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Research Article

Effectiveness of Nano Spray from Extract of Kelubut Leaf (*Passiflora foetida* L.) as Antibiofilm on Catheters of Patients with Urinary Tract Infection

Hasyrul Hamzah[1,2*], Nur Atika Astriani[1], Norma Feti Farida[1], Lila Zulfa Kamilah[1], Aryantinur Azizah[1], Fathur Rahman[1]

[1]Faculty of Pharmacy, University Muhammadiyah Kalimantan Timur, Ir. H. Juanda St. No. 15, Sidodadi, Samarinda, Kalimantan Timur, 75124, Indonesia
[2]Indonesian Biofilm Research Collaboration Centre (IBRCC), Farmako Street, Sekip Utara, Yogyakarta, 55281, Indonesia

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***Corresponding author:**
Hasyrul Hamzah
email: hh241@umkt.ac.id

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ABSTRACT

Catheter-associated urinary tract infections (CAUTI) have increased significantly every year. Approximately 70-80% of these infections involve biofilms. This study aims to determine the effectiveness of nano spray formula of kelubut leaf extract in inhibiting and eradicating biofilm formation on catheters. Kelubut (Passiflora foetida L.) leaf extract was obtained through maceration process to obtain useful compounds. Antibiofilm activity was assessed using microbroth dilution, with scanning electron microscopy for visualization. The nano spray formulation activity of kelubut (Passiflora foetida L.) leaf extract was able to inhibit biofilm growth by (77.74% ± 0.01), while ciprofloxacin was slightly more effective with a value of (80.22% ± 0.01). SEM analysis confirmed the ability of the kelubut leaf extract nano spray formulation to inhibit and damage Escherichia coli biofilms. The nano spray formulation of kelubut leaf extract (Passiflora foetida L.) coded NS1 is able to inhibit biofilm formation activity on catheters and has the potential to be developed as an antibiofilm candidate against bacteria that cause urinary tract infections.

INTRODUCTION

Catheter-associated urinary tract infection (CAUTI) is one of the most common types of healthcare-associated infection (HAI) found in hospitals. Catheters are a major factor in CAUTI with approximately 70-80% of these infections involving biofilm (Hamzah *et al*., 2022; Nicolle 2014). The frequency of CAUTI in the 2013 NHSN report showed that the highest CAUTI rates were found in neurosurgical ICUs (5.3/1000 catheter days), burns (4.8 per 1000 catheter days), and neurological ICUs (4.5 per 1000 catheter days) (Shepard *et al*., 2015).

High CAUTI rates are associated with biofilm growth mode. The persistence of a urinary catheter in the bladder may provide an attachment medium for bacteria to colonize the biofilm (Afrilina *et al*., 2017). Biofilms, which are communities of bacteria attached to a substrate or surface, can grow on catheter surfaces that integrate with organic or inorganic materials and interact with each other. The ability of microorganisms on these surfaces is influenced by electrostatic and hydrophobic interactions as well as ionic forces (Hamzah *et al*., 2022).

The organic entity generally usually connected with CAUTI is Uropathogenic Escherichia coli (UPEC) which is the main normal causative specialist in instances of muddled or simple urinary parcel diseases (Werneburg, 2022; Reid *et al*., 1996). UPEC likewise creates biofilm-based intracellular bacterial networks (IBCs) as insurance against having insusceptible reactions and to defeat bacterial resistance (Wasfi *et al*., 2020). Biofilm eradication is difficult due to the high level of antimicrobial resistance exhibited by these structures. Nanotechnology is becoming a new alternative in biofilm formation

inhibition strategies due to its potential use in medical treatment to deliver drugs to the target site in optimal concentrations, prevent inactivation, and improve therapeutic efficacy with fewer side effects (Wang *et al*., 2020).

