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In-vitro Studies on Bacterial Biofilm Formation Using Urinary Catheters

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Abstract

Though the bacterial united community that lives in a special social pattern, and known as bacterial biofilm is a fascinating phenomenon, yet it might lead to a disastrous outcome. Catheter-associated urinary tract infections due to biofilm formation are rising rapidly, and have a high mortality rate especially in long term catheterized patients. Thus, two types of catheters were used Foley latex 100% silicone coated and, Foley pure silicone size 14 Ch/FR. The most predominate causative agents of catheter-associated urinary tract infection were studied namely *Staphylococcus epidermidis* and uropathogenic *Escherichia coli*. The ability to form biofilm on both catheters was investigated, by inoculating each catheter separately with the bacterial species under study. Growth was studied quantitatively by constructing growth curves, and qualitatively by rolling and imprinting the catheter segment over nutrient agar plates. It was noted that latex catheters supported biofilm formation compared to silicon. The treated catheters with three medicinal plant extracts pomegranate (*Punnica granatum*), guava (*Psidium guajava* Lim) and cranberry (*Vaccinium macrocarpon*) compared to the untreated exhibited reduction in bacterial biofilm formation by four to two folds, depending on the tested bacterium, the incubation period and plant used. Simulating the urinary bladder environment, human urine and artificial urine were used as the nutrient fluid. Interestingly, human urine supported the growth of *S. epidermidis* on latex and silicone catheters, compared to its growth on nutrient broth. However, when *E. coli* was inoculated on both latex and silicone catheters, grown in artificial urine, viable counts varied significantly in view of the type of catheter. Silicone appeared to retard growth compared to Latex since the maximum population reached after12 hours and declined after 336 hours. Negative staining of the studied catheters using transmission Electron Microscope confirmed the formation of biofilms by *S. epidermidis* and *E. coli*. Surprisingly, the same susceptibility pattern of both species was observed before and after forming biofilm.

Keywords: Biofilm; Urinary Catheter; Staphylococcus Epidermidis; *Escherichia Coli*

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Introduction

Although therapeutic prosthetic devices are common medical procedures and a life-saving treatment for many patients, yet, several complications are associated with their use. The latter include bacteraemia, systemic infections, damage of the artificial device, antibiotic resistance and high morbidity and mortality [1-4].

As millions of indwelling devices are implanted in patients yearly, as many as are accompanied by the formation of biofilms that adhere to the surfaces of the medical devices, subsequently leading to treatment failure [2,5-9].

Amazingly and unexpectedly, the first microscopic observation on biofilm formation was around four centuries ago by Anthony van Leeuwenhoek (1683-1708) who described aggregates of microorganisms on materials from his own mouth. Secondly and not surprisingly, came the pioneer of microbiology Louis Pasteur (1822-1895) who proved that the spoilage of wine was due to aggregates of microorganisms, and also reported membrane formation which he noticed in vinegar barrels [10]. In addition, Hoiby in 1970s observed the existence of biofilm in the sputum of cystic fibrosis patients. Since then, the relationship between bacterial biofilm and human infections was documented and approximately 80% of human infections were accordingly related to biofilms. The latter include otitis media, cystitis, periodontitis and serious ocular infections [7,11].

Furthermore, infections associated with prosthetic indwelling devices have increased tremendously, such as infections associated with prosthetic hip and knee joints, since orthopaedic biomaterials provide surfaces for bacteria to adhere to and subsequently form biofilms [12]. Also, infections associated with prosthetic heart valves conferred several problems some with reported fatality [13-15]. Moreover, catheter associated urinary tract infections (CAUTI) have been reported as the most common nosocomial urinary tract infection associated with in dwelling urinary catheter around the world causing bacteraemia [16-18]. Unfortunately, once bacterial biofilm is established, bacterial communities in these biofilms become resistant to antimicrobial treatment and host defense, thereby become the source for recurrent infections [9]. Above all, such infections are difficult to eradicate because these bacteria live in well-developed biofilms [19,20]. Regrettably in the Gulf area, the true burden of CAUTI incidence is unknown because of lack of national and regional surveillance reports in Gulf Cooperation Council countries [16].

The aim of the present work was to perform *in vitro* studies on

the biofilm formation by the most common species that cause urinary tract infections using urinary catheter, in addition, factors that enhance the previous phenomenon were studied, to establish control and prevention strategies thereby minimizing the incidence of infection.

Materials and Methods

Bacterial Species and Media Used

Two bacterial isolates were obtained from King Fahad and King Faisal Hospitals, Jeddah, Saudi Arabia. *Staphylococcus epidermidis* was previously isolated from patient with urinary tract infection, whereas, uropathogenic *Escherichia coli* was previously isolated from catheterized urine. Nutrient Agar, Nutrient Broth, Mueller Hinton Agar, Eosin Methylene Blue Agar were obtained from (Hi media, India). Human urine was used as a fluid medium to simulate the normal environment of the bladder. A urine sample was collected from an apparently healthy female volunteer who had no history of UTI nor given any antibiotics in the prior six months. The pooled urine was sterilized through 0.45 µm porosity filter, using a bottle-top analytical filter system (Thermo-scientific Nalgene 150 ml sterile Analytical Filter unit) and stored at 4°C until used, usually within three days. Artificial urine medium was prepared according to Griffith [21] the composition of the medium was in g\l dist. water: Calcium chloride, 0.49; Magnesium chloride hexahydrate, 0.65; Sodium chloride, 4.6; Trisodium citrate dehydrate, 0.65; Disodium oxalate, 0.02; Potassium dihydrogen phosphate, 2.8; Disodium sulfate, 2.3; Potassium chloride, 1.6; Ammonium chloride, 1.0; Urea, 25 and Gelatin, 5.0 at pH 6.1. The medium was sterilized as previously mentioned for human urine.

