

Iridoid glucosides against *Staphylococcus aureus* infection and inhibit inflammation on bovine mammary epithelial cells

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Abstract

Staphylococcus aureus can cause diseases in humans and livestock, destroy the body barrier, exacerbate inflammation, and seriously threaten human health and animal husbandry development. Iridoid glucosides isolated from *Rehmannia glutinosa* is an important Chinese traditional medicine widely used in various diseases. The aim of this study was to investigate whether the iridoid glucosides were effective against *S. aureus* infection and inhibit inflammation of bovine mammary epithelial cells. The antibacterial potential was determined using the in vitro broth microdilution assay against *S. aureus*. Then, the antibiofilm activity was tested using a BCA protein assay, an electric conductivity assay, and scanning electron microscopy. Finally, the anti-inflammatory activities were investigated by measuring nitric oxide (NO), tumor necrosis factor- α (TNF- α), interleukin 6 (IL-6), and interleukin 1 β (IL-1 β) production in *S. aureus*-stimulated MAC-T cells, respectively. Biofilm results indicated that both 1MIC and 3MIC *S. aureus* groups showed a significant increase in a dose-dependent manner after two hours of iridoid glucosides treatment. This indicated that iridoid glucosides treatment caused the leakage of intracellular electrolytes. NO, ELISA and RT-PCR results showed the iridoid glucosides inhibit the NO, TNF- α , IL-6, and IL-1 β production in *S. aureus*-stimulated MAC-T in a concentration-dependent manner. In summary, iridoid glucosides had antibacterial, antibiofilm, and anti-inflammatory activity against *S. aureus*, and they might have application value and development potential in therapy for bovine mastitis.

Keywords: Iridoid glucosides, Bovine Mammary Epithelial Cells, *Staphylococcus aureus*, antibacterial, anti-inflammatory

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Introduction

Staphylococcus aureus, a very common Gram-positive pathogen, is widely present in the natural environment (Darboe et al., 2019). *S. aureus* can cause a variety of infectious diseases, such as mastitis. Mastitis, an infection or inflammation of the parenchyma of the mammary gland, is considered to be one of the most frequent and troublesome diseases in dairy cows (Bradley, 2002). *S. aureus* is the main pathogenic bacteria cause of mastitis in dairy cows (Farnsworth et al., 2015). Antibiotics were used frequently for the treatment of clinical mastitis in cows (Peng & Gurunathan, 2017). However, due to the extensive use of antibacterial agents in bovine mastitis, the emergence of antibiotic-resistant bacteria and the frequent reoccurrence of chronic bovine mastitis have become a critical issue (Chambers, 2001; Gomes & Henriques, 2016). Another major reason for antibiotic resistance and recurrence of inflammation is bacterial biofilm formation inside the udder tissue (Atulya et al., 2014; Davies, 2003; Olson et al., 2002). Therefore, the capacity of *S. aureus* to form biofilms can result in persistent inflammatory infection in the bovine mammary gland, which is difficult to prevent, control, and eradicate (Labbate et al., 2004). *S. aureus* infections were previously treated with antibiotics such as penicillin, aminopenicillin, tetracycline, and cephalosporin. However, in recent decades, the number of *S. aureus* infections has increased dramatically, accompanied by the emergence of resistant strains. In particular, the emergence of multidrug-resistant (MDR) strains poses a serious threat to public health (Branco et al., 2018).

Many traditional Chinese medicines have anti-inflammatory effects, especially medicinal plants in the families *Scrophulariaceae*, *Rubiaceae*, *Lamiaceae*, *Verbenaceae*, and *Gentianaceae*, which are rich in iridoid compounds (Dong et al., 2011). Iridoid glycosides extracted from the *Rehmannia glutinosa* have a number of pharmacologically active properties, such as anti-cerebral ischemic injury (Li et al., 2004a; Li et al., 2004b; Shieh et al., 2011), anti-senile dementia (Jiang et al., 2008; Wang et al., 2009), anti-inflammatory (Choi et al., 2013; Bi et al., 2013), antimicrobial, diabetes mellitus (Wang et al., 2010; Huang et al., 2010; Li et al., 2014), anti-tumor, and anti-apoptosis actions (Zhang et al., 2013). Iridoid glycosides have a wide range of pharmacological effects, with the most prominent being their anti-inflammatory activity (Li et al., 1999). Catalpol, belonging to iridoid glycosides, is the main active ingredient of an extract from *R. glutinosa*. It has been reported that iridoid glycosides enhance recovery of cerebral function after cerebral ischemia through a mechanism that involves anti-inflammation (Zhu et al., 2015). Studies have also shown the anti-neuroinflammatory and anti-oxidative effects of iridoid glycosides on BV2 microglia and primary cortical neuron cells (Yang et al., 2020). More and more studies have proved that iridoid glycosides play an important role in protecting against Alzheimer's disease (Zhang et al., 2009) and in neuroprotection, microcirculation improvement, and antioxidation (Huang et al., 2013).

