IV. MATERIALS AND METHODS

1. Lactic acid bacteria

1.1 Collection of the microorganisms

The microorganisms were collected from 40 pieces of 2.5 cm-sized goats' intestinal mucosa (Dr. Siriwoot Sookkhee's the former project). Phosphate Buffer solution pH 7.2 (PBS) was used for washing the mucosa. The suspensions were centrifuged at 1,200 rpm at 4°C before collecting the pellets. The microorganisms in these pellets were grown in De Mann-Rogosa-Sharpe broth (MRS; Merck™; Merck KGaA, Darmstadt, Germany) under CO_2 atmosphere at 37°C for 48 hrs. Afterwards, lactic acid bacteria were isolated on Rogosa SL agar (Rogosa; Bacto™; Becton Dickinson, Sparks, MD, USA) under CO_2 atmosphere at 37°C for 48 hrs. Each isolated colony was kept in glycerol medium for storing in the freezer until use. One thousand isolates of lactic acid bacteria were recruited and then recultured in MRS broth and Rogosa agar at the above condition.

1.2 Selection of antimicrobial lactic acid bacteria

Staphylococcus aureus ATCC 25923, *Sarcina lutea* ATCC 9341, *Escherichia coli* ATCC 25922 and *Bacillus subtilis* ATCC 6633 which cultured in Tryptic Soy broth (TSB; Bacto™; Becton Dickinson) were performed as the tested strains (226) for the primary screening of antimicrobial lactic acid bacteria by using the agar cup diffusion method (227). Each tested strain was cultured in TSB at 37°C for 24 hrs before adjusting a turbidity of the culture as equal to McFarland Standard No. 0.5. The suspension was swabbed onto Tryptic Soy agar (TSA; Bacto™; Becton Dickinson). Each isolate of lactic acid bacteria was cultured in MRS broth under CO_2 atmosphere at 37°C for 24 hrs. The cell-free supernatant was harvested from culture by centrifuging with 4,800 rpm at 4°C for 30 min. This cell-free supernatant was filled into the cylinder cup which placed on the swabbed agar and then grown at 37°C for 24 hrs. The antimicrobial activity was assessed according to their inhibition zones. Among 1,000 isolates of lactic acid bacteria, 10% of them were selected for secondary antimicrobial screening. In this procedure, the broad spectrum of

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antimicrobial activity against *S. aureus* ATCC 25923, *S. lutea* ATCC 9341, *E. coli* ATCC 25922, *B. subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus epidermidis*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Enterococcus faecalis*, *Streptococcus mitis*, *Streptococcus mutans* and *Porphyromonas gingivalis* W50 were also determined. *S. aureus, S. lutea, E. coli, B. subtilis, P. aeruginosa, S. epidermidis*, *K. pneumoniae* and *B. cereus* were separately cultured in TSB while *E. faecalis, S. mitis* and *S. mutans* were separately cultured in Brain-Heart Infusion broth (BHI; Difco™; Becton Dickinson, Sparks, MD, USA) under the CO_2 atmosphere at 37°C for 24 hrs and *P. gingivalis* were cultured in BHI broth under the anaerobic atmosphere at 37°C for 48 hrs. The 10% potent antimicrobial lactic acid bacteria were selected. All experiments and results were described in my special problem report. In the present study, three isolates of lactic acid bacteria which possessed the strongest antimicrobial activity were recruited for their characterization on the activity.

1.3 Identification of potent antimicrobial lactic acid bacteria (15)

The potent antimicrobial isolates of lactic acid bacteria were identified according to their biochemical profiles by using the API-50 CHL test kit (API®50 CHL; BioMérieux, Durham, NC, USA). Each isolate was cultured on MRS agar under the CO_2 atmosphere at 37°C for 48 hrs. The bacteria were picked up by using a swab and prepared a heavy suspension in the MRS broth. The inoculum was adjusted the optical turbidity as equal to McFarland Standard No. 2 by transfering a certain number of drops of bacterial suspension into the MRS broth. The twice drops of suspension were inoculated into the API-50 CHL medium test kit (API®50 CHL Medium; BioMérieux) and the suspension was filled into the biochemical test kit and covered with mineral oil. The culture was incubated under aerobic atmosphere at 37°C for 48 hrs. The result was recorded at 24 and 48 hrs of incubating times and the biochemical profile obtained from each strain was identified using the identification software (API®50 CHB/E databases; BioMérieux). BBL anaerobe identification kit (BD BBL CRYSTAL™ Identification Systems; Becton Dickinson) was used to confirm these identifications. Each cultured isolate was inoculated into BBL culture fluid (BBL CRYSTAL™ ANR Inoculum Fluid; Becton Dickinson) and adjusted the

