Antioxidant and Antibacterial Activities of *Pterocarya stenoptera* Bark Extract and its Mechanism on *Staphylococcus aureus* Through Cell Membrane Damage

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Pterocarya stenoptera bark extract (PSBE) was assessed for its chemical constituents, antioxidant activity, and antibacterial activity. The total phenolic content (TPC) in the PSBE was 272.92 ± 3.22 mg gallic acid equivalent (GAE)/g. Three phenolic compounds (quercetin-3-O-(2″-O-galloyl)-rhamnoside, quercetin, and juglone) were identified in the PSBE *via* ultraperformance liquid chromatography/mass spectroscopy (UPLC/MS) analysis. The PSBE possessed remarkable antioxidant activity in the DPPH and $ABTS^+$ radicals scavenging system, with IC_{50} values of 96.25 ± 3.82 μg/mL and 158.26 ± 6.08 μg/mL, respectively. Moreover, the PSBE had high antibacterial activity, which was especially sensitive to *Staphylococcus aureus.* The antibacterial mechanism of the PSBE on the *S. aureus* was related to the deformation of the cell morphology and the destruction of the cell membrane structure, which was confirmed using a BacLight Viability Kit and scanning electron microscope (SEM) observations. These findings suggest that the strong antioxidant and antibacterial properties of the bark extract from *P. stenoptera* make the tree species suitable as a natural additive in the pharmaceutical, food, and cosmetic industries.

Keywords: Pterocarya stenoptera; Antioxidant activity; Antibacterial activity; Cell membrane damage

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INTRODUCTION

Pterocarya stenoptera C. DC. is a native deciduous tree of the maple genus in the Juglans family (Trouern-Trend *et al.* 2020). It is primarily found in the forests along streams, river beaches, and wet mountain slopes below 1500 meters above sea level in China and the Korean Peninsula. In the past 100 years, *P. stenoptera* has been a main source of wood. In addition, the bark of *P. stenoptera* is rich in tannins, which can be used as a raw material to extract tannin compounds (Xu *et al.* 2020). As an important urban landscaping tree, *P. stenoptera* is abundant. However, there is still minimal research of this species, so it may be an underutilized resource.

The World Health Organization (WHO) describes antibiotic resistance as "one of the biggest threats to global health, food security and development today" as the number of multi- and pan-resistant bacteria is dangerously rising (Rigali *et al.* 2018). On the one hand, the globalization of food supply affects the outbreak of foodborne diseases worldwide, and consumers pay more and more attention to the safety of food (Gomes *et*

al. 2013). To control foodborne pathogens, various preservation techniques such as heating, refrigeration, and the addition of chemical preservatives are commonplace in the food industry (Jans *et al.* 2016; Sridhar *et al.* 2020). However, as the demand for food without synthetic preservatives increases, food additives with natural, efficient, non-toxic, and broad antibacterial spectrum characteristics have gained increasing attention (Ju *et al.* 2020). On the other hand, due to the ability that bacteria possess of being able to develop antibiotic resistance by mutation, hospital-related infections, especially in immuno-compromised and cancer patients, are increasingly spreading without appropriate antibiotic treatment (Salesa *et al.* 2019). Therefore, there is a pressing need to look for new antibiotics to replace traditional drugs. Moreover, on this basis, the study of antibacterial mechanism can make the mechanism of drug action clearer and make the use of drugs more targeted.

Screening effective bioactive components from plants, and then using them as the lead compound to synthesize antibacterial drugs with stronger efficacy and less side effects, has always been a hot field in the research and development of antibacterial agents. Based on this, the authors were interested in whether the rich resources of *P. stenoptera* has antibacterial development value. In recent years, the biological activities of the extracts from *P. stenoptera* have been preliminarily studied. It has been reported that the essential oil of *P. stenoptera* has a strong inhibitory effect on *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella* typhimurium, *Vibrio parahaemolyticus*, and *Candida albicans* (Yin *et al.* 2020). In addition, studies show that *P. stenoptera* bark extract has excellent antiviral effects (Cheng *et al.* 2004). However, due to limited research, there is no clear report on the antibacterial mechanism of *P. stenoptera* bark extract. Therefore, the main purpose of this study was to investigate the chemical constituents, antioxidant activity, and antibacterial activity of *P. stenoptera* bark extract (PSBE) and explore its inhibitory mechanism on *S. aureus* bacteria.

