

# Comparison of Activity and Inhibitory Mechanism between (+)-Catechin and Water Extract of Gambier (*Uncaria Gambir* Roxb.) Against Some Bacteria

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## Abstract

Gambier (*Uncaria gambir* Roxb) has been used by many people in Asia as anti-microbial, anti-diarrhea, and agent for the treatment of burns. Gambier is also one component for chewing medicine to strengthen teeth and antimicrobial. This research aims to compare the antibacterial activity as well as the mechanism of inhibition between (+)-catechins and gambier extract, as the basis for choosing a substance to be developed as antimicrobial drug. (+)-Catechin was extracted from gambier, the result of (+)-catechin which obtained through isolation was compared with (+)-catechin standard by using thin layer chromatography. The anti-bacterial activity tests of (+)-catechin and extract of gambier were carried out by using the method of microdilution against some bacteria, namely, *Staphylococcus epidermidis*, *S.aureus*, *S. mutans*, *S. viridans* and *Bacillus subtilis*. For the detection of the mechanism of bacterial cellular destruction, the leakage of proteins and nucleic acids was observed by using UV-Visible spectrophotometer. In addition, the leakage of ions  $Ca^{2+}$  and  $K^{+}$  was observed by using Atomic Absorption Spectrophotometer (AAS). Meanwhile, the cellular morphological changes was observed by using scanning electron microscopy (SEM). Isolation results of (+)-catechin from gambier was obtained the yield 22.55%. The results of antibacterial activity of catechins was obtained minimum inhibitory concentration (MIC) values against *S. epidermidis*, *S. mutans*, and *S. viridans* was 5.5 mg/ mL, 8 mg/ mL, and 8 mg/ mL, respectively. While against *S. aureus* and *B. subtilis* the MIC values were not obtained up to 25 mg/ mL. The MIC of aqueous extract of gambier against the *S. epidermidis* was 22.5 mg/ mL. The MIC was not obtained against the other four bacteria until the concentration of 25 mg/ mL. The leakage of protein, nucleic acid as well as  $K^{+}$  and  $Ca^{2+}$  ions were observed at 1 MIC and 2 MIC. The result SEM assay, it was found that the (+)-catechin and gambier extract causes plasma cell membrane damage and coagulation of the nucleoid. Plasma cell membrane damage and coagulation of the nucleoid by (+)-catechin worse than gambier extract.

**Keywords:** (+)-Catechin, Gambier, Antimicrobial, Gram Positive Bacteria

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## 1. Introduction

Gambier (*Uncaria gambir* Roxb) is a kind of herbal that has been known in Indonesia for a long time and added on betel nut chewing as well as additional substance in traditional herbal medicine. Traditionally has been used for people with diarrhea, sore throat, sores, burns and wound healing.

Based on the research of Nurliana [1] and Anggraini [2], the main content of the *Uncaria gambir* Roxb are catechins (up to 51%), tannin (22-50%), and a number of alkaloids such as gambirtannin, derivatives dihydro and oxo of gambirtannin. There are 9 types of catechins that was found in gambier, namely; (+)-Catechin, (+)-epicatechin, Gambiriin A 1, Gambiriin A2, Gambiriin B2, Epigallocatechin, Catechin-(4 $\alpha$ -8)-ent-epicatechin, Gambirflavan D1 and Gambirflavan. The results of research

by Heitzman [3] and Taniguchi [4], the main content of catechin gambier is (+)-catechin with concentration about 88%.

According to Pambayun [5], extract of gambier could inhibit the growth of *Streptococcus mutans*, *Staphylococcus aureus* and *Bacillus* and according to Voravuthikunchai [6], extract of gambier also could inhibit the growth of *Helicobacter pylori* that resistant to antibiotic.

