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Original Article

Total alkaloids of *Sophora alopecuroides*- and matrine-induced reactive oxygen species impair biofilm formation of *Staphylococcus epidermidis* and increase bacterial susceptibility to ciprofloxacin

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ABSTRACT

Objective: To investigate the mechanism by which total alkaloids of *Sophora alopecuroides* (TASA) and matrine (MT) impair biofilm to increase the susceptibility of *Staphylococcus epidermidis* (*S. epidermidis*) to ciprofloxacin.

Methods: The minimum biofilm inhibitory concentration (mBIC) was determined using a 2-fold dilution method. Structure of biofilm of *S. epidermidis* was examined by Confocal Laser Scanning Microscope (CLSM). The cellular reactive oxygen species (ROS) was determined using a DCFH-DA assay. The key factors related to the regulation of ROS were accessed using respective kits.

Results: TASA and MT were more beneficial to impair biofilm of *S. epidermidis* than ciprofloxacin (CIP) ($P < 0.05$). TASA and MT were not easily developed resistance to biofilm-producing *S. epidermidis*. The mBIC of CIP decreased by 2–6-fold following the treatment of sub-biofilm inhibitory concentration (sub-BIC) TASA and MT, whereas the mBIC of CIP increased by 2-fold following a treatment of sub-BIC CIP from the first to sixth generations. TASA and MT can improve the production of ROS in biofilm-producing *S. epidermidis*. The ROS content was decreased 23%–33% following the treatment of sub-mBIC CIP, whereas ROS content increased 7%–24% following treatment with TASA + CIP and MT + CIP combination from the first to sixth generations. Nitric oxide (NO) as a ROS, which was consistent with the previously confirmed relationship between ROS and drug resistance. Related regulatory factors-superoxide dismutase (SOD) and glutathione peroxidase (GSH) could synergistically maintain the redox balance *in vivo*.

Conclusion: TASA and MT enhanced reactive oxygen species to restore the susceptibility of *S. epidermidis* to ciprofloxacin.

1. Introduction

Staphylococcus epidermidis is a coagulase-negative pathogen that has become a leading source of hospital and community-acquired infections. This is due to the ability of *S. epidermidis* to quickly develop drug resistance via the production of protective biofilms. Indeed, the increasing prevalence of biofilm production in *S. epidermidis* strains has led to a serious threat to public health worldwide (Otto, 2013). Biofilms of *S. epidermidis* are comprised of clusters of cells that are encased in a self-synthesized extracellular polymeric matrix that helps the bacteria attach to support surfaces, which protects them from host defenses and antimicrobial agents (Shih et al., 2010). As a result, bacteria that produce biofilms are 10–1000 times less susceptible to antimicrobial agents than planktonic bacteria partially owing to the fact that extracellular polymeric substances of the biofilm can act as a barrier to prevent the contact of bacteria with antimicrobial agents (Penesyan, Gillings, & Paulsen, 2015). In addition to the physical barrier, biofilm-producing bacteria also maintain low activity levels and low metabolic rates that confer resistance to antibiotics. In recent years, it has been shown that bacterial resistance is related to reactive oxygen species (ROS) produced by bacteria, of which the ROS plays a protective role in bacteria exposed to low antibiotic concentrations, and an antibacterial role in bacteria exposed to high concentration of antibiotics (Kohanski, DePristo, & Collins, 2010).

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There was a common mechanism of cellular death killed by bactericidal antibiotics, regardless of drug-target interaction, which stimulate the production of highly deleterious hydroxyl radicals in bacteria, which ultimately contribute to cell death (Kohanski, Dwyer, Hayete, Lawrence, & Collins, 2007). O^{2-} is an oxygen-containing compound that is particularly active. In addition to O^{2-}, hydrogen peroxide (H_2O_2), hydroxyl radicals (–OH), and nitric oxide (NO) are all ROS (Dwyer, Kohanski, & Collins, 2009). Antibiotics and other antimicrobial agents can exert their antibacterial functions by significantly enhancing bacterial respiration and inducing a sharp increase in ROS production in bacteria (Lobritz et al., 2015). As a result, ROS not only enhances the antibacterial activity of bacterial agents, but also induces gene mutations in bacterial cells (Sakai, Nakanishi, Yoshiyama, & Maki, 2006). Indeed, the ROS was found to affect DNA replication and increase the mutation rate.

It is generally recognized that genetic mutations are the primary cause of bacterial drug resistance (Kohanski et al., 2010). Non-lethal concentrations of antibiotics showed an ability to induce and produce ROS that are sufficient to activate the external drug pump AcrAB-TolC by affecting the multiple antibiotic resistance repressor (MarR) and the multiple antibiotic resistance activator (MarA). Alternatively, the stress protection mechanism of bacteria can be activated through the superoxide response transcriptional regulator (SoxS) and the superoxide response factor (SoxR), which can induce gene mutations and increase bacterial tolerance to antimicrobial agents (Finkel & Holbrook, 2000).

There are a multitude of microbial antioxidant enzymes that are capable of scavenging intracellular ROS, including glutathione peroxidase (T-GSH) and superoxide dismutase (SOD). These antioxidant enzymes are able to scavenge ROS and reduce and/or eliminate oxidative damage to host microorganisms (Han et al., 2011). Antimicrobial agent-induced ROS cause an imbalance in oxidation and reduction. ROS also damage DNA, proteins, lipids, and other macromolecules, which leads to greater permeability of the cell membrane and cell wall (Finkel & Holbrook, 2000). Another study showed that a non-lethal concentration of antibiotics resulted in an increase in the mutation rate, which, in turn, resulted in enhancing drug resistance. Specifically, these mutations were associated with concentrations of antibiotic-induced bacterial ROS production (Ma, Mi, Xue, Wang, & Zhao, 2016).

Studies have found that the antimicrobial components of traditional Chinese medicines (TCMs) typically include flavonoids, alkaloids, organic acids, volatile oils, polysaccharides, saponins, anthraquinones, and terpenoids. It is thought that the antibacterial mechanisms of TCMs arise through bacteria and host double regulation, and TCMs can reverse drug-resistant bacteria to restore bacterial susceptibility to antibiotics (Zhou, Jia, Liu, & Wang, 2012). However, TCM can regulate the content of ROS in bacteria, which may be a mechanism to restore sensitivity to bacteria.

Previous work has demonstrated that total alkaloids extracted from the seeds of *Sophora alopecuroides* (TASA) exhibit a broad range of antibacterial activities. Specifically, TASA was shown to reverse the susceptibility of clinical multidrug resistant *Escherichia coli* to CIP, in part by downregulation of the AcrAB-ToLC efflux pump (Zhou et al., 2012). Further, TASA showed a better inhibitory effect on the late stage of biofilm thickening of clinic *S. epidermidis* (Li et al., 2016). Matrine (MT) is an alkaloid found in the roots of *Sophora* species. MT has been reported to possess a wide range of pharmacological effects, including anti-inflammatory, anti-arrhythmia, anti-viral, antifibrotic, anti-allergy, analgesic, and immunosuppressive properties (Kan, Zhu, Liu, & Zhang, 2013). However, whether TASA and MT are able to exert their antibacterial or antibiofilm functions by inducing intracellular ROS production remain unclear. The objective of the present study was to explore the antimicrobial activity of MT and TASA in the regulation of ROS production in a strong biofilm-producing *S. epidermidis* isolate.

