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# Tube-Test of Antibiofilm Activity of Yellow Root Extract (*Fibraurea tinctoria* Lour.) Against Bacteria Causing Impetigo

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**Abstract:**

Impetigo is a contagious superficial bacterial skin infection that commonly occurs in children. The main causative agents are *Staphylococcus aureus (S. aureus)* and *Streptococcus pyogenes (S. pyogenes*). Both types of bacteria are capable of forming biofilms, making them resistant to antimicrobial agents. Antimicrobial agents that affect the survival of biofilm-forming bacteria are not only derived from synthetic drugs but also from phytochemical compounds. The antibiofilm effect can be assessed through a tube-test. *Fibraurea tinctoria* Lour (*F. tinctoria*), known as yellow root, contains various antibacterial compounds and has the potential as an antibiofilm agent. This experimental study aims to evaluate the ability of *F. tinctoria* root extract as an antibiofilm against *S. aureus* and *S. pyogenes* as the causative agents of impetigo in vitro. The study design used a post-test only with control group design with a tube-test method. The antibiofilm effect of *F. tinctoria* root extract at concentrations of 3.125%-50% and a 0.5% ampicillin control were tested on standard ATCC isolates of *S. aureus* and *S. pyogenes*. The observed parameters were the Minimum Biofilm Inhibitory Concentration (MBIC) and Mean Gray Value (MGV). The result showed *F. tinctoria* extract inhibited the biofilm formation of the tested bacteria. The antibiofilm activity was significantly influenced by extract concentration ($P > 0.05$). The MBIC value of the extract was found at a concentration of 12.5%, with MGV against *S. aureus* ranging from 130.303-157.3833 and 131.9767-160.1833 against *S. pyogenes*. In conclusion, *F. tinctoria* root extract demonstrates antibiofilm effects in vitro against the two tested bacteria that cause impetigo.

Keywords: Antibiofilm; Fibraurea tinctoria; Staphylococcus aureus: Streptococcus pyogenes: Tube-test.

## Introduction

Impetigo is a dermatological disorder with complications that pose a global problem and have the potential to be life-threatening. It is estimated that over 2% of the global population experiences impetigo, with approximately 162 million cases occurring annually.[1] The incidence of impetigo can affect all races, genders, and age groups. However, it is more common in children aged 2-5 years.[1,2] The disease occurs year-round and is more prevalent in tropical climates, particularly in lowlands (warm and humid conditions), and is associated with overcrowding and poor hygiene.[1-4] Bacterial impetigo is a pyoderma that affects the superficial layers of the epidermis, caused by *Staphylococcus aureus* (*S. aureus*) and group A beta-hemolytic *Streptococcus pyogenes* (*S. pyogenes*), or both. Impetigo manifests in either bullous or non-bullous forms.[3,4] In tropical areas, the non-bullous form accounts for 80% of cases, with *S. aureus* and *S. pyogenes* being the main causative agents.[4,5] In Indonesia, there were approximately 2,759 cases of this disorder in 2017, primarily affecting children aged 2-4 years.[6]

Common treatments for impetigo include systemic antibiotics (penicillin and other beta-lactam antibiotics), topical antibiotics (mupirocin, fusidic acid), and topical antiseptics. However, the use of antibiotics to treat pyoderma infections has become a serious issue due to the increasing antibiotic resistance.[8,9] Bacterial resistance to antibiotics is linked to the evolution of bacteria and their ability to form biofilms, which are produced by *S. aureus* and *S. pyogenes*.[9,10] Biofilms are structured bacterial colonies encased in a polymer matrix that adhere to the host tissue surface. Biofilms help pathogens neutralize the effects of antibiotics, as antibiotics fail to penetrate the biofilm, and evade the host immune system.[11,12]

Phytochemical-based antibiotics have been widely studied because of their low toxicity and environmentally friendly nature. Phytochemicals have antimicrobial and antibiofilm activity, and they affect both monospecies and polymicrobial biofilm formation. Compounds that function as antibiofilms include phenolics, flavonoids, alkaloids, tannins, saponins, and steroids.[13,15] In vitro, plant extract administration through a tube-test has been reported to produce antibiofilm effects formed by microbes.[16,17] The tube-test can observe antibiofilm effects both qualitatively and quantitatively.[18,19]

