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RESEARCH ARTICLE

# *Medulla Tetrapanacis* water extract ameliorates mastitis by suppressing bacterial internalization and inflammation via MAPKs signaling *in vitro* and *in vivo*

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## Abstract

*Medulla Tetrapanacis* (MT) is a commonly used herbal ingredient in soup to promote lactation and management of mastitis among lactating mothers worldwide, particularly in Asian countries. However, scientific evidence to support its usage in the management of mastitis is limited. This study aimed to investigate the effects of MT water extract and its underlying mechanisms in *Staphylococcus aureus* (SA)–induced mastitis in human mammary epithelial cells (HuMEC) and lipopolysaccharide (LPS)–induced mastitis in lactating Sprague–Dawley (SD) rats. The anti-inflammatory and antibacterial effects of MT water extract were examined in SA-infected HuMEC by ELISA and plate counting, respectively. The effects of MT water extract on blood-milk barrier and the underlying mechanism of action of the MT water extract were also investigated by transendothelial electrical resistance assay and western blot. The effects and mechanisms of MT water extract on alleviating mastitis on SD rats were evaluated using LPS-induced mastitis rat model. Our results showed that MT water extract could

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suppress SA-induced mastitis by reducing SA internalization and growth, protecting blood-milk barrier integrity, and attenuating release of cytokines (interleukin 6 [IL-6] and tumor necrosis factor-alpha [TNF-$\alpha$]) in HuEMC. Furthermore, the antibacterial effect might be related to the increase of antimicrobial peptides transcription, and the anti-inflammatory effect is at least partly mediated by inactivation of p38 and JNK signaling pathways. Finally, our *in vivo* results showed that MT water extract ameliorated LPS-induced mastitis in SD rats via suppressing inflammatory cytokine release (TNF-$\alpha$, IL-6, and interleukin-1 beta), myeloperoxidase, and alleviating pathohistological damage by downregulation of JNK signaling pathway.

**KEYWORDS**

antimicrobial peptide, blood–milk barrier, inflammation, MAPK-signaling pathway, mastitis, *Medulla Tetrapanacis*

## 1 | INTRODUCTION

Exclusive breastfeeding for first 6 months is highly recommend to mothers by World Health Organization (WHO, 2001) as breast milk is considered the best diet containing adequate nutrient, including macromolecules and minerals for the infants. Breast milk also contains bioactive components, for example, nucleotides, lactoferrin, lysozyme, human milk oligosaccharides, and antibodies derived from breastfeeding mothers that can enhance gut microbiome, promote the development of gastrointestinal system, and strengthen the immune system of the babies (Boquien, 2018; Qu et al., 2023). In addition, it has been reported that breast milk diet could lower the risk of chronic diseases such as obesity, diabetes, asthma, and diarrhea (Carr et al., 2021). Apart from having beneficial effects on infants, when compared with formula-feeding mothers, breastfeeding mothers have lower risk of developing ovarian and breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer, 2002; US Department of Health Human Services, 2011). Although there is no doubt that breastfeeding is the best feeding practice with substantial benefits to both the mother and baby, the current worldwide exclusive breastfeeding rate is only 48%, which is far behind the proposed target aiming to reach 70% in 2030 (WHO, 2023). There are numerous reasons for early cessation of breastfeeding, such as insufficient milk supply, lack of sleep, latch problems, pain from cracked nipples, and breast engorgement. Mastitis and its related discomfort are one of the major causes directly leading to breastfeeding failure (Morrison et al., 2019; Wilson et al., 2020).

Mastitis is referred to a common inflammatory breast condition primarily caused by milk stasis with or without infection with mainly *Staphylococcus epidermidis* and *Staphylococcus aureus* (SA) (Boakes et al., 2018). Manifestations of mastitis, including breast swelling, breast redness, breast pain, and systemic symptoms, including fever and chill (Louis-Jacques et al., 2023)*. A systematic review revealed that mastitis afflicted 25% of lactating women during their 26 weeks postpartum (Wilson et al., 2020). Without appropriate and timely management, mastitis could result in severe complications such as breast abscess and septic shock (Boakes et al., 2018; Pevzner & Dahan, 2020). Currently, treatment of mastitis extensively relies on supportive measures, for instance, milk pumping, message, and administration of analgesic drugs and antibiotics. However, the efficacy of supportive treatments, like massage and antibiotics, is non-conclusive (Anderson et al., 2019; Jahanfar et al., 2013), and the use of antibiotics arouses safety concern from the parents, leading to lower compliance with exclusive breastfeeding (Grzeskowiak et al., 2022). Accumulating investigations have been conducted to study the anti-inflammatory and antimicrobial properties of food and phytochemical constituents isolated from plants (e.g., herbs) for developing functional food or supplements to ameliorating inflammatory diseases (Dhakal et al., 2023; Kaviya et al., 2023; Mei & Chen, 2023; Zeng et al., 2022). This surges people seeking alternative treatments (e.g., herbal food) that are safe and effective in resolving mastitis without compromising exclusive breastfeeding.

The use of herbal medicine, including traditional Chinese (TCM) medicine among breastfeeding mothers for improving lactation and alleviating mastitis, has become prevalent in the world, particularly in Europe (e.g., Switzerland and Italy), Asian countries, and Australia (Bettiol et al., 2018; Kennedy et al., 2013; Sim et al., 2013). *Medulla Tetrapanacis* (MT) belongs to the Araliaceae family, which is the dried stem pith of *Tetrapanax papyrifer* (Hook.) K.Koch, well known as rice paper plant pith and Tongcao in Chinese (Xi & Gong, 2017). MT is traditionally used as a diuretic and galactagogue (Xi & Gong, 2017), whereas it is one of the most commonly used TCM herbs among breastfeeding mothers in Taiwan and Macau for reliving mastitis and promoting lactation, as reported by two recent cross-sectional studies (Chao et al., 2021; Zheng et al., 2020). Despite the popularity of MT among breastfeeding mothers, there is no scientific evidence to support the use of it in alleviating mastitis. Although our recent study has shown that MT water extract possesses antibacterial and anti-inflammatory effects in murine macrophages (Kwok et al., 2023), whether it could protect human mammary epithelial cells (HuMEC) from SA infection and lipopolysaccharide (LPS)–induced mastitis *in vivo* remains to be studied. Mammary epithelial cells (MEC) are widely used to study mastitis and the host response by SA infection (H. Wang et al., 2019; R. Wang et al., 2021). Numerous studies have reported that SA-induced mastitis triggers the release of inflammatory cytokines, such as interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-$\alpha$) to cause mammary

tissue damage (e.g., disrupt the integrity of blood-milk barrier) and inflammation (Wilson et al., 2020; C. Zhao et al., 2021). In addition, the inhibition of SA internalization (non-professional phagocytosis) in MEC has been found to alleviate mastitis by resolving inflammation. In addition, antimicrobial peptides (AMPs), such as beta-defensin-1, lipocalin-2, and cathelicidins secreted by MEC, have also been shown to participate in combating SA invasion during mastitis (Cavalcante et al., 2020; Ochoa-Zarzosa et al., 2009; Sharma et al., 2016). Increasing number of studies revealed that herbs and phytochemical constituents isolated from natural source against mastitis by attenuating inflammation (Ge et al., 2021), repairing damaged blood-milk barrier (Kan et al., 2019), and preventing SA internalization (Wei et al., 2014). Apart from SA, LPS has also been commonly used to induce mastitis in rodents (Li et al., 2018; Mingfeng et al., 2014). Natural products and their constituents have been reported to suppress LPS-induced inflammatory cytokines (TNF-$\alpha$, IL-6, and interleukin-1beta [IL-1$\beta$]) and myeloperoxidase (MPO) in mammary glands and improve the abnormal histopathological changes, including immune cell infiltration and cell necrosis (Li et al., 2018; Zhao et al., 2023). In this study, we hypothesized that MT water extract could protect SA-induced mastitis by repairing tight junction damage, inhibiting SA internalization by stimulating AMPs, and attenuating the release of inflammation mediators by inactivation of the Mitogen-activated protein kinase (MAPK) pathway in HuMEC. This study also aimed to evaluate the pharmacological effects of MT extract in LPS-induced mastitis in rats.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemical and reagents

HuMEC, HuMEC ready medium, HuMEC supplement kit, penicillin/streptomycin (PS), TrypLE express enzyme, and thiazolyl blue tetrazolium bromide (MTT) were obtained from Gibco. TRIzol reagent was purchased from Invitrogen. LightCycler 480 SYBR green I master, LightCycler 480 white multiwell plate 96, and sealing foil were bought from Roche. PrimeScript RT reagent kit with gDNA eraser (Perfect Real Time) was obtained from Takara Biochemicals. Human TNF-$\alpha$ and human IL-6 ELISA kits were obtained from PeproTech.

