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Anti-Biofilm Activity of Ethanol Extract of *Citrus hystrix* Dc. Against Opportunistic Pathogenic Microbes In-Vitro

Lia Yulia Budiarti[1], Husnul Khatimah[2], Muhammad Zaki Ridhoni[3], Ghina Hafizhah[3], Muhammad Fachriyad[3], George Arietama[3]

[1]Department of Microbiology and Parasitology, Faculty of Medicine and Health Science, Universitas Lambung Mangkurat, Banjarmasin, Indonesia
[2]Department Biomedic, Faculty of Medicine and Health Science, Universitas Lambung Mangkurat, Banjarmasin, Banjarmasin, Indonesia
[3]Medical Study Program, Faculty of Medicine and Health Science, Universitas Lambung Mangkurat, Banjarmasin, Banjarmasin, Indonesia
Coresspondence Author: hkhatimah@ulm.ac.id

Abstract:

Opportunistic pathogenic microbes are often a problem in the treatment of infections because of their ability to form biofilms. The structure of the irreversible components of the glycocalyx or microbial capsule plays a role in protecting it from antimicrobial exposure and disinfection. The effectiveness of antibiofilms needs to be evaluated, including those from natural preparations. *Citrus hystrix DC*. (*C.hystrix*) contains antimicrobial compounds and its activity as an antibiofilm needs to be known. This experimental research aims to test the anti-biofilm effect of *C.hystrix* extract against standard microbial isolates. The dilution method with tube-test was used to analyze the antibiofilm effect of a combination of *C. hystrix* leaf and peel extracts (6.25%, 12.5%, 25%, 50%, 75%, and 100%) with 70% ethanol control. Antibiofilm observations are qualitatively based on the Minimum Biofilm Inhibitory Concentration (MBIC) and quantitatively based on the Mean Gray Value (MGV) of biofilm intensity. The results of observations in 3 experiments showed that the MBIC of *C.hystix* extract was 6.25% for gram-positive bacteria and 12.5% for gram-negative bacteria and Candida. The average MGV of *C.hystrix* extract was 75%, equivalent to 70% ethanol for all test microbes (p.0.05). The highest average MGV was produced by 100% combination extracts for *S.epidermidis* (138,09±0,36), *S.aureus* (135,69±2,01), *E.coli* (134,75±0,89), *P.aeruginosa* (130,76±0,24), and *C.albicans* (130,41±0,41). In conclusion, hystrix orange leaf and peel extracts produce anti-biofilm activity against test microbes in-vitro.

Keywords: anti-biofilm, *Citrus hystrix*; in-vitro; mean gray value; minimum biofilm inhibitory concentration

Introduction

Microbes that act as commensals do not cause disease in healthy hosts with a good immune system, but when the host's body condition declines they can cause more serious disorders. Infections by opportunistic, biofilm-forming microbes are increasingly being reported.[1]

Infections caused by microbes with biofilms are a problem that is difficult to overcome, because microorganisms in biofilms are more resistant to treatment and more difficult to reach by antimicrobial agents.[2-3] Biofilms are very important for public health because of their role in infectious diseases and the transmission of infections through the use of medical equipment.[4] Römling and Balsalobre (2012), stated that human infections related to biofilm formation are estimated to reach 80%.[5] Microbial biofilms in persistent tissue that are resistant to antimicrobial agents cause an increase in cases of nosocomial infections.[2]

Biofilm has the property of adhering irreversibly and thickening to the surface and being embedded in the matrix. Biofilms are composed of an extracellular polymeric substance (EPS) matrix, namely a layer of extracellular polymeric substance glycocalyx and capsular material in the form of polysaccharides, proteins and nucleic acids, as well as glycopolysaccharides secreted by the microbes themselves. Its role is to protect microbes against exposure to antimicrobial substances and disinfectant action.[6-7] Resistant penetration of antimicrobial agents through the three-dimensional matrix of the biofilm, with very slow diffusion and difficulty in achieving effective concentrations. The most common biofilm-producing pathogens are *Staphylococcus aureus (S.aureus), Streptococcus epidermidis (S.epidermidis), Pseudomonas aeruginosa (P.aeruginosa), Escherichia coli (E.coli),* serta *Candida albicans (C.albicans).*[8-11] Pathogens with biofilms can survive in the environment in the air and damp surfaces.[12] Transmission of infection that often occurs is through touching the skin of the hands and using equipment that has been contaminated with pathogens.

