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New Strategies for Inhibition of *Listeria monocytogenes* and *Klebsiella pneumoniae* Biofilm Formation and Persistence

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ABSTRACT

Objectives: This study aimed to find new strategies for the prevention of bacterial biofilms and investigate the effect of some plant extracts on the biofilm formation by certain pathogenic bacterial strains in vitro.

Methods: Fourteen different biofilm forming bacterial isolates were collected and their biofilm were quantitatively measured under different temperature, pH and growth medium using Crystal violet staining method. Exopolysaccharides (EPS) produced by the isolates were estimated and a comparison between the tested isolates was made. The effect of some plant extracts on bacterial growth, biofilm formation and exopolysaccharide quantity was determined.

Results: The two isolates, *Listeria monocytogenes* (ATCC13932) and *Klebsiella pneumoniae* (ATCC700613) were among the most active biofilm forming bacterial isolates. The optimum temperature, pH and media for EPS production and biofilm formation were determined. The effect of some plant extracts of Cranberry; Pomegranate peel and, Arak on growth and formation of biofilm and EPS were recorded. Moreover, minimum inhibition concentration of each plant extract was performed.

Conclusion: EPS quantity produced from the tested isolates depends on some effective factors such temperature, media contents and pH. Aqueous-Cranberry, aqueous-Pomegranate Peel and methanolic-Arak extracts have antibacterial and antibiofilm activities on *L. monocytogenes* and *K. pneumonia*. Thus, application of new natural approaches for inhibiting bacterial biofilm is important to prevent persistent and recurrent biofilm related infections.

Keywords: Biofilms, plant extracts, exopolymeric, *Listeria monocytogenes*, *Klebsiella pneumonia*

Introduction

Bacterial biofilm is a structured community of bacterial cells attached to a surface and surrounded in a self-produced Exopolymeric substance (EPS) matrix made up primarily of polysaccharides and other biomolecules such as proteins, lipids, and nucleic acids. Bacterial biofilm identified as a significant virulence mechanism in the pathogenesis of many medically important bacterial pathogens, causing serious life-threatening infections. Bacteria within the biofilm can persist, causing chronic and recurrent infections and developing antibacterial and immunological resistance. The phenomenon of biofilm recalcitrance makes them extremely difficult to treat and eradicate effectively.[1] Protection from the hostile environment, nutritional availability, metabolic cooperation, and the acquisition of new genetic material are all functional advantages of bacterial biofilm. Many pathogenic bacteria, including *Listeria monocytogenes*, *Streptococcus mutans, Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa,* have been shown to form biofilms.[2] The main concern brought on by biofilms is a development in antibiotic resistance that makes it difficult to cure infections caused by biofilms. Globally, public health is at risk due to the rise in microbial resistance to antibiotics, which decreases the efficacy of treatments and raises morbidity, mortality, and medical expense rates.[3] Due to the enhanced resistance provided by sessile cells, many of the control strategies for biofilm control appear to be almost worthless. Consequently, biofilms are not easily eliminated by managing elements like heating, drying, cleaners, and detergents and persist on surfaces, especially in hospitals, where they cause contamination and the spread of infections. Therefore, new strategies other than the traditional ones are urgently required, and a suitable solution for the "biofilm problem" is anticipated in near future.[4]

It has been demonstrated that natural compounds made from numerous plant extracts inhibit adhesion, Quorum Sensing (QS) and subsequently the development of biofilms. The strategy of Quorum Sensing Inhibition (QSI), which targets autoinducers, can be used to prevent the growth of biofilms and represent a novel, natural, widespread, antibacterial approach utilized by plants with significant impact on biofilm formation and consequent inhibition of its related illnesses.[5–8] Numerous workers have recently discovered that many therapeutic and food-related herbal plants have anti-QS properties. Moreover, QS obstruction will reduce the virulence of invasive pathogens, making them more vulnerable to the applied mode of therapy and facilitating simple clearance by host defense mechanism. For example, garlic, chamomile, vanilla, and burdock leaf can block the quorum sensing process.[9] However, research into the development of therapeutically useful and secured anti-QS chemicals is still in its early stages, necessitating more investigation of natural materials.[10]

The ability of several plants to prevent the growth of biofilm in pathogens such *L. monocytogenes, K. pneumoniae, P. aeruginosa, S. pyogenes, S. mutans,* and *S. aureus* has also been observed. Certain plant extracts are efficient at delaying the growth of multi-species biofilm.[5] The majority of investigations to date have concentrated on observing the anti-biofilm activity of plant extracts taken singly rather than in combination.[3,11] Therefore, it is necessary to investigate natural medicinal alternatives with minimal side effects for the treatment and prevention of various biofilm-related infections. This study aimed to investigate the bacterial biofilm formation of some certain isolated pathogenic strains and the effect of some

plant extracts on the in-vitro biofilm formation of the isolates.

