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Oxidative Stress-Mediated Repression of Virulence Gene Transcription and Biofilm Formation as Antibacterial Action of *Cinnamomum burmannii* Essential Oil on *Staphylococcus aureus*

Lingling Shi [1,†], Wei Lin [1,†], Yanling Cai [2], Feng Chen [1], Qian Zhang [2], Dongcheng Liang [2], Yu Xiu [1], Shanzhi Lin [1,*] and Boxiang He [2,*]

[1] College of Biological Sciences and Biotechnology, National Engineering Laboratory for Tree Breeding, Key Laboratory of Genetics and Breeding in Forest Trees and Ornamental Plants, Ministry of Education, Tree and Ornamental Plant Breeding and Biotechnology Laboratory of National Forestry and Grassland Administration, Beijing Forestry University, Beijing 100083, China; shilingling2019@bjfu.edu.cn (L.S.); linwei@bjfu.edu.cn (W.L.); chenfeng1996@bjfu.edu.cn (F.C.); yuxiu@bjfu.edu.cn (Y.X.); szlin@bjfu.edu.cn (S. L.)

[2] Guangdong Provincial Key Laboratory of Silviculture, Protection and Utilization, Guangdong Academy of Forestry, Guangzhou 510520, China; caiyl@sinogaf.cn (Y.C.); zhangq7610@sinogaf.cn (Q.Z.); gzldc@sinogaf.cn (D.L.); heboxiang@sinogaf.cn (B.H.)

* Correspondence: szlin@bjfu.edu.cn (S.L.); Tel.: +86-10-62336114. heboxiang@sinogaf.cn (B.H.).

† These authors contributed equally to this work.

Abstract: This work aimed to identify chemical compounds of *Cinnamomum burmannii* leaf essential oil (CBLEO) and to unravel antibacterial mechanism of CBLEO at molecular level for developing antimicrobial. CBLEO had 37 volatile profiling with abundant borneol (28.40 %), and showed good potential to control foodborne pathogens, of which *Staphylococcus aureu*s had the greatest inhibition zone diameter (28.72 nm) with the lowest values of minimum inhibitory concentration (1.0 μg/mL) and bactericidal concentration (2.0 μg/mL). To unravel antibacterial action of CBLEO on *S. aureus*, a dynamic exploration of antibacterial growth, material leakage, ROS formation, protein oxidation, cell morphology and interaction with genome DNA were conducted on *S. aureus* exposed to CBLEO at different doses (1/2-2×MIC) and times (0-24 h), indicating that CBLEO acts as inducer for ROS production and oxidative stress of *S. aureus*. To highlight antibacterial action of CBLEO on *S. aureus* at molecular level, a comparative association of ROS accumulation with some key virulence-related gene (sigB/agrA/sarA/icaA/cidA/rsbU) transcription, protease production and biofilm formation in *S. aureus* subjected to CBLEO at different levels and times, revealing that CBLEO-induced oxidative stress caused transcript suppression of virulence regulators (RsbU and SigB) and its targeted genes, causing protease level increase destined for biofilm formation and growth inhibition of *S. aureus*, which may be as key bactericidal action. Our findings provide valuable information for studying the antibacterial mechanism of essential oil against pathogens.

Keywords: *Cinnamomum burmannii* essential oil; antibacterial action; oxidative stress; virulence gene; transcriptional expression; biofilm; Staphylococ*cus aureus*

1. Introduction

Food is rich in nutrients and suitable for the growth and reproduction of pathogens. Microbial food spoilage and food-borne disease remain the major issue for public health worldwide [1]. The synthetic preservatives, such as tertiary butylhydroquinone (TBHQ), butylated hydroxytoluene (BHT) and several organic acids and salts), have been widely applied in food industry to guarantee both food safety and security, but various adverse effects (immunity suppression, teratogenicity, carcinogenicity, hypersensitivity, allergic reaction, and acute toxicity) caused by prolonged use have greatly impacted public health, which has become a hot button worldwide [1–3]. Therefore, the

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development of novel and effective natural antimicrobial agent is of paramount importance to assure food safety and public health.

Plant essential oil has been identified with high and extensive biological properties (such as antimicrobial, anticancer, and antiviral and antioxidant activity) [4–9]. In recent years, the development and utilization of novel natural essential oils in food industry has become one research focus, and notably, plant essential oils have been used as new natural source of antimicrobials, food preservatives and packaging [1,2,10–12]. Several studies have indicated that essential oil can cause a range of damage to bacterial, including cell membrane damage and content leakage [6,8,9,13–15], respiratory metabolism depression [9,14,16], redox homeostasis disruption [8,17], DNA topological change and DNA/RNA synthesis) [9,15,18,19]. It is also noted that foodborne pathogens-caused food poisoning and diseases have become one major threat to human health and food safety by secreting enterotoxins [20,21], but the growth and reproduction of pathogens are tightly dependent on biofilm formation, and thus the prevention of pathogen biofilm formation has become an effective way to ensure food safety [22]. The regulation of bacterial biofilm formation may involve in coordinated expression of several virulence genes [23–25]. In recent years, the essential oils have been identified with anti-biofilm effect against fungi and bacteria [18,26–31] and effective inhibition of virulence-related gene expression [9,16,26,28]. Yet, few studies have concerned about cellular response of bacteria to oxidative stress and ROS accumulation induced by essential oil, moreover, the acting mechanism of essential oil involved in virulence attenuation and biofilm formation inhibition remains enigmatic.

The genus *Cinnamomum*, a member of the family Lauraceae, is widely distributed in Southeast Asia with notable economic value owing to its rich essential oil and medicinal utilization [32–34]. The essential oils of *Cinnamomum* plants have been shown with various biological effects (such as antimicrobial, anti-inflammatory and antitumor) [32–36], and widely used in medicine, perfume and chemical industry, especially in food industry for natural preservative, antimicrobial and antioxidant agents, and flavoring agent (such as brewing chocolate, chewing gum and liquors) [37–39]. Of note, *Cinnamomum burmannii* is one of the most promising sources in food, pharmaceutical or cosmetic industry due to its unique compounds (such as borneol, α-terpineol and α-pinene) [32,38]. Presently, based on our studies on different *C. burmannii* germplasms, some accessions have been selected with high yield of essential oil (1.2 %-1.6 %) and high proportion of borneol (45.5 %-64.8 %), and notably, we have established standard system for utilization of *C. burmannii* [40–42]. Yet, the antibacterial mechanism of *C. burmannii* leaf essential oil (CBLEO) is unknown, which has intercepted CBLEO application in modern industry.

The aim of this work was to unravel antibacterial action and to highlight molecular mechanism that governed bacterial growth and biofilm inhibition for developing CBLEO as potential source of natural antibacterial agent. To this end, one plus tree of *C. burmannii* (accession CB01) was used to detect volatile profile of CBLEO and to assess antibacterial activity on 7 representative food-borne pathogens, and antibacterial action of CBLEO was conducted on *Staphylococcus aureus* (susceptible strain). As an initial step toward exploring antibacterial action of CBLEO, we focused on the dynamic effects of CBLEO on bacterial growth, content release, electric conductivity, ROS and MDA formation, protein oxidation, and cell morphology in *S. aureus* subjected to different doses (1/2×MIC, 1×MIC and 2×MIC) and times (0-8 h). Such assay could help to unravel how CBLEO induce oxidative stress. To highlight antibacterial acting mechanism of CBLEO on *S. aureus* at molecular level, a comparative assay was conducted on the association of ROS accumulation with some key virulence-related gene transcription, protease level, biofilm formation and the interaction with genome DNA in response of *S. aureus* to CBLEO at different levels and times. This study presents the effect of essential oil-mediated oxidative stress and ROS accumulation on transcription of virulence-associated regulators as an attempt to elucidate antibacterial mechanism of essential oil.

2. Results

2.1. Identification of volatile compounds in C. burmannii leaf essential oil (CBLEO)

To develop C. burmannii leaf essential oil (CBLEO) as potential source for application, we detected chemical compounds of CBLEO by GC-MS. A total of 37 compositions were identified (Table 1), accounting for 99.56 % of total oil. The main chemical components of CBLEO mainly included 14

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monoterpene hydrocarbon, 29 sesquiterpene hydrocarbon, 6 monoterpene alcohol, 6 sesquiterpene alcohol and 5 sesquiterpene hydrocarbon, of which monoterpene (85.38 %) was the dominant group of components. The rich compound was borneol (28.40 %), followed by bornyl acetate (9.43 %), eucalyptol (9.22 %), D-limonene (7.44 %), α-pinene (3.96 %), β-cymene (3.96 %), β-caryophyllene (3.71 %), α-terpineol (3.15 %), α-phellandren (2.67 %), sabinene (2.53 %), β-myrcene (2.41 %), β-pinene (2.38 %) and camphor (2.14 %). All these indicated a complex of chemical components with rich borneol in CBLEO.

Table 1. Compounds and contents of the essential oils from *Cinnamomum burmami* leaves by GC-MS analysis.

Type	Compound	Formula	RI[a]	Percentage (%)[b]
Monoterpene hydrocarbons (14)	α-Thujene	$C_{10}H_{16}$	902	0.56 ± 0.03^{e}
	α-Pinene	$C_{10}H_{16}$	948	3.96 ± 0.17^{bc}
	(−)-Camphene	$C_{10}H_{16}$	943	2.03 ± 0.12^{c}
	Sabinene	$C_{10}H_{16}$	897	2.53 ± 0.11^{c}
	β-Pinene	$C_{10}H_{16}$	943	2.38 ± 0.10^{c}
	β-Myrcene	$C_{10}H_{16}$	958	2.41 ± 0.10^{c}
	α-Phellandrene	$C_{10}H_{16}$	969	2.67 ± 0.12^{c}
	3-Carene	$C_{10}H_{16}$	948	0.32 ± 0.02^{e}
	4-Carene	$C_{10}H_{16}$	919	0.19 ± 0.01^{f}
	β-Cymene	$C_{10}H_{14}$	1042	3.96 ± 0.19^{bc}
	D-Limonene	$C_{10}H_{16}$	1018	7.44 ± 0.25^{b}
	β-*cis*-Ocimene	$C_{10}H_{16}$	976	0.43 ± 0.02^{e}
	γ-Terpinene	$C_{10}H_{16}$	998	0.50 ± 0.03^{e}
	α-Terpinolen	$C_{10}H_{16}$	1052	0.96 ± 0.06^{e}
Monoterpene alcohol (6)	Eucalyptol	$C_{10}H_{18}O$	1059	9.22 ± 0.29^{b}
	Linalool	$C_{10}H_{18}O$	1082	0.63 ± 0.03^{e}
	Borneol	$C_{10}H_{18}O$	1138	28.40 ± 0.62^{a}
	Terpinen-4-ol	$C_{10}H_{18}O$	1137	1.50 ± 0.09^{cd}
	α-Terpineol	$C_{10}H_{18}O$	1143	3.15 ± 0.18^{bc}
	Guaniol	$C_{10}H_{18}O$	1228	0.23 ± 0.01^{f}
Monoterpene ketone (1)	Camphor	$C_{10}H_{16}O$	1121	2.14 ± 0.10^{bc}
Monoterpene aldehyde (1)	α-Citral	$C_{10}H_{16}O$	1174	0.19 ± 0.01^{f}
Monoterpene ester (2)	Bornyl acetate	$C_{12}H_{20}O_2$	1277	9.33 ± 0.24^{b}
	Geranyl acetate	$C_{12}H_{20}O_2$	1352	0.25 ± 0.01^{f}
Sesquiterpene hydrocarbons (5)	β-Caryophyllene	$C_{15}H_{24}$	1494	3.71 ± 0.19^{bc}
	α-Caryophyllene	$C_{15}H_{24}$	1579	0.99 ± 0.05^{e}
	Germacrene D	$C_{15}H_{24}$	1515	0.58 ± 0.03^{e}
	α-Guaiene	$C_{15}H_{24}$	1469	0.19 ± 0.01f
	Germacrene B	$C_{15}H_{24}$	1603	1.74 ± 0.10^{cd}
Sesquiterpene alcohol (6)	Elemol	$C_{15}H_{26}O$	1522	0.42 ± 0.02^{e}
	trans-Nerolidol	$C_{15}H_{26}O$	1564	1.36 ± 0.07^{cd}
	Spathulenol	$C_{15}H_{24}O$	1536	1.72 ± 0.08^{cd}
	Guaiol	$C_{15}H_{26}O$	1614	1.28 ± 0.08^{cd}
	(−)-Spathulenol	$C_{15}H_{24}O$	1536	0.51 ± 0.03^{e}
	Bulnesol	$C_{15}H_{26}O$	1614	0.44 ± 0.02^{e}
Sesquiterpene ester (1)	Caryophyllene oxide	$C_{15}H_{24}O$	1507	1.00 ± 0.08^{cd}
Others (1)	Cinnamyl acetate	$C_{11}H_{12}O_2$	1367	0.24 ± 0.01^{f}
Total				99.56 ± 0.21

[a] Retention index of present experiment determined on SH-R×ITM-5SIL MS column using n-alkanes (C_8–C_{25}) series. [b] Relative percentage was calculated by peak area, and the data represent the average of three replicates (n = 3). Different lowercase letters represent significant differences ($p < 0.05$).