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optical turbidity as equal to McFarland Standard No. 4. The suspension was placed into the reaction kit and incubated at 37°C in the moist chamber. The reaction was observed with light source for biochemical reactions and UV light for fluorescent reactions after 4 hrs of incubation and interpreted according to the BBL software (BBL CRYSTAL™ System Electronic Codebook; Becton Dickinson). The standard strains of lactobacilli, namely, *Lactobacillus casei* TISTR 390, *Lactobacillus fermentum* TISTR 055, *Lactobacillus plantarum* TISTR 541 and *Lactobacillus rhamnosus* TISTR 108 that provided by TISTR Culture Collection Bangkok MIRCEN, Thailand Institute of Scientific and Technological Research (TISTR) were tested in parallel.

1.4 Determination of casein utilization (15)

This determination was carried out to differentiate two species of *Lactobacillus* namely *L. casei* and *L. paracasei* based on the activity of caseinase enzyme which produced by the tested bacteria. The potent antimicrobial isolate was cultured in MRS broth at 37°C under CO_2 atmosphere for 24 hrs. The culture was filled into each well of Skim Milk agar (see Appendix A). The hydrolysis zone was assessed to the casein utilization activity after incubating at 37°C under CO_2 atmosphere for 48 hrs. *L. casei* TISTR 390 was tested in parallel.

1.5 Determination of antimicrobial susceptibility

The susceptibilities of the potent antimicrobial isolates toward various antimicrobial agents were monitored. The method was performed by the agar-disc diffusion method (228). Blood agar plate was swabbed with each cultured isolate which adjusted the optical turbidity as equal to McFarland Standard No. 0.5. Each tested antibiotic disc was placed on the agar plate. The inhibition zones were measured after incubation at 37°C under CO_2 atmosphere for 24 hrs and the susceptibilities were interpreted according to the table of antibiotic susceptibility provided by Oxoid (Oxoid®; Basingstoke, Hampshire, England). The zone diameter of *S. aureus* ATCC 25923 was used as the interpretive standard indicator. The minimal inhibitory concentration (MIC) of antibiotics which exhibited the resistance pattern in each isolate was determined by using E-test method (E-test®; AB

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BIODISK, Solna, Sweden). This determination was performed according to E-test method (228) as follows. One million cells/ml of the tested suspension were swabbed on the blood agar plate. An E-test antibiotic strip was placed on the agar plate and incubated at 37°C under CO_2 atmosphere for 24 hrs. MIC was determined by observing the inhibition zone.

1.6 Analysis of growth curve and generation time (229)

The amounts of 10^4 CFU/ml of each isolate were performed as the starting inoculum for growth curve analysis by culturing them in MRS broth under CO_2 atmosphere for 48 hrs. Every hour, their cultures were collected and measured the optical density at 600 nm. The growth curve was plotted according to the optimal density of each time. Each viable count of them at early- and mid-log phase was determined by spreading on MRS agar and incubating under CO_2 atmosphere for 36 hrs. The recovered colonies were counted in the colony forming unit and the generation time was also calculated during the period of early- and mid-log phase as the following formula,

$$\text{Generation time} = \frac{0.3 \times T}{\text{Log } B_f - \text{Log } B_i}$$

B_f = final number bacteria (CFU/ml)

B_i = initial number bacteria (CFU/ml)

T = total time

2. Characterization of cell-free supernatants

2.1 Preparation of cell-free supernatants

The amount of 10^3 CFU/ml of each isolate was inoculated into MRS broth and cultured at 37°C under CO_2 atmosphere for 24 hrs. The cell-free supernatant was harvested by centrifuging the fresh culture with 4,800 rpm at 4°C for 30 min before filtering through a 0.45 μm syringe filter (Minisart®; Sartorius AG, Goettingen, Germany).