2. Materials and methods

2.1. Bacterial strains

Strains of *S. epidermidis* were purchased from the American Type Culture Collection (FDA strain Seattle 1946, ATCC 35984; ATCC, Rockville, MD, USA). The clinical *S. epidermidis* was isolated and identified using biochemical characterization and polymerase chain reaction assays from milk samples of a cow with mastitis (D. Liu, Swiatlo, Austin, & Lawrence, 2006), and then producing-biofilm *S. epidermidis* S3 was identified using crystal violet staining (Tremblay et al., 2013) and polymerase chain reaction assays (Mekni, Bouchami, Achour, & Ben Hassen, 2012) by our laboratory.

2.2. Chemicals and kits

TASA (total alkaloids ≥98%) was obtained from Salt Lake Pharmaceutical Factory (Yinchuan, Ningxia, China, 9600169). MT was purchased from Solarbio, Beijing (MT, content ≥98%, 07809703), samples of which were processed according to the Chinese Pharmacopoeia (2005 edition, certificate 040228). TASA and MT were freshly dissolved in distilled water before use (Zhou et al., 2010). CIP was purchased from Pharmaceutical and Biological Products Inc. (Beijing, China, 130451-200302). 2′,7′-Dichlorodihydrofluorescein dictate (DCFH-DA) was purchased from Sigma-Aldrich (D6883-50MG, formula weight 487, dissolved in DMSO; St. Louis, MO, USA). Analysis kits for alkaline phosphatase, nicotinamide adenine dinucleotide phosphate (NADPH oxidase), superoxide dismutase (SOD), catalase (CAT), and glutathione (GSH) were purchased from Nanjing Jiancheng Biotechnology Co., Ltd. (Nanjing, China).

2.3. Antibacterial susceptibility testing

Antibacterial activity was evaluated via minimum inhibitory concentration (MIC), which is a microbroth dilution method developed by the Clinical and Laboratory Standards Institute (CLSI Document M100-S24). For the microbroth 2-fold dilution method, each well of a 96-well microplate was coated with 2-fold serial dilutions of antibiotics. For CIP, a starting concentration of 32 μg/mL and a final dilution concentration of 0.125 μg/mL was used. For TASA and MT, a starting concentration of 50 mg/mL and a final dilution concentration of 0.096 mg/mL was used. The inoculums of *S. epidermidis* field isolate S3 and *S. epidermidis* reference ATCC35984 were adjusted to 1×10^8 colony-forming units (CFU)/mL by comparing them with a 0.5 McFarland turbidity standard, further 1:1000 re-diluted as an inoculant (Njeru et al., 2016). Briefly, bacterial inoculates were incubated on Trypticase Soy Broth (TSB) agar at 37 °C for 12 h in a microaerophilic atmosphere (10% O_2 and 5% CO_2). MIC was defined as the lowest concentration of a test agent that completely inhibited visible bacterial growth (Kobayashi et al., 2004). All experiments were performed in triplicate.

2.4. Effect of TASA, MT and CIP on biofilm

Effect of TASA, MT, and CIP on structure of biofilm of *S. epidermidis* was examined by Confocal Laser Scanning Microscope (CLSM). Concentrations of TASA and MT were used 25 mg/mL in *S. epidermidis* reference ATCC35984 and *S. epidermidis* field isolate S3 according to their MIC values, respectively. Concentration of CIP was 0.25 μg/mL and 8 μg/mL in *S. epidermidis* reference

ATCC35984 and *S. epidermidis* S3 field isolate S3 according to their MIC values, respectively. Staining and CLSM analysis of biofilm were performed based on image structure analyzer (ISA) software (Guan, Luo, Fang, & Zhou, 2018).

2.5. Fractional inhibitory concentration index (FICI)

Values of FICI were used for evaluating antibiotic interactions in a combination of TASA, MT and CIP. The FICI was determined by MICs for each combination and was calculated by using the following equation: FICI = FIC_A + FIC_B = A/MIC_A + B/MIC_B, where A and B are the MIC of each antibiotic in combination (in a single well), and MIC_A and MIC_B are the MIC of each drug individually. The FICI was interpreted as follows: FICI value of <0.5, Synergy: the combination of the compounds increases the inhibitory activity (decrease in MIC) of one or both compounds than the compounds alone; FICI value of 0.5–4, additive or indifference: the combination has no increase in inhibitory activity or a slight increase in inhibitory activity from the additive effect of both compounds combined. FICI value of >4, antagonism: the combination of compounds increases the MIC, or lowers the activity of the compounds (Petersen, Labthavikul, Jones, & Bradford, 2006).

2.6. Resistance to antimicrobial agents induced subinhibitory concentrations

The minimum biofilm inhibitory concentration (mBIC) was defined as the lowest concentration of a test agent that completely inhibited visible biofilm producing bacteria growth. The 1/2mBIC reagents concentration was screened as a sub-minimum biofilm inhibitory concentration (sub-mBIC) by pre-experiment. The changes in the first and sixth generations of mBICs for TASA, MT and CIP were measured with sub-mBIC TASA, MT, CIP, TASA + CIP, and MT + CIP, respectively. A sterile coverslip was placed on the bottom of a 12-well plate, and 1 mL of bacterial suspension at a density of 1×10^6 CFU/mL was added to sub-mBIC TASA, MT, CIP, TASA + CIP, and MT + CIP, respectively. The first generation of bacteria was cultured at 37 °C for 18 h. We discarded any remaining floating bacteria and collected the biofilm-producing cells on the coverslip with 1 mL of sterile TSB. We then centrifuged samples at 1500 rpm for 10 min, washed them three times with phosphate-buffered saline (PBS), and resuspended them in TSB. We then added 10 μL of the resuspension to 10 mL TSB overnight. Then, 1 mL of the bacterial suspension at a density of 1×10^6 CFU/mL was added to a sterile coverslip on the bottom of a 12-well plate with sub-mBIC TASA, MT, CIP, TASA + CIP, and MT + CIP, respectively. The above steps were repeated an additional four times until the sixth generation was reached. The sixth generation of bacterial cells was then cultured at 37 °C for another 18 h. The mBICs of TASA, MT, and CIP after sub-mBIC were determined using a 2-fold dilution method (Sun et al., 2009).

2.7. ROS production of bacteria exposed to antimicrobial agents

ROS concentration was determined using a DCFH-DA assay (Ibanez et al., 2012). DCFH can be oxidized by ROS to produce the fluorescent compound DCF. Green fluorescence intensity is directly proportional to the level of intracellular ROS (Gabriela Ciapetti et al., 1998). Therefore, the fluorescence intensity of DCF is proportional to the reactive oxygen level in cells (Espada, 2016). The bacterial cultures and method of administration were performed as described in section 2.6. The collected bacteria were adjusted to 1×10^8 CFU/mL and 10 μmol/L DCFH-DA fluorescent probe was added. Reactions were incubated in the dark for 20 min and inverted every 5 min to load the probe into the bacteria. We then centrifuged samples at 1500 rpm for 10 min and washed them three times with PBS. Excess DCFH-DA was removed and the bacteria were resuspended in PBS. The detection of cellular ROS was accomplished using a fluorescence spectrophotometer with excitation and emission wavelengths of 488 nm and 525 nm, respectively.

