Bactericidal and antibiofilm properties of *Rumex japonicus* Houtt. on multidrug-resistant *Staphylococcus aureus* isolated from milk

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ABSTRACT

Multidrug-resistant (MDR) *Staphylococcus aureus* and its biofilm formation have been challenging to control in milk and dairy industries. Biofilms formed by *Staph. aureus* may result in the failure of antibacterial agents and disinfectants to penetrate the biofilm in an attempt to control contamination. Novel natural antibacterial agents are required to combat MDR bacteria and biofilms. In this study, we evaluated the bactericidal, antibiofilm, and antimotility effects of *Rumex japonicus* Houtt. (RJH) extract on MDR *Staph. aureus* isolated from milk. The RJH extract exhibited good antibacterial activity against MDR strains with minimum inhibitory concentrations (MIC) ranging from 0.78 to 6.25 mg/mL and minimum bactericidal concentrations ranging from 3.125 to 12.5 mg/mL. The extract showed strong inhibition of biofilm formation (81.9%) at sub-MIC value and eradication of biofilm at higher concentrations. The motility of *Staph. aureus* was effectively blocked by the extract. Major compounds emodin, chrysophanol, and physcion were identified in RJH extract using HPLC-linear trap quadrupole (LTQ)/Orbitrap-mass spectrometry. The extract was nontoxic to human epithelial cell lines such as Caco-2 and HT-29 cell lines at concentrations ranging from 0.1 to 0.5 mg/mL, and from 0.1 to 0.75 mg/mL, respectively. These findings suggest that RJH extract could be an alternative to synthetic preservatives in milk and dairy products.

Key words: multidrug resistance, *Rumex japonicus*, antibacterial activity, antiviral effect, natural preservative

INTRODUCTION

Staphylococcus aureus is one of the major foodborne pathogens worldwide, and its associated food poisoning is attributed to strong virulence factors such as the production of toxins, biofilms, and various resistance proteins (Jørgensen et al., 2005; Kümmel et al., 2016). The major symptoms of *Staph. aureus* infection are associated with gastrointestinal illness such as vomiting, diarrhea, and stomach cramps (Johler et al., 2015; Kümmel et al., 2016). According to the United States Centers for Disease Control, approximately 240,000 cases of *Staph. aureus* are reported annually in the United States, including 1,681 cases of illness and 86 cases of hospitalization (Higginbotham et al., 2014; Johler et al., 2015; Babić et al., 2019). Although it accounts for a small percentage of total food poisoning illnesses, there are still serious problems due to the emergence of multidrug resistance (MDR). The outbreak of staphylococcal food poisoning is mainly associated with consumption of contaminated foods including meat, dairy, bakery products, and other animal-derived foods (Hennekinne et al., 2012). Milk, especially raw milk, is often contaminated with *Staph. aureus* from dairy animals with subclinical mastitis (Aslantaş and Demir, 2016; Felipe et al., 2019). Additionally, low level of viable *Staph. aureus* cells might be still present in pasteurized and UHT milks and other dairy products due to inadequate processing or handling, leading to economic loss and potential risks to public health (Hennekinne et al., 2012; Chen et al., 2020a).

The emergence of MDR *Staph. aureus* is a global problem in food safety. The indiscriminate use of antibacterial agents in agricultural and food industries causes the development of MDR bacteria, which are attributed to the alteration of the drug target site, horizontal gene transfer, acquisition of new resistance genes through mobile genetic elements, and impermeability of antibiotics into bacterial biofilm (Aminov 2009; Munita and Arias, 2016). *Staphylococcus aureus* is a highly virulent pathogen that is capable of forming biofilms on

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a variety of abiotic or biotic surfaces (Lister and Horwill, 2014). Forming biofilm is an important strategy for bacterial survival under unfavorable environments, such as osmotic stress, nutrient scarcity, mechanical and thermal forces, as well as exposure to antimicrobial agents (Frank and Koffi, 1990). The biofilm is a microbial aggregate embedded in a self-produced matrix. It consists of extracellular polymeric substances, including proteins, nucleic acids, and polysaccharides, and provides a physical barrier to the penetration of antibacterial agents and disinfectants (Lister and Horwill, 2014). Moreover, the biofilm can facilitate the emergence and spread of heritable MDR (Ogawara, 2019).

In the milk and dairy industries, it is challenging to control the MDR *Staph. aureus* and its biofilms, leading to serious economic losses. In our previous study, we found that *Staph. aureus* strains isolated from milk have MDR to erythromycin, ciprofloxacin, clindamycin, gentamicin, penicillin, and streptomycin (Zhang et al., 2019). Some synthetic chemicals such as sodium azide have been used to inhibit the growth of *Staph. aureus*, although there are still concerns about undesirable side effects of these chemicals (Upadhyay et al., 2014; Alfaifi et al., 2020). In addition, these available antimicrobial agents have limited efficacy against MDR bacteria (Gatadi et al., 2019). In the case of thermal processing to control pathogens, there is also potential damage to the nutritional and sensory properties of milk and dairy products by overpasteurization (Chen et al., 2020a).