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2.2 Determination of pH sensitivity (89)

The aim of this treatment was to determine the active condition of each cell-free supernatant which still possessed the high residual antimicrobial activity. Because of the lactic acid, other acids and acidic bacteriocins in each cell-free supernatant, the high activity is going to be presented in acid condition. On the other hand, if the activity is going to be presented in alkaline condition, it seems to be caused by the basidic proteins. The cell-free supernatant of each potent antimicrobial isolate was separately adjusted to a pH range from 1 to 14 by using 1N NaOH or 1N HCl. The adjusted supernatants were tested the antimicrobial activity against *P. gingivalis* W50 by using the agar-cup diffusion method. The inhibition zones were analysed as the percentage of residual activity by calculating as follows:

$$\text{Percentage of residual activity} = 100 \times \frac{\text{(zone of pH-adjusted supernatant)}}{\text{zone of original supernatant}}$$

The activity of each test was compared with the original supernatant which were non-adjusted pH (pH 4.5) and MRS broth (pH 6.2) as the controls.

2.3 Determination of heat sensitivity (89)

Due to the high temperature could affect the activity of some compounds containing in cell-free supernatant, heat treatments at high temperatures were performed to determine the residual activity. The aim of this treatment was to determine the antimicrobial acitivity of cell-free supernatant after heated. The cell-free supernatant of each potent antimicrobial isolate was separately incubated in water bath at 60, 80, 100°C for 30 min and at 121°C for 15 min by autoclaving. The antimicrobial activity against *P. gingivalis* W50 of each supernatant was done as described above. The inhibition zones were analysed as the percentage of residual activity by calculating as follows:

$$\text{Percentage of residual activity} = 100 \times \frac{\text{(zone of heated supernatant)}}{\text{zone of original supernatant}}$$

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The activity of each supernatant was compared with the original supernatant and MRS broth as the controls.

2.4 Determination of enzyme sensitivity (89)

The aim of this treatment was to characterize the antimicrobial activity of proteins or bacteriocins containing in each cell-free supernatant. Two proteolytic enzymes, trypsin-EDTA (Gibco™; Invitrogen, Grand Island, NY, USA) and pepsin (Sigma®; Sigma chemical, St. Louis, MO, USA), were performed in this study. It seems to be the activity that caused from proteinaceous compounds. The cell-free supernatant was separately treated with 0.5, 0.25 and 0.125 g/l of trypsin or pepsin at 37°C for 1 hr then inactivated the enzyme by heating at 75°C for 10 min. The antimicrobial activity against *P. gingivalis* W50 of each supernatant was done as described above. The inhibition zones were analysed as the percentage of residual activity by calculating as follows:

$$\text{Percentage of residual activity} = 100 \times \frac{\text{(zone of digested supernatant)}}{\text{zone of non-digested supernatant}}$$

The activity of each supernatant was compared with the non-digested supernatant and MRS broth as the controls.

2.5 Extraction of crude bacteriocins by ammonium sulphate precipitation (13)

Various percentage (w/v) of AR grade ammonium sulphate (Merck™; Merck KGaA), 20%, 40%, 60% and 80%, were separately added into 500 ml of each freshly prepared cell-free supernatant and MRS broth by stirring on magnetic stirrer at 4°C for 1 hr. The precipitant was harvested by centrifuging with 4,200 rpm at 4°C for 30 min. The supernatant was removed. The protein pellet was resuspended with sterilized PBS pH 7.2 in the twice volume of the pellet. The dissolved proteins were dialysed with the sterilized PBS pH 7.2 by using the dialysis membrane under the cut off = 7.5 kDa (Snake Skin® Pleated Dialysis Tubing; Pierce Biotechnology, Rockford, IL, USA) at 4°C for 24 hrs. This dialysis was done on the continuous magnetic