Rat IL-6 uncoated ELISA kit, rat IL-1$\beta$ uncoated ELISA kit, and rat TNF-$\alpha$ uncoated ELISA Kit were purchased from Invitrogen. Rat MPO ELISA kit was obtained from Solarbio. LPS from *Escherichia coli* O55:B5 was obtained from Sigma-Aldrich. Polyester (PET) membrane tissue culture plate inserts were bought from Jet Biofil. Environment-friendly dewaxing transparent liquid and hematoxylin and eosin (H&E) staining kits were purchased from Servicebio.

RIPA (Radioimmunoprecipitation assay buffer) lysis buffer, bovine serum albumin (BSA), and the Sodium dodecyl sulfate (SDS) sample buffer were obtained from Beyotime. Dimethyl sulfoxide was bought from Duksan. Pierce rapid gold BCA protein assay kit, SuperSignal West Pico PLUS Chemiluminescent Substrate, restore plus western blot stripping, phenylmethylsulfonyl fluoride (PMSF), and Halt protease and phosphatase inhibitor cocktail were bought from Thermo Scientific. Antibodies against phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, phospho p44/42 MAPK (Erk1/2) (Thr202/Tyr204), p44/42 MAPK (Erk1/2), phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK, phospho-I$\kappa$B (Ser32/36), I$\kappa$B, phospho-NF-$\kappa$B p65 (Ser536), phospho-NF-$\kappa$B p65 (Ser536) (93H1), and NF-$\kappa$B p65 (D14E12) were bought from Cell Signaling Technology. Primary antibodies against ZO-1 and occludin were obtained from Invitrogen. $\beta$-Actin was purchased from ABclonal. Anti-mouse secondary antibody with horseradish peroxidase (HRP)–conjugated immunoglobulin G (IgG) was purchased from Cell Signaling Technology. Anti-rabbit secondary antibody with HRP-conjugated IgG was bought from Thermo Scientific.

### 2.2 | Preparation of water extract of *Medulla Tetrapanacis* (MT) and quantification of phytochemical constituents

MT water extract was prepared with reference to our previous study (Kwok et al., 2023). Our previous study found that MT water extract contain L-pyroglutamic acid, adenosine, phytosphingosine, and aurantiamide acetate (Kwok et al., 2023). Hence, in this study, the amount of these compounds in MT water extract was determined by high-performance liquid chromatography (HPLC). Waters e2695 HPLC System with a Waters 2489 UV/Vis detector (Waters Corp.) was used to determine the content of aurantiamide acetate (conditions detail in Table S1). Thermo Scientific UltiMate 3000 with a diode-array detector (DAD) (Thermo Fisher) was used to quantify the quantity of adenosine (conditions detail in Table S2). Thermo Scientific Ultimate 3000 with MSQ plus mass detector (Thermo Fisher) was employed to determine the amount of phytosphingosine (conditions detail in Table S3). Ultimate 3000 with a DAD was also used to quantify -pyroglutamic acid (conditions detail in Table S4).

### 2.3 | Cell culture of HuMEC

HuMEC cells were bought from Gibco. HuMEC cells were maintained in HuMEC ready medium with HuMEC supplement kit (HuMEC complete medium) and 1% PS at 37°C in incubator with 5% $CO_2$ and humidified air. They were routinely sub-cultured with TrypLE express enzyme every 5–6 day or seeded into 6 well plates or 96 well plates upon reaching 70%–80% confluency.

HuMEC cells were treated with different treatment conditions (details are indicated in figures legends) to examine the cytotoxicity of MT water extract and the anti-inflammatory and antibacterial effects of MT water extract as well as the ability of MT water extract on repair the blood-milk barrier in SA-induced mastitis.

### 2.4 | Animal study

Animal study was conducted according to the protocol approved by the animal ethics committee of the Hong Kong Polytechnic University (No.: 23-24/715-FSN-R-STUDENT, SZ CAF only). Specific pathogen-free female Sprague–Dawley (SD) rats after 7 days of laboring were purchased (3.5 months old) from Guangdong Sijiajingda Biotechnology Co. Ltd. After 1 week acclimation, a total of 37 lactating female rats

were randomly assigned into the 6 following groups; control (saline, $n = 6$), LPS group (0.1 mg/mL, $n = 6$), dexamethasone + LPS group (Dex, 5 mg/kg, $n = 6$), low-dose MT + LPS group (360 mg/kg, $n = 6$), middle-dose MT + LPS group (720 mg/kg, $n = 6$), and high-dose MT + LPS group (1440 mg/kg, $n = 7$). The experiment scheme is displayed in Figure 8a. Before intramammary injection of LPS injected into the fourth on the right [R4] and the fourth on the left [L4] of the mammary glands under isoflurane anesthesia, different dosages of MT water extract were given to rats oral gavage 2-h before LPS, whereas Dex was intramuscularly injected into rats 1-h before LPS injection. The same practice was also adopted before $CO_2$ sacrifice. After 24-h LPS challenge, all rats were sacrificed by $CO_2$ overdose. The mammary tissue of all rats was collected either snap freezing by liquid nitrogen and storage at −80°C or fixed in 4% paraformaldehyde for subsequence analysis.

## 2.5 | Histopathological examination

Fresh mammary tissues were obtained from rats and fixed in 4% paraformaldehyde for 24–48 h at room temperature, followed by dehydration. After that, fixed tissues were embedded in paraffin followed by sectioning with microtome CryoStar NX50 (Thermo Fisher) at a thickness of 4 µm. The paraffin sections were dewaxed in environment-friendly dewaxing transparent liquid (Servicebio) and were dehydrated with gradient ethanol. Sections were stained with H&E Staining Kit (Servicebio) for 5 min. Eventually, the sections were dehydrated and sealed, and their histological details were visualized under an upright optical microscope Nikon Eclipse E100 equipped with a camera Nikon DS-U3 (Nikon) with 40× magnifications.

## 2.6 | Cell viability assay

The cytotoxicity of MT water extract on HuMEC was examined by MTT assay. Briefly, HuMEC cells (10,000 cells/mL) were treated with various concentrations of MT water extract (0.0625 8 mg/mL) for 24-h in incubator at 37°C. After 24-h, medium was aspirated and replaced with 100 µL of MTT solution (final concentration: 0.5 mg/mL) (Gibco) for 2-h incubation at 37°C. After 2-h of incubation, the MTT solution was discarded and 100 µL of DMSO was added to dissolve the violet crystal with gentle shaking. The absorbance of the samples was measured at 570 nm with 650 nm as a reference wavelength using a microplate reader (BMG Labtech CLARIOstar). Percentages of cell viability were calculated based on the following equation: absorbance of sample/absorbance of control × 100%.

## 2.7 | SA internalization and inhibition assays

HuMEC cells were seeded at 500,000 cells/mL at 6 well plates overnight in HuMEC complete medium without 1% PS. After aspirating culture medium and washing with Phosphate-buffered saline (PBS), HuMEC cells were pre-treated with or without 2 mg/mL MT for 2-h, followed by co-culturing with multiplicity of infection 100 of SA (ATCC 25923, SA) in HuMEC complete medium without 1% PS for 2-h with or without accompanied 2 mg/mL MT treatment. After 2-h post infections, cells were washed twice with 200 µg/mL gentamicin in HuMEC complete medium for 15 min to remove uninternalized SA. Cells were either lysed with 0.1% Triton X-100 in LB for CFU counting (internalized bacteria) or cultured for another 24-h with HuMEC complete medium (PS free) and 2 mg/mL MT (SA inhibition assay). After 24-h post infections, the extracellular SA was collected for CFU counting. Similarly, the internalized bacteria after 24-h were also counted. All the conditioned mediums (both 2- and 24-h treatments) were collected for measurement of cytokine level by ELISA.

## 2.8 | Transepithelial electrical resistance (TEER)

The integrity of tight junction of the monolayer HuMEC was evaluated using the transepithelial electrical resistance (TEER) assay with Millicell ERS-2 Voltohmmeter (Millipore) referencing a previous study with sight modifications (Yang et al., 2018). In brief, HuMEC cells were seeded at 90,000 cells/mL at transwell insert (PET membrane with 1.12 $cm^2$ growth area, Jet Biofil) placing in 24-well plates. After the formation of a monolayer (overnight), the HuMEC cells were subject to SA infection (Section 2.7). The TEER on the inside and outside of the apical wells was measured according to the manufacturer's instructions after 2- and 24-h post infection. TEER values were calculated by TEER = transwell resistance × growth area of the insert ($\Omega\ cm^2$).

## 2.9 | Evaluation of cytokines and MPO secretion

The conditional medium collected from the SA internalization and SA inhibition assays was subject to ELISA assays of human TNF-$\alpha$ and IL-6 using commercially available kits (PeproTech) according to the manufacturer protocol. An amount of 100 mg mammary tissues collected from the animal study were homogenized with beads by Precellys Evolution homogenizer (Bertin) for three milling cycles at 4°C, and each cycle consisted of 30 s milling at 6000 rpm and 10 s pause. After homogenization, samples were centrifugated at 12,000 rpm at 4°C for 10 min. The collected supernatants were subject to ELISA assays of rat IL-6, IL-1$\beta$, TNF-$\alpha$, and MPO using commercially available ELISA (Invitrogen) (MPO: Solarbio) according to the manufacturer protocol.