2.8. Analysis of key factors related to regulation of ROS of S. epidermidis

The bacterial cultures and method of administration were performed as described in Section 2.6. Nitric oxide (NO) levels in bacteria was determined according to the instructions of the kits for three replications in each group. Lipid peroxidation (MDA) in the supernatant was determined using premade kits. The bacterial resuspension was disrupted by ultrasonication in PBS on ice. The bacterial culture was sonicated at 300 w for 20 min with ultrasonic crushing for 5 s, intermittent 5 s. The sonicated solution was centrifuged at 4000 rpm for 10 min at 4 °C and the supernatant was collected and stored at −80 °C until use. SOD and GSH activities were determined according to the instructions of the respective kits.

2.9. Data analysis

Data were analyzed using SPSS version 19.0. The degree of variation of data was represented by the mean ± standard deviation. One-way analysis of variance (ANOVA) was used to compare the differences between multiple samples. Dunnett-*t* and *SNK*-q were used to compare differences between two groups.

3. Results

3.1. Susceptibility of S. epidermidis to antimicrobial agents

The MICs of *S. epidermidis* reference strain ATCC35984 and *S. epidermidis* field isolate S3 for CIP were 0.25 μg/mL and 8 μg/mL, respectively (Table 1). The *S. epidermidis* field isolate S3 was more resistant to ciprofloxacin than *S. epidermidis* reference strain ATCC35984 according to CLSI guidelines. However, *S. epidermidis* field isolate S3 and reference strain ATCC35984 have the same sensitivity to TASA and MT, and their MICs were 25 mg/mL.

3.2. TASA and MT impaired biofilm of S. epidermidis

To investigate the effects of antimicrobial agents on *S. epidermidis* biofilms, the images of the biofilm of *S. epidermidis* field isolate S3 and reference strain ATCC35984 treated with TASA and MT for 24 h were compared with those treated with CIP (Fig. 1). The stained signal green represented live bacteria and the red signal represented dead bacteria. The orange parts in the images were caused by the overlay of dead and live bacteria ones. Picture showed that the biofilm was dense and almost no gap in control group. In contrast, the structure of biofilm was thin and sparse in drug treatment group, and the proportion of live bacteria was significantly decreased, and the majority of areas were impaired by fluorochrome with a significantly increased orange signal. In addition, the number of dead bacteria in TASA and MT group was significantly larger than that in CIP group. These parameters thickness, biovolume (BV), average diffusion distance (ADD), areal porosity (AP) and textual entropy (TE) of biofilm treated with TASA, MT and CIP were analyzed by ISA software (Tables 2). Data displayed that, compared with the control, biomass, average diffusion distance and TE of biofilm were all decreased, except an increased areal porosity. Simultaneously, these data demonstrate

Table 1
MICs of different drugs in *S. epidermis* isolates S3 and ATCC35984.

Drugs	MICs[a]	
	ATCC35984	S3
TASA	25 ($mg{\cdot}mL^{-1}$)	25 ($mg{\cdot}mL^{-1}$)
MT	25 ($mg{\cdot}mL^{-1}$)	25 ($mg{\cdot}mL^{-1}$)
CIP	0.25 ($\mu g{\cdot}mL^{-1}$)	8 ($\mu g{\cdot}mL^{-1}$)

[a] Data represent mean of three independent experiments for each condition.

that CIP has a weaker effect on biofilm formation than TASA and MT ($P < 0.05$).

3.3. Interactions of antimicrobial agents

Interactions of antimicrobial agents by checkerboard analysis is used to determine the impact on potency of the combination of antibiotics in comparison to their individual activities. This comparison is then represented as the FICI value. Table 3 listed antibiotic interactions in a combination of TASA or MT and CIP *in vitro* on *S. epidermidis* ATCC35984 and *S. epidermidis* S3 isolate. It was observed that there was additive or indifference between TASA or MT and CIP on above two strains (ADD or IND). This result suggested that a combination of TASA or MT with CIP has no increase in inhibitory activity or a slight increase in inhibitory activity on biofilm-producing *S. epidermidis*.

3.4. Increase susceptibility of S. epidermidis to ciprofloxacin-induced sub-mBIC of TASA and MT

To research alterations of drug resistance of biofilm-producing bacteria to sub-mBIC of TASA/MT and CIP alone and in combination for six generations. Averages of mBIC to TASA, MT and CIP were obtained from three experiments, and the mean is expressed using the arithmetic mean, with sub-mBIC of TASA, MT, CIP, TASA + CIP and MT + CIP induced after the first and sixth generations of biofilm-producing *S. epidermidis* field isolate S3 and reference strain ATCC35984. Of note, the data showed that the mBIC of biofilm-producing bacteria for CIP was only changed following treatment sub-mBIC chemicals (Table 4) (Plot this data as Fig. 2a). For biofilm-producing *S. epidermidis* reference strain ATCC35984, the mBIC of CIP decreased from 0.125 to 0.0625 following the induction of six generations with mBIC TASA; The mBIC of CIP decreased from 0.167 to 0.025 following treatment of six generations with mBIC MT. Interestingly, there was a clear difference between TASA, MT and CIP, in which the mBIC of CIP increased form 0.5 to 1 following treatment of six generations with sub-mBIC CIP in reference biofilm-producing strain ATCC35984 (Fig. 2a). Biofilm-producing *S. epidermidis* field isolate S3 also had a similar results that the mBIC of CIP decreased from 4 to 2 (2-fold) following induction with sub-mBIC TASA and MT in the sixth generation, whereas the mBIC of CIP increased from 32 to 64, twice as high after sub-mBIC CIP-induced in the sixth generation (Table 4).

In combination, traditional Chinese medicine, TASA and MT were not easily developed resistance to biofilm-producing *S. epidermidis*. The mBICs of CIP following treatment with combine sub-mBIC TASA + CIP and MT + CIP remained constant until the induction of the sixth generation for *S. epidermidis* reference strain ATCC35984, which intimated their resistance no increase. Moreover, for the biofilm-producing *S. epidermidis* field isolate S3, bacterial resistance to ciprofloxacin was not increased but restored sensitivity that the mBICs of CIP following treatment with combine sub-mBIC TASA + CIP and MT + CIP decreased from 8 to 4 (2 fold) following the induction of six generations (Fig. 2a).

3.5. TASA and MT enhanced ROS to restore susceptibility of S. epidermidis to ciprofloxacin

To study alterations of ROS production following treatment with sub-mBIC of TASA/MT and CIP alone and in combination. Following the induction of generations with TASA, MT and CIP, the ROS content of the experimental group of biofilm-producing *S. epidermidis* field isolate S3 and *S. epidermidis* reference strain ATCC35984 were significantly increased compared to the control group ($P < 0.05$). In addition, ROS production following treatment with all chemicals at mBIC was significantly higher than in cells treated with sub-mBIC (Data is not showed). Noteworthy, there were no significant changes in ROS content following treatment with sub-mBIC TASA and MT for biofilm-producing *S. epidermidis* strains ATCC35984 and S3 six generations later. Whereas ROS pro-

