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Research Note

Staphylococcal Enterotoxin A Gene–Carrying *Staphylococcus aureus* Isolated from Foods and Its Control by Crude Alkaloid from Papaya Leaves

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ABSTRACT

Staphylococcus aureus is a known pathogen causing intoxication by producing enterotoxins in food. Staphylococcal enterotoxin A is one of the enterotoxins commonly implicated in staphylococcal food poisoning. The ability of crude alkaloid extract from papaya leaves to inhibit the growth of *S. aureus* and staphylococcal enterotoxin A synthesis was investigated. Staphylococcal enterotoxin A gene–carrying *S. aureus* was isolated from raw milk and ready-to-eat foods. Crude alkaloid was extracted from ground, dried papaya leaves using ultrasonic-assisted extraction, and a MIC of the alkaloid was determined by the broth macrodilution method. Furthermore, *S. aureus* isolate was exposed to the crude alkaloid extract at one- and twofold MIC, and the expression of *sea* was subsequently analyzed using a quantitative reverse transcription real-time PCR. Ten isolates of *S. aureus* were obtained, and nine of those isolates were *sea* carriers. The yield of crude alkaloid extract was 0.48 to 1.82% per dry weight of papaya leaves. A MIC of crude alkaloid to *S. aureus* was 0.25 mg/ml. After exposure to the alkaloid at 0.25 and 0.5 mg/ml for 2 h, a significant increase in cycle threshold values of *sea* was observed. The *sea* was expressed 29 and 41 times less when *S. aureus* was exposed to crude alkaloid at one- and twofold MIC, respectively. This study revealed that crude alkaloid of papaya leaves could control staphylococcal enterotoxin A gene–carrying *S. aureus* by suppressing the expression of *sea*, in addition to the ability to inhibit the growth of *S. aureus*. The expression of *sea* was successfully quantified.

Staphylococcus aureus is a known pathogen, causing intoxication by producing staphylococcal enterotoxin in food. Staphylococcal enterotoxin A is one of the staphylococcal enterotoxins commonly implicated in staphylococcal food poisoning *(8, 28)* that is found in raw milk *(30)* and ready-to-eat food *(26)*. Staphylococcal enterotoxin is expressed from the mid-exponential phase of growth, synthesized from the *sea* gene. Staphylococcal enterotoxin A acts as a superantigen and stimulates the release of inflammatory cytokines, causing symptoms of poisoning, such as nausea and vomiting *(29)*.

Papaya plants (*Carica papaya*) are widely cultivated in tropical countries. In addtion to the usefulness of papaya fruit, papaya leaves are also consumed as vegetables, used to improve the tenderness of meat, and used as traditional medicine *(1, 19)*. To improve meat tenderness, the meat is usually wrapped in papaya leaves or marinated with pastes made from papaya *(1, 25)*. Furthermore, different solvent extracts of papaya leaves have been reported to have antimicrobial activity against both gram-positive and gram-negative bacteria, such as *Escherichia coli, S. aureus, Bacillus cereus, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa (3)*. The antimicrobial activity of plant extracts are usually associated with the phytochemical content, i.e., flavonoids *(13)*, tannins, terpenoids, and alkaloids *(9)*. Tannins and terpenoids can disrupt cell membranes *(3, 9)*, whereas alkaloids have been shown to intercalate into DNA and inhibit DNA synthesis *(6)*. Because papaya leaves have been reported to contain alkaloids, namely, carpaine, pseudocarpaine, and dehydrocarpaine I and II *(19)*, there is a potential action of papaya leaf extract to inhibit staphylococcal enterotoxin A production through alkaloid activity.

The effect of crude alkaloid extract from papaya leaves on the expression of *sea* can be studied by the quantitative reverse transcription PCR (qRT-PCR) technique. The qRT-PCR technique consists of reverse transcription, followed by real-time PCR (qPCR). The qPCR uses fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction *(27)*. In qPCR, the cycle threshold (C_T) will be obtained. C_T is the cycle number at which the reporter dye emission intensity rises through the threshold value. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed, and the lower the C_T will be *(5)*. This study was conducted to investigate the ability of crude alkaloid extract from papaya

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TABLE 1. *Nucleotide sequences of primers used in PCR analysis (20)*

Gene	Primer	Sequence (5′ → 3′)	PCR product
sea	SEA1	TTGGAAACGGTTAAAACGAA	120 bp
	SEA2	GAACCTTCCCATCAAAAACA	
16S rRNA	16sF	CCGCCTGGGGAGTACG	240 bp
	16sR3	AAGGGTTGCGCTCGTTGC	

leaves to inhibit the growth of *S. aureus* and staphylococcal enterotoxin A synthesis. Staphylococcal enterotoxin A synthesis was evaluated by relative quantification of *sea* expression by the qRT-PCR technique.