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stirrer. The dialysis buffer was changed every 6 hrs. The dialysed proteins were ultrafiltered through desalting media column (HiTrap™ Desalting; Amersham Biosciences AB, Uppsala, Sweden). The desalted protein which trapped in the media column was eluted with 1 column volume of the sterilized PBS pH 7.2, and then concentrated with the Vivaspin 6 membrane under the cut off = 10 kDa (Vivascience AG; Hannover, Germany) by using the centrifugation with 3,000 rpm at 4°C for 30 min. The concentrated protein or called as crude bacteriocins of each precipitation was determined the antimicrobial activity against *P. gingivalis* W50 by using agar-cup diffusion method and also determine the total protein assay. Crude bacteriocins from the precipitation which possessed the strongest antimicrobial activity were selected as the potent crude bacteriocins, and this precipitation was selected to perform as the protocol for the bacteriocin extraction. The crude bacteriocins was taken in aliquots and stored at -20°C until use.

2.6 Total protein assay (13)

Total protein concentration was determined by using BCA protein assay (BCA™ Protein Assay Kit; Pierce Biotechnology). Twenty-five µl of each diluted crude bacteriocins and each dilution of bovine serum albumin standard were pipetted into a 96-microplate well (Costar®; Corning Incorporated, Brooklyn, NY, USA). Two hundred µl of BCA working reagent was pipetted into each well and mixed on a plate shaker for 30 sec. The reactions were incubated at 37°C for 30 min. The absorbance of all wells were measured at 590 nm and then corrected with the blank absorbance as called as the blank-corrected measurement. The standard curve was plotted between the blank-corrected measurement of each BSA standard and its concentration in µg/ml. Total protein concentration of crude bacteriocins was determined by the absorbance calculation from the BSA standard curve.

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2.7 One-dimensional polyacrylamide gel electrophoresis (13)

This study was used to analyze the size or protein pattern of bacteriocins in the SDS-PAGE gel. Thirty µg of crude bacteriocins and the precipitated MRS broth were separately denatured with the biotechnology grade sodium dodecyl sulphate, biotechnology grade (AMRESCO®; AMRESCO, Solon, OH, USA) and β-

mercaptoethanol (BIO-RAD®; BIO-RAD Laboratory, Hercules, CA, USA), before boiling at 95°C for 10 min in water bath. These denatured proteins were loaded into the wells of 12% SDS-polyacrylamide gel (BIO-RAD®; BIO-RAD Laboratory) as the separating gel and 4% SDS-polyacrylamide gel (BIO-RAD®; BIO-RAD Laboratory) as the stacking gel. The proteins were electrophoresised in SDS-PAGE chamber (Mini-PROTEAN® III cell; BIO-RAD Laboratory) under 50 volts constant current condition for stacking gel and 90 volts for resolving gel in the running buffer pH 8.3 until the bromophenol tracking dye (Merck™; Merck KGaA) reached the bottom of the resolving gel. The proteins were visualized by staining with 0.1% Coomassie Brilliant Blue R250 (BIO-RAD®; BIO-RAD Laboratory) in acetic acid - methanol solution for 2 hrs on the rocking shaker and 3 times destaining with acetic acid - methanol solution for 30 min.

2.8 Two-dimensional polyacrylamide gel electrophoresis (230)

The molecular size and charge of crude bacteriocins were separated by 2D-PAGE using Immobilized pH Gradients (IPG). The protein samples were desalted 4 times by using 2D clean-up reagent kit (Ettan™ Sample Preparation Kits; Amersham Biosciences). In the first dimension, the isoelectric focusing was performed in IPG gradient strips pH 3-10 for a length of 7 cm (Immobiline™ DryStrip; Amersham Biosciences). Fifty µg of cleaned protein were loaded directly onto the strip in a strip holder and rehydrated in rehydration buffer at room temperature for 12 hrs. The rehydrated IPG strip was rinsed with deionized water for a second and placed on the electrophoresis chamber (Multiphor II Electrophoresis System; Pharmacia Biotech AB™, Uppsala, Sweden). The acidic end of the IPG strip was faced toward the anode and multiple run for focusing. The running condition of IEF was included step-and-hold mode under 300 volts at 0.2 kVh for 30 min, gradient mode under 1,000 volts at 0.3 kVh for 30 min, gradient mode under 5,000 volts at 4.0 kVh for 1 hr and step-and-hold mode under 5,000 volts at 2.0 kVh for 2 hrs. The running condition for pH 3-10 IPG strip was galvanized with 50 µA per strip at 20°C. After running, the IPG strips were equilibrated twices, for 15 min/time in 10 ml equilibration buffer by the rocking shaker. The equilibrated IPG strips were rinsed with deionized water for a second and placed on a filter paper for a few minutes to drain out the excess equilibration buffer.