Materials and Methods

The Used Plants

Three plants, fresh samples of Cranberry (*Vaccinium macrocarpon*) and Pomegranate peel (*Punica granatum)* were collected from local markets of Jeddah while Arak; (*Salvadora persica*) *was* collected from Al Bahah region. All plants were collected in sterile plastic bags, washed and extracted. Agar well diffusion method was used to determine the inhibitory activity of each plant extract. MIC of the plant extracts was performed. The effect of the plant extract on the inhibition of biofilm formation and on EPS formation were accomplished by a spectrophotometric method.

Study Setting

This study was conducted during the period March 2021-March 2022 in the Central Laboratories of Biological Sciences Department, at Faculty of Science in King Abdulaziz University, Jeddah, Saudi Arabia. The ethical approval (Reference No 138-21) was obtained from the unit of Biomedical Ethics Research Committee of King Abdulaziz University Hospital (KAUH) in Jeddah for permitting sample collection.

Sample Collection

Different fourteen biofilm forming bacterial strains were collected in agar culture plates including Gram-positive and Gram-negative pathogenic strains from the Clinical and Molecular Microbiology Laboratory of King Abdulaziz University Hospital; *Staphylococcus aureus* (ATCC 29213), *Methicillin-Resistant Staphylococcus aureus* (MRSA) (ATCC 43300), *Streptococcus pyogenes* (ATCC 12344), *Streptococcus agalactiae* (ATCC 12386), *Streptococcus mutans* (ATCC 25175), *Streptococcus mitis* (Clinical strain), *Enterococcus faecalis* (ATCC 27270), *Listeria monocytogenes* (ATCC 13932), *E. coli* (ATCC 25922), *Klebsiella pneumoniae* (ESBL) (ATCC 700613), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (Clinical strain), *Acinetobacter baumannii* (ATCC19606), and *Serratia marcescens* (ATCC13880).

Culture Conditions and Standardization

Each collected strain was routinely cultured aerobically on Blood Agar plates at 37°C for 24 hrs. and maintained at 4°C until required for the study. The isolates were then subcultured in Tryptic Soy Broth at same conditions with continuous agitation in a shaking incubator prior to each experiment. The tested strains broth cultures were standardized and adjusted with sterile saline to turbidity equivalent to 0.5 on the McFarland standard scale which contains approximately 1.5×10^8 CFU/ml and the diluted 1:10 in sterile broth to obtain the final suitable concentration for final inoculation used for the in-vitro experiments.

Screening of all Isolates for Biofilm Formation

All collected bacterial isolates were screened and assayed for biofilm formation using Crystal Violet Staining (CVS) method described by Christensen et al. (1995)[12] to determine the ability to form biofilm and to measure the biofilm if formed.

Studying the Growth Curve and Biofilm Curve for the Selected Isolates

After inoculating nutrient broth medium with certain isolate, growth was estimated by measuring turbidity using spectrophotometer through recording optical density. Previous step was repeated at 2 hrs. intervals until the absorbance no longer increase. After that, readings were plotted on a graph with time and optical density as absorbance and the changes in growth phases were tracked.

EPS Extraction and Estimation

The biofilm was quantitatively estimated in terms of the quantity of EPS produced by the strain. The EPS was extracted from the overnight broth culture of the tested strains by centrifugation[13]. The phenol-sulphuric acid method (Dubois et al., 1956)[14] was used to estimate the total carbohydrate content in the EPS by spectrophotometric method and comparing with a standard of glucose solutions for confirmation. The comparison between the tested bacterial strains was made.

Biofilm and EPS Estimation under Different Conditions

Biofilm formation by the selected bacteria was estimated after growing bacteria in different conditions. Also, the total carbohydrate content in the EPS of the selected isolate was extracted and estimated by the assay method as mentioned before but the broth culture was incubated under different temperatures; 25, 30, 35, 37, 40 and 45°C. The optimum temperature for EPS and biofilm was determined**.** Also, EPS of the selected isolate was extracted and estimated as mentioned before but the selected isolate was cultured in different broth media; Nutrient broth, Muller-Hinton broth, Tryptic Soy broth, LB broth and Brain Heart Infusion broth. The biofilm was measured in each media and the optimum broth media for EPS and biofilm was determined**.** Similarly, the selected bacterium was grown in broth culture medium with different pH; 5, 5.5, 6, 6.5, 7 and 7.5. At the end, the biofilm was measured in each pH and the optimum pH for EPS and biofilm was determined.

