SUPPLEMENTAL MATERIAL

Chemical constituents, antibacterial activity and mechanism of *Paeonia suffruticosa* Andr. buds extract against *Staphylococcus aureus* and *Escherichia coli* O157:H7

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**Abstract:** Sixteen chemical constituents of *Paeonia suffruticosa* Andr. buds extract (PSABE) were identified by UHPLC-PDA-Q/TOF-MS, belonging to phenolic acids, flavonoids, monoterpene glycosides and gallotannins. PSABE exhibited significant antibacterial activity against six tested microorganisms. Particularly, it showed the most efficient antibacterial effect against *Staphylococcus aureus* and *Escherichia coli* O157:H7, which the minimum inhibition concentration (MIC) and minimum bactericide concentration (MBC) both were 1.56 mg/mL and 6.25 mg/mL, respectively. The results showed that PSABE induced obvious alterations in membrane fatty acid composition of *S. aureus* and *E. coli* O157:H7, such as the decrease of unsaturated fatty acids, leading to the reduce of membrane fluidity. Membrane integrity was destroyed and cell morphology was obviously changed with PSABE. Furthermore, the transcription level of virulence factors was inhibited in the presence of PSABE. These results indicated that PSABE mainly exerted antibacterial effect by damaging cell membrane and inhibiting transcription level of virulence factors.

**Keywords:** *Paeonia suffruticosa* Andr. Buds extract; Antibacterial activity; Antibacterial mechanism; Virulence factor; *Staphylococcus aureus*; *Escherichia coli* O157:H7

**Experimental**
**Tested microorganisms and chemicals**

A total of six food-borne pathogenic bacteria and spoilage bacteria were used for evaluation of antimicrobial activity, including 3 Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Bacillus cereus* ATCC 11778) and 3 Gram-negative bacteria (*Escherichia coli* O157:H7 NCTC 12900, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853). Propidium iodide (PI), 1,6-diphenyl-1,3,5hexatrien (DPH), 14% Boron trifluoride methanol solution, HPLC grade acetonitrile and formic acid, quinic acid, galloyl glucose, gallic acid, paoniflorin, oypaeoniflorin, benzoyloxypaeoniflorin, pentagalloylglucose were purchased from Aladdin (Shanghai, China). All other chemicals used were of analytical grade.

**Plant materials and preparation of extract**

*Paeonia suffruticosa* Andr. buds were purchased from Chinese herb store in Wuxi, Jiangsu, China. The buds were dried to a constant weight using an electric thermostatic blast drying oven (DHG-9075A, Shanghai, China) at 60 °C and then ground into a fine powder. 40 g of the fine powder was extracted with 800 mL of 70% ethanol at 60 °C for 2 h and the filtrate was collected by vacuum filtration device, and repeated twice. Following the solvent was evaporated in a rotary evaporator under reduced pressure and the concentrate was remained stationary at 4 °C for 24 h, then the supernatant was freeze-dried to obtain a dry powder using a freeze dryer at low temperature (12Plus, Labconco Corporation, Kansas City, USA). The dried extract was sealed and stored at 4 °C.

**Identification of chemical constituents**

PSABE was carried out using an ACQUITY ultra-high-performance liquid chromatography with photodiode array detector (Waters Corporation, Milford, MA, USA) coupled with quadrupole time-of-flight mass spectrometry (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) source operating in negative modes (UHPLC-PDA-Q/TOF-MS).

PSABE solution (2 mg/mL) was filtered through a 0.22μm membrane filters and separated on a UPLC BEH C18 column (1.7 μm, 2.1×100 mm, Waters Corporation, Milford, MA; USA) at 30 °C with an injection volume of 10 μL. The mobile phase was composed of 0.1% formic acid in water (eluent A) and acetonitrile (eluent B), flow rate 0.30 mL/min (the elution gradient used as follow: 0–2 min, 100% A; 2–10 min, 70% A;10–20 min, 20% A; 25–30 min,100% A). The PDA spectra
were measured over the wavelength range of 200–600 nm. The mass spectrometric conditions were as follows: capillary voltage 3.5 kV; cone voltage 30 V; ion source temperature 100 °C; desolvation temperature 400 °C; desolvation gas 700 L/h; cone gas flow 50 L/h; detector voltage 1.8 kV and scan range 20–2000 m/z. The data obtained from UHPLC–MS were subsequently entered the MassLynx V4.1 Application Manager software (Waters). The chemical constituents were identified based on the retention time, PDA λmax and MS data compared with the authentic reference compound or reported in the literature.