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In the second dimension, the molecular sizes were analysed by SDS-PAGE. The equilibrated IPG strips were placed on the top of the vertical 12.5% SDS-polyacrylamide gel (RapidGel™-40% Acrylamide/Bis-Acrylamide19:1; Amersham Biosciences) and overlayered with 2 ml of warm agarose solution (BIO-RAD®; BIO-RAD Laboratory). Electrophoresis was done in SDS-PAGE chamber under 100 volts constant current condition in 1X SDS electrophoresis buffer pH 8.3 until the bromophenol blue (PlusOne™; Amersham Biosciences) tracking dye migrated to the lower end of the gel. After running, the overlayered agarose sealing solution was removed from the polyacrylamide gel and separated proteins in the gel were immobilized by fixing with acetic acid-methanol solution. These proteins were visualized by using 0.1 % Coomassie Brilliant Blue G-250 (Coomassie™ Brilliant Blue G-250; Amersham Biosciences) stain for 2 hrs and twice times destained with acetic acid - methanol solution for 30 min.

3. Partial purification of crude bacteriocins

3.1 Anion exchange column chromatography (108)

One ml-size anion exchange column (HiTrap™ Q FF; Amersham Biosciences) chromatography was performed by using Fast Protein Liquid Chromatography apparatus (FPLC; ÄKTA™ explorer; Amersham Biosciences). The column was equilibrated by 5 column volumes of 20 mM Tris-HCl pH 8.0 (buffer A) before 2 ml of crude bacteriocins were applied into column. The unbound substance was washed out by 10 column volumes of buffer A. The anionic proteins were eluted by 20 mM Tris-HCl pH 8.0 supplementing with 1.0 M NaCl (buffer B) in 3 segmented gradients, including a step of 0 column volume at 35% NaCl, 25 column volumes at 55% NaCl and 10 column volumes at 100% NaCl with flow rate 1.0 ml/min. One ml of each eluted fraction was collected into aliquot and then monitored the absorbance at 280 nm. Each collected fraction was concentrated with the above membrane kit. The concentrated fraction proteins were assessed the total protein concentration by using BCA protein assay kit and then observed by SDS-PAGE.

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3.2 Determination of the antimicrobial activity (227)

This experiment was assessed the pooled fraction which possessed the highest antimicrobial activity after the purification. The residual antimicrobial activity of each pooled fraction against *P. gingivalis* W50 was determined by using agar-cup diffusion method as described above. The average inhibition zone of each pooled fraction was compared with the zone demonstrated by the freshly prepared cell-free supernatant of each lactobacillus isolate and calculated the percentage of residual activity as follows:

$$\text{Percentage of residual activity} = 100 \times \frac{\text{(zone of pooled fraction)}}{\text{zone of cell-free supernatant}}$$

The pooled fraction demonstrated the highest percentage of residual activity was carried to the further study.

3.3 Effect of the solvent towards the antimicrobial activity (227)

This experiment was assessed the appropriate diluent which exhibited the strongest antimicrobial activity of the partially purified bacteriocin. The antimicrobial activity against *P. gingivalis* W50 of the partially purified bacteriocins was also determined by using agar-cup diffusion method. The unbound pooled fractions and peak pooled fractions were dissolved in Tris-HCl pH 8.0, sodium acetate buffer pH 5.0, MRS broth and modified MRS broth (mMRS; see Appendix A) as the tested solvents. The proteins were 2-fold serially diluted with each solvent. The forth dilution was determined the antimicrobial activity. The average inhibition zone of a protein solution was compared with the freshly prepared cell-free supernatant of each lactobacillus isolate and calculated the percentage of residual activity as follows:

$$\text{Percentage of residual activity} = 100 \times \frac{\text{(zone of tested solution)}}{\text{zone of cell-free supernatant}}$$

The tested solution demonstrated the highest percentage of residual activity was carried to determine the bacteriocin activity.