However, a protective effect of iridoid glycosides on breast inflammation has not been reported. Whether it can protect against *S. aureus* induced bovine mammary epithelial cell inflammation remains unknown. Therefore, the aim of this study was to investigate the ability of iridoid glycosides against *S. aureus* infection and inhibit inflammation on bovine mammary epithelial cells.

Materials and Methods

Ethics statement: All experimental plant procedures complied with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. All experimental cell procedures and bacterial strains were approved by the Animal Care and Use Committee of Xinxiang University, Xinxiang, China, and performed in accordance with the animal welfare and ethics guidelines.

Reagents: A BCA protein kit was purchased from Biyuntian Biotechnology (Shanghai, P. R. China, cat. P0010). CCK-8 kit was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, P. R. China, cat. CK04). ELISA Kits (TNF- α , IL-6, and IL-1 β) were purchased from Jiangsu Mei Biao Biological Technology Co., Ltd. (Jiangsu, P. R. China, cat. MB-4838A\MB-4905A). Nitric Oxide Assay Kit was purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, P. R. China, cat. S0023).

Iridoid glycosides from *R. glutinosa* were extracted by the reference method (Dang et al., 2017), freeze-dried, and stored at 4 °C.

Preparation of the *Staphylococcus aureus* strain: The *S. aureus* strain (25923) used in this study was obtained from the American Type Culture Collection (ATCC). The *S. aureus* strain was grown on the Nutrient Broth (NB) overnight at 37 °C under aerobic conditions. A single colony was inoculated into LB broth and incubated overnight at 37 °C while shaking at 180 rpm for 10 h. In order to get the bacteria to the logarithmic growth phase, 100 μ L was transferred into 10 ml of fresh LB broth and incubated at 37°C while shaking at 180 rpm for 4 h. Then, 1 mL of *S. aureus* bacteria liquid was in a 1.5-mL centrifuge tube, centrifuged at 12,000 rpm for 1 min, and resuspended the bacterial pellet with phosphate buffer (pH = 7.4). The *S. aureus* bacteria liquid was then serially diluted 10 times, seeded onto LB plates, and placed the LB plates in a 37 °C incubator for 12 h, and counted the number of *S. aureus* per milliliter after growing visible single bacteria. The bacteria were diluted by decimal serial dilutions one time, and 10⁵ CFU/mL was obtained.

Antibacterial activity: The agar diffusion test was used to detect the antibacterial effect of iridoid glycosides on *S. aureus*. *S. aureus* was cultured overnight, and the supernatant was discarded by centrifugation. The bacteria were washed with sterile PBS and resuspended in a smaller volume to concentrate the cells. Next, 100 μ L of the bacterial solution was pipetted into 25 mL of agar and shaken gently to mix. The plate was poured, a puncher was

used to make holes after the agar medium had solidified, and 20 μ L of iridoid glycosides were added to the wells. Sterile water was used as a control. The plates were incubated overnight at 37 °C, and the results were observed.

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of iridoid glycosides able to inhibit the microorganism's growth. To determine the MIC (Ruan et al., 2020), *S. aureus* was cultured overnight and adjusted to 10^5 CFU/mL. Next, 100 μ L of the adjusted bacterial solution was added to each well of a 96-well polypropylene microtiter plate. After serially two-fold dilution of the drug, 100 μ L was added to a 96-well plate to obtain concentrations from 1 to 300 mg/mL. The solutions in the wells were mixed, and controls were established at the same time: positive control with only the bacterial solution without iridoid glycosides and a blank control with only the culture medium. The plates were incubated overnight at 37 °C to observe the antibacterial activity results. Experiments were repeated three times with three parallels.

