Original Article

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Bactericidal action of *Alpinia galanga* essential oil on food-borne bacteria

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ABSTRACT: The use of natural antimicrobial agents is garnering attention due to consumer and producer awareness of health problems. This study found that the essential oil of *A. galanga* had strong bactericidal activity against both Gram-negative and Gram-positive bacteria. The bactericidal action of *A. galanga* oil was extremely rapid. Results of scanning electron microscopy observations suggested that *A. galanga* oil had antibacterial action probably as a result of its modification of the bacterial cell membrane, disrupting the membrane's permeability. This study suggested that the essential oil of *A. galanga* shows promise as a natural antimicrobial agent for use as a food preservative.

Keywords: *Alpinia galanga*, antibacterial, mechanism of action, essential oil, killing time

1. Introduction

Food safety is a highly important issue for both consumers and the food industry due to the rising number of cases of food-associated infections. Hence, good manufacturing practices have been introduced in the food industry in order to control the level of pathogens in food products (*1*,*2*). The most effective way to minimize food contamination by microorganisms is to add an effective antimicrobial agent to food products. The antimicrobial agents used nowadays are both chemical and natural agents but the latter are more increasingly desired by consumers because of their lower toxicity and naturalness.

Various plants have shown the potential to have antimicrobial action (*3-6*). Some have been used to treat infectious diseases caused by pathogenic microorganisms (*7*,*8*). The active substances generally accumulate in a particular part of the plant. Anantaworasakul *et al*. reported that the antibacterial active extract of *Sesbania grandiflora* could be obtained from the stem bark of the plant (*8*). *Alpinia galanga*, a plant in the Zingiberaceae family, is widely distributed in tropical areas. It has been used as a medicine to treat stomachaches in China and Thailand (*9*). The fresh rhizome of *A. galanga* has a characteristic fragrance as well as pungency, hence its rhizome is used as an essential component in Thai curry paste. The crude extract of *A. galanga* has been reported to have antioxidant and antimicrobial activities (*10*,*11*). Janssen and Scheffer reported that the monoterpenes in the essential oil from fresh *A. galanga* rhizomes have antimicrobial activity against *Trichophyton mentagrophytes* (*12*). An ethanol crude extract of *A. galanga* was reported to inhibit *Staphylococcus aureus* (*13*). Certain food-borne diseases originate from foods contaminated by different strains of bacteria. Current knowledge of its bactericidal action is highly lacking. There are even less data on this plant's action against bacteria that contaminate food. Moreover, there is very little information on its antibacterial kinetics and mechanism of action. In order to encourage the use of *A. galanga* as a natural food preservative, more strains of bacteria should be tested in order to guarantee *A. galanga* has antimicrobial activity against these food-borne pathogens.

The purpose of this study was to investigate the antimicrobial activity of the *A. galanga* rhizome against several food-borne bacteria. The antibacterial potency of the essential oil was compared with three crude fractions obtained by solvent extraction. The antibacterial activity of *A. galanga* essential oil on the bacteria in question was studied in detail.

2. Materials and Methods

2.1. *Plant Materials*

Rhizomes of *A. galanga* (6-12 months age) cultured in the northern part of Thailand were collected in October 2008. A voucher specimen was deposited with the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand. Fresh rhizomes were used for extraction of the essential oil. Dried rhizome powder was prepared by slicing the fresh rhizomes into small pieces

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and drying them at 60°C for 48 h. The dried rhizome was ground into a fine powder for use in solvent extraction.

2.2. *Essential oil extraction and GC-MS analysis*

The fresh rhizomes were chopped and subjected to hydro-distillation for 6 h; a Clevenger apparatus was used to obtain the essential oil fraction. The essential oil obtained was dried using anhydrous sodium sulphate and then stored in a dark, airtight bottle at 4°C until needed.

The oil was subjected to GC-MS. The GC-MS analysis was performed on an Agilent 6890 gas chromatograph operating in electron impact (EI, 70 eV) mode. The gas chromatograph was equipped with an HP 5973 mass selective detector and fitted with a fused silica capillary column (HP-5MS) supplied by HP, USA (30.0 m × 250 µm i.d., 0.25 µm film thickness). The oven temperature was programmed to increase from 100 to 280°C at a rate of 3°C/min and finally stay isothermal for 10 min. The carrier gas was helium introduced at a rate of 1.0 mL/min. A diluted sample of 1.0 µL was injected manually and the split ratio was adjusted to 40:1. GC-MS analyses were performed using a Thermo Finnigan-TRACE GC (Waltham, Massachusetts, USA) coupled with a TRACE MS plus (EI, 70 eV) from the same company.

