Antibacterial activity and mode of action of totarol against *Staphylococcus aureus* in carrot juice

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Revised: 8 August 2017 / Accepted: 18 December 2017 / Published online: 3 February 2018
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Abstract Food contaminated with pathogenic bacteria such as *Staphylococcus aureus* (*S. aureus*), represents a serious health risk to human beings. Totarol is an antibacterial novel phenolic diterpenes. In present study, the antibacterial activity of totarol against *S. aureus* was investigated in a food system. The antibacterial activity of totarol was determined by measuring the zones of inhibition and minimum inhibitory concentrations (MICs). The MICs for *S. aureus* strains were in the range of 2–4 µg/ml. The probable antibacterial mechanism of totarol was the alteration in cell membranes integrity and permeability, which leading to the leakage of cellular materials. The electric conductivity showed a time- and dose-dependent increasing manner, and we utilized totarol to induce the production of cytoplasmic β-galactosidase in *S. aureus*. Scanning electron microscopy and transmission electron microscopy analysis further confirmed that *S. aureus* cell membranes were damaged by totarol. The time-kill assay and detection of the kinetics of *S. aureus* deactivation in situ indicated that totarol has good preservative activities in a food model. Totarol successfully inhibited *S. aureus*

development in carrot juice, at room temperature (25 °C) and in refrigerator (4 °C) respectively. Our works provided not only additional evidences in support of totarol being regarded as a natural antibacterial food preservative but also fundamental understanding on the mode of antibacterial action. It is necessary to consider that totarol will become a promising antibacterial additive for food preservative.

Keyword *Staphylococcus aureus* · Totarol · Antibacterial mechanism · Food system · Food preservative

Introduction

Food-borne diseases and food poisoning, the major public health concerns provoked by food pathogens, are important causes of morbidity and mortality around the world (Balaban and Rasooly 2000; Loir et al. 2003). The food-borne diseases and food poisoning are often caused by food contamination with pathogenic bacteria such as *S. aureus*, a commensal and opportunistic foodborne pathogen. The food contamination caused by *S. aureus* resulted largely from poor sanitary habits or storage conditions (Zhao et al. 2016). *S. aureus* can grow in various food products and cause contamination due to the ability to survive in potentially dry and stressful environments (Loir et al. 2003). The continuing emergence of food-borne diseases and food poisoning caused by *S. aureus* strains threatens public health and safety, therefore, control of microorganisms in food products continues to be a worldwide problem (Krishnamoorthy et al. 2012).

Conventional food safety intervention includes the use of cold chain, high salt, high sugar, acidic environments, chemical preservatives, and other modern technologies

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(Miao et al. 2016). Chemical synthetic preservatives have been continued widely used in food industries in recent years (Tian et al. 2014). However, it has been a controversial topic due to the occurrence of respiratory disease or other health risks caused by the use of these artificial chemical compounds in controlling food spoilage and pathogenic bacteria (Fleming-Jones and Smith 2003). As a consequence, it is necessary and urgent to discover novel and safe natural antibacterial agents to extend foods shelf-life and ensure food safety.

The natural product, totarol, a phenolic diterpenoid and a major constituent isolated from the sap of *Podocarpus totara*, has been reported to have a potent antimicrobial activity (Jaiswal et al. 2007). Previous studies have shown that totarol possesses effective activity against Gram-positive bacteria and extraordinary activity against *S. aureus* and could be used to treat clinical illnesses and avoid food spoilage (Muroi and Kubo 1996; Constantine et al. 2001). And other report showed that totarol was a potent efflux pump inhibitor in *S. aureus* and could reduce biofilm formation (Smith et al. 2007). In addition, it is well-known that totarol exhibits antiplasmodial, antifungal activities and neuroprotective effects (Gao et al. 2015). Totarol is also approved for use as an antibacterial additive in several consumer products, including toothpaste and acne treatments (Kim and Shaw 2010). The antibacterial activity of totarol has been previously evaluated, however, to our knowledge, there was little data available explaining the mechanism behind the antibacterial action of totarol against *S. aureus* strains in detail. Therefore, in present study, it was aimed to explore antibacterial activity of totarol and antibacterial preserving properties of totarol were further elucidated after its incorporation in carrot juice contaminated with *S. aureus*. In addition, the antibacterial mechanism of totarol towards *S. aureus* cells was also studied by adopting the mechanistic approaches to provide fundamental understanding on the mode of antibacterial action.