2.2. Assay of antibacterial activity of CBLEO

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The detections of DIZ, MIC and MBC values were applied to determine antibacterial activity of CBLEO. In case of CBLEO, the DIZ values ranged from 7.51 to 28.72 mm across all tested strains, and the MIC and MBC values were in range of 1.0−16.0 μg/mL and 2.0−32.0 μg/mL, respectively (Table 2), indicating that CBLEO displayed good inhibition effects on foodborne pathogens. Of note, the maximum value of DIZ (28.72 nm) and the minimum values of MIC (1.0 μg/mL) and MBC (2.0 μg/mL) were all marked in response of S. aureus to CBLEO, indicating a strong bactericidal effect of CBLEO on S. aureus. Hence, to develop CBLEO as potential antibacterial agent, our following works were focused on the exploration of antibacterial action of CBLEO on S. aureus.

Table 2. Assay of antibacterial activity of *C. burmannii* leaf essential oils (CBLEO) against seven representative food-borne pathogens.

Microorganisms	CBLEO			Antibiotic[a]		
	DIZ[b] (mm)	MIC[b] (μg/mL)	MBC[b] (μg/mL)	DIZ[b] (mm)	MIC[b] (μg/mL)	MBC[b] (μg/mL)
Gram-positive bacteria						
Bacillus subtilis	21.31 ± 0.65[b]	2.0	4.0	40.70 ± 0.71[a]	0.125	0.25
Listeria monocytogenes	13.33 ± 0.45[bc]	4.0	8.0	35.31 ± 0.56[b]	0.125	0.25
Staphylococcus aureus	28.72 ± 0.72[a]	1.0	2.0	30.05 ± 0.56[b]	0.5	1.0
Gram-negative bacteria						
Escherichia coli	9.71 ± 0.61[bc]	8.0	16.0	37.52 ± 0.61[a]	0.25	0.5
Pseudomonas aeruginosa	7.51 ± 0.48[bc]	16.0	32.0	37.31 ± 0.57[a]	0.25	0.5
Enterobacter aerogenes	10.21 ± 0.51[bc]	8.0	16.0	33.71 ± 0.61[b]	0.25	0.5
Salmonella	9.02 ± 0.43[bc]	8.0	16.0	39.51 ± 0.45[a]	0.125	0.25

[a]Ampicillin was used as reference antibacterial agent. [b]DIZ: diameter of inhibition zone; MIC: minimal inhibitory concentration; MBC: minimal antibacterial concentration. The values are mean of triplicate detection (n = 3) ± standard deviation. Different lowercase letters represent significant differences ($p < 0.05$).

2.3. Effect of CBLEO on bacterial growth of S. aureus

To unravel antibacterial action of CBLEO against S. aureus, the assay of antibacterial kinetics curve was conducted on the CBLEO treatments with five different doses (1/8×MIC, 1/4×MIC, 1/2×MIC, 1×MIC, and 2×MIC) and times (0-24 h), all of which displayed a dose/time-dependent inhibition manner for bacterial growth (Figure 1). The greatest inhibitory effect was recorded at 2×MIC for 24 h with the number of viable cells reduced by 96.86 % from 7.0 to 0.2 lg CFU/mL, followed by 93.57 % decrease at 1×MIC, but 38.29 %, 20.28 % and 6.86 % decline was marked for 1/2×MIC, 1/4×MIC and 1/8×MIC, respectively (Figure 1). A significant inhibition of bacterial growth was noted within 1 h incubation of 1×MIC and 2×MIC, and a complete inhibition occurred within the first 8 h and 12 h at 2×MIC and 1×MIC, respectively, but the control cells showed a normal growth pattern (Figure 1), indicating a great bactericidal potential of CBLEO to S. aureus.

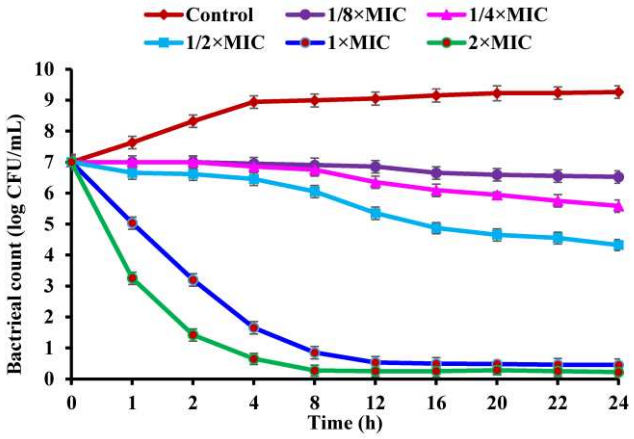

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Figure 1. The growth kinetics curve of Staphylococcus aureus affected by CBLEO. The data represented the mean value ± SD of three parallel replicates.

2.4. Impact of CBLEO on cell structure of S. aureus

The damage of cell structure (cell wall and membrane) in CBLEO-treated S. aureus was evaluated as part of an attempt to understand antibacterial action of CBLEO. Firstly, the effect of CBLEO on cell wall damage was tested by the leakage assay of AKP (cell wall damage-biomarker enzyme). In presence of CBLEO, AKP activity in S. aureus suspensions increased by a dose/time-dependent pattern (Figure 2A). After 8 h of exposure to CBLEO, AKP activity at 2×MIC displayed 1.7- and 0.8-fold greater than that at 1/2×MIC and 1×MIC, respectively, and the notably increased activity was recorded within the first 2 h at both 1×MIC and 2×MIC, whereas the control exhibited no change in AKP activity (Figure 2A), revealing a destruction of cell wall of S. aureus caused by CBLEO.

Next, CBLEO-mediated damage on the permeability of cell membrane was assayed by detecting electric conductivity. During exposure to CBLEO, a dose/time-dependently increased profile for electric conductivity was marked in S. aureus suspensions, of which a rapid increase was all detected within the first 1 h (Figure 2B). After 8 h incubation of CBLEO, the values of electric conductivity at 1/2×MIC, 1×MIC and 2×MIC displayed 4.0-, 7.3- and 8.9-fold greater than those of the control (Figure 2B), indicating that CBLEO could effectively cause cell membrane damage and permeability increase of S. aureus with the intracellular electrolyte release.

As for cell membrane integrity, the releases of intracellular protein and nucleic acid were tested. After incubation of S. aureus with different doses of CBLEO (1/2×MIC, 1×MIC and 2×MIC), the released amount of protein displayed a dose/time-dependent increase, in which the increased degree of protein release at 2×MIC for 8 h was 2.0- and 0.7-fold greater than at 1/2×MIC and 1×MIC respectively (Figure 2C). A similar dose/time-dependently increased pattern was also noted for nucleic acid leakage (Figure 2D). Yet, those in the control showed no significant change (Figure 2C, D). These results indicated that CBLEO could result in irreversible damage of cytoplasmic membrane integrity of S. aureus with a loss of cellular materials.

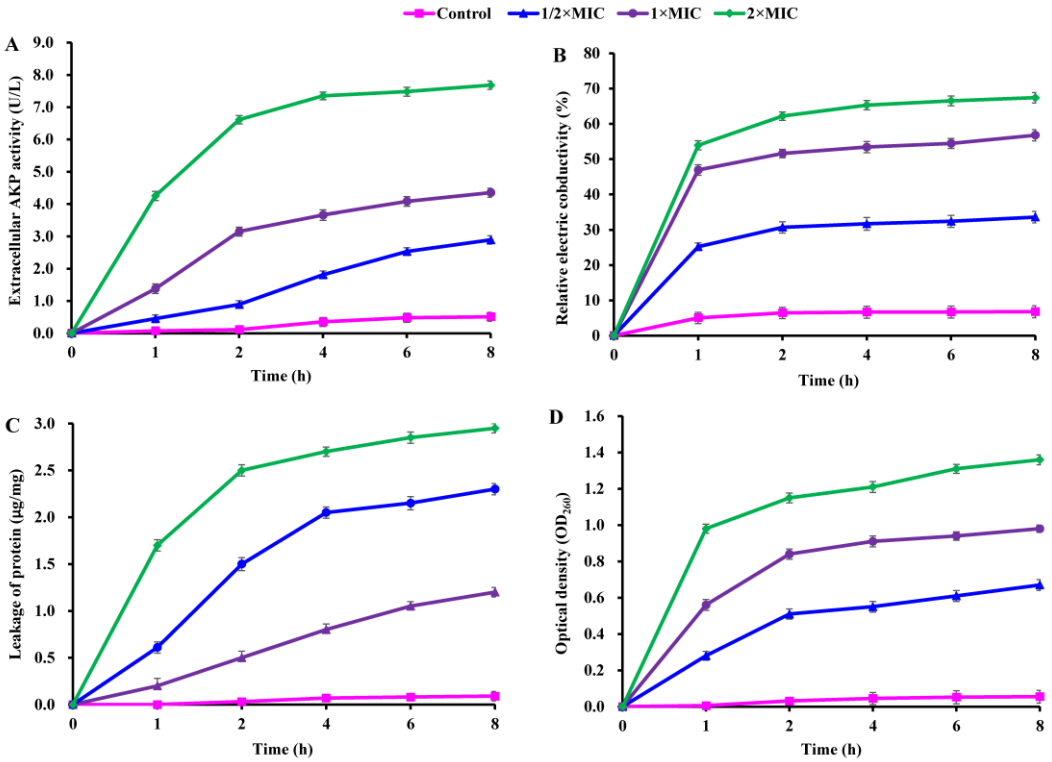

Figure 2. Effect of CBLEO on cell wall and cell membrane of S. aureus. **(A)** Extracellular activity of alkaline phosphatase (AKP); **(B)** Relative electric conductivity; **(C)** Leakage of protein; **(D)** Release of 260-nm absorbing material. The data represented the value ± SD of three replicates.

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To highlight antibacterial action of CBLEO on S. aureus, we further performed SEM analysis to explore the influence of CBLEO on cell morphology of S. aureus. The untreated S. aureus cells retained normal and complete appearance with a smooth surface, intact cell membrane and cell wall structure (Figure 3A), whereas the CBLEO-treated S. aureus cells became irregular, and cell surface collapsed or shriveled (Figure 3B), and thus considered that CBLEO could change cell morphology and damage cell membrane of S. aureus. This further confirmed the above assayed results of CBLEO-caused damage to the permeability and integrity of cell membrane of S. aureus (Figure 2).

