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Research Paper

Star Anise (*Illicium verum* Hook. f.) as Quorum Sensing and Biofilm Formation Inhibitor on Foodborne Bacteria: Study in Milk

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ABSTRACT

Bacteria use quorum sensing (QS) systems to communicate with each other and regulate microbial group behavior, such as the secretion of virulence factors, including biofilm formation. In order to explore safe, edible agents, the potential of star anise (SA) as an anti-QS and antibiofilm agent and its possible application in milk safety were investigated. *Staphylococcus aureus, Salmonella* Typhimurium, *Pseudomonas aeruginosa,* and biosensor strain *Chromobacterium violaceum* were selected as test strains for QS, biofilm, and exopolysaccharide assays. The percent acidities and total plate counts were determined to evaluate the quality of biofilm-inoculated and noninoculated milk. The yield of SA extraction was 25.90% ± 0.2% (w/w). At sub-MIC, SA extract did not show any effect on bacterial growth. The production of violacein was inhibited by 89% by SA extract. The extract also inhibited the formation of biofilm by up to 87% in a dose-dependent manner. Inhibition rates of 70.45%, 42.82%, and 35.66% were found for exopolysaccharide production. The swarming motility of *S. aureus* was reduced by about 95.9% by SA extract. Confocal laser scanning microscopy analysis confirmed that the development of biofilm architecture was hampered. It was found that SA extract could delay the spoilage of milk. In the endeavor to avoid drug resistance, pathogenesis, and resistance to biocides while improving food safety and avoiding health hazard issues arising from synthetic chemicals, SA extract could be used as a potential QS and biofilm inhibitor.

Key words: Biofilm; Foodborne pathogens; Milk; Quorum sensing; Star anise

Many bacterial species regulate gene expression in response to changes in cell population density by the production, release, and subsequent detection of chemical signaling molecules called autoinducers through a mechanism called quorum sensing (QS) *(3, 10, 35)*. By using QS mechanisms, bacteria work like multicellular organisms and express different virulence phenotypes and factors, such as biofilm formation, bioluminescence, sporulation, pathogenesis, etc. *(28)*. Biofilms are three-dimensional, architecturally complex structures that protect microbes from hostile environmental factors and generally consist of exopolymeric substances and glycoprotein matrix. It is now known from many lines of evidence that QS coordinates the transition to a biofilm lifestyle when the population density reaches a threshold level *(23, 30)*.

Biofilm-forming pathogens frequently colonize certain types of fish, dairy, poultry, meat, and ready-to-eat foods and food packaging materials and even all types of equipment surfaces in brewing and food processing plants *(4, 26, 31)*. Microorganisms (foodborne pathogens in biofilm matrix) produce saccharolytic, proteolytic, lipolytic, and pectinolytic enzymes whose metabolic products are associated with microbial spoilage of food *(4)*. Therefore, the QS-regulated formation of biofilm is a safety problem in food industries that needs to be solved.

A large number of anti-QS and antibiofilm agents are available on the market, but most of them are synthetic. According to the regulations for producing and labeling natural food products set forth by the U.S. Department of Agriculture, the application of artificial flavorings and chemical and synthetic preservatives in foods labeled as "natural" is prohibited in the United States *(33)*. At present, all over the world, consumers are becoming more concerned about the issue of carcinogenicity. Considering the harmful effects of many synthetic compounds on human health, it is necessary to seek out QS and biofilm inhibitors from natural sources as natural/green products. Many of these green products are generally recognized as safe (GRAS) *(5, 19)*.

Star anise (SA) (*Illicium verum* Hook. f.) has been widely applied as an ingredient of the traditional five-spice powder of Chinese cooking, as a flavoring agent, and as a medicine for well over 3,000 years. Few reports have been published characterizing SA and its efficacy as an

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antifungal, antimycotoxigenic, antioxidant, and antidiarrheal agent. It is a major industrial source of shikimic acid, a primary ingredient used to prepare the anti-influenza virus drug Tamiflu, which mitigates the severity of the influenza virus *(1, 12, 15)*. However, the development of novel therapy and food preservation strategies based on the anti-QS and antibiofilm properties of crude extract of SA has not been undertaken.

The main objective of this study was to evaluate the anti-QS and antibiofilm efficacies of SA extract. Then, SA extract was applied in a food matrix to evaluate its suitability for ensuring a balance between food safety and quality concerns.

MATERIALS AND METHODS

Collection of SA fruits. Authenticated (specimen no. JNU201409S03) dried SA fruits were purchased from a supermarket (Auchan, Wuxi, People's Republic of China). The fruits were well cleaned, washed with tap water, and dried at room temperature. After this, the SA fruits were ground and transformed to powder by using a grinder (FW100, Tai Si Te Instrument Co., Ltd., Tianjin, China). The powder was preserved in sealed, clean, plastic containers and kept away from light, heat, and moisture until use. SA extract was prepared from this powder.

Preparation of SA extract and determination of yield. Solvent (water-ethanol, 20/80) was blended with SA powder (about 100 g) and agitated for 3 h at 45°C. After that, the extracts were filtered using a Buchner funnel through Whatman no. 1 filter paper. The same procedure was followed a second and third time on the extractions of previous materials to ensure maximum yields. Following vacuum drying (BC-W203, Shanghai Biochemical Equipment Co., Ltd, Shanghai, China) at 40°C, the dried extracts were scraped out, weighed to obtain the percent yield of the extract, and stored in sterile sealed polybags inside desiccators.