Materials and methods

Chemical reagents and bacterial strains

Totarol was purchased from Sigma-Aldrich (St. Louis, USA), and stock solutions at various concentrations were dissolved in dimethyl sulfoxide (DMSO) obtained from Sigma-Aldrich (St. Louis, USA). The Mueller–Hinton (MH) broth and the Tryptic Soy Broth (TSB) were purchased from Qingdao Hope Bio-Technology Co., Ltd (Qingdao, China). Sodium dodecyl sulfate (SDS), acrylamide and O-Nitrophenyl- β -D-Galactopyranoside (ONPG) was purchased from Sigma-Aldrich (St. Louis, USA).

Coomassie Brilliant Blue R-250 was purchased from Beyotime Biotechnology (Shanghai, China). All the reagents were of the highest grade available commercially.

Staphylococcus aureus strain ATCC 29213 used in this study was obtained from the China Medical Culture Collection Center. Thirteen food-borne isolates of *S. aureus* strains were obtained from Jilin Entry-Exit Inspection and Quarantine Bureau (Table 1). All microorganisms were cultured overnight in TSB at 37 °C with constant shaking.

Antibacterial activities

Antibacterial susceptibility testing

To determine the Minimal Inhibitory Concentrations (MICs) of totarol against the above-mentioned *S. aureus* strains, antibacterial susceptibility testing was carried out according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2009). In brief, the bacteria cultured at 37 °C overnight with constant shaking were diluted with MH broth to adjust to a final concentration of 10^5 colony-forming units (CFU)/ml. Totarol was prepared in MH broth to acquire different subinhibitory concentrations (0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16 μ g/ml) by serial microdilutions. Then a 50 μ l aliquot of totarol dilutions of different subinhibitory concentrations and 50 μ l of the bacterial suspension were added into individual wells of a 96-well microtiter plate. Wells without totarol treatment served as positive growth controls. Then the plates were incubated for 16 to 24 h at 37 °C. The MIC was defined as the lowest concentration of drug that inhibited > 90% of the microorganism's growth by optical density (OD) at 600 nm using a microtiter plate reader (Oo et al. 2010).

Table 1 In vitro totarol against *S. aureus* food-borne isolates and *S. aureus* ATCC 29213

Strains	MIC (range) of totarol (μ g/ml)
<i>S. aureus</i> JL-11110	2 (2)
<i>S. aureus</i> JL-11111	2 (1–2)
<i>S. aureus</i> JL-11112	4 (2–4)
<i>S. aureus</i> JL-11113	2 (1–2)
<i>S. aureus</i> JL-11114	4 (4)
<i>S. aureus</i> JL-11115	2 (2)
<i>S. aureus</i> JL-11116	4 (2–4)
<i>S. aureus</i> JL-11117	2 (2)
<i>S. aureus</i> JL-11118	4 (2–4)
<i>S. aureus</i> JL-11119	4 (2–4)
<i>S. aureus</i> JL-11120	2 (1–2)
<i>S. aureus</i> JL-11121	2 (1–2)
<i>S. aureus</i> JL-11122	2 (1–2)
<i>S. aureus</i> ATCC 29213	2 (2)

Agar disk diffusion tests

The antibacterial activities of totarol against *S. aureus* ATCC 29213 were measured by agar disk diffusion method on the basis of previous study (Lv et al. 2011). Briefly, the bacteria cultured overnight were diluted to 10^7 CFU/ml in MH broth. 100 μ l of the bacteria suspension was spread on the MH agar plates uniformly by confluent swabbing of the surface. Sterile paper disks (6 mm in diameter) impregnated with aliquots of 10 μ l of totarol with different final concentrations (0.25, 0.5, 1 and 2 μ g/ml) were placed on the surfaces of seeded agar plates. Paper disks impregnated with PBS served as the control groups. After incubation at 37 °C for 16 h to 24 h, the diameter of the zones of inhibition (ZOI) surrounding the disks (disk diameter included) were used to evaluate the bacterial inhibitions.

Antibacterial mechanisms

Electric conductivity assay

Based on previous studies, cellular leakage from the bacteria was determined by detecting electrolyte leakage into the incubation medium using a conductivity meter (Lee et al. 1998; Shen et al. 2015). After incubating at 37 °C overnight, the bacteria was collected by centrifugation at $11,000\times g$ for 10 min and washed thrice using phosphate-buffered saline (PBS) buffer, and then the OD of the final bacterial suspension was adjusted to 0.5 at 630 nm ($OD_{630\text{ nm}} = 0.5$). Then the diluted bacterial suspension was divided into four flasks, and totarol was added to each flask with different concentrations: 0.25 μ g/ml, 0.5 μ g/ml, 1 μ g/ml, except to the control group (no totarol treatment). After incubation for 1 h, 2 h, 4 h, 6 h and 8 h at 37 °C. A 5 ml sample was removed from each flask, and then filtered by 0.22 mm syringe filters to remove the bacterial cells. The supernatant conductivity was measured using a conductivity meter, and the results were expressed as μ S/cm.

