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Research Article

Phenol Coefficient Test Combination Infusion of *Cananga odorata* – *Averrhoa bilimbi* L. Against *Staphylococcus aureus* and *Salmonella typhi* in Vitro

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ABSTRACT

Cananga odorata (kenanga) flowers and *Averrhoa bilimbi* L. (belimbing wuluh) fruit are plant parts that containrelatively the same antibacterial compounds, namely *flavonoids, saponins,* and *tannins*. Both of these plants can be developed as an alternative natural antiseptic preparation, which is made in the form of a combination infusion.The effectiveness of an antiseptic preparation is measured by the coefficient value of the phenol antiseptic substance compared to 5% phenol against several standard test bacteria. This study aims to determine the antibacterial activity of the combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit against *Staphylococcus aureus* ATCC 25923 and *Salmonella typhi* ATCC 19430 based on the in vitro phenol coefficienttest. The phenol coefficient test method was carried out conventionally. The coefficient value close to 1 can be said that the antiseptic preparation has good activity and is equivalent to the phenol coefficient value as a comparison. The results showed that the mean coefficient of the phenol coefficient of the combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit was 0.91 against *S. aureus* and 0.83 against *S. typhi*. The conclusion of this study, the antibacterial activity of the combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit against *Staphylococcus aureus* obtained a higher phenol coefficient value than *Salmonella typhi*.

Keywords: *phenol coefficient, combination infusion, Cananga odorata, Averrhoa bilimbi L.,Staphylococcus aureus, Salmonella typhi*.

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Introduction

The use of antiseptics to prevent the transmission of infectious diseases is important for everyone. Theuse of antiseptics is intended to reduce or kill bacterial colonization on the surface or skin tissue. Generally, infection transmission often occurs due to the use of water that has been contaminated with bacteria and direct contact with hands containing normal flora or the presence of bacterialcontaminants on the skin surface. Types of bacteria*Staphylococcus aureus* and *Salmonella typhi* arepathogens that cause infectious diseases whose transmission can be prevented by the use of antiseptics. Several previous studies have utilized and developed biodiversity in Indonesia, as herbal preparations with antibacterial properties (18), (17), (4).

Among the types of plants that have been studiedand have antibacterial properties are *Cananga odorata* (kenanga) and *Averrhoa bilimbi* L. (belimbing wuluh). These two types of plants are widely grown in the territory of Indonesia and have been used as medicinal preparations, both in single and combined dosage forms. *Cananga odorata* flowers and *Averrhoa bilimbi* L. fruit contain relatively the same antibacterial compounds, namely *flavonoids, tannins, and saponins* (6), (13), (8), (10). *Cananga odorata* flowers are often used as aromatherapy and as a combination ingredient in making soap or skin antiseptics. *Averrhoa bilimbi* L.fruit is known to have activity in reducing the number of bacterial colonies on hands (14).

The test results of *Cananga odorata* flower essential oil are known to have antibacterial activityagainst gram-positive and gram-negative bacteria (*Bacillus subtilis, Staphylococcus aureus, Salmonella enteritidis, and Escherichia coli*) (3). Test results of *Cananga odorata* flower essential oilin gel dosage forms hand sanitizer, is known to havean inhibitory effect on *E. coli* and *S. aureus* bacteria(8). The test results of a single preparation of the ethanolic extract of *Averrhoa bilimbi* L. fruit are also known to have an antibacterial effect against gram- positive and gram-negative bacteria (*Staphylococcus aureus, Staphylococcusepidermidis, Bacillus cereus, Kocuria rhizophila,* and *Escherichia coli*) (19). Extract test results ethanol fruit of *Averrhoa bilimbi* L. is known to have an inhibitory effect on *S. aureus* and *S. typhi* (10).

Based on the known benefits of antibacterial compounds, a natural antiseptic preparation made from *Cananga odorata* flowers and *Averrhoa bilimbi* L. fruit was made. These two plants could bedeveloped as a combination antiseptic preparation that is easy to make in a simple way, namely infusion. An antiseptic preparation can be said to have an effective way of working if it has a phenol coefficient value that is close to or equal to 1. The phenol coefficient test is a standard test used to compare an antibacterial substance from antiseptic materials, with 5% phenol as a comparison substance (16). The standard laboratory bacteria thatcan be used in the phenol coefficient test are *Staphylococcus aureus* and *Salmonella typhi* (5).

