Original Research

The Inhibition Effect of Capsaicin Extract Against *Staphylococcus aureus*: An *In Vitro* Experimental Study

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Abstract

Aim: *Staphylococcus aureus* can be isolated from contaminated food. A toothbrush is used daily in both planktonic and biofilm cells. No drugs are proven to be effective against *S. aureus* infection; since Methicillin has become resistant in the form Methicillin-Resistant *S. aureus* (MRSA), a search is necessarily made for active herbal compounds related to this infection. One of the herbal agents, capsaicin, has antibacterial effects as it has a composition of flavonoid, carotenoid, phenol, and efflux pump inhibitors (EPIs) activity. Previous studies have shown that capsaicin could be used as anti-*S. aureus*, but it has restricted diffusion. The present study was aimed to evaluate the efficacy of capsaicin as an alternative herbal substance to threat the antibiotic resistance of *S. aureus*. **Materials and Methods:** This research used a serial dilution method in isolating *S. aureus* both from the food sold from the side of road and ten days used toothbrush in the solid media called Blood Agar Plate (BAP). It was replicated to five times. Capsaicin was diluted from 100% to 0.78125%. These data were analyzed by one-way ANOVA. **Results:** The Minimal Inhibitory Concentration (MIC) to *S. aureus* planktonic formation was at 3.125%, whereas the MIC to biofilm formation was at 12.5% ($P < 0.05$). **Conclusion:** Capsaicin extract could inhibit the growth of *S. aureus* (planktonic and biofilm cell). Therefore, capsaicin is an alternative herbal candidate to prevent problems related to *S. aureus*.

Keywords: Biofilm *Staphylococcus aureus*, Capsaicin, MRSA, SDG3 Good Health and Well-Being (Infectious Disease), *Staphylococcus aureus* Planktonic

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Introduction

Living things need uncontaminated food and drinks to survive, but very rarely are food and drinks entirely clean. The main contaminant bacteria in food and drinks are: *Staphylococcus aureus*, Eschericia coli, and Salmonella. This study mainly focuses on discussing *S. aureus*. *Staphylococcus aureus* can be isolated from food and drinks, and they could also be obtained from toothbrushes that are used daily for a long period. A toothbrush can be contaminated, as people use it in the oral cavity and then place it in a container. Such toothbrush contamination is considered aerosol borne.[1-4]

Staphylococcus aureus causes gastroenteritis (the second cause after salmonellosis with its percentage of 31.7%), bacteremia (the second cause after E. coli), and MRSA in 60% patients in the Intensive Care Unit (ICU).[5-8] The MRSA is a type of nosocomial pathogen that increases the risk of: Community acquired-methicillin resistant *S. aureus* (CA-MRSA), health associated-methicillin resistant *S. aureus* (HA-MRSA), and livingstock acquired-methicillin resistant *S. aureus* (LA-MRSA). *Staphylococcus aureus* is one of the normal floras in the oral cavity, and it triggers periodontitis.[8-13]

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No other drugs except Methicillin are resistant to *S. aureus*. Thus, there is a necessity to search for a herbal agent to treat *S. aureus* infection. Capsaicin (*trans*-8-methyl-*N*-vanillyl-6-nonenamide) is a herbal agent in chilli. It is usually used in powder form as an appetite stimulant. Its agonist receptor is Transient Receptor Potential Vanilloid-1 (TRPV-1). TRPV-1 can be found in all of sensory nerve endings. Once sensitized, it can secrete both Substance P (SP) and Calcitonine Gene Related Peptide (CGRP). Capsaicin ($C_{18}H_{27}NO_3$) is effective in inhibiting the growth of Bacillus subtilis by the use of both diffusion and serial dilution methods. The other studies related to *S. aureus* have concluded that capsaicin can inhibit the growth of *S. aureus*, when only combined with antibiotics.[14-17]

Materials and Methods

Study setting

This study is an experimental laboratory research. The sample used in this study was obtained from isolated *S. aureus* both from food sold from the side of road and ten days used toothbrush in BAP. It was replicated to five times. The capsaicin extract in 500-g hot peppers (*Capsicum frutescens*) samples was obtained from UPT Laboratorium Herbal Materia Medica, located in Jalan Lahor No. 87, Batu sub-district, Malang district. Capsaicin was then diluted in 70% of ethanol so that the total capsaicin extract was at 150 mL. This study was conducted in the Research Centre Laboratorium, Microbiology Division, Faculty of Dental Medicine, Universitas Airlangga from July until October 2019.