The nano formulation's small size, large surface area, and highly sensitive properties allow it to penetrate biological barriers such as biofilms and selectively target bacteria rather than other cells (Harjai *et al*., 2020). Nanoparticles are strong colloidal particles with a measurement of 1-1000 nm (Wirasti *et al*., 2021) and they contain macromolecular materials and can be used for treatment as drug carriers whose active compounds have been dissolved, entangled, and encapsulated (Juliantoni, Hajrin and Subaidah 2020). Chitosan is a polysaccharide that is widely found in nature after cellulose. Chitosan has specific properties, namely the existence of bioactive, biocompatible, chelating, antibacterial and biodegradable properties (Román-Doval *et al*., 2023; Ode *et al*. 2010). One of the uses of chitosan in biological and biomedical systems is in drug delivery and drug release systems (Baharlouei and Rahman 2022). Nanoparticles with chitosan polymer can protect active compounds from physical and microbiological degradation (Mohammed *et al*., 2017). So it is necessary to make a new breakthrough in making nanoparticles using chitosan polymers with plant extracts that are potential candidates in biofilm inhibition.

Currently, many plants have been utilized and developed to find new potential antibiofilms. (Sari and Puspitasari, 2021) reported that kelubut leaves have anti-inflammatory, antitumor, anticancer, antihepatotoxicity, and antimicrobial activities. However, its antibiofilm activity has never been reported. This study aims to determine the optimum formula of kelubut (Passiflora foetida L.) leaf extract nanoparticles using chitosan polymer and Na-TPP cross-linker as a new strategy in fighting infections generated by biofilms on catheters in nano spray.

METHODS

Materials

The materials in this study are kelubut leaf (*Passiflora foetida* L.), biofilm-forming *E.coli* ATCC 25922, sterile aquadest, crystal violet 1% (Merck, Germany), acetic acid (Merck, Germany), Brain Heart Infusion (BHI), ethanol 96% (Merck, Germany), ethanol 70% (Merck, Germany), chitosan (Merck, Germany), glutaraldehyde solution (Sigma, USA) and catheter (Merck, Germany) Laminar Air Flow, incubator (IF-2B) (Sakura, Japan), micropipette (Gilson, France), multichannel micropipette (Socorex, Switzerland), flat bottom polystyrene microplate 24-well (Iwaki, Japan), microplate reader (Optic Ivymen System 2100-C, Spain), autoclave (Sakura, Japan), phosphate buffered saline (PBS-Fisher-UK), Scanning Electron Microscopy (Hitachi, Japan), and analytical balance (AB204-5, Switzerland).

Plant Determination

Plant assurance was completed at the Mulawarman Herbarium, Lab of Tropical Timberland Environment and Biodiversity Preservation, Personnel of Ranger service, Mulawarman College.

Preparation of Simplisia Powder

Kelubut leaves were gathered toward the beginning of the day, washed and depleted, then dried in a broiler (40°C for 6-8 hours), and mixed.

Plant Extraction

Kelubut leaf powder macerated with ethanol for 24 hours. The macerate is separated by precipitation or filtration and then evaporated with a rotary evaporator to obtain a liquid extract and continued with the waterbath process until a thick extract is obtained.

Screening of Phytochemical Compounds

Phytochemical screening was carried out through the principle of color test using appropriate reagents for each compound found. Phytochemical screening was carried out on test tubes using detection reagents (Maulana, Triatmoko and Nugraha, 2020).

Alkaloid Test

For the alkaloid test, two reagents were used: Mayer and Dragendorff. 1 g of extract was dissolved in 100 ml of ethanol, then 1 ml of each reagent was added.

Flavonoid Test

The extract was weighed 1 g, boiled with 100 ml water for 5 minutes, filtered, and 5 ml filtrate was taken. 0.05 mg magnesium powder and 2 ml concentrated HCL were added.

Saponin Test

A total of 1 g of extract was weighed, then added 100 ml of warm water, then shaken for 1 minute and add 2 drops of 1 N HCI. if the foam formed remains stable for about 7 minutes, then the extract is positive for saponins.