EXPERIMENTAL

Plant Material and Extract Preparation

The *P. stenoptera* bark sample (Lot: 20180930) was collected from the bank of the Qiantang River (30° 20’ 50’’ N, 120° 15’ 21’’ E) in Southeast China at an altitude of 68 m to 75 m. The *P. stenoptera* bark was identified by the associate professor Chen Kongrong of Zhejiang Chinese Medical University. The extraction was performed according to the methods outlined by Othman *et al.* (2011) with some modifications. The plant powder was smashed through a forty-mesh sieve and mixed with solvent (ethanol: water, 9:1, v/v) at a 1:10 (w/v) ratio at 60 °C for 3 h. The solids were separated from the extractant using filter paper, and the residues were re-extracted with fresh solvent twice more with the previously described methods. The combined extractant was dried using a rotary evaporator and treated with a VirTis AdVantage 2.0 vacuum freeze dryer (SP Scientific, Gardiner, NY, USA) to obtain the PSBE powder.

Total Phenolics Content (TPC)

The total phenolics content (TPC) was determined by the Folin-Ciocalteu (FC) method. The PSBE was dissolved in dimethyl sulfoxide (DMSO) (neoFroxx, Einhausen, Germany) and mixed with deionized water and the FC phenol reagent. After 5 min of incubation in the dark, 1 mL of 0.7 M sodium carbonate solution was added, and the

solution was quickly diluted to the scale with deionized water. The mixture was incubated in the dark for 1 h at room temperature.

The absorbance of the mixture was measured at 760 nm using an ultraviolet-visible (UV-VIS) 3600 spectrophotometer (Shimadzu, Tokyo, Japan). The TPC of the PSBE was expressed as the milligram gallic acid equivalents per gram of dry extract (mg GAE/g). The appropriate blank (deionized water) and solvent control (DMSO + reagents) were included in each trial.

Ultraperformance Liquid Chromatography/Mass Spectroscopy (UPLC/MS) Analysis

The PSBE was dissolved with methanol and centrifuged to obtain the supernatant. The sample was analyzed by a UPLC SYNAPT G2-S mass spectrometer (Milford, MA, USA). For the chromatography analysis, a Waters ACQUITY UPLC BEH C_{18} column (100 mm × 2.1 mm, 1.6 μm) was used at 30 °C. The mobile phase consisted of 0.1% formic acid aqueous solution (A) and 100% acetonitrile (B). The gradient elution program was as follows: 0 to 2 min, 10% B; 2 to 26 min, 10% to 90% B; 26 to 28 min, 90% B; 28 to 28.1 min, 90% to 10% B; 28.1 to 30 min, 10% B. The injected sample volume flow rate was 0.3 mL/min.

The MS was conducted in the MS^E continuum mode with a scan range of *m*/*z* 50 to 1500. In the negative ion mode, the capillary voltage and the taper voltage were 3.0 kV and 40 V, and the taper hole flow rate was 50 L/h at 120 °C. When the temperature was 400 °C, the dissolvent flow was 800 L/h. The measurement of the mass was based on 1 ng/μL leucine-enkephalin (*m*/*z* 554.2615) solution as the calibration standard solution.

Determination of the Antioxidant Activity

The capacity of the PSBE to remove the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical and the 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^+$) radical was determined at 517 nm or 734 nm using the UV-VIS 3600 spectrophotometer according to the methods described by Zhao *et al.* (2020). Standard ascorbic acid was used for comparison.

Determination of the Antibacterial Ability

The minimum inhibitory concentration (MIC) of the PSBE was determined based on the methods described by Othman *et al.* (2011). Briefly, two-fold serial dilution of the PSBE sample was performed with sterile Luria-Bertani (LB), and 100 μL of diluted PSBE sample (0.03 mg/mL to 1 mg/mL final concentrations) was mixed with 100 μL of diluted microbial culture (10^5 CFU/mL final inoculum). In this study, *Escherichia coli*, *S. aureus*, *Salmonella*, and *B. subtilis* were used in the determination of the antibacterial activity *in vitro*. The *E. coli*, *S. aureus*, *Salmonella*, and *B. subtilis* were kindly provided by the Food Microbiology Laboratory of the Zhejiang Chinese Medical University of China. Ampicillin (Amp) and gentamicin (Gen) were used as the positive control samples. The highest DMSO concentration was 2.5%, which has been shown to have no influence on the microbial growth of the tested microorganisms (data has not shown). The 96-well plate was incubated in a 37 °C microbial incubator for 24 h. The absorbance was measured and compared before and after the culture, and the lowest concentration of the extract that corresponded to the well without an obvious increase in the absorbance (increase in absorbance < 0.1) was MIC.