Results of research that was carried out by Fatimah et al [7] on lozenges of mixture gambier and betel leaf with a ratio 0.636:0.333 g. Lozenges was given to six healthy volunteers for 7 days. The results of measurements of CD4 volunteers before and after 7 days administration of lozenges, T test showed significantly different compared with normal controls ( $p \leq 0.05$ ) and lozenges of mixture gambier and betel leaf also have the potential to against virus, namely by increasing the levels of CD4 on volunteers

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In this study, (+)-Catechin was isolated from extract of gambier, the result of (+)-catechin which obtained through isolation was compared with (+)-catechin standard by using thin layer chromatography. The anti-bacterial activity tests of (+)-catechin and extract of gambier were carried out by using the method of microdilution against 5 Gram-positive bacteria, namely, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus viridans* and *Bacillus subtilis*. For the detection of the mechanism of bacterial cellular destruction, the leakage of proteins and nucleic acids was observed by using UV-Visible spectrophotometer. In addition, the leakage of  $K^+$  and  $Ca^{2+}$  ions was observed by using Atomic Absorption Spectrophotometer (AAS). Meanwhile, the cellular morphological changes was observed by using scanning electron microscopy (SEM)

## 2. Methodology

### 2.1. Materials for Experiment

Gambier (*Uncaria gambir*, Roxb) was obtained from Haraw region, Payakumbuh, West Sumatra, The largest producer of gambier in Indonesia. While the (+)- catechins was obtained by carrying out isolation from the Gambier extract.

### 2.2. Separation of (+)-Catechin and Semi-Polar Compounds from Gambier extract with Partition Process by Using Water and Ethyl Acetate as a Solvents

The Gambier (250 g) was dissolved in 500 mL of hot water. Allow samples to reach room temperature and partition with 500 ethyl asetat, Allow a few minutes, until water phase with ethyl acetate phase separate. Then separate both of phases with separating funnel. Repeat the work several times. Until phase of ethyl acetate looks clearly. Ethyl acetate phase obtained was concentrated with a rotary vacuum evaporator, then dried with a freeze drier until was obtained a dry powder.

### 2.3. Isolation of (+)-Catechin from Gambier ekstrak

Isolation of (+)-catechin was carried out by using column chromatography. Silica gel was used as stationary phase, the mixture of chloroform and methanol by ratio 4 : 1 was used as the mobile phase.

The water content existing in the silica gel was removed by heating in an oven at a temperature of 100 oC for 30 minutes. Silica gel was suspended in the eluent to form a pasty and input into the column. The Column faucets was opened, let the eluent flow to the limit of the adsorbent, then the column faucet was closed. Gambier dry extract obtained was dissolved in eluent.

Collect the liquid droplets that come out from the column and add eluent until was obtained liquid 30 mL for each fraction. Analysis (+)-catechin for each fraction was carried out by using Thin Layer Chromatography. Chloroform and

methanol with a ratio (3:1) was used as the mobile phase. To look spots was used solution 10% of vanillin in concentrated  $H_2SO_4$ . Then, spots that formed were compared with (+) - catechin standard.

### 2.4. Preparation of Bacterial Suspension

The bacterial used were *S. epidermidis*, *S. aureus*, *Str. mutans*, *Str. viridans* and *B. subtilis*. Culture stocks were obtained from microbiology laboratory, Indonesian Institute of Sciences, Cibinong, Bogor. The stocks of these bacteria were kept in nutrient agar, then inoculated into the Mueller-Hinton Broth, incubation at shaker incubator with speed 120 rpm, temperature 37 0C, for 24 hours until reach the active phase.

The amount of bacteria was adjust approximately  $1 \times 10^5$  CFU/ mL by using metode of McFarland. Bacteria *Streptococcus mutans* was inoculated into agar slope medium, added 5% blood by using a Dose that was sterilized by heat in the Bunsen flame, and then incubated at 37 0C for 24 hours.

### 2.5. Preparation of Test Solutions for the Gambier Extract and (+)-Catechin

Preparation of solution concentration the Gambier extract and (+)-catechin was made from 5 mg/mL - 25 mg/mL. For testing diameter of inhibition area, the test solution (+)-catechin and the Gambier was dissolved in DMSO 10% and the concentration of solution used was 7 mg/mL. Determination of minimum inhibition concentration(MIC) was used the concentration of solution as follow 5; 5,5; 6; 6,5; 7; 7,5; 8; 10; 12,5; 15; 17,5; 20; 22,5 mg/ mL and 25 mg/ mL

### 2.6. Determination of Diameter of Inhibition Area

Determination of diameter of inhibition was carried out by using paper disc diffusion method used by Supprakul et al [8] and Caburian et al [9]. As much as 20 mL of sterile Mueller-Hinton Agar was poured into each petri dish (except for *Streptococcus mutans* blood added as much as 5%). Let freeze at room temperature. Bacteria was suspended 0.1 mL into petri dishes. Spread bacterial suspension inoculum over entire surface with glass rod spreader and allowed to stand for approximately 15 minutes. Paper discs was placed on solid agar medium that has been frozen. Drop test solutions of gambier and (+)-catechin as mention above as much as 10  $\mu$ l on paper discs. Then incubated for 24 hours at 37 0C. Measure diameter of inhibition was formed around the discs.