Terpenoid Test

Weigh 1 g of extract, dissolve with 100 ml of ethanol, take 2 ml of dissolved extract, add 3 drops of concentrated HCI and 1 drop of

concentrated H_2SO_4. Red and purple colors indicate positive results.

Tannin Test

Dissolve 1 g of extract in 100 ml of ethanol. Take 2 ml of dissolved extract and add a few drops of $FeCl_3$ 10%.

Steroid Test

A total of 1 g of extract was weighed, then dissolved with 100 ml of ethanol, then the extract that had dissolved was taken as much as 2 ml, and a few drops of Liebermann Burchard reagent were added. Positive results responded with the formation of green or blue color.

Bacterial Strain

Escherichia coli (ATCC 25922) was refined into BHI medium and brooded at 37°C for 24 hours. Bacterial thickness was inspected utilizing a spectrophotometer and acclimated to McFarland 0.1 norm (0.5-1.5x108 CFU/ml-1) (Wijianto and Hamzah, 2022).

Biofilm Formation Inhibition Test on Kelubut Leaf Extract *(Passiflora foetida* L.*)*

In this test, a 96-well level base polystyrene microtiter plate was utilized. For the biofilm attachment phase, each well of the microtiter plate received 100 L of E. coli suspension (107 CFU/mL) and was incubated at 37°C for 90 minutes. After the hatching period the plate was washed with 150 µL of sterile refined water multiple times to eliminate follower cells as much as 100 µL of kelubut leaf remove was added to each washed well. As a control medium, media without microbial development was utilized and tension was utilized as a negative control. The plates were brooded at 37°C for 24 hours for biofilm arrangement (Hamzah *et al*., 2018).

Preparation of kelubut (Passiflora foetida L.) leaf extract nano spray

The preparation of the nano spray begins with making a chitosan solution which is weighed as much as 0.1 g, 0.3 g, and 0.4 g of chitosan, each of which is dissolved with 0.2 M acetic acid in a 100 mL volumetric flask and stirred for 24 hours. Then the preparation of 0.1% b/v NaTPP solution was carried out. Weighed 0.1 g of NaTPP dissolved with distilled water in a 100 mL volumetric flask. In the preparation of nano spray formula, 0.2 g of kelubut leaf extract was dissolved into 10 mL of chitosan solution with various concentrations (1%; 0.5%; 0.25% and 0.125% b/v). After that, 2.5 mL of 0.1% b/v NaTPP solution was added dropwise with a syringe pump while being homogenized using an aerator and stirrer for 30 minutes.

Biofilm inhibitory activity on catheters

The catheter was cut one centimeter then sanitized with 70% ethanol and permitted to dry. A sum of 200 µL of media was placed into each microplate well, then brooded at 37°C for 24 and 48 hours. After hatching, the plate was washed with PBS. 200 ml of medium containing the cleaned disengage with a progression of fixations (1%, 0.5%, 0.25% and 0.125% b/v) was added to all the washing openings. Media containing 1% ethanol was utilized as a dissolvable control, and microbial suspension was utilized as a negative control. Microbial suspensions recently utilized as antibacterial (ciprofloxacin 1% b/v) were utilized as certain controls, while media with no microbial development were utilized as control media. The plates were then hatched at 37°C for 24 hours for the mid-period of biofilm arrangement and 48 hours for the development stage. Then the plate was washed with PBS. Then, 125ml of precious stone violet 1% arrangement was added to each opening, then, at that point, brooded at room temperature for 15 minutes. After hatching, the microplate was washed with PBS and 200ml of ethanol 96% was added to each well to weaken the biofilm framed. Optical Density (OD) assessment was performed with a microplate peruser at a frequency of 620 nm (Hamzah *et al*., 2022).

