**BIOINFORMATICS AND BIOMEDICAL RESEARCH JOURNAL**
2022, Vol. 5, No. 1, 6 - 12
http://dx.doi.org/10.11594/bbrj.05.01.02

**Research Article**

# In Vitro Test of Combination of Leaf Extracts and Bark of *Xylocarpus granatum* as Antiseptic Candidates

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*Article history*:
Submission April 2022
Revised May 2022
Accepted June 2022

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**ABSTRACT**
Antiseptics can be made from plant extracts that are antibacterial. Parts of leaves and bark *Xylocarpus granatum* contains antibacterial compounds such as *saponins, flavonoids, tannins, phenols, and alkaloid*, so that it can be developed as a natural antiseptic candidate. This experimental study aims to analyze the activity of a combination of leaf and bark extracts *X. granatum* against several test bacteria in vitro. The plan uses *a post-test only with a control group design* with a combination treatment of leaf ethanol extract (L) and bark (B) X. granatum 25%, 50%, 75%, and 100% (1:1 ratio) and 70% alcohol control. The dilution method was used to observe the activity of the extract with antiseptic control on the number of test bacterial colonies. Comparison of the effect as an antiseptic was analyzed using Duncan's ANOVA and post-hoc tests with a confidence level of 95%. L+B combination extract *X. granatum* produces significantly different inhibitory effects on the colonization of test bacteria; number of colonies *S.aureus* and *S.epidermidis* relatively less than *E.coli, P.aeruginosa, S.typhi*. L+B Extract *X. granatum* 100% produces an effect equivalent to 70% alcohol on all test bacteria, thus acting as an antiseptic candidate. in conclusion, a combination of leaf and stem bark extracts *X. granatum* has activity as an antiseptic against some test bacteria.

**Keywords:** *antiseptic, number of bacterial colonies, combination of extracts, Xylocarpus granatum.*

## Introduction

Unhygienic behavior, especially in terms of the habit of washing hands can reduce a person's immune system and transmit pathogenic microbes. The use of river water or water that has been polluted for washing hands is also a trigger factor for the colonization of pathogenic bacteria on the hands. The results of the identification of the hand swab samples and feces of the people around the riverbanks of the City of Banjarmasin found that the types of bacteria are often pathogenic like *Staphylococcus aureus (S. aureus), Streptococcus epidermidis* (*S.epidermidis*) and *Escherichia coli (E.coli)* on hand swab samples as well bacteria *E. coli* and *Salmonella typhii* (*S. typhii*) in stool samples.[1],[2],[3]

Washing hands using antiseptic and clean water can inhibit the growth of bacteria on the hands, thus preventing the transmission of bacteria between people and through objects or equipment. Microbial colonization on the surface or in the skin tissue can be effectively reduced or eradicated with antiseptics.[4] Antimicrobial content is antiseptic to prevent infectious agents from spreading from person to person.[3] Antiseptic content, generally alcohol which is bactericidal, works by

*How to cite:*
Budiarti, L. Y., Heriyani, F & Nuriyah, S. (2022) In Vitro Test of Combination of Leaf Extracts and Bark of *Xylocarpus Granatum* as Antiseptic Candidates. *Bioinformatics and Biomedical Research Journal 5*(1): 6 - 12. doi: 10.11594/bbrj.05.01.02

denaturing cell proteins and breaking down bacterial cells.[5] Antiseptics have the ability to either kill or prevent the growth of microorganisms that live on the body's surface, particularly on the skin of the hands; However, prolonged use can irritate the hands' skin. Natural antiseptics have undergone a number of innovations to reduce the negative effects of synthetic medicinal ingredients and to be somewhat safer for the skin.[6]

*Xylocarpus granatum* or known to the public as 'nyirih mangrove', empirically used to treat fever and as a medicine for wounds, as well as disorders of the skin. [7] Pharmacologically *X. granatum* can be used to treat asthma, diabetes, rheumatism, hepatitis, skin diseases, as an antidote to snake venom, leukemia, cancer, eye disease, tumors, cholera, malaria, dysentery, fever, analgesic, antiseptic, and as an antibiotic.[7],[8] This plant contains various bioactive compounds in the form of flavonoids,[9],[10] saponin,[10] tannin,[9],[10] alkaloid,[7] and phenol.[10],[11] Secondary compound content is mostly found in the leaves and bark.[12],[13]