Sample size calculation

From statistical calculations, it was observed that the number of samples determined from Federer calculation, so that there were 5 times in its replications taken from isolate Staphylococcus bacteria obtained from both sold food near the road and ten days used toothbrushes. There were inclusion and exclusion in this experimental laboratory research.

Dilution of capsaicin

The dilution process of capsaicin was conducted by using 10 test tubes placed in a rack. The first test tube contained 10 mL of pure capsaicin extract at full concentration. It is similar to the previous method which has been explained in the first test tube. To obtain half of the capsaicin extract, the capsaicin from the first test tube was mixed with BHIB medium in the second test tube. The concentration of capsaicin was at 50% in the second test tube. A similar method was followed to obtain 25% of capsaicin concentration in the third test tube. In the second test tube, 50% of capsaicin concentration was mixed with the BHIB in the third test tube. The final concentration of capsaicin in the third tube was at 25%. This same method was applied starting from the fourth test tube until the eight test tube. Some concentrations of capsaicin included 12.5%, 6.25%, 3.125%, 1.5625%, and 0.78125%. These dilution methods were based on the dilution formulation: $C1 \times V1 = C2 \times V2$ (C1 and V1 were the initial concentration and volume, respectively, before the dilution, and C2 and V2 were the concentration and volume, respectively, after the dilution).

Staphylococcus aureus strain

Staphylococcus aureus strain was obtained from isolated sold food near the road and from 10 toothbrushes from 10 respondents that were used for 10 days.

Staphylococcus aureus determination

The successful isolated *S. aureus* was grown in BHIB, and it was incubated for 1 × 24 hours at 37°C. After turbidity was observed, the BHIB culture that was presumed to be *S. aureus* was moved and grown in solid media, namely BAP. The transfer was done by using a streaking technique and spreader tools followed by an incubation process in 2 × 24 hours at an optimal temperature of 37°C. The observation of the colony-shaped growth in the BAP was presumed as *S. aureus* if there was a white colony and hemolyzed the BAP. If these two conditions observed in the BAP, then one osse of the colony was observed using a microscope.[18] Once purple gram-positive coccus were found, they were then identified using the VITEC method in Laboratorium Mikrobiologi Balai Besar Laboratorium Kesehatan Surabaya located in Jalan Karangmenjangan No. 18, Surabaya city, East Java province.

The biofilm growth of *S. aureus* was observed to be due to the successful and confirmed planktonic bacteria, which were moved to the BHIB and saved in an anaerobic jar in 1 × 24 hours by using the Tissue Culture Plate (TCP) method. This method began with isolating *S. aureus* from the BAP, inoculating it into 10 mL BHIB with 1% glukosa, and incubating it at a temperature of 37°C in 1 × 24 hours. The ratio of culture media in the dilution process was 1:100, where this cultured media was then put into ELISA media. Both biofilm and capsaicin extracts were also put into ELISA media and then a staining process was performed by using Crystal Violet (0.1% of concentration). Afterward, the media were cleared by using the Phosphate Buffer Saline (PBS). This ELISA was then observed by using an autoreader using a 540 nm auto reader and measured at an optical density cut value (ODc), which is the average of the negative OD control added by three times of standard deviation (SD) for the negative control value. The ODc was used to interpret the biofilm strength. The biofilm was categorized as weak if the ODc value was ODc -≤~≤ 2× ODc; as medium if the ODc was 2× ODc < ~ ≤ 4× ODc; and finally as strong if it was > 4× ODc.[19]