The aim of this study was to determine the antibacterial activity of the combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit against *Staphylococcus aureus* and *Salmonella typhi*based on in vitro phenol coefficient test. The concentration used was a combination infusion of 100% *Cananga odorata* flower and 100% *Averrhoabilimbi* L. fruit. This research is a laboratory experiment conducted at the Microbiology Laboratory, Faculty of Medicine, University of Lambung Mangkurat in October 2021. The phenol coefficient test method was carried out conventionally, using test bacteria isolates, namely *Staphylococcus aureus* ATCC 25923 and*Salmonella typhi* ATCC 19430. 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 753/KEPK-FK ULM/EC/VIII/2021.

Materials and Methods

Tools and Materials

The equipment used in this study consisted of a test tube (Pyrex Brand®), test tube rack, autoclave (All American®), aerobic incubator (Carbolite®), laminary flow table (Labconco®), Erlenmeyer glass (IWAKI®), sterile knife (stainless), water bath, spatula, filter paper, spirit lamp, sterile ose, measuring pipette, volume pipette, micropipette, infusion pan,

analytical balance, hot plate,stopwatch, aluminum foil, stirring rod, measuring cup, and a beaker.

The main ingredients used in this study were thetest plant *Cananga odorata* flower and *Averrhoa bilimbi* L. fruit as well as samples of pure isolates ofbacterial colonies of *S. aureus* ATCC 25923 and *S. typhi* ATCC 19430 from the Microbiology Laboratory of FK ULM. The chemicals used were Nutrient Broth (NB) media, Brain Heart Infusion (BHI) media, sterile distilled water, 5% phenol solution, and 0.5 *Mc Farland* standard solution (equivalent to 1.5 X 10^8 cfu/mL).

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Preparation of Test Bacteria

Pure isolates of the test bacteria were prepared, then 1 ose was taken and put into the BHI media to make a suspension of the test bacteria cultures. Bacteria were incubated at 37°C for 24 hours. Then,sterile distilled water was added to the bacterial suspension in BHI media so that the turbidity was the same as the standard 0.5 *Mc Farland* solution (equivalent to 1.5 X 10^8 cfu/mL).

Making a Combination Infusion ofCananga odorata Flower - Averrhoa bilimbi L. Fruit

The freshly prepared *Cananga odorata* flowers and *Averrhoa bilimbi* L. fruit were washed thoroughly with running water until they were separated from dirt, then drained. 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 for15 minutes starting from the temperature reaches 90oC while stirring occasionally. The stew is filtered through filter paper while hot. Then combine the infusion of *Cananga odorata* flowers - *Averrhoabilimbi* L. fruit in a 1:1 ratio.

Preparation of 5% Phenol StockSolution

Phenol stock solution was prepared bydissolving 5 grams of phenol crystals with 100 ml ofsterile distilled water, so that a phenol solution witha concentration of 5% was obtained.

Dilution of Standard Phenol SolutionTest

Previously 26 sterile tubes were prepared with labels 1-26. Tubes 1-13 were given 2 ml of 5% phenol solution each. Then sterile distilled water was added from tubes 1-13 in a row, namely 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 13, 18, and 23 ml. Each tube was homogenized. Next, transfer the mixture of 5% phenol solution with distilled water to tubes 14-26 as much as 2 ml each. Obtained a 1:20 dilution of phenol solution; 1:30; 1:40; 1:50; 1:60; 1:70; 1:80; 1:90; 1:100; 1:110; 1:150; 1:200; and 1:250.

Dilution of The Combination Infusion of Cananga odorata Flower - Averrhoa bilimbi L. Fruit.

26 sterile tubes were prepared labeled 1-26.Tubes 1-13 were given a combination of 2 ml of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruitinfusion solution each. Then sterile distilled water was added from tubes 14-26, respectively, namely 0,1, 2, 3, 4, 5, 6, 7, 8, 9, 13, 18, and 23 ml. Each tube was homogenized. Next, transfer the combination solution of *Cananga odorata* flower infusion - *Averrhoa bilimbi* L. fruit that has been added with distilled water to tubes 14-26 each as much as 2 ml. Obtained dilution combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit 1:20; 1:30; 1:40; 1:50; 1:60; 1:70; 1:80; 1:90; 1:100; 1:110; 1:150; 1:200; and 1:250.