One medicinal plant from the family *Menispermaceae* that has been utilized by communities in the Kalimantan islands of Indonesia is yellow root or *Fibraurea tinctoria* Lour. (*F. tinctoria*). The root of this plant is known for its hepatoprotective properties and is also used to treat headaches, diarrhea, diabetes, dysentery, malaria, boost stamina, and as an antioxidant.[20,21] The bioactive compounds in the root extract of this plant include alkaloids, flavonoids, tannins, saponins, and steroids/triterpenoids.[22] The alkaloid group in *F. tinctoria* root consists of isoquinoline compounds, with 25.8% being berberine.[23] Berberine has a wide range of pharmacological and biological activities, such as anti-diarrheal, antibacterial, antiviral, anticancer, and anti-inflammatory effects.[20,24] Berberine shows broad-spectrum effectiveness against microorganisms and has the potential to address synthetic antibiotic resistance issues.[25] Vuddanda et al. (2010) reported that berberine's activity against Gram-positive bacteria is stronger compared to Gram-negative bacteria.[26] Berberine is effective against *S. aureus* as an anti-MRSA.[27] Molecular studies have proven the antibacterial effectiveness of berberine against *S. pyogenes*.[28] In vitro studies have reported the antibacterial activity of ethanol

and infusion extracts of *F. tinctoria* against *S. aureus* and *S. pyogenes*.[22,25,29]

We conducted phytochemical screening as a preliminary test on ethanol extract of *F. tinctoria* and identified the presence of alkaloids, phenolics, flavonoids, tannins, saponins, and terpenoids. The presence of these antibacterial compounds suggests that *F. tinctoria* extract may also have antibiofilm properties. Based on this, the aim of this study is to evaluate the ability of *F. tinctoria* root extract as an antibiofilm agent against the bacteria that cause impetigo in vitro, specifically against *S. aureus* and *S. pyogenes*.

## Research Method

This true experimental study used a posttest-only with control group design. The test method used was the tube test. The treatments tested were *F. tinctoria* root extract with 0.5% ampicillin and 10% DMSO as controls. The observed parameters were the minimum biofilm inhibitory concentration (MBIC) and the Mean Gray Value of the biofilm formed by the bacteria.[17,18,19]

### Preparation of Bacterial Inoculum

Bacterial isolates of *S. aureus* ATCC 25923 and *S. pyogenes* ATCC 19615 were obtained from the collection of the Microbiology Laboratory, Faculty of Medicine, Universitas Lambung Mangkurat (ULM). Each bacterial isolate was used to prepare an inoculum by placing it in TSB medium with 10% glycerol (5 ml) and incubating it at 37°C for 24 hours. The inoculum was then homogenized to achieve turbidity corresponding to the MacFarland 0.5 standard.

### Preparation of *Fibraurea tinctoria* Extract

The yellow root plant was collected from the Tamiang Layang area, Central Kalimantan. The results of taxonomic identification in the Biology Laboratory, Faculty of Mathematics and Natural Sciences, ULM, confirmed it as the *Fibraurea tinctoria* Lour species. The extraction process was carried out in the Pharmacology Laboratory of FKIK ULM. The yellow root was extracted using 70% ethanol with the following steps: 1.5 kilograms (kg) of dried yellow root were ground. Four hundred grams of the powder were weighed and macerated in ethanol for 3 days, with three repetitions. The macerate was then evaporated using a rotary evaporator at 60°C. The remaining ethanol was removed by placing the residue in a desiccator for 24 hours. The extract was diluted with 10% DMSO to make concentrations of 50%, 25%, 12.5%, 6.25%, and 3.125%. Each extract was prepared in a volume of 10 milliliters (ml).