Determination of capsaicin extract with *S. aureus* planktonic and biofilm cells

The determination of MIC and MBC capsaicin extract with *S. aureus* planktonic cells was done by placing as

much as 0.1 mL of isolated *S. aureus* customized by using 0.5 of McFarland (1.5×10^8 CFU/mL)[20] into test tubes with capsaicin extract. In spite of determinating the MIC and MBC of *S. aureus* with capsaicin extract into the test tube, this research also used live dead assay using SYBR Green I and propidium iodide to differ capsaicin's ability to penetrate to the bacterial cells. The former test to determine the MIC and MBC of *S. aureus* with capsaicin extract was done in the solid plate namely Muller Hilton by streaking method. It had been marked before the *S. aureus* and capsaicin extract were put into the Muller Hilton. This Muller Hilton incubated for 24 h at 37°C. It was then placed in an incubator for 2 × 24 hours at an optimal temperature of 37°C. The number of colonies growing in the Muller Hilton was then counted by using a colony counter.

The MIC and MBC capsaicin extract against *S. aureus* biofilm cells was obtained when the bacteria grew in the ELISA media using the dilution process for 40 times. In the dilution process, 5 µL of Si of biofilm cells was placed from ELISA to 200 µL of BHI, during which the incubation process took place in an aero pack system for 3 × 24 hours at 37°C. During the incubation process, the extract was washed once by using 200 µl PBS and given 50 µL Crystal Violet (CV) at 0.1% concentration. After 15 min, a CV was washed once by using PBS 200 µL and mixed with 50 µL ethanol. Then, it was transferred into new ELISA media. From the processes, the optical density (OD) obtained was at 540 nm.[21]

Statistical analysis

The data of MIC and MBC between capsaicin extracts against *S. aureus* planktonic bacteria were obtained by calculating the concentration in each treatment. The mean value was calculated to obtain the average number of *S. aureus* colonies in each treatment. Besides, the data of MIC and MBC between capsaicin extracts against the *S. aureus* biofilm cells were collected by comparing the mean with the OD value of 540 nm. The data obtained were then analyzed by using the one-way ANOVA parametric statistical test.

Results

The MIC and MBC capsaicin against *S. aureus* planktonic cells was collected by comparing the colony counter with concentrations ranging from 100% to 0.78125%, as well as negative and positive controls. Meanwhile, the MIC and MBC capsaicin against *S. aureus* biofilm cells was collected by comparing the OD with each capsaicin concentration, as well as negative and positive controls.

Determination of *S. aureus* planktonic and biofilm cells

Figure 1 shows the bacteria predicted as *S. aureus* on the BAP as they hemolyzed the BAP and were proven by VITEC examination conducted by Laboratorium Mikrobiologi Balai Besar Laboratorium Kesehatan Surabaya. The laboratory confirmation letter explains that in the probability of *S. aureus* being isolated, the BAP was at 99%.

Figure 2 presents the process of obtaining MIC and MBC capsaicin against *S. aureus* planktonic form by using the serial dilution method. It shows that capsaicin with concentrations ranging from 100% to 0.78125% was mixed with Staphylococcus on the Muller Hilton agar that had already been marked earlier.

Figure 3A presents the live/dead assay of control positive contains of *S. aureus* bacteria without capsaicin. Figure 3B shows the live/dead assay of 3.125% capsaicin concentration as an MIC against *S. aureus*. Figure 3C

BBLK Surabaya

Microbiology Chart Report

Printed Jul 15, 2019 08:39 ICT

Patient Name: BAP, Kode 3
Location: FKG UNAIR
Lab ID: 49 Vi

Patient ID: 49 Vi
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Staphylococcus aureus

Source: Isolat

Collected:

Comments:

Identification Information	Analysis Time:	3.85 hours	Status:	Final
Selected Organism	99% Probability	Staphylococcus aureus		
	Bionumber:	01040206256123 1		
ID Analysis Messages				

Biochemical Details

2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	-	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	-	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	-	39	ILATk	+	42	LAC	-	44	NAG	+	45	dMAL	-	46	BACI	+
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	-	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