Killing curve: *S. aureus* was cultivated, the OD₆₀₀ of the bacterial solution was adjusted to approximately 1.0, and iridoid glycosides (1×MIC, 3×MIC) were added. In addition, sterile water was used as a control. The samples were incubated with shaking at 37 °C. The OD₆₀₀ value of the bacterial solution was measured every 1 h, and plate counting was performed at the same time to obtain the antibacterial time-killing curve of iridoid glycosides against *S. aureus* (Wang et al., 2019). Experiments were repeated three times with three parallels.

BCA protein assay: *S. aureus* was cultured overnight, washed, and resuspended in sterile PBS. Iridoid glycosides (1×MIC, 3×MIC) were added to the bacterial solution. Sterile water was added as a control. An iridoid glycoside-only group was a negative control. The mixture was allowed to stand for 5 min, and the supernatant was collected after centrifugation. The effect of iridoid glycosides on the permeability of *S. aureus* for protein was analyzed according to the instructions of the BCA kit.

Electric conductivity assay: *S. aureus* was cultured overnight, washed, and resuspended in sterile PBS. Iridoid glycosides (1×MIC, 3×MIC) were added to the bacterial solution. Sterile water was added as a control. An iridoid glycosides-only group was a negative control. The mixture was allowed to stand for 5 min, and the supernatant was collected after centrifugation. The supernatant was taken to test the solution, which was diluted to measure the conductivity with a Lieci conductivity meter.

Scanning electron microscopy: Bacteria were allowed to grow naturally on the sterile slides in 6-well cell culture plates to form a biofilm. Overnight culture broth and fresh broth were added at a volume ratio of 1:100 and incubated at 37 °C for 24 h. Then, different final concentrations of iridoid glycosides (1×MIC, 3×MIC) were added, and continue incubation. A 2.5% glutaraldehyde solution was added to each well, and the cells were fixed at room temperature for 30 min.

The plate was rinsed 3 times with PBS for 10 min each time. Gradient dehydration was performed with increasing concentrations of an ethanol solution (30, 50, 70, 80, 90, 95, and 100%). After ion spray gold coating, biofilm formation was observed by scanning electron microscopy (SEM) (Zhong et al., 2020).

Cell culture: Bovine mammary epithelial cell line MAC-T is epithelial cells that were isolated from breast tissue and donated by Northwest A&F University. Briefly, the MAC-T was cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) (Gibco, USA, New York, cat. 12400-024) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel, Kibbutz Beit-Haemek, cat. 04-011-1A/B) in 25 cm² culture dishes. Cells were maintained in an incubator at 37 °C and 5% CO₂. Cells were routinely passaged at a rate of 70–80% for all experiments. The Cells were cultured in DMEM/F12 without FBS and then treated with catalpol for 3 h. Then, the cells were further cultured with *S. aureus* for 24 h. Phosphate buffer saline (PBS) was used as a positive control.

Cytotoxicity studies: Cell viability was determined by the CCK-8 kit in accordance with the manufacturer's instructions. First, MAC-T cells were cultured in 96-well plates at 1×10^4 cells/well and treated with the indicated concentration (5 to 200 mg/mL) of iridoid glycoside for 24 h. Then, the MAC-T cells were incubated with 10 μ L of CCK-8 per well in a cell culture incubator for 2 h, and the absorbance of each well was detected at 450 nm with a microplate reader (Dynatech Laboratories, United States).

Griess assay: Nitric oxide (NO) levels production was tested in the supernatant of MAC-T cultures using the Griess reagent Nitric Oxide Assay Kit by detecting nitrite levels (Kierner & Vollmar, 1997). Briefly, MAC-T cells were cultured in 96-well plates at 1×10^4 cells/well and treated with the indicated concentration (0.5, 1, 3 MIC) of iridoid glycosides at 37 °C and 5% CO₂ for 24 h. In addition, the MAC-T cells were pretreated with iridoid glycosides for 3 h and infected with *S. aureus* for 12 h. The absorbance was read at 540 nm in a microplate reader. Nitrite level represents the NO production.