Prevention of infections due to biofilms means that the use of disinfectants (EDTA, formalin, hydrogen peroxide and ethanol) which are routinely used in clinics needs to be evaluated. The commonly used ethanol is bactericidal. The mechanism of action is to denature proteins, damage cell membranes, and also inhibit enzyme synthesis and microbial cell metabolism.[13] Ethanol at high concentrations is not recommended for open wound infections and risk of burns.[7] The impact of biofilm resistance on disinfection is a great opportunity to find alternative anti-biofilm compounds made from natural ingredients.

Citrus hystrix D.C (*C.hystrix*) has been used for a long time as a food product and alternative medicine. The phytochemical content in leaves was found to be greater than in fruit and fruit skin.[14] Bioactive antimicrobials in *C. hystrix* include the flavonoids, phenols, tannins, alkaloids, saponins, terpenoids, glycosides and steroids.[15-16] The activity of *C. hystrix* ethanol extract has been proven to be effective against *S. aureus, S. epidermidis, E. coli,* and *P. aeruginosa*.[15] Compounds that play a role in fungi are phenols, flavonoids, and terpenoids.[16]

Phytochemical compounds are known to produce antimicrobial and antibiofilm effects.[17] Several solvents, namely, water, methanol, ethanol, chloroform, ether, dichloromethanol, and acetone, are used for extraction of natural compounds from various sources for anti-biofilm activity. Phytocompounds having fewer side effects may be better therapeutic agents for biofilm-related infections.[18] The inhibitory effect of C.hystrix alcohol extract against *C.albicans* is greater than that of water extract.[19] Several bioactive compounds can have an anti-biofilm effect, for example phenolics, glycolics, tannins and flavonoid saponins, alkoloids, tannins, saponins and steroids. [20-21] Narigen is

a class of polyphenolic compounds in orange peel that also has an anti-biofilm effect. Antibiofilm from *C.hystrix* oil spray is known to inhibit the formation of *S.mutans* bacterial biofilms.[22] The use of combined drugs for infections caused by biofilms is recommended rather than single preparations.[18] The antibiofilm (anti-adhesion) activity of a combination of herbs with different targets can produce a synergistic effect.[23]

The aim of the research was to test the anti-biofilm effect *of C.hystrix* leaf and peel extracts against microbial isolates that are often pathogenic. A simple in-vitro method for observing biofilms is a qualitative test using a tube-test (liquid dilution) and Congo Red Agar (CRA).[3,24] Analysis of Minimum Biofilm Inhibitory (MBIC) produced by a treatment, characterized by ring thinning and a bluish purple layer on the walls. bottom of the tube.[25] The quantity of biofilm formed on the tube is calculated based on the Mean Gray Value (MGV).[26-27] This study has passed the ethical test based on the letter statement of approval of the ethics committee of the

Research Method

This study was approved by Health Research Ethics Commissions Faculty of Medicine, University of Lambung Mangkurat (FMULM) with letter 146/KEPK-FK ULM/EC/VII/2023. A true experimental model was schemed for this study with post test only and control group design that was conducted at Pharmacology and Microbiology Laboratory of FM ULM.

Materials

The plants kaffir lime used have been identified and determined based on a letter from the MIPA Biology Laboratory of Lambung Mangkurat University. The fresh leaves and fruits of *Citrus hystrix* D.C were collected from Banjarbaru City, located in South Kalimantan Province, in Agust 2023.

The microbial organisms determined in this study contained 6 American Type Culture Collection (ATCC) bacterial and *Candida albicans* isolates. The ATCC bacterial strains were composed of *S.aureus* ATCC 25923, *S.epidermidis* ATCC 35984, *E.coli* ATCC 25922, *P.aeruginosa* ATCC 27333, and *C. albicans* ATCC 10231. These microbial were obtained from Faculty of Medicine, Lambung Mangkurat University, Indonesia. The bacteria were cultivated on blood agar at 37°C for 18-24 hrs and *C.albicans* on Sabaraud dextrose agar at 37°C for 24 hrs. The positive control test was 96% ethanol.