Urinary Catheters Preparation

Foley latex 100% silicone coated, 2- way, size 14 Ch/FR with a diameter of 4.7 mm (Euromed for medical industries, Egypt) and 2-way Foley 100% silicone size 14 Ch/FR with a diameter of 4.7 mm (Saudi Mais Co. for Medical Products, Saudi Arabia) were used in this study. Catheters were divided into three groups namely: untreated, treated and control. The latter means that the catheter has not been inoculated i.e. free of bacteria. All catheters were cut into 1 cm segments for performing the biofilm formation experiments. Each catheter was removed from the external nylon bag under aseptic conditions and left with the internal sterile bag. Using a sterile scissor, the tip of the catheter which contains the deflated balloon was removed, and the catheter was cut into eight segments, each equals1 cm length. These segments were transferred into a sterile container until used. In addition, segments of 2.5 cm length were also prepared.

Preparation of Plant Extracts

Pomegranate (*Punnica granatum*), guava (*Psidium guajava*) and cranberry (*Vaccinium macrocarpon*) were used as antimicrobial medicinal plants. The pomegranate rind and guava leaves were obtained as a dried powder from an herbal remedy shop in Jeddah City, whereas, cranberry fruits were obtained as pure extract from Bulk Supplements, USA. All plants were identified at Biology Department, Faculty of Science, King Abdulaziz University. Aqueous extracts were prepared by suspended 30 g of each plant powder in 250 ml of sterile distilled water, boiled for 30 minutes on a hotplate, filtered using Whatman filter paper no. 1 and stored at 4°C until used [22] (Table 1).

Formation of Biofilm through Catheter Bridge

Nutrient agar plates were used, and each Petri dish was divided into two sectors. By using a sterile scalpel, 9 mm width of the agar at the centre of the plate was cut and removed, resulting in an empty space [23,24]. Catheter segment (2.5 cm) was placed at right angle over the centre of each Petri dish, thus acting as a bridge (Figure 1). The catheter segment was inoculated at one of its edges with 10 µl of *S. epidermidis* 24-hour old culture. The plates were incubated at 37°C for 24-48 hours. After incubation, the catheter segment was removed and placed on another nutrient agar plate where the growth was examined by imprinting and rolling the segment over the agar surface, after which the plates were incubated for 24 hours.

Table 1: The used plants, their families and used parts.

Binomial name	Common name	Family	Used Part
Punnicagranatum	Pomegranate	Lythraceae	Rinds
Psidium guajava	Guava	Myrtaceae	Leaves
Vaccinium macrocarpon	Cranberry	Ericaceae	Fruits

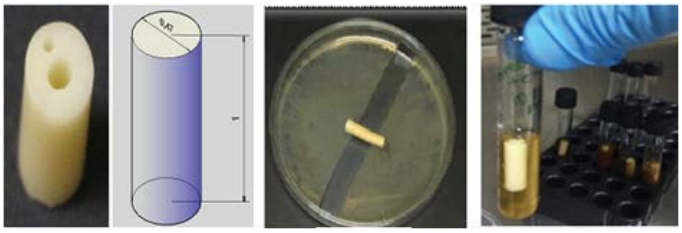

Figure 1: The catheter diameter, catheterbridge on nutrient agar plate, and in nutrient broth.

Preparation of Bacterial Inoculum

From a 24 hrs. subculture of *S. epidermidis,* and *E. coli*, 1 ml of each broth was transferred to 10 ml sterile distilled water. Different dilutions were made to obtain the turbidity visually comparable to the turbidity of the standard 0.5 McFarland tube. Moreover, the accuracy of bacterial turbidity was determined using a spectrophotometer (OPTIMA SP-300, Japan) at a wavelength of 625 nm [25]. The bacterial suspension approximately equaled 2.6 CFU/ml for *S. epidermidis* and 1.9 CFU/ ml for *E. coli,* which represented the initial inoculum used in all experiments.

Biofilm Formation on Foley Urinary Catheter

Each 1 cm catheter segment was aseptically replaced in a sterile Petri dish. The catheter segment was then held with sterile forceps during which 10µl of the standardized suspension was seeded on. Nutrient broth, human urine, and artificial urine were used as the nutrient fluids for growing the bacterial species under study on different catheters.

Seven Petri dishes were prepared for viable counts readings. Readings were taken at intervals to enable the construction of growth curves of the bacterial specie forming the biofilm on the catheter. Seven 10 ml test tubes were used for each growth medium as follows:one tube as a control, which is contains the catheter without inoculation, readings for zero time (immediately after inoculation), 3, 6, 9,12, 24, and 48 hours. However, in case of HU and AU viable counts were taken after 168 and 336 hours, i.e. after one and two weeks respectively. All inoculated tubes were incubated for the required time as mentioned above, except for zero time. The previous procedures were undertaken for both types of catheters.

Enumeration of Bacterial Cells (biofilm)

The catheter was then inserted into a microfuge tube containing 1 ml of sterile 0.05% Tween 80 and was mixed in a rotary mixer for one minute (vortex mixer Vup ELP, Italy); this procedure was performed

to dislodge and disrupt bacterial aggregation [26]. The serial dilution technique was carried out and viable counts were determined [27]. The total area of the catheter segment was determined and the equation used to determine and enumerate bacterial cells that are attached on the external surface of 1 cm catheter segment was as the following:

- CFU/ cm^2 = Cylinder total area = surface area + 2 (base area) = 3.14 = 1.8 cm^2

Catheter Impregnation Method

Catheter segments were immersed in 3 ml of 12% aqueous plant extract of pomegranate rind, guava leaves, and, cranberry fruits for 24 hours. These were considered as the treated catheters, which were then transferred to sterile containers and allowed to dry overnight at room temperature. On the other hand, untreated catheter segments were considered as control.