MATERIALS AND METHODS

Isolation and identification of *S. aureus* from food samples. *S. aureus* was isolated from raw cow's milk and ready-to-eat foods, i.e., egg dishes, sautéed chicken cuts, and chicken satay, purchased from a livestock and street food vendor in Bogor, Indonesia. The isolation procedure was according to the modified *Bacteriological Analytical Manual (4)*. A sample (25 g or 25 ml) was added to 225 ml of normal saline and homogenized in a stomacher (BagMixer 400P, Interscience, Saint Nom, France). The homogenate was serially diluted and spread onto Baird-Parker agar (Oxoid Ltd., Hampshire, UK) supplemented with egg yolk tellurite. Plates were incubated at 37°C for 18 to 24 h, and then presumptive colonies were picked and streaked on mannitol salt agar (Oxoid Ltd.). Typical colonies on mannitol salt agar were then tested for the production of catalase using Staphylase test kit (Oxoid Ltd.) and identified using API Staph (bioMérieux, Inc., Durham, NC), according to the manufacturer's instructions.

Detection of 16S rRNA and staphylococcal enterotoxin A encoding gene. The DNA of *S. aureus* ATCC 25923 and *S. aureus* isolates from foods was isolated using a method adopted from Mason et al. *(23)*, with minor modification, i.e., the use of 5 μl of lysostaphin (10 mg/ml) was substituted with 100 μl of lysozyme (Bio Basic Canada Inc., Markham, Ontario, Canada) solution (10 mg/ml). The DNA pellet was then dried and resuspended in 30 μl of sterile water.

The amplification of the gene encoding 16S rRNA and staphylococcal enterotoxin A was performed using the Thermal Cycler 2720 (Applied Biosystems, Foster City, CA). PCR master mix consisted of 12.5 μl of DreamTaq Green master mix (Thermo Fisher Scientific, Waltham, MA), 1 μl of each primer (10 μM; Table 1), 2 μl of DNA template, and 8.5 μl of nuclease-free water (Thermo Fisher Scientific). Cycling parameters were one denaturation cycle for 5 min at 95°C and 30 amplification cycles for denaturation (1 min at 95°C), annealing (1 min at 55°), extension (1 min at 72°C), and termination for 5 min at 72°C, adopted from Lee et al. *(20)*. The amplification product was analyzed by 1.5% agarose gel electrophoresis at 120 V for 35 min.

The 16S rRNA gene sequence of *S. aureus* isolates was obtained by sequencing and analyzed by nucleotide BLAST program. Phylogenetic analysis was conducted between 16S rRNA sequence of *S. aureus* isolates and 16S rRNA sequence of *S. aureus* ATCC 25923 that was obtained from the GenBank database. *S. aureus* isolates that had the closest relationship with *S. aureus* ATCC 25923 would be used in the next stage of this study.

Preparation of crude alkaloid. Healthy, disease-free, and mature fresh leaves of Calina papaya (IPB9) were collected from University Farm, Bogor Agricultural University (Bogor, Indonesia). The fresh leaves were rinsed thoroughly two to three times under running tap water and once with sterile water and dried at 55°C for 22 h using a vacuum oven (VWR A143 A-143, Sheldon Manufacturing, Inc., Cornelius, OR) *(7)*. Dried leaves were ground into a fine texture using an electric blender. The dried leaves were stored in sealed and labeled containers for later use. Moisture content of fresh and dried leaves was determined using an oven method, according to the AOAC International *(2)*.