Membrane permeabilization assay

The membrane permeabilization was determined by measuring β -galactosidase release activity from *S. aureus* into the culture medium using ONPG as the substrate (Falla et al. 1996; Ibrahim et al. 2000). Logarithmic phase bacteria grown in TSB was harvested by centrifugation at $12,000\times g$ for 10 min, washed thrice using PBS buffer and then resuspended in 0.5% NaCl solution. The final cell suspension was adjusted to the test concentration ($OD_{420\text{ nm}} = 1.2$). Then different concentrations of totarol with 0.25, 0.5 and 1 μ g/ml were added to the test groups respectively, and the group without totarol treatment served as the control. After incubation for 1, 2, 4, 6 and 8 h at

37 °C, no totarol treatment served as the control group. A 10 ml of each sample was removed from each flask and centrifuged at $12,000\times g$ for 10 min at 4 °C immediately. About 1 ml of supernatant was transferred to a new flask, and 4 ml of 0.05 mol/l ONPG was added subsequently. The mixture reacted in a water bath at 37 °C for about 40 min, and terminated by adding 5 ml of 0.5 mol/l Na_2CO_3 . After 1 min, the production of o-nitrophenol over time was measured by monitoring the increase in absorbance at 420 nm using a spectrophotometer.

Detection of membrane disruption by SDS-PAGE

Bacterial cell membrane integrity was detected by determining the release of intracellular components referring to the relevant literature (Laemmli 1970) with slight changes. Logarithmic phase cells of *S. aureus* ATCC 29213 ($\sim 10^7$ CFU/ml) were treated with totarol at 1 μ g/ml, and then the cells were incubated at 37 °C with constant shaking. Aliquots of 10 ml were withdrawn at 1, 2, 4, 6 and 8 h respectively, on totarol treatment served as the control group, then the bacterial suspensions were centrifuged at $14,000\times g$ for 10 min. The pellet was subjected to SDS-PAGE analysis, which was performed with a 12% separating gel and a 4% stacking gel. Proteins bands were stained with 0.1% Coomassie Brilliant Blue R-250 and destained in destaining solution (25% of ethanol and 9% of acetic acid, v/v). The image was observed by Gel Doc XR System (Bio-Rad, USA).

Scanning electron microscopy (SEM) analysis

Morphology changes of the bacterial after treatment with totarol at different concentrations were observed using a SEM (Hitachi S-3400 N, Japan) according to previous methods (Shen et al. 2015; Wang et al. 2016). Logarithmic phase bacteria were allowed to adhere to polylysine-coated coverslips in a 24-well plate for approximately 12 h and then 1 μ g/ml totarol was added. After incubation for 1, 2, 4, 6 and 8 h respectively, no totarol treatment served as the control group. The cells were washed with PBS buffer and fixed in 2.5% glutaraldehyde at 4 °C for about 12 h. After this, the cells were dehydrated using sequential exposure ethanol concentrations ranging from 30 to 100% and the ethanol was replaced by tertiary butyl alcohol at last. The resulting samples were placed on a silicon wafer and subjected to vacuum freeze-drying (Hitachi ES-2030, Japan). Finally, the dried samples were mounted on aluminum stubs with sticky double-side conductive metal tape and gold-coated by ion sputtering (Hitachi E-1010, Japan) for about 2 min.

Transmission electron microscopy (TEM) analysis

The preparation and treatment of target indicators in TEM were the same as those in SEM analysis, and TEM analysis was performed following the guidelines in the literature with slight modifications (Li et al. 2012; Tyagi et al. 2013). The overnight bacterial cells were centrifuged at $8000 \times g$ for 10 min at 4 °C, and the pellets were washed and suspended in PBS buffer to the test concentration ($OD_{600\text{ nm}} = 0.4$). Then the cell suspensions were incubated with totarol at 1 µg/ml for 1, 2, 4, 6 and 8 h incubation respectively, no totarol treatment served as the control group. After washed by PBS buffer, the pellets were collected by centrifugating at $11,000 \times g$ for 10 min, and fixed with 2.5% glutaraldehyde overnight at 4 °C. The resulting pellets were subjected to a series of treatments according to the guidelines in the literature to perform TEM analysis.