Inokulum Preparation.

The ATCC standard type bacterial and Candida test isolates are from the collection of the Microbiology Laboratory of the ULM Banjarbaru Faculty of Medicine. The microbial inoculum suspension was made according to standard procedures, namely inserting a full circle of inoculant into liquid medium TSB + 10% glycerol (5ml) then incubating at 370C for 24 hours. Next, the level of turbidity was homogenized according to the Mac Farland 0.5 standard solution (1x108 CFU/ml) and each microbial isolate was used in the test.[28]

Preparation of Simplicia and *C.hystrix* Extract

C.hystrix leaves are cleaned of dirt, washed with running water, and air-dried until dry; Next, powder is made with a size of 40 mesh. Prepare the peel of the fruit which has been separated, wash it with running water, peel it, slice it thinly, and make it into powder with a size of 40 mesh. All simplicia were macerated with the addition of 96% ethanol for 4x24 hours. The extract was then thickened with a rotary evaporator at 50°C. 37 Extracts of leaves and fruit peel in a combination preparation (ratio 1:1) 100% were made into serial dilutions of 75%, 50%, 25%, 12.5% and 6.25% (V/V).

Phytochemical Analysis

Phytochemical analysis was carried out on concentrated extracts of leaves and fruit peel of *C.hystrix*. Quantitative phytochemical tests were carried out using standard procedures according to Harborne (1998).[29] Results of phytochemical screening. The contents of the ethanol extract of leaves and

fruit peels are alkaloids, phenolics, flavonoids, tannins, saponins, steroids and terpenoids.

Antibiofilm Detection Method

Tube-test and *MBIC* observation

The suspension of each test microbe (1x108 CFU/ml) was put into a tube containing TSB medium + 10% glycerol. Tubes 1-6 contained 2 ml of microbial suspension in TSB + 10% glycerol, 2 other tubes (control) were filled with 4 ml and 2 other tubes contained TSB + 10% glycerol and tubes containing the test microbial suspension without treatment. A total of 2 ml of *C. hystrix* extract was mixed into tubes 1-6 to obtain a solution of 4 ml with the following concentration in each tube: Tube 1: 0% (control), Tube 2: 100%, Tube 3: 75%, Tube 4 : 50%, Tube 5: 25%, Tube 6: 12.5%, and Tube 7: 6.25%. The other tube contains the test microbial suspension with positive control (96% ethanol), suspension with 10% DMSO control, TSB+glycerol 10% medium without treatment, and the test microbial suspension without treatment. All tubes were incubated at 37°C, 24 hours.[1,11]

Anti-biofilm assessment is based on the level of clarity in the tube using value categories: 0 = Clear, no biofilm formation, +1 = Slightly clear, starting to have weak barriers to biofilm formation, +2 = Slightly cloudy, moderate barriers to biofilm formation, +3 = Turbid, there is strong biofilm formation, and +4 = Very turbid, very strong and lots of biofilm is formed.[30] The MBIC value is the smallest concentration that indicates biofilm formation in the +1 category (starting to clear/slightly clear).

Test Congo Red, a biofilm-producing microbe

Congo Red Agar (CRA) media was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes. Congo Red Agar plates are inoculated with test microbes and incubated aerobically (37°C and 24 hours).[11] Positive results on biofilm-forming microbes show black colonies and red colonies on non-biofilm ones.[31] The colonies of each microbe tested on CRA media before extract treatment were black.