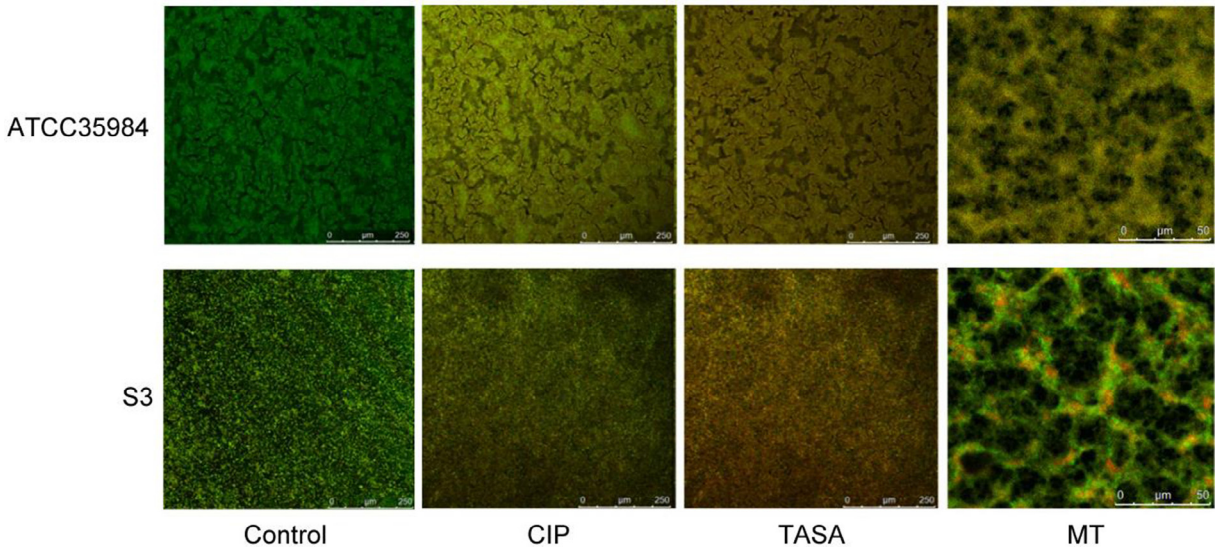

Fig. 1. Effects of antimicrobial agents on biofilm formation in *S. epidermidis* determined by a CLSM analysis. *S. epidermidis* reference strain ATCC35984 *and S. epidermidis* S3 cells were treated with TSB control, 25 mg/mL of TASA and MT, 0.25 μg/mL (ATCC35984) and 8 μg/mL(S3) of CIP at 37 °C for 24 h, and morphological structure of biofilm was visualized under a CLSM.

Table 2
Analysis results of biofilm structural parameters under effect of chemicals.

Strains	Drugs	BV	AP	ADD	TE
ATCC 35984	Control	5682622 ± 10938	0.535 ± 0.03	1.843 ± 0.06	9.038 ± 0.29
	TASA	4618854 ± 14376$^{*\#}$	0.638 ± 0.06$^{*\#}$	1.600 ± 0.04$^{*\#}$	8.151 ± 0.27$^{*\#}$
	MT	4590844 ± 14789$^{*\#}$	0.613 ± 0.04$^{*\#}$	1.6569 ± 0.03$^{*\#}$	8.365 ± 0.30*
	CIP	5300309 ± 12149	0.543 ± 0.08	1.722 ± 0.08	8.702 ± 0.20
S3	Control	3179886 ± 10938	0.747 ± 0.05	1.241 ± 0.07	8.506 ± 0.22
	TASA	1387996 ± 14376$^{**\#}$	0.890 ± 0.08$^{*\#}$	1.061 ± 0.04$^{*\#}$	7.289 ± 0.31*
	MT	127809 ± 12368$^{**\#}$	0.869 ± 0.06$^{*\#}$	1.034 ± 0.03*	7.129 ± 0.26*
	CIP	1878617 ± 12099*	0.751 ± 0.07	1.154 ± 0.05	7.456 ± 0.28*

Note: biovolume (BV), average diffusion distance (ADD), areal porosity (AP) and textual entropy (TE); [a]:Biofilm structure was analyzed using ISA software., $^{**}P < 0.05$ and $^{**}P < 0.01$ *vs* control group; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ *vs* CIP group

Table 3
Antibacterial activity and combined effects of TASA, MT and CIP alone or in combination.

Drug combinations	Bacterial strains	Individual MICs[a]	Combination MICs[a]	Combined FICIs	Interaction
TASA($mg{\cdot}mL^{-1}$)/CIP($\mu g{\cdot}mL^{-1}$)	S3	25/8	3.125/8	1.125	ADD or IND
	ATCC35984	25/0.25	1.56/0.25	1.00625	ADD or IND
MT($mg{\cdot}mL^{-1}$)/CIP($\mu g{\cdot}mL^{-1}$)	S3	25/8	3.125/8	1.125	ADD or IND
	ATCC35984	25/0.25	1.56/0.25	1.00625	ADD or IND

[a] Results represented means of three independent experiments. SYN: synergy; ADD or IND: additive or indifference. ANT: antagonism.

Table 4
mBICs of standard strains following drug treatment.

Strains	Sub-mBICs	mBIC values					
		TASA		MT		CIP	
		FG	SG	FG	SG	FG	SG
ATCC 35984	TASA	20.83	20.83	25	25	0.125	0.0625
	MT	25	25	25	19.84	0.167	0.025
	CIP	20.83	20.83	25	25	0.5	1
	TASA + CIP	25	25	20.83	25	0.33	0.42
	MT + CIP	25	20.83	25	25	0.42	0.42
S3	TASA	25	25	20.83	25	4	2
	MT	25	25	25	25	4	2
	CIP	25	25	20.83	25	32	64
	TASA + CIP	20.83	20.83	25	25	8	4
	MT + CIP	25	20.83	25	25	8	4

[a]Data represent mean of three independent experiments for each condition. FG, first generation; SG, sixth generation.

duction was decreased 23%–33% in sixth generation biofilm-producing *S. epidermidis* strains ATCC35984 and S3 treated with sub-mBIC CIP anti-microbial agents compared with the first generation (Fig. 2b).

In the case of a combination of drug sub-concentrations, we found that ROS content increased 7%–24% following treatment with TASA + CIP and MT + CIP combination for biofilm-producing *S. epidermidis* isolates S3 and *S. epidermidis* strains ATCC35984 (Fig. 2b), indicating that CIP combined with TASA and MT can improve the generation of ROS.

3.6. TASA and MT regulated key factors NO, MDA, SOD and GSH related to ROS to increase susceptibility of S. epidermidis to ciprofloxacin

To investigate changes of NO, MDA, SOD and GSH content treatment with sub-mBIC of TASA/MT and CIP alone and in combination. As a free radical, similar to ROS content, the amount of NO in the sixth generation of cells following sub-mBIC alone CIP treatment in biofilm-producing *S. epidermidis* isolates S3 and *S. epidermidis* strains ATCC35984 was lower than that in the first generation (Fig. 2c). An indicator of bacterial oxidative damage, the MDA content of biofilm-producing *S. epidermidis* strains ATCC35984 and S3 increased following treatments with the different agents for different time periods. Within 2–4 h, bacterial MDA content increased gradually, for the two strains, the trend has increased until the first generation (18 h). The antimicrobial agents worked for the fourth generations until 90 h, distinctive reduction showed with subinhibitory concentrations CIP (Fig. 3). SOD and GSH are enzyme and non-enzyme antioxidants. SOD activity in biofilm-producing *S. epidermidis* S3 isolates was significantly higher following the introduction of antimicrobial agents. SOD activity in the sixth generation was higher following treatment with sub-mBIC CIP in the first generation of biofilm-producing *S. epidermidis* isolates S3 and ATCC35984, but SOD activity in the sixth generation was lower than that in the first generation of cells treated with sub-mBIC TASA and MT (Fig. 2d). Similar results to SOD, GSH activity in the sixth generation of *S. epidermidis* was higher than that in the first generation of bacteria treated with sub-mBIC CIP but GSH activity in the sixth generation was lower than that in the first generation of cells treated with sub-mBIC TASA and MT (Fig. 2e).