The crude alkaloid was extracted by adopting the extraction method, described by Djilani et al. *(10)*. Ten grams of dried papaya leaves was suspended in 400 ml of sodium dodecylsulfate (Merck & Co., Kenilworth, NJ) solution (2%, mass/vol) in an Erlenmeyer flask and sonicated for 2.5 h at a temperature of 25 to 35°C in an ultrasonic bath (Bransonic Ultrasonic Cleaner model 8510E MTH, Branson Ultrasonic Corporation, Danbury, CT). The extract was separated by Whatman no. 1 filter paper (Sigma-Aldrich, St. Louis, MO), and the residual materials were washed with 20 ml of pure water. The solution of combined filtrates was acidified with sulfuric acid (Merck & Co.) solution (2%, vol/vol) to a pH of 3 to 4, and the alkaloids were precipitated with 15 ml of Mayer's reagent. The precipitate was then dissolved in sodium carbonate (Merck & Co.) solution (5%, mass/vol) and extracted by $CHCl_3$ (J.T. Baker, Center Valley, PA). The organic layer was washed by water to neutral pH, dried with Na_2SO_4 (Merck & Co.) to remove the remaining water, and concentrated to dryness using a rotary evaporator (Butchi Rotavapor R-210, BÜCHI Labortechnik AG, Flawil, Switzerland) and N_2 gas to obtain alkaloids. The extracts were collected from several extraction processes and combined into one solution using dimethyl sulfoxide (Merck & Co.) as the solvent.

Preparation of bacterial inoculum. The 18- to 24-h-old staphylococcal enterotoxin A gene–carrying *S. aureus* isolate culture in tryptic soy broth (TSB; Oxoid Ltd.), incubated at 37°C, was centrifuged at 9,500 × *g* for 10 min (Hermle Z383K, Hermle Labortechnik GmbH, Wehingen, Germany). The supernatant was discarded, and the pellet was resuspended in normal saline solution. The cell suspension was adjusted to an optical density at 625 nm of 0.08 that was equivalent to the McFarland 0.5 turbidity standard (10^8 CFU/ml).

MIC determination. MIC of crude alkaloid was determined using the broth macrodilution method *(24)*. One hundred microliters of bacterial suspension (10^6 CFU/ml) was inoculated into 1 ml of TSB containing crude alkaloid at 0.125, 0.25, 0.5 and 1 mg/ml. A tube containing inoculum and TSB (0 mg/ml of crude alkaloid) was used as a control. After incubation at 37°C at 150 rpm for 24 h, bacterial culture at each crude alkaloid concentration and control were diluted and spread on a tryptic soy agar (TSA) plate. The plates were incubated at 37°C for 48 h, and the bacterial cell number was counted. MIC_{90} was determined as the lowest concentration that resulted in inhibition of 90% of tested isolates compared with the number of the initial inoculum *(12)*. Each experiment was carried out three times.

Exposure of *S. aureus* to crude alkaloid of papaya leaves. One hundred microliters of staphylococcal enterotoxin A gene–

carrying *S. aureus* suspension (10^8 CFU/ml) was inoculated into 5 ml of TSB without crude alkaloid (control) and with crude alkaloid at one- and twofold MIC. The tubes were incubated at room temperature at 150 rpm for 2 h. The short exposure was designed to give mild exposure to the cells so that the reduction of cell number did not occur, but it could still affect the gene expression. Bacterial cell number, before and after the 2-h exposure, was determined using the spread plate technique on a TSA plate.

RNA extraction and cDNA synthesis. Two milliliters of treated *S. aureus* culture with crude alkaloid was centrifuged at 5,000 × *g* for 5 min. The *S. aureus* pellet was then suspended in 100 μl of 1× TE buffer (PEQLAB Biotechnologie GmbH, Erlangen, Germany) and 30 μl of lysozyme (PEQLAB Biotechnologie GmbH) solution (10 mg/ml). The RNA was extracted using a peqGOLD Bacterial RNA Kit (PEQLAB Biotechnologie GmbH), according to the manufacturer's instructions. The residual DNA was digested using a DNaseI, RNase free (Thermo Fisher Scientific) treatment. RNA concentration and purity (A_{260}/A_{230} and A_{260}/A_{280} ratios) were assessed using a UV-1800 Spectrophotometer (Shimadzu, Kyoto, Japan).

One microliter of total RNA was then applied in a 20-μl reverse transcription to synthesize cDNA using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The reverse transcription reactions were performed for 1 h at 42°C, and the reverse transcriptase inactivated for 5 min at 70°C, as described in the manufacturer's protocol.

Relative quantification of *sea* expression. The qPCR analysis was performed using Swift Spectrum Themal Cycler 48 (Esco Healthcare Pte. Ltd., Singapore). PCR master mix consists of 1 μl of the relevant cDNA, 0.8 μl of each primer (10 μM; Table 1), 10 μl of KAPA SYBR FAST qPCR Kit Master (Kappa Biosystems, Woburn, MA), and nuclease-free water, up to 20 μl of reaction volume. Cycling parameters were one denaturation cycle for 5 min at 95°C and 45 amplification cycles for denaturation (1 min at 95°C), annealing (1 min at 55°C), extension (1 min at 72°C), and termination for 5 min at 72°C *(20)*. Fluorescence readings were taken after each extension step.