## 2.10 | RNA extraction and reverse transcription

HuMEC (500,000 cells/mL) was seeded into six well plates then followed by the treatment mentioned above (SA inhibition assays). After 24-h incubations, total RNA extraction in HuMEC was done by adding TRIzol with reference to the manufacturer's protocol. NanoDrop One Microvolume UV–Vis Spectrophotometer was employed to determine the concentration and quality of purified RNA. PrimeScript RT Reagent Kit with gDNA Eraser was used to convert 500 ng RNA into complementary DNA (cDNA) according to the manufacturer's protocol.

### 2.11 | Real-time quantitative polymerase chain reaction (qPCR)

Real-time quantitative polymerase chain reaction (qPCR) was performed using converted cDNA with LightCycler 480 SYBR Green I Master and LightCycler-480 II Instrument (Roche) to quantify AMP-related genes, including the beta-defensin-1, lipocalin-2, and cathelicidin levels. The results were normalized to the beta actin as an internal control for RNA expressions through Ct $2^{-\Delta\Delta}$ value. The primers for qPCR were listed in Table S5.

### 2.12 | Western blot assay

After 2-h post infection (details are indicated in figures legends), HuMEC cells were lysed in RIPA lysis buffer with 1 mM PMSF and protease and phosphatase inhibitor cocktail on ice for 5 min followed by homogenization with needle, then centrifugation at 12,000 rpm at 4°C for 10 min to obtain protein extract. Mammary tissue from rats was lysed and homogenized with beads in RIPA lysis buffer with 1 mM PMSF and protease and phosphatase inhibitor cocktail by Precellys Evolution homogenizer (Bertin) for 3 milling cycles at 4°C, each cycle consisted of 30 s milling at 6500 rpm and 10 s pause. After homogenization, samples were centrifugated at 12,000 rpm at 4°C for 10 min to collect protein supernatants. Protein concentrations were determined using Pierce rapid gold BCA Protein Assay Kit (Thermo Fisher). Samples were equal loaded and separated on SDS-polyacrylamide gel. After electrophoresis (100 V, 90 min), the separated proteins were transferred to polyvinylidene difluoride membranes using wet transfer. Non-specific sites were blocked with 5% BSA for 1 h, and the blots were then incubated with phospho-NF-κB p65 (Ser536), NF-κB p65, phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), p44/42 MAPK (Erk1/2), phospho-p38 MAPK (Thr180/Tyr182), p38 MAPK (Cell Signaling Technology), ZO1, occludin (Invitrogen), and β-actin (ABclonal) at 1:1000 in 5% BSA in TBST overnight at 4°C. Corresponding anti-rabbit and anti-mouse secondary antibodies with HRP-conjugated IgG, 1:2000 (Thermo Fisher) in 5% BSA in TBST (120 min, room temperature), were used to detect the binding of its correspondent antibody. β-Actin was used to verify equal loading of protein in each lane. The protein expression was detected with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher) and quantified with ChemiDoc Imaging System (Bio-Rad Laboratories).

### 2.13 | Statistical analysis

All data were expressed as means ± SEM. One-way ANOVA followed by Tukey's multiple comparison test or Student's *t* test were performed to compare the differences between groups. All statistical analyses were performed by GraphPad Prism 5.01 for Windows (GraphPad Software). $p < .05$ was considered statistically significant.

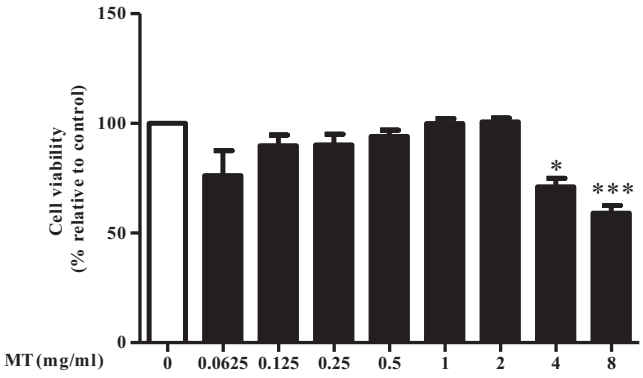


**FIGURE 1** Cytotoxicity of *Medulla Tetrapanacis* (MT) water extract on human mammary epithelial cells (HuMEC). Cells were treated with different concentrations of MT (0.25, 0.5, 1, or 2 mg/mL) for 24 h. Data were expressed as mean ± SEM, $n = 3$. One-way ANOVA followed by Tukey's multiple comparison test was performed to compare the differences between groups $^{*}p < .05$ and $^{***}p < .001$.

## 3 | RESULTS

### 3.1 | Quantification of chemical constituent in MT water extract

Our previous study showed that MT water extract contains L-pyroglutamic acid, adenosine, phytosphingosine, and aurantiamide acetate (Kwok et al., 2023). Hence, in this study, the amount of these compounds in MT water extract was determined by HPLC system. Table 1 showed that 1 g of MT water extract contained 1637.9 μg L-pyroglutamic acid, 101.19644 μg adenosine, 7.74879 μg phytosphingosine, and 57.06298 μg aurantiamide acetate.

### 3.2 | Effects of MT water extract on the cell viability of HuMEC

The cytotoxicity of various concentration of MT water extract (0.0625–8 mg/mL) on HuMEC cells was determined using MTT assay. MT water extract at 4 and 8 mg/mL showed significant cytotoxic effects to HuMEC, whereas 0.0625–2 mg/mL MT water extract did not show any cytotoxic effects (Figure 1). Therefore, 2 mg/mL MT water extract was used as the highest dosage in all the subsequent experiments.

### 3.3 | MT water extract attenuated SA invasion (internalization) and inhibited bacterial growth in SA-infected HuMEC

The effects of MT water extract on internalization and inhibition of SA in HuMEC cells were assessed by CFU plate counting. When comparing with infection group, MT water extract (2 mg/mL) significantly attenuated the internalization of SA in HuEMC cells after 2-h post infection ($p < .01, n = 5$, Figure 2a). Similarly, after 24-h post infection, MT water extract markedly reduced the number of bacteria when compared to the untreated group ($p < .01, n = 7$, Figure 2b).

**TABLE 1** Quantification of chemical constituent in *Medulla Tetrapanacis* (MT) water extract.

| Compound | Quantity (μg/g) |
|---|---|
| L-Pyroglutamic acid | 1637.92356 |
| Adenosine | 101.19644 |
| Phytosphingosine | 7.74879 |
| Aurantiamide acetate | 57.06298 |

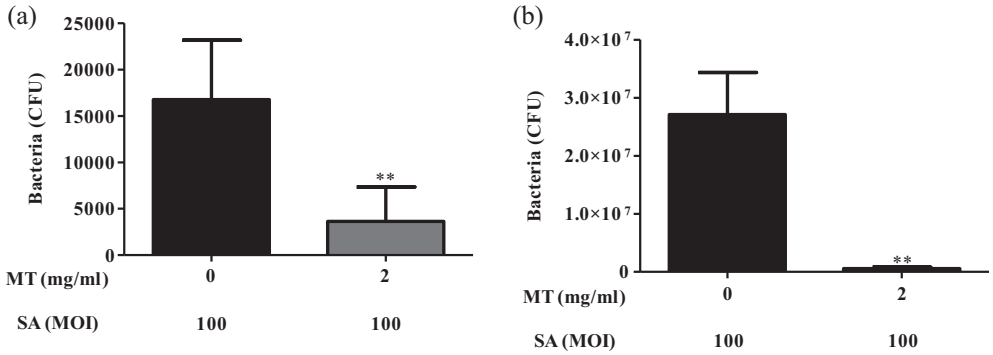


**FIGURE 2** Bactericidal effect of *Medulla Tetrapanacis* (MT) water extract (2 mg/mL) on *Staphylococcus aureus* (SA) in human mammary epithelial cells (HuMEC). (a) 2 h post infection and (b) inhibitory effect at the end of the infection period (24 h). Data were expressed as mean ± SEM: (a) invasion of SA $n = 5$ and (b) inhibitory effect on SA $n = 7$. Student's $t$ test was performed to compare the differences between groups, $^{**}p < .01$ and $^{*}p < .05$ versus SA group. MOI, multiplicity of infection.