### 2.7. Determination of Minimum Inhibition Concentration (MIC)

Determination of minimum inhibition concentration was used micro-dilution method by using sterile mikroplate with the method used by Supprakul [8]. Each wells for test solution was filled with 100 $\mu$ l Mueller-Hinton Broth and into each of wells was inoculated 100 mL suspension of test

bacteria. Then add 50 mL of test solution of gambier extract and (+)-catechin as mention above. As a control was used two types control, namely, wells A containing medium and bacterial suspension, wells B containing medium, the solvent and the suspension of test bacteria. Then microplate was incubated on shaker incubator with 150 rpm for 24 hours at 37 °C. Determination of minimum inhibition concentration (MIC) was carried out by culturing the test sample on Nutrien Agar medium after incubation for 24 hours. The smallest concentration that causes the bacteria did not grow on bacterial culture was expressed as the MIC for the test solution. Each experiment was conducted with two times.

### 2.8. Analysis of Proteins and Nucleic Acids

Suspension of test bacteria that had been incubated for 24 h in Muller-Hinton Broth (MHB) medium with the method used by Carson et al [2002]. Centrifuged at 3500 rpm for 20 minutes. Subsequently the filtrate discarded and the pellets in the tubes were washed with phosphate buffer pH 7.0. Washing conducted 2 times. Then add test solution of (+)-catechin with treatment 1 MIC and 2 MIC which add phosphate buffer up to 10 mL. Incubated for 24 hours in a shaker 150 rpm. Then the suspension was centrifuged again for 15 minutes at 3500 rpm, and supernatant fluid was taken. Perform measurement of absorbance with spectrophotometer UV/Vis at a wavelength of 260 nm and 280 nm.

For this testing was only done to the bacteria *Staphylococcus epidermidis*, because *Staphylococcus epidermidis* most sensitive among the test bacteria. In this case. So also for the Analysis of Metal Leakage and scanning of changes in cell morphology by SEM.

### 2.9. Analysis of Metal Leakage

Analysis of leakage of metals was measured in the form ions  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  used methods Carson et al [10]. Leakage of ions  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  from the bacterial cell membrane due to treatment with (+)-catechin. Preparation of samples for analysis of leakage of ions is the same as preparation for the leakage of proteins and nucleic acids. Leakage was expressed with the measurability of metal ions contained in the test bacteria after contact with (+)-catechin in treatment 1 MIC and 2 MIC. Leakage of ions  $\text{Ca}^{2+}$  and  $\text{K}^{+}$  was detected by using Atomic Absorption Spectrophotometer (AAS).

### 2.10. Scanning of Changes in Cell Morphology by

Suspension of test bacteria that had been incubated for 24 h in MHB medium SEM by the method of Carson et al [10]. Centrifuged at 3500 rpm for 20 minutes. Filtrate discarded and the pellets in the tubes was washed with phosphate buffer (pH 7.0) twice. Added the (+)-catechin with treatment

1 MIC and 2 MIC up to 10 mL. Incubated for 24 hours in a shaker 150 rpm. Bacterial cell suspension results of contact with (+)-catechin was centrifuged at 3500 rpm for 15 minutes.

Pellet was washed with a buffer solution fospat and the liquid separated by centrifuge, the treatment was repeated twice. Filtrate discarded and the pellet was immersed in glutaral cocodhilate dehide and buffer for 4 hours.