During relative quantification by the qRT-PCR technique, the changes in gene expression in the sample treated with crude alkaloid were measured relative to the calibrator sample and the reference gene. The C_T values of the target gene were compared directly to an internal reference C_T, and the results were expressed as ratios of the target-specific signal to the internal reference *(36)*. The expression of *sea* was calculated relative to the calibrator sample and an internal control using a comparative C_T method ($2^{-\Delta\Delta C_T}$) *(33)*. In this study, the 16S rRNA gene was used as the reference gene (internal control), whereas the sample without the crude alkaloid addition was used as the calibrator sample. The experiment was performed three times.

Data analysis. Data were analyzed by SPSS version 16.0 (Chicago, IL) using one-way analysis of variance. Significance was defined at $P < 0.05$ by using Duncan's multiple range test. The results were expressed as means ± standard deviations.

RESULTS AND DISCUSSION

Presence of *sea* carrier *S. aureus*. All food samples were found to be contaminated with *S. aureus*. There were a total of 78 presumptive *S. aureus* isolates on Baird-Parker agar, but only 65 isolates showed a typical colony of *S. aureus* on mannitol salt agar, i.e., could change the color of agar to yellow. All 65 tested isolates were coagulase positive, and from 13 isolates tested using API Staph, 10 isolates (76.9%) were positive for *S. aureus*, followed by *Staphylococcus lentus* (15.4%) and *Staphylococcus xylosus* (7.7%).

TABLE 2. *The presence of 16S rRNA and* sea *gene in* S. aureus *isolates and* S. aureus *ATCC 25923*[a]

	Food source	16SrRNA	*sea*
S. aureus ATCC 25923	—	+	−
S. aureus S1	Raw milk	+	+
S. aureus S4	Raw milk	+	+
S. aureus S10	Raw milk	+	+
S. aureus TB1	Egg dishes	+	+
S. aureus TB10	Egg dishes	+	−
S. aureus UA1	Sautéed chicken cuts	+	+
S. aureus UA2	Sautéed chicken cuts	+	+
S. aureus UA13	Sautéed chicken cuts	+	+
S. aureus SJ1	Chicken satay	+	+
S. aureus SJ4	Chicken satay	+	+

[a] +, present; −, absent.

Furthermore, all of the obtained *S. aureus* isolates exhibited 240 bp of the 16S rRNA gene PCR product after amplification using 16sF and 16sR3 primers, the same as the *S. aureus* ATCC 25923 as a positive control (Table 2). Nine of 10 *S. aureus* isolates showed 120 bp of *sea* PCR product that indicated the ability to produce staphylococcal enterotoxin A. On the other hand, *S. aureus* TB10 from egg dishes, such as *S. aureus* ATCC 25923, did not have *sea*, confirming that this strain did not produce staphylococcal enterotoxin A (Table 2). The results of the 16S rRNA gene sequence analysis using BLAST showed that the 10 isolates were *S. aureus* (data not shown). This case confirmed the identification results, using API Staph. Phylogenetic analysis results showed that *S. aureus* SJ1 had the closest relationship with *S. aureus* ATCC 25923 (data not shown).

Primers SEA1 and SEA2 were also used by Rall et al. *(32)* to detect the presence of *sea* in *S. aureus* isolates from raw and pasteurized milk using PCR. Among the genes that encode the classic enterotoxins (A through E), *sea* was the most commonly found (41%), followed by *sec* (20.5%), *sed* (3.7%), *seb* (7.7%), and *see* (5.1%).

MIC of crude alkaloid of papaya leaves to *S. aureus*. The moisture content of fresh mature papaya leaves used in this study was 78.17% ± 0.10%, while the dried leaves was 12.06% ± 0.29%. The crude alkaloid extract, collected from seven different extraction processes, yielded a range of 0.48 to 1.82% per dry weight of papaya leaves. Crude alkaloid at a concentration of 0.25, 0.5, and 1 mg/ml was able to reduce the cell number of *S. aureus* SJ1. MIC_{90} observed for crude alkaloid to *S. aureus* SJ1 was 0.25 mg/ml (Fig. 1). At this concentration, crude alkaloid could reduce by 1 log CFU/ml (90%) the bacterial cell number from 4.65 to 3.67 log CFU/ml (Fig. 1). MIC of crude alkaloid to *S. aureus* in this study was lower than MIC of ethanolic extract of papaya leaves found by Rahman et al.