Scanning Electron Microscope (SEM) Investigations of the Cell Membrane Damage by PSBE

The *S. aureus* cells (1×10^9 CFU/mL) were exposed to PSBE for 24 h at concentrations of 2×MIC. The cells were washed with phosphate-buffered saline (PBS) (pH 7.2), fixed with 2.5% glutaraldehyde, dehydrated with ethanol, dried by CO_2, and coated with gold. The ultrastructure of the indicators was observed with a high-resolution SU-8010N scanning electron microscope (SEM) (Hitachi, Tokyo, Japan).

Fluorescence Microscopy Analysis of Cell Membrane Damage by PSBE

The membrane damage of the *S. aureus* by the PSBE was examined with a Ti-S fluorescence microscopy (Nikon, Tokyo, Japan). The bacteria were prepared in 0.85% saline at 10^9 CFU/mL inoculum and treated with the PSBE at MIC and 4×MIC for 30 min and 24 h. Live (in 0.85% saline), and dead (70% ethanol, 10 min) cells were included as the controls. The treated cells were centrifuged, washed, and stained with the LIVE/DEAD® BacLight Viability Kit (ThermoFisher Scientific, USA) which consisted of SYTO9 dye and PI dye. It is a popular fluorescence-based method for evaluating bacterial cell viability and membrane damage. The SYTO9 can penetrate the whole cell membrane and react with nucleic acid to show a strong green fluorescence. The PI can enter only in the case of a damaged cell membrane and replace the SYTO9 in nucleic acid, emitting a red fluorescence signal.

Briefly, the same amount of SYTO9 dye was mixed with PI dye, and 3 μL of mixed dye was added to 1 mL of the treated bacterial solution. The mixtures were incubated at room temperature for 15 min in the dark. After rinsing with 0.85% saline, the stained microbial samples were placed on microscope slides and observed using fluorescence microscope immediately.

Statistical Analysis

All the experiments were carried out in independent triplicate with duplicate sampling (n=3). The data were expressed as the mean ± the standard deviation (SD). Significant differences ($p < 0.05$) among the mean values were determined by one-way analysis of variance (ANOVA) followed by a t-test, using the SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Extraction Yield and the TPC of the PSBE

Phenolics have been shown to have antioxidant, antibacterial, antitumor, anti-inflammatory, hypoglycemic, and other effects (Ryu and Koh 2018; Ferhi *et al.* 2019). Due to the sustainable nature and broad spectrum of plant phenolics, increased research has been devoted to them. Therefore, the TPC of the PSBE was determined in this experiment. The data showed that the extraction yield of the PSBE was 12.86% ± 1.43%, and the TPC of the PSBE was 272.92 ± 3.22 mg GAE/g PSBE.

Though the TPC in *P. stenoptera* was not reported, there have been similar studies in other plants of the Juglans family. For example, the TPC of walnut kernel septum membranes was 122.78 ± 2.55 mg GAE/g (Li *et al.* 2019), and the TPC of *Cyclocarya paliurus* (Batal) Iljinskaja leaves was 20.80 to 52.69 mg GAE/g (Zhou *et al.* 2019). In this study, the TPC of *P. stenoptera* was lower than that of the above plants, which may be due

to the different sources of the extracts. For example, Cabreúva (*Myrocarpus frondosus* Allemao, Fabaceae) bark extract has been reported to have antioxidant and anti-inflammatory effects, but its TPC is only 158.77 ± 8.6 mg GAE/g of extract (Bottamedi *et al.* 2020), which is lower than that of the PSBE reported in this study. These findings suggest that PSBE may have interesting biological activities to be developed as a natural product.

UPLC-MS/MS Analysis

To further understand the chemical constituents of the PSBE, the main compounds in the PSBE were analyzed *via* UPLC/MS. As shown in Table 1 and Fig. 1, it was found that the main active compounds in PSBE were quercetin-3-O-(2″-O-galloyl)-rhamnoside, quercetin, and juglone.