2.3. *Identification of essential oil constituents*

The components of the obtained essential oil were identified by comparison of their mass spectra with those of NIST98 library data in the GC-MS system and Adams libraries spectra. The order in which compounds were eluted was compared with their retention indices as reported in the literature (*14*). Retention indices of the components were determined relative to the retention times of a series of *n*-alkanes with linear interpolation.

2.4. *Preparation of crude extracts*

A dried, powdered sample of *A. galanga* was separately weighed and macerated in a different-polarity solvent, *i.e.*, hexane (non-polar), ethyl acetate (semi-polar), and ethanol (polar) for 4 cycles at room temperature. Each cycle lasted 7 days with 1 h of mechanical stirring every day. The filtrates of the same solvent from each macerated cycle were pooled. The solvent was removed under reduced pressure at 45°C using a rotary evaporator. The weight of the resulting extracts was measured and extracts were stored in dark bottles at 4°C until use.

2.5. *Microbial strains*

The food-borne microorganisms used in this study consisted of 7 reference strains, *i.e.*, *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Salmonella enteritidis* (ATCC 13076), *Salmonella typhimurium* (ATCC 14028), *Salmonella typhi* (DMST 5784), *Listeria monocytogenes* (DMST 1730), and *Shigella sonnei* (DMST 561), and three field strains of *E. coli*. Tryptic soy broth (TSB) was used to culture the bacteria. All strains were stored at −20°C in glycerol and subcultured twice in TSB at 37°C 24 h before testing.

2.6. *Screening for antimicrobial activity*

Comparative antimicrobial potency of the essential oil and the three extracts was studied by using the disc diffusion method according to Najjaa *et al*. and Arias *et al*. with minor modifications (*15,16*). *E. coli* (ATCC 25922), *S. typhimurium* (ATCC 14028), and *S. aureus* (ATCC 25923) were used as the test strains in this study. Briefly, a single colony of the respective test bacterium was transferred to TSB and incubated overnight. Three milliliters of each culture were mixed with 100 mL of melted Mueller Hinton Agar (MHA, Difco, USA) at about 45°C and poured onto the surface of an agar plate containing 2 g agar in 100 mL distilled water. The test samples (80 µL) were placed on 8-mm discs of sterile filter paper (Advantec, Tokyo, Japan) twice with air-drying in between. Control discs were similarly prepared using distilled water and pure solvents. Each loaded disc was placed on the aforementioned bacterial culture plate and incubated at 37°C for 18-24 h. Diameters of inhibition zones were measured and recorded.

2.7. *Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)*

The MIC and MBC of *A. galanga* essential oil were determined by a broth dilution method (*17,18*). Tween 20 was used to solubilize the extracts. All tests were performed in Mueller Hinton broth. Serial two-fold dilutions of the oil ranging from 0.05 to 200 mg/mL were prepared in 96-well microtiter plates. The final concentration of each strain was adjusted to 4×10^4 cfu/mL. Plates were incubated at 37°C for 24 h. The MIC was defined as the lowest concentration of the essential oil at which the microorganism did not demonstrate visible growth. Microorganism growth was indicated by turbidity. To determine the MBC, broth was taken from each well and incubated in Mueller Hinton Agar at 37°C for 24 h. The MBC was defined as the lowest concentration of the essential oil at which the incubated microorganism was completely killed. Each test was performed in triplicate. Gentamicin served as a positive control.