### 3.4 | MT water extract suppressed TNF-α and IL-6 secretion in SA-infected HuMEC

The effect of MT water extract suppressing SA-induced inflammatory cytokines (TNF-α and IL-6) in SA-infected HuMEC was examined using ELISA. After 2-h post infection SA markedly increased TNF-α ($p < .01$) and IL-6 ($p < .001$) secretion from HuMEC ($n = 5$), whereas 2 mg/mL MT water extract significantly reduced SA-induced TNF-α ($p < .05$) and IL-6 ($p < .001$) (Figure 3a,b). Similarly, after 24-h post infection, SA also significantly increased TNF-α ($p < .01$, $n = 7$) and IL-6 ($p < .01$, $n = 6$) release that were significantly inhibited by 2 mg/mL MT (Figure 3c,d).

### 3.5 | MT water extract upregulated antimicrobial peptides in SA-infected HuMEC

It has been shown that AMPs, such as cathelicidins, lipocalin-2, and beta-defensin-1, inhibit SA internalization and their growth (Cavalcante et al., 2020; Kraemer et al., 2011; Sharma et al., 2016). Hence, we examined if MT water extract modulated gene expression of AMPs (e.g., cathelicidins, lipocalin-2, and beta-defensin-1) to reduce the SA internalization by qPCR. As shown in Figure 4, when compared with SA alone group, 2 mg/mL MT water extract upregulated the transcription level of beta-defensin-1 ($p < .05$, $n = 3$, Figure 4a) and lipocalin-2 ($p < .05$, $n = 4$, Figure 4b) by approximately 4.3-fold. However, MT water extract only caused a tendency to increase the gene expression of cathelicidins ($n = 4$, Figure 4c).

### 3.6 | MT water extract restored SA disrupted blood-milk barrier in HuMEC

Reduced blood-milk barrier integrity is one of the major characteristics of mastitis (Bao et al., 2023; Kan et al., 2019). The effect of MT water extract on SA interrupted blood-milk barrier was first evaluated by measuring TEER. TEER has been widely used to assess the integrity of tight junction dynamics in cell culture of endothelial or epithelial monolayers (Srinivasan et al., 2015). Figure 5a shows that SA significantly lowered the TEER of HuMEC after 2-h post infection, whereas MT water extract significantly restored the SA suppressed TEER in concentration-dependent manner ($p < .05$, $n = 4$). Similar observations were also found after 24-h post infection (Figure 5b, $p < .05$, $n = 4$). Subsequently, we evaluated the effects of MT water extract on tight junction protein expression by western blot. Figure 5c,d shows that SA significantly reduced occludin expression in HuMEC ($p < .05$, $n = 3$), whereas MT water extract restored the SA-downregulated occludin protein expression ($p < .001$, $n = 3$). However, both SA infection and MT water extract did not significantly alter ZO1 expression in HuMEC (Figure 5c,d).

### 3.7 | MT water extract inactivated MAPKs (JNK, p38) signaling pathways in SA-infected HuMEC

Previous study has shown that MT water extract suppressed inflammation in murine macrophage via reduction of MAPKs and NF-κB

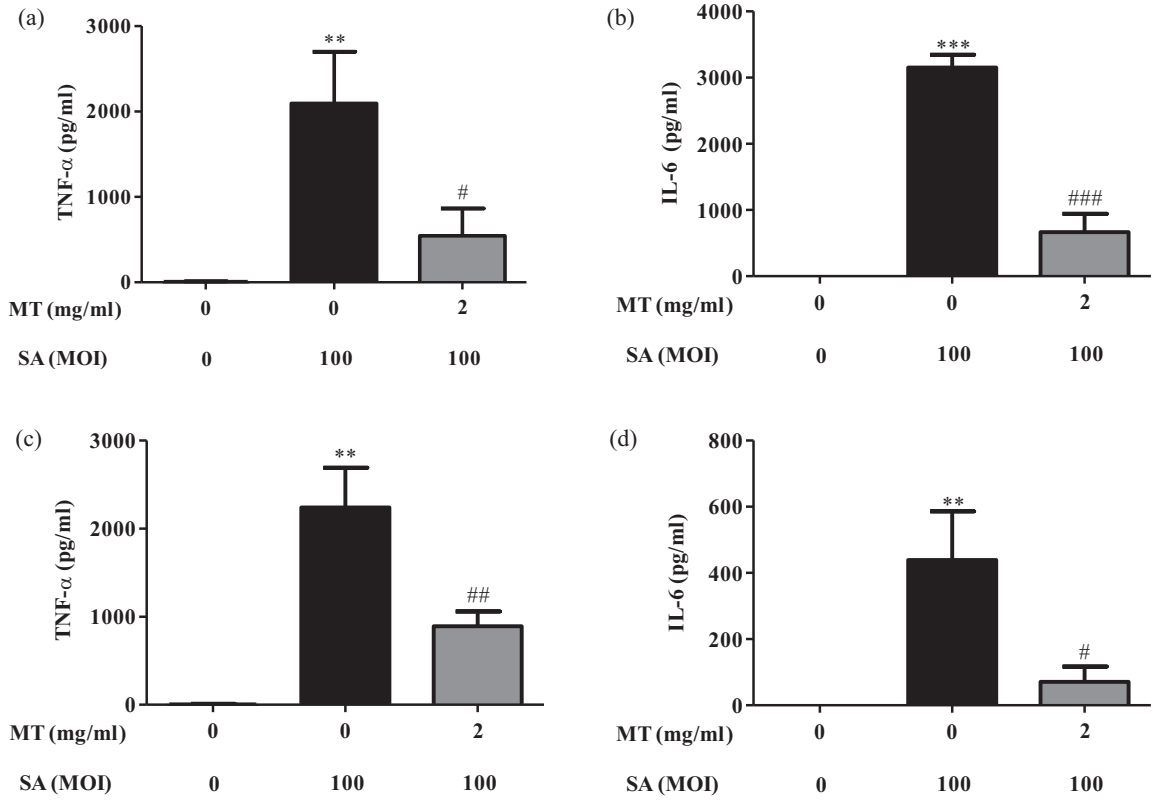


**FIGURE 3** Effects of *Medulla Tetrapanacis* (MT) water extract on *Staphylococcus aureus* (SA)–induced tumor necrosis factor-alpha (TNF-$\alpha$) and interleukin 6 (IL-6) secretion in human mammary epithelial cells (HuMEC) after 2 h of post infection (a and b) and at the end of 24 h treatment (c and d). Data were expressed as mean ± SEM; $n = 5$ (a and b), $n = 7$ (c), and $n = 6$ (d). One-way ANOVA followed by Tukey's multiple comparison test was performed to compare the differences between groups. $^{**}p < .01$ and $^{***}p < .001$ versus control and $\#p < .05$, $\#\#p < .01$ and $\#\#\#p < .001$ versus SA group. MOI, multiplicity of infection.

signaling activities (Kwok et al., 2023). Hence, we examined the effects of MT water extract on these signaling pathways in HuMEC via Western blot. Our results showed that SA significantly increased the phosphorylation of p38 ($p < .01$) and JNK1/2 ($p < .001$), MT markedly suppressed the SA-induced activation of MAPK signaling (JNK1/2 and p38, $p < .01$, $n = 3$ Figure 6a,b). However, both SA and MT did not significantly alter phosphorylation of ERK1/2 in HuMEC. MT water extract only showed modest reduction of NF-$\kappa$B p65 activity (Figure 6a,b).

## 3.8 | MT water extract improved histopathological changes in mammary tissue of LPS-induced mastitis in rats

The pharmacological effects of MT water extract on LPS-induced mastitis in rats were evaluated by histopathology via H&E staining. As shown in Figure 7a, the control group showed normal histology with a regular alignment of epithelial cells in acinar of mammary tissues (black arrow). When compared to control group, LPS induced excessive histopathological damage in mammary tissue (Figure 7b) such as severe lesion in the mammary gland cavity (green arrow), infiltration of inflammatory cells (red arrow), and extensive epithelial cell death (gray arrow). Positive control Dex suppressed LPS-induced pathological changes. Similarly, MT water extract attenuated LPS-induced pathological damages of the mammary tissue in a dose-dependent manner (Figure 7c–f).

## 3.9 | MT water extract suppressed proinflammatory cytokines and MPO in mammary tissues of LPS-induced mastitis in rats

The effects of MT water extract on LPS-induced inflammatory cytokines (TNF-$\alpha$ and IL-6) and MPO in mammary tissues from LPS-induced mastitis in rats were examined using ELISA. After 24-h of LPS (0.1 mg/mL) intramammary injection, TNF-$\alpha$ (Figure 8b), IL-6 (Figure 8c), IL-1$\beta$ (Figure 8d), and MPO (Figure 8e) were markedly increased in the mammary tissue ($p < .001$, $n = 6$). When compared with LPS group, MT water extract significantly suppressed LPS-induced TNF-$\alpha$, IL-6, IL-1$\beta$, and MPO levels in mammary tissue of rats in dose-dependent manner (Figure 8b–e, $p < .001$, $n = 6$ for all groups except 1440 mg/kg MT $n = 7$). Positive control Dex (5 mg/kg) also significantly suppressed LPS-induced TNF-$\alpha$, IL-6, IL-1$\beta$, and MPO in mammary tissue of rats (Figure 8b–e, $p < .001$).