Bacterial strains and culture conditions. Commonly found foodborne and spoilage bacterial strains *Staphylococcus aureus* ATCC 6538 (gram positive), *Salmonella* Typhimurium ATCC 50013 (gram negative), and *Pseudomonas aeruginosa* ATCC 9027 (gram negative) were purchased from China General Microbiological Culture Collection Center (Beijing, China). Biosensor strain *Chromobacterium violaceum* ATCC 12472 was purchased from Spanish Type Culture Collection (CECT), Valencia, Spain. They were cultured according to the procedures given by the purchase center. All strains were maintained at 37°C except for *C. violaceum,* which was maintained at 30°C.

Determination of MICs. Serial dilutions (0.5, 1.0, 2.0, 3.0, and 4.0 mg/mL) of a 100-μL aliquot of SA extract were added to the wells of a 24-well culture plate (Sarstedt, Newton, NC) containing 800 μL of fresh broth medium and 100 μL of bacterial suspension (10^8 CFU/mL). After incubation for 24 h, the growth medium in every well was observed very carefully and MICs were calculated *(24)*. Wells containing only broth and only bacteria (without extract) were the negative and positive controls, respectively. All further experiments were performed at the sub-MIC of SA extract.

Growth curve observation. In order to confirm the non-antibacterial activity (anti-QS is not a killing mechanism) of SA extract, the bacteria were grown in batch cultures with and without SA extract. Overnight cultures of *C. violaceum, S. aureus, Salmonella* Typhimurium, and *P. aeruginosa* (1%; optical density of 0.4 at 595 nm) were inoculated into 250-mL Erlenmeyer flasks containing 100 mL of the required broth with SA extract (at the highest sub-MIC) and without SA extract (positive control). The flasks were incubated at the optimum temperature for the respective pathogen at 250 rpm in a shaking incubator. The optical density at 595 nm was measured hourly to trace bacterial growth up to 15 h *(7)*. Finally, growth curves were plotted, and levels of inhibition in comparison with the results for the positive control were determined.

Bioassay for anti-QS: preliminary screening (disk diffusion assay). To evaluate the anti-QS capability of the SA extract, the qualitative agar disk diffusion method was used as previously published, with some modifications *(17)*. An aliquot of 100 μL of fresh *C. violaceum* culture dilution was plated on Luria-Bertani (LB) agar (LB broth supplemented with 1.5% bacteriological agar) in petri dishes. Sterile paper disks (Whatman No. 1, 6.0 mm in diameter) loaded with SA extract at a concentration of 4 mg/mL (10 μL of extract per disk) were placed on the agar. The petri dishes were incubated at 30°C for 30 h. The growth inhibition ring (colorless) around the SA extract-loaded paper disk was measured. LB broth–loaded paper disks and a known QS inhibitor (cinnamaldehyde) were used as the negative control and positive control, respectively.

Flask incubation assay for quantification of violacein production. The previously described procedure of Truchado et al. *(32)* was used to quantify the anti-QS activity of SA extract. *C. violaceum* (1×10^8 CFU/mL) was inoculated into Erlenmeyer flasks containing Luria-Bertani broth supplemented with SA extract to obtain different concentrations (0.5, 1, 2, and 4 mg/mL). The flasks were incubated at 30°C in a shaking incubator for 30 h. One milliliter of culture from each flask was transferred to a test tube and centrifuged at 18,630 × *g* (Scilogex D3024R, Scilogex, Rocky Hill, CT) for 15 min to precipitate the insoluble pigment. The pellet was resuspended in 1 mL of dimethyl sulfoxide (Aladdin Industrial Corporation, Shanghai, China) and homogenized by vortexing for 3 min. Violacein absorbance was determined at 585 nm by using a UV-visible light spectrophotometer (Epoch, BioTek Instruments, Inc., Winooski, VT). The positive-control sample consisted of *C. violaceum* in LB broth without extract. Inhibition of violacein production was calculated with respect to the results for the control, and triplicate measurements were performed.

Antibiofilm assay. A crystal violet assay was performed as previously described by Gao et al. *(14)* to determine the antibiofilm activity. Bacterial cultures were grown in 24-well culture plates with SA extract at sub-MIC and incubated for 24 h. Then, suspension cultures were removed from the wells. The wells were rinsed three times with phosphate-buffered saline and fixed by drying for 3 h at 37°C in an incubator. Once the wells were fully dried, 1.000 μL of 0.1% crystal violet (Sinopharm Chemical Reagent Co. Ltd., Shanghai, China) stain was added to each well for 15 min. The excess stain was rinsed off with tap water, and 1 mL of 95% (v/v) ethanol was added to each well for 1 h to release the stain. A 100-μL amount of stained ethanol from each well was then transferred to a 96-well plate for spectrophotometric (Epoch, BioTek Instruments, Inc.) analysis (optical density at 570 nm). For all of the assays, a positive control (without an SA fraction) and a negative control (without bacterial strain inoculation) were

prepared. The procedure was performed in triplicate, and the data are expressed as the mean values ± standard deviations.