Challenge antibacterial tests in fresh carrot juice

Time-kill curves assay

The fresh carrots used in this assay was purchased from the local market (Changchun, China), washed, peeled and squeezed to obtain carrot juice. And then the carrot juice was autoclaved at 110 °C for 10 min to inactivate the naturally existing bacterial population. The bactericidal activity of totarol against *S. aureus* ATCC 29213 was evaluated by measuring the reduction in the numbers of CFU according to previous studies (Carson et al. 2002; Ananda et al. 2009). The bactericidal kinetics of totarol were studied in TSB containing 0.25, 0.5 and 1 µg/ml of totarol with an initial bacteria inoculum of 1×10^5 CFU/ml. In brief, the samples were cultured in TSB with or without totarol treatment at 37 °C. No totarol treatment served as the control group. At each predetermined time point (0, 3, 6, 9, 12 and 24 h), a 100 µl sample taken out from each test bacterial suspension was serially diluted in 0.85% NaCl sterile saline and plated on TSB agars. A viable count was performed after incubation overnight at 37 °C and the number of CFU was recorded. Bactericidal activity (99.9% kill) was defined as a $\geq 3 \log_{10}$ CFU/ml reduction in colony count from the initial inoculum (LaPlante 2007).

In situ antibacterial test

Food preserving property was evaluated with the methods according to previous research (Stojković et al. 2013) with slight modifications and the results were expressed as percentage inhibition. The bacteria was added to the sterile carrot juice and adjusted to a final concentration of

1×10^6 CFU/ml. The samples mixed with different concentrations of totarol ranging from 0.25 to 2 µg/ml thoroughly. Experimental microplates were divided into two groups: one group was kept at room temperature (25 °C) and the other group was kept at 4 °C. Both groups contained equal amounts of totarol. The inhibition percentages of the samples at 25 °C (1) and 4 °C (2, 3) were calculated by the reduction of bacterial colonies numbers using the equations as following:

$$\% \text{inhibition} = 100 - (\text{CFU}_{\text{sample}} / \text{CFU}_{\text{blank}}) \times 100 \quad (1)$$

$$\% \text{inhibition} = 100 - (\text{CFU}_{\text{sample}} / \text{CFU}_{\text{blank}}) \times 100 - T_{\text{inhibition}} \quad (2)$$

$$T_{\text{inhibition}} = 100 - (\text{CFUT}_{\text{growth}} / \text{CFUT0}_{\text{growth}}) \times 100 \quad (3)$$

where $\text{CFU}_{\text{sample}}$ and $\text{CFU}_{\text{blank}}$ is the CFU of the antimicrobial samples and the blank sample incubation at 25 °C respectively. $\text{CFUT}_{\text{growth}}$ and $\text{CFUT0}_{\text{growth}}$ presented the growth of strains at 4 °C after and before incubation respectively.

Statistical analysis

All the experiments in the present study were conducted in triplicate, and the average values with standard deviation (SD) were revealed. Values are expressed as mean $\pm$ SD of three different experiments performed in triplicate. Error bars indicate the SD. And the statistical analyses were accomplished using PASW Statistics 18.0 software and EXCEL program.

Results and discussion

MIC values and ZOI values determination

The MIC and ZOI values of totarol against *S. aureus* strains are presented in Tables 1 and 2. Results showed that the totarol had antibacterial effects on tested strains. The MIC values for *S. aureus* food-borne isolates were in the range of 2–4 µg/ml, and the MIC value of totarol against *S.*

Table 2 ZOI values of totarol against *S. aureus* ATCC 29213

Totarol	ZOI values (mm)
Control	5.80 $\pm$ 0.20
0.25 µg/ml	10.58 $\pm$ 0.20
0.5 µg/ml	14.35 $\pm$ 0.15
1 µg/ml	19.88 $\pm$ 0.10
2 µg/ml	34.40 $\pm$ 0.20

The results are expressed as mean $\pm$ SD

aureus ATCC 29213 was 2 µg/ml. In addition, the antibacterial activity of totarol against *S. aureus* was determined by agar diffusion assay. the ZOI values of totarol against *S. aureus* ATCC 29213 was 5.80, 10.58, 14.35, 19.88 and 34.40 mm in the discs impregnated with 0.25, 0.5, 1 and 2 µg/ml totarol respectively.

According to our study, our research has determined that the MIC values of totarol against *S. aureus* strains ranged from 2 to 4 µg/ml. Previous study have shown that totarol exhibited good antibacterial activity, with an MIC of 2 µg/ml against *S. aureus* (Smith et al. 2007), and it is in accordance with our results. Furthermore, it has been demonstrated that totarol possessed low toxicity, the half maximal inhibitory concentration (IC₅₀) of totarol was 7.5 µg/ml (Gordien et al. 2009). The concentrations of totarol used in this study were lower than the IC₅₀ of totarol.