Biofilm degradation activity on catheters

This method is similar to the method of inhibiting biofilm activity on catheters. Biofilm was inoculated into a microplate in the same way as described above. After incubating at 37°C for 48 hours, cultures from each well were decanted, and planktonic cells were reduced by washing them with PBS. Ciprofloxacin was used as a positive control with a concentration of 1% w/v. After incubation, the microplate was washed three times with 200 ml sterile PBS to remove adherent cells. Biofilm degradation was added with 125uL of 1% crystal violet solution in each well, then incubated at room temperature for 15 minutes. After incubation, the microplate was washed with PBS and 96% ethanol was added into each hole to dilute the biofilm formed. The OD examination was performed with a microplate reader at a wavelength of 620 nm (Hamzah *et al*., 2020).

Particle size analysis and zeta potential measurement

Particle size and zeta potential were -

determined using a nano DTS zeta sizer and TEM (Amyliana and Agustini, 2021).

***Scanning Electron Microscopy* (SEM)**

The catheter was inserted into microplate 24 containing the test suspensions that had been treated similarly to the biofilm inhibition test. The catheter was then incubated at 37°C for 24 and 48 hours, followed by washing the catheter three times with sterile distilled water, then fixed with glutaraldehyde for ± 24 hours. The dehydration process was then carried out using methanol for 30 minutes to minimize the amount of water so that the observation process was not disturbed. The samples were then observed using SEM with a voltage of 10 Kv (Hamzah *et al*., 2022).

Stastical Method

The exploration results went through measurable investigation through the ANOVA ordinariness test, which used the Shapiro-Wilk strategy. The SPSS 23 (IBM Corp., Chicago, USA) was used to evaluate the data, and the test's normality level was *P 0.05.

RESULTS AND DISCUSSION

As per a new authority explanation from the Public Establishments of Wellbeing, over 65% of all microbial contaminations are brought about by biofilms. One of them is urinary tract infection caused by E. coli. This process occurs because the bacteria form a biofilm on the catheter that provides a relatively strong defense and the bacteria in the biofilm have been shown to communicate with each other and initiate cell detachment and attachment (Hamzah *et al*., 2022). Based on the literature, it is explained that bacteria can form biofilms synergistically with other bacterial species, and physically and physiologically the biofilm structure gets thicker and stronger (Smith, 2017). This poses a challenge for healthcare workers in controlling and treating biofilm-related diseases.

Plant determination

The research begins with the process of determining the plants to be used as samples. Plant determinations aim to determine if the plant sample used is the expected plant and to prevent sample errors for the research (Devi and Mulyani, 2017).

Samples in this study were taken in Rapak Dalam Village, Samarinda City, East Kalimantan. The determination results show that the kelubut leaves used in the study can be confirmed to be the type (Passiflora foetida L.). The determination results have been tested at the Mulawarman University Forestry Laboratory, with the determination number: 111.UN17.4.08/LL/2023.

Phytochemical Compounds Contained in Kelubut Leaf Extract (Passiflora foetida L.)

Phytochemical screening test aims to identify secondary metabolite compounds contained in the test sample. Based on phytochemical screening, kelubut (*Passiflora foetida* L.) leaf extract contains alkaloids, flavonoids, saponins, terpenoids, tannins, and -

Table 1. Phytochemical Screening Results

Secondary Metabolites	Reagent	Parameter	Identification Results	+/-
Alkaloid	Mayer	White precipitate or solution that turns cloudy	Solution turns cloudy	+
	Dragendorff	Brownish orange precipitate	Formation of brownish orange precipitate	+
Flavonoid	Mg + HCl 2 N	Orange, yellow or red	Formation of red color	+
Saponin	Aquadest + 1 N HCl	Foam remains stable +7 minutes	Foam remains stable +7 minutes	+
Terpenoid	Concentrated HCl + concentrated H_2SO_4	Red or purple color	Solution turns red	+
Tannin	$FeCl_3$1%	Dark blue or greenish black	Formation of greenish black color	+
Steroid	Liebermann Burchard	Green or blue	Formation of green color	+

and steroids. These compounds were identified through phytochemical screening tests using specific reagents. The results are shown in Table 1.