CLSM observation. To observe the biofilm architecture, samples were prepared according to a previously reported method *(36)* and observed under confocal laser scanning microscopy (CLSM; Leica TCS SP2; Leica Microsystems, Heidelberg, Germany). Cover slips treated with and without SA extract (control) were inoculated and cultured overnight. The L13152 LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Inc., Eugene, OR) was used to stain the biofilm on the cover slips according to the manufacturer's protocol. Finally, three-dimensional views confirmed the surface coverage of biofilm on cover slips and the density of bacteria.

Swarming motility assay. Swarming motility assay were carried out by a previously described method *(22),* with slight modifications. Amounts of 25 μL of molten soft top agar (0.3% agar, 1% tryptone, 5% yeast extract powder, and 5% sodium chloride) with SA extract at the sub-MIC for the final concentration were immediately poured over the surface of a solidified LB agar plate as an overlay. Different plates' center points were inoculated with an overnight culture of *S. aureus, Salmonella* Typhimurium, or *P. aeruginosa* separately. Then, plates were incubated at 37°C for 30 h. Swarming migration was observed. The control plates were prepared without extract. Photographs were taken using an iPhone 4s at the same distance from each disk, and picture contrasts were adjusted. The first time, swarming areas were measured by using ImageJ software (analyze > set scale > Image > adjust > color threshold > analyze > tool > ROI manager > Wand [tracing] tool > add). Finally, the levels of inhibition of swarming motility in comparison with that of the control were calculated.

EPS inhibition assay. SA-treated overnight culture was added to vials and centrifuged at 12,000 × *g* for 30 min at 4°C to remove bacterial cells. The supernatant obtained was collected and precipitated with two volumes of absolute chilled ethanol by incubating the mixture at 4°C overnight. The precipitated exopolysaccharide (EPS) was collected by centrifugation at 12,000 × *g* for 30 min at 4°C, and the supernatant was decanted. The pellet containing EPS was dried at room temperature. The total carbohydrate content in the EPS was estimated by the phenol-sulfuric acid method *(18)*. The absorbance was measured at 490 nm.

Application in a food matrix. As a food matrix, pasteurized milk (milk in a plastic bag, 3.5% fat; Yili Company, China) was purchased from the school supermarket, and the experiment was performed 15 to 18 days before its expiration date. *P. aeruginosa* biofilms were grown on cover slips (24 h). Then, milk in petri dishes was inoculated with biofilm-containing cover slips, with or without SA extract, at sub-MIC. Samples were incubated at 37°C for 48 h. Then, samples were observed very carefully with the naked eye to identify spoiled samples, and the acidity test was performed *(25)*. Total plate counts of SA-treated and nontreated milk were calculated according to the method of Anderson et al. *(2)*.

Statistical analysis. The differences between control and test results were analyzed using Student's *t* test at the 95% confidence level ($P < 0.05$). Statistical data analysis was undertaken using IBM SPSS Statistics 20 and Origin Pro. 9.0.

RESULTS

Yield of SA extract. The yield of crude extract from the dried fruit of SA was 25.90% ± 0.2%.

Effect of SA extract on bacterial growth. By visual inspection, the MICs of SA extract were evaluated against 3 tested foodborne bacteria (*S. aureus, P. aeruginosa,* and *Salmonella* Typhimurium) and the biosensor strain *C. violaceum*. The MIC of SA extract was higher than 3 mg/mL for all bacteria tested. The results obtained (Fig. 1) revealed that the cell densities (bacterial growth) did not differ between the untreated control and cultures treated with SA extract at the concentration (sub-MIC, 3 mg/mL) used.

Anti-QS property of SA extract and inhibition of violacein production. For preliminary screening of the anti-QS property of SA extract, the disk diffusion method was performed using the sensor strain *C. violaceum*. The positive control and SA extract showed the same behavior, with a colorless, opaque ring visible around each, while the negative control did not show any inhibition activity (Fig. 2). This confirmed that SA extract has appreciable anti-QS potential. Then, the inhibitory effect of SA extract on violacein pigment production was measured and quantified. From the results shown in Figure 3, it can be concluded that with an increase in the concentration of SA extract, the production of violacein was decreased. The production of violacein was inhibited by about 89% at the SA extract concentration of 3 mg/mL, while 0.5 mg/mL inhibited violacein production by about 45%.

Effect of SA extract in antibiofilm assay. The QS circuit regulates the initiation and maturation of biofilms. In the present study, antibiofilm assays for *S. aureus, Salmonella* Typhimurium, and *P. aeruginosa* were evaluated. The results obtained (Table 1) show concentration-dependent inhibition of biofilms of test bacterial pathogens. In this study, the highest (87%) biofilm inhibition was shown in *S. aureus* at the 3-mg/mL concentration, while the *P. aeruginosa* and *Salmonella* Typhimurium biofilms were inhibited by around 80 and 60%, respectively.

Image analysis of biofilm by CLSM. SA extract was found to be very effective in disrupting the biofilm architecture of *S. aureus, Salmonella* Typhimurium, and *P. aeruginosa*. From the results shown in Figure 4, it is clear that the three-dimensional structure of *S. aureus* biofilm treated with 3 mg/mL SA extract was more disturbed than that of the other biofilms.