Plant Extract Preparation

Certain plants; Cranberry, Pomegranate peel, Arak were collected from the markets according to their importance in the scientific research articles. To obtain the extracts, the fresh plant was initially air-dried at room temperature and ground to a fine powder in the blender. Grinded powder from each plant were soaked in Methanol solvent separately. This step was repeated but with soaking in distilled water instead of methanol under shaking condition for one day, then filtered in whatman filter paper no. 1. Then the methanol-solved filtrates were evaporated using the rotary evaporator and the water-solved filtrates were lyophilized. Finally, dried extracts were stored at 4°C in an airtight screw-cap bottles. Before applying the extract in any experiment, it was dissolved in distilled water or DMSO depending on the solvent used and then filtered by 0.22 µm syringe filter and stored in amber bottles at 4°C.

The Inhibitory Activity Plant Extracts

Agar well diffusion method (Heatley, 1944)[15] was used to determine the inhibitory activity of both aqueous and organic (methanolic) extracts from each plant (Cranberry,

Pomegranate peel and Arak). Each plate well was filled with the extract and then the plates were incubated at 37°C for 24 h. Inhibition of bacterial growth was measured as inhibition zone diameters and the average value was calculated.

Determination of Minimum Inhibition Concentration of the Plant Extracts

Minimum inhibition concentration (MIC) of the plant extracts was performed in 96- well microtiter plates against the tested isolates. Each selected plant extract was diluted to two-fold with MHB in each well. Afterward, bacterial suspensions and the indicator phenol red were added into the wells and the plates were then incubated at 37°C for 24 h.[16] Microplates were read using a microplate reader. MIC was recorded as the lowest concentration of the plant extract and absolute inhibition of observable growth. The plates were incubated following reading for another more 24 hrs. and the biofilm was stained and assessed as mentioned before by crystal violet staining assay to detect the MIC that inhibit the biofilm also.

Antibiofilm Assay of Plant Extracts

The effect of the plant extract on the inhibition of biofilm formation was accomplished by a spectrophotometric method as stated by Plyuta et al. (2013).[17] Broth cultures and the plant extracts with the MIC were incubated in 96-well microplates under a standard condition. The biofilm biomass was assayed as described before and the percentage of inhibition was estimated.

The Effect of Plant Extracts on EPS Formation

The broth culture of the selected isolate was incubated with the sub-inhibitory concentration of the extract in the optimum medium and pH at the optimum temperature for 48 hrs. The EPS was extracted and estimated as described before and the values were compared with that recorded before treating by the plant extracts.

Statistical Analysis

All the experiments were performed in triplicate. All values are expressed as the mean. The significance of the results was analyzed by one way analysis of variance (ANOVA), through the Statistical Package for Social Science (SPSS) program (Version 25.0) to analyze the quantitative data collected; this allowed identification of the relationships between several variables, as well as testing of the assumptions of the research. The main data analysis consisted of descriptive statistics in the form of indicators of central tendencies (means) and variability (standard deviations). Otherwise, we use alternative non-parametric tests such as Kruskal–Wallis tests and results with P-value <0.05 were considered significant.

Results

From the Clinical and Molecular Microbiology Laboratory of King Abdulaziz University Hospital, Different fourteen biofilm forming bacterial strains were collected in agar culture plates. The samples include Gram-positive and Gram-negative pathogenic strains. The study was conducted in the Central Laboratory of Biological Sciences Department in the Faculty of Science in the period from March 2021 to March 2022.

Screening of the Bacterial Biofilm Forming Isolates and Biofilm Measurements

All collected bacterial strains were assayed to test the ability to form biofilm and to measure the biofilm if formed. All isolates were confirmed as biofilm forming strains and their biofilms are ranged according to the strength. Table 1 showed that the scale range of the mean of the biofilm formation is between 0.1777 and 0.6427. MRSA was the least biofilm forming isolates (0.1777) and *A. baumannii* was the most biofilm forming isolates (0.6427) within the group. Two strains among the most active biofilm forming bacterial isolates were selected to be tested in the further experiments; *Listeria monocytogenes* (ATCC 13932) and *Klebsiella pneumoniae* (ESBL) (ATCC 700613) according to their importance.