The compounds contained in kelubut leaf extract have been shown to have antibacterial properties and can inhibit bacterial growth effectively. These compounds can disrupt bacterial cell membranes, affect DNA function, inhibit protein synthesis, damage cell walls and membranes (Hamzah *et al*., 2022).

Antibacterial Effect of Kelubut Leaf Extract (Passiflora foetida L.) against E.coli

The study found that kelubut (*Passiflora foetida* L.) leaf extract had 80.43% ± 0.01% inhibitory activity against the growth of *E. coli* at a concentration of 1%. Ciprofloxacin which was used as control drug gave an inhibitory activity of 82.00% ± 0.01. (Figure 1).

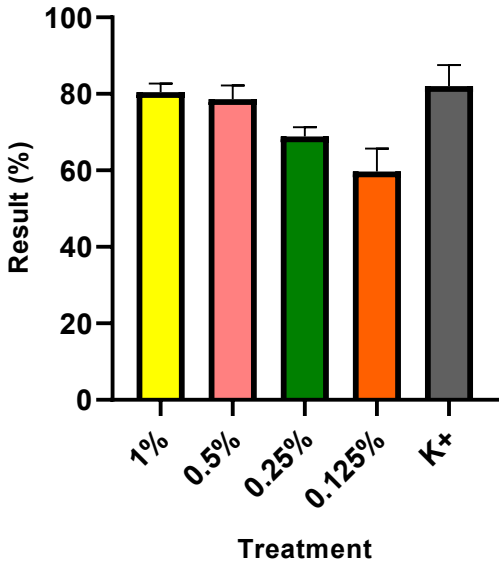


Figure 1. Percentage of Antibacterial Activity of Kelubut Leaf *(Passiflora foetida L.)* against *E.coli*

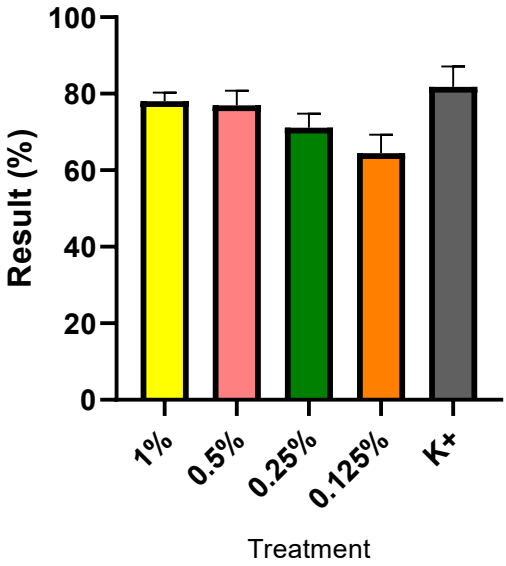


Figure 2. Inhibitory Activity of Kelubut *(Passiflora foetida* L.*)* Leaf Extract against *E.coli* Biofilm Middle Phase (24 hours)

These results show that the antibacterial activity of kelubut (*Passiflora foetida* L.) leaf extract is not significantly different from the control drug ciprofloxacin. After data analysis, significant differences were found between each concentration of 1%, 0.5%, 0.25%, and 0.125 b/v, with p <0.05. The viability of the concentrate relies upon its focus in view of information examination, with higher fixations bringing about more noteworthy hindrance of *E. coli* development. Alkaloids and flavonoids are two important compounds in antibacterial activity against various types of bacteria (Changestu *et al*., 2023). The mechanism of action of both in antibacterial activity is very diverse, including disrupting the mechanism of nucleic acid synthesis, damage to the cell wall, and inhibiting protein synthesis. Antibacterial activity in kelubut (*Passiflora foetida* L.) leaf extract has the potential to be developed into antibiofilm candidates in the world of health.