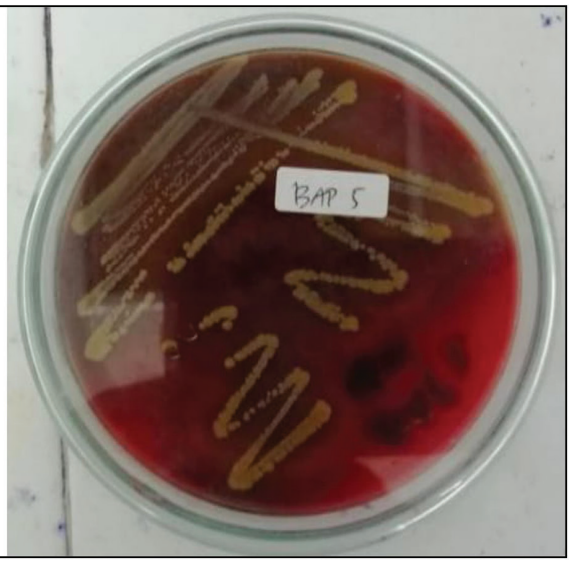

Figure 1: The results of VITEC examination on *S. aureus*

presents the live/dead assay of 6.25% capsaisin concentration as a Minimal Bactericidal Concentration against *S. aureus*.

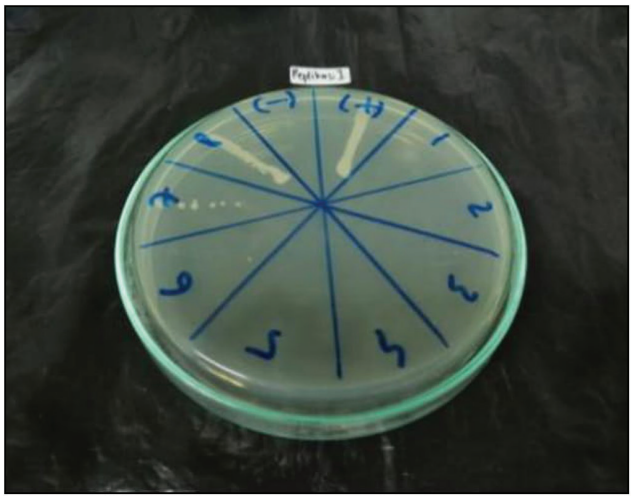

Figure 2: MIC and MBC capsaicin against *S. aureus* planktonic form

Table 1 shows the significant difference in increasing capsaicin concentrations to the number of the *S. aureus* colonies. It can be determined that the MIC capsaicin against *S. aureus* was 3.125%. It means that the capsaicin concentration higher than 3.125% could inhibit the growth of *S. aureus* but concentrations below that did not inhibit the growth of *S. aureus*.

Table 2 shows the significant difference in variable capsaicin concentrations against *S. aureus* biofilm. It can be said that the MIC of capsaicin against *S. aureus* biofilm was at 12.5%. It means that the capsaicin concentration higher than this concentration could inhibit *S. aureus* biofilm, whereas concentrations below that did the opposite. The strength of *S. aureus* biofilm based on the TCP method is presented in Table 2. This method determines that 100% until 12.5% concentrations of capsaicin extract could form low biofilm, whereas 6.25% to 1.5625% concentrations formed moderate biofilm. Meanwhile, 0.78125% concentration formed strong biofilm.