Enzyme-linked immunosorbent assay (ELISA): The serum levels of TNF- α , IL-6, and IL-1 β in the supernatants were determined using ELISA Kits. All samples were measured according to the manufacturer's instructions. The intra-assay tested one serum 6 times in the same plate at the same period. The inter-assay tested one serum 6 times in each plate and each time.

Real-time PCR: The primer sequences for real-time PCR are shown in Table 1. Total RNA was extracted from the cells using RNAiso Plus (TaKaRa, Dalian, P. R. China, cat. 9109) in accordance with the manufacturer's instructions. The assessment of the quantity and quality of RNA was verified using a NanoDrop 1000 (Thermo Scientific, Co., Ltd., P. R. China). The 260: 280 nm optical density value was

between 1.8 and 2.0. The first-strand cDNA was synthesized using a PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Dalian, P. R. China, cat. RR047A). Quantitative real-time PCR was performed using TB Green Premix Ex Taq™ II (TaKaRa, Dalian, P. R. China, cat. RR820B) on a 7500 Real-Time PCR system (Applied Biosystems Inc., Foster City, CA, USA). GAPDH was used as the reference gene. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1 Quantitative real-time PCR primer information.

Genes	Accession Number	Sequence
TNF- α	NM_173966.3	F:5'-GGTGGTGGGACTCGTATGCCAATGC-3' R:5'-GTGAGGAACAAGGGGGTGG-3'
IL-6	NM_173923.2	F:5'-ACAGCTATGAACTCCCGCTT-3' R:5'-TCTCACATATCTCCTTTCTCATTGC-3'
IL-1 β	XM_005889988.2	F:5'-TCGAAACGTCCTCCGACGAG-3' R:5'-TGAGAGGAGGTGGAGAGCCT-3'
GADPH	NM_001034034.2	F:5'-AGATGGTGAAGGTCGGAGTG-3' R:5'-CGTTCTCTGCCTTGACTGTG-3'

Result

Iridoid glycosides show bactericidal activity against *S. aureus*: The antibacterial activity of iridoid glycosides against *S. aureus* was detected by the agar diffusion method. It was found that iridoid glycosides produced a zone of inhibition against *S. aureus*, while the control had no zone of inhibition, indicating that iridoid glycosides have antibacterial activity against *S. aureus*. Furthermore, the MIC results showed that the

Statistical analysis: All data are expressed as the mean and standard error. Group comparisons were performed by one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons, which was used to explore the effect of Iridoid glycosides on *S. aureus* and *S. aureus*-induced inflammation in MAC-T cells. Statistical analyses were performed using GraphPad Prism version 6.01 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was declared as $*p<0.05$, $**p<0.01$, and $***p<0.001$.

MIC of iridoid glycoside against *S. aureus* was 10.4 mg/mL (Figure 1A). The killing curve results show that Iridoid glycosides (1×MIC) had relatively stable inhibition of *S. aureus* within 5 h. Iridoid glycosides (3×MIC) killed 99.9% of *S. aureus* within 1 h and produced good bactericidal activity. The above experiment results showed that iridoid glycosides have good bactericidal activity against *S. aureus* (Figures 1B and 1C).