Antibiotic and Plant Extracts Susceptibility Tests

The standard paper disk diffusion method was performed using commercial antibiotic discs, to determine the susceptibility pattern of *Staphylococcus epidermidis* and uropathogenic *Escherichia coli* before and after biofilm formation [22,28]. Agar well diffusion method was used to determine the antimicrobial activity of the plant extracts using Mueller Hinton agar [29].

Transmission Electron Microscope Studies

S. epidermidis was inoculated on latex catheter segments (untreated and treated with pomegranate extract). Negative contrast transmission electron microscopy was used according to [30]. Investigation was under taken at zero time and 9 hours.

Statistical Analysis

Three replicates were carried out and the mean value for the three readings were expressed as mean ± standard deviation (±SD). Statistical analysis was performed using IBM SPSS program. One-way analysis of variance (ANOVA) and unpaired t- test were used to determine significant differences. The statistical difference was considered significant atp-value <0.05 and highly significant at p-value <0.001.

Results

Staphylococcus epidermidis was sub-cultured on nutrient agar for 24 hours at 37°C. The obtained colonies showed white, opaque and flat colonies. Under light microscope the examined bacterium was Gram-positive cocci arranged in grape like clusters. Escherichia coli was sub cultured on the selective Eosin methylene blue (EMB) agar medium for 24 hours. The examined colonies appeared as metallic green while colonies on nutrient agar appeared greyish white (opaque). Under light microscope, the examined cells of *E. coli* were Gram-negative bacilli appearing as a short and straight rod. The antimicrobial activity of the aqueous plant extracts of pomegranate, guava and cranberry against *S. epidermidis* and *E. coli* was determined using Agar well diffusion method. Pomegranate aqueous extract showed the highest inhibition activity against *S. epidermidis* with an inhibition zone of 40 mm diameter, while a weak inhibition was observed against *E. coli* (mean diameter of inhibition zone of 19 mm) as shown in (Figure 2). Guava aqueous extract exhibited a less inhibitory effect on S. epidermidis with a zone of 31 mm while slight inhibition activity *on E. coli* was observed. Cranberry aqueous extract showed weak inhibitory effect on both *S. epidermidis* and *E. coli* with a zone of 12 mm in diameter. As shown in (Figure 3), the ability of *S. epidermidis* to form biofilm

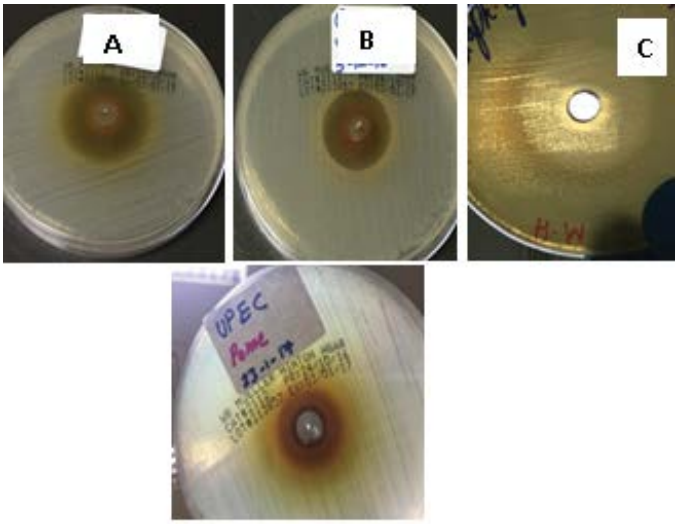

Figure 2: Antimicrobial activity of pomegranate extract, guava extract, cranberry extract, against S. epidermidis, pommegranate extract against uropathogenic *E. coli.*

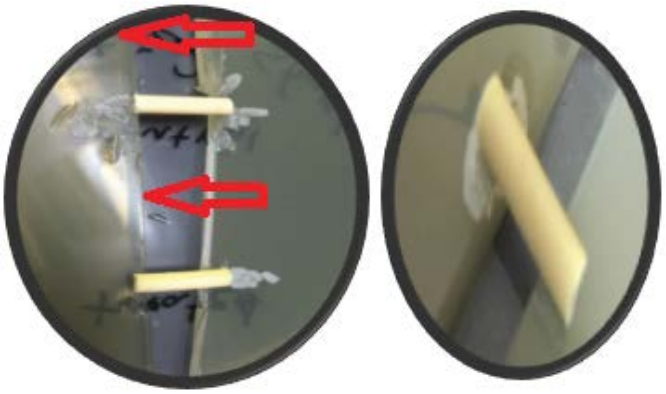

Figure 3: Migration of *S. epidermidis* across the catheter bridge. untreated latex catheter, latex catheter treated with cranberry extract.

through migration over the untreated catheter segments was apparent and successfully migrated across the catheter bridge to the other side. After 24 hours incubation, biofilm formation was documented by the appearance of bacterial growth on agar surface and on the edge of the inoculated catheters.

In this work, both *S. epidermidis* and *E. coli* were grown in nutrient broth for different incubation periods as a control, whereas, Latex and Silicon catheters were seeded separately with *S. epidermidis* and *E. coli* for biofilm formation studies. The viable plate count was obtained to estimate the bacterial growth curve whether before or after bacteria forming biofilm (Figure 4). Growth curves of *S. epidermidis* and *E. coli* were compared. The viable counts of both *bacteria* decreased in number after 6, 9, 12 and 24 hours when growing on latex catheter i.e. as a biofilm- compared to its growth in broth. However, when the two tested bacteria were grown on silicon catheter, inhibition of growth was more apparent when compared to their growth in nutrient broth, and silicon catheter appeared to retard bacterial growth more than latex catheter. Interestingly, *S. epidermidis* and *E. coli* growth as biofilm was reduced approximately by two folds or more compared to their growth in nutrient broth as shown in (Figure 4).