Plant-derived antimicrobials have received attention as a promising alternative for the development of new preservatives due to their antibacterial properties and safety (Ma et al., 2018). The plant extracts have multitargeted inhibitory effects such as cellular mechanism, disturbance of cell membranes, and modulation of virulence factors, contributing a broad spectrum of antibacterial activity against MDR pathogens (Gyawali and Ibrahim, 2014; Silva et al., 2016). *Rumex japonicus* Houtt. (**RJH**) belongs to the Polygonaceae family, and it contains a variety of phytochemical constituents, including anthraquinones, phenolic compounds, flavonoids, and triterpenoids (Sun et al., 2020). With various chemical scaffolds and complex structures, these phytochemicals in RJH could potentiate its antibacterial activity against MDR bacteria (Gyawali and Ibrahim, 2014).

This present study was carried out to examine virulence factors of *Staph. aureus* isolates from milk and to investigate bactericidal and antivirulence effects of RJH extract on MDR *Staph. aureus*. To the best of our knowledge, we are the first to report that hydroethanolic RJH extract has antibacterial and antivirulence effects and is applicable in a food matrix. In addition,

we identified the phytochemical compounds present in RJH extract and evaluated the cytotoxicity of RJH extract on human colon epithelial cell lines. This study suggested that RJH extract is a promising biopreservative with activity against MDR *Staph. aureus*.

MATERIALS AND METHODS

Plant Material and Extraction

Rumex japonicus Houtt. was collected from a market in Shanghai, China. The dried roots were ground into powder using a laboratory-scale mill (S025, IKA). Powder (4.0 g) was extracted twice with 40 mL of 60% (vol/vol) ethanol using an ultrasound-assisted extraction method (1 h, 40°C, and 480 W). The extract was centrifuged at room temperature (900 × *g*, 15 min) and the supernatant concentrated using a rotary evaporator (Shanghai Ya Rong Co. Ltd.) at 40°C under vacuum. The concentrated extract was dried using a vacuum freeze-dryer (Ningbo Shuang Jia instrument Co., Ltd.). The freeze-dried extract was dissolved in 1% dimethyl sulfoxide (Beyotime) for further use.

Qualitative Analysis by HPLC-Linear Trap Quadrupole/Orbitrap-MS

The HPLC analyses were performed on a HPLC 1260 system (Agilent) equipped with quaternary pump, an autosampler, a thermostatically controlled column compartment, and a vacuum degasser unit. The RJH extract (20 µL) dissolved in methanol (15 mg/mL) was injected into an HPLC column (C18 5 µm, 4.6 × 250 mm; Shimadzu) at a flow rate of 1 mL/min in 70 min from 30% methanol and 70% H₂O (0.1% formic acid) to 90% methanol and 10% H₂O (0.1% formic acid) at 35°C. A hybrid linear trap quadrupole (**LTQ**)-orbitrap XL MS (Thermo Fisher Scientific) was connected to an HPLC system equipped with a binary pump and an autosampler (Thermo Fisher Scientific) via an electrospray ionization (ESI) interface in a post-column splitting ratio of 1:4. For MS detection, high-purity nitrogen (N₂) was used as the sheath gas and auxiliary gas, and ultra-high pure helium (He) was used as the collision gas. The optimized ESI parameters in the positive and negative ion modes were as follows: resolution of the orbitrap mass analyzer was set as 30,000; capillary temperature was 320°C; sheath gas flow was 35 arbitrary units (arb.); auxiliary gas flow was 20 arb.; source voltage was 4.0 kV; capillary voltage was 24 V; tube lens voltage was 65V. The analysis was performed in both negative and positive ion mode with the mass range of *m/z* 50 to 2,000. The MS full scan was detected by high-resolution and MS/MS analysis by an ion trap

dynode. The MS scan functions and HPLC solvent gradients were controlled by the Xcalibur data system (Thermo Scientific), and all the data acquisition and processing were conducted by Xcalibur 2.1 software (Thermo Scientific).

Bacterial Strains and Culture Conditions

A total of 11 isolates of *Staph. aureus* from milk were used in the study. *Staphylococcus aureus* ATCC 25923 was used as the reference strain. The isolates were evaluated for the antibiotic sensitivity in our previous study (Zhang et al., 2019). All bacterial strains were grown at 37°C in tryptic soy broth (TSB; Oxoid).

Biofilm Formation

The biofilm-forming ability of *Staph. aureus* strains was evaluated as described by Ackerman et al. (2018) with modifications. Briefly, 100 µL of bacterial suspension in TSB supplemented with 1% glucose (1×10^6 cfu/mL) was added into each well of 96-well microtiter plate (Corning). After 24 h of incubation, planktonic cells in each well were removed. Then, wells were gently washed with PBS (pH 7.5) twice, air dried, and stained with 0.1% crystal violet (Aladdin) for 5 min. Excess crystal violet was removed by washing with PBS 3 times. Then, 33% (vol/vol) glacial acetic acid (Sigma-Aldrich) was used to dissolve the precipitates in wells. The biofilm was quantified by measuring absorbance at 595 nm using a microtiter reader (Molecular Devices). The biofilm-forming ability of strains was categorized by comparing optical density (OD) value of each strain with that of the negative control (OD_{NC}), as follows: weak (OD_{NC} < OD ≤ 2 × OD_{NC}), moderate (2 × OD_{NC} < OD ≤ 4 × OD_{NC}), or strong (OD > 4 × OD_{NC}) biofilm producers (Lee et al., 2014).