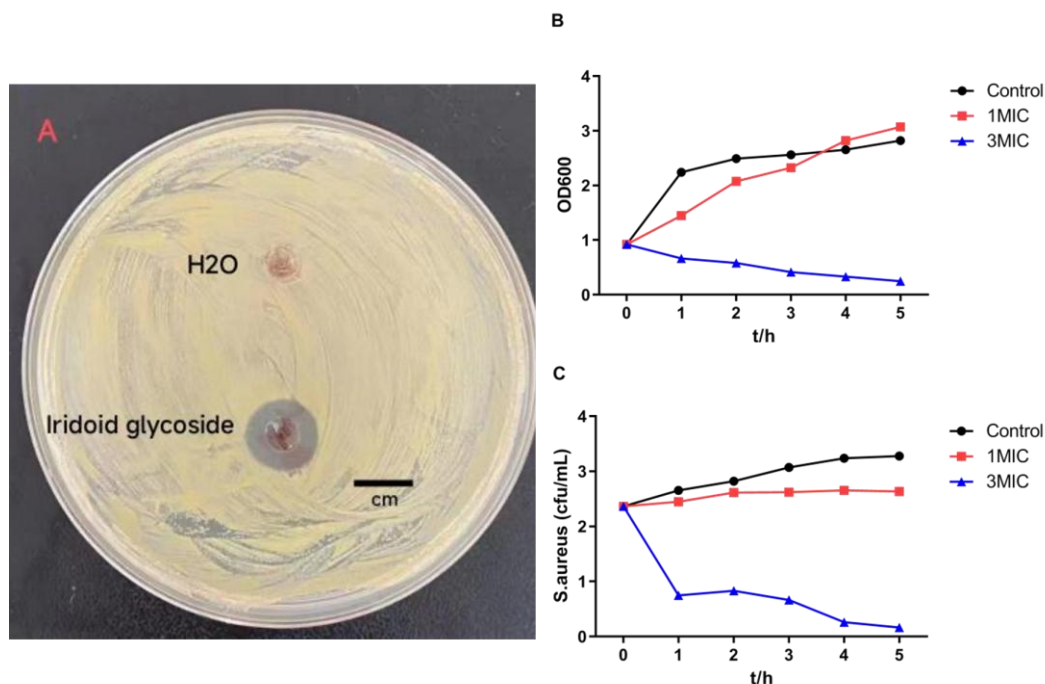


Figure 1 Detection of the bactericidal activity of iridoid glycosides. (A) The agar diffusion test of the antimicrobial activity of iridoid glycosides against *S. aureus*. (B) OD600 measurement of bacterial culture after the action of iridoid glycosides at different times. (C) The determination of the number of viable bacteria after the action of iridoid glycosides at different times.

Iridoid glycosides destroy the integrity of the bacterial cell membrane, change the membrane potential, and release cell contents: In the previous experiments, it was shown that iridoid glycosides have killing activity

against *S. aureus*. To further explore the *S. aureus* killing mechanism of iridoid glycosides, the effect of iridoid glycosides on the cell membrane structure of *S. aureus* was studied. A BCA protein quantitation kit was used

to determine the changes in the total protein content of the bacterial supernatant after the action of iridoid glycosides. The result showed that the total protein content of the bacterial culture supernatant was significantly higher than that of the control group after 5 min of iridoid glycosides acting on *S. aureus* ($P < 0.01$) (Figure 2A).

The effect of iridoid glycosides on the integrity of *S. aureus* biofilms by electric conductivity assay. Biofilm results indicated that during the whole experiment period, there was little change in the relative electric conductivity of the control group. However, both 1×MIC and 3×MIC groups showed a significant increase in a dose-dependent manner after 2 h of iridoid glycosides treatment (Figure 2B). These results indicated that iridoid glycosides treatment caused the leakage of intracellular electrolytes, including K^+ , Ca^{2+} , Na^+ , and so on.

The effect of iridoid glycosides on *S. aureus* biofilm formation was further observed by SEM. Iridoid glycosides (3×MIC) were incubated with *S. aureus* for 12 h. In the blank control, the biofilm formed by *S. aureus* was dense, and the gaps between bacteria were small. But after the action of iridoid glycosides (3×MIC), *S. aureus* biofilm formation was significantly reduced, and the structure was loose (Figure 2C).

Iridoid glycosides reduce NO release and inhibit inflammatory cytokine expression: Iridoid glycosides cytotoxicity was evaluated by CCK-8 assay after incubating MAC-T for 24 h. The results showed that iridoid glycoside (<100 mg/mL) had no cytotoxic effects on MAC-T (Figure 3A).

In this study, we measured the effects of iridoid glycosides on NO production induced by *S. aureus* (Figure 3B). The results showed that stimulation of MAC-T with *S. aureus* significantly increased the production of NO, and the increase in NO production was suppressed by iridoid glycosides extract in a concentration-dependent manner. The persistent expression of iNOS leads to the production of NO.

Cytokines play a dominant role in inflammatory response. To investigate the anti-inflammatory effects of iridoid glycosides, the expression of inflammatory factors in cell supernatants was measured by RT-PCR and ELISA. As shown in Figures 3(C, D, E, F, G, H), *S. aureus* significantly upregulated TNF- α , IL-6, and IL-1 β gene and protein expression compared to control and iridoid glycosides slightly increased TNF- α , IL-6, and IL-1 β gene and protein expression with an. However, the addition of iridoid glycosides reduces the gene and protein expression levels of inflammatory factors, and iridoid glycosides inhibited the expression of inflammatory mediators in *S. aureus*-stimulated MAC-T in a concentration-dependent manner.