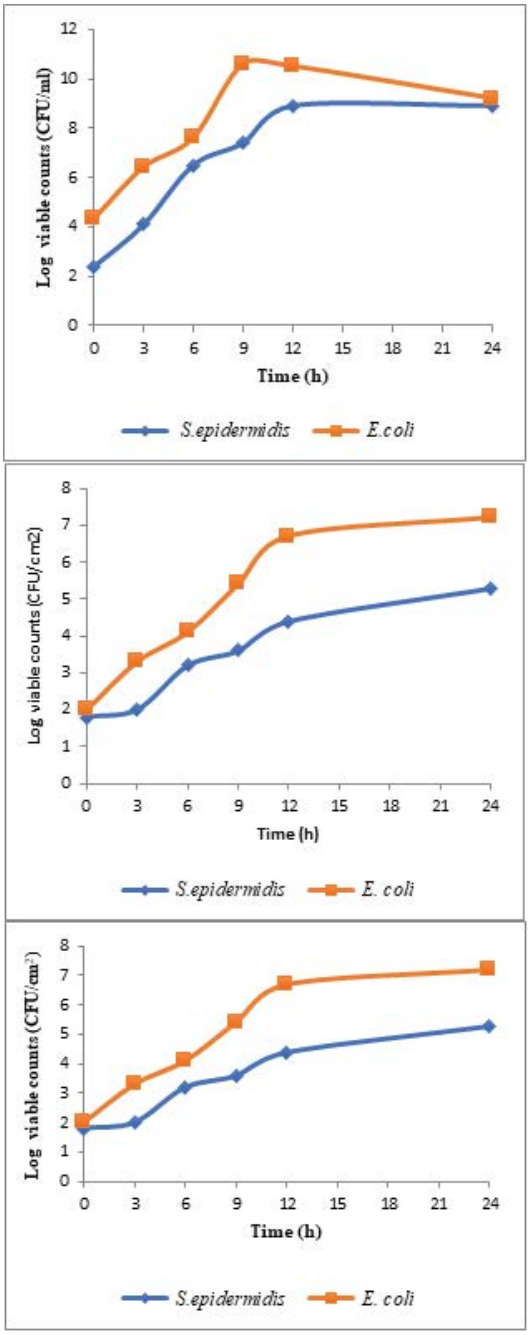

Figure 4: A comparison between growth curve of *S. epidermidis* and *E. coli* in nutrient broth, Seeded on latex catheter, and on silicone catheter.

Significant differences were apparent between viable counts at intervals of the two species, in respect, to time and types of catheters using ANOVA test at $P \leq 0.05$. Turning to time as a factor controlling the growth of *S. epidermidis* and *E. coli* grown as a control or when seeded on silicon and latex catheters, significant differences were found after 24 hours at $P \leq 0.05$ using unpaired t- test.

Growth curves of *S. epidermidis* grown on untreated latex and silicone catheters. *S. epidermidis* was seeded with the same size of inoculum on latex and silicone catheters. Bacterial counts were determined using agar plate dilution method. The results revealed that latex catheters were more favorable for bacteria to form biofilms and provides an appropriate environment to *S. epidermidis* to grow and flourish, compared to the silicone catheter (Figure 4). On other words, lower population levels were recorded for biofilms on silicone catheters compared to the population levels on latex catheters. The effects of pomegranate and guava aqueous extracts on growth of *S. epidermidis* that grown on treated catheters was determined for 0 to 48 hours. Impregnated silicone and latex catheters in pomegranate aqueous extract showed decreases in the viable counts by more than two four folds (Figure 5 and Figure 6). On the other hand, there were significant differences at $P \leq 0.05$ between *S. epidermidis* viable counts on untreated and treated catheters with pomegranate or guava extracts when grown on silicone or on latex catheters, after 12 and 24 hours.

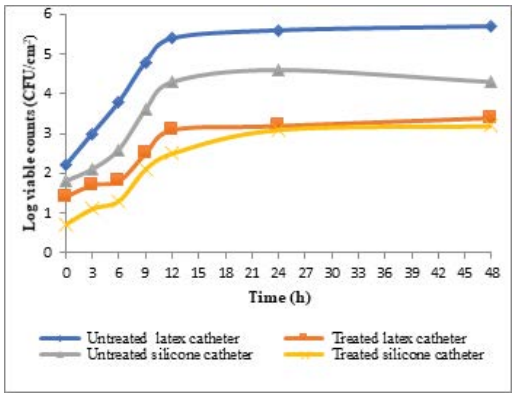

Figure 5: Growth of *S. epidermidis* seeded on untreated and treated catheter with pomegranate extract.

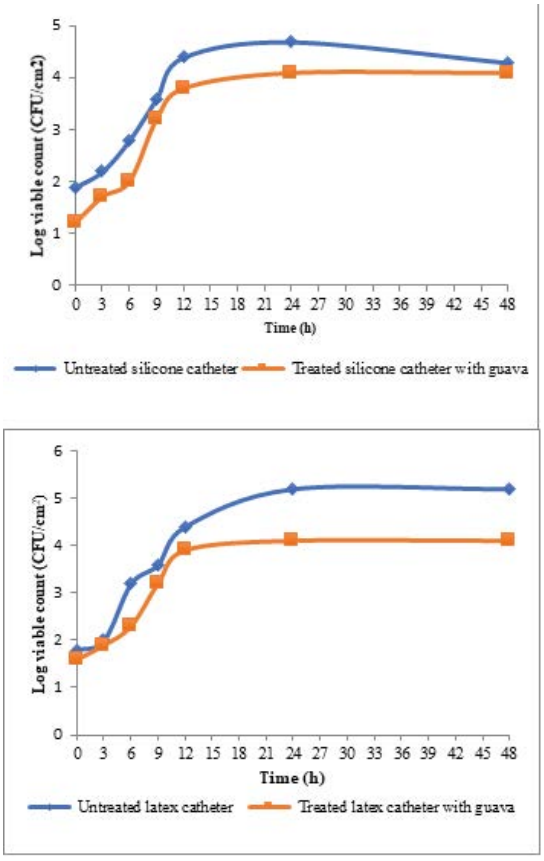

Figure 6: Growths of *S. epidermidis* grown on untreated and treated catheters with guava extract. Silicone catheter, Latex catheter.