Confirm MBIC on CRA media by pouring CRA media (55°C) into the cup and adding the microbial suspension and test extract (+1), then the cup is incubated (37°C, 24 hours). The microbial colony that grows the least or does not grow on the plate is MBIC (Minimum Biofilm Inhibitory Concentration).[31]

Pengukuran Mean Gray Value (MGV)

After completing the determination of MBIC through a test tube, the MGV measurement is then carried out. Each tube was washed 3 times with 0.2ml phosphate buffer saline (PBS) pH 7.3 and dried. The dried tube was treated with 0.5 ml of crystal violet (0.1%) then the excess color was removed and the tube was washed with sterile distilled water (deionized water) and dried.[1,11]

The results of biofilm formation on the tube were then photographed using a digital camera. The photo of the tube with purple film was observed using the Adobe Photoshop CS6 program and the MGV intensity was measured using the measurement tool. The selected photo file is entered on the Window tab and select Measurement Log, block the area where the color intensity will be seen using the Rectangular Marquee Tool then click Record Measurements, then you will get the MGV value (scale 0 – 255). A high MGV value indicates a thinner color intensity.[26,32,33] Treatments that produce an inhibitory effect on biofilm formation will show a higher MGV compared to negative controls.

Data analysis

The research design used a true experimental posttest only control group design. Data obtained from 3 repetitions. MBIC values were analyzed descriptively. MGV data was statistically analyzed using SPSS version 26.0 for Windows. Data distribution analysis used the Shapiro-Wilk test and Levene's test. Parametric data analysis used the One-way ANOVA test and Duncan's Post-hoc test at a confidence level of 95%.

Results

All microbes tested were biofilm formers. The results of observing the level of clarity in the Tube-test showed that the MBIC of *C.hystric* leaf and rind extract (L+PCH) in *S.aureus* and *S.epidermidis* was 6.25% while in *E.coli, P.aeruginosa*, and *C. albicans* by 12.5%. The antibiofilm effect produced by L+PCH extract and control is shown in Figure 1. Comparison of the mean and standard deviation of L+PCH extract in inhibiting biofilm is shown in Table 1.

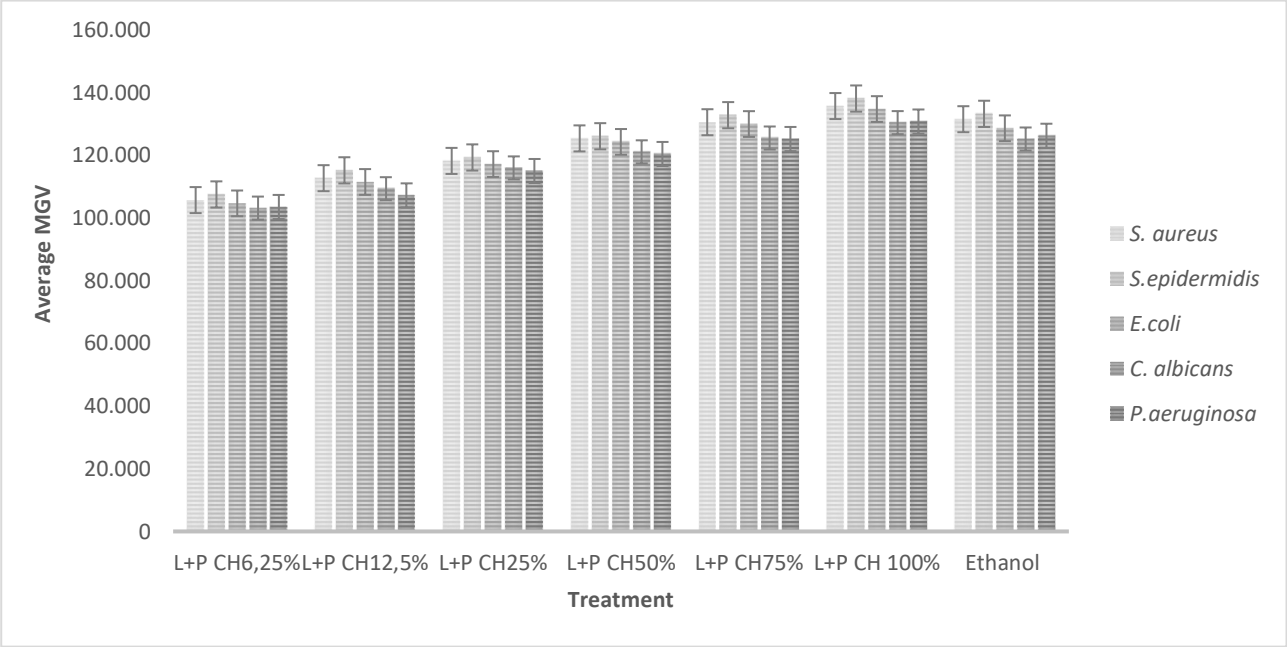

Figure 1. Average Mean Gray Value (MGV) Anti-biofilm of the combination extract of leaves and peel of *Citrus hystrix* fruit (L+P CH) and control on the formation of test microbial biofilms