### Tube-test and MBIC Observation

The tube-test is a qualitative method for determining the Minimum Biofilm Inhibitory Concentration (MBIC), marked by the thinning of the purple-blue ring and layer on the walls/bottom of the tube. The test was carried out by introducing the inoculum of each bacterium into tubes containing TSB medium with 10% glycerol. Tubes 1-6 were prepared with microbial suspensions in 2 ml of TSB + 10% glycerol, with 4 ml added to 2 control tubes containing TSB + 10% glycerol, and 2 more control tubes containing untreated bacterial suspensions. A total of 2 ml of yellow root extract (3.125%, 6.25%, 12.5%, 25%, and 50%) was mixed into tubes 1-6, resulting in 4 ml of solution per tube. The control tubes contained 0.5% ampicillin, 10% DMSO, and TSB + 10% glycerol without treatment. All tubes were incubated at 37°C for 24 hours.[31] The test was repeated three times.

The anti-biofilm assessment was based on the clarity of the tubes using the following scoring categories: 0 = Clear, no biofilm formation, +1 = Slightly clear, beginning of weak biofilm formation inhibition, +2 = Slightly cloudy, moderate biofilm formation inhibition, +3 = Cloudy, strong biofilm formation, +4 = Very cloudy, very strong and abundant biofilm formation.[32]

### Congo Red Agar test and MBIC Confirmation

This test is to confirm biofilm-producing bacteria. The Congo Red Agar (CRA) medium was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes. The Congo Red Agar plates were inoculated with the test bacteria and incubated aerobically (37°C, 24 hours). Positive results for biofilm-forming microbes are indicated by black colonies, while non-biofilm producers show red colonies. Each bacterial colony on the CRA medium before extract treatment appeared black. MBIC confirmation on the CRA medium was done by pouring CRA medium (55°C) into a plate, adding the bacterial suspension and test extract (+1), and incubating the plate (37°C, 24 hours). Microbial colonies with minimal or no growth on the plate are considered as MBIC.[33]

### Mean Gray Value Measurement

Quantitatively, biofilm intensity can be observed through the Adobe Photoshop CS6 program by measuring the Mean Gray Value (MGV) from the digital biofilm photo.[17] Bacterial biofilm formation from the tube-test is indicated by the presence of a biofilm adhesion ring on the walls and bottom of the tube. MGV measurement begins by opening the Adobe Photoshop CS5 application, selecting a file, and importing the photo. Next, select the Window tab and choose Measurement Log, then block the area to measure color intensity using the Rectangular Marquee Tool. Click Record Measurements, and the MGV will be obtained on a scale of 0–255. A lower MGV value indicates thicker biofilm formation, while a higher MGV value suggests thinner biofilm formation or that biofilm formation has been inhibited by the anti-biofilm agent.[34]

### Analysis Data

The quantitative data, MGV was normally distributed and homogeneous. Data analysis to determine the difference in effects between treatments was tested using a One-way ANOVA and a Post-hoc Duncan test at a 95% confidence level.[35]

## Results

Visual observations from the tube-test are shown in Table 1. The biofilm formation of S. aureus and S. pyogenes bacteria can be inhibited by the administration of F. tinctoria extract at a MBIC of 12.5%.

Increased extract concentration was able to inhibit bacterial biofilm formation more effectively, resulting in a clearer suspension in the tube-test. The administration of ampicillin and 6.25% extract had less effect on biofilm inhibition, while DMSO and TSB+glu showed no antibiofilm effect.

Table 1 Tube-test, effect of *F. tinctoria extract* on the formation of test bacterial biofilm

| Treatment | *Biofilm on* tube-test | |
|---|---|---|
| | *S.aureus* | *S.pyogenes* |
| Yellow root extract 3.25% | ++ | ++ |
| Yellow root extract 6.25% | + | + |
| Yellow root extract 12.5% | - | - |
| Yellow root extract 50% | - | - |
| Ampicillin 0.2% | + | + |
| DMSO 10% | ++++ | ++++ |
| TSB+glu | ++++ | ++++ |

Description: - = Clear, no biofilm formation, + = Slightly clear, beginning of weak biofilm formation inhibition, ++ = Slightly cloudy, moderate biofilm formation inhibition, +++ = Cloudy, strong biofilm formation, ++++ = Very cloudy, very strong and abundant biofilm formation.