Motility

Motility of *Staph. aureus* strains was evaluated according to a previous study (O'May and Tufenkji, 2011). An overnight culture of *Staph. aureus* (5 µL) was inoculated onto the center of the TSB plates containing 0.25% agar. The plates were incubated at 37°C for 24 h, and the zones of motility were observed.

Antibacterial Activity

Determination of MIC and Minimum Bactericidal Concentration. The MIC and minimum bacterial concentration (MBC) of RJH extract against *Staph. aureus* isolates were determined by a serial di-

lution microplate method and Mueller-Hinton (MH) agar counting, respectively (Kim et al., 2020).

Time-Kill Analysis. To evaluate the antibacterial activity of RJH extract against *Staph. aureus* strains, time-kill analysis was conducted. *Staphylococcus aureus* ATCC 25923, SJTUF 20745, SJTUF 20758, and SJTUF 20841 (1×10^6 cfu/mL) at the exponential phase were cultured in MH broth (Oxoid) supplemented with various concentrations of the extract (1/8 MIC, 1/4 MIC, 1/2 MIC, MIC, and MBC) at 37°C with shaking at 180 rpm for 24 h in an incubator. Aliquots were taken at different time intervals (0, 2, 4, 8, 12, and 24 h), and viable bacterial colonies were counted by plating aliquots on MH agar.

Nucleic Acid and Protein Leakage Assay. Cytoplasmic components such as nucleic acids and proteins released from bacterial cells after extract treatment were analyzed to evaluate the integrity of the cell membrane (Chen et al., 2020b). Exponential growth phase cells of *Staph. aureus* strains ATCC 25923, SJTUF 20745, SJTUF 20758, and SJTUF 20841 were washed with PBS 3 times, harvested by centrifugation at $2,400 \times g$ for 10 min at 4°C, resuspended in PBS (1×10^6 cfu/mL) containing various concentrations of RJH extract (0, MIC, and MBC), and cultured at 37°C for 12 h. The samples were collected at 12 h and filtered through a 0.22-µm Millipore filter. The DNA and RNA released from bacterial cells were measured by OD at 260 nm with Nanodrop (Allsheng). The protein content was determined by bicinchoninic acid protein assay (Takara Bio).

Antibiofilm

Inhibition of Biofilm Formation. *Staphylococcus aureus* SJTUF 20758 (1×10^6 cfu/mL) was incubated with various concentrations of RJH extract (1/16 MIC, 1/8 MIC, 1/4 MIC, 1/2 MIC, and MIC mg/mL) in a 96-well microtiter plate at 37°C for 24 h. Biofilm was quantified by using crystal violet assay as mentioned above.

Eradication of Mature Biofilm. *Staphylococcus aureus* SJTUF 20758 (100 µL, 1×10^6 cfu/mL) was inoculated into each well of a microtiter plate (Corning). Biofilms were established at 37°C for 24 h. Preformed biofilms were treated with various concentrations (1/2 MIC, MIC, 2 MIC, and MBC mg/mL) of RJH extract. After treatment at 37°C for 24 h, the remaining biofilms were quantified using the crystal violet assay as described above, and the bacterial viability in biofilm was analyzed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay (Feng et al., 2018). After removal of supernatant

in each well, 20 μ L of MTT (5 mg/mL) was added and incubated for 4 h at 37°C. The purple MTT formazan precipitate was dissolved in 150 μ L of dimethyl sulfoxide and absorbance determined at 570 nm using a microplate reader.

Microscopic Visualization of Biofilm. Antibiofilm activity of the RJH extract was evaluated by microscopic visualization of biofilm as described by Bazargani and Rohloff (2016) with a minor modification. For inhibition of biofilm formation, biofilm was established for 24 h on cover glass slides placed in a 6-well polystyrene plate with or without RJH extract. For the eradication study, preformed biofilm on cover glass slides was treated with RJH extract for 24 h. After treatment, biofilms were stained with 0.1% crystal violet and visualized under light microscopy at 100 $\times$ magnification.

Motility Inhibition

For motility inhibition assay, 5 μ L of overnight culture suspension of *Staph. aureus* (SJTUF 20772 and 20862) was inoculated onto 0.25% agar plates containing 1/8 MIC, 1/4 MIC, 1/2 MIC, and MIC (mg/mL) concentrations of RJH extract and incubated at 37°C for 24 h. The motility was observed by its movement pattern and distance.