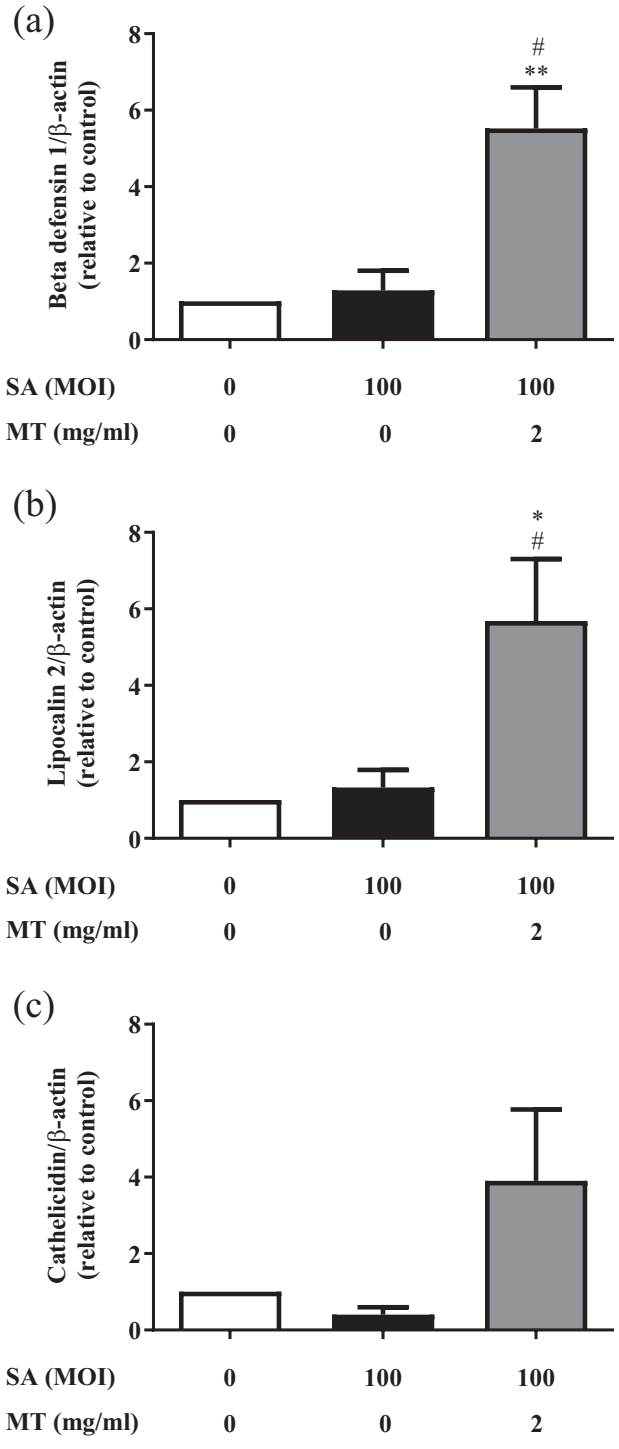


**FIGURE 4** Effect of *Medulla Tetrapanacis* (MT) water extract on antimicrobial peptide gene expression in *Staphylococcus aureus* (SA)–infected human mammary epithelial cells (HuMEC) after 24-h infection. Data were expressed as mean ± SEM; $n = 3$ (a), $n = 4$ (b and c). One-way ANOVA followed by Tukey's multiple comparison test was performed to compare the differences between groups. $^{*}p < .05$ and $^{**}p < .01$ versus control group and $\#p < .05$ versus SA group. MOI, multiplicity of infection.

### 3.10 | MT water extract inactivated MAPKs (JNK) signaling pathways in LPS-induced mastitis in rats

The underlying mechanism of MT water extract against LPS-induced mastitis in rats was studied by examining protein expression of JNK and NF-κB p65 via western blot. Figure 9 showed LPS significantly increased phosphorylation of JNK1/2 ($p < .001$, $n = 4$) and p65 ($p < .01$, $n = 3$) in mammary tissue of rats, whereas MT extract suppressed the LPS-induced activation of JNK1/2 in dose-dependent manner (720 mg/kg MT: $p < .05$ and 1440 mg/kg MT: $p < .01$, Figure 9a,b, $n = 4$). Similar to our *in vitro* results, MT water extract slightly reduced the LPS-induced phosphorylation of NF-κB p65 (Figure 9a,b, $n = 3$).

## 4 | DISCUSSION

Among numerous reasons for early cessation of breastfeeding, such as insufficient milk supply, lack of sleep, latch problems, pain from cracked nipples, and breast engorgement, mastitis and its related discomfort is one of the major obstacles to high compliance with exclusive breastfeeding (Wilson et al., 2020). Mastitis is a condition referring to inflammation of the breast that principally caused by milk stasis, which may or may not accompanied by infection (Louis-Jacques et al., 2023). A prospective cohort study reported that mastitis occurred in 20% of women during the first 6 months (Kinlay et al., 2001) and delayed or mismanagement of mastitis can lead to severe conditions such as breast abscess and sepsis, where hospitalization will be required (Pevzner & Dahan, 2020). Antibiotics are commonly used to control possible infections (Pevzner & Dahan, 2020). However, studies have showed that the use of antibiotics increased the risk of abrupt breastfeeding cessation in women with mastitis, partly due to the concerns about the side effects of antibiotics to the infants (Grzeskowiak et al., 2022; Jahanfar et al., 2013). A large number of studies have demonstrated that herbs and their active compounds, such as ginsenoside Rg1, could ameliorate a wide range of inflammatory diseases such as atherosclerosis, traumatic brain injury, and chronic obstructive pulmonary disease (W. Wang et al., 2024; Zhai et al., 2021, 2023). Hence, intervention from natural source is more welcomed by the lactating parents that could be instrumental in reducing abrupt breastfeeding cessation associated with mastitis (Bettiol et al., 2018; Kennedy et al., 2013; Zheng et al., 2020). In this study, we investigated the pharmacological effects and underlying mechanism of action of MT against mastitis using well established *in vitro* and *in vivo* models of mastitis (Cai et al., 2021; Mingfeng et al., 2014). MT is a popularly used herb for the management of mastitis among Asian breastfeeding mothers. Our result showed that MT possessed antibacterial and anti-inflammatory effects and protected the blood-milk barrier integrity in the SA-induced mastitis model in HuMEC. Our results suggested that the anti-inflammatory and antibacterial effects were mediated by the inactivation of the JNK and p38 signaling pathways and upregulation of AMPs transcription, respectively. Furthermore, our results also showed that MT water extract attenuated LPS-induced mastitis