Both bacteria are biofilm producers; bacterial colonies on CRA appeared black before the extract administration and turned red without bacterial growth after the extract

was applied (Figure 1). This highlights that *F. tinctoria* extract has an antibiofilm effect.[33]

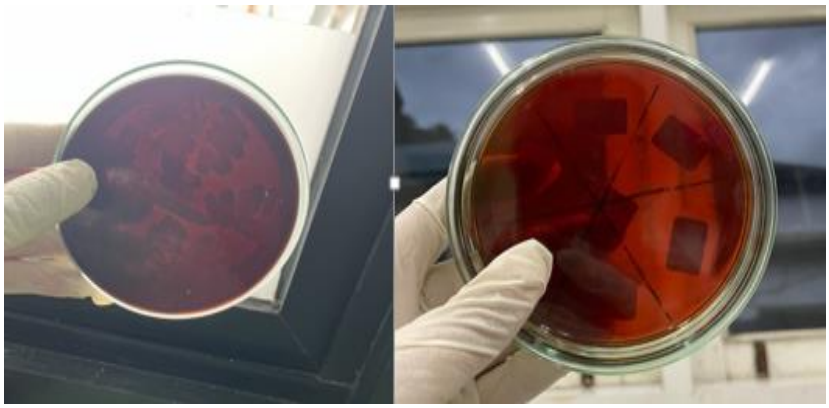
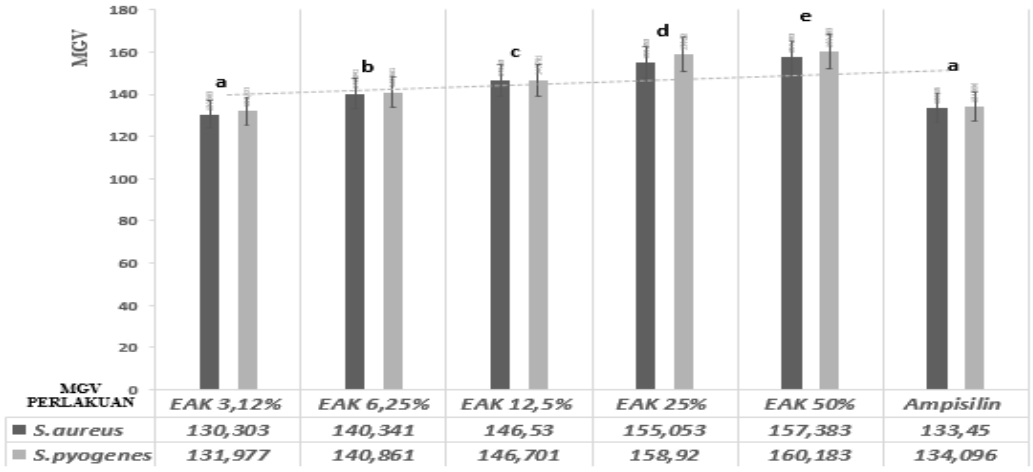


**Figure 1** Bacterial biofilm colonies before and after administration of *F. tinctoria extract*

Quantitative observations were made by assessing biofilm intensity based on MGV. The average MGV of *S. aureus* and S*. pyogenes* biofilms with extract treatment and control is shown in Figure 2. Statistical analysis showed that the administration of *F. tinctoria* extract significantly affected biofilm intensity ($p<0.05$). The antibiofilm effect of the extract was significantly different and influenced by the extract concentration ($p<0.05$). Increasing the concentration enhanced the antibiofilm intensity in the tube-test and raised the MGV.[34]




| MGV PERLAKUAN | EAK 3,12% | EAK 6,25% | EAK 12,5% | EAK 25% | EAK 50% | Ampisilin |
|---|---|---|---|---|---|---|
| S.aureus | 130,303 | 140,341 | 146,53 | 155,053 | 157,383 | 133,45 |
| S.pyogenes | 131,977 | 140,861 | 146,701 | 158,92 | 160,183 | 134,096 |

Description: EAK : yellow root extract;
*) notification of letters a,b,c,d,e showing the difference in MGV in each treatment