Antibacterial mechanisms

Electrical conductivity of cell suspensions for totarol-treated S. aureus

The electrical conductivity was applied to indicate the membrane permeability of bacteria in order to illustrate the mechanism of antibacterial mechanism. In this assay, we determined electric conductivity of the cell suspensions for *S. aureus* treated with different concentrations of totarol at different time points. As shown in Fig. 1a, the electric conductivity showed a time- and dose-dependent increasing manner. When the bacteria exposed to totarol with 0.25 µg/ml, the electric conductivity of suspensions for *S. aureus* increased sharply at 1 h (21.7 µs/cm), and when the incubation time was 2, 4 and 6 h, the electric conductivity increased to 21.7, 22.7 and 23.6 µs/cm which increased by 59.6, 66.9 and 73.5% respectively compared with the controls. And after incubation for 8 h, the electric conductivity of suspensions increased to 24.3 µs/cm, which increased by almost 2-fold compared with the control. When the bacteria treated with totarol with 0.5 µg/ml at different time points, the electric conductivity of suspensions rose to 22.3, 23.1, 25.9, 28.1 and 29.3 µs/cm at 1, 2, 4, 6 and 8 h which increased by 63.97, 69.85, 90.44, 106.61 and 115.44% respectively compared with the controls. In addition, samples from *S. aureus* exposed to totarol with 1 µg/ml showed higher conductivity values than that of all the other groups and displayed a rapid rise trend. The conductivity values of *S. aureus* samples treated with totarol with 1 µg/ml at 1, 2, 4, 6 and 8 h was 22.7, 24.4, 28.3, 29.8 and 30.9 µs/cm, which increased by 66.91, 79.41, 108.09, 119.12 and 127.21% respectively. These data indicated that varying degrees of increase for conductivity could be observed in the bacterial suspension

Fig. 1 Electric conductivity of cell suspensions for totarol-treated *S. aureus* at different concentrations and time points (a). Release of cytoplasmic β-galactosidase activity (measured by absorbance at 420 nm) of *S. aureus* cells treated with totarol at different concentrations and time points. Values are expressed as mean of three different experiments performed in triplicate (b). SDS-PAGE patterns of cellular soluble proteins from totarol-treated *S. aureus* at different time points (c). Values are expressed as mean ± SD of three different experiments performed in triplicate. Error bars indicate the SD

after totarol treatment, and totarol had various degrees of effects on the integrities of the bacterial cell membranes. With the increase of totarol concentrations, damage also increased gradually. Compared to the controls, the electric conductivity values of the suspensions increased immediately after the addition of totarol at different concentrations and it also increased rapidly with the increasing treatment time and concentrations of totarol.

The bacterial plasma membrane provides a permeability barrier to the passage of small ions such as K^+ and Na^+ which are necessary electrolytes, facilitate cell membrane functions and maintain proper enzyme activity. When the bacterial cells meet biocidal challenges, such as exposure to antimicrobial agents, the cytoplasmic cell membrane may become compromised, damaged and functionally invalid, and the small ions tend to leach out first, and then followed by large molecules such as nucleic acids, proteins and other materials (Chen and Cooper 2002). Increases in the leakage of electrolytes will indicate a disruption of this permeability barrier. In addition, maintaining ion homeostasis is integral to the maintenance of energy status, solute transport, metabolic regulation, control of turgor pressure and motility of cell (Cox et al. 2001). Therefore, even relatively minor variations to the structure of membranes can hurtfully affect cell metabolism and result in bacteria death (Sharma et al. 2013). The results in this assay indicated that the electric conductivity of the suspension increased rapidly with the increasing treatment time and concentration of totarol, which demonstrated that the permeability of bacteria membrane would be increased correspondingly, causing the leakage of electrolytes and leading to cell death.

Release of cytoplasmic β -galactosidase from *S. aureus* treated with totarol

In the present study, we utilized totarol to induce the production of cytoplasmic β -galactosidase in *S. aureus*. After adding totarol to the culture medium, the enzymatic activity of β -galactosidase was measured. Totarol-mediated *S. aureus* membrane permeability was presented in Fig. 1b, the β -galactosidase activity (OD_{420nm}) detected after totarol treatment kept increasing during the test time period (up to 8 h). In the control group, the values of the β -galactosidase activity stabilized at a relatively steady and lower state. However, after totarol treatment, the values of the β -galactosidase activity in the three test groups from *S. aureus* increased dramatically not only in a dose-dependent manner but also in a time-dependent manner compared with the control group. The β -galactosidase activity of *S. aureus* treated with totarol with 0.25 μ g/ml at different time points was 0.093, 0.258, 0.287, 0.363, 0.408 and 0.381 respectively. And those treated with 0.5 μ g/ml of totarol

was 0.108, 0.332, 0.301, 0.416, 0.374 and 0.446 respectively. When treated with 1 μ g/ml of totarol, the β -galactosidase activity was 0.088, 0.347, 0.385, 0.439, 0.519 and 0.543 respectively. These results suggested that totarol increased the cell membrane permeability of *S. aureus*, which led to the leakage of cytoplasmic content to the culture medium.