Table 1 Standard Deviation Average MGV Anti-biofilm of the combination extract of leaves and peel of *Citrus hystrix* fruit (L+P CH) and control

Treatment	Average MGV Anti-biofilm				
	S.aureus	*S.epidermidis*	*E.coli*	*P.aeruginosa*	*C.albicans*
DMSO1%	96,44 ±0,94[g]	97,32 ±0,62[g]	96,28±0,62[g]	95,99± 0,873[g]	97,41 ±0,46[g]
L+P CH 6,25%	105,70±0,64[f]	107,50 ±1,29[f]	104,65 ±0,47[f]	103,50 ±1,00[f]	103,14±0,97[f]
L+P CH 12,5%	112,69±0,41[e]	115,19±0,62[e]	111,49±0,53[e]	107,19 ±0,61[e]	109,33±0,52[e]
L+P CH 25%	118,20±1,11[d]	119,30 ±0,59[d]	117,19±0,16[d]	114,97±0,23[d]	115,97±0,35[d]
L+P CH 50%	125,39±1,11[c]	126,07±0,33[c]	124,27±0,74[c]	120,40±0,58[c]	121,09±1,20[c]
L+P CH 75%	130,55±1,24[b]	132,78±0,02[b]	129,96±0,09[b]	125,18±0,52[b]	125,50±1,11[b]
L+P CH 100%	135,69±2,01[a]	138,09±0,36[a]	134,75±0,89[a]	130,76±0,24[a]	130,41±0,41[a]
Ethanol	131,50±1,05[b]	133,22±1,08[b]	128,61±0,43[b]	126,22±0,45[b]	125,19± 0,32[b]

a, b, c, d, e, f, g superscript in the same column shows no significant difference (P>0.05)

Figure 1 shows that MGV biofilm increased when using increasing concentrations of L+P CH extract. DMSO treatment produced the lowest MGV. This means that DMSO does not affect the biofilm formed by the test microbes, the thick biofilm blocks the light intensity. L+P CH 100% extract produced the highest MGV value. The concentration of the extract affects the antibiofilm effect, so that increasing the concentration can increase the barrier to a higher level. The antibiofilm activity of *C.hystix* extract against gram-positive bacteria is

greater than against gram-negative bacteria and Candida.

Table 1 shows the results of statistical analysis of the average MGV antibiofilm produced by LP+P CH extract at a concentration of 75% which is not significantly different from the positive control for all test microbes. *Citrus hystrix* 75% extract has effectiveness as an antibiofilm equivalent to 96% ethanol (p.005). The antibiofilm activity produced is due to the role of bioactive compounds contained in the *C.hysrix extract.* The groups of compounds identified in the extract are alkaloids, phenolics, flavonoids, tannins, saponins, steroids and terpenoids.

Discussion

Biofilm is a major virulence factor that contributes to infectious diseases.[6] The largest component of biofilms in bacteria is EPS which is composed of polysaccharide intercellular adhesin (PIA).[34] *C. albicans* biofilms are composed of crossed hyphal structures. The antibiofilm response during adhesion, namely ergosterol as a component of yeast cell membranes, is downregulated due to the obstruction of carbon flow to ergosterol. The cycle of biofilm formation begins with planktonic cell attachment, then forms colonization and biofilm formation, then undergoes a maturity process and finally a dispersion process (detachment of cells from the biofilm). [34] As a result, it inhibits the growth of hyphae and inhibits the formation of biofilm. The subsequent process of adhesion failure causes the release of the biofilm from the abiotic substrate.[35]