Cytotoxicity

Cytotoxicity of RJH extract was evaluated in human colon adenocarcinoma cell models, Caco-2 and HT-29, which have typical features of healthy human intestinal epithelial cells (Feng et al., 2018). Caco-2 and HT-29 cells were purchased from the American Type Culture Collection (ATCC) and used within passages 4 to 35. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco) and supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (10,000 U/mL; Gibco). Cells were maintained in a humidified environment with 5% CO₂ at 37°C. To evaluate cytotoxic effect, cells were placed (5×10^3 cells/well) in a 96-well microtiter plate for 4 h at 37°C with 5% CO₂ before treatment with various concentrations of RJH extract (0.1, 0.25, 0.5, 0.75, and 1 mg/mL). After treatment for 24 h, cell viability was evaluated using the MTT colorimetric assay as mentioned above.

Time-Kill Assay of RJH Extract Against MDR *Staph. aureus* in UHT Whole and Skim Milk

The antibacterial activity of RJH extract in commercial UHT whole and skim milk was determined

based on time-kill assay as described by Chang et al. (2017) with modifications. Briefly, 5 mL of whole or skim milk was inoculated with 1×10^6 cfu/mL *Staph. aureus* SJTUF 20758 at exponential growth phase and incubated at 4°C and 25°C for 1 h before RJH extract treatment (0 MIC, MIC, and MBC) to adapt to each condition. Treated samples were kept for 7 d at 4°C and 25°C. Viable bacterial cells were enumerated at each day by plating aliquots on Baird-Parker selective agar (Qingdao Haibo Biotechnology Co. Ltd.).

Statistical Analysis

All experiments were performed in triplicate, and experimental data are presented as mean values $\pm$ standard deviation. Statistical analysis was performed using SPSS 22.0 software (IBM Corp.) and GraphPad Prism 8 (GraphPad Software). The data were analyzed with Student's *t*-test and one-way ANOVA followed by Tukey's multiple comparison test, as appropriate. Significance was considered at $P < 0.05$ and $P < 0.01$.

RESULTS

Classification of *Staph. aureus* Isolates Based on Biofilm Formation and Motility

Staphylococcus aureus ATCC 25923 and 11 *Staph. aureus* strains isolated from raw milk were tested for their biofilm-forming abilities using the crystal violet assay. The biofilm-forming ability of strains was categorized by comparing the OD value of each strain with a negative control, as follows: weak ($OD_{NC} < OD \leq 2 \times OD_{NC}$), moderate ($2 \times OD_{NC} < OD \leq 4 \times OD_{NC}$), or strong ($OD > 4 \times OD_{NC}$) biofilm producers (Lee et al., 2014). The results showed *Staph. aureus* strains varied in strong, moderate, and weak biofilm-forming abilities (Table 1). Of the tested *Staph. aureus* strains, 9 (SJTUF 20758, SJTUF 20978, SJTUF 20745, SJTUF 20841, SJTUF 20862, SJTUF 20772, SJTUF 20755, SJTUF 20746, and SJTUF 20927) were strong biofilm producers. Reference strain (ATCC 25923) formed moderate biofilms, whereas 2 strains (SJTUF 20973 and SJTUF 20991) formed weak biofilms.

Motilities of *Staph. aureus* strains were evaluated based on movement patterns and distances (Table 1). The motility patterns were divided into 2 groups, spreading or comet gliding. *Staphylococcus aureus* SJTUF 20746, SJTUF 20755, and SJTUF 20758 showed comet gliding movements, whereas *Staph. aureus* SJTUF 20772 and SJTUF 20862 exhibited spreading pattern motility on soft agar. The mobile distances of *Staph. aureus* strains were also measured. *Staphylococcus aureus* SJTUF

Table 1. Biofilm formation and motility of *Staphylococcus aureus* isolated from milk and antibacterial activity of *Rumex japonicus*

Strain	Biofilm formation		Motility		Antibacterial activity	
	Optical density	Category ¹	Distance (mm)	Pattern ²	MIC (mg/mL)	MBC ³ (mg/mL)
ATCC 25923	0.52 ± 0.10 ^{hi}	Moderate	9.9 ± 0.9 ^{gh}	—	1.56	3.125
SJTUF 20745	2.27 ± 0.18 ^{abc}	Strong	12.8 ± 0.5 ^{fg}	—	1.56	3.125
SJTUF 20746	1.11 ± 0.19 ^{fg}	Strong	45.8 ± 0.7 ^b	Gliding	6.25	>25
SJTUF 20755	1.50 ± 0.10 ^{ef}	Strong	41.3 ± 1.8 ^c	Gliding	1.56	3.125
SJTUF 20758	2.73 ± 0.21 ^a	Strong	32.1 ± 2.4 ^d	Gliding	1.56	6.25
SJTUF 20772	1.72 ± 0.09 ^{de}	Strong	81.1 ± 1.3 ^a	Spreading	6.25	12.5
SJTUF 20827	0.96 ± 0.17 ^{gh}	Strong	17.1 ± 0.7 ^e	—	1.56	12.5
SJTUF 20841	2.09 ± 0.04 ^{bcd}	Strong	14.9 ± 0.6 ^{ef}	—	6.25	12.5
SJTUF 20862	1.87 ± 0.09 ^{cd}	Strong	79.1 ± 0.9 ^a	Spreading	1.56	3.125
SJTUF 20973	0.29 ± 0.04 ^{ij}	Weak	11.5 ± 0.4 ^{gh}	—	0.78	3.125
SJTUF 20978	2.52 ± 0.22 ^{ab}	Strong	9.9 ± 0.6 ^{gh}	—	6.25	6.25
SJTUF 20991	0.24 ± 0.09 ⁱ	Weak	9.6 ± 0.4 ^h	—	1.56	6.25

^{a–j}Different letters within columns indicate significant difference ($P < 0.05$) from one-way ANOVA plus post hoc Tukey test.