Swarming motility is affected by SA extract. SA extract inhibited the swarming motility of *S. aureus* in a dose-dependent manner (Table 2). A 3-mg/mL concentration reduced the swarming motility zone of *S. aureus* by 95.5%. At the same concentration, the effect of SA extract on *Salmonella* Typhimurium's swarming motility was smaller (80% reduction).

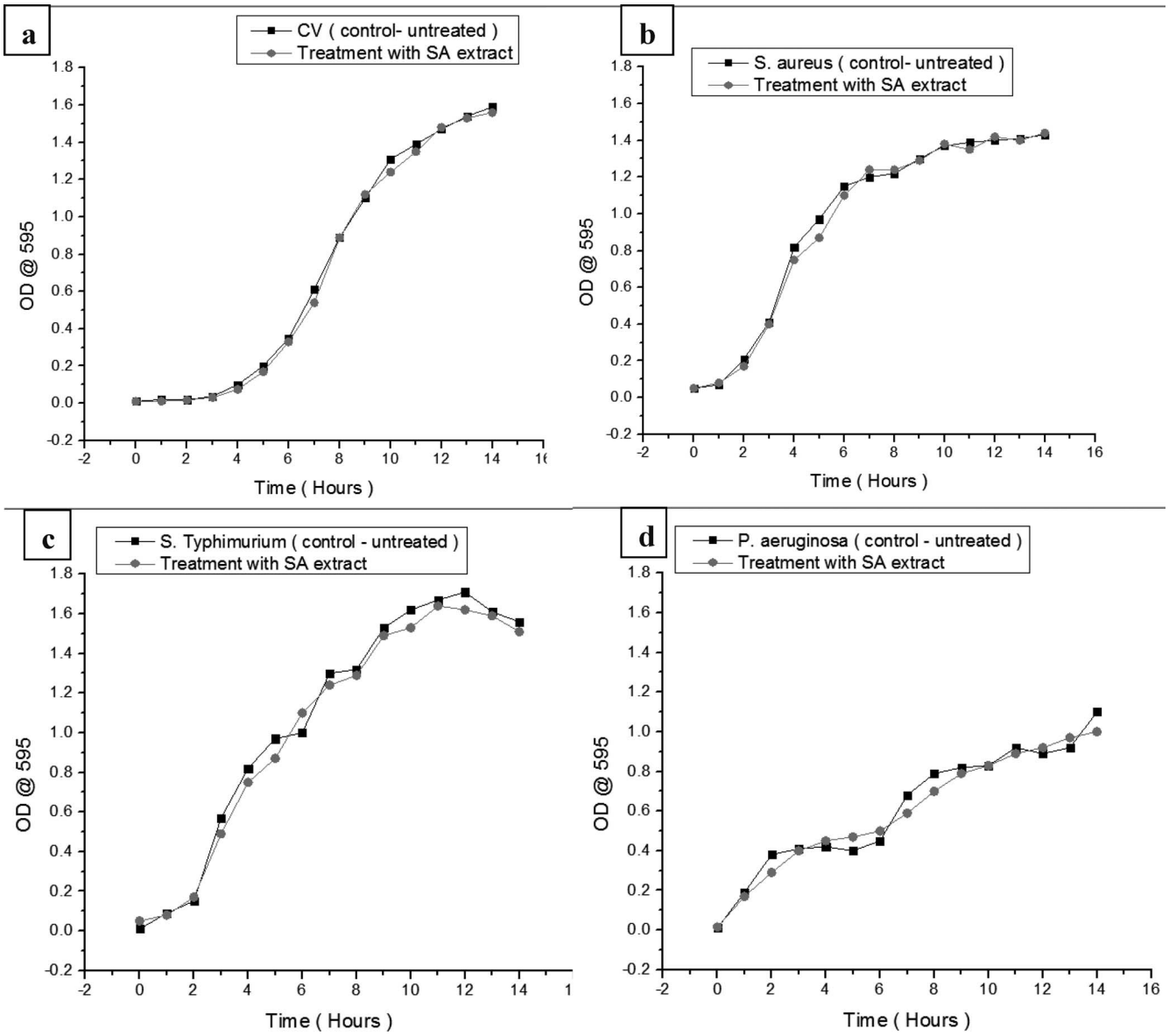


FIGURE 1. *Growth curves of (a)* Chromobacterium violaceum, *(b)* Staphylococcus aureus, *(c)* Salmonella *Typhimurium, and (d)* Pseudomonas aeruginosa, *treated with (●) and without (■) SA extract at 3 mg/mL.*

Inhibition of EPS production. EPS production was significantly inhibited by SA extract, in a dose-dependent manner. The highest inhibition of EPS production, around 70.45%, was observed in *S. aureus* at 3-mg/mL SA extract. At the same concentration, SA extract inhibited the EPS production of *Salmonella* Typhimurium and *P. aeruginosa* by about 42.82% and 35.66%, respectively (Fig. 5).

