

Antibacterial Activity of Mixed Infusion of *Averrhoa bilimbi* L. Fruit and *Cananga odorata* Flowers Against Several Numbers of Bacterial Colonies in Vitro Test

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Abstract:

Antiseptic alcohol 70% is known to be effective in inhibiting the growth of bacterial colonization on the hands, but its use in the long term can cause skin disorders. An alternative antiseptic can be made from a mixture of star fruit (*Averrhoa bilimbi* L.) and flower *Cananga* (*Cananga odorata*) which contains several antibacterial compounds. The purpose of this study was to analyze the antibacterial activity of the infusion mixture of *Averrhoa bilimbi* and *Cananga odorata* flowers in inhibiting the growth of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Salmonella typhi*, and *Shigella sonnei* bacteria. This in vitro research design used a posttest only with control group design with the observation parameter being the number of test bacteria colonies that grew on the observation medium. The treatments tested were a mixture of 50%, 75%, and 100% *Averrhoa bilimbi* infusion and 100% *Cananga odorata*, and 70% alcohol control. The results showed that the infusion mixture of *Averrhoa bilimbi* and *Cananga odorata* had different inhibitory effects on Gram-positive and Gram-negative bacteria; the number of colonies of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli* was not significantly different from the mixed treatment concentration of 100% (1:1) with 70% alcohol treatment. In conclusion, the infusion mixture of *Averrhoa bilimbi* fruit and *Cananga odorata* flower had antibacterial activity against Gram-positive and Gram-negative bacteria, and the best inhibitory effect was obtained in the 100% infusion mixture treatment.

Keywords: Antibacterial; *Averrhoa bilimbi* L.; *Cananga odorata*; Gram-positive bacteria; Gram-negative bacteria

Introduction

People's behavior that is not hygienic can transmit various bacterial infectious diseases due to the use of polluted water, direct contact with hands, or equipment. The types of bacteria that cause infections that often occur in people living on riverbanks include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella sonnei*.¹⁻³ Transmission of infection by bacteria can be prevented by the use of antiseptics. 70% alcohol is an antiseptic that is commonly used in the community. Alcohol is a bactericidal antiseptic, that works by denaturing proteins and damaging cell membranes, thereby inhibiting the performance of enzymes and metabolic processes of bacterial cells.^{4,5} Excessive use of alcohol will have a negative impact in the form of irritation and burning on the skin.⁶ So that to reduce the negative impact of using chemical-based antiseptics, it can be overcome by using natural antiseptics. Natural herbs contain several phytochemical compounds that are antibacterial. Phytochemical compounds can act as a shield against infectious diseases.³⁶ Among the plants that have the potential to be developed as alternative antiseptics and have been studied to contain antibacterial compounds are star fruit *Averrhoa bilimbi* L. (*A. bilimbi*) and flower *Cananga odorata* (*C. odorata*).

Averrhoa bilimbi L. fruit contains various secondary antibacterial compounds such as flavonoid compounds, apigenin, and luteolin compounds as flavonoids, saponins, tannins, alkaloids, and terpenes.⁷⁻¹¹ Secondary compounds found in *A. bilimbi* fruit have activity in inhibiting several pathogenic bacteria so that they can be developed as disinfection preparations.¹¹ A single dosage of *A. bilimbi* fruit extract was found to affect inhibiting the bacteria *S. aureus*, *S. epidermidis*, *S. typhi*, *P. aeruginosa*, *S. typhimurium*, and

V. parahaemolyticus.^{11,12} Giving *A. bilimbi* extract can also inhibit bacterial colonization on the hands.

Cananga odorata flowers contain several antibacterial compounds in the form of flavonoids, tannins, saponins, steroids, phenolic compounds, and essential oil.^{13,14} *C. odorata* flowers can be used as a soap ingredient and hand sanitizer preparations.^{15,16} The test results of the ethanolic extract of *C. odorata* flowers are known to be able to inhibit *S. aureus*, *S. epidermidis*, and *E. coli*.⁸ Pure preparations of *C. odorata* flower essential oil are said to have activity against *S. aureus*, *E. coli*, and *S. enteritidis*.¹⁷ *S. aureus* and *E. coli* bacteria can also be inhibited by treatment with *C. odorata* flower hand sanitizer gel.¹⁶