¹Categorized by comparison of each strain's optical density (OD) with the mean OD value of negative controls ($OD_{NC} = 0.174 \pm 0.01$), according to Lee et al. (2014), as follows: weak ($OD_{NC} < OD \leq 2 \times OD_{NC}$), moderate ($2 \times OD_{NC} < OD \leq 4 \times OD_{NC}$), or strong ($OD > 4 \times OD_{NC}$) biofilm producers.

²Categorized by the pattern of movement on 0.25% swimming agar, according to Pollitt and Diggle (2017).

³MBC = minimum bactericidal concentration.

20772 and SJTUF 20862 moved the farthest, followed by SJTUF 20746, SJTUF 20755, and SJTUF 20758. Other strains showed weak motility.

methyl ether isotectorigenin), terpenoids (myrianthnic acid), and naphthalenes (trachrysone and nepodin).

Phytochemical Compounds in RJH Extract

The phytochemical compounds in RJH extract were identified using HPLC-LTQ/Orbitrap-MS analysis (Table 2). The RJH extract contained anthraquinones (emodin, chrysophanol, physcion, and emodin-8-glucoside), flavonoids (4',5,6,7-tetramethoxyflavone and

Antibacterial Activity of the RJH Extract

The MIC and MBC of RJH extract were determined against 1 reference and 11 MDR *Staph. aureus* strains (Table 1). The results showed that the RJH extract had antibacterial activities against all 12 *Staph. aureus* strains. The MIC values ranged from 0.78 to 6.25 mg/mL. Except for SJTUF 20746 (MBC >25 mg/mL), the

Table 2. Compounds identified in *Rumex japonicus* Houtt. extract using HPLC-linear trap quadrupole/orbitrap-MS

Peak	Rt ¹ (min)	Identified compound	Formula	MS ² (<i>m/z</i>)	MS/MS ³ (<i>m/z</i>)	Adduct	Area%	Reference
1	3.34	4',5,6,7-Tetramethoxyflavone	C ₁₉ H ₁₈ O ₆	341.28	179.01, 160.97, 142.99, 118.90, 112.89	–H	5.05	
2	17.56	Myrianthnic acid	C ₃₀ H ₄₈ O ₆	504.31	342.19, 382.25, 185.04		8.31	Jang et al., 2005
3	36.13	Isotectorigenin, 7-methyl ether	C ₁₈ H ₁₆ O ₆	327.24	312.05, 284.17	–H	1.55	
4	37.14	Emodin-8-glucoside	C ₂₁ H ₂₀ O ₁₀	431.25	271.06, 287.00, 329.04, 389.15	–H	3.17	Wang et al., 2018b
5	37.86	Trachrysone	C ₁₄ H ₁₄ O ₄	245.15	230.15, 231.14	–H	0.54	Wang et al., 2018b
6	43.07	Nepodin	C ₁₃ H ₁₂ O ₃	215.13	173.04, 215.17	–H	4.83	Orbán-Gyapai et al., 2017
7	54.69	Emodin ⁴	C ₁₅ H ₁₀ O ₅	271.03	229.06, 201.03, 225.16, 230.09, 239.20, 270.95	+H	20.42	Xie et al., 2019
8	57.83	Chrysophanol ⁴	C ₁₅ H ₁₀ O ₄	255.02	227.02, 223.03, 209.03, 237.21, 255.15, 199.09	+H	12.12	Xie et al., 2019 and Liu et al., 2015
9	62.70	Physcion ⁴	C ₁₆ H ₁₂ O ₅	285.06	285.05, 211.03, 228.95, 253.10, 266.96, 239.18	+H	5.27	Liu et al., 2015 and Wang et al., 2018b

¹Rt = retention time.

²Precursor ion.

³Product ions, after fragmentation of precursor ion.

⁴Confirmed with a standard compound.

MBC values for *Staph. aureus* strains ranged from 3.125 to 12.5 mg/mL. In the time-kill analysis (Figure 1), RJH extract showed good antibacterial activity against *Staph. aureus* strains in a concentration-dependent manner. At the MBC, the viable cells of *Staph. aureus* SJTUF 20841 decreased by 99.9% within 12 h. For all other *Staph. aureus* strains, the number of viable cells was reduced within 24 h. The extract at the MIC concentration exhibited bacteriostatic activities against *Staph. aureus*.