The effect of ethanol on biofilms is that it causes the release of eDNA. The role of eDNA is to be involved in microbial survival and as a matrix component in the biofilm structure. The release of eDNA from biofilm components which is a mixture of eDNA that has been accumulated with that accumulated during biofilm growth.[36]

The antibiofilm activity produced by C. hystrix extract in this study was due to the role of the bioactive compounds contained in the extract. The groups of compounds identified in the extract are alkaloids, phenolics, flavonoids, tannins, saponins, steroids and terpenoids. Phytochemical compounds have antibiofilm effects. Essential oils from the leaves and fruit skin of C.hystrix produce synergistic effects as antibacterial and antibiofilm in vitro.[37] In-vitro, polyphenol content produces anti-biofilm activity in biofilms formed by *S. aureus, S. pyogenes, P. aeruginosa* and *C. albicans*.[38] Tannin compounds have been proven to inhibit biofilm formation formed by *E. coli, S. aureus, P. aeruginosa,* and *C. albicans.*[37]

Complete inhibition of biofilm could not be achieved with any one phytochemical. Phytochemicals show the potential to act as an antibiofilm synergist.[39] The synergistic effect is influenced by their ability to disrupt cell membrane formation, limit the diffusion of hydrophobic compounds in the biofilm, and restrain cell adhesion in the biofilm.[5,40] This study shows the effect of a combination of extracts from the leaves and fruit peel of C. hystrix produces antibiofilm formed by *S. aureus, S. epidermidis, E. coli, P. aeuginosa,* and *C. albicans.*

Increasing the extract concentration allows more bioactive compounds to be dissolved in the extract and work synergistically to produce a more optimum effect. In this study, the antibiofilm effect of a combination of *C. hystrix* leaf and rind extract was 75%, equivalent to 96% ethanol, so that *C. hystrix* extract has the potential to be developed as an alternative disinfection agent.

The phytochemical content prevents the ability of biofilm formation in Gram-positive, Gram-negative bacteria and *C. albicans.*[41] Phytochemical mechanisms for inhibiting biofilm formation include inhibition of quorum sensing (QS), anti-initial adhesion, maturation and inhibiting microbial colonization.[16,42] Quorum- sensing which is a communication system between cells with various cellular bacteria such as pathogenesis,

nutrient acquisition, conjugation, motility, and secondary metabolite production from bacteria. The biofilm inhibition process begins with the initial adhesion stage, maturation and formation of the biofilm form.[16,41]

The flavonoids that act as antibiofilms are the flavanones narirutin, hesperidin, the flavonol quercetin, and the flavones sinensetin, nobiletin, and tangeretin. Phenolic compounds and flavonoids interfere with the quorum-sensing mechanism of bacterial biofilms.[8,16,41] The mechanism of phenolic and flavonoid groups in fungi is to prevent adhesion, disrupt the transition mechanism of yeast to hyphae and reduce the hydrophobicity of the cellular surface of biofilm cells, thus preventing the formation of fungal biofilms.[35] Saponin compounds play a role in reducing the production of bacterial biofilm slime, increasing the permeability of cell membranes through the diffusion process so that disrupt the stability of bacterial cells.[43] In fungi, saponin compounds play a role in inhibiting mycelial transformation, preventing aggregation, and destroying the surface morphology of fungal cells.[44] inhibition of biofilm by alkaloid compounds, namely reducing initiator genes for forming biofilms, inhibiting quorum-sensing, and reducing regulatory factors for forming biofilms.[36,45] In fungi, these alkaloid compounds play a role in inhibiting and initiating biofilm formation.[44] The mechanism of biofilm inhibition by tannin compounds is disrupting the adhesion mechanism, reducing the production of bacterial biofilm slime and damaging the polymicrobial EPS matrix.[46-47]

Conclusions

The conclusion from the in-vitro test results was that it was proven that Citrus hystrix DC leaf and peel extracts were present. produces anti-biofilm activity as does ethanol against opportunistic pathogenic microbes. The synergistic effect comes from the mechanism of action of the bioactive compounds in the combination of extracts and the anti-biofilm activity is influenced by concentration. Sustainability that can be developed from this extract is the stability and organoleptic tests according to SNI standards.

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