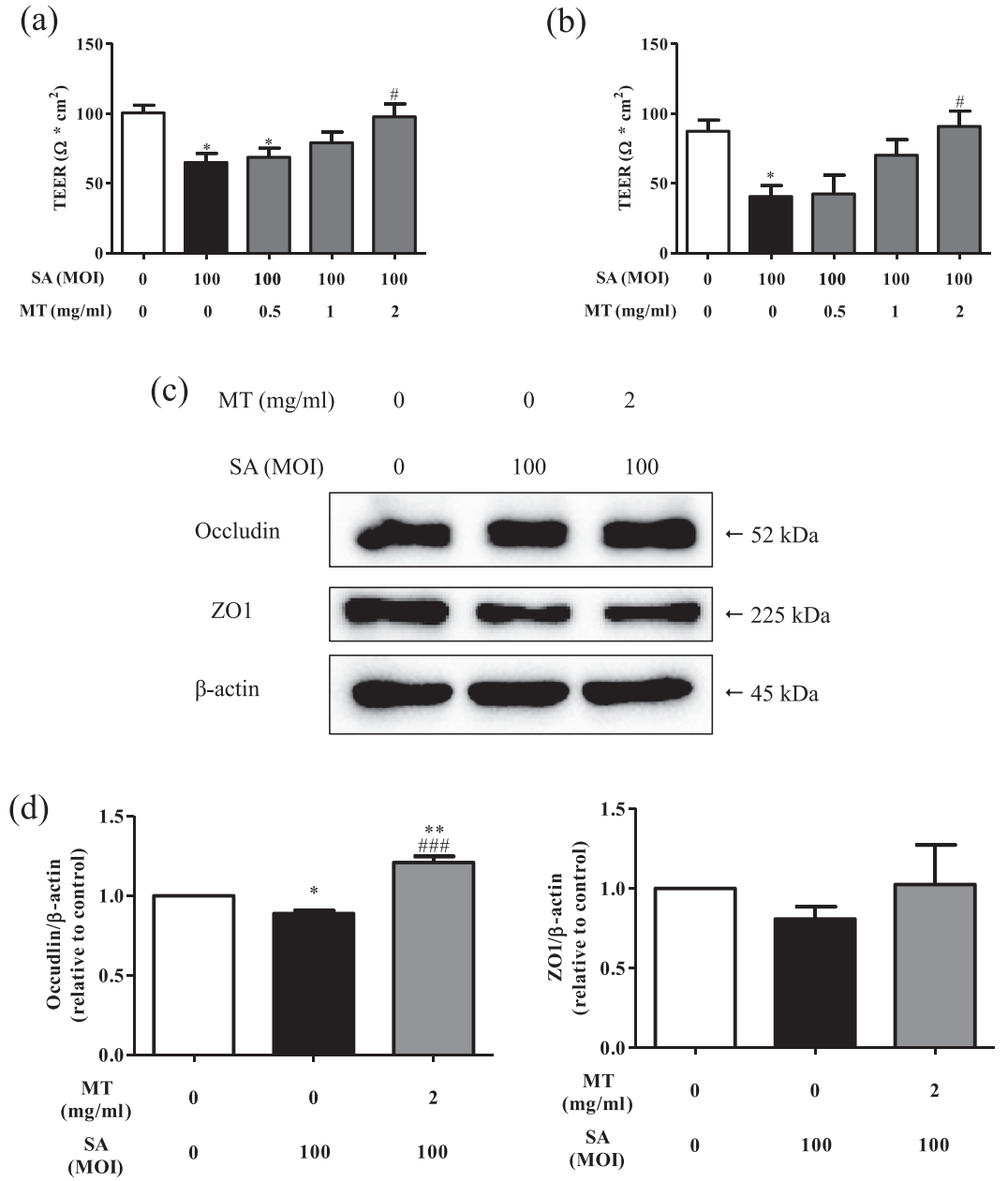


**FIGURE 5** Effects of *Medulla Tetrapanacis* (MT) water extract on blood-milk barrier in *Staphylococcus aureus* (SA)–stimulated human mammary epithelial cells (HuMEC). (a) Transepithelial electrical resistance (TEER) in HuMEC after 2 h post infection and (b) 24 h post infection. (c) Representative bands of occludin and ZO1. Densitometric quantification of (d) occludin and ZO1 was normalized $\beta$-actin. Data were expressed as mean ± SEM; $n = 3$. One-way ANOVA followed by Tukey's multiple comparison test was performed to compare the differences between groups. $^{*}p < .05$ and $^{**}p < .01$ versus control group and $^{###}p < .001$ verses SA group $^{\#}p < .005$. MOI, multiplicity of infection; TEER, Transepithelial electrical resistance.

in lactating SD rats by its anti-inflammatory effects and amelioration of pathohistological damage via inactivation of the JNK signaling pathway.

SA is a normal human flora that is also a gram-positive bacterium commonly found in breastfeeding mothers with mastitis. A study found that more than 92% of SA developed antibiotic resistance to at least single antibiotic, whereas 11% were found to be multidrug-resistance (Marín et al., 2017). This greatly limited the effectiveness of antibiotics on managing mastitis clinically. MEC are the first defense of the mammary gland against infection. In contrast, the invasion of SA in MEC by internalization enables it to colonize and replicate in the mammary gland, a main process that aggravates the development of mastitis (Zaatout et al., 2020). There is a large body of evidence to show that herbs and their phytochemical constituents have high efficacy in preventing SA internalization and inhibiting their growth in MECs to ameliorate mastitis (Beccaria et al., 2018; Mordmuang et al., 2019; Wei et al., 2014). Similarly, results from this study found that MT water extract remarkably reduced SA internalization in HuMEC

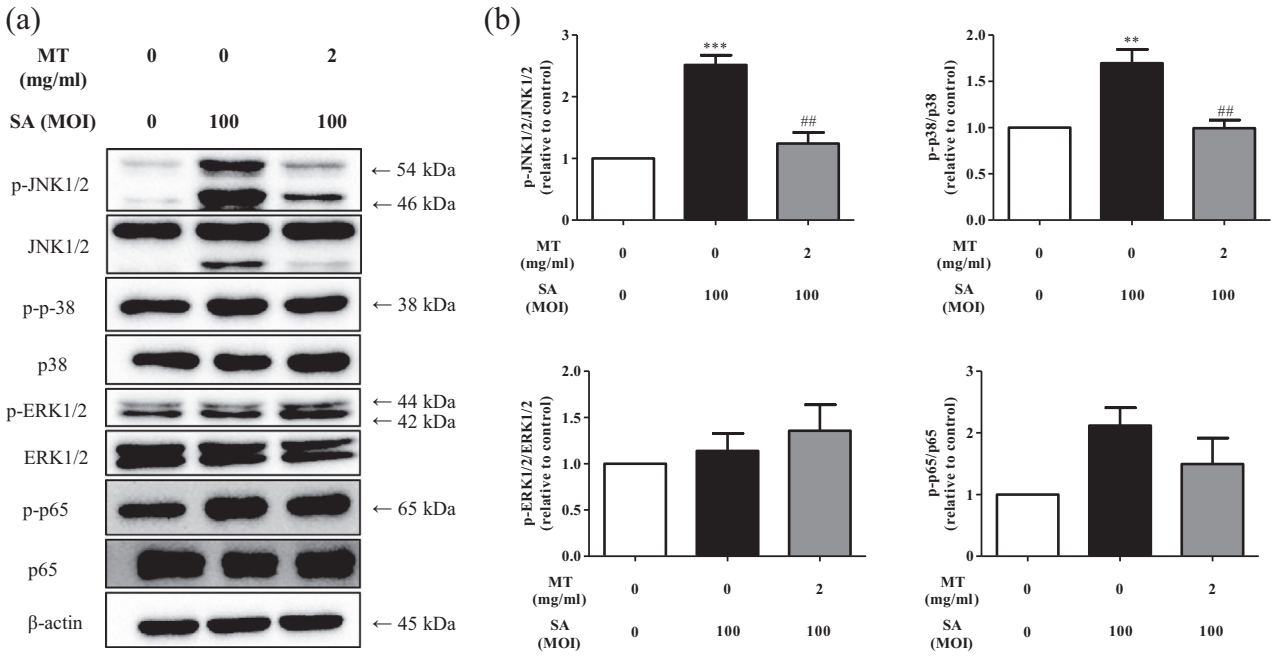


**FIGURE 6** Effects of *Medulla Tetrapanacis* (MT) water extract on p38, JNK1/2, ERK1/2, and p65 activation in *Staphylococcus aureus* (SA)–stimulated human mammary epithelial cells (HuMEC). (a) Representative bands of p-p38, p-JNK1/2 and p-ERK1/2, p-p65. (b) Densitometric quantification of p-p38, p-JNK1/2 and p-ERK1/2, p-p65 was normalized with p38, JNK1/2, ERK1/2, or p65. Data were expressed as mean ± SEM; $n = 3$. One-way ANOVA followed by Tukey's multiple comparison test was performed to compare the differences between groups. $^{**}p < .01$ and $^{***}p < .001$ versus control and $\#\#p < .01$ versus SA group.

after 2-h post infection and at the end of 24-h treatment, MT water extract significantly reduced the number of bacteria when compared to SA model group. These results indicated that MT water extract could prevent SA-induced mastitis by suppressing SA infection in HuMEC via both bacteriostatic and/or bactericidal activities and reduction of SA internalization in HuMEC.

AMPs are key part of the innate immune system of MECs against infection. MECs can produce various AMPs, including beta-defensin-1, lipocalin-2, and cathelicidins (Sharma et al., 2016). All of them have been reported to retard SA invasion or infection in MECs (Cavalcante et al., 2020; Jia et al., 2001; Sharma et al., 2016). The modes of actions of AMPs against pathogens are direct antimicrobial activity (e.g., interrupting cell wall, poring cell membrane, inhibiting protein, DNA, RNA synthesis, and permeabilizing bacterial membrane), anti-biofilm effect, and stimulating immune response (Saeed et al., 2022). AMPs are considered a novel therapeutic alternative against rapid development of multidrug-resistant pathogens. For example, nisin, a commonly used as a food preservative produced by *Lactococcus lactis* is approved by FDA for treating mastitis in dairy cows (Saeed et al., 2022). Moreover, several studies have showed that herbs extract and natural food such as green tea–induced secretion of AMP (e.g., beta-defensin-1–3) from skin (Lee et al., 2022), gingival epithelial (Lombardo Bedran et al., 2014), and intestinal epithelial cells (Wan et al., 2016). Similarly, our results showed that MT water extract upregulated the gene expression of AMPs (beta-defensin-1, lipocalin-2) in the HuMEC, suggesting that upregulation of these AMPs could partly contribute to the antibacterial activity of MT water extract against SA infection in HuMEC.