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3.4 Determination of bacteriocin activity (231)

The bacteriocin activity or bacteriocin unit was determined by the serial dilution microtiter plate assay. The samples, including freshly cell-free supernatant, ammonium sulphate precipitated crude and partially purified bacteriocin were tested. Fifty μl appropriate diluent of each bacteriocin sample was dropped into each microtiter well. Each bacteriocin-containing sample was diluted in two-fold serial dilutions and 50 μl of 100-fold diluted *P. gingivalis* W50 which cultured in BHI broth were then dropped. The tested microtiter plate was incubated at 37°C under anaerobic atmosphere for 48 hrs. The growth inhibition of *P. gingivalis* was assessed by using a microplate reader (MultiskanEX; Labsystems, Helsinki, Finland) for measuring the absorbance at 590 nm. One bacteriocin unit was defined as the amount of bacteriocin which could inhibit the fifty percent growth of tested bacteria compared with the culture without bacteriocin. It was calculated from the reciprocal of last dilution demonstrating inhibititory activity and presented as bacteriocin unit or bacteriocin activity (BU) per milliliter as follows:

$$\text{Bacteriocin activity (BU/ml)} = \frac{\text{Titer at 50\% growth}}{\text{Bacteriocin sample volume}} \times 1{,}000 \times 2$$

The total activity (BU), the total protein (mg), the specific activity (BU/mg), the purification fold and the recovered activity (%) of bacteriocin were calculated as follow:

$$\text{Total activity (BU)} = \text{Bacteriocin activity} \times \text{Final sample volume}$$

$$\text{Total protein (mg)} = \text{Protein concentration} \times \text{Final sample volume}$$

$$\text{Specific activity (BU/mg)} = \frac{\text{Total activity}}{\text{Total protein}}$$

$$\text{Purification folds} = \frac{\text{Specific activity of each sample}}{\text{Specific activity of supernatant}}$$

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$$\text{Recovered activity (\%)} = \frac{\text{Total activity of each sample}}{\text{Total activity of supernatant}} \times 100$$

The partially purified bacteriocin of each isolate demonstrated the highest percentage of recovered activity was carried to determine the minimal inhibitory concentration and time killing assay.

3.5 The determination of minimum inhibitory concentration (MIC) (232)

The MIC of the partially purified bacteriocin was used to the concentration of protein in the time killing assay. The broth microdilution technique was performed to determine MICs of *P. gingivalis* W50. The two-fold serial dilutions of B85/4 supernatant and active anionic fractions in 100 µl BHI broth were prepared. Ampicillin was used as the parallel experiment. The bacterial suspension was then added to each well to achieve final bacterial cells of 5×10^5 CFU/ml. Wells containing bacterial cultures without antibiotic were also tested as the growth control. The cultures were inspected for OD. after incubation at 37°C under anaerobic atmosphere for 48 hrs. Wells shown OD. equal to media alone was defined as MIC.

3.6 Time killing assay (232)

This experiment was used to determine the minimal exposure time of active compound to kill the tested bacteria. One million CFU/ml of *P. gingivalis* W50 cultured in BHI broth was carried to determine the killing time. Each test tube containing the BHI broth and MIC of B85/4's supernatant or MIC or 2-folds MIC of the partially purified bacteriocin or MIC of ampicillin or the sterilized PBS pH 7.2 (as the control) were inoculated with 10^5 CFU/ml of the tested bacteria. The cultures were then incubated at 37°C under anaerobic atmosphere for various times of incubation. At the end of each time point, ten-fold serial dilutions of the tested experiments were prepared with the sterilized PBS pH 7.2 and then 20 µl of them were dropped onto anaerobic blood agar plates in duplicate. The viable count in CFU/ml for tested bacteria at different time points were counted after 24 hrs of the incubation and compared with the culture control in BHI broth. The CFU in the tested wells were calculated the percentage of growth inhibition as following formula.