**Agar diffusion assay**

The antibacterial activity of PSABE was described by the slightly modified according to previous methods (Zhang et al. 2016). 100 µL of bacterial suspension (1×10^8 cfu/mL) was spread on LB agar medium surface. Then filter paper discs (6 mm) soaked with 10 µL of 100 mg/mL PSABE (equivalent to 1 mg dose) were placed onto the top layer of the plates. The antibacterial effect of PSABE was evaluated by measuring the diameter of inhibition zone (DIZ) after plates were incubated for 24 h at 37 °C.

**Determination of minimum inhibition concentration (MIC) and minimum bactericide concentration (MBC)**

The MIC and MBC of the PSABE were determined based on the Clinical and Laboratory Standards Institute (CLSI) microdilution method (Cockerill et al. 2012) with a few modifications. 100 µL diluted PSABE (final concentrations of 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0 mg/mL) was mixed with 100 µL bacterial suspension (1×10^8 cfu/mL) on a 96-well plate. The inhibition of growth was observed by optical density at 600 nm using a multimode reader (Bio-Tek, USA) after incubated at 37 °C for 24 h. The change of optical density less than 0.05 was considered to be the lowest concentration at which inhibited the bacterial growth and was defined as the MIC. For MBC determination, 100 µL of bacterial suspension from the well greater than MIC without visible bacterial growth were plated onto fresh LB agar plates and were incubated for 24 h at 37 °C, and the MBC was defined as the lowest concentration that less than five colonies in a plate.

**Growth curves**

The growth curves of S. aureus and E. coli O157:H7 cells were constructed by the slightly modified according to previous methods (Wu et al. 2017). The PSABE was added to the cultures to maintain
the final concentrations of 1/8MIC, 1/4MIC, 1/2MIC, MIC and MBC. Then, all cultures were further incubated and the cell growth was monitored by optical density at 600 nm every 2 h.

**Membrane fatty acid analysis**

PSABE was added to the culture to maintain a final concentration of 0, MIC and MBC and incubated at 37 °C for 3 h after *S. aureus* and *E. coli* O157:H7 were cultured to the mid-logarithmic phase. The obtained pellets were washed twice with PBS and used for analysis of membrane fatty acids.

The membrane fatty acids were esterified by previous method (Huang et al. 2018) and the fatty acid methyl esters were analyzed by the GC-MS system (Trace1310 gas chromatograph-Thermo TSQ8000) (Thermo Fisher Scientific, Basingstoke, UK) equipped with capillary column TG-5 (30 mm×0.25 mm×0.25 μm). The inlet temperature was 220 °C, the split ratio was 5:1, and the injection volume was 1 μL. The carrier gas was high-purity helium with a flow rate of 2.0 mL/min and the program temperature rise: the initial temperature was 50 °C for 1 min, then the temperature was rose to 200 °C at 10 °C/min and 250 °C at 3 °C/min, respectively, and finally increased to 280 °C at 10 °C/min, kept it for 8 min. Electron impact ion source (EI), electron energy 70 eV, ion source temperature 300 °C, quadrupole rod temperature 150 °C, interface temperature 280 °C, scan mode, scanning relative atomic mass range: 29 to 450 amu, solvent delay 1 min.