Table 1. The Chemical Constituents of PSBE

No.	Component	Molecular Formula	Molecular Weight (g/mol)	t_r (min)
1	Quercetin-3-O-(2″-O-galloyl)-rhamnoside	$C_{21}H_{20}O_{12}$	464.10	6.40
2	Quercetin	$C_{15}H_{10}O_7$	302.23	7.33
3	Juglone	$C_{10}H_6O_3$	174.15	12.52

Note: t_r stands for retention time

Previous studies have identified 39 compounds from the essential oil of *P. stenoptera* by researchers (Yin *et al.* 2020). However, there is still little research on the non-volatile chemical composition of *P. stenoptera*. By comparing the plants of different genera in the same family, the chemical constituents identified in PSBE were also identified in *Juglans regia* L. extract (Li *et al.* 2019) and *C. paliurus* extract (Yang *et al.* 2019). This study only analyzed and identified three compounds in PSBE. However, the chemical components in *P. stenoptera* are complex, which also requires further exploration in the future.

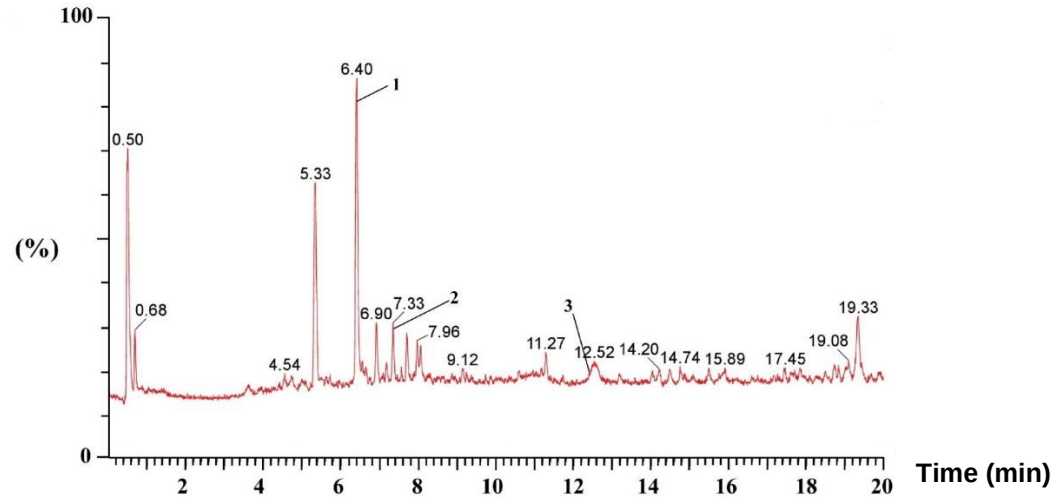

Fig. 1. The total ion chromatogram (TIC) of PSBE. (1) Quercetin-3-O-(2″-O-galloyl)-rhamnoside; (2) Quercetin; (3) Juglone

Antioxidant Activity of the PSBE

Plant phenolics have been shown to possess strong antioxidant capacity, the ability to improve physical discomfort, and health benefits (Cheng *et al.* 2020). This study used DPPH and $ABTS^+$ radical systems to explore the antioxidant activity of PSBE. As shown in Fig. 2, the PSBE showed strong DPPH radical and $ABTS^+$ radical scavenging activity with half-maximal inhibitory concentration (IC_{50}) values of 96.25 ± 3.82 μg/mL and 158.26 ± 6.08 μg/mL, respectively.

Plant extracts with antioxidant activity have been widely used in the pharmaceutical, food, and cosmetic industries. For example, potato peel extract, which is rich in phenolic substances and has good antioxidant properties, can form active film with potato starch and be used as a substitute for active food packaging (Gebrechristos *et al.* 2020). This study demonstrated that PSBE has a certain antioxidant capacity and can be developed to a novel food antioxidant.