Inhibitory Activity of Kelubut Leaf Extract *(Passiflora foetida L.)* against *E.coli* Biofilm in Middle Phase (24 Hours)

Kelubut leaf extract (*Passiflora foetida* L.) at a concentration of 1% b/v showed strong antibiofilm activity against *E. coli* in the middle phase, with a value of 78.04% ± 0.01. According to the classification referred from (Setyowati *et al*., 2024) antibiofilm activity is categorized as having strong activity if biofilm inhibition exceeds 70% ± 0.01. Ciprofloxacin with a concentration of 1% b/v has a comparative value of 81.81% ± 0.01 (Figure 2).

The effectiveness of biofilm inhibition is associated with the administration of 1% b.v. kelubut (*Passiflora foetida* L.) leaf extract compounds that cause a decrease in the number of cell densities characterized by cells experiencing division, deformation and cell leakage (Hamzah *et al*., 2023). This is because the kelubut leaf extract compound (*Passiflora foetida* L.) is able to damage the Extracellular Polymeric Substance (EPS) which is a protective biofilm.

Activity of Nano Spray Formula on Middle Phase (24 hours) *E.coli* Biofilm on Catheters

In this study, we evaluated the antibiofilm potential of nano spray formula of kelubut (*Passiflora foetida L.*) leaf extract against *E. coli* biofilm inhibition on catheters. The results showed that the nano spray formula with code NS1 gave an activity of 77.74% ± 0.01, while the control drug ciprofloxacin gave an activity of 80.22% ± 0.01 (Figure 3).

The results showed that the comparison of nano spray formula of kelubut leaf extract (*Passiflora foetida* L.) with NS1 code was able to inhibit 70% of *E. coli* biofilm growth on the catheter and was not much different from the control drug ciprofloxacin. Based on the results presented in Figures 3, during the middle phase (24 hours), the nano spray formulation showed varying levels of inhibitory activity for each concentration. The concentration of the extract affects the size of the inhibition, because the increase in the concentration of the extract given causes the greater content of active ingredients that are antibacterial in nature, resulting in more effective inhibition of bacterial growth. Compounds found in kelubut leaf extract can prevent the formation of complex structures and

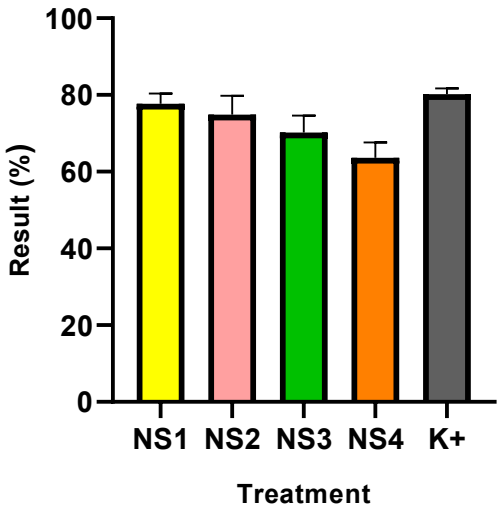


Figure 3. Activity of Nano Spray Formula on Middle Phase (24 Hours) *E.coli* Biofilm on Catheters

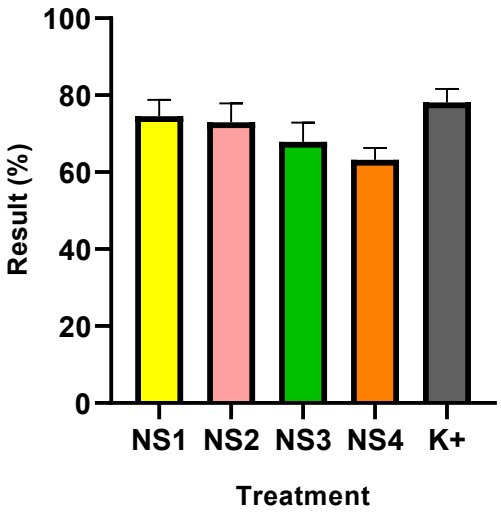


Figure 4. Activity of Nano Spray Formula on Maturation Phase (24 Hours) *E.coli* Biofilm on Catheters