Application of SA extract in preventing milk spoilage. A simple preliminary assay of the QS-inhibitory activity of SA extract was performed in pasteurized whole milk. The untreated milk (control, without extract) inoculated with *P. aeruginosa* biofilm exhibited spoilage and a higher plate count (196×10^6 CFU/mL) (Table 3), whereas milk inoculated with the test biofilm and treated with SA extract showed minimal spoilage and a lower plate count (138×10^4 CFU/mL) after 48 h. It was also found that the

TABLE 1. *Percentages of inhibition of biofilm formation of* Staphylococcus aureus, Salmonella *Typhimurium and* Pseudomonas aeruginosa *are shown*[a]

	% inhibition of:		
	Staphylococcus aureus biofilm	*Salmonella* Typhimurium biofilm	*Pseudomonas aeruginosa* biofilm
Positive control	0	0	0
0.5 mg/mL	16 ± 0.9	7.8 ± 0.5	4.88 ± 1.1
1 mg/mL	32.3 ± 0.3	43 ± 0.3	13.6 ± 0.3
2 mg/mL	81 ± 0.2	54.5 ± 0.2	28.8 ± 0.6
3 mg/mL	87 ± 0.9	60 ± 0.8	48 ± 0.4
Negative control	Nill	Nill	Nill

[a] Results are calculated compared with the results for the control. Mean values from triplicate independent experiments ± standard deviations are shown.

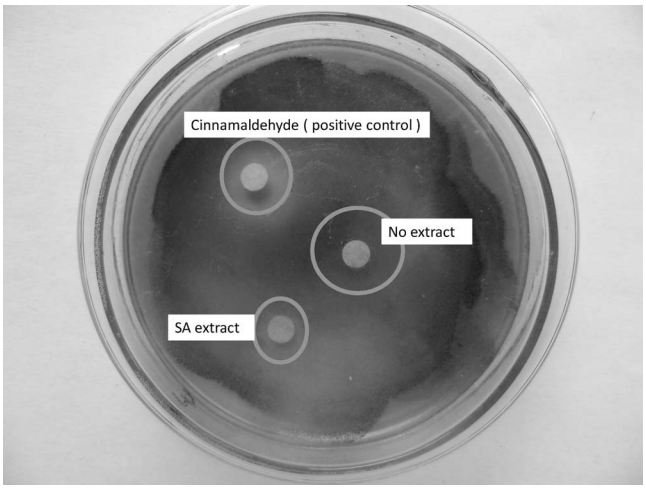


FIGURE 2. *Anti-QS activity of SA extract in agar plate disk diffusion assay. Inhibition rings (colorless) are found around positive control (cinnamaldehyde) and targeted-sample (SA extract at 3 mg/mL concentration)–loaded disks. There was no change around the negative control (no extract, LB broth–loaded paper).*

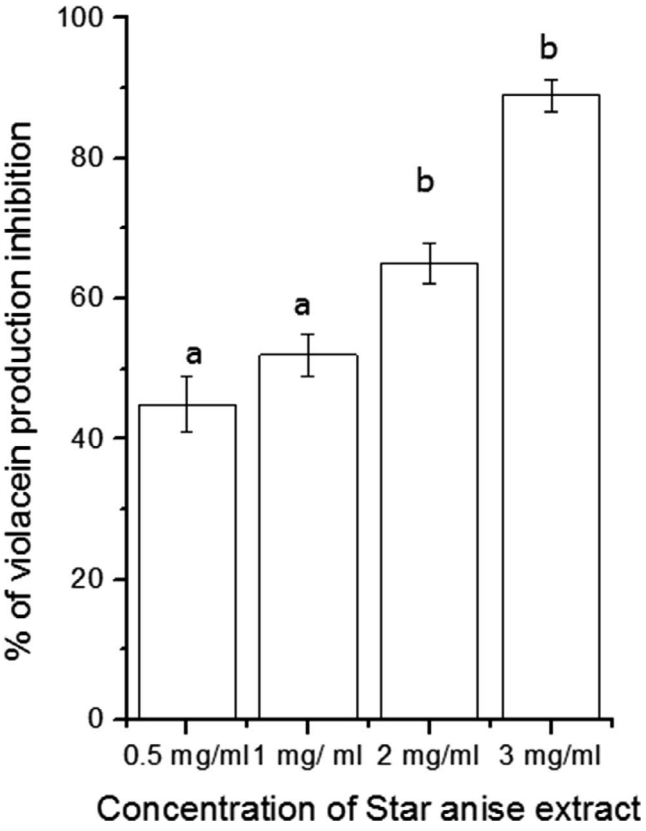


FIGURE 3. *Inhibition of violacein production by SA extract in flask incubation assay (concentrations of 0.5, 1, 2, and 3 mg/mL). Violacein production was measured using a microplate reader as described in "Materials and Methods." Error bars show the standard deviations of the results of triplicate experiments. Different letters show results that are significantly different at a* P *value of <0.05.*

percent acidity of the treated sample was lower than that of the positive sample (Table 3). We also noticed that the milk samples containing SA extract had a brownish color (Fig. 6).