2.8. *Study of bactericidal kinetics*

In this study, 5 reference strains of bacteria (*L. monocytogenes* (DMST 1730), *S. aureus* (ATCC 25923), *S. typhi* (DMST 5784), *S. sonnei* (DMST 561), and *E. coli* (ATCC 25922)) and 3 clinical strains of *E. coli* were used as the test organisms. The killing kinetics of the essential oil were studied at oil concentrations equivalent to the

MBC of the bacterial strains. Bacterial cells were grown to logarithmic phase during 1 h of pre-incubation in fresh broth prior to the addition of the essential oil solution. A bacterial concentration between 6 and 8 log cfu/mL was used. The cultures were then incubated in a shaker (Julabo, Allentown, PA) at 37°C for a certain period of time or until no viable cells were found. Viable cell counts were determined by plating 50 µL of known dilutions of the culture samples onto tryptic soy agar. Cell count plates were incubated for up to 48 h before they were deemed to have no growth. Plates with 30-300 colonies were used for cfu counts. Log cfu was plotted with respect to time to create bactericidal kinetic curves. Gentamicin obtained from Sigma-Aldrich (St. Louis, MO, USA) was used as a positive control. All assays were performed in triplicate.

2.9. *Observation of bacterial morphology*

The bacterial morphology before and after exposure to *A. galanga* oil was examined using scanning electron microscopy (SEM, JSM 5410 LV, Jeol, Japan). Sample preparation for SEM was done as follows. The bacterial suspension before or after a certain amount of exposure to the oil was dropped onto a filter membrane and air dried. Next, the bacteria were fixed with 2.5% glutaraldehyde in PBS and rinsed with the same buffer solution. Subsequently, the fixed bacteria were stained with 1% OsO_4 in PBS for 1 h and dehydrated with different mixtures of water and ethanol. The membrane was coated with gold and analyzed with SEM.

3. Results and Discussion

3.1. *Yields of extraction*

Hydrodistillation of the fresh *A. galanga* rhizomes yielded essential oil of 3.0 mL/kg. In solvent extraction of the dried rhizome, hexane had an extract yield of 33.9 g/kg, which was the highest. This yield was approximately 2-fold higher than that for the two extracts obtained with the other two extracting solvents, ethyl acetate and ethanol, as shown in Figure 1. A previous report (*19*) indicated that the yield of essential oil obtained from the distillation of *A. conchigera*, a plant known as small galanga, was 1.6 mL/kg. The current results indicate that *A. galanga* possesses greater essential oil content than *A. conchigera*.

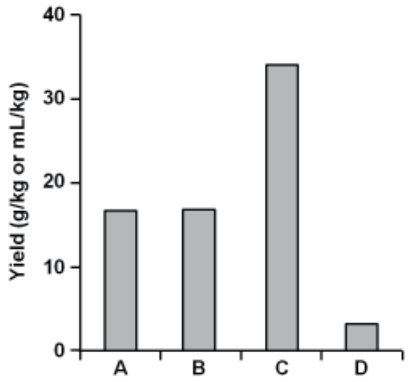

Figure 1. The percent yield of *A. galanga* hexane (A), ethyl acetate (B), ethanol extracts (C) and essential oil (D). Yield of extracts is given in g/kg while that of the essential oil is given in mL/kg.

3.2. *Chemical composition of the essential oil*

Chromatography allowed identification of 27 compounds, representing 93.1% of the oil. Results of quantitative and qualitative analysis by GC-MS are shown in Table 1. The essential oil consisted mainly of two cyclic terpenes; piperitenone (33.3%) and limonene (29.6%). As far as a search of the literature could ascertain, only one report mentioned the chemical composition of the essential oil of *A. galanga* grown in Malaysia (*20*). The previously identified compounds represented 83-93% of the oil, depending on its method of preparation, but the two main compounds in the previous report differed from those in this study. This may be due to the fact that the rhizomes were grown in different regions, which could have caused differences in chemical composition.

3.3. *Antibacterial activity of the extracts*

The growth inhibition zones of the essential oil in comparison to the crude extracts from different solvents measured by using agar disc diffusion assay are shown