Growth Curve for the Selected Bacterial Isolates

The growth for the selected isolates was estimated by spectrophotometer and growth curve was made according to the time intervals (0, 2, 4, 6, 8, 24, 36, 48, 60 hrs.) starting with certain concentration for each isolate. Figure 1 showed that number of cells of the two bacterial isolates were increased gradually until reach to stationary phase after 24 hours and the growth gradually deceased until constant while the cells remaining metabolically active. It was noticed from the curve that the mean growth rate of *Klebsiella pneumoniae* is much higher than that of *Listeria monocytogenes* within the same time interval. After 8 hours, the growth rate of *L. monocytogenes* is increasing suddenly at variance with the growth rate of *Klebsiella pneumoniae* which is increasing gradually within all intervals.

Biofilm Formation for the Selected Isolates at Different Incubation periods

Biofilm formation for the selected isolates was estimated by spectrophotometer after staining with crystal violet and biofilm curve was made according to the time intervals (0, 2, 4, 6, 8, 24, 36, 48, 60 hrs), starting from the zero point before

Table 1. **Screening of the bacterial biofilm forming isolates and measuring the formed biofilm**

Bacterial isolate	Biofilm (Mean ± SD)	Biofilm type	Bacterial isolate	Biofilm (Mean ± SD)	Biofilm type
S. aureus	0.23 ±. 0.68	Moderate	***E. coli***	0.24 ± .0.02	Moderate
MRSA	0.17 ±. 0.13	Weak	***K. pneuomoniae***	0.64 ± .0.03	Strong
S. pyogenes	0.29 ± .0.14	Moderate	***P. aeruginosa***	0.60 ± .0.06	Strong
S. agalactiae	0.24 ± .0.15	Moderate	***S. marcescens***	0.54 ± .003	Strong
S. mutans	0.27 ± .0.06	Moderate	***P. mirabilis***	0.21 ± .016	Moderate
S. mitis	0.19 ± .0.22	Weak	***E. faecalis***	0.19 ± .002	Weak
L. monocytogenes	0.65 ± .0.01	Strong	***A. baumannii***	0.64 ± .005	Strong

biofilm formed. Figure 2 showed that both isolates bacterial cells were increased gradually until reach to stationary phase after 24 hours and the biofilm became matured and stable with metabolically active cells. It was noticed from the curve that the biofilm formation rate of *Klebsiella pneumoniae* is higher than that of *Listeria monocytogenes* within short time intervals.

4- Antimicrobial Sensitivity Testing for the Selected Isolates

The selected isolates were examined for sensitivity to several important commercial antibiotics by disc- diffusion method according to Kirby-Baur technique described by Baur et al. (Wayne, 2017).[18] using Clinical and Laboratory Standards Institute Guidelines to interpret diameter of growth inhibition zone. Table 2 showed that both isolates were resistant to about half of the antibiotics and they were slightly different from each other in the sensitivity results.

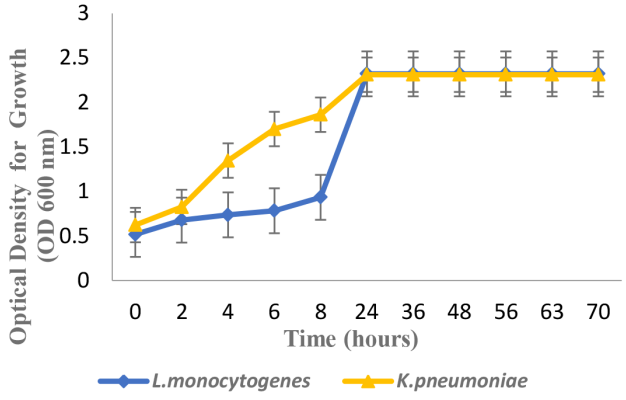

Fig. 1 **Bacterial growth curves estimation during definite time intervals for the two selected isolates, *L. monocytogenes* and *K. pneumoniae*.**

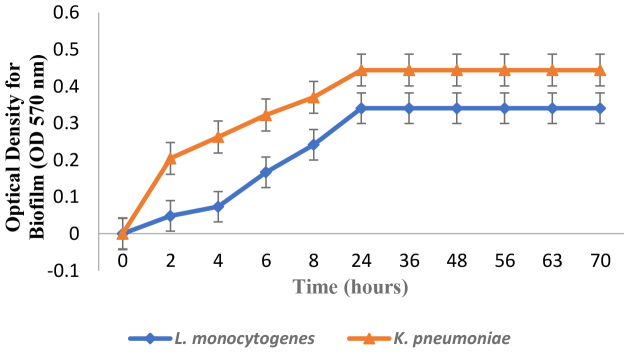

Fig. 2 **Biofilm formation progress estimation during definite time intervals for the selected isolates; *L. monocytogenes* and *K. pneumoniae*.**

Exopolysaccharides Extraction and Estimation for the Selected Isolates

Biofilm was quantitatively estimated in terms of the quantity of Exopolysaccharides (EPS) produced by the strains by estimation of the total carbohydrate content in the EPS of the selected isolates by spectrophotometric method and comparing with a standard of glucose solutions for confirmation *L. Monocytogense* has more carbohydrate content in the EPS layers (OD: 1.523) than *K. pneumoniae* (OD:1.351) where *L. Monocytogense* carbohydrate content is equal to 0.21 ± 0.02 µg/mg of cells while *K. pneumoniae* carbohydrate content is approximately equal to 0.18 ± 0.03 µg/mg of cells (Table 3).