DISCUSSION

Quorum signals are identified as being involved in bacterial pathogenesis and in food spoilage. Thus, the anti-QS mechanism might open a new era of action against bacterial pathogenesis. Rather than being a direct killing mechanism, the anti-QS mechanism is a smart strategy to inhibit food spoilage. Considering the unique properties of SA spices (e.g., SA spices are edible and popular due to their unique flavors and colors, they have been used as medicines from ancient times, they are GRAS, and they have antifungal and antimycotoxigenic effects), we hypothesized that SA should have anti-QS and antibiofilm activities. Therefore, we studied the efficacy of the crude extract of SA as a QS and biofilm inhibitor. We also performed assays of the effects of SA extract on EPS production and swarming motility with three common foodborne and spoilage pathogens. CLSM was performed to obtain an overview of the biofilm destruction.

For commercial prospects, the yield of crude extract is very important. The ratio of solvent, extraction time and temperature, extraction methods, etc., are related to the percent yields of extract *(6)*. In a previous study *(20),* it was found that the yields of hot-water extraction of *Cinnamomum cassia* and *Brassica nigra* were 27.66% ± 0.42% and 26.33% ± 0.44%, respectively. This is similar to our results.

TABLE 2. *Effects of SA extract on swarming motility of tested foodborne pathogens* Staphylococcus aureus, Salmonella Typhimurium, *and* Pseudomonas aeruginosa[a]

	Staphylococcus aureus		*Salmonella* Typhimurium		*Pseudomonas aeruginosa*	
SA extract concn (mg/mL)	Total area of swarming motility	% decrease of swarming motility zone	Total area of swarming motility	% decrease of swarming motility zone	Total area of swarming motility	% decrease of swarming motility zone
Positive control	15.34 ± 2.6	0	9.68 ± 1.9	0	22.78 ± 1.4	0
0.5	2.12 ± 0.9	86.1	3.94 ± 0.1	59.3	19.09 ± 0.2	16.2
1	1.64 ± 0.1	89.3	3.03 ± 0.01	68.7	8.96 ± 0.9	60.7
2	0.82 ± 0.1	94.7	2.40 ± 0.3	75	6.56 ± 0.4	71.2
3	0.62 ± 0.2	95.9	1.87 ± 0.7	80	3.96 ± 0.7	82.6

[a] ImageJ software was used to calculate the swarming zone. The percentage of decrease of the swarming motility zone was calculated compared with the result for the control. Mean values from triplicate independent experiments ± standard deviations are shown.

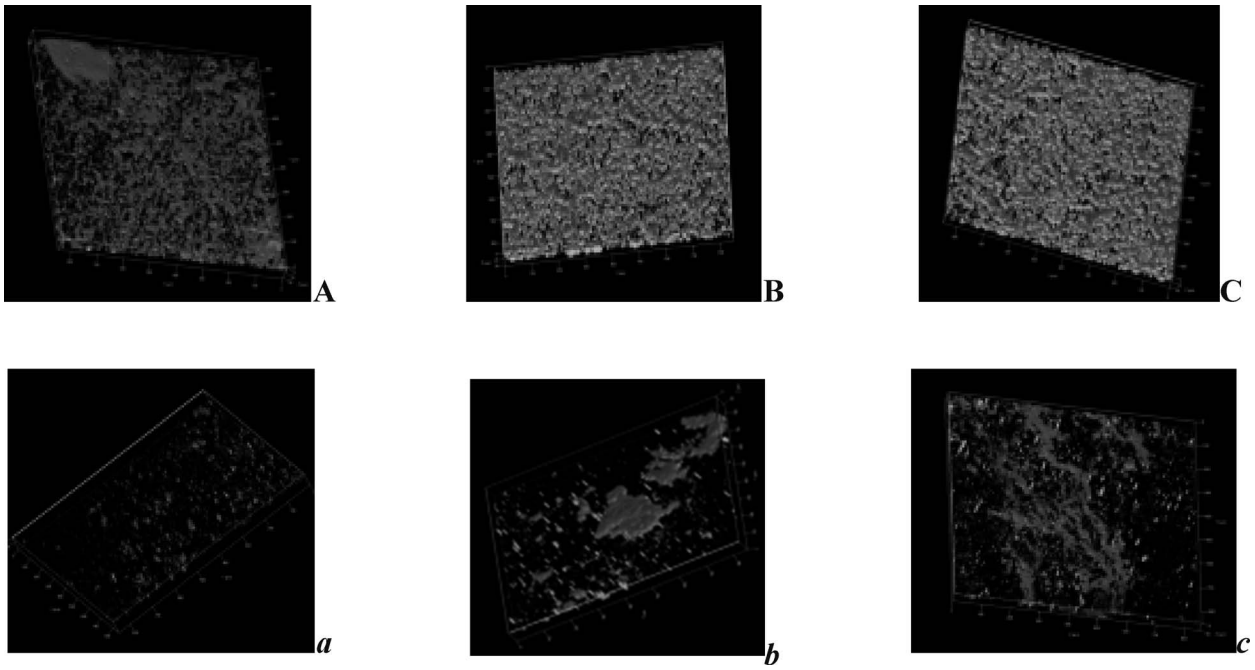


FIGURE 4. *Confocal laser scanning microscopy (CLSM) analysis: three-dimensional images of control and treated biofilms show the effect of SA extract. (A, B, and C) Control (no extract added) biofilms of* Staphylococcus aureus, Salmonella *Typhimurium, and* Pseudomonas aeruginosa. *(a, b, and c) Biofilms treated at sub-MIC of SA extract (3 mg/mL).*

In this study, SA did not disturb the growth of tested bacteria at concentrations up to 3 mg/mL (sub-MIC) (Fig. 1). Thus, the inhibition of QS and biofilm is due to the anti-QS mechanism. This property is very important in the development of medicines for the treatment of biofilm-related infectious disease, in the treatment of antibiotic-resistant bacteria, and in food processing to prevent food spoilage *(26, 31)*. Because the SA extract exhibited antibiofilm and anti-QS activity that was not due to antibacterial activity, it may be very favorable for the prevention of bacterial drug resistance as well.