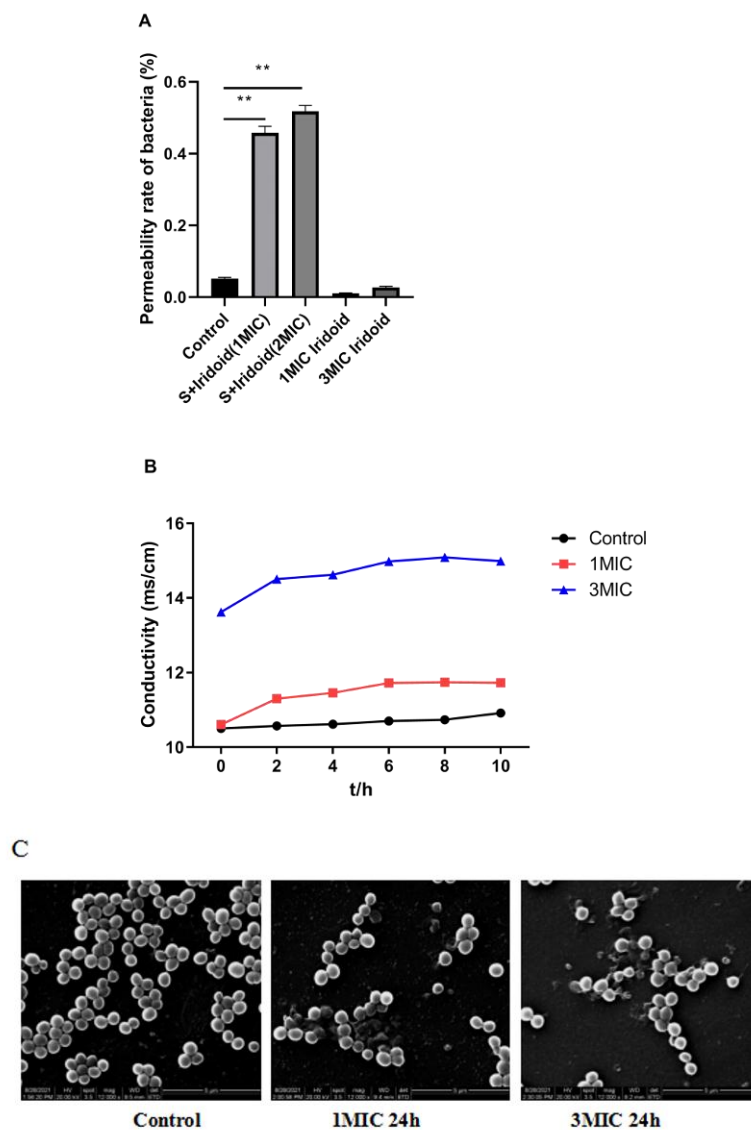


Figure 2 The mechanism of action of iridoid glycosides activity on *S. aureus*. (A) The BCA kit was used to detect the results of total protein content in bacterial supernatant. (B) An electric conductivity assay was used to detect changes in the membrane potential of *S. aureus* after iridoid glycosides treatment. (C) After *S. aureus* was exposed to iridoid glycosides, scanning electron microscopy was used to observe the biofilm. Error bars indicate the mean $\pm$ SEM, n = 3. Statistical significance was defined as $**p < 0.01$.