Several studies have stated that there are differences in antibacterial activity between drugs in single (pure) preparations and mixed preparations. A mixture of herbal preparations of different types can have a better effect if they contain antibacterial compounds that work synergistically and can increase their activity as antibacterial.¹⁸ A mixture of star fruit extract gel with aloe vera can provide a greater inhibitory effect on *S. aureus*.¹⁹ The mixture of *Cananga* flower oil extract with lavender and clary sage has a better effect in inhibiting the bacteria *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa*.²⁰

Based on the description above, the mixture of *A. bilimbi* fruit and *C. odorata* flower has the potential to be developed as an alternative antiseptic preparation. To determine the antibacterial activity of a mixture of *A. bilimbi* fruit and *C. odorata* flowers, an in vitro trial was carried out on several bacterial isolates that could be prevented from spreading by administering an antiseptic. The purpose of this study was to analyze the antibacterial activity of the mixed treatment of *Averrhoa bilimbi* fruit and

Cananga odorata flowers against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Salmonella typhi*, and *Shigella sonnei* bacteria in vitro. In this study, the herbal preparation used was in the form of an infusion, because several compounds can be dissolved both in water solvents, and making infusions is easy to make using simple equipment. so that it can be developed as an alternative antiseptic preparation. This study has passed the ethical test based on the letter statement of approval of the ethics committee of the Faculty of Medicine, University of Lambung Mangkurat Number 801-02/KEPK-FK ULM/EC/IX/2021⁷

Research Method

Materials

The *Averrhoa bilimbi* L. and *Cananga odorata* are obtained around Banjarbaru, South Kalimantan. The plants used have been identified and determined based on a letter from the MIPA Biology Laboratory of Lambung Mangkurat University. The fruit of *A.bilimbi* used in this study is a fresh green color that is not too ripe and soft; While the *C.odorata* flower used is the yellow petal part, selected flowers that are ripe, fresh, and picked in the morning.

Tested bacterial isolates namely *Staphylococcus aureus* ATCC 29523, *Staphylococcus epidermidis* ATCC 35983, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Shigella sonnei* ATCC 25931, and *Salmonella typhi* ATCC 19430 are collections of microbiology laboratories of the Faculty of Medicine ULM Banjarbaru. The positive control test was 70% alcohol.

Tested Bacteria Preparation

Pure isolates of *S.aureus*, *S. epidermidis*, *E. coli*, *S.typhi*, and *S.sonnei* which grew on nutrient agar (NA) at 37°C (24 hours) were used as test bacteria isolates. 1 ose of test bacterial isolates were taken aseptically and put into the nutrient broth (NB) from several samples of pure test isolates of bacterial.

Next, incubation was carried out for 5 hours at 37°C, then homogenized and the turbidity equalized with Mc Farland 0,5 standard solution ($1,5 \times 10^8$ CfU/ml). Each suspension of the test bacteria in the NB media in this tube was prepared as a test bacterial.

Preparations of Infusion of Averrhoa bilimbi L. Fruit and Cananga odorata Flower

Then, the plants were sliced into small pieces and then weighed 100 grams each, and added 100 ml of distilled water to make a liquid preparation in a beaker and a concentration of 100% w/v was obtained. Then put into an infusion pan and heated over a water bath for 15 minutes starting from the temperature reaches 90°C while stirring occasionally. The stew is filtered through filter paper while hot. Subsequently, a series of dilutions were carried out for each infusion of the test *A.bilimbi* fruit with a concentration of 50%, 75%, and 100% (w/v) and 100% *C.odorata* flowers, just placed into a 10 ml test tube, to be prepared as a treatment test sample. Then combine the infusion of *A.bilimbi* fruit and *C.odorata* flowers in a 1:1 ratio.