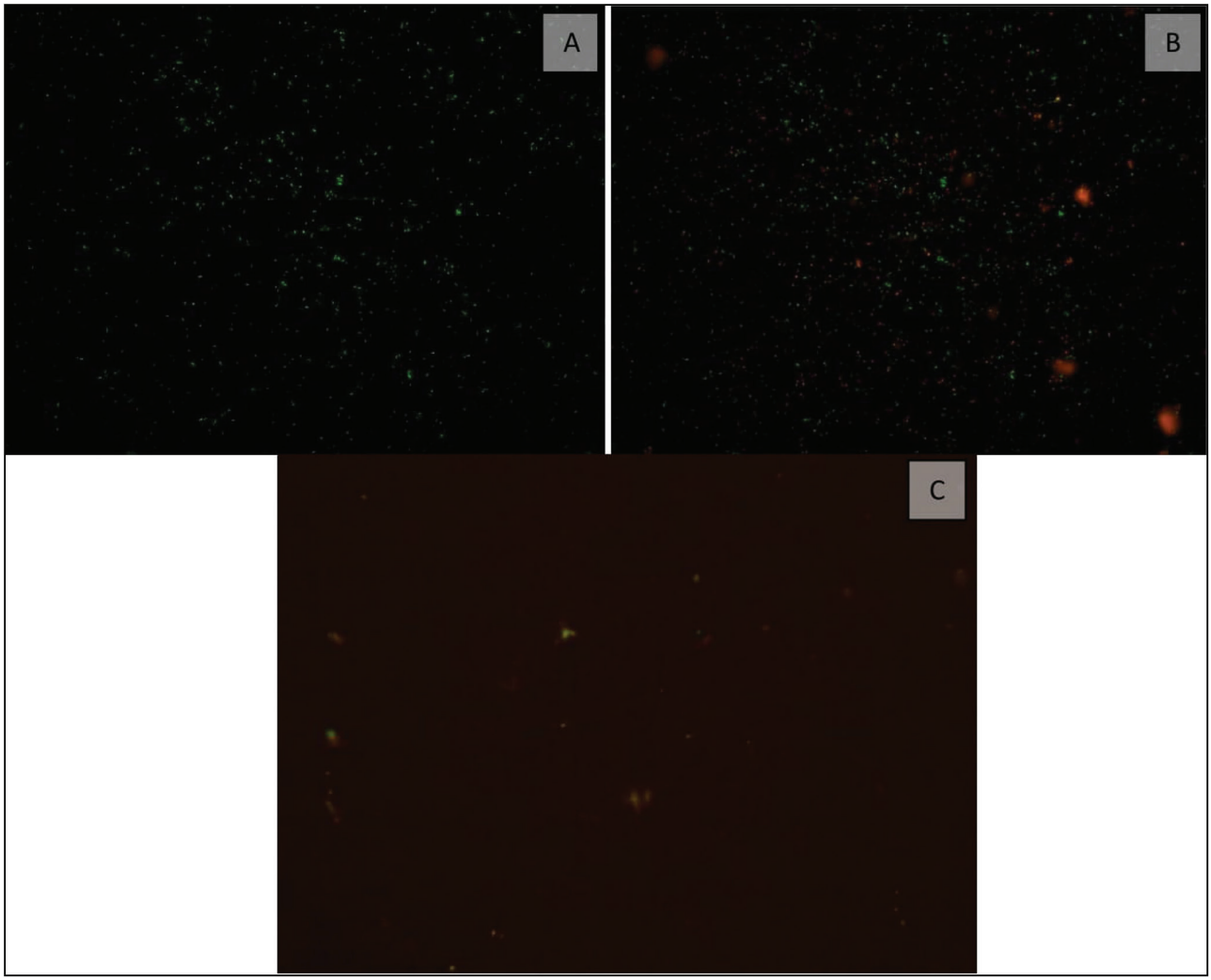

Figure 3: MIC and MBC capsaicin against *S. aureus*

Discussion

Capsaicin is one of the herbal medications that is suitable for the WHO's recommendations. The WHO suggests therapy using traditional agents, which is done on 80% of the population to determine the demands of health. Herbal agents can produce more and various active metabolites that are different from each other. These active metabolites are usually called alkaloid, flavonoid, saponin, terpenoid, steroid, glicoside, tannin, and essential oil, and these derivations with each metabolite exhibit different activity. Alkaloid functions as antioxidant, antibacterial, diuretic, antispasmodic, antimalaria, and analgesic compound.[22]