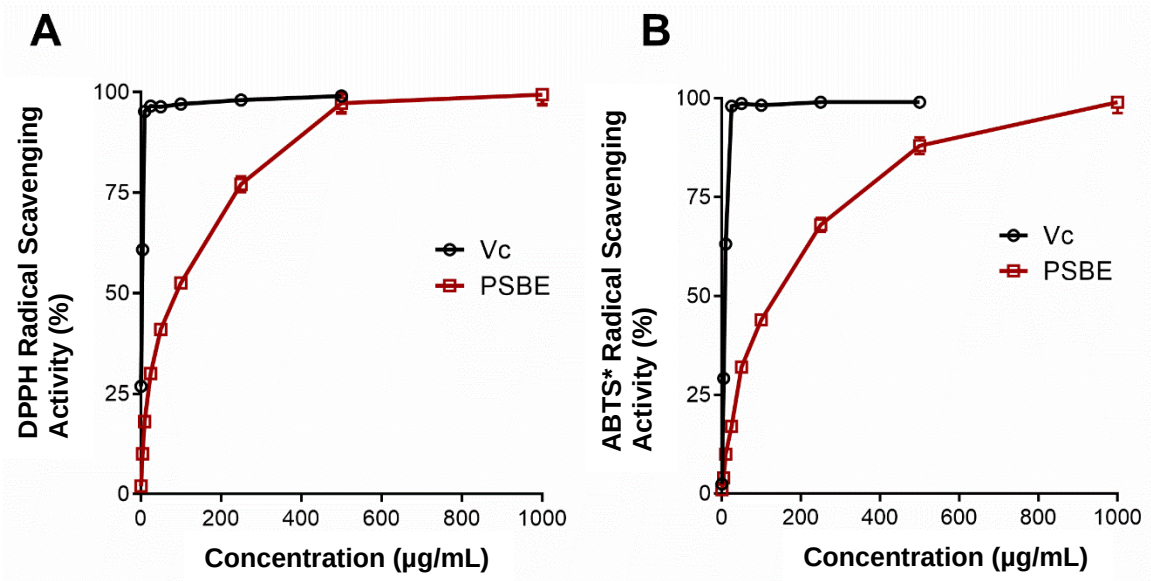

Fig. 2. a) DPPH radical scavenging activity and the b) $ABTS^+$ radical scavenging activity of PSBE. The data are expressed as means ± SD (n = 3)

Antimicrobial Activity of the PSBE

As potential natural antibacterial agents, phenolic compounds have always been a popular research topic. Through the determination of the TPC, this work confirmed that PSBE is rich in phenolics. Additionally, the mass spectrum results also showed the existence of quercetin-3-O-(2″-O-galloyl)-rhamnoside and quercetin, which have been shown to possess strong antibacterial activity (Adnan *et al.* 2019; Zhang *et al.* 2020a). Moreover, studies have shown that juglone identified from PSBE has significant antibacterial activity (Nitulescu *et al.* 2019). Based on this research, it was assumed that PSBE had antibacterial activity. In order to prove this hypothesis, this work determined the MIC of the PSBE. As shown in Table 2, the MIC of PSBE for *Salmonella* and *B. subtilis* was determined to be 125 μg/mL. Compared with other pathogens, the bacteriostatic effect of PSBE for *E. coli* was the weakest, with an MIC of 250 μg/mL. The *S. aureus* was the most effective, with an MIC of 31.25 μg/mL.

Table 2. MIC of the PSBE and the Positive Control

Bacteria Type	PSBE	Amp	Gen
E. coli	250.0	10.0	< 2.0
S. aureus	31.25	< 2.0	
Salmonella	125.0	15.625	
B. subtilis	125.0	2.50	

Note: The MIC was determined using the micro-dilution method.

S. aureus is a common pathogen that can cause food poisoning, so it is a worldwide contributor to illnesses such as intestinal infections and food-borne poisoning. Therefore, the ability to better inhibit *S. aureus* has been a topic of concern. In this study, it was confirmed that PSBE can inhibit the growth of *S. aureus* to a better extent than the previously reported *Newtonia buchananii* leaf extract. The MIC of *N. buchananii* leaf extract is 62.5 μg/mL, at which level it is considered as a potential antibacterial agent (Motlhatlego *et al.* 2020). This further suggests that PSBE can be used as a natural antibacterial agent with great practical value.