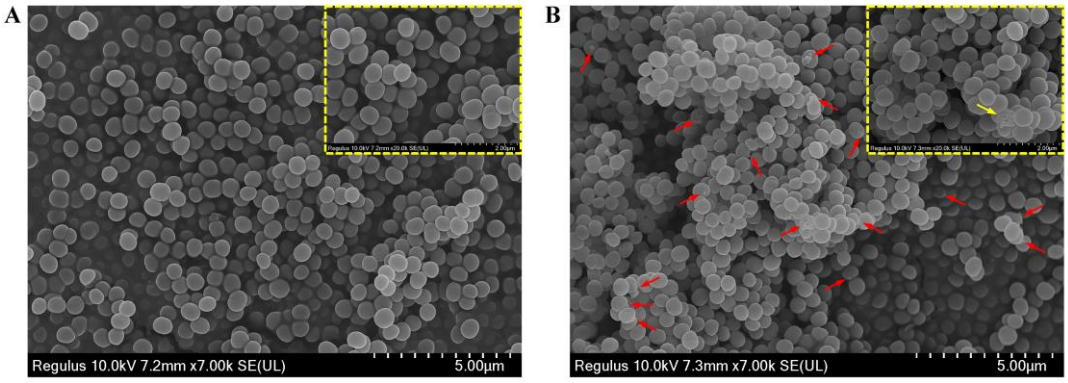

Figure 3. Effect of CBLEO on cell morphologically of S. aureus by scanning electron microscope (SEM) assay. **(A)** SEM image of the untreated S. aureus; **(B)** SEM image of S. aureus treated with CBLEO (1×MIC) for 2 h. The red arrows represented cell morphology change and cell membrane damage.

2.5. Effect of CBLEO on cellular MDA and ROS generation and protein oxidation of S. aureus

Considering that CBLEO could damage cell structure and control bacterial growth of S. aureus (Figures 1-3), it was vital to determine the potential of CBLEO to induce oxidative stress destined for cell membrane damage and growth inhibition of S. aureus. Firstly, MDA was selected as lipid peroxidation-biomarker to evaluate the temporal amount change in S. aureus cells during exposure to CBLEO, and a dose/time dependently increased pattern was identified for intracellular MDA (Figure 4A), in which MDA content was much higher at 2×MIC than at both 1×MIC and 1/2×MIC after 8 h exposure, while the control remained almost no change, implying that CBBLEO could induce cell lipid peroxidation of S. aureus. Hence, we then focused on exploring intracellular ROS generation and protein oxidation of S. aureus cells as oxidative stress-indicator. The intracellular levels of ROS and protein carbonyl formation increased in S. aureus cells by a dose/time dependent manner during exposure to CBLEO, both of which all showed the maximum value after 8 h of exposure to the CBLEO at 2×MIC, but no notable change was detected for the control (Figure 4B, C), emphasizing that CBLEO-induced oxidative stress could result in an accumulation of ROS and protein oxidation product in S. aureus cells.

2.6. Effect of CBLEO on cellular total protein concentration of S. aureus

Given the effect of CBLEO-induced oxidative stress on cellular lipid peroxidation and protein oxidation of S. aureus (Figure 4B, C), it was essential for us to investigate whether CBLEO-induced oxidative stress affected total protein of S. aureus cells. Hence, a dynamic analysis of cellular total protein was performed in S. aureus exposed to CBLEO. Compared with the control, the amount of cellular total protein exhibited a dose/time-dependently decreased profile in S. aureus exposed to CBLEO, and 25.79 %, 34.37 % and 53.01 % decline was observed at 1/2×MIC, 1×MIC and 2×MIC after 8 h incubation of CBLEO respectively (Figure 4D), referring an induction of bacterial protein fragmentation or its biosynthesis disturbance by CBLEO.

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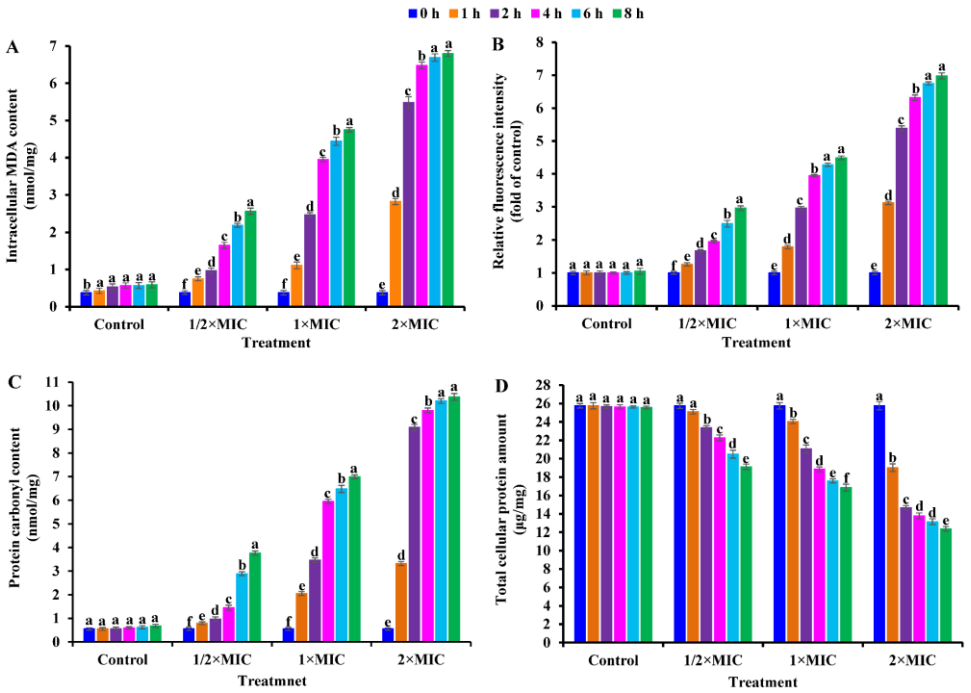

Figure 4. Effect of CBLEO on cell lipid peroxidation and oxidative stress in response of S. aureus to different doses and times. **(A)** Intracellular mallondialdehyde (MDA) level; **(B)** Intracellular ROS generation; **(C)** Protein carbonyl content; **(D)** Total cellular protein. The data represented the mean value ± SD of three parallel replicates, and different letters denoted significant differences ($p < 0.05$).

2.7. Effect of CBLEO on cellular biofilm development and protease activity of S. aureus

Another concern was that whether CBLEO has anti-biofilm activity against S. aureus. A dose/time-dependent decrease in biofilm biomass was marked in S. aureus cells exposed to CBLEO, of which the reduction of biofilm biomass was 43.64 %, 63.75 % and 85.42 % after 8 h of exposure to 1/2×MIC, 1×MIC and 2×MIC respectively, but the increased biofilm formation was observed for the control (Figure 5A), emphasizing that CBLEO had good anti-biofilm effect on S. aureus. Also noteworthy was an association of biofilm formation decline with protease production [43]. Compared with biofilm biomass (Figure 5A), the protease activity exhibited a dose/time-dependent increase in S. aureus exposed to CBLEO, but a decrease in protease activity was detected for the control (Figure 5B), indicating that CBLEO-induced low capacity of biofilm formation may be associated with an increase of protease activity.

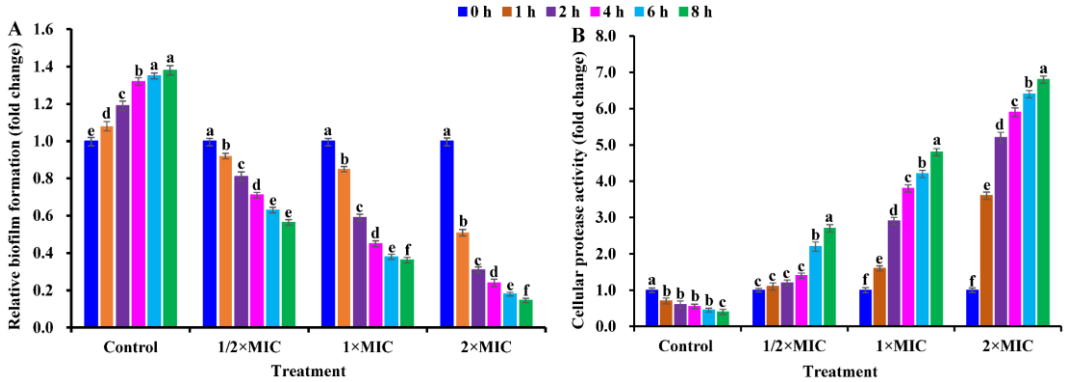

Figure 5. Effect of CBLEO on biofilm formation and protease activity in response of S. aureus cells to different doses and times. **(A)** Assessment of inhibitory capacity of CBLEO on biofilm formation by microtiter plate assay; **(B)** Change of cellular protease activity. The values of biofilm formation and protease production in S. aureus cells from the control and CBLEO-treated with different doses at 0 h

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was arbitrarily set to 1.00 for standardization, and the data represented the mean value ± SD of six parallel replicates and different letters denote significant differences ($p < 0.05$).

2.8. Effect of CBLEO on genome DNA of S. aureus

It was reported that main component (carvacrol or citral) of essential oil could be chimeric with bacterial DNA and break DNA structure of S. aureus and E. coli [9,14,16]. In attempt to highlight antibacterial action of CBLEO on S. aureus at the molecular level, we explored the binding activity of CBLEO to genomic DNA of S. aureus during exposure to CBLEO at different concentrations and times by agarose gel electrophoresis assay. After incubation, the same single band of genomic DNA was marked for S. aureus from all the CBLEO-treated and control samples, and the band brightness of them showed no notable change (Figure 6), suggesting that CBLEO may have no direct effect on genome DNA of S. aureus.

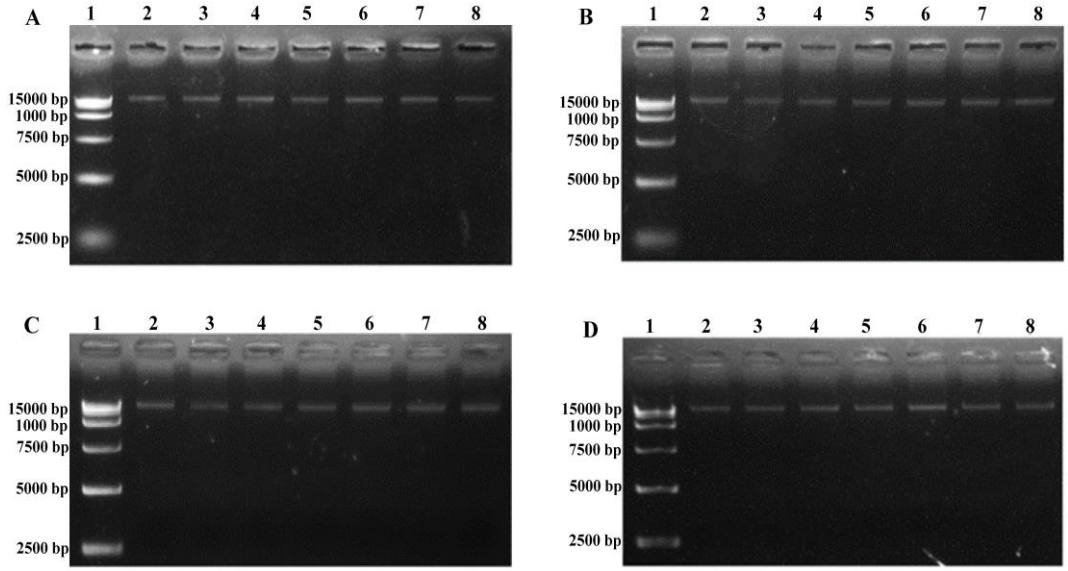

Figure 6. Effect of CBLEO on genome DNA of S. aureus cells by agarose gel electrophoresis assay. **(A)** After 0.5 h incubation with different levels of CBLEO; **(B)** After 1 h incubation with different levels of CBLEO; **(C)** After 3 h incubation with different levels of CBLEO; **(D)** After 5 h incubation with different levels of CBLEO. Lane 1 signified DNA Marker; Lane 2 represented genome DNA of S. aureus treated by PBS (as the control); Lanes 3-8 signified genome DNA of S. aureus treated with 1/2×MIC, 1×MIC, 2×MIC, 4×MIC, 8×MIC and 16×MIC of CBLEO, respectively.