Phenol Coefficient Test Combination Infusion of Cananga odorata Flower -Averrhoa bilimbi L. Fruit and 5% Phenol against S. aureus and S. typhi

Previously we prepared a tube rack and sterile test tubes containing NB media that had been labeled according to the dilution (1:20; 1:30; 1:40; 1:50; 1:60; 1:70; 1:80; 1 :90; 1:100; 1:110; 1:150; 1:200; and 1:250) along with contact times (5, 10, and 15 minutes). In addition, also prepare other tools such as a spirit lamp, a micropipette, ose, and a suspension of *S. aureus* and *S. typhi* bacteria. Then pipette 0.5 ml of the test bacterial suspension which is equivalent to 0.5 *Mc Farland* solution into each sterile tube (a combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit and 5% phenol) starting from the tube with a dilution of 1:20to 1:250, then homogenized. After 5

minutes, take 1ose from each dilution tube (a combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit and 5% phenol) into a test tube containing NB on a 5 minute contact time label rack. Next, the ose is sterilized with a spirit lamp. After the second 5 minutes, take 1 ose from each dilution tube (a combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit and 5% phenol) into a testtube containing NB on a 10 minute contact time label rack. Then the ose is sterilized with a spirit lamp. After the third 5 minutes, take 1 ose from eachdilution tube (a combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit and 5% phenol) into a test tube containing NB on a 15minute contact time label rack. Furthermore, all thetubes were incubated at 37°C for 24 hours and then observed for turbidity. The presence of bacterial growth (+) was indicated by the medium becoming cloudy, and the absence of bacterial growth (-) indicated by the medium remaining clear.

Results and Discussion

Based on the results of observations on test tubes from all treatments can be seen in tables 1 and2, which did not show any turbidity (no growth of test bacteria was found), then the phenol coefficientvalue was calculated and tabulated the data which can be seen in table 3.

The formula for the phenol coefficient value can be seen as follows:

$$Phenol\ Coefficient = \frac{\text{Highest dilution of antiseptic that is lethal within 10 minutes but not lethal in 5 minutes}}{\text{The highest dilution of lethal phenol solution in time 10 minutes but not turn off in 5 minutes}}$$

Table 1. Results of Observation of Antibacterial Activity Combination Infusion of Cananga odorata Flower - Averrhoa bilimbi L. Fruit and 5% Phenol against S. aureus

Dilutions	Combination Infusion of 100% *Canage odorata* flower – 100% *Averrhoa bilimbi* L. Fruit									Phenol 5%								
	Contact Time									Contact Time								
	5th mins			10th mins			15th mins			5th mins			10th mins			15th mins		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1:20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:60	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:70	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:90	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:100	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
1:110	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-
1:150	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1:200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1:250	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Information:
[+] = There is growth of *S. aureus* bacteria (cloudy medium)
[-] = No growth of *S. aureus* bacteria (clear medium)
1,2,3 = Deuteronomy 1, 2, and 3

Table 2. Results of Observation of Antibacterial Activity Combination Infusion of Cananga odorata Flower - Averrhoa bilimbi L. Fruit and 5% Phenol against S. typhi

Dilutions	Combination infusion of 100% Cananga odorata flower - 100% Averrhoa bilimbi L. Fruit									Phenol 5%								
	Contact time									Contact time								
	5th mins			10th mins			15th mins			5th mins			10th mins			15th mins		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
1:20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:30	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:50	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:60	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1:70	+	+	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-
1:80	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-
1:90	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1:100	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1:110	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1:150	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1:200	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1:250	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Information:
[+] = There is growth of *S. typhi* bacteria (cloudy medium)
[-] = No growth of *S. typhi* bacteria (clear medium)
1,2,3 = Deuteronomy 1, 2, and 3

Table 3. Mean Phenol Coefficient Value Combination Infusion of Cananga odorata Flowers - Averrhoa bilimbi L. fruit against S. aureus and S. typhi

Treatments	Mean Phenol Coefficient Value	
	S. aureus	*S. typhi*
Combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit	0.91±0,01	0.83±0.07
Phenol 5%	1.00±0,00	1.00±0,00
Negatif Control	0.00±0,00	0.00±0,00

The mean value of the phenol coefficient of the combination infusion of *Cananga odorata* (kenanga) flowers - *Averrhoa bilimbi* L. (belimbingwuluh) fruit against *Staphylococcus aureus* and *Salmonella typhi*, respectively, was 0.91 and 0.83. Based on the data obtained, the phenol coefficient value which is closer to 1 indicates that the type of antiseptic tested against *S. aureus* has better antiseptic power than *S. typhi*.