Then the centrifuged and the supernatant was discarded. Pellets immersed again with 2% tannin acid in the buffer chocodilate for 12 hours, then centrifuged again, supernatant discarded and pellets was immersed in 1% osmium tetraoksida solution for 2-4 hours. Washed with buffer cocodilte, then centrifuged and pellets washed with ethanol 50%, allow 10 minutes and centrifuged again (done twice). Then washed again with 70% ethanol, 80% and 95% respectively for 10 minutes and then centrifuged. Pellet was washed with absolute ethanol and allowed to stand for 10 minutes and the centrifuged again (this treatment was carried out 2 times). Washed again with terbutanol, allowed to stand for 10 minutes and centrifuged (done twice).

Added a little terbutanol in the sediment cell. Applied to cell smears on a glass slip, then placed on the stub for coating with gold for 1 hour in a vacuum. Observed by using Scanning Electron Microscopy (SEM)

## 3. Results and Discussions

From this study was obtained, Identification of fractions isolation results from gambier with thin layer chromatography (TLC) showed that there were other compounds that were not (+)-catechin, that occurred on fraction 1 to 9 and fraction 41 to 70, as shown in Figure 1.

In fractions 10 to 40, the results of thin layer chromatography showed only one spot that was formed. This spot has the same Rf value with Rf value of standard catechin, as shown in the Figure 2. Fractions isolation results from 10 to 40 fractions was collected and the solvent was evaporated. The result was expressed as the yield of (+)-catechin from gambier, after calculated the yield was obtained 22.68%.

According to result of research conducted by Amos [11], the yield of total catechin gambier extract (bongkahan) come from Payakumbuh 40-60% and the Result of research that was conducted by Taniguchi et al [4] was got 9 types of catechin, namely, (+)-catechin, (-)-epicatechin Gambiriin A1, Gambiriin A2, Gambiriin B1, Gambiriin B2, Catechin-(4 $\alpha$ -8)-ent-epicatechin, Gambirflavan D1 and Gambirflavan D2. In this study we were got 22.68% (+)- the yield of (+)-catechin. If it is taken analogous based on the result of research by Amos et al (2010) with content total catechin 50%.

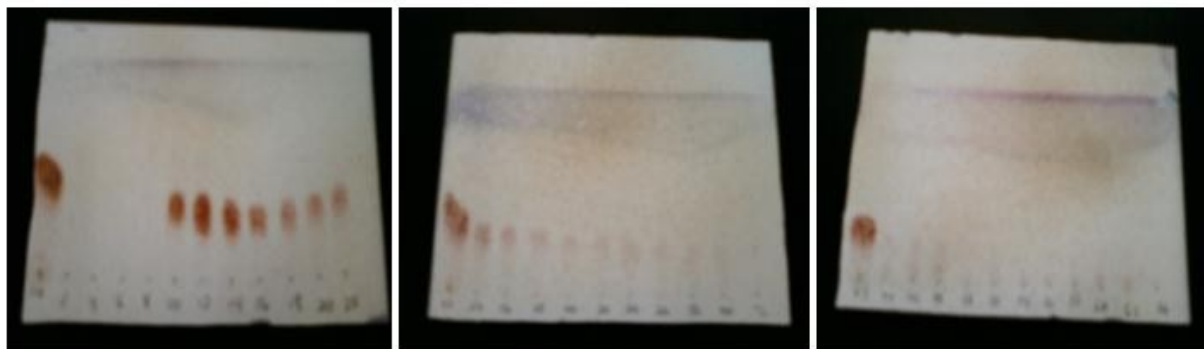


Fig 1. Thin Layer Chromatogram (TLC) fractions isolation results of gambier

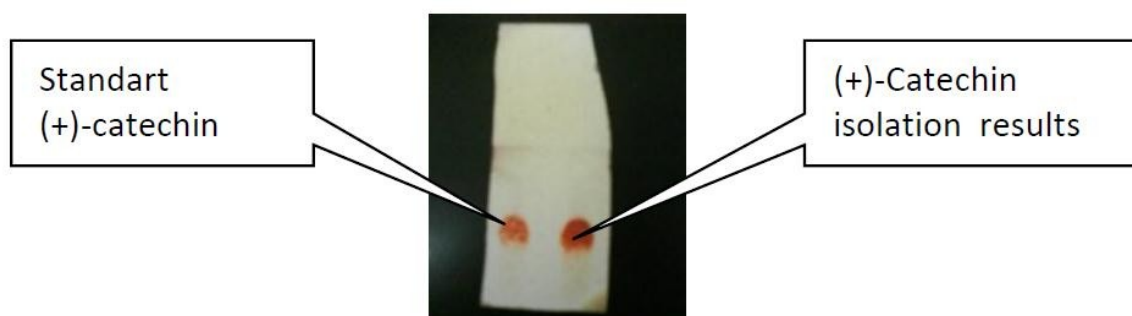


Figure 2. Thin Layer Chromatography of 10-40 Fraction of Isolation Results of Gambier