Antibacterial Activity Test

The Dilution methods of testing the antibacterial activity against bacterial culture use standard culture methods. Each test treatment has been prepared (infusion of the rind of the test plant at various test concentrations, 70% alcohol, and distilled water) and the bacterial test suspension. 0.1 ml of the test bacterial suspension was taken and added to a tube containing 10 ml of the test treatment and homogenized by shaking slowly for about 5 minutes. Next, 0.1 ml of the test bacteria suspension was taken and poured on a petri dish, then a warm NA media was poured in. The petri dish was shaken slowly and evenly, until the NA media solidified, after that each test media was

incubated at 37°C for 24 hours. The number of bacterial colonies growing on NA media was calculated using a colony counter.

Analysis Data

Data from the infusion activity of *A.bilimbi* fruit and *C.odorata* flowers against the number of bacterial colonies tested were analyzed using the ANOVA method with a confidence level of 95% ($\alpha = 0.05$), followed by the Duncan test.

Results

The results of the antibacterial activity test from the mixed treatment of infusion of *A. bilimbi* fruit and *C. odorata* flowers against various Gram-positive and Gram-negative test bacteria are shown in Figure 1 and Table 1. Good antibacterial activity was shown by the growth of colonies of various test bacteria incubation results on nutrient agar (NA) media with the least number of bacterial colonies.

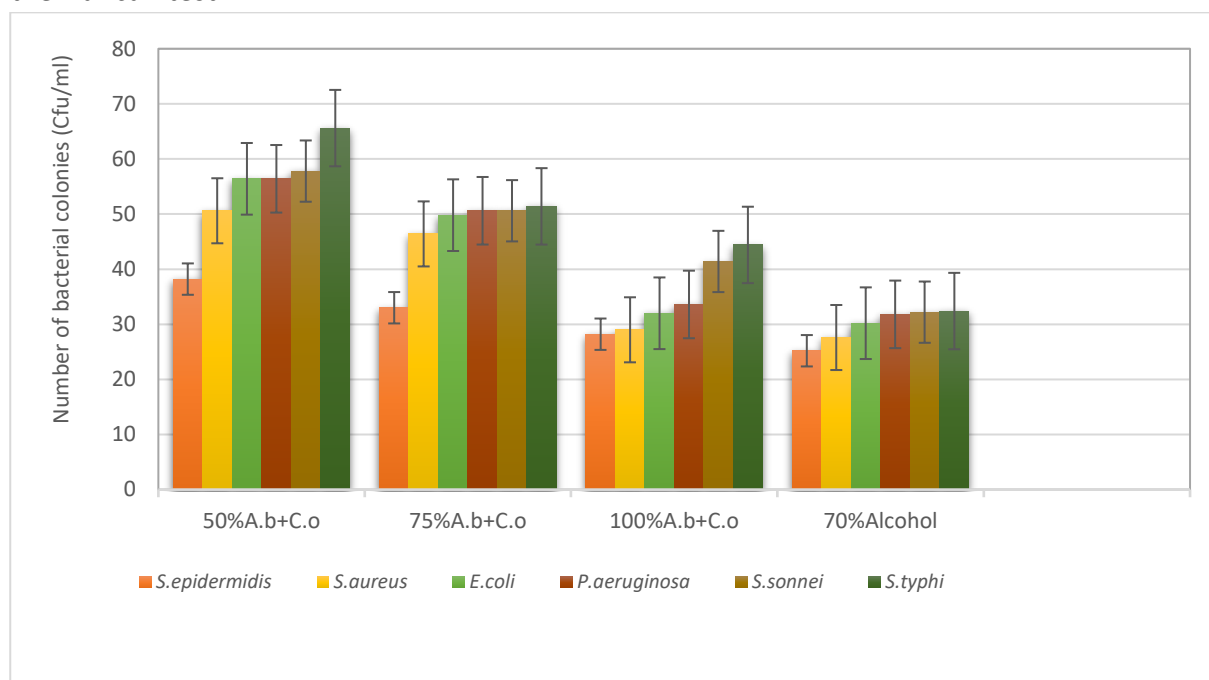


Figure 1. Average Number of Bacterial Colonies Tested on Mixed Treatment of Infusion of *Averrhoa Bilimbi* Fruit (A.b) and *Cananga odorata* Flower (C.o) as well as control

Table 1 Standard Deviation Average Number of Bacterial Colonies Tested on Mixed Treatment of Infusion of *Averrhoa bilimbi* Fruit (A.b) and *Cananga odorata* Flower (C.o)