In sub-mBIC combination, NO content did not alter inducing by TASA + CIP and MT + CIP form the first to the sixth generation of the reference bacteria and biofilm-producing *S. epidermidis* strains S3. This increase in MDA extended to the sixth generation including sub-mBIC TASA + CIP and MT + CIP for the reference bacteria

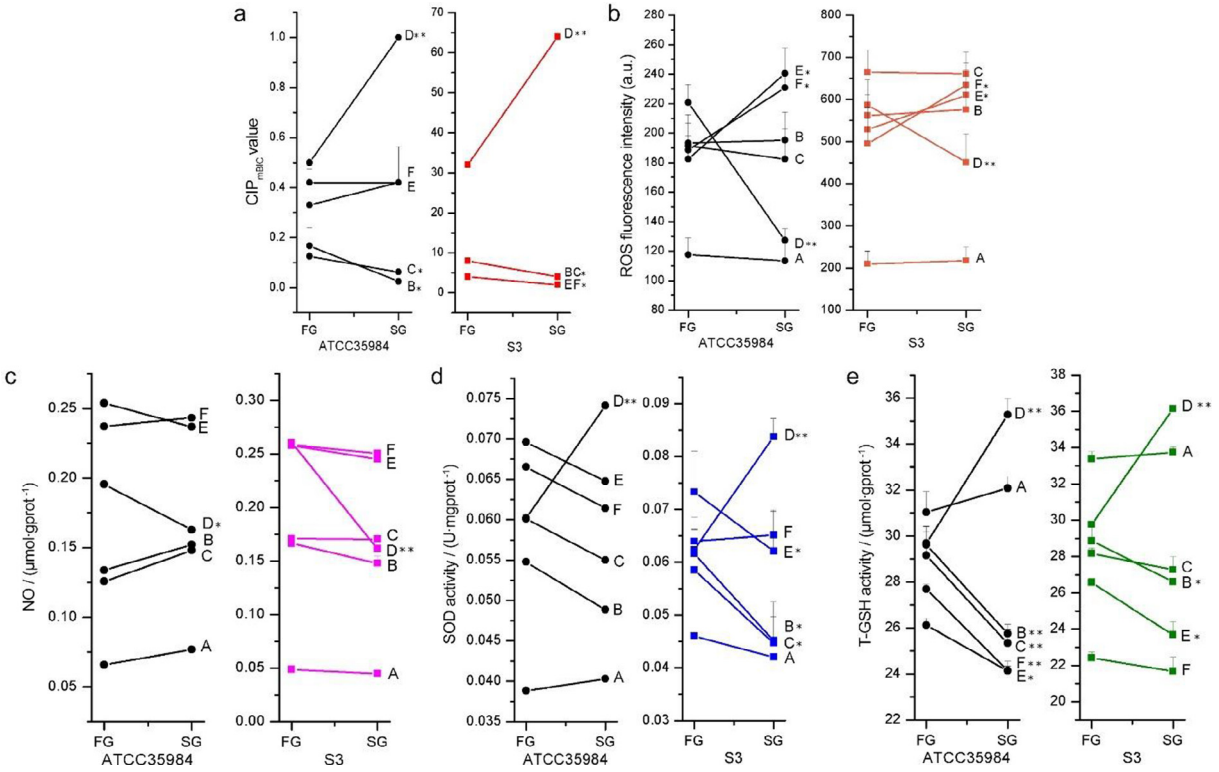

Fig. 2. CIP_{mBIC} changes, levels of ROS and NO, activities of SOD and T-GSH in biofilm-producing *S. epidermidis* treated with sub-mBIC TASA/MT and CIP alone and in combination from the first to the sixth generation. Bacteria treated with sub-mBIC TASA, MT, and CIP were cultured at 37 °C for 18 h (first generation). These steps were repeated until the sixth generation was obtained. $^{*}P < 0.05$; $^{**}P < 0.01$. A, control; B, sub-mBIC TASA; C, sub-mBIC MT; D, sub-mBIC CIP; E, sub-mBIC TASA + CIP; F, sub-mBIC MT + CIP. a. CIP_{mBIC} values following treatment with sub-mBIC TASA, MT, CIP, TASA + CIP, and MT + CIP were determined by a 2-fold dilution method. b. First and sixth generation ROS levels of *S. epidermis* following treatment with sub-mBIC TASA, MT, CIP, TASA + CIP, and MT + CIP. The detection conditions were excitation and emission wavelengths of 488 nm and 525 nm, respectively. c. NO levels in the first and sixth generation *S. epidermis* treated with sub-mBIC TASA, MT, CIP, TASA + CIP, and MT + CIP. d. SOD activity of first and sixth generation *S. epidermis* treated with sub-mBIC TASA, MT, CIP, TASA + CIP, and MT + CIP. e. T-GSH activity of first and sixth generation *S. epidermis* treated with sub-mBIC TASA, MT, CIP, TASA + CIP, and MT + CIP. Note: CIP, ciprofloxacin; TASA, total alkaloids of *S. alopecuroides*; MT, matrine; mBIC, minimum biofilm inhibitory concentration; ROS: reactive oxygen species; NO, nitric oxide; SOD, superoxide dismutase; GSH, glutathione; FG, first generation; SG, sixth generation.