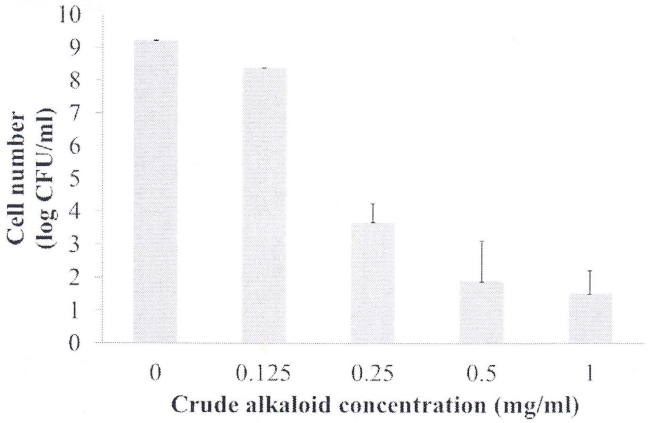

FIGURE 1. *Ability of crude alkaloid of papaya leaves at various concentrations, by macrodilution test after 24-h incubation, to inhibit and or reduce the cell number of* S. aureus *SJ1, with an initial level of 4.65 log CFU/ml. Values are means of three replicates, and error bars represent standard deviations.*

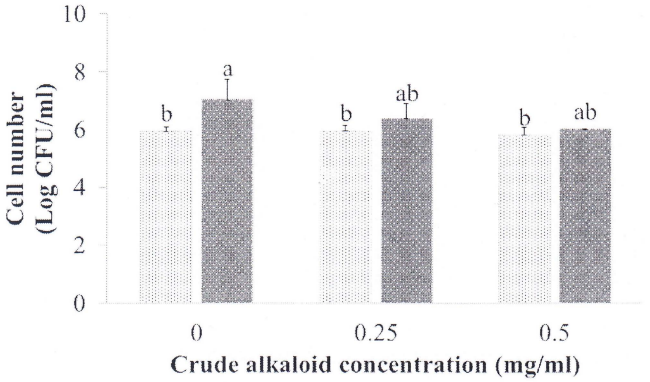

FIGURE 2. *Cell number of* S. aureus *SJ1 before (▨) and after (▩) exposure with crude alkaloid of papaya leaves at zero-, one- and twofold MIC for 2 h. Values are means of three replicates, and error bars represent standard deviations. Means with different letters are significantly different* ($P < 0.05$).

(31), i.e., in a concentration of 1.25 mg/ml. This crude alkaloid MIC was also lower than the crude alkaloid extract from *Tribulus terrestris* leaves against *S. aureus* that was in a concentration of 0.625 mg/ml *(16)*. On the other hand, a MIC of papaya leaf crude alkaloid extract was higher than that of the alkaloid extract from *Aconitum elwesii* and *Sida acuta* Burm. f. leaves, i.e., 0.094 and 0.078 mg/ml, respectively *(15, 34)*.

Effect of crude alkaloid to the cell number of *S. aureus* and expression of *sea*. Exposure of crude alkaloid to *S. aureus* SJ1 at one- and twofold MIC for 2 h resulted in a lower bacterial number in comparison to the control (Fig. 2). In contrast with the results of MIC determination in which the cell number without alkaloid exposure increased 4.56 log CFU/ml, the cell number of the control (no alkaloid extract) increased only 1.09 log CFU/ml after 2-h incubation (Figs. 1 and 2). On the other hand, the bacterial cell number at 0.25 and 0.5 mg/ml decreased 0.98 and 2.77 log CFU/ml, respectively, after the 24-h incubation with MIC determination, whereas the cell number slightly increased with 0.42 and 0.21 log CFU/ml, respectively, after 2-h incubation. However, these increases were not significantly different (Fig. 2). This short incubation (2 h) gave mild exposure, so the alkaloid did not inactivate and decrease the cells but still affected the expression of *sea*. The increase of bacterial cell number at the early exposure time with the antimicrobial compound was also found in Hermsen et al. *(14)*. Methicilin-susceptible *S. aureus* cell number increased until the fourth hour of exposure time and decreased about 3 log CFU/ml at the 16th hour when it was exposed to the cefazolin antibiotic in cation-adjusted Mueller-Hinton broth media at pH 7.4.