**Membrane fluidity**

Membrane fluidity were estimated by measuring fluorescence polarization of DPH based on the previously reported method slightly modification (Wu, et al. 2017). Logarithmic phase cells were washed with PBS and adjusted to a cell density of 1×10⁸ cfu/mL. Then, bacterial suspensions were exposed to different concentrations of PSABE (0, MIC and MBC). After incubation at 37 °C for 1, 2, 3h, bacterial cells were washed twice with PBS and adjusted to a cell density of 1×10⁸ cfu/mL, and DPH was added to bacterial suspensions to maintain the final concentration of 1 × 10⁻⁶ M and incubated at room temperature for 30 min in the dark. Fluorescence polarization was examined by a fluorescence spectrophotometer (F-7000, Hitachi, Tokyo, Japan) equipped with 360/40 nm fluorescence excitation and 460/40 nm fluorescence emission, respectively. The fluorescence anisotropy (P) value is calculated by the following formula:

\[
P = \frac{(I_{VV}-GI_{VH})}{(I_{VV}+GI_{VH})}
\]
In the formula, the subscripts represent the direction of the polarizer and the analyzer, \( v \) is the vertical direction and \( h \) is the horizontal direction, respectively. And \( G \) is the corrected factor, \( G = \frac{I_{HV}}{I_{HH}} \).

**Membrane integrity**

Membrane integrity was examined by the PI fluorescence probe on the basis of the previously reported method slightly modification (Guo et al. 2017). Logarithmic phase cells were washed with PBS and adjusted to a cell density of \( 1 \times 10^8 \) cfu/mL. Different concentrations of PSABE (0, MIC and MBC) were added to bacterial suspensions and incubated at 37 °C for 1, 2, 3 h, respectively. The cells were adjusted to a cell density of \( 1 \times 10^8 \) cfu/mL with PBS, and PI was added to the suspension at a final concentration of 30 \( \mu \)M and incubated for 15 min in a dark ice bath. The PI fluorescence intensity was measured using fluorescence spectrophotometer in the excitation wavelength of 530 nm and emission wavelength of 620 nm.

**Scanning electron microscope (SEM) observations**

The morphological changes of *S. aureus* and *E. coli* O157:H7 were determined by SEM (Shi et al. 2016). Different concentrations of PSABE (0, MIC and MBC) were added to bacterial suspensions and incubated at 37 °C for 3 h. Cells were centrifuged at 2000 \( \times \) g at 4 °C for 15 min and washed twice with PBS. Then, cells were fixed in 2.5% glutaraldehyde at 4 °C for 12 h and dehydrated in a sequential graded ethanol (30%, 50%, 80%, 90%, 100%), at 15 min intervals, followed by final 100% tertiary butyl alcohol incubated for 30 min. All samples were vacuum freeze-drying in silver paper and the changes of cell morphology were observed by using a scanning electron microscope (SU8220, Hitachi, Tokyo, Japan).

**Analysis of transcription level of virulence factors**

Logarithmic phase cells were transferred to fresh cultured LB medium with different concentrations PSABE (0, 1/8MIC, 1/4MIC and 1/2MIC) added and incubated at 37 °C for 12 h. The total RNA was extracted using total RNA extraction kit following the manufacturer's instructions, followed cDNA was synthesized by reverse transcription using the HiScript® QRT SuperMix (Vazyme, Nanjin, China). The primer pairs used for real-time RT-PCR were listed in Table S1 and the 16S rRNA housekeeping gene served as an internal control. The PCR reaction was performed on fluorescence real-time quantitative PCR instrument (CFX96, Bio-Rad, California Hercules,
America) with AceQ® Universal SYBR® qPCR Master Mix (Vazyme, Nanjin, China), and cycle parameters: at 95 °C for 5 min for 1 cycle, at 95 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s for 40 cycles, and the melting curve was established in the range of 65 to 95 °C. The folding induction in target gene expression was transformed into critical threshold cycle (CT), which values were calculated by using the Delta-Delta Ct algorithms.