Like ginger extract *(18)* and Amomum tsaoko extract *(27),* SA showed significantly effective anti-QS and antibiofilm activity against the foodborne pathogens tested, which could significantly reduce bacterial virulence and prevent bacterial resistance. Vasavi et al. *(34)* found that the ethyl acetate fraction (0.75 to 1.00 mg/mL) of *Syzygium cumini* and *Pimenta dioica* extracts completely inhibited violacein. However, our study reported 89% reduction of violacein production. This observation in relation to SA extract may be due to blockage of *N*-acyl homoserine lactone (AHL) production, inactivation of signal molecules, interference with the signal receptor, or synergistic effect.

The regulation of rhamnolipids, swarming motility, siderophores, the surrounding environment, etc., are important factors for the formation of *P. aeruginosa* biofilm which are correlated with QS *(14, 29)*. It is considered that AHL is one of the most important cell-to-cell communication systems in *Salmonella* bacteria. The regulatory protein SdiA, a counterpart of LuxR (in AHL systems), activates the genes of *Salmonella* pathogenicity island 1 and other virulence genes *(9)*.

One of the important characteristics of a biofilm is its strong architecture. CLSM analysis of the foodborne-pathogen biofilms demonstrated (Fig. 4) that SA extract has a great destructive effect on the architecture of *S. aureus* biofilm compared to its effects on *Salmonella* Typhimurium and *P. aeruginosa* biofilms. *Escherichia coli* K-12 and *P. aeruginosa* PAO1 biofilms were inhibited by *Rosa rugosa* tea polyphenol extract *(36)* in the same manner. As the QS

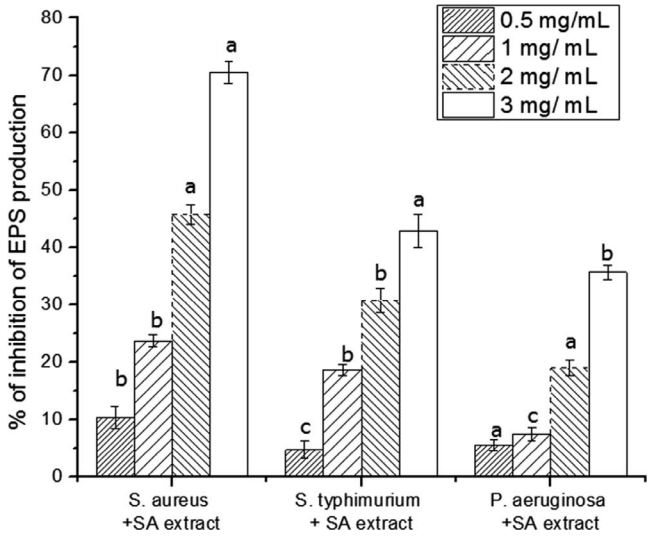


FIGURE 5. *Exopolysaccharide (EPS) inhibition of* Staphylococcus aureus, Salmonella *Typhimurium, and* Pseudomonas aeruginosa *following treatment with SA extract at sub-MIC. Error bars show the standard deviations of the results of triplicate experiments. Different letters show results that are significantly different at a P value of <0.05.*

TABLE 3. *Total plate counts and percent acidities of whole milk samples at 0 h and after 48 h*[a]

Parameter	Pasteurized whole milk	Whole milk + *P. aeruginosa* biofilm (48 h)	Whole milk + *P. aeruginosa* biofilm + SA extract (48 h)
TPC (CFU/mL)[b]	$(138 \pm 15) \times 10^2$	$(196 \pm 07) \times 10^6$	$(138 \pm 12) \times 10^4$
% acidity	0.16	0.22	0.19

[a] Mean values of triplicate independent experiments ± standard deviations are shown.
[b] TPC, total plate count.