The antibacterial working mechanism on herbal agents may be through direct, indirect, and antibiotic potentiation. The direct antibacterial activity works through the inhibition of cell walls and protein synthesis, the change in cell membrane, the nucleic acid inhibition, and the biofilm inhibition process. The indirect antibacterial activity involves immunomodulation and prevention of bacterial adherence to host cells. Capsaicin consists of some active compounds, such as: carotenoid, phenol, flavonoid, alkaloid, and vitamin C. Flavonoid is a chemical compound that contains an antibacterial agent, synergistic antibiotic agent, and it can eliminate bacterial virulence. It is also the biggest phenol group and the most polar, thereby easily penetrating to the peptidoglycan layer in *S. aureus* planktonic and biofilm cells. Moreover, it can bind to bacteria's peptidoglycan so that it destroys the cell wall.[7,14,23-27]

The other compounds of capsaicin are phenol, carotenoid, and alkaloid. Phenol functions to destroy the cell membrane, inactivate enzymes, denature protein, and decrease the permeability cell membrane. Carotenoid binds to porin, a transmembrane protein in the outer layer of cell wall bacteria. Carotenoid and porin bind to a strong polimer chain that can destroy porin, followed by a decrease in the cell membrane permeability. Porin is the metabolite entrance in a bacterium. Alkaloid has an antibacterial activity that inhibits the synthesis of the cell membrane. It will cause bacterial cells to become more permeable, and the content of the cytoplasm will easily come out and kill the bacteria.[25,26,28]

Capsaicin may affect *S. aureus* biofilm due to the combination of direct, indirect, and potentiation of drugs for antibiotic use. All of this activity is related to the activity changes in modulation in the host immunity system through the elimination in Stapyhlococcus aureus adherence into the host cell. One of the basic principles of *S. aureus* to form its biofilm is by adhering to the host cell due to Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMMS) and Secreted Expanded

Table 1: MIC and MBC capsaicin extract against *Staphylococcus aureus* planktonic form

Capsaicin concentration (%)	$X \pm SD$	*P* value							
		100%	50%	25%	12.5%	6.25%	3.125%	1.5625%	0.78125%
6.25%	0	1.00	1.00	1.00	1.00	1.00	1.00	0.000*	0.000*
3.125%	0	1.00	1.00	1.00	1.00	1.00	1.00	0.000*	0.000*
1.5625%	11.60 ± 1.14	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
0.78125%	27.40 ± 2.302	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
Control (+)	157 ± 6.189	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*

x = the average of the *S. aureus* colonies; SD = standard deviation
*$P < 0.05$

Table 2: MIC and MBC capsaicin extract against *S. aureus* biofilm

Capsaicin concentration (%)	X ± SD	*P* value									
		100%	50%	25%	12.5%	6.25%	3.125%	1.5625%	0.78125%	CP	CN
100	0.51 ± .02	0.000^{1}	0.000^{2}	0.000^{3}	0.000^{4}	0.000^{5}	0.000^{6}	0.000^{7}	0.000^{8}	0.000^{9}	1.00
50	0.89 ± .03	0.000^{10}	0.000^{11}	0.000^{12}	0.000^{13}	0.000^{14}	0.000^{15}	0.000^{16}	0.000^{17}	0.000^{18}	0.000^{19}
25	1.32 ± 0.01	0.000^{20}	0.000^{21}	0.000^{22}	0.000^{23}	0.000^{24}	0.000^{25}	0.000^{26}	0.000^{27}	0.000^{28}	0.000^{29}
12.5	1.56 ± 0.01	0.000^{30}	0.000^{31}	0.000^{32}	0.000^{33}	0.000^{34}	0.000^{35}	0.000^{36}	0.000^{37}	0.000^{38}	0.000^{39}
6.25	2.31 ± 0.08	0.000^{40}	0.000^{41}	0.000^{42}	0.000^{43}	0.000^{44}	0.000^{45}	0.000^{46}	0.000^{47}	0.000^{48}	0.000^{49}
3.125	2.54 ± 0.02	0.000^{50}	0.000^{51}	0.000^{52}	0.000^{53}	0.000^{54}	0.000^{55}	0.000^{56}	0.000^{57}	0.000^{58}	0.000^{59}
1.5625	3.71 ± 0.11	0.000^{60}	0.000^{61}	0.000^{62}	0.000^{63}	0.000^{64}	0.000^{65}	0.000^{66}	0.000^{67}	0.000^{68}	0.000^{69}
0.78125	4.08 ± 0.62	0.000^{70}	0.000^{71}	0.000^{72}	0.000^{73}	0.000^{74}	0.000^{75}	0.000^{76}	0.000^{77}	0.000^{78}	0.000^{79}
CP	4.08 ± 0.57	0.000^{80}	0.000^{81}	0.000^{82}	0.000^{83}	0.000^{84}	0.000^{85}	0.000^{86}	0.000^{87}	0.000^{88}	0.000^{89}
CN	0.47 ± 0.16	0.000^{90}	0.000^{91}	0.000^{92}	0.000^{93}	0.000^{94}	0.000^{95}	0.000^{96}	0.000^{97}	0.000^{98}	0.000^{99}