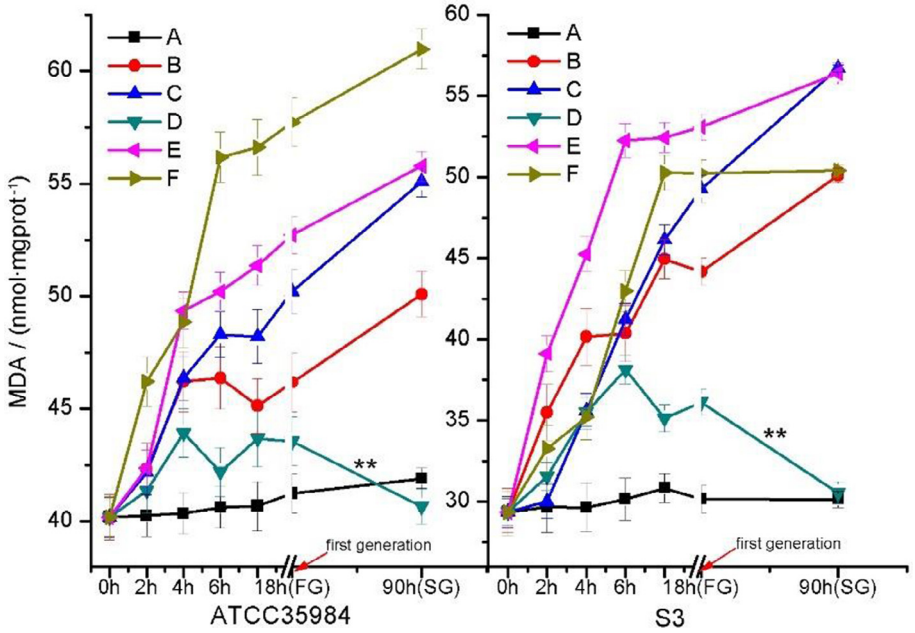

Fig. 3. Lipid peroxidation (MDA levels) of *S. epidermis* (ATCC35984 and S3) treated with sub-mBIC TASA/MT and CIP alone and in combination. First and sixth generation lipid peroxidation (MDA) levels of *S. epidermis* treated with sub-mBIC TASA, MT, CIP, TASA + CIP, and MT + CIP. Bacteria treated with sub-mBIC TASA, MT, and CIP were cultured at 37 °C for 18 h (first generation). These steps were repeated until the sixth generation was obtained. $^{*}P < 0.05$; $^{**}P < 0.01$. A, control; B, sub-mBIC TASA; C, sub-mBIC MT; D, sub-mBIC CIP; E, sub-mBIC TASA + CIP; F, sub-mBIC MT + CIP. Note: MDA, lipid peroxidation.

and biofilm-producing *S. epidermidis* strains S3 (Fig. 3). SOD activity and GSH activity in the sixth generation was lower than that in the first generation of cells treated with TASA + CIP on biofilm-producing *S. epidermidis* S3 isolates and *S. epidermidis* reference strains ATCC35984 (Fig. 2d). In particular, SOD activity and GSH activity in the sixth generation arise no decrease or increase treated with MT + CIP for the two strains (Fig. 2e).

4. Discussion

Image structure analyzer (ISA) software was used to analyze the biofilm structure of the 12-layer image scanned by CLSM along the Z axis. The higher the BV and ADD was, the thicker the biofilm was. Higher structural entropy (TE) indicates that the structure of BF is more complicated and the uniformity is poor. The regional porosity (AP) reflects the density of the biofilm structure. The lower the AP is, the denser the structure of the membrane is. This research revealed that TASA and MT are more effective in inhibiting biofilms. Previous research also showed that TASA displayed the most effective effect on inhibition of biofilm formation (Li et al., 2016). However, the mechanism of this apparent effect is unclear. In this context, we found that TASA and MT induced reactive oxygen species (ROS) to impair biofilm and increase the susceptibility of *S. epidermidis* to ciprofloxacin.

A number of studies have found that antibiotics can increase the oxidative stress of bacteria via the production of ROS. Excess ROS is the key cause of bacterial cell death (Becerra, Paez, Larovere, & Albesa, 2006), although the anti-bacterial mechanisms of ROS dependence are nonspecific (Wainwright, Smalley, Scully, & Lotfipour, 2012). We found that under conditions of mBIC and sub-mBIC, TASA, MT and CIP are able to enhance ROS concentrations in bacteria. At bactericidal concentrations, TASA, MT, and CIP likely share a similar bactericidal mechanism. For antibacterial sensitivity and induction of ROS, antibiotics and herbal compounds were different under the different treatments, such as sub-mBIC and mBIC drug concentrations. The mBIC of CIP decreased 2 (0.125/0.0625) (4/2) to 6 (0.167/0.025)-fold following treatment with sub-mBIC TASA and sub-mBIC MT for six generations in *S. epidermis* strains ATCC35984 and S3. By contrast, the mBIC of CIP increased 2-fold following sub-mBIC CIP treatment for six generations in *S. epidermis* strains ATCC35984 (1/0.5) and S3 (64/32). These results suggested that resistance to TCM is not easily developed, and to some degree, can even reverse CIP resistance in *S. epidermis*. Interestingly, ROS production following treatment with all chemicals at 1 × mBIC was significantly higher than in cells treated with sub-mBIC. And using a sub-mBIC of CIP, ROS production declined over multiple generations. ROS content decreased by 1.3 (220.8/127.5)–1.5 (587.1/451) fold under sub-mBIC CIP from the first to sixth generations. These results suggested that a high concentration of ROS induced by antibiotics favored antibacterial activity, but resulted in the development of drug resistance at low concentrations (Foti, Devadoss, Winkler, Collins, & Walker, 2012; Kohanski et al., 2010; Liu et al., 2016). However, the mBICs of TASA and MT following treatment with sub-mBICs TASA + CIP and MT + CIP did not change until the induction of the sixth generation. The mBIC of CIP decreased 2-fold following sub-mBICs TASA + CIP and MT + CIP induction in the sixth generation of isolated strains of *S. epidermidis* S3. ROS content was ascendant in the sub-mBICs TASA + CIP and MT + CIP tested agents from the first to sixth generations. Overall, our results suggested drug resistance to CIP may be delayed by the use of TCM prior to the use of antibiotics, and combined TASA + CIP and MT + CIP could increase the antibacterial effect.

Studies have shown that endogenous NO in bacteria can directly modify some antibiotics and help to protect bacteria against oxidative damage caused by antibiotics (Cirz et al., 2005). This, in turn, allows bacteria to develop a broad spectrum of antibiotic resistance. *S. epidermis* attacks its host by increasing the availability of NO, which induces host cell damage. This results in a reduction in immune cells needed to kill the bacteria (Chakraborty, Pramanik, & Roy, 2012). The test found that the levels of NO decreased from the first generation to the sixth generation following treatment with sub-mBIC CIP in the reference strain and isolates. In line with this result, bacterial resistance to sub-mBIC CIP increased in the sixth generation standard strains from 0.5 (up to 1) isolates to 32 (up to 64). NO content was reduced in the sixth generation of bacteria-treated sub-mBIC CIP, suggesting that NO content was not sufficient to kill bacteria, resulting in bacterial drug resistance. Exogenous NO has inhibitory effects on bacterial growth, and high concentrations of NO can inhibit *S. epidermis*. *Staphylococcus aureus* can perceive and respond to NO through the SrrAB two-component system and activate SrrAB regulators to repair damage and reduce the toxicity of exogenous NO (Kinkel, Roux, Dunman, & Fang, 2013). Endogenous NO is formed by the oxidation of *L*-arginine under the catalysis of bacterial NOS. Nitrate reductase (NR) reduces nitrate ions to nitrite ions, and nitrate can be further reduced to NO by nitrite reductase (Gordon, 2003). This study found that the content of NO in the cell was related to resistance of bacteria to antibacterial drugs. When bacteria produced a proper amount of NO to protect themselves, it was easy to form drug resistance. When a large amount of NO was produced, it was not good for themselves, but it caused damage to itself. NO is a free radical and is considered a ROS, which is consistent with the previously confirmed relationship between ROS and drug resistance.