Exopolysaccharides (EPS) Estimation for the Selected Isolates under Different Temperatures

The total carbohydrate content in the EPS of the selected isolate was estimated under different temperatures; 25°C, 30°C, 35°C, 37°C, 40°C and 45°C. Figure 3 showed that both isolates were similar in the optimum temperature for EPS and the sequent biofilm formation which was 37°C. It was clear that the two temperatures 35°C and 37°C were very approximate in the effect on EPS measurements for both isolates and they were both optimum temperatures. Temperature 25°C was the minimum for both isolates EPSs. As shown in Figure 3, *K. pneumoniae* has mean of Exopolysaccharides less than *L. monocytogenes* under different temperatures.

Exopolysaccharides Estimation for the Selected Isolates under Different Media

The total carbohydrate content in the EPS of the selected isolate was estimated under different media. In Figure 4, it was shown that *K. pneumoniae* has mean of Exopolysaccharides less than *L. monocytogenes* under different media including Tryptic Soy Broth, Luria-Bertani Broth and Brain Heart Infusion Broth. Bacteria grown in Tryptic Soy Broth and Brain Heart Infusion have the largest values of the Exopolysaccharides for both isolates and they are considered as the optimum media while Nutrient broth and Muller-Hinton Broth have the lowest values of EPS for both isolates.

Exopolysaccharides (EPS) Estimation for the Selected Isolates under Different pH Measures

The total carbohydrate content in the EPS of the selected isolate was estimated under different pH measures. As shown in

Table 2. **Antimicrobial sensitivity testing for *L. monocytogenes* and *K. pneumoniae***

Antimicrobial agents	Dose	Inhibition zones (mm)		Antimicrobial agents	Dose	Inhibition zones (mm)	
		L. monocytogenes	*K. pneumoniae*			*L. monocytogenes*	*K. pneumonia*
Bacitracin	10 units	**R**	**R**	Amikacin	30 mcg	18	16
Chloramphenicol	30 mcg	**R**	**R**	Ceftazidime	30 mcg	**R**	**R**
Penicillin G	10 units	**R**	**R**	Aztreonam	30 mcg	**R**	**R**
Polymyxin B	300 units	**R**	10	Piperacillin	100 mcg	17	**R**
Gentamicin	10 mcg	17	8	Imipenem	10 mcg	35	30
Neomycin	30 mcg	14	9	Ciprofloxacin	5 mcg	**R**	19

Figure 5, *K. pneumoniae* has mean of Exopolysaccharides less than *L. monocytogenes* under different measures of pH. It was found that pH 7 and pH 7.6 have the largest values of the Exopolysaccharides for both isolates and they are considered as the optimum pH while the cells grown at pH 6 and pH 8 have the lowest values of EPS for both isolates.

Table 3. **The quantity of exopolysaccharide in the selected isolates; *L. monocytogenes* and *K. pneumoniae***

Bacteria	Absorbance (490 nm)	EPSs (µg/mg of cells)
L. monocytogenes	1.523	0.21 ± 0.02
K. pneumoniae	1.351	0.18 ± 0.03

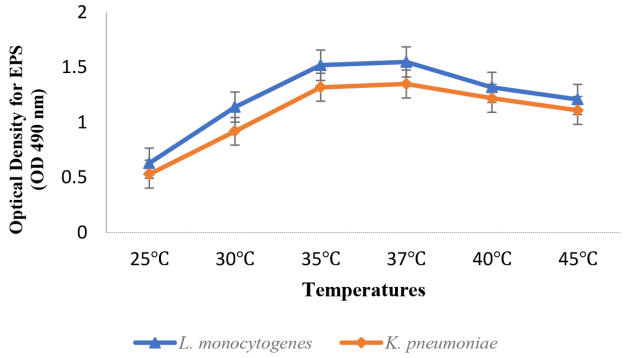

Fig. 3 **Exopolysaccharides (EPS) measurements for *Listeria monocytogenes* and *Klebsiella pneumoniae* under different temperatures.**