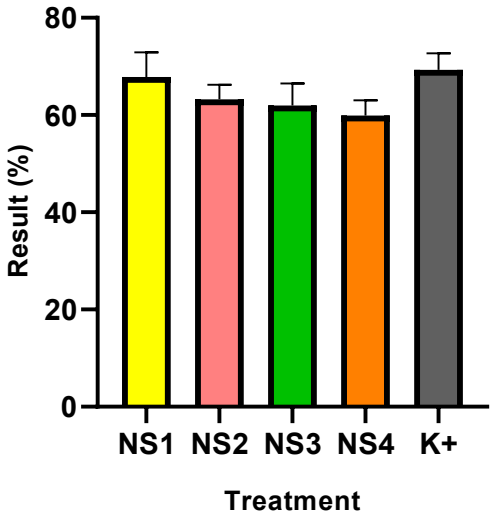


Figure 5. Activity of Nano Spray Formula on Degradation Phase (24 Hours) *E.coli* Biofilm on Catheters

production of extracellular matrix that persists against *E. coli* inhibition of biofilm growth can occur by disrupting communication signals between bacteria or inactivating genes that stimulate matrix synthesis.

Activity of Nano Spray Formula on Maturation Phase (48 hours) E.coli Biofilm on Catheters

As shown in Figure 4, the nano spray formulation of kelubut leaf extract (Passiflora foetida L.) code NS1 inhibited by 74.51% 0.01 and the control drug ciprofloxacin by 78.09% 0.01. These data indicate that the nano spray formulation with code NS1 has less activity than in the middle phase (24 hours). This is on the grounds that in this stage the EPS network of E. coli biofilm created more and the biofilm structure framed is denser and more perplexing, this should be visible from the E. coli biofilm sludge layer appended to the wells ring.

This is likewise supported by (Hamzah *et al*., 2018) explanation that the 48-hour period of biofilm development makes some lengthy memories contrasted with the 24-hour stage, hence the biofilm local area shaped in this stage is progressively various and coordinated with one another, subsequently framing a sort of 3-layered bunch that will speak with one another when an unfamiliar item will enter their local area.

Activity of Nano Spray Formula on the Degradation of *E. coli* Biofilm on Catheters

In the degradation phase, the nano spray formula with code NS1 was only able to provide inhibition of 66.85% ± 0.01 while the control drug ciprofloxacin provided inhibition of 71.00% ± 0.01 (Figure 5). In this phase, the nano spray formula compound of kelubut leaf extract (*Passiflora foetida* L.) has more difficulty to penetrate the biofilm defense of *E. coli* on the catheter because the growth of biofilm formation in this phase is longer than the maturation phase (48 hours), this can be seen from the mucus produced in the degradation phase is very thick and dense. Therefore, the nano spray formula compound of kelubut leaf extract has more difficulty to destroy biofilms in this phase.

This result is reinforced by (Hamzah *et al.* 2018) the statement that in the degradation phase the biofilm EPS structure formed is more numerous, thick and very complex so that the protection of *E. coli* from antibiofilm agents is getting stronger.

Results of Particle Size Analysis (PSA) and Zeta Potential of Nano Spray Formula of Kelubut Leaf Extract *(Passiflora foetida* L.*)*

According to (Rachmawati and Surini, 2018) Nanoparticles are colloidal structures that have sizes between 10-1000 nm. The Table 2 shows that formulas NS1, NS2, NS3 and NS4 have sizes of more than 10 nm and less than 1000 nm, so the four formulas fulfil the requirements of nanoparticle preparations. The PDI values in formulas NS1 and NS2 meet the requirements where the PDI value is generally below 0.3, so it can be said to be good. When the PDI value is above 0.3, the average particle size cannot be used. While the potential zeta values for Formulas NS2, NS3 and NS4 do not meet the criteria for good nanoparticle stability where the potential zeta must be smaller than -30 mv and greater than + 30 mv (Juliantoni *et al*., 2020). This means that the formula that meets the requirements and criteria is formula NS1 (Table 2).