Table 1. Main chemical composition of *A. galanga* essential oil

Components	% Area	RT.[a]	RI.[b]
Limonene	29.64	3.32	1,041
gamma-Terpinene	1.22	3.56	1,058
alpha-Terpinolene	0.44	3.99	1,087
1-Undecene	0.20	4.09	1,093
(−)-Borneol	0.72	5.48	1,166
para-Cymen-8-ol	3.06	6.03	1,189
alpha-Terpineol	0.20	6.25	1,198
Z-Citral	1.23	7.50	1,248
(−)-Bornyl acetate	0.39	8.51	1,282
Piperitenone	33.31	10.56	1,349
alpha-Cubebene	0.15	10.78	1,355
Decanoic acid	1.31	11.55	1,377
beta-Elemene	1.91	12.30	1,398
alpha-Gurjunene	0.20	12.83	1,414
trans-beta-Caryophyllene	3.38	13.39	1,431
trans-beta-Farnesene	0.42	15.44	1,487
beta-Selinene	0.46	15.56	1,490
delta-Selinene	0.31	15.71	1,494
Pentadecane	5.62	15.95	1,500
alpha-Amorphene	3.01	16.50	1,517
7-*epi*-alpha-Selinene	0.83	16.65	1,521
trans-gamma-Bisabolene	2.25	16.79	1,525
alpha-Cadinol	0.61	21.08	1,661
gamma-Selinene	0.40	21.62	1,681
beta-Bisabolene	0.84	22.21	1,702
Apiol	0.65	22.45	1,708
alpha-*trans*-Bergamotol	0.30	27.46	1,828

[a] Retention times; [b] Retention indices.

in Table 2. *S. aureus* was more sensitive to the ethanol and hexane extracts than *E.coli* and *S. typhimurium*. These results are in substantial agreement with a previous study (*13*) that reported that ethanol extract of *A. galanga* inhibited *S. aureus* but did not inhibit *E. coli*. The ethyl acetate extract inhibited *E. coli* and *S. typhimurium* less than the essential oil did. This extract exhibited slightly higher inhibitory activity against the Gram-positive *S. aureus*, whereas *A. galanga* essential oil had the strongest inhibition against the Gram-negative *E. coli* and *S. typhimurium*. These results demonstrate the strong potency of *A. galanga* essential oil in terms of inhibiting the test strains of both Gram-negative and Gram-positive bacteria. The essential oil of *Zingiber officinale*, a plant which belongs to the same family as *A. galanga*, is reported to not inhibit *E. coli* (*21*). The current results indicate that *A. galanga* oil is advantageous in that it inhibits many Gram-negative bacteria, including *E.coli* and *S. typhimurium*. Given its high antibacterial potency, the essential oil was selected for further investigation to determine its MIC and MBC. More strains of bacteria were used in this experiment, the results of which are shown in Table 3. The oil was found to have strong bactericidal activity against *E. coli*, *S. aureus*, *S. sonnei*, and *S. typhi*, a finding that was echoed by the oil's MIC and MBC against each strain of 4.0, 8.0, 2.0, and 2.0 mg/mL, respectively. The MIC of the oil against *L. monocytogenes* was 2.0 mg/mL, indicating bacteriostasis, whereas the oil's bactericidal activity against this strain was 4.0 mg/mL. The MIC and MBC of gentamicin against *E. coli* (ATCC 25922) and *S. typhi* (DMST 5784) were 16 and 8 µg/mL, respectively. However, gentamicin's MIC values against *S. aureus* (ATCC 25923), *S. sonnei* (DMST 561), and *L. monocytogenes* (DMST 1730) were 8, 8, and 4 µg/mL, respectively, and its corresponding MBC values were 16, 16, and 8 µg/mL, respectively. A point worth mentioning is that *A. galanga* essential oil had strong activity against Gram-negative bacteria, which are known for their insensitivity to many antibacterial agents (*22-24*). Moreover, all tested strains contaminate food and are causes of food-borne diseases. Consequently, *A. galanga* essential oil showed promise as a natural food preservative to minimize bacterial growth in food products. The current results also indicated the considerable potential of *A. galanga* essential oil to inhibit food-borne pathogens, which trend to be resistant to antibiotics.