**Figure 2** Average MGV of *S.aureus* and *S.pyogenes* biofilms on *F.tinctoria extract treatment*

## Discussion

Non-bullous impetigo causes multiple lesions in children, often relapsing and showing a poor response to antibiotics.[36] The bacteria *S. aureus* and *S. pyogenes*, as the causative agents of impetigo, adapt well to their human host, as they are equipped with virulence factors, including biofilm formation. This protects the bacteria from the host's systemic defense and antibiotic action.[11,12] Biofilm formation is generally divided into four stages: adhesion, aggregation, maturation, and dispersion. During the adhesion stage, planktonic cells of *S. aureus* and *S. pyogenes* use various extracellular matrix-binding factors and serum proteins

through MSCRAMMs, which are microbial surface components recognizing adhesive matrix molecules.[37] MSCRAMMs mediate bacterial adhesion to natural tissues and biomaterial surfaces. Once attached to the surface, the bacterial cells begin to divide and accumulate with sufficient nutrient sources. The bacteria continue to proliferate and thicken, forming a biofilm. During the maturation stage, the biofilm structure becomes more complex and is embedded in a slime layer. In the dispersion stage, planktonic cells are released, contributing to biofilm spread.[38,39]

In this study, the exposure of *F. tinctoria* extract at 12.5% as MBIC was demonstrated by the clear tube-test, and no growth of both bacterial strains was found on the CRA medium. The MBIC value indicates that biofilm biomass metabolism was disrupted by extract exposure. In MGV observations, biofilm intensity increased with extract exposure above the MBIC concentration. This suggests that the plant extract may interfere with biofilm formation of both bacterial isolates at various stages of biofilm destruction.[40] The application of plant extract as a source of antibiofilm agents against both bacterial isolates has been reported. The antibiofilm activity of specific plant parts may be due to the presence of polyphenols, flavonoids, alkaloids, tannins, steroids, and certain saponins.[13,15] The antibacterial activity of the phytochemical content in *F. tinctoria* decoction has a strong effect on both bacterial isolates.[29] We have previously evaluated the content of *F. tinctoria* ethanol extract and identified the presence of alkaloids, phenolics, flavonoids, tannins, saponins, and terpenoids."

Generally, antibiofilm activity on bacterial biofilms is thought to occur by suppressing the expression of genes encoding proteins and enzymes used for biofilm formation.[13,15] Alkaloid compounds work by reducing the expression of biofilm-forming initiation genes, inhibiting quorum-sensing, and decreasing biofilm-regulating factors.[41] Berberine, an alkaloid compound in F. tinctoria, acts as a DNA ligand causing DNA damage and also disrupts cell membranes.[41,42] Phenolic compounds in bacterial cells play a role in disrupting metabolism.[43] Phenolic compounds act as antibiofilm agents by inhibiting motility that precedes biofilm formation and directly inhibiting the biofilm itself.[44] Flavonoids work by inhibiting biofilm formation through interference with the anchoring process of biofilm-associated proteins (BAP) and the polymerization of BAP.[43,45] Saponins work by binding to the active sites of mannitol dehydrogenase enzymes and extracellular DNA (eDNA), which is a key component of the extracellular polysaccharide matrix.46 Terpenoids have anti-cell-adhesion properties. Tannins work by reducing slime biofilm production, decreasing the expression of genes icaA and icaD, thereby inhibiting the quorum sensing RNAIII regulator.[47]

Based on this study, the use of F. tinctoria root herbal remedies by the community can be scientifically validated as having antibiofilm potential. However, further research is needed to develop it as a phytopharmaceutical to ensure its safety and efficacy for human use."

## Conclusions

The results from in vitro testing indicate that *F. tinctoria* root exhibits antibiofilm activity against both *S. aureus* and S. *pyogenes* bacterial strains. *F. tinctoria* root has the potential to be developed into a treatment for chronic wounds. The plant extract consists of numerous components, and it is expected that its mechanism of action involves multiple targets within bacterial cells, which warrants further investigation.

Additionally, identifying non-toxic concentrations for normal human cell lines is

important for developing natural health products or antibiofilm therapies in the future.

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