**Statistical analysis**

All the experiments were performed in triplicate. The data were expressed as the mean ± SD. SPSS 18.0 software was used for statistical analysis. One-way analysis of variance (ANOVA) and Duncan's multiple range test were used to determine significant differences between means (P < 0.05).
| primer name | gene | oligonucleotide primer sequence (5′–3′) |
|-------------|------|--------------------------------------|
| sea-F       | sea  | ATGGTGCTTATTATGGTTATC                |
| sea-R       | sea  | CGTTTCCAAAGGTACTGTATT               |
| hla-F       | hla  | TTGGTGCAAATGTTTC                    |
| hla-R       | hla  | TCACCTTCCAGCCTACT                   |
| tst-F       | tst  | ACCCCTGTCCCTTATC                    |
| tst-R       | tst  | AAAAGCGTCAGACCCACCTAC               |
| agrA-F      | agrA | TGATAATCTTATGAGGTGCTT               |
| agrA-R      | agrA | TGATAATCTTATGAGGTGCTT               |
| stx2-F      | stx2 | CTTCGGTATCCTTATCCC                  |
| stx2-R      | stx2 | GGGTGTGGTAAATAACAG                  |
| eae-F:      | eae  | GTC TGT TTA ATC CAA GCA             |
| eae-R       | eae  | GTC GTA TAT GAT AAA AGT GGA         |
| ygaG-F      | ygaG | CCGAACAAAAGAAGTGATG                 |
| ygaG-R      | ygaG | CGTACCAATCAGAACTCATA                |
| 16S rRNA-F  | 16S rRNA | GCTGCCCTTTGTATTGTC               |
| 16S rRNA-R  | 16S rRNA | AGATGTGGTGTTAAGTCCC             |
| NO | RT (min) | PDA λ<sub>max</sub> (nm) | MS[M-H]<sup>−</sup> (m/z) | MS/MS Fragments (m/z) | identification and tentative identification | molecular formula | references |
|----|---------|------------------------|-----------------|---------------------|------------------------------------------|----------------|-----------|
| 1  | 3.69    | 278                    | 191             | 174,85              | quinic acid<sup>a</sup>                    | C<sub>7</sub>H<sub>12</sub>O<sub>6</sub> | (Draths et al. 1999) |
| 2  | 4.49    | 271                    | 343             | 191,169             | galloylquinic acid<sup>b</sup>            | C<sub>14</sub>H<sub>16</sub>O<sub>10</sub> | (Michael et al. 2007) |
| 3  | 4.80    | 278                    | 331             | 169                 | galloyl glucose<sup>a</sup>              | C<sub>13</sub>H<sub>16</sub>O<sub>10</sub> | (Zhu et al. 2015) |
| 4  | 5.78    | 272                    | 169             | 125                 | gallic acid<sup>a</sup>                   | C<sub>7</sub>H<sub>6</sub>O<sub>5</sub>  | (Engstrom et al. 2015) |
| 5  | 6.74    | 271                    | 483             | 331,169,125         | digalloyl glucose<sup>b</sup>            | C<sub>20</sub>H<sub>20</sub>O<sub>14</sub> | (Li et al. 2016) |
| 6  | 10.37   | 260                    | 495             | 465,321,169         | oypaeoniflorin<sup>a</sup>               | C<sub>23</sub>H<sub>28</sub>O<sub>12</sub> | (Chen et al. 2009) |
| 7  | 11.35   | 266,346                | 609             | 447,285,167         | kaempferol 3,7-di-O-glucoside<sup>b</sup> | C<sub>28</sub>H<sub>34</sub>O<sub>15</sub> | (Francisco et al. 2012) |
| 8  | 13.24   | 210,270                | 415             | 269                 | apigenin 7-O-rhamnoside<sup>b</sup>       | C<sub>21</sub>H<sub>20</sub>O<sub>9</sub>  | (Çulhaoğlu et al. 2015) |
| 9  | 13.83   | 235                    | 479             | 959,525,283,121     | paeoniflorin<sup>a</sup>                  | C<sub>23</sub>H<sub>28</sub>O<sub>11</sub> | (Chen et al. 2009) |
| 10 | 14.40   | 271,354                | 639             | 477,315             | isorhamnetin 3,7-di-O-glucoside<sup>b</sup> | C<sub>28</sub>H<sub>24</sub>O<sub>16</sub> | (Francisco et al. 2012) |
| 11 | 14.70   | 272,358                | 615             | 463,301,165         | quercetin 3-O-galloylglucoside<sup>b</sup> | C<sub>28</sub>H<sub>32</sub>O<sub>17</sub> | (Li et al. 2009) |
| 12 | 15.96   | 217,279                | 939             | 769,617,463,301,169,125 | pentagalloylglucose<sup>a</sup>       | C<sub>41</sub>H<sub>32</sub>O<sub>26</sub> | (Chen et al. 2009) |
| 13 | 16.78   | 214,270                | 599             | 567,313             | benzoyloxypaeoniflorin<sup>a</sup>       | C<sub>30</sub>H<sub>32</sub>O<sub>13</sub> | (Chen et al. 2009) |
| 14 | 17.33   | 216,280                | 1091            | 939,769,617,447,169,125 | hexagalloylglucose<sup>b</sup>       | C<sub>48</sub>H<sub>36</sub>O<sub>30</sub> | (Chen et al. 2009) |
| 15 | 17.95   | 218,279                | 1243            | 1091,939,769,169,125 | heptagalloylglucose<sup>b</sup>         | C<sub>55</sub>H<sub>40</sub>O<sub>34</sub> | (Frohlich et al. 2002) |
| 16 | 20.43 | 215,277 | 349 | 197,169 | ethyl m-digallate<sup>b</sup> | C<sub>16</sub>H<sub>14</sub>O<sub>9</sub> (Li, et al. 2016) |