2.9. Effect of CBLEO on transcript of virulence-related genes and regulatory proteins in S. aureus

The above findings that oxidative stress could effectively induce ROS accumulation (Figure 4B) and biofilm formation reduction (Figure 5A) with no direct effect on bacterial genome DNA (Figure 6) in response of S. aureus cells to CBLEO, prompted us to highlight mechanism for how CBLEO cause virulence attenuation and biofilm formation inhibition at the molecular level. Biofilm formation, one key virulence determinant, is controlled by several virulence genes in response to ROS. To ascertain the anti-biofilm mechanism of CBLEO at molecular level, some vital virulence-associated genes responsible for biofilm formation, including agrA (accessory gene regulator A), sigB (sigma factor B, involved in biofilm formation and stress response), sarA (staphylococcal accessory regulator A), cidA (murein hydrolase regulator, involved in cell lysis and extracellular DNA release), icaA (intercellular adhesin A, involved in cell wall and biofilm formation), and rsbU (involved in regulation of sigB and biofilm formation) were selected as potential antibacterial targets to detect dynamic changes of their transcriptional levels in response of S. aureus cells to CBLEO by qRT-PCR.

As shown in Figure 7, the transcriptional levels of sigB/rsbU/agrA/sarA/icaA/cidA in CBLEO-treated S. aureus cells were all down-regulated by a dose/time-dependent pattern, of which the lowest transcript level was recorded for 8 h at 2×MIC, but an increase of them was marked in the

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control, as also noted for biofilm formation in CBLEO-treated S. aureus cells (Figure 5A), indicating that CBLEO could inhibit transcription of virulence-related genes destined to reduction of S. aureus biofilm formation. Of note, the down-regulated degree of transcriptional level of sigB (88.02 %) and rsbU (92.01 %) were much higher than that of agrA/sarA/icaA/cidA (68.89 %–73.97 %) after 8 h of exposure at 2×MIC (Figure 7), implying that SigB and RsbU may be as crucial regulatory protein for controlling biofilm formation in CBLEO-treated S. aureus.

Together, CBLEO-induce collaborative transcription repression of virulence-related genes was mainly responsible for the inhibition of biofilm formation, of which both RsbU and SigB) may be as antibacterial targets of CBLEO against S. aureus (Figure 8).

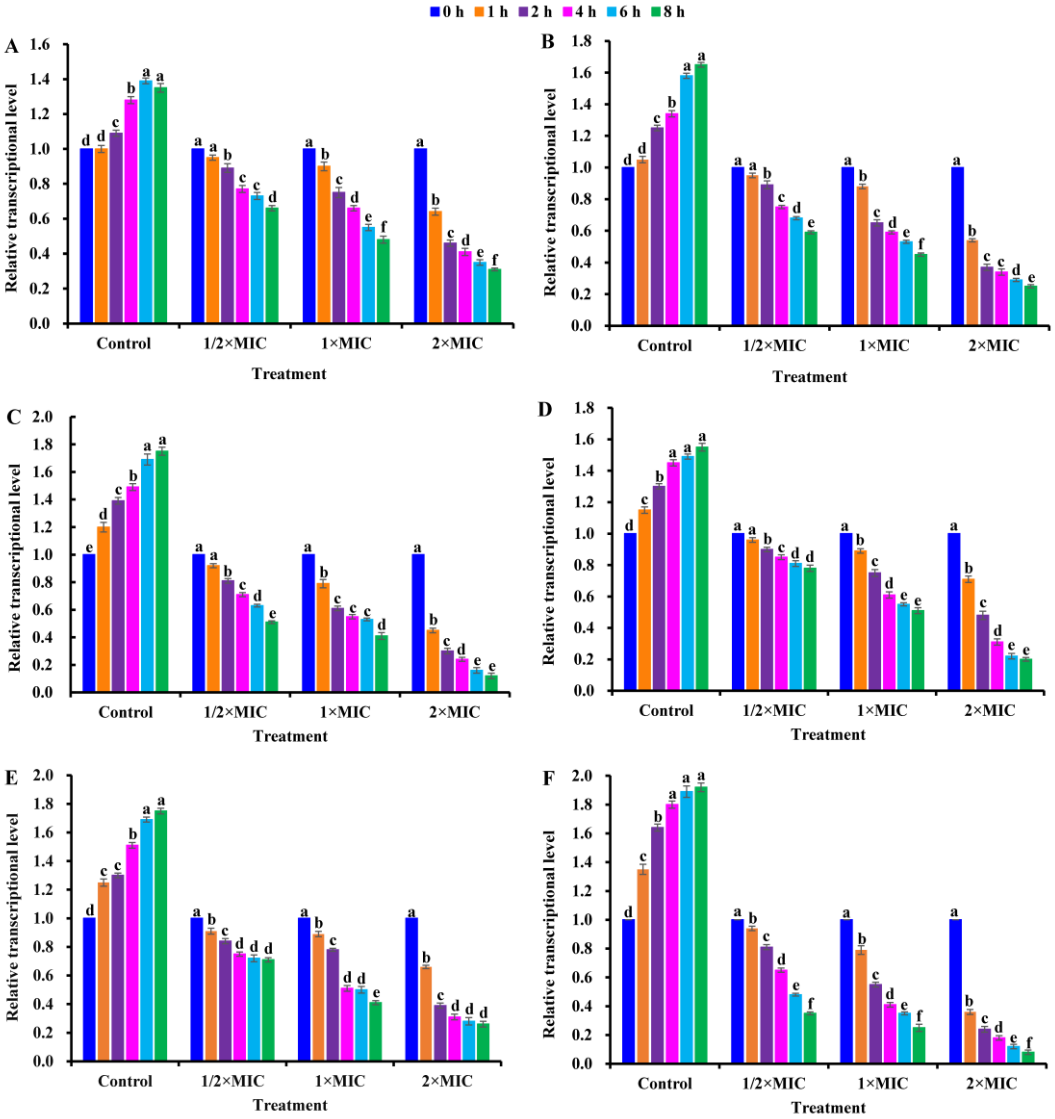

Figure 7. Effect of CBLEO on transcriptions of virulence-associated regulators of S. aureus under exposure to different concentrations and times by qRT-PCR detection. **(A)** Relative transcription of agrA (accessory gene regulator A); **(B)** Relative transcription of sarA gene (staphylococcal accessory regulator A); **(C)** Relative transcription of sigB (sigma factor B); **(D)** Relative transcription of icaA (intercellular adhesin A); **(E)** Relative transcription of cidA gene (encoding for holin); **(F)** Relative transcription of rsbU (SigB activator). The relative expression values were counted as $2^{-\triangle\triangle Ct}$, and 16S RNA was used as the internal control. The transcription level in S. aureus cells from the control and CBLEO-treated with different concentrations at 0 h was arbitrarily set to 1.00 for standardization, and the data represented the mean value ± SD of three parallel replicates and different letters denote significant differences ($p < 0.05$).

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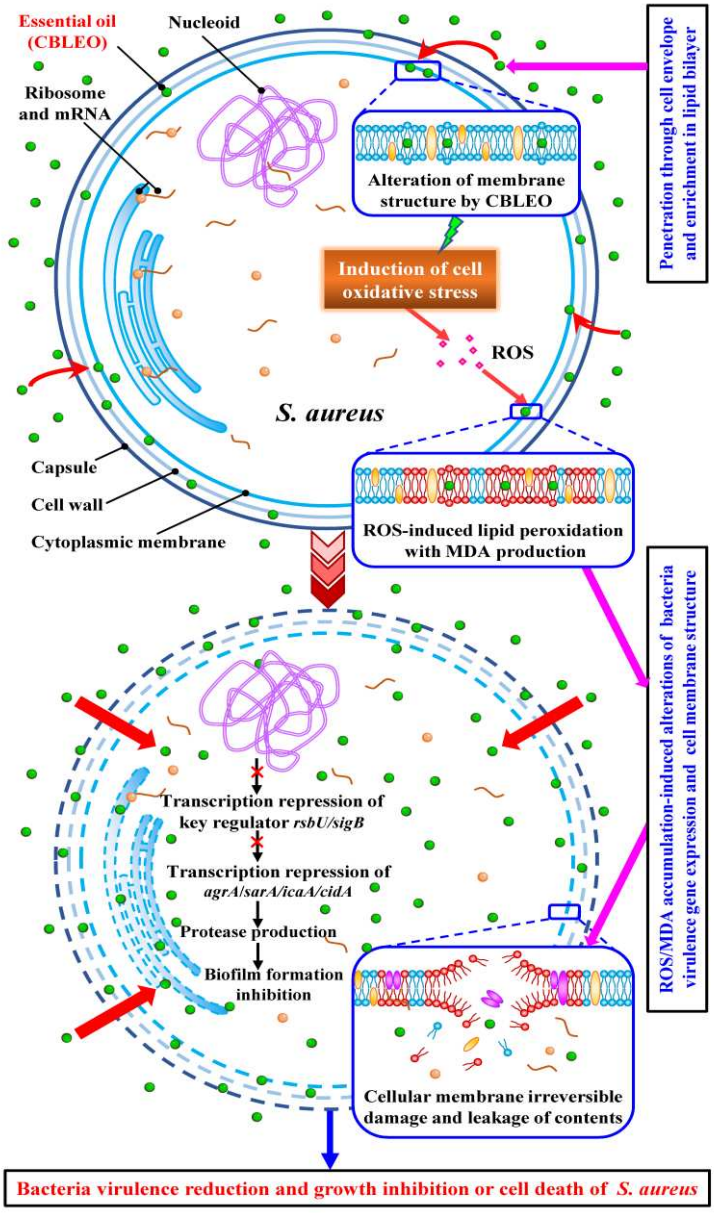

Figure 8. The Antibacterial acting mode of C. burmami leaf essential oil (CBLEO) on S. aureus. The identified antibacterial mechanism of CBLEO against S. aureus are based on the present work is summarized from two perspectives of virulence-related gene transcription regulation and cellular structure destruction. The abbreviations are shown as the follow: agrA, accessory gene regulator A; sarA, staphylococcal accessory regulator A; sigB, sigma factor B; icaA, intercellular adhesin A; ROS, reactive oxygen species; MDA, mallondialdehyde.

3. Discussion

3.1. Rich volatile profiling and high antibacterial activity for CBLEO

Essential oils from plants have been widely used as natural antimicrobials in food field [10–12]. In this work, 37 volatile compositions were identified in C. burmannii leaf essential oil (CBLEO) (Table 1), of which many compounds and its contents were different from those reported for other Cinnamomum species (such as C. pauciflorum, C. zeylanicum and C. camphora) [26,37,44,45], indicating a difference in volatile profiling and its contents among different Cinnamomum species. Several works have revealed a strong antimicrobial activity for some volatile components (such as α-

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pinene, α-terpineol, β-pinene, β-ocimene, β-caryophyllene, 1,4-cineole, eucalyptol, limonene, borneol, bornyl acetate, and linalool) [4–8,26,36,37,46–51]. Here, given rich compounds of borneol (28.31%), bornyl acetate (9.43 %), eucalyptol (9.22 %), limonene (7.44%), α-pinene (3.96 %), β-caryophyllene (3.71%) and α-terpineol (3.15%) in CBLEO (Table 1), integrated with a good inhibitory effect of CBLEO on all tested food-borne pathogens (Table 2), It seems certain that these chemical compositions may be potential antimicrobial compounds of CBLEO. Of note, borneol was identified as the richest compound of CBLEO (Table 1), suggesting that it may be the most promising antibacterial agent, which could be evidenced by recently published results of pharmacological studies [32,47–49]. All findings indicated that the essential oil (especially borneol) from C. burmannii leaf may be as novel source of natural antimicrobial agent. Of note, S. aureus was identified as the most susceptible pathogen (Table 2), and thus the following work focused our attentions on highlight antibacterial action of CBLEO against S. aureus for development of CBLEO as natural antibacterial agent for potential utilization.