There is content others catechin of 27.32% (50% minus 22.68%), or content of (+)catechin of total catechin is 45,36%. On the other hand, the result of research was conducted by Anggraini et al, (2011) was conducted about gambier leaves, the yield of total catechin gambier leaves was 13,74% and the result of research was conducted by Das N.P., (1967), the yield of total (+)-catechin gambier leaves was 9,4%. Content catechins in gambier extract higher than gambier leaves, because on the manufacturing process of gambier extract (bongkahan) after heating with boiling water was carried out filtering. Where the residue and the fibers that exist in gambier leaves was discarded and the main compound which dissolved in hot water is catechins.

The result diameter of inhibition zone of antibacterial activity test of gambier extract with concentration 7 mg/ mL was obtained for *S. epidermidis*, *S. aureus* and *B. Subtilis*; 5 mm, 1 mm and 1 mm respectively. While for *Str.mutans* and *Str.viridans* was not obtained inhibition zone. For (+)-catechin was obtained inhibition zone for all test bacteria and diameter of inhibition zone for *Str.mutans* and *Str.viridans* larger than *S. epidermidis*, *S. aureus* and *B. subtilis*, as shown in Table 1.

In this study for gambier extract against *Str.mutans* and *Str.viridans* was not obtained inhibition zone as has been obtained by Das et al [12] and Heitzman et al [3], the main content of gambier is (+)-catechin. In this case, caused by differences in the level of sensitivity, where the content of (+)-catechin was lower in the gambier extract compared with (+)-catechin. As shown in Table 1.

Table 1  
The Result Diameter of Inhibition Area (mm) of Antibacterial Activity Test between Gambier Extract Compared with (+)-Catechin at Concentration 7 mg/ml

Name	Gambier Extract (mm)	(+)-Catechin (mm)
<i>S.epidermidis</i>	5	2
<i>S.aureus</i>	1	2
<i>Str.mutans</i>	-	4
<i>Str.viridans</i>	-	4
<i>B. subtilis</i>	1	2

Results of research that was conducted by Pambayun [5], by doing isolation gambier with ethyl acetate was obtained inhibition zone for *Str. Mutans*, *S. aureus* and *B. Subtilis*.

The results isolation of Gambier extract with ethyl acetate as much as 30 mL was put onto the paper discs, it was obtained diameter of inhibition zone for *Str. Mutans*, *S. aureus* and *B. Subtilis*; 9,67 mm, 9,33 mm and 8,33 mm respectively.

It is caused, catechins are compounds that dissolve in the semi-polar solvent, where ethyl acetate is a semi-polar solvent. Or it can be said, the compounds which have antibacterial activity in gambier that isolated with ethyl acetate is catechins. While, gambier extract was obtained from the solvent water will separate only slightly catechins than other compounds that exist in Gambier, because water is a polar solvent and catechins are not isolated by polar solvents. In other words, compounds that are polar in gambier do not have antibacterial properties.

The comparison Minimum Inhibitory Concentration (MIC) values of gambier extract compared with (+)-catechin also showed, that (+)-catechin has antibacterial activity more sensitive than gambier extract. *S. epidermidis* is the most sensitive, both to the gambier extract and (+)-catechin.

For gambier extract only *S. epidermidis* was obtained MIC value of 5 Gram-positive were tested until a concentration of 25 mg/ mL. While for (+)-catechin only *S. aureus* and *B. subtilis* were not obtained MIC values until a concentration of 25 mg/ mL, while for *S. epidermidis*, *Str. aureus* and *B. subtilis* were obtained MIC values 5.5 mg/ mL, 8 mg/ mL and 8 mg/ mL respectively as shown in Table 2.