X = the average of the capsaicin OD and *S. Aureus*; SD = standard deviation; CP = control positive; CN = control negative
$^{1,2,3,4,5\ dst}$The similar numbers show the significant difference ($P < 0.05$)

Repertoire Adhesive Molecules (SERAMS). These two adhesion factors are related to the adherence of *S. aureus* to the host cell and induction of acyl-homoserine lactones (AHL) and quorum sensing. As a result, the bacteria can adhere to each other. One of the strategies in the inhibition of biofilm is breaking down the peptidoglycan as one of the *S. aureus* cell wall materials.[22,27]

The elimination process of antibiotics from the inner layer of biofilm is called efflux pump, and it contributes directly or indirectly to the antibiotic resistance. It pumps out the relevant antibiotic to the outer layer of the biofilm directly and the inhibition process in the biofilm formation indirectly. *S. aureus* has an efflux pump to eliminate antibiotic resistance. Efflux pumps can be inhibited by efflux pumps-inhibitors (EPIs) to mainly block and disturb the biofilm formation, thereby increasing the antibiotic potentiation and reversing antibiotic resistance. Capsaicin is a material that has as an EPI. The EPI is a novel inhibitor in the efflux pumps of *S. aureus* (NorA). Therefore, it will excessively produce NorA efflux pump, mainly affecting the inhibition of efflux pumps. The inhibition causes *S. aureus* that are unable to produce the antibiotic agent in the biofilms and possibly kill them. Capsaicin as an EPIs indirectly prevents the biofilm formation in the host cell through inhibition of adhesion factors (MSCRAMMs and SERAMs).[29-31]

Conclusions

This study finds that capsaicin can be used as an alternative herbal substance to threat the antibiotic resistance of *S. aureus* (planktonic and biofilm cells). The bacteria grow because of the consistent alternative substances, such as: flavonoid, phenol, carotenoid, and alkaloid. Despite this, capsaicin is an EPI that eliminates *S. aureus* as it enables the production of antibiotics, thereby reaching the biofilm environment.

Future scope

The major limitation of the study was the difficulty to grow and isolate the Staphylococcus bacteria, which is the most important thing in this study. Inspite of this, the other things that were disadvantageous were the COVID-19 pandemic and buying the Capsaicin Extract in Malang.

Acknowledgment

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Author contributions

Data analysis, interpretation, and article preparation was done by FS; data collection and laboratory work was done by ER; data were checked by AY and JS; and finally, the article and submission process of the article was done by DAW and AB.

Ethical policy and Institutional Review board statement

This laboratory experimental study with a randomized posttest only control group design was conducted in Research Centre, Microbiology Laboratorium Division, Faculty of Dental Medicine, Universitas Airlangga Surabaya and it had received approval for an ethical fit test. All of the protocols were approved by the Ethics Committee of Faculty of Medicine, Universitas Airlangga Surabaya (No: 168/EC/KEPK/FKUA/2019).

Patient declaration of consent

This study is an *in vitro* study, so this part is not applicable.

Data availability statement

Data are available on reasonable request to the corresponding author's mail.

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