Cell walls and cell membranes are the first barriers of microorganisms to resist external damage. Increased production of ROS induced by antibiotics will disrupt the redox balance in bacteria and damage DNA, proteins, lipids, and other macromolecules. ROS produced in large quantities will also destroy bacterial structure and increase permeability of the cell membrane and cell wall. These changes facilitate the combination of drugs with the target (Kohanski et al., 2007). TCMs and extracts can change the permeability of cell membranes and inactivate bacteria. For example, anthocyanines can inhibit biofilm formation in *S. epidermidis* by changing the permeability of the cell wall (Cabiscol, Tamarit, & Ros, 2000; Hernández-Hierro et al., 2014). Likewise, *Galla chinensis* extract can alter the morphological size of *S. epidermidis* and rupture bacterial cell walls (Naghmouchi et al., 2006). The cell wall and cell membrane of *S. epidermidis* contain lipids and phospholipids (MDA). MDA content can reflect the degree of oxidative damage of bacteria. To observe the accumulation of MDA over time, we measured MDA content at 0, 2, 4, 6, 8, 18 (18 h as the first generation, 36 h as the second generation, and so on, and as the sixth category after 90 h), and 90 h as well as in the first and sixth generations. As shown in Fig. 3, MDA increased over time and remained relatively stable after 18 h. Surprisingly, only treatment with sub-mBIC CIP showed a downward trend in the sixth generation. This suggested that MDA accumulation decreased when drug resistance was generated in the sixth generation and that MDA may also be related to bacterial drug resistance (Yuan, Peng, & Gurunathan, 2017).

SOD was the main regulators of ROS changes in bacteria. SOD catalyzes the super oxygen anion free radical O_2- disproportionation reaction, resulting in the production of O_2 and H_2O_2. CAT will then catalyze H_2O_2, resulting in O_2 and H_2O. Our study also found that SOD levels were induced to a greater extent in the sixth generation following treatment with sub-mBIC CIP compared with the first generation of *S. epidermidis* strains ATCC35984 and S3. However, SOD levels following treatment of sixth generation of *S. epidermidis* S3 with sub-mBIC TASA + MT were lower compared

with the first generation. The relationship between SOD and ROS levels was analyzed and the change in SOD levels was the inverse of the change in ROS levels in *S. epidermidis* strains ATCC35984 and S3 treated with sub-mBIC CIP anti-microbial agents compared with the first generation. On the contrary, the change in SOD levels was consistent with the change in ROS content in *S. epidermidis* S3 treated with sub-mBIC TASA + CIP combination anti-microbial agents compared with the first generation. The SOD dismuting reaction is the first line of defense of bacteria against excessive ROS; changes in SOD and ROS levels have a dynamic balance (Nakonieczna et al., 2010).

GSH is an antioxidant enzyme that can restore H_2O_2; it is also an effective hydroxyl free radical scavenger, and can directly react with ROS. GSH activity following treatment with sub-mBIC CIP was higher in sixth generation *S. epidermidis* strains ATCC35984 and S3 compared with the first generation. SOD and GSH were representative enzymes and non-enzymatic antioxidants, consistent with our results, which can synergistically maintain the redox balance *in vivo* (Masip, Veeravalli, & Georgiou, 2006).

5. Conclusions

TASA and MT are more conducive to the elimination the biofilms of *S. epidermis*. TASA and MT are not easily developed resistance, and to some degree, TASA and MT can reverse CIP resistance in *S. epidermis*. TASA and MT can restore sensitivity to ciprofloxacin by relying on enhanced ROS production. ROS induced by antibiotics at high concentrations favored sterilization, while at low concentrations, the production of ROS was conducive to bacterial resistance. NO is a ROS and thus our results were consistent with the previously confirmed relationship between ROS and drug resistance. Our results showed that SOD and GSH were representative enzymes and non-enzymatic antioxidants, which could synergistically maintain the redox balance *in vivo*. SOD, and GSH also showed differences in the regulation of ROS changes in TCM and antibiotics.

Authors' contributions

X.Z. conceived and designed the program; F.J., M.S. and J.Z. verified the experiments and acquired data; F.J. and M.S. analyzed the data and drafted the manuscript; X.Z. checked the data and critically revised the manuscript. All authors proofread and approved carefully the final version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Becerra, M. C., Paez, P. L., Larovere, L. E., & Albesa, I. (2006). Lipids and DNA oxidation in *Staphylococcus aureus* as a consequence of oxidative stress generated by ciprofloxacin. *Molecular and Cellular Biochemistry, 285*(1–2), 29–34. https://doi.org/10.1007/s11010-005-9051-0.

Cabiscol, E., Tamarit, J., & Ros, J. (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. *International Microbiology, 3*(1), 3–8.

Chakraborty, S. P., Pramanik, P., & Roy, S. (2012). *Staphylococcus aureus* infection induced oxidative imbalance in neutrophils: Possible protective role of nanoconjugated vancomycin. *International Scholarly Research Notices Pharmacology, 2012*. https://doi.org/10.5402/2012/435214 435214.

Cirz, R. T., Chin, J. K., Andes, D. R., de Crecy-Lagard, V., Craig, W. A., & Romesberg, F. E. (2005). Inhibition of mutation and combating the evolution of antibiotic resistance. *PLOS Biology, 3*(6). https://doi.org/10.1371/journal.pbio.0030176 e176.

Dwyer, D. J., Kohanski, M. A., & Collins, J. J. (2009). Role of reactive oxygen species in antibiotic action and resistance. *Current Opinion in Microbiology, 12*(5), 482–489. https://doi.org/10.1016/j.mib.2009.06.018.

Espada, J. (2016). Current methods to unravel ROS biology. *Methods, 109*, 1–2.

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature, 408*(6809), 239–247. https://doi.org/10.1038/35041687.

Foti, J. J., Devadoss, B., Winkler, J. A., Collins, J. J., & Walker, G. C. (2012). Oxidation of the guanine nucleotide pool underlies cell death by bactericidal antibiotics. *Science, 336*(6079), 315–319.

Gabriela Ciapetti, D. G., Verri, E., Savarino, L., Cenni, E., Savioli, F., & Pizzoferrato, A. (1998). Fluorescent microplate assay for respiratory burst of PMNs challenged *in vitro* with orthopedic metals. *Journal of Biomedical Materials Research, 5*(41), 455–460.

Gordon, S. (2003). Alternative activation of macrophages. *Nature Reviews Immunology, 3*, 23. https://doi.org/10.1038/nri978.

Guan, C. P., Luo, H. X., Fang, H. E., & Zhou, X. Z. (2018). Global transcriptome changes of biofilm-forming *Staphylococcus epidermidis* responding to total alkaloids of *Sophorea alopecuroides*. *Polish Journal of Microbiology, 67*(2), 223–226.

Han, Y. T., Chen, X. H., Xie, J., Zhan, S. M., Wang, C. B., & Wang, L. X. (2011). Purple sweet potato pigments scavenge ROS, reduce p53 and modulate Bcl-2/Bax to inhibit irradiation-induced apoptosis in murine thymocytes. *Cellular Physiology and Biochemistry, 28*, 865–872.

Hernández-Hierro, J. M., Quijada-Morín, N., Martínez-Lapuente, L., Guadalupe, Z., Ayestarán, B., Rivas-Gonzalo, J. C., & Escribano-Bailón, M. T. (2014). Relationship between skin cell wall composition and anthocyanin extractability of *Vitis vinifera* L. cv. Tempranillo at different grape ripeness degree. *Food Chemistry, 146*, 41–47.

Ibanez, I. L., Bracalente, C., Notcovich, C., Tropper, I., Molinari, B. L., Policastro, L. L., & Duran, H. (2012). Phosphorylation and subcellular localization of p27Kip1 regulated by hydrogen peroxide modulation in cancer cells. *PLoS One, 7*(9). https://doi.org/10.1371/journal.pone.0044502 e44502.