<sup>a</sup> Compounds identified by comparison with the authentic reference compound

<sup>b</sup> Tentatively identified based on PDA \( \lambda_{\text{max}} \) and MS data reported in the literature
Table S3 DIZ, MIC and MBC of PSABE against tested microorganisms

| Test microorganisms       | DIZ (mm)<sup>a</sup> | MIC (mg/mL)<sup>b</sup> | MBC (mg/mL)<sup>c</sup> |
|---------------------------|-----------------------|-------------------------|--------------------------|
| *S. aureus*               | 18.0 ± 1.0            | 1.56                    | 6.25                     |
| *B. subtilis*             | 15.7 ± 0.6            | 3.13                    | 12.5                     |
| *B. cereus*               | 14.3 ± 0.6            | 1.56                    | 6.25                     |
| *E. coli* O157:H7         | 16.3 ± 0.6            | 1.56                    | 6.25                     |
| *K. pneumoniae*           | 15.0 ± 1.0            | 1.56                    | 6.25                     |
| *P. aeruginosa*           | 15.3 ± 0.6            | 3.13                    | 6.25                     |

<sup>a</sup> DIZ, diameter of the inhibition zone, data are expressed as means ± SD (n =3), including diameter of paper disc 6 mm. Tested at a concentration of 100 mg/mL PSABE.

<sup>b</sup> MIC, minimum inhibition concentration.

<sup>c</sup> MBC, minimum bactericide concentration.
Fig. S1 Growth curves for *S. aureus* (A) and *E. coli* O157:H7 (B) cultured in LB with PSABE at different concentrations. Data are expressed as means ± SD (n = 3).

Fig. S2 The changes of cell membrane fatty acid of *S. aureus* (A, B) and *E. coli* O157:H7 (C, D) with PSABE treatment. Values of each column are means ± SD (n = 3).
**Fig. S3** Effects of PSABE on the membrane fluidity of *S. aureus* and *E. coli* O157:H7 cells. Values of each column are means ± SD (n = 3).

**Fig. S4** Effects of PSABE on the membrane integrity of *S. aureus* and *E. coli* O157:H7 cells. Values of each column are means ± SD (n = 3).
Fig. S5 SEM photomicrographs of *S. aureus* and *E. coli* O157:H7 cells. SEM of *S. aureus*: (A) untreated group, (B) MIC treated group, (C) MBC treated group; SEM of *E. coli* O157:H7: (D) untreated group, (E) MIC treated group, (F) MBC treated group.
Fig. S6 PSABE inhibits the expression of virulence factors of *S. aureus* (A) and *E. coli* O157:H7 (B). Relative expression levels of virulence factors in LB incubated with different concentrations of PSABE. Data are expressed as the mean ± SD of three independent experiments.
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