No biofilm inhibition was recorded for *S. epidermidis* on treated latex catheters by cranberry aqueous extracts. No clear inhibition in the biofilm formation by *S. epidermidis* was recorded for treated latex catheters by cranberry.

The effect of pomegranate aqueous extract on growth curve of *E. coli* grown on treated latex catheters was determined. Regardless of the effect of pomegranate aqueous extract on *E. coli* was less and decreased by only one-fold for first nine hours from 48 hrs. Moreover, counts of *E. coli* on treated latex catheters for 12 or 24 hs in broth significantly differed in viable bacterial counts at P≤ 0.05. On contrast, no clear inhibition was recorded for guava aqueous extract on *E. coli* grown on treated latex catheter. Effect of cranberry aqueous extract on growth of *E. coli* on treated catheter was determined. Decrease in the viable counts of *E. coli* was recorded compared to untreated latex catheter after 2 hrs. Furthermore, cranberry aqueous extract has reduced the colony-forming unit of *E. coli* that seeded on treated catheter with one-fold at 3 hrs to 6 hrs and with two folds starting from 9 hours to 48 hours comparing to untreated catheters at the same time, as shown in (Figure 7).

Using unpaired t- test, significant difference was recorded at P ≤ 0.05 between *E. coli* viable counts on untreated and treated catheters with cranberry water extracts after 12 and 24 hours. Concerning silicone catheters, no biofilm formation was recorded by *E. coli* on treated catheters with either pomegranate or guava aqueous extracts.

Growth of *S. epidermidis* and *E. coli* seeded on silicone and latex catheters and grown in urine media was recorded and compared. Significantly, *S. epidermidis* grown on Silicone catheters showed no growth at 336 hrs (two weeks). On the other hand, *S. epidermidis* that grown on latex catheters have been persisted until 336 hrs (two weeks) as shown in (Figure 8). Growth of *E. coli* in human urine medium grown on silicone and latex catheters were determined for 0 to 336 hrs. Moreover, growths of *E. coli* that seeded on silicone catheters were gradually increased for the first 6 hrs and log cell numbers increased from 1.9 to 5.1. Then, from 9 hrs to 12 hrs the growth of *E. coli* was stable with log cell numbers of about 5.4. However, the growth of *E. coli* started to increase again from 24 hrs and log the cells was 5.5 until 168 hrs. After 336 hrs i.e. 2 weeks, the log number of *E. coli* decreased to reach 5. On the contrary, *E. coli* that grown on latex catheters showed increasingly viable counts until 24 hrs here the log of cells was 7.1 while, the growth of *E. coli* that seeded on latex catheters was stable from 48 hrs to 336 hrs with log number of 7.3 (Figure 8).

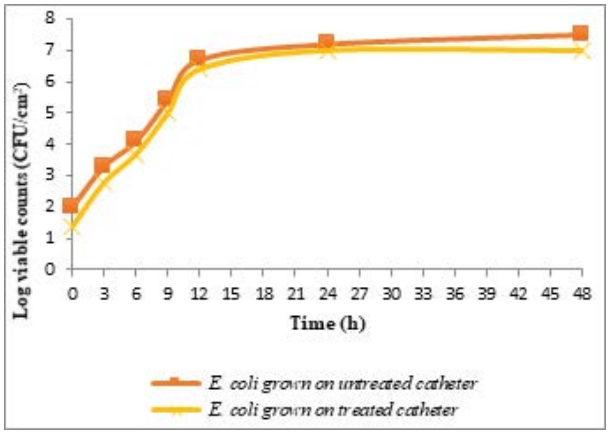

Figure 7: Growths of *E. coli* on untreated and treated latex catheters with pomegranate extract.

Growth of *E. coli* that seeded on silicone and latex catheters in artificial urine medium was studied and log of the obtained viable counts were compared. Numbers of *E. coli* grown on silicone catheters were increased rapidly from zero to 12 hrs. Otherwise, the growth of *E. coli* after 24 and 48 hrs was steady with log viable counts 6.5 and 6.6, respectively. Similarity, the viable counts of *E. coli* after 168 hrs and 336 hrs i.e. 1 week and 2 weeks were decreased slowly with log counts of 5.1 and 5. Unlikely, *E. coli* that seeded on latex catheters were stubborn and persistent until 36 hrs while *E. coli* that grown on silicone catheters showed less colony forming units than latex (Figure 9).