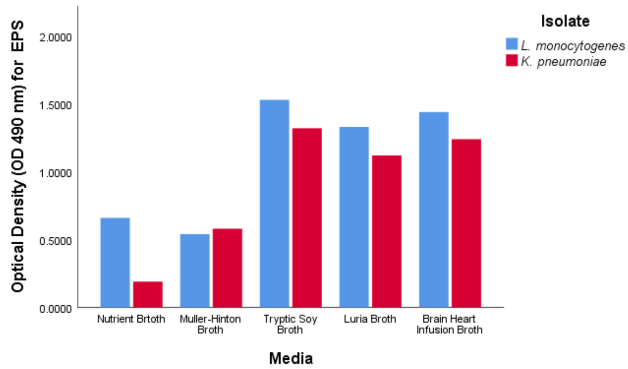

Fig. 4 **Exopolysaccharides (EPS) measurements for *Listeria monocytogenes* and *Klebsiella pneumoniae* under different media.**

Antibacterial Effect of the Plant Extracts on the Selected Isolates with Minimum Inhibitory Concentrations (MIC) and Sub-Inhibitory Concentrations (Sub-MIC)

It was reported from the test that not all solved plant extract were effective with all isolates. Both isolates were resistant to aqueous solved of Arak extract (Aa) and less sensitive to organic-solved Cranberry (Co) and Pomegranate peel (Po) extracts. Thus, organic-solved Arak (Ao), aqueous-solved Cranberry (Ca) and Pomegranate peel (Pa) extracts were chosen.

Table 4 showed the antibacterial effect of the plant extracts on the selected isolates, the minimum inhibitory concentrations and the sub-inhibitory concentrations. The effect of plant extracts on *L. monocytogense* growth is more than on *K. pneumoniae* growth. Both isolates were affected by the extract of arak and cranberry respectively. *K. pneumoniae* growth was not affected by pomegranate peel extract and was resistant. Arak extract made the biggest inhibition zone on *L. monocytogenes* and the smallest inhibition zone on *K. pneumoniae* comparing to other selected plant extracts.

The Effect of the Plant Extracts on Bacterial Growth, EPS Production and Biofilm Formation

Figures 6–8 showed that there is an effect of the plant extracts; Cranberry, Pomegranate Peel and Arak respectively on bacterial growth, EPS production and biofilm for both selected isolates. It was clear in Figures 9 and 10 that the effect of plant extracts varies from one extract to another in the two different types of bacteria where EPS and biofilm were the least possible with the extract of arak and cranberry respectively. Table 5 provides all the descriptive measures (mean, SD, SE, 95% CI, etc.)

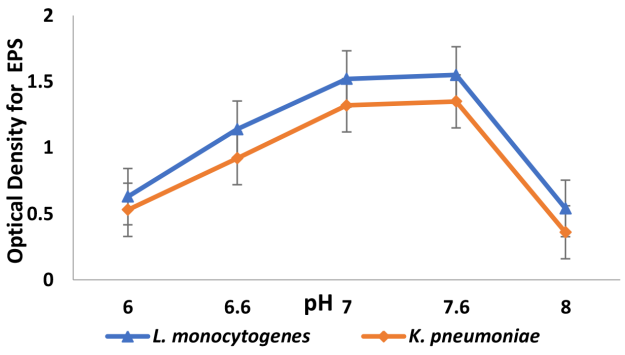

Fig. 5 **Exopolysaccharides (EPS) measurements for *Listeria monocytogenes* and *Klebsiella* pneumoniae under different pH measures.**

Table 4. **Antibacterial effect of the plant extracts on the selected isolates with Minimum Inhibitory Concentrations (MIC) and Sub-Inhibitory Concentrations (Sub-MIC)**

Isolate	Plant extract								
	Cranberry (aqueous)			Pomegranate Peel (aqueous)			Arak (organic)		
	IZ (mm)	MIC (mg/ml)	SIC (mg/ml)	IZ (mm)	MIC (mg/ml)	SIC (mg/ml)	IZ (mm)	MIC (mg/ml)	SIC (mg/ml)
L. monocytogenes	18.0	12.5	6.0	17	25	12.5	20	29	14
K. pneumoniae	16.0	25.0	12.5	0.0	ND	ND	19	29	14
Difference	Sig at $P \leq 0.05$			Sig at $P \leq 0.05$			Not Sig at $P \leq 0.05$		

IZ, Inhibition Zone; MIC, Minimum Inhibitory Concentration; SIC, Sub-Inhibitory Concentration; ND, Not detected.

of biofilm and Exopolysaccharides of the bacterial isolates with all plant extracts. The value of F-statistic by ANOVA for the effect of plant extracts on biofilm is 19.516 and the *P*-value is 0.008. Since the *P*-value is <0.05, this means that not all means of the biofilm for all groups are the same and thus there was significance difference between them. The value of F-statistic by ANOVA for the effect of plant extracts on Exopolysaccharides is 44.766 and the *P*-value is 0.002. Since the *P*-value is <0.05, this means that not all means of Exopolysaccharides for all groups are same and there was significant difference between them.