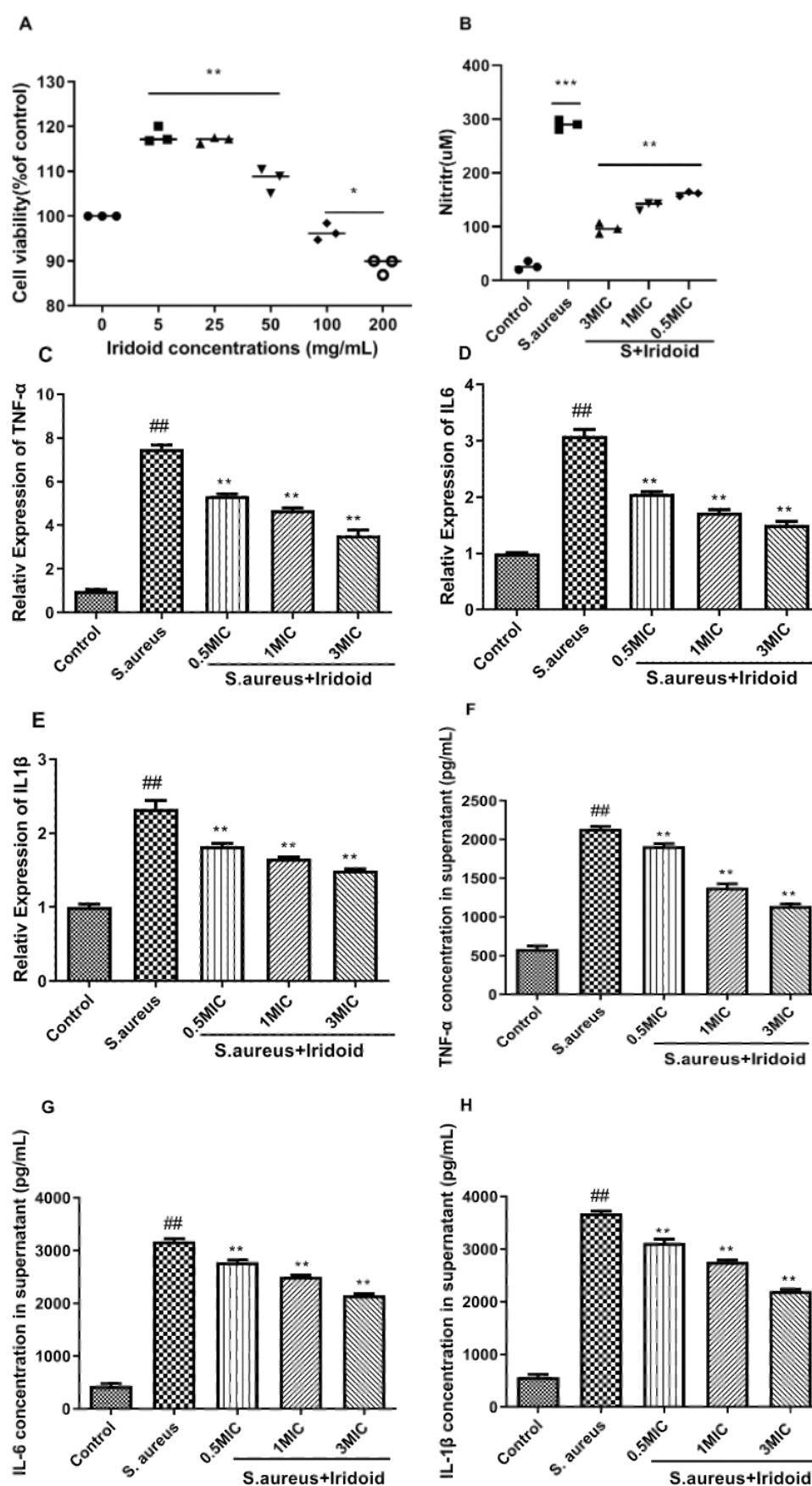


Figure 3 Iridoid glycosides do not induce cytotoxicity and alleviate inflammation in MAC-T cells. (A) Cell viability was measured using the CCK-8 assay. (B) The release of NO from MAC-T cells after treatment with different concentrations of iridoid glycosides. (C, D, E) The mRNA expression of TNF- α , IL-6, and IL-1 β after iridoid glycosides treatment. (F, G, H) The protein expression of TNF- α , IL-6, and IL-1 β after iridoid glycosides treatment. Error bars indicate the mean $\pm$ SEM, $n = 3$. Statistical significance was defined as $**p < 0.01$ and $***p < 0.001$. $##p < 0.01$ vs. control group, $*p < 0.05$, $**p < 0.01$ vs. *S. aureus* group.