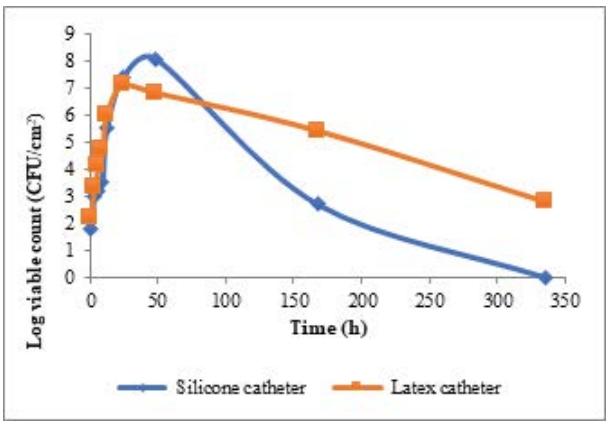

Figure 8: The viable counts of bacteria grown on silicone and latex catheters in human urine medium, *S. epidermidis*, *E. coli.*

Unpaired t- test was used to compare the means of the two bacterial species under study. Significant difference was found in viable counts of *S. epidermidis* and *E. coli*, seeded on latex catheters and grown in nutrient broth and sterile urine at P≤ 0.05. Comparing the growth of *S. epidermidis* and *E. coli* which were seeded on latex catheters and grown in urine, the viable counts differed significantly at P≤ 0.001. Concerning the growth of *E. coli* on latex catchers, in sterile urine and in artificial urine; the counts were significantly different at P≤ 0.05.

Similarly, the bacterial counts of *S. epidermidis* or *E. coli* seeded on silicone catheter and grown in either broth medium or urine were determined and statistically compared. Significant difference at P≤ 0.05 was found for *S. epidermidis* and highly significant difference at P≤ 0.001 was found for *E. coli*. The counts of *S. epidermidis* and *E. coli* varied significantly and significant difference at P≤ 0.05 was shown between the growths of the two bacterial species in sterile urine seeded on silicone catheters. In urine and after 24 hours growth, the counts of *S. epidermidis* seeded on latex and silicone catheters were compared and significant difference at P≤ 0.05 was found. The difference was highly significant at P≤ 0.0001 for viable counts of *E. coli*. Antibiotic susceptibility of *S. epidermidis* and *E. coli* were determined before and after the study by measurement of inhibition zones obtained by different antibiotics (Table 2) and the antibiotic patterns were the same (no significant difference was found). *E. coli* was sensitive to six out of eleven tested antibiotics while *S. epidermidis* was resistant to 5 out of ten antibiotics. Examination of *S. epidermidis* grown on untreated and treated catheters with pomegranate aqueous extract using transmission electron microscope was carried out. The images of negative staining by transmission electron microscope have revealed that the *S. epidermidis* obtained from control catheter at zero time showed bacterial aggregations more than the treated catheter at the same time (Figure 10).

Table 2: The antibiotics susceptibility pattern (Diameter of inhibition zone, mm) of *S. epidermidis* and *E. coli* after forming biofilm.

	S. epidermidis*							
Antibiotics	PG	E	FOX	KF	CD	TS	FA	TAXO
24 hrs.	R	R	R	26	35	25	R	R

	E. coli										
Antibiotics	IMI	SAM	AK	CAZ	PRL	CIP	ATM	TN	GM	CM	LEV
24 hrs.	40	R	25	R	R	30	30	R	22	R	38

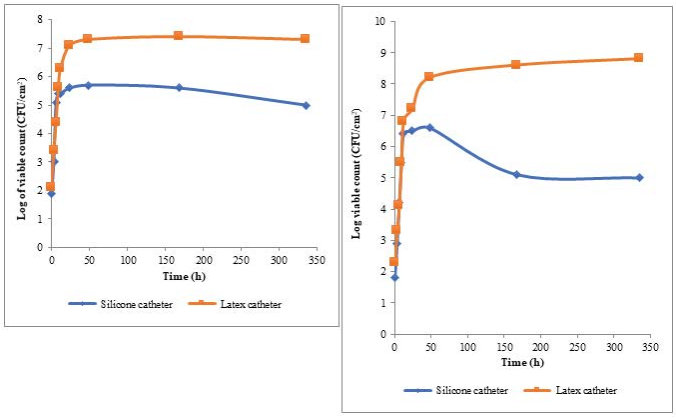

Figure 9: The viable counts of *E. coli* grown on silicone and latex catheters in artificial urine medium.

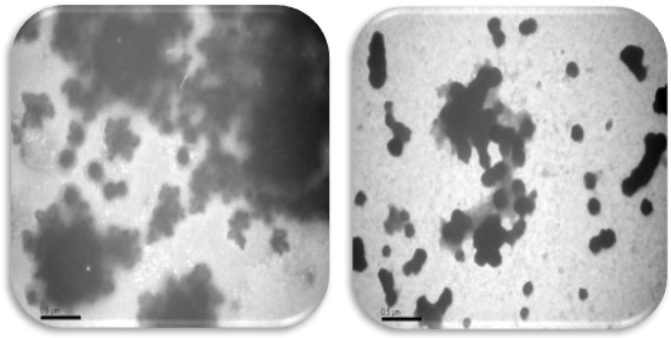

Figure 10: *S. epidermidis* collected from untreated latex catheter and treated catheter with pomegranate after 9 hours of growth in NB medium.

Antibiotics for S. epidermidis*: PG 10 units, Penicillin; E 15 µg, Erythromycin; FOX 30 µg, Cefoxitin; KF 30 µg, Cephalothin; CD 30 µg, Clindamycin; TS 25 µg, Cotrimoxazole; FA 10 µg, Fusidic acid; TAXO A 0.04 units, Bacitracin. Diameter of inhibition zone in mm / ≤ 19 Resistant / > 20. R; resistant (inhibition zone < 20 mm). Antibiotics for E. coli**: IMI 10 µg, Imipenem; SAM 20 µg, Ampicillin; AK 30 µg, Amikacin; CAZ 30 µg, Ceftazidime; PRL 100 µg, Piperacillin; CIP 5 µg, Ciprofloxacin; ATM 30 µg, Aztreonam; TN 10 µg, Tobramycin; GM 10 µg, Gentamycin, CM 2 µg, Clindamycin; LEV 5 µg, Levofloxacin. Inhibition zone* was determined based on BASC standardized disc susceptibility testing clinical breakpoints for enterococci. Diameter of inhibition zone in mm / ≤15 Resistant / > 20. R; resistant (inhibition zone < 30 mm).