Inflammation is one of the key processes in the development of mastitis. SA invasion on MEC will trigger the innate immune response of MEC to resolve the SA infection via generation of reactive oxygen species (ROS) and secretion of proinflammatory cytokines such as IL-6 and TNF-$\alpha$. On one hand, the ROS and cytokines can kill the SA directly, and on the other hand, they can recruit immune cells to the inflammatory site to eradicate the SA (Ingman et al., 2014; Zhang et al., 2022). However, unresolved inflammation results in excess proinflammatory cytokines that could aggravate the mastitis by exacerbating the damage of mammary tissue and disrupting the integrity of the blood-milk barrier (C. Zhao et al., 2021). In this study, MT water extract significantly suppressed SA-induced proinflammatory cytokines (i.e., IL-6 and TNF-$\alpha$) release in HuMEC. Similarly, indirubin, a Chinese herb extract from indigo, has been shown to improve LPS-induced mastitis in both mouse and MEC via reduction of proinflammatory cytokines (IL-6, TNF-$\alpha$, and IL-1$\beta$) and MPO (a hallmark of immune cells infiltration) as well as improving abnormal histopathological changes (Lai et al., 2017). Our results also showed that MT water extract significantly attenuated MPO and proinflammatory cytokines, including IL-6, TNF-$\alpha$, and IL-1$\beta$ in LPS-induced mastitis in rats as well as reduced the infiltration of immune cells and excessive lesions in mammary glands of rats.

An intact blood-milk barrier on MEC is important to maintain a proper exchange of nutrients and milk and acts as a physical barrier to prevent pathogen invasion. The integrity of blood-milk barrier is maintained by various membrane components, including tight junctions, adherens junction proteins, and desmosomes (Wellnitz & Bruckmaier, 2021). Mastitis increases blood-milk barrier permeability and disrup-

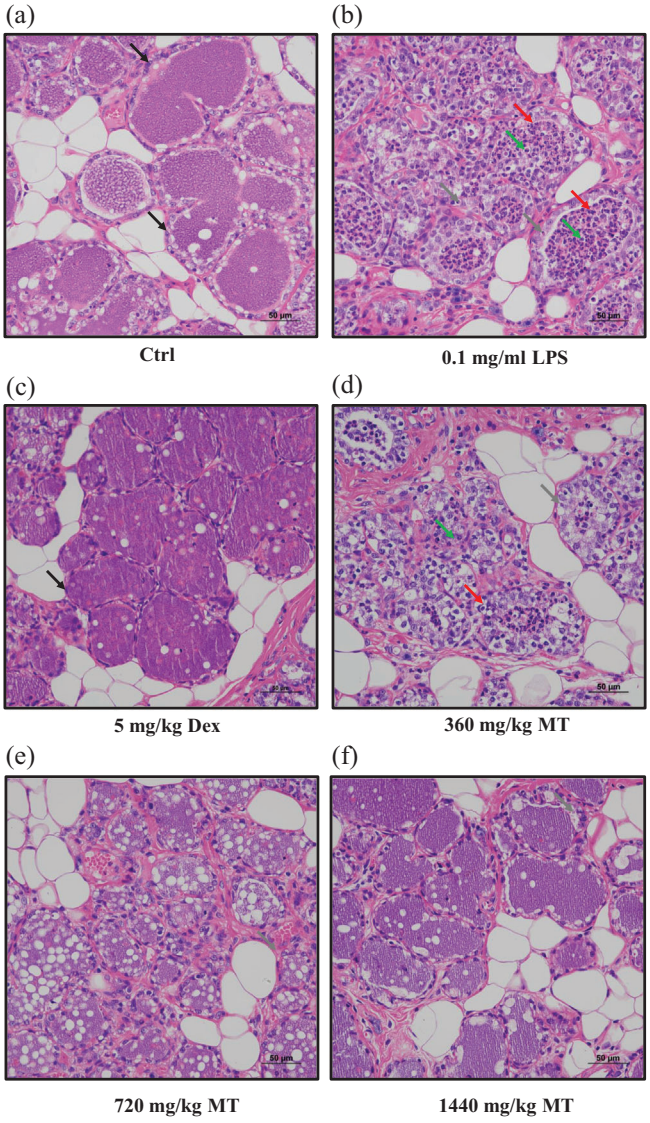


**FIGURE 7** Effects of *Medulla Tetrapanacis* (MT) water extract on pathological changes of mammary gland of lactating Sprague–Dawley (SD) rats (a–f). Sections were stained with hematoxylin and eosin (H&E) staining followed by capturing under 40×. (a) Control, (b) 0.1 mg/mL lipopolysaccharide (LPS), (c) 5 mg/kg Dex, (d) 360 mg/kg MT, (e) 720 mg/kg MT, and (f) 1440 mg/kg MT. Scale bars represented 50 μm in 40× magnification. Black arrow indicated a regular alignment of epithelial cells in acinar of mammary tissues. Green arrow showed severe lesion in the mammary gland cavity. Red arrow showed infiltration of inflammatory cells. Gray arrow indicated extensive epithelial cell death.

tion of the tight junction, which in turn facilitate the entry of SA to and neutrophils infiltration to the breast tissues, resulting in extensive inflammation and eventually tissue damage (Ingman et al., 2014). Membrane proteins, such as occludinand ZO1, are the major tight junction proteins in MEC where their expressions have been shown to be reduced in damaged blood-milk barrier (Wellnitz et al., 2014). In our experiment, we showed that SA infection significantly reduced the TEER values and protein expression of occludin in the HuMEC. More importantly, MT water extract enhanced the TEER values and occludin expression in the SA-infected HuMEC, indicating that MT water extract could alleviate mastitis at least partly by protecting the blood-milk barrier integrity.

Several studies have demonstrated the importance of MAPK and NF-κB in regulating inflammation response in both SA- and LPS-induced mastitis in MEC and *in vivo* (Ge et al., 2021; Jiang et al., 2017). Our previous study has showed that MT water extract could suppress the phosphorylation of MAPK-signaling pathways in LPS-stimulated murine macrophage (Kwok et al., 2023), but its anti-inflammatory mechanism in SA-induced mastitis in HuMEC- and LPS-induced mastitis in lactating SD rats has not been reported. Hence, we examined the molecular mechanism of MT water extract in the SA-infected HuMEC- and LPS-induced mastitis in rats by accessing the activation of the MAPK- and NF-κB-signaling pathways. Our results showed that MT water extract significantly reduced SA-induced phosphorylation of p38 and JNK in HuMEC cells, but not ERK and NF-κB p65, and similarly, our *in vivo* results also showed that MT water extract significantly attenuated LPS-induced phosphorylation of JNK, but not NF-κB p65. Collectively, our experiments indicated that the anti-inflammatory effect of MT water extract in HuMEC and lactating SD rats was at least partly mediated by MAPK-signaling pathway but not NF-κB, which aligned with our previous findings in macrophage (Kwok et al., 2023).

## 5 | CONCLUSION

In conclusion, our results showed that MT water extract alleviated SA-induced mastitis in HuMEC via attenuating SA internalization and inhibiting SA growth. These protective effects are mediated by multiple mechanisms, including upregulation of AMPs transcription, reduction of pro-cytokines release, and blood-milk barrier protection. Moreover, our study revealed that MT water extract ameliorated LPS-induced mastitis in lactating SD rats via suppressing inflammatory cytokine MPO and mitigated pathohistological damage of mammary tissues. Finally, our *in vitro* and *in vivo* results indicate that the protective effects of MT water extract against mastitis are mediated via inactivation of the MAPK-signaling pathways. This study provides clear pre-clinical evidence to support the use of MT water extract for the management of mastitis.

### AUTHOR CONTRIBUTIONS

Franklin Wang-Ngai Chow, Jing-Jing Li, and Sai-Wang Seto conceived the project and designed the experiments. Carsten Tsun-Ka Kwok, Yuanhao Hu, Bun Tsoi, Fiona Wong, Pak-Ting Hau, and Emily Wan-Ting Tam preformed the experiment and data analysis. Carsten Tsun-Ka Kwok, Franklin Wang-Ngai Chow, Jing-Jing Li, and Sai-Wang Seto wrote the manuscript. Yiu-Wa Kwan, George Pak-Heng Leung, Daniel Kam-Wah Mok, and Simon Ming-Yuen Lee contributed to the experimental technical consultations. All authors reviewed and revised the manuscript.