Ultrastructure Observation of *S. aureus* by SEM Analysis

The *S. aureus* was observed with the SEM, which presented a three-dimensional effect so that the morphological changes of the cell wall and membrane could be better observed (Lenardon *et al.* 2020). As shown in Fig. 3, the cell wall and membrane of the *S. aureus* in the control group was smooth, with no obvious adhesion between the cells. After the PSBE treatment, there was adhesion between the bacterial cells and the cell wall and membrane were damaged and sunken, which caused the cell content to flow out. The results showed that the structure of the *S. aureus* cell wall and membrane was changed by PSBE.

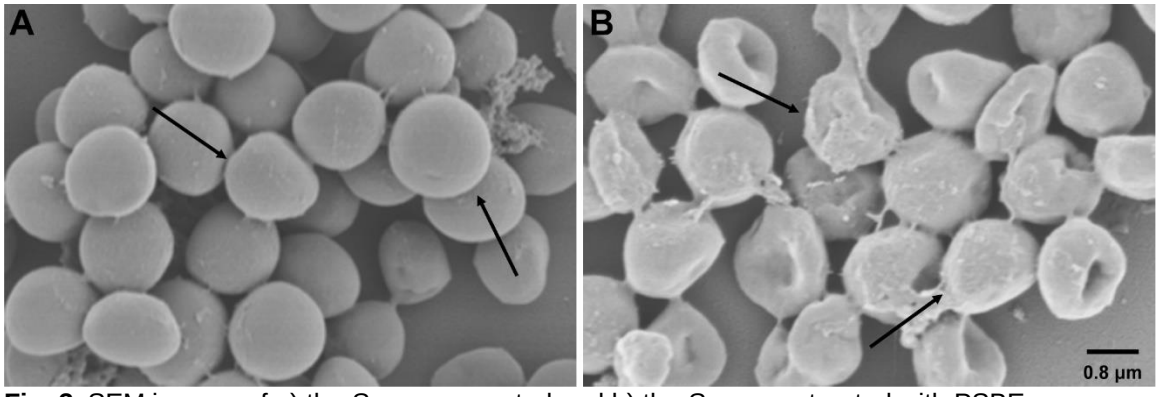

Fig. 3. SEM images of a) the *S. aureus* control and b) the *S. aureus* treated with PSBE

Some studies have shown that when the integrity of the bacterial cell membrane was destroyed, the cell permeability increased and the cell content flowed out, which led to cell death (Xue *et al.* 2019; Dai *et al.* 2020). The antibacterial activity of plant extracts was mostly achieved by destroying the bacterial cell wall and membrane (de Souza *et al.* 2020; Zhang *et al.* 2020b). For example, *Magnolia officinalis* extract damaged the cell wall

and membrane of the *S. aureus*, as revealed by the SEM micrographs. This caused the *S. aureus* to increase its permeability, lose the cell membrane or cell wall components, damage the intracellular structural components, and change the bacterial cell morphology (Hu *et al*. 2011). These investigations were similar to the results that were obtained in the present study, which was verified as the antibacterial mechanism of the PSBE was related to the destruction of the bacterial cell wall and membrane.

Effect of the PSBE Treatment on the *S. aureus* Membrane Integrity

In order to further show the damaging effect of the PSBE on the bacterial cell membrane, the effect of the PSBE on the *S. aureus* membrane integrity was tested with the LIVE/DEAD BacLight Viability Kit. Based on the observed fluorescence changes, the viability and membrane damage of the bacterial cells were indirectly reflected. As shown in the Fig. 4, there was no obvious membrane damage of the *S. aureus* in the 0.85% saline solution (control) after 2 h (Fig. 4A) and 24 h (Fig. 4D) of incubation. Similarly, the *S. aureus* that was exposed to MIC and 4×MIC PSBE for 2 h did not cause obvious damage to the bacterial cells (Figs. 4B and 4C). However, after 24 h of treatment with MIC PSBE for 24 h, the red fluorescence increased obviously, which indicated that the bacterial cell membrane was damaged and the bacterial viability decreased (Fig. 4E). Furthermore, after 24 h of the 4×MIC treatment, all the cells in the visual field showed red fluorescence and the damage of the bacterial cell membrane was aggravated (Fig. 4F). These results indicated that the antibacterial activity of the PSBE for the *S. aureus* may have been caused by the membrane damage. The effect of the PSBE was both dose-dependent and time-dependent, which was consistent with the experimental results of the SEM observation.