Discussion

The bacterial united community that lives in a special social pattern-almost similar to our own human community- and known as bacterial biofilm is a real beautiful and fascinating phenomenon. Nonetheless, this beauty confers a great hazard to human lives, thus this hazard was the motive behind the present work. It is well known that bacterial biofilms are the monsters that initiate infections through indwelling prosthetic devices, and the cause of losing many lives comprising a high rate of morbidity and mortality [5,31,32]. The use of different indwelling prosthetic devices became mandatory for therapeutic purposes in many and various diseases. In fact, the use of catheters nowadays is a normal daily routine, such as central line catheters, intravenous catheters, and urinary catheterization [33]. Furthermore, catheter associated urinary tract infections have been recorded as the most common nosocomial infections worldwide [16,17]. In addition, urinary catheterization is an essential process that is routinely used preoperatively, whether in simple or major operations; accordingly, urinary catheters were used at the present study to investigate the formation of biofilms by a Gram positive and a Gram-negative bacterial species that are incriminated in urinary tract infections.

Foley's catheters are said to be the most popularly used in clinical practice, hence two types were used namely; Foley latex 100% silicone coated and Foley 100% silicone [34,35]. It has been also reported that *E. coli* and *S. epidermidis* are frequently isolated from catheter associated urinary tract infections, thus the *E. coli* and *S. epidermidis* were collected from clinical specimens as the bacterial species under study [6,36]. The method used in this work, for investigating the formation and enumeration of biofilm gave reproducible results. Also, other researchers used the same technique with fruitful findings [24,27,37 and 38]. The ability of bacteria to form biofilm on Foley urinary catheters in the current work was proven by rolling and imprinting the catheter segment over a nutrient agar in each reading. Since, at the beginning of the experiment, starting from 0 to 6 hours, colonies were tiny and discrete, while after 9 to 24 confluent growth was observed. The latter may indicate that the formation of biofilm is directly proportional to the incubation time. Furthermore, statistical analysis proved significant differences between the variables.

Interestingly, the formation of biofilm by *S. epidermidis* and *E. coli*, on the tested catheters whether untreated or treated differed tremendously, compared to the control. Also, after 12 hours the population level of *E. coli* biofilm on untreated latex was higher compared to control; whereas, the maximum level reached for both organisms and maintained this level for up to 48 hours. In contrast, the growth of *S. epidermidis* was hindered on silicon catheter, and dropped by three folds after 12 hours compared to latex. This may be attributed to the smooth surface of Silicone catheter, unlike the irregular nature of latex catheter surfaces which facilitate bacterial attachment and growth [39]. The pathogenicity of *S. epidermidis* is mostly due to its ability to colonize indwelling polymeric devices and form a thick, multilayered biofilm. Biofilm formation is a major problem in treating *S. epidermidis* infection as biofilms provide significant resistance to antibiotics and to components of the innate host defenses. Various cell surface associated bacterial factors play a role in adherence and accumulation of the biofilm such as the polysaccharide intercellular adhesion. Furthermore, many genes have an important function in the regulation of biofilm formation which involves complex mechanisms, that needs further research for new anti-staphylococcal therapeutics [40]. Moreover, biofilm formation by *S. epidermidis* was found to cause infections related to peritoneal dialysis and its adherence to serum-coated catheters was four times greater than to uncoated ones, suggesting that *S. epidermidis* binds to serum proteins on the catheter surface [41]. In addition, some authorities stated that the ability of bacteria to aggregate, forming biofilms, is strictly related to the capacity

of producing an extracellular mucoid substance known as slime layer composed of polysaccharide. New molecular techniques based on PCR have come alongside more traditional methods for identification of virulent biofilm-forming strains, by detection of the genes controlling the production of this extracellular polysaccharide [42].

On the other hand, *E. coli* managed to grow on latex catheter, by one-fold more than *S. epidermidis* and less than the control by 4 folds after 12 hours, where significant differences were found. Pathogenic *E. coli* strains possess specific adherence factors that allow them to colonize sites that *E. coli* does not normally inhabit, such as the small intestine and the urethra. Most frequently these adhesions form distinct morphological structures called fimbriae (also called pili) or fibrillae, which can belong to one of several different classes and are either long and wiry or curly and flexible [43,44]. The *Afa*adhesins that are produced by many diarrhoeagenic and uropathogenic *E. coli* are described as a fimbrial adhesions, but in fact seem to have a fine fibrillar structure that is difficult to visualize. Adhesions of pathogenic *E. coli* can also include outer-membrane proteins, such as intimin (eae) of EHEC, and other non-fimbrial proteins. Some surface structures trigger signal transduction pathways or cytoskeletal rearrangements that can lead to disease.

Comparing the susceptibility pattern of *S. epidermidis* and *E. coli* before and after forming biofilm, results obtained at the present study, showed that *S. epidermidis* and *E. coli* after forming biofilm on both catheters had almost the same susceptibility pattern after 24, 168- and 336-hours old biofilm. Though it is well known that bacterial biofilms develop higher resistance, however, in this study this could be attributed to the type of the strains used or to the period of incubation, and the antibiotic used. In addition, it is known that *in vivo* behaviour of microorganisms is different from that *in vitro*. In contrast, bacteria growing in a biofilm are characterized by higher resistance towards antibiotics. Therefore, routine determination of bacterial antibiotic susceptibility from a mid- stream urine specimen during infection, is not a good prediction for the response of biofilm-associated bacteria. Until now, methods to evaluate the effects of antibiotics on biofilm formation in routine clinical laboratories are still missing. Previous studies proved that biofilm-associated bacteria are less sensitive to killing by antibiotics compared with their free bacterial cells; in addition to the extracellular matrix which remains attached to the surface, constituting a risk for re-colonization [45]. Therefore, the biggest fear and concern in medical wards nowadays is bacterial biofilm resistance against currently available antibiotic. Buhmann et al. strikingly reported that, bacterial biofilm can tolerate up to 1000-folds higher concentrations of antibiotics compared to control bacterial cells. More importantly, some plants have been reported to be able to prevent the formation of biofilm in some pathogens such as *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes*, *Streptococcus mutans*, and *S. aureus* [47-50]. So far, most studies have focused on the observation of anti-biofilm activity of herbs taken as a single unit but not in combination, such as in herbal recipes. No attention has been paid to the antibacterial or anti-biofilm activity of traditionally used herbal recipes.