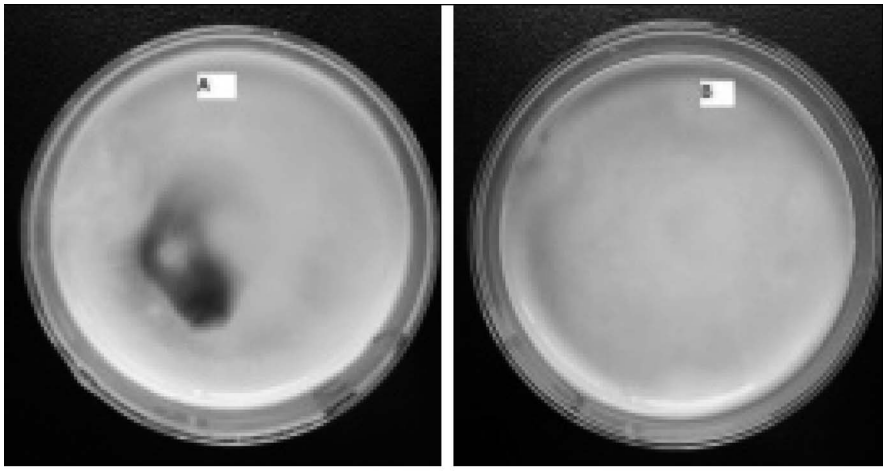
circuit is disrupted by SA extract, the development of the biofilm architecture is hampered.

Another QS-regulated phenotype is swarming motility, by which bacteria can spread as a biofilm over a surface by flagella-driven movement. SA extract significantly decreased the swarming ability of tested bacteria and significantly inhibited the formation of biofilms. In general, the swarmer cells of *E. coli, Salmonella,* and *Yersinia* are regulated by the *flhDC* master operon, which regulates the swarmer cell differentiation in swarming motility. These motile cells share signals within the intracellular network to colonize the entire available surface. The integrated and colonized motile cells are preceded by extracellular slimelike material *(11)*. This study showed that higher doses of SA extract decreased swarming motility on a larger scale (Table 2). Zhang et al. *(36)* also found dose-dependent activity of *Rosa rugosa* tea polyphenol extract in inhibition of the swarming motility of *E. coli* K-12 and *P. aeruginosa* PAO1, with about 84.90 and 78.03% inhibition, respectively, at 640 μg/mL. SA extract appeared to reduce the swarming motility of the tested pathogens by interfering with their ability to reach the substratum and made a considerable change in the spacing of the radiating tendrils or reduced the proton motive force that is needed for flagellar movement.

The importance of QS-dependent EPS production in biofilm development and its maturation is well reported. Moreover, increased biofilm formation often correlates with increased EPS production *(13)*. In this study, SA extract (3 mg/mL) demonstrated effective interruption of EPS production (Fig. 5). Santhakumari et al. *(28)* found that marine cyanobacterium *Synechococcus* sp. extract resulted in significant reductions, 66 and 68%, of EPS production in *Vibrio harveyi* and *Vibrio vulnificus,* respectively. Their results support the findings of this study.

As SA extract has anti-QS and antibiofilm activity against foodborne pathogens, it was applied in pasteurized whole milk. *Enterobacter* spp., *Pseudomonas* spp., and *Serratia* spp. have shown AHL production ability in raw and pasteurized milk. Thus, QS might play a role in the spoilage of milk and dairy products *(4)*. In a successful result, this study found that SA extract–treated milk was minimally spoiled after 48 h; the color of the milk was brownish. The bacterial biofilm–inoculated control milk without SA extract was spoiled (Fig. 5). As the crude extract of SA has its own brown color, the milk also got the color. Thus, SA extract could potentially be used in various cheese products which have color and aroma, so the color and aroma of the extract would have less effect on the products. The SA extract might also be used in formulated yogurt products, such as strawberry or banana yogurt blends. These formulated yogurt products contain fruit pulp or jam, which have some color, so the color from the SA extract might be ignored. In a future study, we will demonstrate the quality parameters of food products treated with SA extract. Our results agreed well with the findings of Cava et al. *(8)*. They concluded that milk samples with higher fat content reduce the antimicrobial activity of the essential oils of cinnamon bark, cinnamon leaf, and cloves. Their study indicated the possibility of using these three essential oils in milk beverages as natural antimicrobials. Our findings also show that SA extract is a promising natural alternative for the preservation of cheese, formulated yogurt, flavored milk, and other foods.

The anti-QS and antibiofilm activities of SA extract could be owing to the action of any of its phytoconstituents



A B

FIGURE 6. *In vivo anti-QS activity of SA extract was observed in pasteurized milk. Control consisting of milk inoculated with* Pseudomonas aeruginosa *biofilm, which is showing spoilage (A), and milk treated with SA extract showing minimal spoilage and brownish color after 48 h (B).*

or the synergistic effect of the various ingredients on the bacterial QS circuit. But it is very difficult to comment on the exact mode of action of a crude extract unless the main compounds are separated and purified. Plant extracts have been found to act as QS inhibitors because of similarities in their chemical structures to those of QS signals (AHLs) and also because of their ability to degrade signal receptors (LuxR/LasR) *(16)*.

Clove extract inhibited QS-regulated phenotypes in *P. aeruginosa* PA01, including the expression of *lecA*::*lux* (by hexane extract). Certain compounds contained in the spices and bark can affect the adhesion of microorganisms as well *(21)*. *I. verum* (SA) contains essential oils, prenylated C-6–C-3 compounds, lignans, sesquiterpenes, and flavonoids *(15)*. So the results of this study suggest that the above-mentioned constituents and other compounds of SA extracts might interfere with the QS-regulated *lux*, *rhl,* and *las* systems.

In conclusion, this study has found that SA extract has the potential to be used in the food industry as a food preservative (green product) to control the spoilage of food and even as the baseline for researching SA compounds in drug development industries.

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