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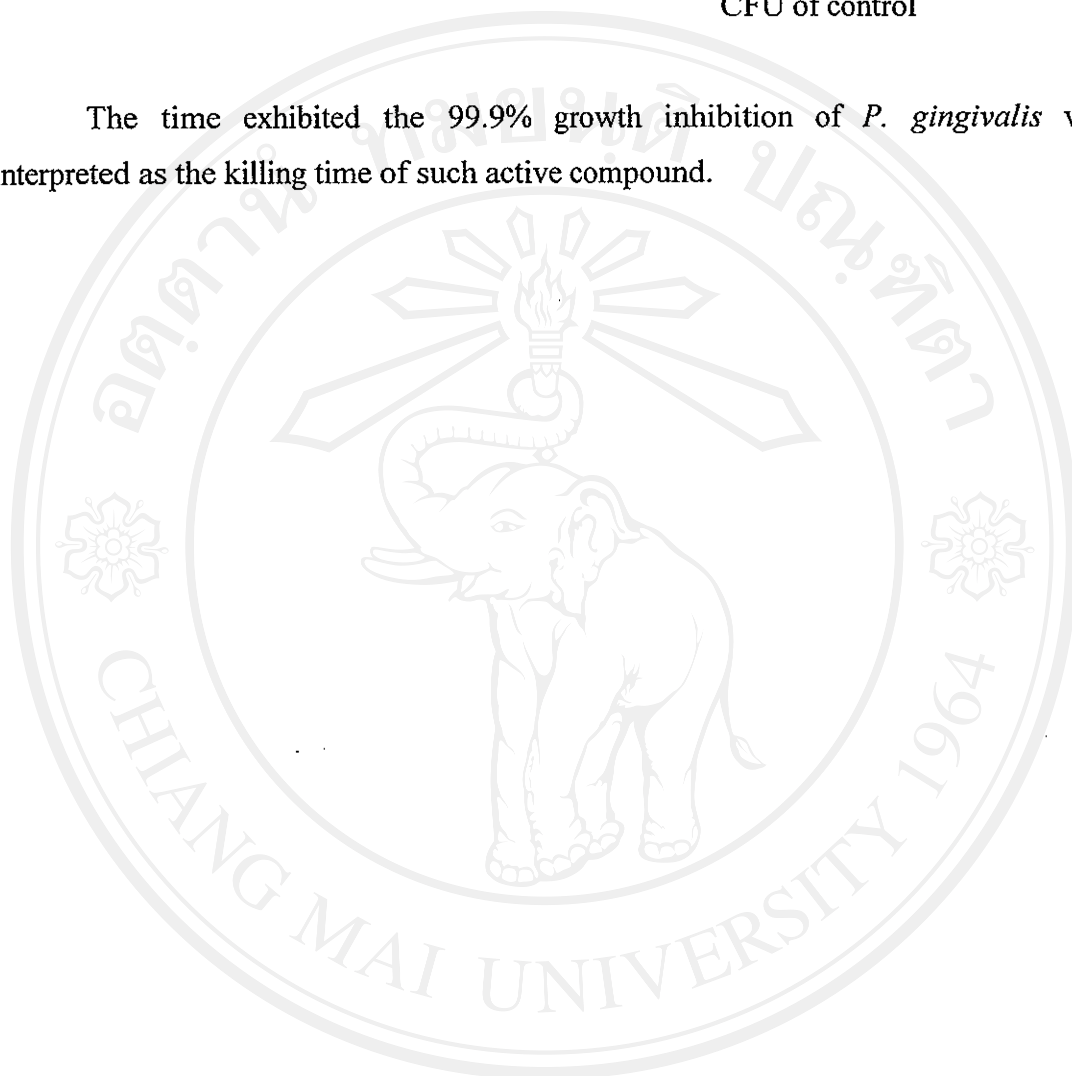
$$\text{Percentage of growth inhibition} = 100 \times \frac{\text{(CFU of control} - \text{CFU of tested)}}{\text{CFU of control}}$$

The time exhibited the 99.9% growth inhibition of *P. gingivalis* was interpreted as the killing time of such active compound.



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