Extract *X. granatum* on a single preparation, reported to have effects on some fish pathogenic bacteria such as *P.aeruginosa, E.coli, V.alginolyticus,* and *S.epidermidis*[13],[14] as well as opportunistic pathogenic bacteria in humans, namely *S.aureus* and *E.coli*.[12] The antibacterial effect produced by *X. granatum* extract can be developed as a natural antiseptic candidate. The origin of the plant's habitat and the type of test bacteria influence antibacterial activity.[15] Combination preparations, intended to enhance the effect of a drug, can also be applied to herbs. The combination effect is good, because it has the same ingredients and mechanism of action, resulting in a synergistic effect.[16]

This research informs the pharmacological effects *X. granatum* from Bird Island, South Kalimantan. This study aims to analyze the antibacterial activity of a combination of leaf and bark extracts *X. granatum* as an alternative antiseptic preparation. The test method uses the dilution method, which is treated on several laboratory bacterial isolates.

## Methods

This experimental study used a posttest only with a control group design, this was approved by the ethical commission of the Faculty of Medicine, University of Lambung Mangkurat (ULM); letter number: 205/KEPK-FK ULM/EC/VII/2022 and 234/KEPK-FK ULM/EC/VIII/2022. The research was conducted at the Pharmacology and Microbiology Laboratory, Faculty of Medicine, University of Lambung Mangkurat Banjarbaru, from August to November 2021.

### Research Procedures

### Collection of plant material

The test plant has been identified as a type of *X.granatum* by the ULM Faculty of Mathematics and Natural Sciences Biology Laboratory. Leaves and bark of *Xylocarpus granatum* are a collection of mangrove forests on Bird Island, Batu Licin, South Kalimantan, which were acquired in June 2022.

### Sample preparation of plant extracts

The obtained bark and leaves were washed with tap water to remove visible dust and then were shade dried at room temperature. Then crushed to a fine powder using a mortar and pestle and stored in the refrigerator (4$^{o}$ C) for further use. 100g of leaves and bark samples were taken and extracted individually with ethanol. Samples were kept for 72 hours at room temperature and it's were stirred with a glass rod after every 2 hours. After 3 days, the mixtures were filtered using the whatman's filter papers no.1. The extracts obtained were dried and concentrated in rotary vacuum evaporator and concentrated extracts were stored in refrigerator (4$^{o}$C).[12] The filtrate was evaporated at 40 °C using a rotary evaporator.[17] Then extract solutions were made with concentrations of 12.5%, 25%, 50% and 100% (w/v) and combined *X.granatum* extract preparations from leaves and bark were made with a ratio of 1:1. For positive control, 70% alcohol and 1% DMSO were used as negative controls.

### Test bacteria preparation

Bacterial isolates tested in this study were *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Salmonella typhii* ATCC 19430. These bacteria are a collection of the Microbiology Laboratory of the Faculty of Medicine, ULM. In order to multiply and homogenize the suspension of the test bacteria culture, each bacterial isolate was taken up to one ose and placed in a tube containing BHI media. In the suspension of the test bacteria that has been stored in the incubator for 8 hours (37°C), sterile distilled water is

added and homogenized so that the suspension of each test bacteria is equivalent to the turbidity of the Mc Farland solution 0.5 or the number of test bacteria is equivalent to 1.5 x 108 CFU/ml. [18],[19]

**Bacterial activity test**

The method of testing the antibacterial activity against bacterial culture uses standard culture methods. The extracts were combined by homogenizing 0.5 ml each of stem bark extract and 0.5 ml each of *X. granatum* leaves in the same test tube. In a sterile petri dish, prepare 1 ml of each test bacterial culture. Warm Nutrient Agar media at 46$^{o}$ C was added to the culture of the test bacteria in a petri dish. The media was immediately poured into a petri dish that already had the test bacteria suspended in it. The NA is slowly shaken in the petri dish and freezes for 24 hours in an incubator at 37$^{o}$C. On NA media, the results of the incubation will show bacterial growth colonies. Utilize a colony counter to count the number of living bacterial colonies.[20]

**Statistic Analysis**

The distribution of the research data from the three repetitions was normal and uniform. At a confidence level of 95 percent, the One-way Anova test and the Duncan test were used to analyze the data. [20]

**Result and Discussion**

The results of the combined treatment of *X.granatum* leaf (L) and bark (B) extracts and control of all test bacteria are shown in Figure 1 and Table 1. This study proves the presence of antibacterial activity from the combination of *X.granatum* extracts in inhibiting bacterial colonization.