3.4. *Bactericidal action of A. galanga oil*

To study the bactericidal action of *A. galanga* essential oil, test microorganisms were subjected to the oil and gentamicin at a concentration of their corresponding MBC. The kinetic bactericidal action was expressed as a time versus cell death curve as shown in Figure 2. The essential oil of *A. galanga* killed all tested bacteria faster than gentamicin. The results shown in Figure 2A revealed that within 10 min *A. galanga* essential oil (4 mg/mL) killed about 6 log cfu/mL of *E.coli* (ATCC 25922) whereas in the same period of time gentamicin (16 µg/mL) killed less than 1 log cfu/mL of the bacteria. Further, the time for *A. galanga* essential oil (4 mg/mL) to completely kill *E. coli* (ATCC 25922) was only 40 min whereas gentamicin (16 µg/mL) took 120 min. As shown in Figures 2B-2D, the rapid killing of bacteria by *A. galanga* oil was observed with the three field strains of *E.coli* as well. The crude extract of *Azadirachta indica* was previously reported to be a potent antibacterial agent, but it did not kill *E. coli* after

Table 2. Bacterial inhibitory zone after exposure to the extracts (20 mg) and gentamicin (10 µg) using the disc diffusion method (*n* = 3)

Samples	Inhibition Zone (mm)*		
	E. coli (ATCC 25922)	*S. typhimurium* (ATCC 14028)	*S. aureus* (ATCC 25923)
Ethanol extract	NZ	NZ	11.8 ± 0.4
Ethyl acetate extract	9.0 ± 0.1	7.8 ± 0.4	16.5 ± 1.4
Hexane extract	NZ	NZ	21.7 ± 2.9
Essential oil	10.2 ± 0.4	9.5 ± 1.0	10.0 ± 0.2
Gentamicin	16.3 ± 0.9	10.4 ± 1.2	23.1 ± 2.5

* NZ: no inhibition zone. Data were expressed as mean ± S.D.

Table 3. MIC and MBC of *A. galanga* oil in comparison to gentamicin obtained by the broth dilution method (*n* = 3)

Bacterial Strain	MIC		MBC	
	Essential oil (mg/mL)	Gentamicin (µg/mL)	Essential oil (mg/mL)	Gentamicin (µg/mL)
E. coli (ATCC 25922)	4	16	4	16
S. aureus (ATCC 25923)	8	8	8	16
S. sonnei (DMST 561)	2	8	2	16
S. typhi (DMST 5784)	2	8	2	8
L. monocytogenes (DMST 1730)	2	4	4	8

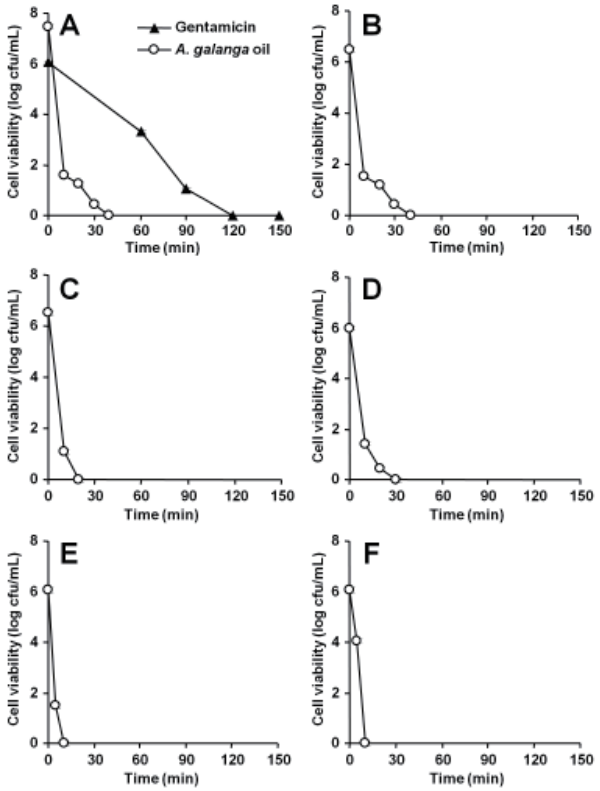

Figure 2. Killing kinetics of Gram-negative bactericidal action of *A. galanga* oil against *E. coli* (ATCC 25922) (A), *E. coli* clinical strains (B-D), *S. typhi* (DMST 5784) (E), and *S. sonnei* (DMST 561) (F) (*n* = 3).