For analyze the leakage of nucleic acids and proteins from the bacterial cell wall was observed only on bacteria *S. epidermidis* with 1 MIC (5.5 mg/mL) and 2 MIC (11 mg/mL). While for other bacteria that are not carried out, because the antibacterial effects of work (+)-catechin against *S. epidermidis* is estimated similar with antibacterial effects of (+)-catechin against other bacteria. The test results for (+)-catechin was obtained for nucleic acids with 1 MIC at absorbance 1,003, with 2 MIC at absorbance 1,015 and on

normal control at absorbance 0,423. While for proteins with 1 MIC at absorbance 1,994, with 2 MIC at absorbance 2.01 and on normal control at absorbance 0.437. This results shown, the greater the concentration the greater the leakage that occurred, as shown in Figure 3.

For analyse the leakage of ion  $Ca^{2+}$  and  $K^{+}$  from the bacterial cell wall also was observed only on bacteria *S. epidermidis* with 1 MIC (5,5 mg/mL) and 2 MIC (11 mg/mL).

Table 2  
The MIC Values (mg/ mL) of Gambier Extract and (+)-Catechin against Gram Positive

Bacteri a	Gambier Extract (mm)	(+)-Catechin (mm)
<i>S. epidermidis</i>	22,5	5,5
<i>S. aureus</i>	>25	>25
<i>Str. mutans</i>	>25	8
<i>Str. viridans</i>	>25	8
<i>B. subtilis</i>	>25	>25

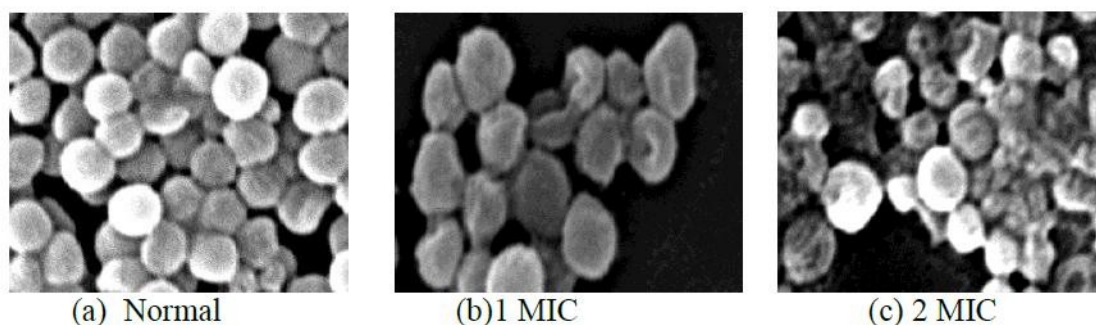


Figure 3. The assay of *Staphylococcus epidermidis* by SEM (magnification 15,000 X); (a) normal, (b) given 1 MIC (+)-catechin and (c) given 2 MIC (+)-catechin

The test results for (+)-catechin was obtained for  $Ca^{2+}$  with 1 MIC at concentration 4,7 ppm, with 2 MIC at concentration 5,2 ppm and on normal control at concentration 0,1 ppm, while for  $K^{+}$  with 1 MIC at concentration 80,5 ppm, with 2 MIC at concentration 89,6 ppm and on normal control 64 ppm. This results shown, the greater the concentration the greater the leakage that occurred, as shown in Figure 4.

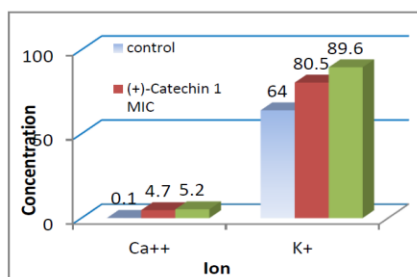


Fig 4. Ion Leakage Level of  $Ca^{2+}$  and  $K^{+}$  by (+)-Catechin against *S. epidermidis* with Doses of 1 MIC and 2 MIC

#### 4. Conclusion

The yield (+)-catechin from extract of gambier was found about 22.55%. The result of leakage measurement of proteins, nucleic acids and  $K^{+}$  and  $Ca^{2+}$  ions from the cell membrane and result of SEM assay, it were found that the (+)-catechin and gambier extract with 1 MIC and 2 MIC causes plasma cell membrane damage and coagulation of the nucleoid. Plasma cell membrane damage and coagulation of the nucleoid by (+)-catechin worse than gambier extract.

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