Cell Membrane Integrity

The leakage of cell constituents was measured to study the effect of RJH extract on the integrity of *Staph. aureus* cell membrane. As shown in Figure 2, compared with untreated cells, *Staph. aureus* ATCC 25923, SJTUF 20745, SJTUF 20758, and SJTUF 20841 treated with MBC concentrations of RJH extract had significantly more leakage of nucleic acids and proteins from cells ($P < 0.01$). However, the *Staph. aureus*

Figure 1. Time-kill assay of *Rumex japonicus* Houtt. extract at 1/8 MIC, 1/4 MIC, 1/2 MIC, MIC, and minimum bacterial concentration (MBC) values against one reference (A) and 3 MDR *Staphylococcus aureus* strains, SJTUF 20745 (B), SJTUF 20758 (C) and SJTUF 20841 (D). Error bars represent standard deviations.

Figure 2. Leakage of DNA (A), RNA (B) and protein (C) from one reference and 3 multidrug-resistant *Staphylococcus aureus* strains treated with *Rumex japonicus* Houtt. extract at 0, MIC, and minimum bacterial concentration (MBC) for 12 h. *** $P < 0.01$ compared with control group. Error bars represent standard deviations.

strains treated at MIC concentration exhibited less leakage, compared with MBC concentration, after 12 h of treatment.

Antibiofilm Effects of RJH Extract

To evaluate the antibiofilm effects of RJH extract, *Staph. aureus* SJTUF 20758 was used because it was shown to have the strongest biofilm production. As shown in Figure 3A, sublethal concentrations of RJH extract inhibited biofilm formation of *Staph. aureus* in a concentration-dependent manner. The RJH extract treatments at 1/2 MIC and MIC showed reduction (81.9% and 86.7%, respectively) in biofilm formation. The RJH extract at 1/4 MIC exhibited 38.7% inhibition of biofilm formation. Light microscopy analysis demonstrated that *Staph. aureus* treated with RJH extract at 1/2 MIC concentration showed poor biofilm development compared with the untreated control (Figure 4A and B).

Mature biofilm of *Staph. aureus* was used to investigate the biofilm-disruption levels by RJH extract. Mature biofilm was treated with various concentrations of RJH extract (MIC, 2 MIC, and MBC) for 24 h, and biofilm was quantified using the crystal violet assay. As shown in Figure 3B, RJH extract was effective to eradicate biofilm in a concentration-dependent manner. The percentages of biomass reduction by RJH extract exposure were 27.4%, 66.5%, and 78.6% at MIC, 2 MIC, and MBC values, respectively. Light microscopy images showed that the mature biofilm was destroyed by RJH extract at 2 MIC compared with untreated control (Figure 4C and D). In addition, the MTT assay was used to measure metabolically active cells in biofilm after RJH exposure (Figure 3C). After 24 h of treatment with various concentrations (1/2 MIC, MIC, 2 MIC, and MBC) of the RJH extract, the reductions

in metabolic activity of viable cells in mature biofilm were 25.2%, 15.2%, 4.0%, and 3.2%, respectively.

Antimotility

Because of their different motility patterns, *Staph. aureus* SJTUF 20758 and SJTUF 20772 strains were used to evaluate the antimotility effects of RJH extract. The RJH extract inhibited the spreading and comet gliding migration of *Staph. aureus* in a concentration-dependent manner (Figure 5). For *Staph. aureus* SJTUF 20758, which has comet gliding movement, the extract exhibited a strong effect in blocking its movement at 1/4 MIC, 1/2 MIC, and MIC (Figure 5A). However, the extract had good spreading inhibition of *Staph. aureus* SJTUF 20772 only at the MIC (Figure 5B).

Cytotoxicity of RJH Extract

The cytotoxic effect of RJH extract in Caco-2 and HT-29 cells was evaluated by MTT assay after 24 h of exposure (Figure 6). The results indicated that RJH extract did not affect the multiplication capacity of Caco-2 or HT-29 cells at concentrations ranging from 0.1 to 0.5 mg/mL and from 0.1 to 0.75 mg/mL, respectively.

Application of RJH Extract in UHT Whole Milk and Skim Milk

To investigate the antibacterial activity of RJH extract against *Staph. aureus* SJTUF 20758 in a food matrix, UHT whole milk and skim milk were used as food models. *Staphylococcus aureus* was treated with the MIC and MBC values of RJH extract and incubated at 4°C or 25°C for 7 d. As shown in Figure 7, in whole and skim milk treated with MBC and stored

Figure 3. Antibiofilm effect of *Rumex japonicus* Houtt. (RJH) extract on *Staphylococcus aureus* SJTUF 20758. Inhibition of biofilm formation by sublethal concentration of RJH extract (A), eradication of biofilm by RJH extract (B), and effect of RJH extract on metabolic activity of bacterial cells within biofilm (C). MBC = minimum bacterial concentration. *** $P < 0.01$ compared with untreated (control) group. Error bars represent standard deviations.

at 4°C, *Staph. aureus* viable cells decreased by 4.8 and 4.2 log₁₀ cfu/mL, respectively. In the case of storage at 25°C, the reduction in cell viability was not observed in whole milk, whereas bacterial growth was inhibited (from 5.8 to 4.7 log₁₀ cfu/mL) in skim milk after 3 d of administration in a time-dependent manner. The antibacterial activity of RJH extract was relatively effective in skim milk compared with whole milk.