3.2. ROS generation-mediated oxidative stress and cell membrane damage involved in antibacterial action of CBLEO

During exposure of bacteria to the essential oil, lipophilic components could bind to bacterial cell surface and penetrate outer membrane, and then its lipid bilayer may contact with hydrophobic part of cell membrane and subsequent cause toxic effect, leading to cell wall and membrane damage, function destruction, material release, ROS generation and cell death [6,8,9,14,16]. However, the essential oil-mediated mechanism of bacterial cell membrane damage is still unclear. Several studies have shown that intracellular material leakage may be a good biomarker of irreversible damage to cell membrane [6,8,13]. Here, a close negative correlation was established between inhibitory effect of bacterial growth (Figure 1) and significant increase of material release, extracellular AKP activity (cell wall damage-marker enzyme) and electric conductivity in S. aureus during exposure to CBLEO (Figure 2), implying that a destruction of cell membrane structure induced by CBLEO may be as one pivotal cause for growth inhibition of S. aureus. This was in line with previous antibacterial effect of essential oil on several foodborne pathogens (such as Bacillus subtilis, B. cereus, Escherichia coli, E. faecalis, Listeria monocytogenes, Pseudomonas aeruginosa, Shigella dysenteriae, S. aureus, Salmonella typhimurium, and Shigella flexneri) [6,8,9,14,15].

ROS-induced lipid peroxidation during oxidative stress is known as one key initiator to cause cell membrane damage. MDA, the most abundant product of lipid peroxidation, can induce cellular protein oxidation [52], and thus widely serves as indicator of oxidative stress for studying microbial growth, cell death and disease incidence [8,53]. However, the association of oxidative stress with MDA variation in bacterial cell caused by essential oil remains unclear. Given that a similar accumulative pattern of MDA (Figure 4A) and ROS (Figure 4B) in CBLEO-treated S. aureus cells was positively associated to the degree of bacterial growth inhibition, material release and cell membrane damage (Figures 1-3), it seems certain that CBLEO-induced oxidative stress may be one crucial bactericidal effect, as was the case for the effect of essential oil on S. flexneri, S. aureus, E. coli and P. digitatum [8,9,14,17]. This could also be evidenced by previous results showing that the effects of antibacterial agents (such as catechin, fluconazole, miconazole and hypocrellin A) may be attributed to induction of oxidative stress with ROS production and membranous damage destined for cell death [54–56]. Also noteworthy was the impact of ROS accumulation on cellular protein oxidation [57]. Here, the increased amount of cellular protein carbonyl in CBLEO-treated S. aureus (Figure 4 C) ran parallel to a similar accumulation of ROS and MDA (Figure 4 A, B), but both of which showed a negative correlation with the declined content of total cellular protein (Figure 4D), and thereby concluded that CBLEO-induced oxidative stress could cause cellular protein oxidation and biosynthesis disturbance, as also noted for the effect of essential oil on P. digitatum and S. aureus [14,17].

Together, CBLEO-mediated oxidative stress and ROS accumulation may be as the primary driver of cell membrane damage of S. aureus. Yet, little attention has been paid to a cross-talk between

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oxidative stress and virulence-associated factor expression required for biofilm formation during exposure of bacteria to the essential oil.

3.3. CBLEO-induced transcription repression of virulence-associated genes in S. aureus as pivotal antibacterial action

Foodborne disease has become a serious issue affecting human health and food safety. S. aureus, one of the most common foodborne pathogens, can grow in various foods and cause food poisoning by secreting enterotoxins that cause various disease symptoms (such as nausea, vomiting and diarrhea) [20,21], and thus pose a serious threat to human health [22]. The S. aureus enterotoxins, as a superfamily of secreted virulence factors, is generally regulated by quorum-sensing agr system via autoinduce peptide (AIP) and two divergent transcripts (called RNAII and RNAIII), of which RNAII transcript is an operon of agr genes (agrBDCA) that encode key factors for agr regulatory activation [58]. Of these, AIP is produced from AgrD precursor, and then processed and exported as quorum signal by AgrB to activate sensor kinase AgrC and response regulator AgrA, subsequently leading to induction of agr system and upregulation of RNAII/RNAIII transcription essential for virulence production [58–60]. Also, AgrA is known as key virulence regulator, but SarA has been identified as positive regulator of agr activity in S. aureus [23–25]. Yet, it is unclear to whether essential oil can affect bacterial AgrA or SarA activity. Here, the coordinately repressed transcriptions of both sarA and agrA in the response of S. aureus cells to CBLEO (Figure 7A, B) were temporally and positively correlated with bacterial growth inhibition (Figure 1), indicating that CBLEO-induced repression of the transcriptions of sarA and its targeted agrA may contribute to growth inhibition of S. aureus, which was the case for the effect of essential oil on C. albicans, C. violaceum, P. aeruginosa, P. arotovorum, P. aroidearumor and S. aureus [16,18,26–28]. AgrA and SarA, two key global regulator of virulence genes were tightly controlled by transcription regulator SigB [23,61], which may in turn regulate transcriptions of several virulence-related factors crucial for cell processes (such as stress response and biofilm formation) of B. subtilis, L. monocytogenes, P. aeruginosa and S. aureus [25,61,62]. Given the fact that a similar dose/time-dependently repressed transcription of sigB/agrA/sarA/icaA/cidA in CBLEO-treated S. aureus cells (Figure 7) was temporally and positively correlated to an inhibition of bacterial growth (Figure 1) and biofilm formation (Figure 5A), but concomitantly with an increase of protease activity (Figure 5B), it seems likely that CBLEO-mediated coordinate repression of sigB/agrA/sarA/icaA/cidA transcript could activate expression of protease gene destinted to increase its production in S. aureus cells during exposure to CBLEO, which may contribute to inhibition of biofilm formation and bacterial growth. This was consistent with antibacterial effect of essential oil on E. coli, A. baumannii, C. violaceum, C. albicans, P. aroidearumor, P. aeruginosa, and P. arotovorum [9,18,26–29,31]. In support of our results, sigB, sarA, agrA, icaA or cidA mutation in S. aureus could increase protease amount with a significant decline of biofilm formation [43,63–69], and exogenous addition of protease notably limited biofilm formation of S. aureus [70,71].

Also of note was a role of RsbU in SigB activity activation [72,73]. Mutation of rsbU could repress transcription of sigB and its targeted downstream gene (ica/sarA/agr) crucial for bacterial biofilm formation [62,74]. Our findings that a similar dose/time-dependently repressed transcription of rsbU/sigB/agrA/sarA/icaA/cidA in the response of S. aureus cells to CBLEO (Figure 7) ran parallel to the reduced biofilm formation (Figure 5A), of which the transcriptions of rsbU (64.94 %–92.01 %) and sigB (48.98 %–88.02 %) were significantly down-regulated (Figure 7C, F), revealed that both RsbU and SigB may be key regulator in controlling biofilm formation of S. aureus cells exposed to CBLEO. This fact was supported by previous results that rsbU or sigB mutation increased expression of protease gene and reduced biofilm formation [71,73]. Hence, CBLEO-mediated transcriptional repressions of RsbU and SigB may be as pivotal antibacterial targets against S. aureus. Yet, the actual mechanism by which essential oil repressed transcription of virulence regulators destined for biofilm formation inhibition remains unknown.

ROS-mediated oxidative stress could cause AgrA oxidation to loss regulatory activity [24,25], and the mutation of rsbU, sigB, agrA or sarA in S. aureus increased susceptibility to oxidative stress

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and inhibited biofilm formation [24,71]. Given a negative correlation among massive accumulation of ROS and protein oxidation (Figure 4B, C), low transcript of rsbU/sigB/agrA/sarA/icaA/cidA (Figure 7), less formation of biofilm (Figure 5A) and notable inhibition of bacterial growth (Figure 1) in S. aureus exposed to CBLEO, it seems clear that CBLEO-induced growth inhibition of S. aureus was attributed likely to limitation of biofilm formation via depressing transcriptions of virulence-related regulators caused by ROS accumulation, which may confer the key acting mechanism of CBLEO on S. aureus, coincided with antibacterial effect of essential oil on P. aeruginosa, C. violaceum and E. coli [9,18]. Our results were also consistent with previous results showing that the treatments of antibacterial agents (linezolid, benzimidazole and vancomycin) could cause oxidative stress, leading to transcription repression of virulence-related gene with protease activity increase destined for biofilm formation inhibition in several pathogens [71,75,76]. All these indicated that ROS-mediated oxidative stress could be a critical initiator to cause transcription repression of key regulators (RsbU and SigB) and its targeted genes during exposure of S. aureus to CBLEO with biofilm formation decrease, and eventually leading to bacterial growth.

Altogether, collaborative transcription repression of virulence-related genes, effective increase of protease activity, and notable inhibition of biofilm formation, may responded specifically to an increase of ROS-induced oxidative stress in S. aureus during exposure to CBLEO, of which RsbU/SigB-mediated transcription regulatory system may contribute mostly to bactericidal effect of CBLEO against S. aureus.

4. Materials and Methods

4.1. Plant materials

The *C. burmannii* leaves were collected from plus tree with high borneol (accession CB01) locating at our planting base (Guangdong Huaqingyuan Biotechnology Co. LTD) of Guangdong Province (E115°50′1", N24°28′28"), China [41].

4.2. Extraction of essential oil and analysis of chemical components of CBLEO

Essential oil was extracted by steam distillation, and the fresh leaves (about 50 g) were powdered and subjected to hygro-distillation using a modified Clevenger-type apparatus for 5 h [77]. The obtained oils were collected, measured and dried over with anhydrous Na_2SO_4, and then stored in a sealed tube at −20 °C for further use. The essential oil yield (1.61 %) was calculated as the percentage (%, w/w) of fresh leaves [8].

Detection of chemical components by GC-MS was performed on GCMS-QP2020W/O gas chromatograph (Shimadzu, Japan), equipped with SH-R×ITM-5SIL MS column (30 m × 0.25 mm, 0.25 um) [77]. The temperature program was as follows: from 70 °C to 160 °C at 2 °C/min and hold for 2 min, then increased to 220 °C at 10 °C/min and kept for 5 min. The carrier gas was nitrogen at 1.19 mL/min. The GC inlet was set in a splitting mode with split ratio 1:20 and at 230 °C and 1.0 μL of diluted samples (1/10, v/v, in hexane) were injected. The quadrupole MS operating parameters: interface temperature 200 °C; electron impact ionization at 70 eV with scan mass range of 45−450 m/z. Identifications of volatile compounds were based on the Mass Spectral Library database and retention indices of authentic reference standards.

4.3. Bacterial strains and culture

Seven representative food-borne pathogens were used for antibacterial activity assay, including four Gram-negative bacteria of *Pseudomonas aeruginosa* (CICC 21636), *Escherichia coli* (CICC 10389), *Salmonella* (CICC 10982) and *Enterobacter aerogenes* (CICC 10293), and three Gram-positive bacteria of *Staphylococcus aureus* (CICC 10384), *Bacillus subtilis* (CICC 10275) and *Listeria monocytogenes* (CICC 21633).

To assess antibacterial activity of CBLEO on foodborne pathogens, the tested bacterial suspensions were prepared in nutrient broth (NB), expect for brain heart infusion (BHI) for *L.*

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monocytogenes, and incubated at 37 °C for 24 h. Each strain inoculum was suspended in 0.85 % of sterile saline to obtain a standard microbial density (about 10^7 CFU/mL).

4.4. Assessment of antibacterial activity of CBLEO

4.4.1. Detection of diameter of inhibition zone (DIZ)

DIZ detection by agar disc diffusion was applied to screen antimicrobial activity of CBLEO [8]. Each strain inoculum (100 μL, 10^7 CFU/mL) was coated on the culture medium surface (BHI for *L. monocytogenes*, NB for other bacteria), and the sterile 6 mm paper disc impregnated with CBLEO (10 μL, 50 mg/mL) was placed on medium surface. After the incubation at 37 °C for 24 h, antimicrobial activity was determined by a clear zone around the disc, and the DIZ was detected. Ampicillin (10 μg/disc, 10 mg/mL) and sterile distilled water (10 μL) were used as the positive and negative controls, respectively.

4.4.2. Determination of minimum inhibitory (MIC) and bactericidal concentration (MBC)

Microdilution method was used to detect the MIC and MBC of CBLEO [8]. A series of two-fold dilutions of CBLEO (0.125–32 μg/mL) were added into bacteria suspension (1×10^7 CFU/mL) in the wells of a sterile microplate, and cultured overnight at 37 °C. The lowest concentration of CBLEO that showed no visible bacteria growth was defined as the MIC, and the MBC was expressed as the lowest concentration of CBLEO required to kill bacteria. Ampicillin was applied as reference antibacterial agent.