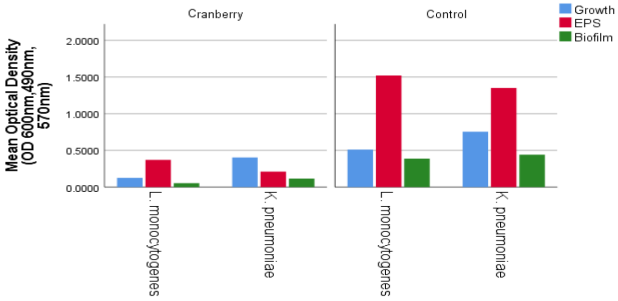

Fig. 6 **The effect of the plant extracts Cranberry on bacterial growth, EPS production and biofilm for both selected isolates; *Listeria monocytogenes* and *Klebsiella pneumoniae*.**

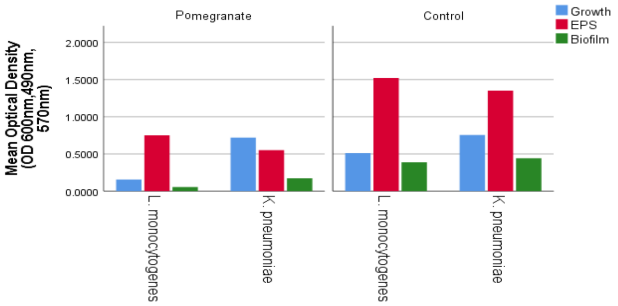

Fig. 7 **The effect of the plant extracts Pomegranate Peel on bacterial growth, EPS production and biofilm for both selected isolates; *Listeria monocytogenes* and *Klebsiella pneumonia*.**

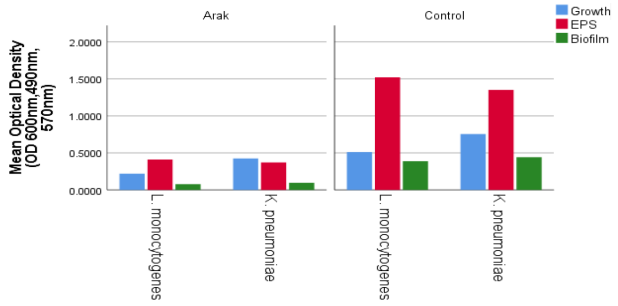

Fig. 8 **The effect of the plant extracts Arak on bacterial growth, EPS production and biofilm for both selected isolates; *Listeria monocytogenes* and *Klebsiella pneumoniae*.**

Discussion

L. monocytogenes and *K. pneumonia* produced biofilms which is influenced by physical factors such as nutrient media composition, pH, temperature, and biological factors. Bacterial biofilms are strongly linked to and controlled by bacterial quorum sensing (QS), a cell-to-cell communication system that allows bacteria to monitor population density and control physiological processes by releasing and receiving small signal molecules called autoinducers to trigger the expression of specific genes for biofilm formation.[19] The significance of biofilm-related infections caused by indwelling medical implants and appliances, such as contact lenses, artificial joints, and catheters, has been emphasized. Bacteria within the biofilm can endure, leading to repeated, chronic infections and the development of immunological and antibacterial resistance. Compared to planktonic cells, bacteria that are in biofilms are more resistant to host defenses and conventional antibiotics. In fact, when cells are embedded in biofilms, they can become 10–1000 times more resistant to the actions of antimicrobial drugs.[20] Biofilm also enables gene transfer among bacteria which can lead to increase in the number of virulent strains[2] that means transfer resistance genes within members of the biofilm micro-community.[20] The pharmaceutical industry is actively researching plant-derived substances such as crude extracts of leaves, roots, and stems, as well as individual compounds isolated from these, essential oils and essential oil constituents for potential applications in the treatment. Although there is now a lot of study on plants and their active ingredients, the main emphasis is on the antibacterial qualities against bacteria that form biofilms.[21] Plant extracts are increasingly being studied as alternative antimicrobial agents since they are safe, accessible, affordable, effective, and have a variety of phytochemicals with little to zero chance of building resistance.[11] Due to their nature-based methodology and affordability, products like these that combine conventional therapy with the most recent research find wider consumers.[22]