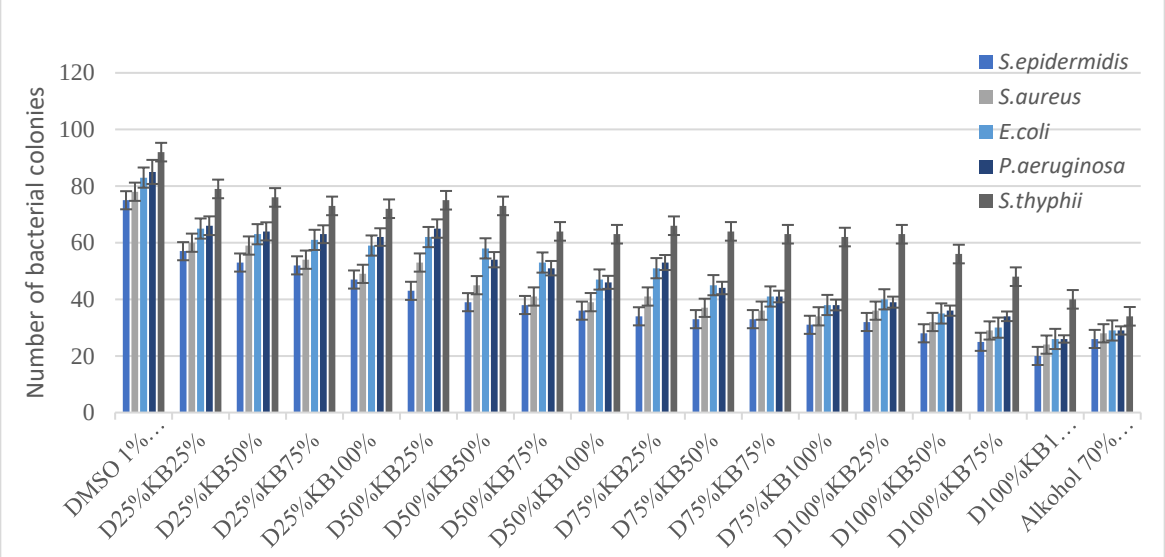


Figure 1. Number of Test Bacterial Colonies in Combination Treatment of *X.granatum* Leaf Extract (L) and Bark (B) and Control

Increasing the concentration of the extract appeared to have an effect on inhibiting the colonization of the test bacteria, so that the number of bacterial colonies decreased (Figure 1). The effect of 70% alcohol treatment and the combination of L+B *X.granatum* 100% (1:1) produced the best inhibitory effect than the lower L+B concentration treatment. In tested extract preparations, increasing the concentration can increase the solubility and stability of antibacterial secondary compounds, thereby increasing their antibacterial efficacy.[4] The 70% alcohol treatment resulted in effective inhibition so that the average number of bacteria growing on nutrient media was the least. 70% alcohol has been shown to inhibit and kill bacteria. The bactericidal properties of alcohol work by damaging cell membranes and denaturing bacterial cell proteins.[5] This means that extracts that have antibacterial activity such as alcohol can be used as candidates for natural antiseptics.

The research data is normally distributed and homogeneous (p>0.05). Based on the analysis of One-way Anova and Duncan's Post Hoc tests, a significantly different effect was obtained for each treatment tested in inhibiting bacteria (p<0.05). The average number of bacteria in the treatment of leaf and bark extracts *X. granatum* to the test bacteria is shown in Table 1. In the treatment of leaf and bark extracts *X.granatum* 70% and alcohol produced an effect on the number of gram-positive bacteria colonies *(S.aureus, S.epidermidis*) less than gram negative bacteria ((*E. coli, P.aeruginosa* and *S. thyphii*).

Table 1. Comparison of the Number of Test Bacteria Colonies at Treatment of Several Concentrations of the Combination of Leaf (L) and Bark (B) Extract *X.granatum* Based on Duncan's BNT Test