4.5. Analysis of bacterial growth kinetics of S. aureus

Antibacterial kinetics assay was used to assess antibacterial mechanism of CBLEO on *S. aureus* (the most susceptible strain) [6]. In order to increase CBLEO solubility, the stock CBLEO was prepared in 4 % dimethyl sulphoxide (DMSO) [51] to obtain five different doses (1/8×MIC, 1/4×MIC, 1/2×MIC, 1×MIC, and 2×MIC), and then was respectively added into *S. aureus* (100 mL, 107 CFU/mL). After incubating for 3, 6, 9, 12, 15, 18, 21 and 24 h, the collected suspension was used to detect optical density (OD) at 600 nm by ultraviolet spectrophotometer (Agilent Cary 3500, USA). The growth kinetic curve of *S. aureus* was elaborated by drawing the lg number of CFU/mL versus incubated time [8].

4.6. Analysis of antibacterial mechanism of CBLEO on S. aureus

4.6.1. Cell membrane permeability

Detection of electric conductivity using an electrical conductivity meter (DDS-11D, China) was used to evaluate impact of CBLEO on cell membrane permeability of *S. aureus*, and the result was defined as the relative electric conductivity. All analyzed procedures (including *S. aureus* cell isolation, isotonic bacteria preparation and electric conductivity detection) were based on our previous method [8].

4.6.2. Integrity of cell membrane

The membrane integrity of *S. aureus* cells was evaluated by detecting the leakage of intracellular nucleic acid and protein [8]. *S. aureus* cells (1×107 CFU/mL) were incubated at 37 °C with CBLEO at different levels (1/2×MIC, 1×MIC and 2×MIC) and times (0-8 h), and the samples were collected at different times for centrifugation (10,000 rpm) at 4 °C for 10 min. The obtained supernatants were used to detect the amounts of nucleic acid and protein using ultraviolet spectrophotometer (Agilent Cary 3500, USA). The detected result of nucleic acid was expressed as the absorbance value at 260 nm (OD_{260}), and the amount of protein was standardized to the used amount of *S. aureus* cells (μg/mg).

4.6.3. Cell wall damage

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The impact of CBBLEO on cell wall damage of *S. aureus* was analyzed by detecting release of alkaline phosphatase (AKP) [78]. Different doses (1/2×MIC, 1×MIC and 2×MIC) of CBLEO were added into *S. aureus* cells (107 CFU/mL) and incubated at 37 °C, and then the supernatants collected from different times (0-8 h) were used for the detection of AKP activity by commercial kit (RS0904F, Redshineen Biotech, China). The AKP activity was standardized to the used amounts of *S. aureus* cells and expressed as U/L.

4.6.4. Scanning electron microscope (SEM) analysis

The impact of CBLEO on cell morphology of *S. aureus* was tested by SEM assay [16]. The *S. aureus* suspension (10^7 CFU/mL) was added into CBLEO (1×MIC), and the group added equal amount of absolute ethanol was applied as the control. After 2 h of incubation, the collected cells were washed with phosphate-buffered saline buffer, and then fixed in glutaraldehyde (2.5 %) at 4 °C for 12 h, followed by dehydration under the different levels of ethanol gradient (30, 50, 80, 90 and 100 %). The specimens were dried at critical point of CO_2 and coated with gold-palladium by Polaron E5100 II (Polaron Instruments Inc., Hatfield, CA). Finally, the samples were observed with a scanning electron microscopy (SEM, JSM-7001F, JEOL, Japan) at voltage of 10 kV.

4.6.5. Analyses of cellular protein oxidation and ROS and MDA production

Oxidative stress and lipid peroxidation of *S. aureus* induced by CBLEO were assessed by detecting the amounts of ROS generation and protein oxidation (two key markers of oxidative stress) and MDA accumulation (a marker of lipid peroxidation) [8,57]. *S. aureus* cells (10^7 CFU/mL) were detected at incubation (37 °C) with different doses (1/2×MIC, 1×MIC and 2×MIC) and times (0−8 h) of CBLEO, and the samples were collected and then centrifugated at 4 °C for 10 min under 10,000 rpm. The obtained cell pellets were used to determine protein carbonyl, ROS and MDA by using assay kits of ab126287, ab113851 and ab118970 (Abcam, China), respectively. The ROS amount was given in fold of untreated controls, and the protein carbonyl content (protein oxidation product) was standardized to the used amount of *S. aureus* cells and defined as nmol/mg. The MDA content was also standardized to the used amounts of *S. aureus* cells, and expressed as nmol/mg.

4.7. Change in bacterial total protein

The *S. aureus* cells (10^7 CFU/mL) were incubated with different doses of CBLEO at 37 °C, and the samples were collected respectively from different times (0-8 h), and then centrifuged at 10,000 rpm for 10 min under 4 °C, the obtained cell pellets were used for the detection of total protein amount by using BCA Protein Assay Kit (102536, Abcam). The result was standardized to the used amounts of *S. aureus* cells and expressed as μg/mg.

4.8. Total protease assay

Total protease activity was detected in the above obtained cell pellets of *S. aureus* by analysis kit (ab111750) according to the manufacturer's instructions, and the fluorescein isothiocyanate (FITC)-labeled casein was used as a general substrate. The protease activity was standardized to the used amounts of cell total protein and expressed as U/mg protein using BSA as standard. One unit (U) was defined as the amount of protease that cleaves substrate to yield an amount of fluorescence equivalent to 1.0 μmol of unquenched FITC per min at 25 °C.

4.9. Analysis of virulence-associated gene expression

Total RNA of *S. aureus* was extracted by RNAprep Cell/Bacterial kit (Tiangen, China), and was reverse transcribed by PrimeScriptTM RT reagent kit (Takara, Japan). The qRT-PCR was performed on BIO-RAD CF× ConnectTM Real-Time System using SYBR Green qPCR Mix (Biomarker, China). All amplified primers used for the detection for virulence genes were listed in Table S1, and 16S rRNA was used as internal reference. The relative expression value of target genes in comparison with

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reference gene was counted by $2^{-\triangle\triangle Ct}$ method [9], and the expression level in *S. aureus* from the treatments of different doses of CBLEO at 0 h was arbitrarily set to 1.00 for standardization.

4.10. Assay of antibiofilm activity

The impact of CBLEO on biofilm formation of *S. aureus* was assessed by a microtiter plate assay [18]. An overnight culture of *S. aureus* was added into a 96-well dish containing 200 μL of LB broth supplemented with various levels of CBLEO. After incubation at 37 °C for 1−8 h, the bacterial cells were washed with PBS to remove all unattached cells and media components, and then 250 μL crystal violet staining solution (0.1 %) was added and incubated for 20 min at 25 °C. After this, the plates were washed with PBS buffer for 2−3 times, and solubilized with ethanol. The absorbance at 570 nm (OD_{570}) was detected in CBLEO-treated (different concentrations) or control sample from 0−8 h, of which the value of OD_{570} at 0 h was arbitrarily set to 1.00 for standardization, and the result was defined as fold change.

4.11. Assay of binding activity of CBLEO to bacteria genome DNA

The binding activity of CBLEO to genomic DNA of *S. aureus* was tested by agarose gel electrophoresis method [79]. Genomic DNA of *S. aureus* was isolated by TIANamp Bacteria DNA Kit (Tiangen, China), and the value of OD_{260} was measured to calculate DNA concentration. An aliquot (100 ng) of genome DNA was incubated with CBLEO at different doses (1/2×MIC, 1×MIC, 2×MIC, 4×MIC, 8×MIC and 16×MIC) at 37 °C for 0.5, 1, 3 and 5 h under darkness, and each incubated mixture (5.0 μL) was loaded to run agarose gel electrophoresis. The bacteria treated by PBS was used as the control.

4.12. Statistical analysis

All the results were recorded as mean ± SD (standard deviation) three independent replicates, and analyzed by ANOVA employing Student's test at $P < 0.05$. All statistical assays were performed by IBM SPSS Statistics 25 software.

5. Conclusions

In this work, the *C. burmannii* leaf essential oil (CBLEO) was identified with diverse volatile compounds and high amount of borneol as well as good antibacterial activity, and *S. aureus* was the most susceptible pathogen. CBLEO could act as a strong inducer for ROS accumulation and oxidative stress of *S. aureus*, causing cell structure damage, giving rise to effective repression of virulence-related gene transcription with a significant inhibition of biofilm formation destined for growth inhibition of *S. aureus*. Notably, the comparative association among ROS accumulation, virulence-associated gene transcription, protease production, biofilm formation and bacterial growth in *S. aureus* cross different doses and times of CBLEO treatment had led to the identification of RsbU and SigB as important transcriptional regulator crucial for bacterial biofilm formation and growth inhibition. RsbU/SigB-mediated repression of biofilm formation caused by CBLEO-oxidative stress may be as key antibacterial target against *S. aureus*. Our findings should provide valuable information for those studying the acting mechanism of essential oil against pathogens. Further research focused on exploration of functional attribute (especially for borneol) of essential oil from *C. burmannii* leaf in food practical utilization as a natural antibacterial agent.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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References

1. Bondi, M.; Lauková, A.; de Niederhausern, S.; Messi, P., Papadopoulou, C. Natural preservatives to improve food quality and safety. *J. Food Qua*l. **2017**, *2017*, 1090932. doi:10.1155/2017/1090932
2. Gutiérrez-del-Río, I.; Fernández, J., Lombó, F. Plant nutraceuticals as antimicrobial agents in food preservation: terpenoids, polyphenols and thiols. *Int. J. Antimicrob. Agents*. **2018**, *52*, 309-315. doi:10.1016/j.ijantimicag.2018.04.024
3. Erickson, M.C., Doyle, M.P. The challenges of eliminating or substituting antimicrobial preservatives in foods. *Annu Rev. Food Sci. Technol*. **2017**, *8*, 371-390. doi:10.1146/annurev-food-030216-025952
4. Dahham, S.S.; Tabana, Y.M.; Iqbal, M.A.; Ahamed, M.B.; Ezzat, M.O.; Majid, A.S., Majid, A.M. The anticancer, antioxidant and antimicrobial properties of the sesquiterpene β-caryophyllene from the essential oil of *Aquilaria crassna*. *Molecules*. **2015**, *20*, 11808-11829. doi:10.3390/molecules200711808
5. Akarca, G. Composition and antibacterial effect on food borne pathogens of *Hibiscus surrattensis* L. calyces essential oil. *Ind Crop Prod*. **2019**, *137*, 285-289. doi:10.1016/j.indcrop.2019.05.043
6. Bouyahya, A.; Assemian, I.C.C.; Mouzount, H.; Bourais, I.; Et-Touys, A.; Fellah, H.; Benjouad, A.; Dakka, N., Bakri, Y. Could volatile compounds from leaves and fruits of *Pistacia lentiscus* constitute a novel source of anticancer, antioxidant, antiparasitic and antibacterial drugs? *Ind Crop Prod*. **2019**, *128*, 62-69. doi:10.1016/j.indcrop.2018.11.001
7. de Almeida, W.S.; de Lima, S.G.; Barreto, H.M.; Andrade, L.M.d.S.; Fonseca, L.; Athayde Sobrinho, C.; Santos, A.R.B., Muratori, M.C.S. Chemical composition and antimicrobial activity of the essential oil of *Lippia lasiocalycina* Cham. (Verbenaceae). *Ind Crop Prod*. **2018**, *125*, 236-240. doi:10.1016/j.indcrop.2018.09.007
8. Chen, F.; Miao, X.; Lin, Z.; Xiu, Y.; Shi, L.; Zhang, Q.; Liang, D.; Lin, S., He, B. Disruption of metabolic function and redox homeostasis as antibacterial mechanism of *Lindera glauca* fruit essential oil against *Shigella flexneri*. *Food Control*. **2021**, *130*, 108282. doi:10.1016/j.foodcont.2021.108282
9. Dai, J.; Li, C.; Cui, H., Lin, L. Unraveling the anti-bacterial mechanism of *Litsea cubeba* essential oil against *E. coli* O157:H7 and its application in vegetable juices. *Int. J. Food Microbiol*. **2021**, *338*, 108989. doi:10.1016/j.ijfoodmicro.2020.108989
10. Pandey, A.K.; Kumar, P.; Singh, P.; Tripathi, N.N., Bajpai, V.K. Essential oils: Sources of antimicrobials and food preservatives. *Front Microbiol*. **2017**, *7*, 2161. doi:10.3389/fmicb.2016.02161
11. Sharma, S.; Barkauskaite, S.; Jaiswal, A.K., Jaiswal, S. Essential oils as additives in active food packaging. *Food Chem*. **2021**, *343*, 128403. doi:10.1016/j.foodchem.2020.128403
12. Ardjoum, N.; Chibani, N.; Shankar, S.; Salmieri, S.; Djidjelli, H., Lacroix, M. Incorporation of *Thymus vulgaris* essential oil and ethanolic extract of propolis improved the antibacterial, barrier and mechanical properties of corn starch-based films. *Int. J. Biol. Macromol*. **2023**, *224*, 578-583. doi:10.1016/j.ijbiomac.2022.10.146
13. Bajpai, V.K.; Sharma, A., Baek, K.-H. Antibacterial mode of action of *Cudrania tricuspidata* fruit essential oil, affecting membrane permeability and surface characteristics of food-borne pathogens. *Food Control*. **2013**, *32*, 582–590. doi:10.1016/j.foodcont.2013.01.032
14. Hu, W.; Li, C.; Dai, J.; Cui, H., Lin, L. Antibacterial activity and mechanism of *Litsea cubeba* essential oil against methicillin-resistant *Staphylococcus aureus* (MRSA). *Ind Crop Prod*. **2019**, *130*, 34-41. doi:10.1016/j.indcrop.2018.12.078
15. de Souza Moura, W.; de Souza, S.R.; Campos, F.S.; Sander Rodrigues Cangussu, A.; Macedo Sobrinho Santos, E.; Silva Andrade, B.; Borges Gomes, C.H.; Fernandes Viana, K.; Haddi, K.; Oliveira, E.E.; Nascimento, V.L., de Souza Aguiar, R.W. Antibacterial activity of *Siparuna guianensis* essential oil mediated by impairment of membrane permeability and replication of pathogenic bacteria. *Ind Crop Prod*. **2020**, *146*, 112142. doi:10.1016/j.indcrop.2020.112142
16. Cui, H.; Zhang, C.; Li, C., Lin, L. Antibacterial mechanism of oregano essential oil. *Ind Crop Prod*. **2019**, *139*, 111498. doi:10.1016/j.indcrop.2019.111498