Phenol is the standard of antiseptic power. The mechanism of action of phenol as a bacteristatic antiseptic works by the interaction between phenoliccompounds and bacterial cells through an absorption process involving hydrogen bonds. At lowconcentrations, phenol will form protein complexeswith weak bonds and immediately undergo decomposition, followed by penetration of phenol into bacterial cells and cause precipitation and protein denaturation. Meanwhile, at high concentrations of phenol will cause coagulase of bacterial cell proteins and cytoplasmic membranes to undergo lysis (12).

The results of previous studies stated that *Cananga odorata* flowers are known to contain

secondary compounds including *flavonoids, tannins, saponins, steroids*, and essential oils (6), (13), (8). While the secondary compounds containedin the fruit of *Averrhoa bilimbi* L. are *flavonoids, alkaloids, tannins, and saponins* (10). The solubility of a compound in a solvent depends on the group attached to the solvent. 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 test bacterial cell membrane.In this study, the method used was infusion using sterile aquadest as the solvent, so that the secondarycompounds that are polar in the two plants such as *flavonoids, saponins, and tannins* will dissolve easily and can work optimally as antibacterial (11).

The mechanism of action of *flavonoids* as antibacterial is by inhibiting cell membrane function, nucleic acid synthesis, and bacterial energy metabolism. When inhibiting cell membranefunction, *flavonoids* form complex compounds with extracellular proteins that can damage bacterial cellmembranes, followed by the release of thesebacterial intracellular compounds. *Flavonoids*inhibit the synthesis of nucleic acids that play a role in the process of interclassification or hydrogen bonding by accumulating bases in nucleic acids thatinhibit the formation of DNA and RNA. This causesdamage to the permeability of the bacterial cell walland lysosomes. *Flavonoids* can inhibit energy metabolism by inhibiting the use of oxygen by bacteria. Energy is needed by bacteria for the biosynthesis of macromolecules, so that if their metabolism is inhibited, these bacterial molecules cannot develop into complex molecules (1), (15).

The mechanism of action of *saponins* as antibacterial is by causing leakage of proteins and enzymes from the bacterial cell. *Saponins* are activesubstances that can increase membrane permeability so that cell hemolysis occurs. When *saponins* interact with bacterial cells, the bacteria will break down or lyse which interferes with the survival of the bacteria (15).

Tannins work as antibacterial by inhibiting the enzyme reverse transcriptase and DNA topoisomerase so that bacterial cells cannot be formed. *Tannins* can inactivate enzymes and interfere with protein transport in the inner layer of cells. *Tannins* also have a target on cell wall polypeptides so that the formation of cell walls is less than perfect. As a result, bacterial cells becomelysed due to osmotic and physical pressure so that bacterial cells will die (2).

The antiseptic effectiveness of the combination infusion of *Cananga odorata* flower - *Averrhoabilimbi* L. fruit against *S. aureus* showed an averagephenol coefficient of 0.91. Determination of thephenol coefficient value with *S. aureus* test bacteriashowed that phenol gave effectiveness against antiseptics at a 1:110 dilution from the 1st and 3rd replications but in the 2nd replication the bacteria that died at 10 minutes occurred at a 1:100 dilution,while the combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit gave effectivenessagainst antiseptics at 1:100 dilutions from the 1st and 3rd replications, but in the 2nd replication the bacteria that died at 10 minutes occurred at 1:90 dilutions. The antiseptic effectiveness of the combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit against *S. typhi* showed anaverage phenol coefficient of 0.83. Determination ofthe phenol coefficient value with the *S. typhi* bacteria test showed that phenol gave effectiveness against antiseptics at a 1:80 dilution from the 1st and 3rd replications but in the 2nd replication the bacteria that died in 10 minutes occurred at a 1:70 dilution, while the combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit gave effectiveness against antiseptic at 1:60 dilution fromthe 2nd and 3rd replications, but in the 1st replication the bacteria that died at 10 minutes occurred at 1:70 dilution.