| Treatment | Rerata Jumlah ±SD Koloni Bakteri (CFU/ml) | | | | |
|---|---|---|---|---|---|
| | ***S.aureus*** | ***S.epidermidis*** | ***E.coli*** | ***S.typhi*** | ***P.aeroginosa*** |
| DMSO 1%* | **78,33±2,08$^{k}$** | **75,00±2,00$^{l}$** | **82,67±4,93$^{i}$** | **92,00±5,29$^{h}$** | **85,00±5,00$^{j}$** |
| L25% +B25%* | **60,33±1,53$^{j}$** | **57,00±2,65$^{k}$** | **65,00±2,00$^{h}$** | **78,67±5,69$^{g}$** | **66,33±3,05$^{i}$** |
| L25% + B50%* | **59,00±2,65$^{j}$** | **53,00±1,00$^{j}$** | **62,67±2,08$^{gh}$** | **75,67±9,50$^{fg}$** | **64,00±1,00$^{hi}$** |
| L25% + B75%* | **53,67±2.08$^{i}$** | **52,00±1,00$^{j}$** | **61,33±1,53$^{fgh}$** | **73,00±1,00$^{fg}$** | **63,00±1,00$^{hi}$** |
| L25% + B100%* | **48,67±1,58$^{h}$** | **47,00±2,00$^{i}$** | **59,00±2,65$^{fg}$** | **71,67±3,05$^{ef}$** | **61,67±1,58$^{h}$** |
| L50% + B25%* | **53,00±2,65$^{i}$** | **43,00±2,65$^{h}$** | **62,00±2,00$^{fgh}$** | **75,00±2,00$^{fg}$** | **65,00±2,00$^{hi}$** |
| L50% + B50%* | **45,00±2,00$^{g}$** | **39,00±1,00$^{g}$** | **58,00±2,00$^{f}$** | **73,00±3,00$^{fg}$** | **54,00±1,00$^{g}$** |
| L50% + B75%* | **41,00±1,00$^{f}$** | **38,00±1,00$^{fg}$** | **53,00±2,65$^{e}$** | **64,33±4,04$^{d}$** | **51,00±1,00$^{g}$** |
| L50% + B100%* | **38,67±1,53$^{ef}$** | **36,00±1,00$^{ef}$** | **47,00±2,00$^{d}$** | **63,00±2,65$^{d}$** | **46,00±2,00$^{f}$** |
| L75% + B25%* | **41,33±1,58$^{f}$** | **34,00±1,00$^{de}$** | **51,00±1,00$^{e}$** | **66,00±2,65$^{de}$** | **53,00±1,00$^{f}$** |
| L75% + B50%* | **36,67±1,58$^{de}$** | **33,00±1,00$^{de}$** | **45,00±2,00$^{d}$** | **64,00±1,00$^{d}$** | **44,00±2,65$^{ef}$** |
| L75% + B75%* | **36,00±1,00$^{de}$** | **33,33±1,53$^{de}$** | **41,00±2,65$^{c}$** | **63,00±2,65$^{d}$** | **41,00±1,00$^{de}$** |
| L75% + B100%* | **34,00±1,00$^{cd}$** | **31,00±1,00$^{d}$** | **37,67±2,52$^{bc}$** | **62,00±1,00$^{d}$** | **38,00±1,00$^{cd}$** |
| L100% + B25%* | **36,33±1,58$^{de}$** | **32,00±2,00$^{d}$** | **39,67±1,58$^{c}$** | **63,00±2,65$^{d}$** | **39,00±1,00$^{cd}$** |
| L100% + B50%* | **32,33±2,52$^{c}$** | **28,00±1,00$^{c}$** | **35,33±2,08$^{b}$** | **55,67±4,04$^{c}$** | **36,00±2,00$^{bc}$** |
| L100% + B75%* | **28,67±1,53$^{b}$** | **25,00±1,00$^{b}$** | **29,67±2,52$^{a}$** | **47,67±2,52$^{b}$** | **34,00±1,00$^{b}$** |
| L100% + B100%* | **24,00±1,00$^{a}$** | **20,00±1,00$^{a}$** | **26,00±2,00$^{a}$** | **39,67±1,53$^{a}$** | **25,67±1,58$^{a}$** |
| Alcohol 70%* | **28,00±1,00$^{b}$** | **26,00±3,60$^{bc}$** | **28,67±1,53$^{a}$** | **34,00±2,65$^{a}$** | **28,67±1,58$^{a}$** |

L+B combination *X. granatum* at various concentrations produced a better effect on Gram-positive than Gram-negative bacteria (Table 1). The number of bacterial colonies most to the least: *S.typhi* (66,33 -25,67), *P.aeruginosa* (78,67-39,67), *E.coli* (65,00-26,00), *S.aureus* (60,33-24,00), and *S.epidermidis* (57,00-20,00). In general, extract preparations produce more effective antibacterial activity against gram-positive bacteria than gram-negative bacteria, due to differences in the properties and components of the bacterial cell membrane.[21] gram-negative bacteria (*E. coli, P.aeruginosa, S.thyphi*) have a more complex cell wall structure than gram-positive bacteria