17. OuYang, Q.; Tao, N., Zhang, M. A damaged oxidative phosphorylation mechanism is involved in the antifungal activity of citral against *Penicillium digitatum*. *Front Microbiol*. **2018**, *9*, 239. doi:10.3389/fmicb.2018.00239
18. Mansuri, A.; Lokhande, K.; Kore, S.; Gaikwad, S.; Nawani, N.; Swamy, K.V.; Junnarkar, M., Pawar, S. Antioxidant, anti-quorum sensing, biofilm inhibitory activities and chemical composition of Patchouli essential oil: *in vitro* and *in silico* approach. *J. Biomol. Struct. Dyn*. **2020**, *40*, 154-165. doi:10.1080/07391102.2020.1810124
19. Wu, Y.; Bai, J.; Zhong, K.; Huang, Y., Gao, H. A dual antibacterial mechanism involved in membrane disruption and DNA binding of *2R,3R*-dihydromyricetin from pine needles of *Cedrus deodara* against *Staphylococcus aureus*. *Food Chem*. **2017**, *218*, 463-470. doi:10.1016/j.foodchem.2016.07.090
20. Wu, S.; Duan, N.; Gu, H.; Hao, L.; Ye, H.; Gong, W., Wang, Z. A review of the methods for detection of *Staphylococcus aureus* enterotoxins. *Toxins*. **2016**, *8*, 176. doi:10.3390/toxins8070176
21. Fisher, E.L.; Otto, M., Cheung, G.Y.C. Basis of virulence in enterotoxin-mediated Staphylococcal food poisoning. *Front Microbiol*. **2018**, *9*, 436. doi:10.3389/fmicb.2018.00436
22. Zhao, X.; Zhao, F.; Wang, J., Zhong, N. Biofilm formation and control strategies of foodborne pathogens: food safety perspectives. *RSC advances*. **2017**, *7*, 36670-36683. doi:10.1039/c7ra02497e
23. Cheung, A.L.; Nishina, K.A.; Trotonda, M.P., Tamber, S. The SarA protein family of *Staphylococcus aureus*. The international *journal of biochemistry & cell biology*. **2008**, *40*, 355-361. doi:10.1016/j.biocel.2007.10.032
24. Sun, F.; Liang, H.; Kong, X.; Xie, S.; Cho, H.; Deng, X.; Ji, Q.; Zhang, H.; Alvarez, S.; Hicks, L.M.; Bae, T.; Luo, C.; Jiang, H., He, C. Quorum-sensing *agr* mediates bacterial oxidation response via an intramolecular disulfide redox switch in the response regulator AgrA. *Proc. Natl. Acad. Sci. U. S. A*. **2012**, *109*, (23), 9095-9100. doi:10.1073/pnas.1200603109
25. Jenul, C., Horswill, A.R. Regulation of *Staphylococcus aureus* virulence. *Microbiology spectrum*. **2019**, *7*, 1-21. doi:10.1128/microbiolspec.GPP3-0031-2018
26. Wang, W.; Li, D.; Huang, X.; Yang, H.; Qiu, Z.; Zou, L.; Liang, Q.; Shi, Y.; Wu, Y.; Wu, S.; Yang, C., Li, Y. Study on antibacterial and quorum-sensing inhibition activities of *Cinnamomum camphora* leaf essential oil. *Molecules*. **2019**, 24. doi:10.3390/molecules24203792
27. Manoharan, R.K.; Lee, J.-H.; Kim, Y.-G.; Kim, S.-I., Lee, J. Inhibitory effects of the essential oils α-longipinene and linalool on biofilm formation and hyphal growth of *Candida albicans*. *Biofouling*. **2017**, *33*, 143-155. doi:10.1080/08927014.2017.1280731
28. Joshi, J.R.; Khazanov, N.; Senderowitz, H.; Burdman, S.; Lipsky, A., Yedidia, I. Plant phenolic volatiles inhibit quorum sensing in pectobacteria and reduce their virulence by potential binding to ExpI and ExpR proteins. *Sci. Rep*. **2016**, *6*, 38126. doi:10.1038/srep38126
29. Alves, S.; Duarte, A.; Sousa, S., Domingues, F.C. Study of the major essential oil compounds of *Coriandrum sativum* against *Acinetobacter baumannii* and the effect of linalool on adhesion, biofilms and quorum sensing. *Biofouling*. **2016**, *32*, 155-165. doi:10.1080/08927014.2015.1133810
30. Souza, C.M.C.; Pereira Junior, S.A.; Moraes, T.d.S.; Damasceno, J.L.; Amorim Mendes, S.; Dias, H.J.; Stefani, R.; Tavares, D.C.; Martins, C.H.G.; Crotti, A.E.M.; Mendes-Giannini, M.J.S., Pires, R.H. Antifungal activity of plant-derived essential oils on *Candida tropicalis* planktonic and biofilms cells. *Med. Mycol*. **2016**, *54*, 515-523. doi:10.1093/mmy/myw003
31. Lee, K.; Lee, J.-H.; Kim, S.-I.; Cho, M.H., Lee, J. Anti-biofilm, anti-hemolysis, and anti-virulence activities of black pepper, cananga, myrrh oils, and nerolidol against *Staphylococcus aureus*. *Appl. Microbiol. Biotechnol*. **2014**, *98*, 9447-9457. doi:10.1007/s00253-014-5903-4
32. Al-Dhubiab, B.E. Pharmaceutical applications and phytochemical profile of *Cinnamomum burmannii*. *Pharmacognosy reviews*. **2012**, *6*, 125-131. doi:10.4103/0973-7847.99946
33. Ribeiro-Santos, R.; Andrade, M.; Madella, D.; Martinazzo, A.P.; de Aquino Garcia Moura, L.; de Melo, N.R., Sanches-Silva, A. Revisiting an ancient spice with medicinal purposes: Cinnamon. Trends *Food Sci. Technol*. **2017**, *62*, 154-169. doi:10.1016/j.tifs.2017.02.011
34. Vasconcelos, N.G.; Croda, J., Simionatto, S. Antibacterial mechanisms of cinnamon and its constituents: A review. *Microb. Pathog*. **2018**, *120*, 198-203. doi:10.1016/j.micpath.2018.04.036
35. Shan, B.; Cai, Y.; Brooks, J., Corke, H. Antibacterial properties and major bioactive components of cinnamon stick (*Cinnamomum burmannii*): activity against foodborne pathogenic bacteria. *J. Agric. Food Chem*. **2007**, *55*, 5484-5490. doi:10.1021/jf070424d
36. Huang, J.; Yang, L.; Zou, Y.; Luo, S.; Wang, X.; Liang, Y.; Du, Y.; Feng, R., Wei, Q. Antibacterial activity and mechanism of three isomeric terpineols of *Cinnamomum longepaniculatum* leaf oil. *Folia Microbiol*. **2021**, *66*, 59-67. doi:10.1007/s12223-020-00818-0
37. Muhammad, D.R.A.; Lemarcq, V.; Alderweireldt, E.; Vanoverberghe, P.; Praseptiangga, D.; Juvinal, J.G., Dewettinck, K. Antioxidant activity and quality attributes of white chocolate incorporated with

Cinnamomum burmannii Blume essential oil. J *Food Sci Technol*. **2020**, *57*, 1731-1739. doi:10.1007/s13197-019-04206-6