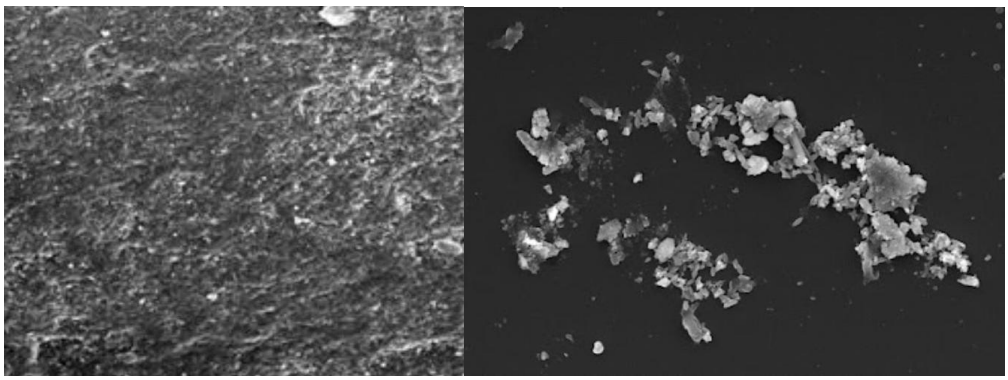
Scanning Electron Microscopy (SEM) Results

The *E. coli* biofilm on the untreated catheter showed an accumulated and very dense cell density and exhibited very thick EPS production (Figure 6(a)). In the above figure, *E. coli* forms a highly structured and complex biofilm on the catheter tract; *E. coli* adheres to and alters the surface of the urinary catheter by blocking the receptor areas of uropathogens. This causes many compounds to have difficulty in providing maximum inhibition due to the thick EPS matrix that protects the biofilm on the catheter.

The application of nano spray formula of kelubut leaf extract code NS1 on the catheter caused a decrease in the number of cell densities characterized by cells that experienced division, changes in shape and cell leakage. This is because

Table 2. Results of PSA and Zeta Potential testing on Nano Spray Formula of Kelubut Leaf Extract *(Passiflora foetida L.)*

	NS1	NS2	NS3	NS4
Particle Size (nm)	175.3	310.0	469.0	664.0
PDI	0.187	0.110	0.580	0.2494
Zeta Potential	-1 .2 mv	27 mv	19 mv	10.3 mv



(a) (b)

Figure 6. (a) SEM on untreated catheters; (b) SEM results on the catheter after treatment

the compound of nano spray formula of kelubut leaf extract NS1 code is able to damage EPS which is a protective biofilm (Hamzah *et al*., 2024; Huang *et al*., 2022; Olivera and Luengo 2019; Sun *et al*., 2018). The mechanism of the nano spray formula of kelubut leaf extract *(Passiflora foetida* L.) code NS1 in inhibiting and killing biofilm on the catheter is based on changes in the biofilm layer due to the entry of the nano spray formula compound of kelubut leaf extract which results in nutrient deprivation and decreased oxygen levels in the biofilm, causing cells to become abnormal and die. This cell leakage is caused by the breaking of hydrophobic bonds consisting of membrane-forming components such as proteins and phospholipids (Hamzah *et al*., 2022) (Figure 6 (b)).

Statistical Results

Statistical tests using SPSS 23 (IBM Corp, Chicago, USA) in this study showed significant differences in each concentration, namely 1%, 0.5%, 0.25% and 0.125% b/v, with a *p* value <0.05.

CONCLUSIONS

This study evaluated the activity of nano spray of kelubut leaf extract (*Passiflora foetida* L.) as an antibiofilm on catheters. The results showed strong inhibitory activity against biofilm formation in the middle phase and biofilm maturation in the nano spray formula of kelubut leaf extract (*Passiflora foetida* L.) with code NS1. In light of the consequences of the SEM study, kelubut leaf separate had the option to harm the EPS grid of *E. coli* biofilm on the catheter. This recommends that the kelubut plant can possibly be created as an antibiofilm applicant on catheters.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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