(*S aureus and S.epidermidis*).[22] The components of the cell wall and outer membrane of gram-negative bacteria are lipoproteins, lipopolysaccharides, and a small amount of peptidoglycan layer. High lipid content and non-polar, making it difficult for antibacterial compounds to penetrate. The structure of the cell wall of gram-positive bacteria consists of peptidoglycan and teichoic acid. The components that make up the gram-positive cell wall are polar and more easily penetrated by antibacterial compounds which are also polar.[23]

The inhibitory effect is due to the role of the active compounds contained in the leaves and bark *X. granatum*; the leaf effect was more influential than the bark (Table 1). These results are different from the research by Shaheb et.al, the content of secondary compounds was found in the ethanol extract of the bark, namely alkaloids, terpenoids, flavonoids, tannins, and saponins, whereas saponins were not found in the leaves.[12] These results indicate that the antibacterial activity is influenced by the origin of different plant habitats. In addition to differences in the nature and types of bacteria, the polarity factor of secondary compounds, both polar, semipolar, and nonpolar in different types of extract solvents, can also affect their antibacterial power.[24],[25]

Antibacterial bioactive compounds in the ethanol extract of leaves and bark *X. granatum* as flavonoid, alkaloid, tannin, phenol, and saponin.[12] Flavonoids prevent bacterial growth by inhibiting the enzyme topoisomerase II (DNA Gyrase). This enzyme plays a crucial role in the transcription and replication of bacterial DNA.[26] Alkaloid works as an antibacterial by interfering with the peptidoglycan component in bacterial cells, preventing the formation of the cell wall layer entirely and resulting in cell death.[27] Besides that, alkaloid they also inhibits the formation of protein synthesis so that they can interfere with bacterial metabolism. Compound class alkaloid It can inhibit the growth of both gram-positive and gram-negative bacteria.[28] Work mechanism soil as an antibacterial which has the ability to form complex compounds with proteins through hydrogen bonds, if hydrogen bonds are formed between soil and protein, it is possible that the bacterial cell protein will be denatured, causing the bacterial metabolism to be disrupted.[26] Phenol has a working mechanism with denatures cell proteins bacteria and hinders the function cell membrane (transport substances from cell to cell other) until inhibits acid synthesis nucleate and hinders the growth of bacteria.[29] While the mechanism of action of saponin compounds is by lowering the surface tension which causes an increase permeability or cell leakage. As a result, the essential component of bacteria is lost and can cause bacterial cell death.[30] Treatment with 70% alcohol (K+) also proved effective in reducing the number of bacterial colonies. Alcohol is an antiseptic that is bactericidal and works by damaging the bacterial cell membrane, so that intracellular components will come out.[5]

In the treatment of the combination of leaf extract and bark, *X. granatum* produces a synergistic effect, resulting in inhibition of the growth of the tested bacterial colonies which are less in number or relatively equal to the number of test bacterial colonies with 70% alcohol treatment. The number of test bacterial colonies was less in the extract combination treatment *X. granatum* than the alcohol treatment, namely the combination of 100% extract (1:1) against gram-positive bacteria (*S.aureus and S.epidermidis*). The number of bacterial colonies that were relatively the same between the combined extract treatment with 70% alcohol was 100% leaf extract with 75% bark (1:1) in gram-positive bacteria and E.coli, as well as a combination of extracts 100% (1:1) against gram-negative bacteria (*E.coli, P.aeruginosa, and S.thyphii*).

The results of this study can prove the effectiveness of the combined treatment of leaf and bark extracts *X. granatum* 100%-75% and 100%-100% in inhibiting the growth of test bacterial colonization. Effectiveness of extract combinations *X. granatum* which is higher or equal to the effectiveness of 70% alcohol, allows it to be developed as a natural antiseptic candidate. Combination treatment of leaf and bark extracts *X. granatum* can produce a synergistic effect, this is influenced by the concentration of extracts and secondary compounds that have the same mechanism of action. A combination of leaf and bark extracts *X. granatum* which has an effectiveness equal to and higher than the effectiveness of 70% alcohol, presumably due to the working effects of secondary compounds which are relatively similar to the mechanism of action of alcohol, namely compounds flavonoids, phenols, and tannins. The results of this study allow the combination of extracts *X.granatum* to be further developed and researched as natural antiseptic candidates, through in vivo and organoleptic tests.

## Conclusion

A combination of leaf and bark extracts X. granatum at various concentrations resulted in the activity of inhibiting test bacterial colonization. There is a combination extract treatment X. granatum which produces an effect equivalent to 70% alcohol, so this extract can be developed as a natural antiseptic candidate. In conclusion, a combination of leaf and bark extracts X. granatum has activity as an antiseptic against some test bacteria.

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