A large variety of phytochemicals with antibacterial activities against therapeutically relevant pathogens are produced by numerous plant species.[21] Literature has already documented experimental evidence of the use of the plant

Table 5. **The effect of the plant extracts on biofilm and exopolysaccharides**

Biofilm	Sum of squares	DF	Mean square	F	Sig.
Between groups	0.155	3	0.052	19.516	0.008*
Within groups	0.011	4	0.003		
Total	0.166	7			
Exopolysaccharides					
Between groups	1.613	3	0.538	44.766	0.002*
Within groups	0.048	4	0.012		
Total	1.661	7			

*: Significant difference at $P \leq 0.05$.

phytochemicals such as peptides, unsaturated long-chain aldehydes, phenols or secondary plant metabolites, such as alkaloids, flavonoids, tannins, terpenes, and terpenoids, separated from essential oils, water extracts, or methanol and butanol soluble compounds for their potential antibacterial significance in the biomedical, and food industries.[23] The main chemical components of the plant including polyphenolics, flavonoids, and triterpenoids[10] can penetrate the bacterial cell wall and cytoplasmic membrane as typical lipophiles, disrupt the structure of the different layers of polysaccharides, fatty acids, and phospholipids and permeabilize them.[24] Plant extracts' ability to dissolve biofilms relies on the type of solvent utilized, specifically whether it is methanol- or ethanol-derived extracts.[25]

In earlier investigations, the ability of plant extracts to reduce biofilm adherence and development on the surfaces was demonstrated.[26] In some studies, it was reported that cinnamon essential oils can reduce or eliminate bacterial biofilms on a stainless-steel surface.[24] Lemon essential oils can eliminate biofilm on polypropylene surfaces.[27] Neem, cranberry extracts, peppermint and coriander essential oils can prevent bacterial cell adhesion.[28] Ginger extract can lower the signal molecules that bacteria use to communicate with each other, lower the formation of EPS, and make it easier to separate biofilm from any surface.[26] Due to the emergence of bacteria strains that cause urinary tract diseases that are resistant to antibiotics, some specialized medicinal plant extracts may offer an alternative to short-term coating for preventing biofilm formation on urinary catheters.[29]

Pharmacological investigations revealed that 's Arak stem extract (*Salvadora persica* L.) has potent antibacterial and anti-plaque effects on oral microorganisms.[30] Green tea polyphenols have been shown by Schneider-Rayman et al. (2021)[31] to have an inhibitory effect on *S. mutans* dental biofilm by preventing the development of Q.S and EPS. Additionally, one study demonstrated that plant extracts from moringa, curry, and guava were the most efficient in delaying the formation of dental multi-species biofilms by focusing on growth, adhesion, and coaggregation without interfering with the oral cavity's homeostasis.[22]

According to Quelemes et al. (2015),[32] neem leaf extract had an inhibitory effect on the growth of MRSA biofilm and planktonic aggregations. With a lower dosage and no risk of resistance developing, myrrh oil has the potential to be a commercially viable antibiotic that eliminates persistent cells in the biofilm.[33] Cranberries exhibit anti-biofilm characteristics against *P. aeruginosa* and *Escherichia coli* by inhibiting motility and blocking adhesion.[34] According to one study, it was showed that pomegranate peel extract prevented biofilm formation as a result of preventing quorum sensing and swimming motility of *Y. enterocolitica*.[35] *Acinetobacter baumannii*-related nosocomial infections require an alternative therapy because carbapenem resistance has increased. Norwogonin, a component from the plant *Scutellaria baicalensis*, appears to exhibit antibacterial properties against *A. baumannii* and methanol extract of the plant *Actinidia deliciosa* reduced production of bacterial biofilm components.[36]

Conclusion

EPS quantity produced from the bacteria depends on some effective factors such temperature, media contents and pH. Aqueous-Cranberry, aqueous-Pomegranate Peel and methanolic-Arak extracts have antibacterial and antibiofilm activities. Aqueous-Cranberry and methanolic-Arak extracts have antibiofilm activities on *L. monocytogenes* and *K. pneumonia* much higher than aqueous-Pomegranate Peel. Although aqueous-Pomegranate Peel doesn't have antibacterial effect on *K. pneumonia,* but it has antibiofilm effect on the same strain. Thus, application of new natural approaches for inhibiting bacterial biofilm is urgently needed and important to prevent persistent and recurrent biofilm related infections.

Conflict of Interest

None.

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