DISCUSSION

The prevalence of MDR *Staph. aureus* with strong virulence has become a major concern due to the increasing cases of failure of antimicrobials in the milk and dairy industries, leading to economic loss and food safety issues (Founou et al., 2016). In our previous study, we reported that *Staph. aureus* isolated from milk has shown resistance to antibiotics including erythromycin, ciprofloxacin, clindamycin, gentamicin, penicillin, and streptomycin, and most isolates have MDR (Zhang et al., 2019). Forming bacterial biofilms is one of the survival strategies of *Staph. aureus* to tolerate antimicrobial agents and other external stress by interfering with the penetration of antimicrobials into biofilm (Rabin et al., 2015). Many studies have reported the correlation of MDR and biofilm formation of *Staph. spp.* isolated from milk and dairy industries (Wang et al., 2018a; Meroni et al., 2019; Bissong and Ateba, 2020). In this study, a similar finding was observed that 9 MDR isolates had strong biofilm formation ability among 11 isolates (Table 1). Motility, one of the virulence factors, is also important for biofilm formation because motility is involved in the initial adhesion on abiotic and biotic surfaces (Bai et al., 2019). Although *Staph. aureus* is not flagellated, spreading or comet gliding motility has been observed (Pollitt and Diggle, 2017). Among the isolates, bacterial strains with spreading motility moved farther compared with strains with gliding motility (Table 1).

The elimination of MDR *Staph. aureus* and its biofilms in milk and dairy products have remained a major concern (Aslantaş and Demir, 2016; Unlu et al., 2018). In attempts to discover new antimicrobials, plant extracts have been studied because of their significant antibacterial properties, safety, and accessibility (Silva et al., 2016; Ma et al., 2018). In addition, plant extracts contain various phytochemical compounds with structural diversity, which can efficiently combat MDR bacteria by targeting multiple sites (Silva et al., 2016). The plant RJH belongs to the Polygonaceae family, which has several nutraceutical effects such as antioxidant, antimicrobial, anti-inflammatory, anticancer, and antidiabetic activities (Sun et al., 2020). Nishina et al. (1993) reported that an *n*-hexane extract of RJH root

had broad-spectrum antibacterial activity against food-borne pathogens including *Staph. aureus*. Elzaawely et al. (2005) demonstrated that the ethyl acetate extract of aerial parts of RJH had strong antibacterial activity against *Bacillus subtilis*, *Bacillus cereus*, and *Escherichia coli*. In the current study, ethanol was used as an extraction solvent due to its safety and good extraction efficiency to obtain antibacterial compounds. We identified the phytochemical constituents of RJH by HPLC-MS, which are 4',5,6,7-tetramethoxyflavone, myrianthric acid, 7-methyl ether isotectorigenin, emodin-8-glucoside, trachrysone, nepodin, emodin, chrysophanol, and physcion (Table 2). Nishina et al. (1993) demonstrated the antibacterial activity of trachrysone in RJH against *Staph. aureus*, *B. subtilis*, *E. coli*, and *Salmonella typhimurium* (MIC values ranged from 100 to 200 mg/L). In addition, many studies have been investigated on the antibacterial activities of emodin, chrysophanol, and physcion (Basu et al., 2005; Wang et al., 2010; Sun et al., 2019; XunLi et al., 2019). Basu et al. (2005) reported that emodin and physcion isolated from *Ventilago madraspatana* exhibited broad-spectrum

antibacterial activities against gram-positive (*B. cereus*, *B. pumilus*, and *B. subtilis*) and gram-negative bacteria (*Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) with MIC values ranging from 0.5 to 2.0 µg/mL and from 70.0 to 250.0 µg/mL, respectively. Nakano et al. (2012) identified chrysophanol, physcion, nepodin, and emodin in *Atraphaxis laetevirens* that showed antibacterial activity against *Flavobacterium columnare* and *Streptococcus iniae*. Consistent with the previous studies, the strong antibacterial activity of RJH extract could be attributed to the inclusion of antibacterial compounds, trachrysone, nepodin, emodin, chrysophanol, and physcion.

The antibacterial activity of RJH extract has been studied (Nishina et al., 1993; Elzaawely et al., 2005), but its bactericidal activity against MDR bacteria, its mechanism of antibacterial action, and antivirulence effects had remained uninvestigated. In the present study, the hydroethanolic RJH extract showed good bactericidal effects on MDR *Staph. aureus* isolates, having different antibiotic-resistant patterns (Table 1). The extract presented bactericidal effects on 4 MDR

Figure 4. Light micrographs (100× magnification; scale bars = 100 µm) of *Staphylococcus aureus* SJTUF 20758 biofilms. Inhibition of biofilm formation: control (A) and treatment of *Rumex japonicus* Houtt. (RJH) extract at 1/2 MIC (B). Eradication of established biofilms: control (C) and treatment of RJH extract at minimum bacterial concentration (D).