Treatment	Average Number of Bacterial Colonies (Cfu/ml)					
	<i>S. aureus</i>	<i>S. epidermidis</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. sonnei</i>	<i>S. typhi</i>
Ab.50%+Co.100%	50,6±1,82 ^c	38,2±2,95 ^c	56,4±2,61 ^c	56,4±3,36 ^c	57,8±1,64 ^d	65,6±1,14 ^d
Ab.75%+Co.100%	46,4±1,52 ^b	33±2,24 ^b	49,8±2,17 ^b	50,6±4,93 ^b	50,6±1,82 ^c	51,4±2,61 ^c
Ab.100%+Co.100%	29±2,92 ^a	28,2±2,78 ^a	32±1,58 ^a	33,6±1,74 ^a	41,4±4,16 ^b	44,4±3,51 ^b
Alkohol 70%	27,6±2,51 ^a	25,2±3,70 ^a	30,2±2,17 ^a	31,8±1,48 ^a	32,2±1,48 ^a	32,4±5,23 ^a

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

Figure 1 shows the mixed treatment of infusion of *Averrhoa bilimbi* fruit and *Cananga odorata* flowers at various test concentrations and 70% alcohol control gave different inhibitory effects on the bacteria *S.aureus*,

S.epidermidis, *E.coli*, *P.aeruginosa*, *S.typhi*, and *S.sonnei*. In general, the infusion mixture treatment at a higher test concentration could reduce the number of test bacteria colonies, while 70% alcohol was able to give the effect

with the least number of test bacterial colonies. The smaller number of test bacteria colonies corresponded to an increase in the concentration of the infusion mixture treatment given to several test bacteria. Increasing the concentration allows a lot of antibacterial substances to be extracted and can increase antibacterial activity so that more bacteria are inhibited from growing.^{10,21} 70% alcohol contains 70% ethyl alcohol and 30% water; alcohol is an antiseptic that works by damaging bacterial cell membranes so that intracellular components will come out.^{4,6,22} Differences in the activity of different antibacterial substances in inhibiting growth / Bacterial colonization are influenced by the number of bacteria, the type of bacteria, and the virulence or resistance of the bacteria.^{4,6}

Table 1 shows that the least mean number of bacteria was obtained from the mixed treatment of 100% *A.bilimbi* infusion and 100% *C.odora* (ratio 1:1) on *S.aureus* 29 CFU/ml, *S.epidermidis* 28,2 CFU/ml, *E.coli* 32 CFU/ml, *P.aeruginosa* 33.6 CFU/ml, *S.typhi* 44.4 CFU/ml and *S.sonnei* 41.4 CFU/ml. The mean number of relatively large bacteria was obtained from the mixed treatment of 50% *A. bilimbi* and 100% *C.odora* infusion, the bacteria *S. aureus* 50.6 CFU/ml, *S.epidermidis* 38.2 CFU/ml, *E.coli* 56.4 CFU/ml, *P.aeruginosa* 65.6 CFU/ml, *S.typhi* 57.8 CFU/ml and *S.sonnei* 56.4 CFU/ml. These results show that the infusion mixture of *A.bilimbi* and *C.odorata* has antibacterial activity and its inhibitory effect on various test bacteria. Various secondary metabolites contained in the infusion mixture of *A.bilimbi* and *C.odorata* can interact actively in inhibiting the growth of various test bacteria. The active compounds found in *A. bilimbi* are saponins, flavonoids, and tannins. The content of active compounds in *C.odorata* other than essential oils, namely saponins and flavonoids.^{17,23} Flavonoid compounds, saponins, tannins, and steroids have the ability as antibacterial.^{9,13}

Discussion

In this study, the treatment of the test plant mixture used the infusion method and alcohol control. The solvent of distilled water or distilled water has polar characteristics so that only polar or semi-polar secondary compounds can be dissolved in aqueous solvents. Alcohols that have a hydroxyl group also have polar characteristics. The solubility of a compound in a solvent depends on the group to which the solvent is attached. The type of solvent used in the herbal extraction process is influenced by the type of material extracted, as well as the structure and composition of the bacterial cell membrane.²⁴ Based on their polarity, flavonoids are classified as polar compounds; saponins, and phenolic compounds including polar and semipolar compounds.²⁴ Terpenoids are nonpolar compounds. Polar compounds present in plants can be extracted with semi-polar and polar solvents.²⁵ In the infusion method, the active compounds from plants that are easily dissolved in water are flavonoids, saponins, and tannins.²⁶ The interaction of various active compounds contained in herbal preparations makes it effective as an antibacterial with various mechanisms of action.