The cell membrane is a biological membrane that separates the cell interior from the outside environment (Singleton 1999). The layer of bacteria consists of phosphatidyl glycerol and cardiolipin as well as embedded proteins (Je and Kim 2006). β -Galactosidase, a normally endoenzyme, could permeate the cytoplasmic membrane. It has demonstrated that the presence of lactose and galactose in the culture medium could induce *S. aureus* cells to produce β -galactosidase in the cytoplasm (Creaser 1955). Generally, cytoplasmic β -galactosidase can not pass through the integral cell membrane of bacteria. However, if the cell membrane is damaged, cytoplasmic β -galactosidase can be detected extracellularly due to its leakage through damaged cell membrane. Therefore, the cytoplasmic β -galactosidase is released as a consequence of change in membrane permeability. In addition, whether totarol permeabilizes the *S. aureus* membranes could be determined by detecting cytoplasmic β -galactosidase in the bacterial suspension.

SDS-PAGE patterns of cellular soluble proteins from totarol-treated *S. aureus*

To further determine the effects of totarol on the integrity of cell membrane of *S. aureus* cells, the amount of cellular soluble proteins released by *S. aureus* cells were measured. The cell protein electrophoresis bands of *S. aureus* were shown in Fig. 1c. The cell protein electrophoresis bands of non-treated *S. aureus* appeared strong and clear. When *S. aureus* was treated with totarol for different time points, the protein contents decreased to lower levels compared to the control group. Furthermore, the extracellular levels of cellular soluble proteins released by *S. aureus* cells treated with totarol kept decreasing during the test time period (up to 8 h). Furthermore, after treatment with totarol for 6 h and 8 h, bands of all molecular weight proteins for *S. aureus* appeared obviously shallow, even almost disappearing altogether. These results indicated that totarol enable to cause irreversible damage to the cytoplasmic membranes, and decrease the content of cellular soluble proteins by permeating and disrupting cell membranes, which led to cell death.

Proteins, the macromolecules of microbial cells, which constitute key structural components, were released from *S. aureus* after exposed to totarol. The measurements of specific leakage markers, proteins, is an indicator of bacterial cell membrane integrity in comparison to unexposed cells (Bajpai

et al. 2013). The results from this study indicated rapid loss of proteins from treated bacteria, due to irreversible damage to the cytoplasmic membranes. Further research is needed to find the target damage sites on bacteria cells to make sure that either antimicrobial effect was from damage to lipopolysaccharide or membrane proteins in cell wall.

SEM observation

Physical and morphological changes may encounter the cell wall surface deterioration of *S. aureus* when treated with a suitable antibacterial agent. Therefore, a SEM analysis was performed to further visualize the effect of totarol on the morphology of *S. aureus* ATCC 29213 cells as compared to control group. The *S. aureus* bacteria exposed to totarol at 1 µg/ml for 1, 2, 4, 6 and 8 h respectively. The SEM images of the untreated and treated bacteria were showed in Fig. 2a. These images illustrated the destructive effects of totarol on *S. aureus* directly. The surfaces of the treated *S. aureus* cells underwent obvious morphological changes compared with the untreated *S. aureus* cells. Untreated *S. aureus* cells, showing the distinctive characteristics of ball-shaped, intact, regular and smooth cell membrane. In contrast, when the bacterial cells were treated with totarol at different test time period, the cells became deformed, wrinkled, shriveled, irregular, pitted, adhesive to each other and some swelling cells were even broken, which may give rise to the leaching out of nutrient and genetic materials. In conclusion, larger populations of sunken and malformed cells were observed in the microphotograph of *S. aureus*. Furthermore, the increase of treatment time of totarol, the changes were more frequent. This observation was in agreement with the results of the SDS-PAGE assay, and demonstrated that totarol may have severe effects on the cell wall and cytoplasmic membrane.

The SEM micrograph of *S. aureus* cells treated with totarol showed that severe morphological alterations appeared in the cell wall and membrane, and the SEM micrograph of treated cells showed many fragmentary bacteria. The changes in physical and morphological of bacterial cells might have occurred due to the effect of essential oil on the permeability and integrity of membrane, thereby resulting in the lysis of bacterial cell wall followed by the losses of intracellular dense materials on the surface of treated cells. However, a more detail cell wall changes still need to be further observed by TEM.