The study of Chursi et al. aimed to investigate antibiofilm formation and mature biofilm eradication ability of ethanol and water extracts of Thai traditional herbal recipes against *S. epidermidis*. A biofilm forming reference strain, *S. epidermidis* was employed as a model for searching anti-biofilm agents by MTT reduction assay and the results revealed that the ethanol extract could inhibit the formation of *S. epidermidis* biofilm on polystyrene surfaces. Furthermore, treatments with the extract efficiently inhibit the biofilm formation of the pathogen on glass surfaces determined by scanning electron microscopy and crystal violet staining. In addition, ethanol extract (0.6–5 μg /ml) could decrease 30 to 40% of the biofilm development. Almost 90% of a 7-day-old staphylococcal biofilm was destroyed after treatment (250 and 500 μg/ml). Therefore, their results clearly demonstrated that herbal recipes could prevent the staphylococcal biofilm development and possessed remarkable eradication ability on the mature staphylococcal biofilm. This is in agreement with the present study in which pomegranate aqueous extract, showed amazing inhibitory activities against biofilm formation by *S. epidermidis* and *E. coli*. Several compounds responsible for the antimicrobial action of each pomegranate extract depending on their abundance. One example is tannins (abundant in pomegranate), which are considered to be toxic to bacteria [52,53]. In addition, Milyani and Ashy reported the inhibitory activity of three combined aqueous extracts of green tea, pomegranate rinds and guava leaves against 20 clindamycin resistant *S.aureus* isolates compared to each extract alone, which indicated synergistic interaction between the three plant extracts.

Milyani and Ashy stated that pomegranate (*Punnicagranatum)* and guava (*Psidium guajava)* aqueous extracts had an inhibitory effect against *S. aureus* using agar well diffusion method. The previous technique was used in the present work to determine the antimicrobial activities of the three tested plants. Surprisingly, guava extract had less inhibition activity against biofilm formation by *S. epidermidis* compared to that formed on control and treated silicone catheters within first three hours. On the other hand, cranberry (*Vaccinium macrocarpon*) have been identified as possible inhibitors of *E. coli* adherence to uroepithelial cells, in this search the effect of a cranberry powder have been tested. It has been found that the cranberry can reduce biofilms formations by *E. coli*. Previous studies have been shown that there is the potential for cranberry compounds to prevent adherence of *E. coli* to vaginal as well as to bladder epithelium, potentially preventing UTI by interrupting colonization before the organisms ascend to the bladder [54,55]. Early research suggested that cranberry extract was effective due to its ability to increase urinary acidity through the excretion of hippuric acid. However, further studies showed only transient increases in urine acidity after cranberry extract consumption. A more likely mechanism by which cranberry might prevent UTI is by inhibiting the adherence of *E. coli* to the bladder epithelium. In this study, the cranberry aqueous extract showed decreasing in viable counts of *E. coli* which means that cranberry aqueous extract inhibited bacterial adhesion. Then, most of the following readings (from 3 to 48 hrs), manifested a decrease within two folds. Surprisingly cranberry aqueous extract showed no clear inhibition on Mueller Hinton agar using agar well diffusion method. Broomfield et al. suggested that the dietary strategy would be an effective means of controlling catheter biofilm.

In an attempt to simulate the urinary bladder environment from the nutrients aspect, human urine and artificial urine were used as the nutrient fluid at the current study. Interestingly, human urine supported the growth of *S. epidermidis* on latex and silicon catheters for 48 hrs, compared to its growth in nutrient broth. However, viable counts began to decline on both catheters, and one week later the population level dropped on silicone and latex segments. Moreover, after two weeks viable counts were indictable on silicone and declined on latex. Since the latter experiment is similar to batch culture, the drop in viable count was expected after such period due to deficiency in nutrients, and also, to accumulation of toxic byproducts. It is naturally important to emphasize that *in vivo* bacterial growth i.e. urinary

bladder, is similar to a continuous culture, where fresh nutrients are continuously supplied, and toxic materials are being flushed away. The viable counts increased by one-fold than its growth in nutrient broth. However, when *E. coli* was inoculated on both latex and silicon catheters, in artificial urine, viable counts varied significantly in view of type of catheter. Silicone appeared to retard growth compared to latex, since the maximum population was reached after 12 hrs and declined after 336 hrs. Negative staining of the studied catheters using transmission Electron Microscope confirmed the formation of biofilms by *S. epidermidis* and *E. coli* and also the counts obtained by culture and by Gram stain. Moreover, it indeed confirmed the inhibitory effect of pomegranate extract on biofilm formation by *S. epidermidis* on Foley urinary catheters. Other researchers investigated biofilm formation using the same technique [30].

In conclusion, there is an urgent need to identify therapeutic strategies that are directed toward the inhibition of bacterial performing biofilms and there is a critical need for the development of alternative treatment to combat the growing number of multidrug resistant pathogen-associated infections, especially in situation where biofilms are involved.

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