Flavonoid compounds contain phenolic compounds that can interfere with bacterial growth. Phenol is acidic alcohol that can denature proteins and damage bacterial cell membranes. Several working mechanisms of flavonoid compounds, namely: (1) forming complex compounds with extracellular proteins that will damage bacterial cell membranes, (2) inhibiting bacterial oxygen, so that inhibiting the biosynthesis of macromolecules in the bacterial energy metabolism process and making them complex compounds, and (3) inhibiting the enzyme topoisomerase II (DNA gyrase) which is an important enzyme in the process of

replication and transcription of bacterial DNA.^{9,27,28}

Saponins include semipolar compounds that can dissolve in lipids and water so that these compounds will be concentrated in the microbial cell membrane. Saponins are active substances whose surfaces are similar to detergents, can reduce the surface tension of bacterial cell walls and damage membrane permeability, thereby interfering with the survival of bacteria. When the surface tension is disturbed, saponins will easily enter cells and interfere with metabolism, causing denaturation of membrane proteins. Saponins have the ability as an antiseptic that functions to prevent the growth of microorganisms or kill them.¹⁸ As an antimicrobial, saponins work by destabilizing and damaging cell membranes so that bacterial cells lyse.²⁹

Tannins have properties as astringents that trigger the complexity of enzymes and substrates in microbial cells, resulting in inhibition of microbial enzyme activity as well as astringent properties that inhibit bacterial permeability to thrive.^{18,29} The activity of tannins is also by damaging the polypeptides of the bacterial cell wall, resulting in the cell wall becoming incomplete and the bacterial cell lysis or death.³⁰

The results of statistical tests (table 1) showed that there was a significant difference in the mean number of colonies of Gram-positive bacteria *S. aureus* and *S. epidermidis* and Gram-negative bacteria *E. coli*, *P. aeruginosa*, *S. typhi*, and *S. sonnei* from each treatment tested. The mixed treatment of *A. bilimbi* infusion below 50% and 75% and *C. odora* 100% gave a significantly different effect with various other treatments in inhibiting each of the tested bacteria, but the treatment of *A. bilimbi* 100% and *C. odora* 100% gave an effect. the inhibition was not significantly different from 70% alcohol in inhibiting *S. epidermidis*, *S. aureus*, *E. coli*, and

P. aeruginosa bacteria. The effect of the mixed infusion treatment on the number of opportunistic normal flora colonies of *S. epidermidis*, *S. aureus*, *E. coli*, and *P. aeruginosa* was also different compared to the number of colonies of pathogenic bacteria *S. sonnei* and *S. typhi*. *S. sonnei* bacteria as the cause of shigellosis/bacillary dysentery and *S. typhi* as the cause of salmonellosis and typhoid fever. The antibacterial effect of the infusion mixture of *A. bilimbi* and *C. odorata* has not been able to optimally inhibit the growth of the two pathogenic test bacteria. This is as discussed above because the activity of an antibacterial substance is influenced by the virulence and resistance of the test bacteria. In opportunistic normal flora bacteria, can produce exotoxins and various virulence factors when there is the influence of predisposing factors that cause the number of bacteria to exceed normal limits or are invasive, so they are easily influenced by antimicrobial substances. Meanwhile, pathogenic bacteria in an optimum environment naturally have endotoxins and produce various extracellular components to defend themselves from various antimicrobial substances.