Discussion

The virulence of *S. aureus* is primarily due to its ability to produce enterotoxins and bacterial biofilm (Figueiredo *et al.*, 2017). Moreover, biofilm formation can generate bacterial resistance to several classes of antimicrobial agents, which has led to the control of *S. aureus* infections increasingly difficult (Stevane, 2003; Dantas *et al.*, 2008; Davies, 2003). Additionally, the emergence of antibiotic-resistant strains imposes a high risk to consumers (Muto *et al.*, 2003). Hence, there is an urgent need to inhibit bacterial biofilm formation to control bovine mastitis.

Plant-derived drugs are new biologically active agents with antimicrobial activity (Gomes & Henriques, 2016) and are used in the prevention and treatment of mastitis (Xu *et al.*, 2021a). For example, the plant extract of ursolic acid inhibited the biofilm formation of *E. coli* (Ren *et al.*, 2005), and the ethanol extract of *Sanguisorba officinalis* strongly inhibited the biofilm formation of methicillin-resistant *Staphylococcus aureus* (MRSA) (Chen *et al.*, 2015). The phenolic extract of *T. Officinale* has an inhibitory effect on *Staphylococcus aureus* by destroying the integrity of the cell walls and cell membranes (Xu *et al.*, 2021b). In this study, we demonstrated the antibacterial and antibiofilm activity of iridoid glycoside against *Staphylococcus aureus*. The minimum inhibitory concentration (MIC) of iridoid glycosides against *S. aureus* was 10.4 mg/mL. Furthermore, after the action of iridoid glycosides, the electrical conductivity of *S. aureus* (1MIC and 3MIC) was increased. The conductivity of bacteria is closely related to the permeability and integrity of their cell membranes. When the bacterial cell membrane is damaged, electrolytes (such as potassium ions) inside the cell might leak out, leading to an increase in the conductivity of the culture medium. This phenomenon can indirectly reflect changes in bacterial intracellular conductivity by measuring changes in the conductivity of the culture medium. This result indicated that iridoid glycosides have inhibitory effects on the biofilm of *S. aureus*. At the same time, it weakened the inflammatory effect induced by *S. aureus*.

Inflammation is an essential protective response to the host against internal and external environmental stimuli (Vendramini-Costa & Carvalho, 2012). Through toll-like receptors (TLRs), macrophages can recognize pathogen-associated molecular patrons (PAMP) as bacterial lipoteichoic acid (LTA). In response to LTA, macrophages release soluble pro-inflammatory mediators such as IL-1, IL-6, TNF- α , and NO (McGettrick & O'Neill, 2013), which activate the mobilization and infiltration of leukocytes at the site of injury. NO is a pro-inflammatory mediator synthesized from L-arginine by the action of the inducible nitric oxide synthase (iNOS). In pathological conditions, NO acts as bactericidal tumoricidal and can induce cytotoxicity and apoptosis (Vilar *et al.*, 2014; Coleman, 2001). In addition to NO, TNF- α , IL-6, and IL-1 β are important cytokines responsible for the regulation of multiple immunological functions (Turner *et al.*, 2014). A study performed by Jin *et al.* (2009) revealed that ethyl acetate extract of *R. glutinosa* in LPS-induced RAW 264.7 Cells had anti-

inflammatory activities. In this study, we demonstrated the ability of iridoid glycoside from *R. glutinosa* to inhibit the NO, TNF- α , IL-6, and IL-1 β production.

In conclusion, the antibacterial constituents and antibiofilm anti-inflammatory activity of iridoid glycosides were investigated in this study. Our results showed that iridoid glycosides significantly reduced the biomass and metabolic activity of *S. aureus* biofilm in a dose-dependent manner, indicating the excellent inhibitory effect of iridoid glycosides on the biofilm formation by *S. aureus*. SEM analysis verified that iridoid glycosides induced dramatic destruction of the architecture and morphology of *S. aureus* biofilm. Iridoid glycosides could be an alternative treatment for mastitis to simultaneously regulate the spread or infection of bacteria and to reduce the adverse effect of *S. aureus*. Owing to the safety of this extract, it can be applied as an intramammary infusion to treat local infection or as a dietary product to control systemic infection. A field study based on these laboratory results is still required to confirm the safety and to evaluate the efficacy of iridoid glycosides to reduce the degree of *S. aureus*-induced cell death in vivo, which, if successful, could provide a basis for a novel approach for bovine mastitis therapy in the future.

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Conflict of interest: All authors had no conflicts of interest to declare.

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