 to 35 years.[22,23] In our study, figure 3 represents the presences of biofilm formers among Escherichia coli, which is approximately 90%, 135 out of 150 isolates. A biofilm is a colony of bacteria living on surfaces enclosed by an extracellular matrix. Similar findings were per the study conducted by Zara Rafaque et., al 2020, in which a total of 155 UPEC strains were scrutinized. Samples were collected from a tertiary care hospital in Islamabad, out of which which113 (73%) were strong biofilm formers.[24] Another study's findings by L Wang, K Zhang et., al 2020 are consistent with ours that a total of 44 E. coli isolates, 39 E. coli isolates were biofilm formers.[25] The above finding shows that most E.coli strains that cause infection are biofilm formers. In the case of Escherichia coli, our findings in Tables no 2 and 3 show that the pomegranate peel extract and mango leaf extract show individual antibiofilm activity in a concentration of 24.46mg/ml and 14.9mg/ml, respectively. Whereas in Table 4, findings showed that both extract combinations show significant synergistic effects. When combined, changes to 3.46mg/ml and 3.56mg/ml instead of concentrations 24.46mg/ml and 14.9mg/ml, respectively. The above finding shows that the abovementioned natural products decrease the concentration required for antibiofilm activity so that we can use the\m in variety. However, numerous novel and intriguing compounds with bioactive compounds have been discovered in the past few years. We anticipate that if plant-derived products are evaluated for desirable therapeutic properties, tremendous progress towards discovering novel therapeutic drugs will be made. Future clinical trials should be done further to analyze the safety and efficacy of natural product extract, this combination of techniques, including antibiotics and potential anti-biofilm agents, should indeed be utilized for better outcomes and requires additional investigation in the future.

LIMITATIONS

The study is conducted in only one setting. It should be multi-centred, keeping with the rampant level of infections by Escherichia coli in the general population and all age groups. The sample size was small. Biofilm formation and antibiofilm-forming activity of natural product extracts were evaluated against only one member of the gram-negative rod, Escherichia coli.

CONCLUSIONS

The ineffectiveness of present antibiotics for managing biofilm-related illnesses is a significant setback. This is due to the layers of protection created by bacteria in the biofilm. Our research study aimed at developing a new anti-biofilm agent from Pomegranate peel extract and mango leaf extract that is inexpensive, utilizes waste resources and can be used as biofilm inhibitory agents. Hence provide an alternative regimen for the treatment of biofilm-forming Escherichia coli-related infections.

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