Kan, Q. C., Zhu, L., Liu, N., & Zhang, G. X. (2013). Matrine suppresses expression of adhesion molecules and chemokines as a mechanism underlying its therapeutic effect in CNS autoimmunity. *Immunologic Research, 56*(1), 189–196. https://doi.org/10.1007/s12026-013-8393-z.

Kinkel, T. L., Roux, C. M., Dunman, P. M., & Fang, F. C. (2013). The *Staphylococcus aureus* SrrAB two-component system promotes resistance to nitrosative stress and hypoxia. *Molecular Biology and Physiology, 4*(6), e00696–00613.

Kobayashi, I., Muraoka, H., Saika, T., Nishida, M., Fujioka, T., & Nasu, M. (2004). Micro-broth dilution method with air-dried microplate for determining MICs of clarithromycin and amoxycillin for *Helicobacter pylori* isolates. *Journal of Medical Microbiology, 53*(Pt 5), 403–406.

Kohanski, M. A., DePristo, M. A., & Collins, J. J. (2010). Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. *Molecular Cell, 37*(3), 311–320.

Kohanski, M. A., Dwyer, D. J., Hayete, B., Lawrence, C. A., & Collins, J. J. (2007). A common mechanism of cellular death induced by bactericidal antibiotics. *Cell, 130*(5), 797–810.

Li, X., Guan, C., He, Y., Wang, Y., Liu, X., & Zhou, X. (2016). Effects of total alkaloids of *Sophora alopecuroides* on biofilm formation in *Staphylococcus epidermidis*. *Biomed Research International, 2016*, 4020715. https://doi.org/10.1155/2016/4020715.

Liu, D., Swiatlo, E., Austin, F. W., & Lawrence, M. L. (2006). Use of a putative transcriptional regulator gene as target for specific identification of *Staphylococcus epidermidis*. *Letters in Applied Microbiology, 43*(3), 325–330.

Liu, Y., Zhou, J., Qu, Y., Yang, X., Shi, G., Wang, X., & Zhao, X. (2016). Resveratrol antagonizes antimicrobial lethality and stimulates recovery of bacterial mutants. *PLoS One, 11*(4). https://doi.org/10.1371/journal.pone.0153023 e0153023.

Lobritz, M. A., Belenky, P., Porter, C. B., Gutierrez, A., Yang, J. H., Schwarz, E. G., & Collins, J. J. (2015). Antibiotic efficacy is linked to bacterial cellular respiration. *Proceedings of the National Academy of Sciences of the United States of America, 112*(27), 8173–8180.

Ma, L. N., Mi, H. F., Xue, Y. X., Wang, D., & Zhao, X. L. (2016). The mechanism of ROS regulation of antibiotic resistance and antimicrobial lethality. *Yi Chuan, 38*(10), 902–909.

Masip, L., Veeravalli, K., & Georgiou, G. (2006). The many faces of glutathione in bacteria. *Antioxidants & Redox Signaling, 8*(5–6), 5–6.

Mekni, M. A., Bouchami, O., Achour, W., & Ben Hassen, A. (2012). Strong biofilm production but not adhesion virulence factors can discriminate between invasive and commensal *Staphylococcus epidermidis* strains. *APMIS, 120*(8), 605–611.

Naghmouchi, K., Drider, D., Kheadr, E., Lacroix, C., Prevost, H., & Fliss, I. (2006). Multiple characterizations of Listeria monocytogenes sensitive and insensitive variants to divergicin M35, a new pediocin-like bacteriocin. *Journal of Applied Microbiology, 100*(1), 29–39.

Nakonieczna, J., Michta, E., Rybicka, M., Grinholc, M., Gwizdek-Wisniewska, A., & Bielawski, K. P. (2010). Superoxide dismutase is upregulated in *Staphylococcus aureus* following protoporphyrin-mediated photodynamic inactivation and does not directly influence the response to photodynamic treatment. *BMC Microbiology, 10*, 323. https://doi.org/10.1186/1471-2180-10-323.

Njeru, S., Obonyo, M., Nyambati, S., Ngari, S., Mwakubambanya, R., & Mavura, H. (2016). Antimicrobial and cytotoxicity properties of the organic solvent fractions of *Clerodendrum myricoides* (Hochst.) R. Br. ex Vatke: Kenyan traditional medicinal plant. *Journal of Intercultural Ethnopharmacology, 5*(3), 226.

Otto, M. (2013). Coagulase-negative staphylococci as reservoirs of genes facilitating MRSA infection: Staphylococcal commensal species such as *Staphylococcus epidermidis* are being recognized as important sources of genes promoting MRSA colonization and virulence. *Bioessays, 35*(1), 4–11.

Penesyan, A., Gillings, M., & Paulsen, I. T. (2015). Antibiotic discovery: Combatting bacterial resistance in cells and in biofilm communities. *Molecules, 20*(4), 5286–5298.

Petersen, P. J., Labthavikul, P., Jones, C. H., & Bradford, P. A. (2006). *In vitro* antibacterial activities of tigecycline in combination with other antimicrobial agents determined by chequerboard and time-kill kinetic analysis. *Journal of Antimicrobial Chemotherapy, 57*(3), 573–576.

Sakai, A., Nakanishi, M., Yoshiyama, K., & Maki, H. (2006). Impact of reactive oxygen species on spontaneous mutagenesis in *Escherichia coli*. *Genes Cells, 11*(7), 767–778.

Shih, Y. T., Chen, P. S., Wu, C. H., Tseng, Y. T., Wu, Y. C., & Lo, Y. C. (2010). Arecoline, a major alkaloid of the areca nut, causes neurotoxicity through enhancement of oxidative stress and suppression of the antioxidant protective system. *Free Radical Biology and Medicine, 49*(10), 1471–1479.

Sun, L., Sun, S., Cheng, A., Wu, X., Zhang, Y., & Lou, H. (2009). *In vitro* activities of retigeric acid B alone and in combination with azole antifungal agents against Candida albicans. *Antimicrobial Agents and Chemotherapy, 53*(4), 1586–1591.

Tremblay, Y. D. N., Lamarche, D., Chever, P., Haine, D., Messier, S., & Jacques, M. (2013). Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. *Journal of Dairy Science, 96*(1), 234–246.

Wainwright, M., Smalley, H., Scully, O., & Lotfipour, E. (2012). Comparative photodynamic evaluation of new phenothiazinium derivatives against *Propionibacterium acnes*. *Photochemistry and Photobiology, 88*(3), 523–526.

Yuan, Y. G., Peng, Q. L., & Gurunathan, S. (2017). Effects of silver nanoparticles on multiple drug-resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from mastitis-infected goats: An alternative approach for antimicrobial therapy. *International Journal of Molecular Sciences, 18*(3). https://doi.org/10.3390/ijms18030569.

Zhou, X., Jia, F., Liu, X., & Wang, Y. (2012). Total alkaloids of *Sophorea alopecuroides*-induced down-regulation of AcrAB-TolC efflux pump reverses susceptibility to ciprofloxacin in clinical multidrug resistant *Escherichia coli* isolates. *Phytotherapy Research, 26*(11), 1637–1643. https://doi.org/10.1002/ptr.4623.

Zhou, Y., Wang, H., Liang, L., Zhao, W. C., Chen, Y., & Deng, H. Z. (2010). Total alkaloids of *Sophora alopecuroides* increases the expression of CD4+ CD25+ Tregs and IL-10 in rats with experimental colitis. *American Journal of Chinese Medicine, 38*(2), 265–277.