TEM observation

Previous study has reported that antibacterial agents are able to interact with bacteria cell membranes directly, and then increase the membrane permeability, cause rapid cell death (Li et al. 2012). In addition, electron microscopy observations

are powerful tools to better understand the impact of a stressor on bacterial cells (Helander et al. 2001). Therefore, to further characterize the bactericidal effects of totarol, a TEM analysis was used to visualize the morphological changes of *S. aureus* cells exposed to totarol at 1 µg/ml for different test time period. As shown in Fig. 2b, without totarol treatment, the normal *S. aureus* cells were surrounded by the cell membranes with compact surface, showing a well-defined cell membrane and a uniform cytoplasm region, and without release of intracellular components. However, as compared to the untreated *S. aureus* cells, totarol-exposed bacterial cells exhibited a wide range of remarkable modifications of bacterial cell membranes after a period of incubation. After *S. aureus* cells exposed to totarol for 1 h and 2 h, the cells gradually lose the clear boundary of cell membrane. Moreover, totarol treatment significantly disturbed uniformity of the cytoplasm region, and caused some slight leakages of cellular cytoplasmic contents. After 4, 6 and 8 h of treatment with totarol, the *S. aureus* cells were lysed and lost their cellular morphology even the cytoplasmic membranes of totarol-treated cells completely collapsed. The results indicated that totarol caused significant damages to the cellular structures including cell membrane and cytoplasm region, which eventually led to bacterial cells rapid lyses and death. There are many reports showed the antibacterial mechanism of increasing membrane permeability and changing cell morphology. Yan et al. (2011) reported that treatments with chitosan increased membrane permeability and changed cell morphology of *Pseudomonas aeruginosa* (*P. aeruginosa*) and *S. aureus*, and the effect of chitosan on membrane permeability of *S. aureus* was more obvious than on that of *P. aeruginosa*, which was partly consistent with the report of our present study.

These morphological alterations in bacterial cells observed by TEM observation might have occurred due to the effect of totarol on membrane integrity, thereby resulting in the lysis of bacterial cell wall followed by the loss of intracellular dense material on the surface of treated cells. Changes in membrane fluidity usually occur due to alterations in membrane lipid composition and are thought to be a compensatory mechanism to counter the lipid disordering effects of the treatment agent (Sikkema et al. 1995). Our studies suggested that totarol might bind to the cell surface and then penetrate to the target sites possibly the plasma membrane and membrane-bound enzymes, resulting in the disruption of cell wall structure (Bajpai et al. 2009).

Challenge antibacterial tests in fresh carrot juice

Effect of totarol on the rate of kill of S. aureus

In vitro time-kill curves test is one of the most commonly used experimental models to evaluate antibacterial activity.

Fig. 2 SEM (a) and TEM (b) of *S. aureus* ATCC 29213 exposed to totarol at 1 $\mu\text{g}/\text{ml}$ treatment with different time points. (a) Control, (b) treatment with totarol for 1 h, (c) treatment with totarol 2 h, (d) treatment with totarol 4 h, (e) treatment with totarol 6 h, (f) treatment with totarol 8 h

Fig. 3 Time-kill curves of totarol treatment with different concentrations against *S. aureus* ATCC 29213. Values are expressed as mean $\pm$ SD of three different experiments performed in triplicate. Error bars indicate the SD

This method could efficiently characterize the rate, extent, and timing of bacterial killing and regrowth (Cheah et al. 2015). In this assay, the bactericidal activity of totarol against the *S. aureus* ATCC 29213 was measured by time-kill curves, which determines how quickly an antibacterial agent acts on an organism. As shown in Fig. 3, the bactericidal effect of different concentrations of totarol reported as the $\log_{10}$ reduction in viable bacterial cells compared to the initial inoculum. Treatment with totarol at 0.25 $\mu\text{g/ml}$ did not result in reduced viability. And the bacteria exposed to totarol at 0.5 $\mu\text{g/ml}$ resulted in a 3.22 $\log_{10}$ reduction over 24 h. While treatment with totarol at 1 $\mu\text{g/ml}$ reduced the viability of *S. aureus* by 5.31 $\log_{10}$ over 24 h. These time-kill results suggested that totarol at a concentration of 1 $\mu\text{g/ml}$ has bactericidal activity. At each time point, totarol at 0.25 $\mu\text{g/ml}$ against *S. aureus* ATCC 29213 indicated no reduction in the number of viable cells, followed by 0.5 $\mu\text{g/ml}$, which demonstrated a greater

reduction, and at 1 $\mu\text{g/ml}$, which indicated the greatest reduction in the number of viable cells. In this assay, the experimental results demonstrated that totarol inhibited bacteria growth in a dose-dependent manner in food system.