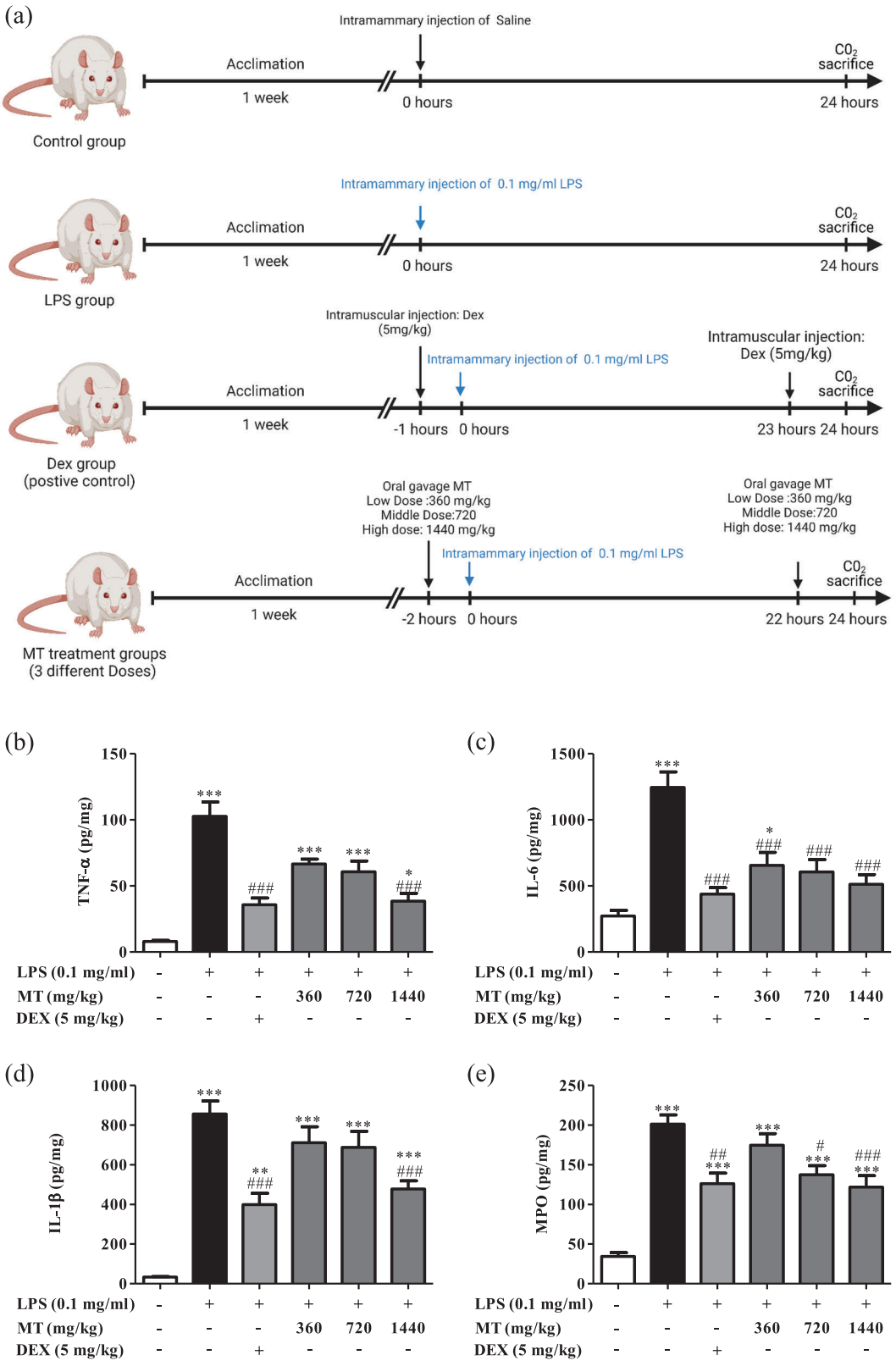


**FIGURE 8** Effects of *Medulla Tetrapanacis* (MT) water extract on lipopolysaccharide (LPS)–induced proinflammatory cytokines and myeloperoxidase (MPO) in LPS-induced mastitis in mammary tissues of lactating Sprague–Dawley (SD) rats. (a) Study design for animal study, (b) tumor necrosis factor-alpha (TNF-$\alpha$), (c) interleukin 6 (IL-6), (d) interleukin-1beta (IL-1$\beta$), and (e) MPO. Data were expressed as mean ± SEM; $n = 6$ except 1440 mg/kg MT water extract $n = 7$. One-way ANOVA followed by Tukey's multiple comparison test was performed to compare the differences between groups. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ versus control and $\#p < .05$, $\#\#p < .01$, and $\#\#\#p < .001$ versus LPS group. *Source*: (a) Created with Bio Render.

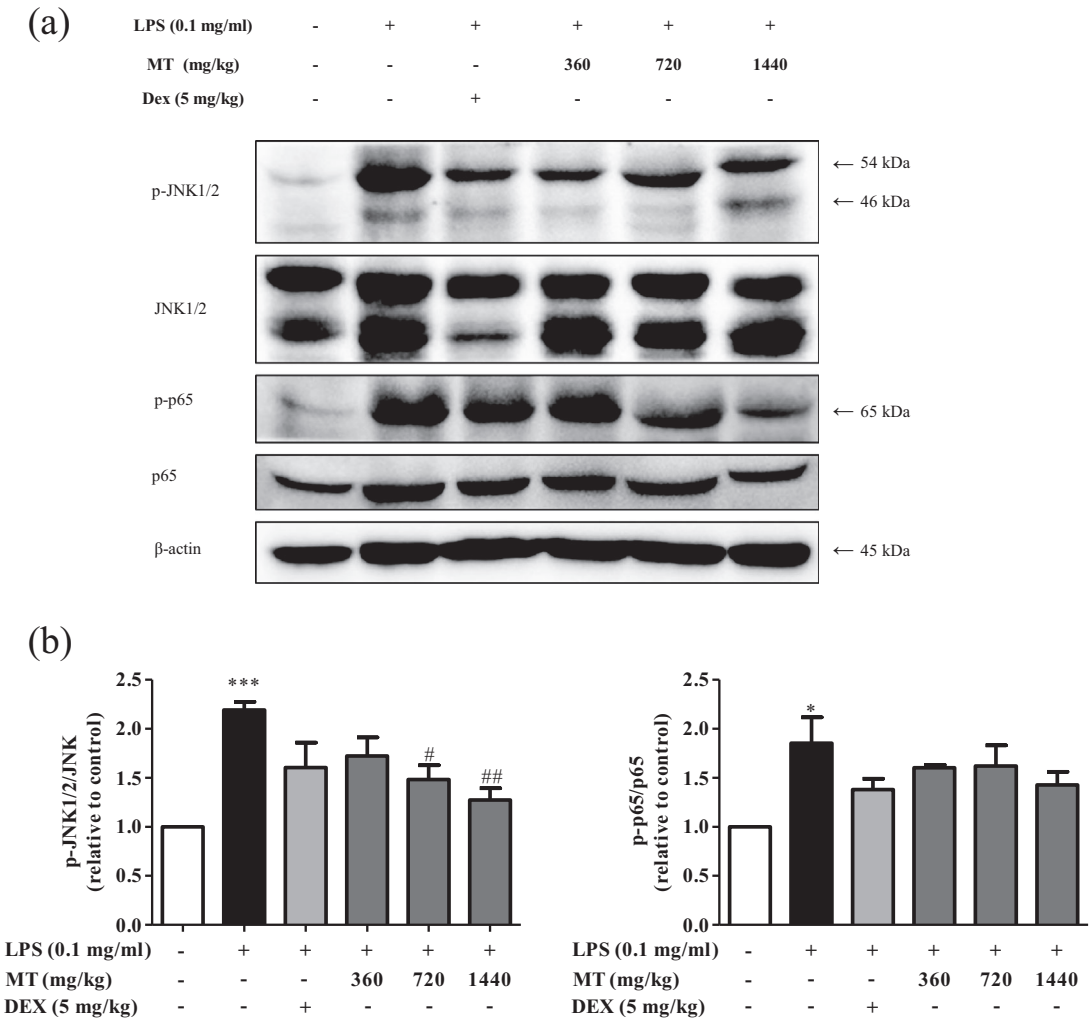


**FIGURE 9** Effects of *Medulla Tetrapanacis* (MT) water extract on JNK1/2 and p65 activation in lipopolysaccharide (LPS)-induced mastitis in mammary tissues of lactating Sprague–Dawley (SD) rats. (a) Representative bands of p-JNK1/2 and p-p65. (b) Densitometric quantification of p-JNK1/2 and p-p65 was normalized with JNK1/2 or p65. Data were expressed as mean ± SEM; $n = 4$ for JNK1/2 and $n = 3$ for p65. One-way ANOVA followed by Tukey's multiple comparison test was performed to compare the differences between groups. $^{*}p < .05$ and $^{***}p < .001$ versus control and $\#p < .05$ and $\#\#p < .01$ versus LPS group.


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## CONFLICT OF INTEREST STATEMENT

The authors confirm that they have no conflicts of interest to declare for this publication.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, Sai-Wang Seto, upon reasonable request.

## ETHICS STATEMENT

Animal study was conducted according to the protocol approved by the animal ethics committee of the Hong Kong Polytechnic University (No.: 23-24/715-FSN-R-STUDENT, SZ CAF only).


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## SUPPORTING INFORMATION

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