The results of this study are by previous studies, namely, the treatment of mixed infusion of *A. bilimbi* and *C. odorata* at the highest test concentration (100%) showed better inhibitory activity against several test bacteria and could be equivalent to the inhibitory activity produced by 70% alcohol. Mixtures of herbal infusions that have relatively the same active compounds can produce greater antibacterial power than the single preparations; the test results of the infusion mixture of *Stenochlaena palustris* and *Sauropus androgynous* at the highest test concentration can produce an inhibitory effect equivalent to the positive control treatment against *S. aureus* and *E. coli* bacteria.³¹ The test results of a mixture of gel

extracts of *A.bilimbi* and *Aloe vera* (*A.vera*), resulted in a better inhibitory effect against *S.aureus* bacteria.¹⁹ In the study of Tadtong et.al. (2012), the results of the test of a mixture of *C.odorata* essential oil with lavender and clary sage (ratio 3: 4: 3) gave a better effect on several test bacteria (*S.aureus*, *S.epidermidis*, *E.coli*, and *P.aeruginosa*) compared to the single preparation.²⁰ The compounds linalool and linalyl acetate are components that play a role in the essential oil mixture.¹⁸ Herbal mixtures can increase the synergistic effect of antibacterial, thus providing a better inhibitory effect on the tested bacteria.^{18,32}

The antibacterial activity of the mixed treatment of *A.bilimbi* and *C.odorata* against various test bacteria in this study gave relatively the same results as the previous study, namely getting the effect of its antibacterial power on Gram-positive better than on Gram-negative so that the number of Gram-positive bacteria that grew in the test media is less than the number of Gram-negative bacteria. Bacteriostatic activity is generally more able to inhibit the growth of gram-positive bacteria than gram-negative bacteria. This is because gram-positive bacteria have a simpler composition. cell wall compared to gram-negative bacteria. Several factors that influence the difference in antibacterial activity are resistance properties and differences in the composition of the cell membranes of the two groups of bacteria.³³ Gram-negative bacteria have a higher lipopolysaccharide composition than gram-positive bacteria. Generally, gram-negative bacteria produce mucus as a form of defense against chemicals. harmful compounds. The outer membrane of gram-negative bacteria acts as a barrier for the entry of compounds that are not needed by the cell, such as bacteriocins, enzymes, and their compounds are hydrophobic.³⁷

The polar secondary compounds contained in the infusion mixture of *A.bilimbi* and *C.odorata* gave different effects on different groups of test bacteria. Polar secondary compounds are effective in inhibiting the growth of gram-positive bacteria, penetration of the peptidoglycan layer which is also polar in gram-positive bacteria occurs more easily than non-polar lipid layers. The cell wall structure of gram-positive bacteria has more peptidoglycan which plays a role in maintaining osmotic stability and antiphagocytosis with less lipid and contains polysaccharides (teichoic acid). Teichoic acid is a water-soluble polymer that acts as a positive ion transport to enter or exit. The nature of the solubility in water (polar) indicates that the cell walls of gram-positive bacteria are more easily penetrated by antibacterial compounds.³⁴ Polar compounds that have entered bacterial cells immediately work to destroy bacteria by denaturing proteins that can cause metabolic activity. Bacterial cells stop because all the metabolic activities of bacterial cells are catalyzed by enzymes which are proteins. Cessation of this metabolic activity will result in bacterial cell death.²⁴ The outer cell membrane of Gram-negative bacteria is a bilayer consisting of phospholipids (inner layer) and lipopolysaccharides (outer layer) with a high lipid composition and little peptidoglycan. The outer membrane is composed of lipid A which is nonpolar. This causes antibacterial compounds to be more difficult to penetrate Gram-negative bacterial cells.³⁵

Conclusions

Averrhoa bilimbi fruit and *Cananga odorata* flower contain antibacterial compounds and can be developed into alternative antiseptic preparations. As a result of the mixed treatment of infusion of *A.bilimbi* fruit and *C.odorata* flowers in this study, there was antibacterial activity in inhibiting the

colonization of several test bacteria, namely Gram-positive bacteria (*S.aureus*, *S.epidermidis*) and Gram-negative bacteria (*E.coli*, *S.typhi*, and *S.dysenteriae*). Increased concentration of treatment produces better effects, with the number of bacterial colonies growing on the test medium the least. The best inhibitory effect of the infusion mixture of *A.bilimbi* fruit and *C.odorata* flower on various test bacteria was obtained at a concentration of 100%.

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