In situ antibacterial activity

Regarding the receivable antibacterial activity of totarol against *S. aureus* ATCC 29213, the antibacterial activity of totarol against *S. aureus* ATCC 29213 was tested under 4 and 25 $^{\circ}\text{C}$ in carrot juice (Table 3). The antibacterial activity was better under refrigerated conditions, 4 $^{\circ}\text{C}$. For all the concentrations at temperature of 4 $^{\circ}\text{C}$ during period of storage, the inhibition percentage was stabilized relatively. At 0.25 $\mu\text{g/ml}$ of totarol, the percentages of inhibition of *S. aureus* was in the range of 80.8–92.5%. At 0.5 $\mu\text{g/ml}$ of totarol, the percentages of inhibition of *S. aureus* was in the range of 92.8–94.5%. With the higher tested concentrations (1 and 2 $\mu\text{g/ml}$), it was achieved 100% inhibition in 12 h in spite of the incubation temperature. The effect was also dose dependent, increasing at higher doses with period of storage. However, compared with refrigerated conditions (4 $^{\circ}\text{C}$), at room temperature (25 $^{\circ}\text{C}$) with the lowest tested concentrations (0.25 $\mu\text{g/ml}$), 12.6% inhibition was achieved in 12 h, and no inhibition was found at 24 h. With the concentration of totarol increasing, the inhibition percentage had an ascending tendency. It indicated that the effect was also in a dose-dependent manner, decreasing at lower doses with period of storage. It is a sustainable way to get over the use of antibiotics contributes which increased antibiotic resistance and the subsequent transmission of these resistances to the environment. Overall, the activity of totarol provided with a strong evidence that it is an efficient and safe way to control the growth of *S. aureus*.

Table 3 The antibacterial activity of totarol in carrot juice against *S. aureus* ATCC 29213

Concentrations of totarol ($\mu\text{g/ml}$)	Temperature ($^{\circ}\text{C}$)	Percentage of inhibition (%) of <i>S. aureus</i> ATCC 29213				
		0 h	12 h	24 h	36 h	48 h
0.25	25	0 $\pm$ 0.00	12.6 $\pm$ 0.80	0 $\pm$ 0.00	0 $\pm$ 0.00	0 $\pm$ 0.00
	4	0 $\pm$ 0.00	92.5 $\pm$ 0.50	89.5 $\pm$ 0.50	84.5 $\pm$ 0.50	80.8 $\pm$ 0.40
0.5	25	0 $\pm$ 0.00	76.7 $\pm$ 0.80	72.5 $\pm$ 0.50	66.9 $\pm$ 0.60	59.8 $\pm$ 0.80
	4	0 $\pm$ 0.00	94.5 $\pm$ 0.50	93.9 $\pm$ 0.20	93.2 $\pm$ 0.80	92.8 $\pm$ 0.50
1	25	0 $\pm$ 0.00	92.5 $\pm$ 0.50	90.4 $\pm$ 0.80	90.5 $\pm$ 0.50	90.6 $\pm$ 0.80
	4	0 $\pm$ 0.00	100 $\pm$ 0.20	100 $\pm$ 0.20	100 $\pm$ 0.20	100 $\pm$ 0.20
2	25	0 $\pm$ 0.00	100 $\pm$ 0.20	100 $\pm$ 0.10	100 $\pm$ 0.10	100 $\pm$ 0.00
	4	0 $\pm$ 0.00	100 $\pm$ 0.00	100 $\pm$ 0.00	100 $\pm$ 0.00	100 $\pm$ 0.00

Values represent means of three independent replicates $\pm$ SD

Conclusion

Overall, totarol showed to be effective against *S. aureus* strains using in vitro model. Furthermore, totarol successfully preserved carrot juice by inhibiting *S. aureus* growth during the product storage period. The probable major mechanism of action of totarol towards *S. aureus* appears to be the disruption of the cytoplasmic membrane, which changed its integrity and permeability. According to our results, we may presume that totarol is a promising antibacterial food preservative, which may be useful for preservation and/or extension of the shelf life of vegetable juice.

Acknowledgments Financial support for this work came from the following sources: the National Nature Science Foundation of China (Nos. 31271951 and 81573448), China Postdoctoral Science Foundation (2013M530142), the Program for New Century Excellent Talents in University (NCET-13-0245) and Natural Science Foundation of Jilin Province (20150101009JC).

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