Figure 5. Antimotility of *Staphylococcus aureus* SJTUF 20772 (A: gliding movement) and SJTUF 20862 (B: spreading movement) treated with 0, 1/8 MIC, 1/4 MIC, and 1/2 MIC.

Figure 6. The viability of Caco-2 (A) and HT-29 (B) cells exposed to various concentrations (0.1, 0.25, 0.5, 0.75, and 1 mg/mL) of *Rumex japonicus* Houtt. extract for 24 h assessed by the colorimetric assay using MTT. ** $P < 0.05$ and *** $P < 0.01$ compared with untreated group. Error bars represent standard deviations.

Staph. aureus within 12 h at MBC, as well as bacteriostatic effects at MIC levels (Figure 1). Many studies have reported the mechanism of antibacterial action of plant extracts; for example, changing cell morphology, disrupting cell wall, and altering membrane permeability eventually lead to cell death (Bai et al., 2015; Kang and Song, 2019; Zheng et al., 2019; Zhu et al., 2020). In agreement with previous studies, we observed that the RJH extract at MBC triggered the leakage of intracellular contents such as nucleic acids and proteins in 4

MDR *Staph. aureus*, indicating that RJH extract disintegrated bacterial cytoplasmic membrane, increased its permeability, and thereby caused cell death (Figure 2). Aside from the bactericidal activity with multiple targets, restraining bacterial virulent factors is a good strategy to combat MDR bacteria and to inhibit the development of MDR bacteria in the food industry (Silva et al., 2016). In this study, we evaluated antivirulence effects of RJH extract, including antibiofilm and antimotility activities, against MDR isolates from milk.

Figure 7. Antibacterial activity of *Rumex japonicus* Houtt. (RJH) extract at 0, MIC, and minimum bacterial concentration (MBC) in whole milk (A, B) and skim milk (C, D). Effect of RJH extract on growth of *Staphylococcus aureus* SJTUF 20758 in milk incubated at 4°C (A, C) and 25°C (B, D) for 7 d.

At sub-MIC concentration (0.78 mg/mL), the biofilm formation was inhibited approximately 81.8% without affecting the growth of planktonic cells (Figure 1 and Figure 3A). This indicated that the biofilm formation inhibitory effect is more likely to involve inhibiting the initial attachment of bacteria and reducing biomass production, and not due to death of cells by the RJH extract. The antimotility activity of RJH extract could result in inhibition of staphylococcal adhesion capacity, thereby disturbing the surface colonization and biofilm formation (Figure 5). Biofilms are composed of extracellular polymeric substances secreted by adhesion cells and act as a natural barrier by blocking the penetration of antimicrobials owing to the integrity of biofilm architecture (Lister and Horswill, 2014). Additionally, biofilm-associated bacteria are metabolically inactive and at dormant state, leading to more resistance to the antimicrobial agents compared with planktonic bacteria (Harrison et al., 2005). Interestingly, we found that the treatment of mature biofilm with RJH extract (2 MIC and MBC) reduced biofilm biomass and viable cells in biofilms (Figure 3B and C).

We next evaluated the antibacterial activity of RJH against MDR *Staph. aureus* in food matrix using UHT whole milk and skim milk as the example models. The extract exhibited a reduction of *Staph. aureus* viable cells in skim milk more than in whole milk (Figure 7), supporting previous studies that also found a weak antibacterial activity in whole milk (Cava et al., 2007; Higginbotham et al., 2014; Chang et al., 2017). Less efficacy of RJH extract in whole milk might be attributed to interference with milk fat globules, proteins, and carbohydrates, providing the protection of bacteria (Higginbotham et al., 2014). In general, RJH extract at MBC concentration showed the bacteriostatic effect of MDR strain in skim milk for long-term storage.

Last, we evaluated the cytotoxic effects of RJH extract on human epithelial cell lines such as Caco-2 and HT-29 cells (Figure 6). The RJH extract showed nontoxic effects on Caco-2 cell line and HT-29 cell line, demonstrating that RJH extract may be safe to human within a certain concentration.

This study showed that MDR *Staph. aureus* isolated from milk exhibited strong virulent properties. Our results demonstrated that RJH extract exhibited bactericidal activity against MDR bacteria by disrupting the integrity of the cell membrane and could be directly applicable in the food matrix. The strong antivirulence effects of RJH extract, such as antibiofilm and antimotility, were also observed, suggesting a potential antibiofilm agent. To overcome MDR pathogens and to meet consumers demand for food free from artificial agents, the RJH extract could be an alternative to synthetic preservatives in the food industry. In spite of

nontoxic effects on epithelial cell lines in the present study, further studies are necessary to evaluate the toxicity of RJH extract in vivo for safety assurance in milk and dairy industries. Furthermore, physicochemical and sensory properties of milk containing RJH extract need to be evaluated their application in milk and dairy products.

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