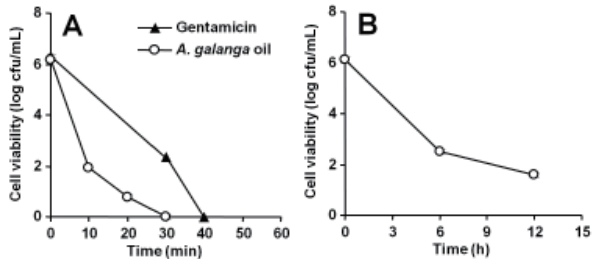

Figure 3. Killing kinetics of Gram-positive bactericidal action of *A. galanga* oil against *L. monocytogenes* (DMST 1730) (A) and *S. aureus* (ATCC 25923) (B) (*n* = 3).

24 h of exposure (*25*). Therefore, the present results indicated that the essential oil of *A. galanga* had greater bactericidal activity than the other plants previously investigated. In addition, other Gram-negative bacteria, including *S. typhi* (DMST 5784) and *S. sonnei* (DMST 561), were also highly sensitive to *A. galanga* oil. This bacterial sensitivity was expressed in a significantly decreased number of bacteria after a short period of exposure to the oil, as shown in Figures 2E-2F. The bactericidal action of *A. galanga* essential oil on Gram-positive bacteria over time is shown in Figure 3. Within 10 min, *A. galanga* essential oil (4 mg/mL) caused a decrease in *L. monocytogenes* (DMST 1730) of about 5 log cfu/mL whereas gentamicin (8 µg/mL) decreased that bacterium no more than 1 log cfu/mL. Moreover the time required for *A. galanga* oil to completely kill the bacterium was only 30 min. Therefore, *A. galanga*

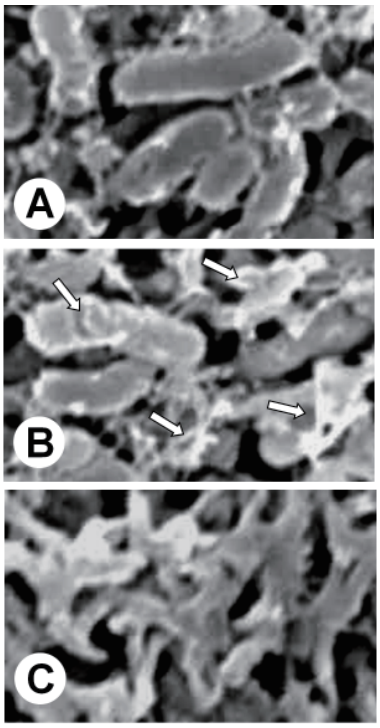

Figure 4. Morphology of *E. coli* (ATCC 25922) before exposure to *A. galanga* oil (A), 10 min afterwards (B), and 40 min afterwards (C) (Arrows point to the lesions of cell destruction).

essential oil completely killed *L. monocytogenes* (DMST 1730) faster than gentamicin did at their corresponding MBCs. An earlier kinetic study of the action of tea tree oil on pathogenic bacteria by LaPlante showed that tea tree oil did not completely kill the test strains of Gram-positive bacteria within 24 h (*26*). The current results demonstrated that *A. galanga* oil has greater potency in terms of killing Gram-positive bacteria. A kinetic study of *S. aureus* (ATCC 25923) revealed a slight decrease in the number of bacteria after exposure to the essential oil; the bacterium was completely killed after more than 24 h. These results indicated that *A. galanga* essential oil was more effective at killing Gram-negative than Gram-positive bacteria.

3.5. *Bacterial morphology study*

Changes in bacterial morphology after different periods of exposure to the essential oil were studied in order to understand the mechanism of antibacterial action of *A. galanga* essential oil. Results demonstrated that *E. coli* was the most sensitive to *A. galanga* oil among the bacteria tested. The oil clearly modified *E. coli* cells. Bacterial cells were found to rapidly shrink within 10 min. The morphology of normal bacteria in comparison to that of shrunken cells is shown in Figures 4A-4B. After shrinking, the cells fragmented and disintegrated. Most cell destruction occurred within 40 min. Destroyed cells are shown in Figure 4C. This

destruction is presumably attributed to *A. galanga* oil, which is prone to interact with lipopolysaccharide on the bacterial cell membrane. The interaction altered the structure of the cell membrane. Cell shrinkage was important evidence of cell membrane alteration caused by the oil. This led to disruption of the bacterial cell membrane in terms of its normal permeability. The leakage of essential bacterial cell components caused cell lysis and ultimately death in a short period of time.

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