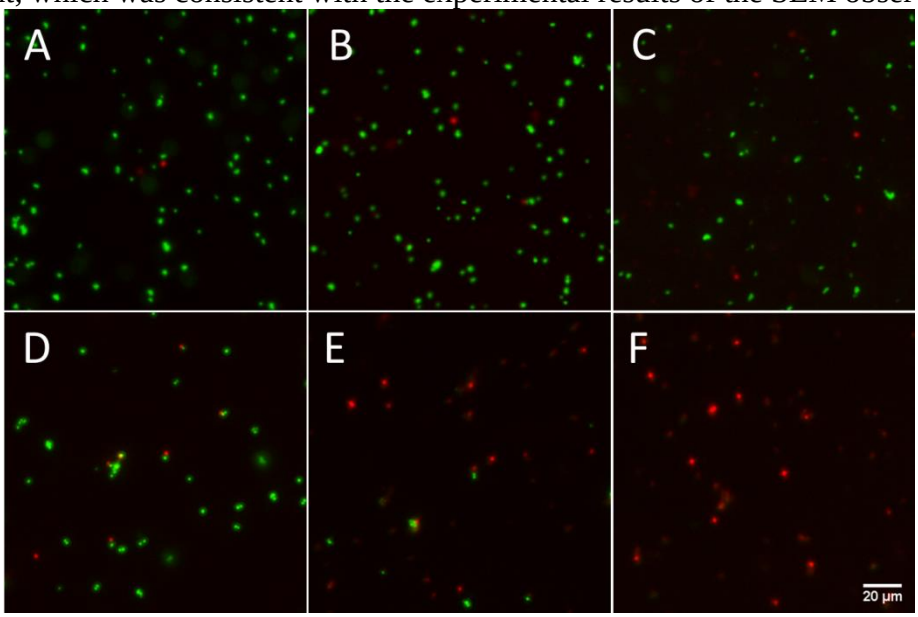

Fig. 4. Fluorescence micrograph of the *S. aureus* a, d) in the 0.85% saline solution (control), b, e) treated with 0.13 mg/mL of PSBE, and c, f) treated with 0.5 mg/mL of PSBE for a, b, c) 2 h or d, e, f) 24 h

A large amount of data showed that quercetin and juglone inhibited *S. aureus* by destroying its biofilm. Quercetin changed the permeability of the microbial biofilm through the influence of Ca^{2+}, K^{+}, Na^{+} (Zhang *et al.* 2020). The juglone inhibited the formation of the microbial biofilm by creating a peroxidation environment in the cells (Wang *et al.* 2016). In this study, PSBE showed a strong inhibitory effect on the *S. aureus*, which was

related to the destruction of the bacterial cell wall and membrane. However, it was not clear whether other mechanisms were involved, such as the cell protein synthesis, DNA synthesis, or RNA synthesis. Further study is needed on this subject.

Food safety problems caused by microorganisms still occur on a large scale in the world (Krishnasamy *et al.* 2020), and *S. aureus* is the main pathogen to blame. The addition of preservatives to control food safety has become commonplace. However, there are concerns about the safety of the existing preservatives, so safer and effective preservatives must be investigated. In this study, PSBE was shown to have good antibacterial and antioxidant effects. The fact that PSBE is a potential natural plant preservative further broadens the use of *P. stenoptera* and provides a reference for the research on the antioxidant and antibacterial properties of other wood species.

CONCLUSIONS

1. The total phenolic content (TPC) in *Pterocarya stenoptera* bark extract (PSBE) was 272.92 ± 3.22 mg gallic acid equivalents per gram (GAE/g) DE. Three phenolic compounds (quercetin-3-O-(2″-O-galloyl)-rhamnoside, quercetin, and juglone) were found in the PSBE.
2. The PSBE possessed remarkable antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+}$) radicals scavenging system, with half-maximal inhibitory concentration (IC_{50}) values of 96.25 ± 3.82 μg/mL and 158.26 ± 6.08 μg/mL, respectively.
3. The antibacterial activity of the PSBE was especially sensitive to the *S. aureus* bacteria. This was likely related to the deformation of the cell morphology and the destruction of the cell membrane structure.
4. This study indicated that the strong antioxidant and antimicrobial properties of the bark extract from *P. stenoptera* make the tree species suitable as a natural additive in the pharmaceutical, food, and cosmetic industries.

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