Based on these studies, it can be seen that the antibacterial activity of the combination infusion of*Cananga odorata* flower - *Averrhoa bilimbi* L. fruiton gram-positive bacteria (*S. aureus*) is greater thanthat of gram-negative bacteria (*S. typhi*). The type ofbacteria inhibited, the structure of the bacterial cell wall, penetration and bonding of antibacterial compounds can affect the activity of antibacterial substances based on the variation of the phenolcoefficient value obtained. Gram-positive bacteria

tend to be more sensitive to antibacterial compounds such as *flavonoids, saponins,* and *tannins*. This is because the structure of the cell wall of gram- positive bacteria is relatively simpler than the structure of the cell wall of gram-negative bacteria. The composition of the cell wall of gram-positive bacteria consists of more than 50% peptidoglycan content and teichoic acid which are both polar and low lipid content (1-4%) which is non-polar. Meanwhile, *flavonoids, saponins* and *tannins* are polar compounds (11). Therefore, these antibacterial compounds penetrate the cell membrane of gram- positive bacteria more easily. Whereas gram- negative bacteria have bacterial cell walls that are more difficult to penetrate compounds by *flavonoid*,*saponins* and *tannins* because they consist of a highcontent of lipid (11-22%) which is non-polar and thecontent of peptidoglycan is only about 5-10% whichis polar. and the outer cell membrane which functions as a selective defense of toxic compoundsthat enter and leave the cell. In addition, gram-negative bacteria have an outer membraneconsisting of phospholipids (inner layer) and non- polar lipopolysaccharides (outer layer). This is whatcauses polar secondary compounds (*flavonoids, saponins* and *tannins*) to be more difficult to enter into gram-negative bacteria cells so that their antibacterial activity is less strong than gram- positive bacteria (7).

The gram-negative outer membrane consists of three layers, namely lipopolysaccharides (LPS), lipoproteins, and phospholipids. In phospholipids there are porins which are formed from proteins. Porins are channels through which some molecules can pass. This outer membrane serves as a barrier against antibiotics, digestive enzymes, and dry conditions, but cannot be a barrier to all substances. The main factors of cell wall damage are lipopolysaccharide (LPS) and porin. Antibacterial compounds that work by penetrating LPS (lipopolysaccharide), hydrophilic molecules will more easily pass through LPS than hydrophobicmolecules (9).

Gram-negative bacteria have hydrophilic properties, namely carboxyl, amino acids and hydroxyl. The mechanism of antibacterial action of each secondary metabolite compound is different. Secondary metabolite compounds inhibit the growthof bacteria starting by damaging the cell wall. Polar compounds can penetrate polar peptidoglycan, as well as some polar compounds such as phenolic compounds that can break the peptidoglycan bond in the bacterial wall. Antibacterial compounds that areable to react with porins (trans membrane proteins) on the outer membrane of bacteria and the bacterialcell wall will form strong polymer bonds, resulting in the destruction of the porin. Damage to the porinwhich functions as a place of entry and exit ofnutrients will cause the permeability of the bacterialcell wall to decrease so that bacterial growth isinhibited or the bacterial cell will die (7).

From the results of this study, further research can be carried out on the phenol coefficient test in assessing the antibacterial activity of the combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit using other solvents. The *flavonoid* compounds, *saponins* and *tannins*contained in both plants are polar, so other solventsthat have polar properties are also needed, such as ethanol. Ethanol is considered as a liquid filter because it is more effective, has a similar level of polarity, is miscible with water in all ratios, and is heat treated for less concentration. In addition, ethanol is a solvent that is often used because it is cheaper and is not toxic. Ethanol can dissolve alkaline alkaloids, volatile oils, *glycosides, curcumin, coumarins, anrakinones, flavonoids, steroids,* resins and chlorophyll.

Conclutions

The antibacterial activity of the combination infusion of *Cananga odorata* flower - *Averrhoa bilimbi* L. fruit against *Staphylococcus aureus* obtained a higher phenol coefficient value than *Salmonella typhi.*

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