In comparison to the growth of control, crude alkaloid at 0.25 mg/ml reduced 61% of the cell numbers and crude alkaloid at 0.5 mg/ml reduced 81% of the cell numbers. These findings supported the results of other researches that found decreasing *S. aureus* numbers after treatment with alkaloids *(18, 21)*. Although the exposure to crude alkaloid up to 0.5 mg/ml for 2 h resulted in a slight increase of the cell numbers, the decline of *sea* relative expression was observed. The results indicated that exposure to the papaya leaf crude alkaloid caused a significant increase in the C_T value of *sea*, but the C_T values of 16S rRNA gene did not significantly increase (Table 3). The C_T value was inversely proportional to the initial number copies of DNA: the higher the C_T value, the lower the initial amount of DNA. Because the C_T values of the 16S rRNA gene were relatively constant (increased insignificantly) indicated that the cell numbers were similar in all samples. The 16S rRNA gene is a housekeeping gene that is always expressed because it codes for 16S ribosomal RNA and is involved in protein metabolism. This gene is used as an internal standard (control) in qPCR and can serve as a marker for the cell numbers in a sample *(35)*.

Furthermore, amplification of *sea* without exposure to crude alkaloid (0 mg/ml) required 17.38 cycles until the fluorescence signal could pass through the threshold. After exposure to 0.25 and 0.5 mg/ml of crude alkaloid for 2 h, the amplification of *sea* required more cycles to pass through the threshold, indicating a decrease of the initial amount of *sea* cDNA, which meant less mRNA was synthesized from *sea*. Lee et al. *(20)* comprehensively studied the expression of enterotoxin genes in *S. aureus* by a reverse transcription qPCR that facilitates detection on an RNA level. Various levels of expression were found, depending on the species and enterotoxin gene. Duquenne et al. *(11)* used qRT-PCR to study the expression of *sea* and *sed* during cheese manufacture. Expression levels of *sea* remained essentially unchanged during the first 72 h of cheese manufacture, whereas *sed* expression decreased.

Relative expression value of *sea* was represented by the value of $2^{-\Delta\Delta C_T}$. ΔC_T was obtained from the difference between C_T of *sea* and 16S rRNA gene. The $\Delta\Delta C_T$ could be obtained from the difference between ΔC_T of the exposed sample (0.25 and 0.5 mg/ml of crude alkaloid) and ΔC_T of the calibrator sample. Then, the $\Delta\Delta C_T$ value was entered into the $2^{-\Delta\Delta C_T}$ equation to obtain the relative expression value (Table 3). The *sea* was expressed 29 times less in *S. aureus* SJ1 that was exposed to 0.25 mg/ml crude alkaloid and 41 times less in *S. aureus* SJ1 that was exposed to crude

TABLE 3. *Relative expression of* sea *of* S. aureus *SJ1 exposed to crude alkaloid of papaya leaves for 2 h, calculated using a comparative* C_T *method*

Crude alkaloid concn (mg/ml)	C_T *sea*[a]	C_T 16S rRNA gene[a]	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta C_T}$
0	17.38 ± 0.28 B	15.26 ± 0.41 C	2.12	0	1.000
0.25	22.58 ± 1.07 A	15.63 ± 0.17 C	6.95	4.83	0.035
0.5	23.15 ± 0.62 A	15.69 ± 0.58 C	7.47	5.35	0.025

[a] Values are means ± standard deviations of three replicates. Means with different letters are significantly different ($P < 0.05$).

alkaloid at 0.5 mg/ml, compared with *S. aureus* SJ1 without crude alkaloid exposure. This comparative C_T method ($2^{-\Delta\Delta C_T}$) was also fruitfully used to calculate the relative expression of target genes in human peripheral blood mononuclear cells by Kharaji and Haghparast *(17)* and target genes in the human glioblastoma cell line by Malvandi et al. *(22)*.

This study highlighted that the crude alkaloid of papaya leaves possessed antibacterial activity against *S. aureus* that not only inhibited bacterial growth but also inhibited the expression of *sea*. In particular, the present study successfully examined the expression of *sea* quantitatively. Considering that papaya leaves are often applied to improve tenderness of meat in Asian and African countries, this practice will give an additional benefit to improve the safety of meat. During the wrapping of meat with papaya leaves or marinating with papaya leaf pastes, the active compound, including alkaloid, will be in contact with bacteria and might result in the inhibition of the growth and forming of enterotoxins.

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