38. Muhammad, D.R.A.; Tuenter, E.; Patria, G.D.; Foubert, K.; Pieters, L., Dewettinck, K. Phytochemical composition and antioxidant activity of *Cinnamomum burmannii* Blume extracts and their potential application in white chocolate. *Food Chem*. **2021**, *340*, 127983. doi:10.1016/j.foodchem.2020.127983
39. Muhammad, D.R.A.; Saputro, A.D.; Rottiers, H.; Van de Walle, D., Dewettinck, K. Physicochemical properties and antioxidant activities of chocolates enriched with engineered cinnamon nanoparticles. *Eur. Food Res. Technol*. **2018**, *244*, 1185-1202. doi:10.1007/s00217-018-3035-2
40. Chen, X.; Lian, H.; Chen, J.; Wang, Y.; He, B.; Chen, Y., Chen, H. Study on influencing factors of essential oil yield in leaves of *Cinnamomum burmannii* chvar. *borneol*. *Forestry Environ Sci*. **2020**, *36*, 18-23. doi:10.3969/j.issn.1006-4427.2020.03.03
41. Wu, G.; Lian, H.; Zhang, C.; Li, B.; Chen, J.; He, B.; Zhang, Q., Wang, Y. Content variation and evaluation of essential oil and its main chemical components of *Cinnamomum burmannii* in Guangdong province. *Forestry Environ Sci*. **2020**, *36*, 88-95. doi:10.3969/j.issn.1006-4427.2020.06.015
42. Xie, P.; He, B.; Wang, Y., Zhang, Q. Standardization of exploration and utilization of *d*-borneol type *Cinnamomum camphora*. *Forestry Environ Sci*. **2019**, *35*, 94-100. doi:10.3969/j.issn.1006-4427.2019.01.017
43. Zielinska, A.K.; Beenken, K.E.; Mrak, L.N.; Spencer, H.J.; Post, G.R.; Skinner, R.A.; Tackett, A.J.; Horswill, A.R., Smeltzer, M.S. *sarA*-mediated repression of protease production plays a key role in the pathogenesis of *Staphylococcus aureus* USA300 isolates. *Mol. Microbiol*. **2012**, *86*, 1183-1196. doi:10.1111/mmi.12048
44. Wang, R.; Wang, R., Yang, B. Extraction of essential oils from five cinnamon leaves and identification of their volatile compound compositions. *Innov Food Sci Emerg*. **2009**, *10*, 289-292. doi:10.1016/j.ifset.2008.12.002
45. Yu, H.; Ren, X.; Liu, Y.; Xie, Y.; Guo, Y.; Cheng, Y.; Qian, H., Yao, W. Extraction of *Cinnamomum camphora* chvar. *Borneol* essential oil using neutral cellulase assisted-steam distillation: optimization of extraction, and analysis of chemical constituents. *Ind Crop Prod*. **2019**, *141*, 111794. doi:10.1016/j.indcrop.2019.111794
46. Chebet, J.J.; Ehiri, J.E.; McClelland, D.J.; Taren, D., Hakim, I.A. Effect of d-limonene and its derivatives on breast cancer in human trials: a scoping review and narrative synthesis. *BMC Cancer*. **2021**, *21*, 902. doi:10.1186/s12885-021-08639-1
47. Bansod, S.; Chilvery, S.; Saifi, M.A.; Das, T.J.; Tag, H., Godugu, C. Borneol protects against cerulein-induced oxidative stress and inflammation in acute pancreatitis mice model. *Environ. Toxicol*. **2021**, *36*, 530-539. doi:10.1002/tox.23058
48. Chen, N.; Wen, J.; Wang, Z., Wang, J. Multiple regulation and targeting effects of borneol in the neurovascular unit in neurodegenerative diseases. *Basic Clin. Pharmacol. Toxicol*. **2022**, *130*, 5-19. doi:10.1111/bcpt.13656
49. Liu, S.; Long, Y.; Yu, S.; Zhang, D.; Yang, Q.; Ci, Z.; Cui, M.; Zhang, Y.; Wan, J.; Li, D.; Shi, A.; Li, N.; Yang, M., Lin, J. Borneol in cardio-cerebrovascular diseases: Pharmacological actions, mechanisms, and therapeutics. *Pharmacol. Res*. **2021**, *169*, 105627. doi:10.1016/j.phrs.2021.105627
50. Salehi, B.; Upadhyay, S.; Erdogan Orhan, I.; Kumar Jugran, A.; S, L.D.J.; D, A.D.; Sharopov, F.; Taheri, Y.; Martins, N.; Baghalpour, N.; Cho, W.C., Sharifi-Rad, J. Therapeutic potential of α-and β-Pinene: A miracle gift of nature. *Biomolecules*. **2019**, *9*, 738. doi:10.3390/biom9110738
51. Nafis, A.; Kasrati, A.; Jamali, C.A.; Mezrioui, N.; Setzer, W.; Abbad, A., Hassani, L. Antioxidant activity and evidence for synergism of *Cannabis sativa* (L.) essential oil with antimicrobial standards. *Ind Crop Prod*. **2019**, *137*, 396-400. doi:10.1016/j.indcrop.2019.05.032
52. Zhou, F.; Zhao, M.; Su, G.; Cui, C., Sun, W. Gelation of salted myofibrillar protein under malondialdehyde-induced oxidative stress. *Food Hydrocolloids*. **2014**, *40*, 153-162. doi:10.1016/j.foodhyd.2014.03.001
53. Hsu, K.-C.; Hsu, P.-F.; Chen, Y.-C.; Lin, H.-C.; Hung, C.-C.; Chen, P.-C., Huang, Y.-L. Oxidative stress during bacterial growth characterized through microdialysis sampling coupled with HPLC/fluorescence detection of malondialdehyde. *J. Chromatogr. B*. **2016**, *1019*, 112-116. doi:10.1016/j.jchromb.2016.01.044
54. Fathima, A., Rao, J.R. Selective toxicity of Catechin-a natural flavonoid towards bacteria. *Appl. Microbiol. Biotechnol*. **2016**, *100*, 6395-6402. doi:10.1007/s00253-016-7492-x
55. Du, W.; Sun, C.; Liang, Z.; Han, Y., Yu, J. Antibacterial activity of hypocrellin A against *Staphylococcus aureus*. *World J. Microbiol. Biotechnol*. **2012**, *28*, 3151-3157. doi:10.1007/s11274-012-1125-z
56. Yan, L.; Li, M.; Cao, Y.; Gao, P.; Cao, Y.; Wang, Y., Jiang, Y. The alternative oxidase of *Candida albicans* causes reduced fluconazole susceptibility. *J. Antimicrob. Chemother*. **2009**, *64*, 764-773. doi:10.1093/jac/dkp273
57. Qian, J.; Wang, C.; Zhuang, H.; Zhang, J., Yan, W. Oxidative stress responses of pathogen bacteria in poultry to plasma-activated lactic acid solutions. *Food Control*. **2020**, *118*, 107355. doi:10.1016/j.foodcont.2020.107355

58. Thoendel, M.; Kavanaugh, J.S.; Flack, C.E., Horswill, A.R. Peptide signaling in the staphylococci. *Chem. Rev.* **2011**, *111*, 117-151. doi:10.1021/cr100370n
59. Le, K.Y., Otto, M. Quorum-sensing regulation in staphylococci-an overview. *Front Microbiol*. **2015**, *6*, 1174-1174. doi:10.3389/fmicb.2015.01174
60. Queck, S.Y.; Jameson-Lee, M.; Villaruz, A.E.; Bach, T.-H.L.; Khan, B.A.; Sturdevant, D.E.; Ricklefs, S.M.; Li, M., Otto, M. RNAIII-independent target gene control by the agr quorum-sensing system: Insight into the evolution of virulence regulation in *Staphylococcus aureus*. *Mol. Cell*. **2008**, *32*, 150-158. doi:10.1016/j.molcel.2008.08.005
61. Bischoff, M.; Dunman, P.; Kormanec, J.; Macapagal, D.; Murphy, E.; Mounts, W.; Berger-Ba¨chi, B., Projan, S. Microarray-based analysis of the *Staphylococcus aureus* σ^B regulon. *J. Bacteriol*. **2004**, *186*, 4085-4099. doi:10.1128/JB.186.13.4085-4099.2004
62. Guldimann, C.; Boor, K.J.; Wiedmann, M., Guariglia-Oropeza, V. Resilience in the face of uncertainty: Sigma factor B fine-tunes gene expression to support homeostasis in gram-positive bacteria. *Appl. Environ. Microbiol*. **2016**, *82*, 4456-4469. doi:10.1128/AEM.00714-16
63. Atwood, D.N.; Loughran, A.J.; Courtney, A.P.; Anthony, A.C.; Meeker, D.G.; Spencer, H.J.; Gupta, R.K.; Lee, C.Y.; Beenken, K.E., Smeltzer, M.S. Comparative impact of diverse regulatory loci on *Staphylococcus aureus* biofilm formation. *MicrobiologyOpen*. **2015**, *4*, 436-451. doi:10.1002/mbo3.250
64. Harapanahalli, A.K.; Chen, Y.; Li, J.; Busscher, H.J., van der Mei, H.C. Influence of adhesion force on *icaA* and *cidA* gene expression and production of matrix components in *Staphylococcus aureus* biofilms. *Appl. Environ. Microbiol*. **2015**, *81*, 3369-3378. doi:10.1128/AEM.04178-14
65. Savage, V.J.; Chopra, I., O'Neill, A.J. Population diversification in *Staphylococcus aureus* biofilms may promote dissemination and persistence. *PloS One*. **2013**, *8*, e62513. doi:10.1371/journal.pone.0062513
66. Lauderdale, K.J.; Boles, B.R.; Cheung, A.L., Horswill, A.R. Interconnections between sigma B, *agr*, and proteolytic activity in *Staphylococcus aureus* biofilm maturation. *Infect. Immun.* **2009**, *77*, 1623-1635. doi:10.1128/IAI.01036-08
67. Rice, K.C.; Mann, E.E.; Endres, J.L.; Weiss, E.C.; Cassat, J.E.; Smeltzer, M.S., Bayles, K.W. The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. *Proc. Natl. Acad. Sci. U. S. A*. **2007**, *104*, 8113-8118. doi:10.1073/pnas.0610226104
68. Tsang, L.H.; Cassat, J.E.; Shaw, L.N.; Beenken, K.E., Smeltzer, M.S. Factors contributing to the biofilm-deficient phenotype of *Staphylococcus aureus sarA* mutants. *PLoS One*. **2008**, *3*, e3361. doi:10.1371/journal.pone.0003361
69. Beenken, K.E.; Blevins, J.S., Smeltzer, M.S. Mutation of *sarA* in *Staphylococcus aureus* limits biofilm formation. *Infect. Immun*. **2003**, *71*, 4206-4211. doi:10.1128/iai.71.7.4206-4211.2003
70. Loughran, A.J.; Atwood, D.N.; Anthony, A.C.; Harik, N.S.; Spencer, H.J.; Beenken, K.E., Smeltzer, M.S. Impact of individual extracellular proteases on *Staphylococcus aureus* biofilm formation in diverse clinical isolates and their isogenic *sarA* mutants. *MicrobiologyOpen*. **2014**, *3*, 897-909. doi:10.1002/mbo3.214
71. Mootz, J.M.; Malone, C.L.; Shaw, L.N., Horswill, A.R. Staphopains modulate *Staphylococcus aureus* biofilm integrity. *Infect. Immun*. **2013**, *81*, 3227-3238. doi:10.1128/iai.00377-13
72. Palma, M., Cheung, A.L. σ^B activity in *Staphylococcus aureus* is controlled by RsbU and an additional factor (s) during bacterial growth. *Infect. Immun. 2001*, *69*, 7858-7865. doi:10.1128/IAI.69.12.7858-7865.2001
73. Knobloch Johannes, K.M.; Jäger, S.; Horstkotte Matthias, A.; Rohde, H., Mack, D. RsbU-dependent regulation of *Staphylococcus epidermidis* biofilm formation is mediated via the alternative sigma factor σ^B by repression of the negative regulator gene *icaR*. *Infect. Immun*. **2004**, *72*, 3838-3848. doi:10.1128/iai.72.7.3838-3848.2004
74. Conlon, K.M.; Humphreys, H., O'Gara, J.P. Inactivations of *rsbU* and *sarA* by IS256 represent novel mechanisms of biofilm phenotypic variation in *Staphylococcus epidermidis*. *J. Bacteriol*. **2004**, *186*, 6208-6219. doi:10.1128/jb.186.18.6208-6219.2004
75. Kong, C.; Chee, C.-F.; Richter, K.; Thomas, N.; Abd. Rahman, N., Nathan, S. Suppression of *Staphylococcus aureus* biofilm formation and virulence by a benzimidazole derivative, UM-C162. *Sci. Rep*. **2018**, *8*, 2758. doi:10.1038/s41598-018-21141-2
76. Shrestha, L.; Kayama, S.; Sasaki, M.; Kato, F.; Hisatsune, J.; Tsuruda, K.; Koizumi, K.; Tatsukawa, N.; Yu, L., Takeda, K. Inhibitory effects of antibiofilm compound 1 against *Staphylococcus aureus* biofilms. *Microbiol. Immunol*. **2016**, *60*, 148-159. doi:10.1111/1348-0421.12359
77. Zhu, B.; Hou, X.; Niu, J.; Li, P.; Fang, C.; Qiu, L.; Ha, D.; Zhang, Z.; Sun, J., Li, Y. Volatile constituents from the fruits of *Lindera glauca* (Sieb. et Zucc.) with different maturities. J. Essent. Oil-Bear. *Plants*. **2016**, *19*, 926-935. doi:10.1080/0972060X.2014.981598

78. Zhao, M.; Bai, J.; Bu, X.; Tang, Y.; Han, W.; Li, D.; Wang, L.; Yang, Y., Xu, Y. Microwave-assisted aqueous two-phase extraction of phenolic compounds from *Ribes nigrum* L. and its antibacterial effect on foodborne pathogens. *Food Control*. **2021**, *119*, 107449. doi:10.1016/j.foodcont.2020.107449
79. Pan, Y.; Zheng, L.-b.; Mao, Y.; Wang, J.; Lin, L.-s.; Su, Y.-q., Li, Y. The antibacterial activity and mechanism analysis of piscidin 5 like from *Larimichthys crocea*. *Dev. Comp. Immunol*. **2019**, *92*, 43-49. doi:10.1016/j.dci.2018.10.008