Inhibition of Neutrophil Adhesion and Antimicrobial Activity by Diluted Hydrosol Prepared from Rosa damascena

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Hydrosol prepared from the flowers of Rosa damascena (rose water) has been traditionally used for various health-related issues, including skin troubles such as erythema, itchiness, swelling. For the care of these skin troubles caused by microbial infection, both antimicrobial and anti-inflammatory effects are required. Here, we investigated the effects of rose water on the growth of Candida albicans and methicillin-resistant Staphylococcus aureus (MRSA), which cause skin infections, and on the function of neutrophils, which play a major role in the regulation of inflammatory reactions. To assess its modulatory effects on neutrophils, the effects of rose water against neutrophil adhesion response were evaluated. Rose water inhibited mycelial growth of C. albicans at a concentration of ca. 2.2%, and reduced viability of MRSA within 1 h. Rose water suppressed neutrophil activation induced by lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF-α), and N-formyl-Met-Leu-Phe (fMLP) at 5–15%. It also reduced the LPS- and TNF-α-induced cell surface expression of the adhesion-related molecule, cluster of differentiation (CD) 11b, but did not affect the migratory capacity of neutrophils with or without chemotactic stimulation. These results suggest that rose water may reduce the pathogenicity of microbes, and attenuate neutrophil stimulation, which is involved in inflammatory responses. These findings suggest that rose water has a potential effect to inhibit skin inflammation caused by microbes.

Key words hydrosol; Rosa damascena; neutrophil; inflammation; Candida albicans

Hydrosols, also known as herbal water, floral water, and hydrolate, are aqueous products co-produced during the steam distillation of various plant materials. Hydrosol obtained from flowers of Rosa damascena (Rosaceae) (rose water) has pleasant scent and is one of the most popular of these products. It has been traditionally used in treatment of various health-related issues, such as oral, ophthalmic, digestive, circulatory, hormonal, and skin complaints by cutaneous and mucosal application or by ingestion. Its main uses are in daily skin care and in treatment of skin troubles such as erythema, itchiness, swelling, pus and blisters. However, there is only anecdotal evidence regarding its efficacy and there have been few experimental studies to examine its activities.

One of the main causes of these skin troubles is microbial infection. Cutaneous treatment for infectious conditions usually focuses on antimicrobial effects. Suppression of microbes is important because infection is not improved if the microbes stay activated. However, inflammatory symptoms, such as edema, pain, and itching, accompanying infection are serious under clinical conditions and therefore suppression of inflammation is also very important. Neutrophils play major roles in the regulation of inflammatory responses. Neutrophils accumulate around the lesional area infected by microbes, and play a role in host defense. However, activated neutrophils may induce excessive inflammatory responses and damage tissues around the infected area, and exacerbate symptoms by the secretion of superoxides, proteases, and other antibacterial substances. Therefore, regulation of the overstimulated neutrophil function is thought to be effective for the suppression of inflammatory process.

We reported previously that essential oils, such as geranium oil and lemongrass oil, suppressed neutrophil function and anti-inflammatory conditions, and had anti-Candida effects. As rose water contains small amounts of essential oil components, some of which are similar to the active components of geranium oil, rose water may suppress neutrophil function and have antimicrobial activity.

In the present study, we investigated the effects of rose water on Candida albicans and methicillin-resistant Staphylococcus aureus (MRSA), which cause skin and mucosal infections, and on neutrophil function. Neutrophil activation is known to occur through two steps, priming and triggering. Yakuwa et al. reported that the priming response of neutrophils can be experimentally estimated by a rapid and simple in vitro method using neutrophil adhesion to plastic culture plates. We evaluated the effects of rose water against neutrophil adhesion response to assess the modulatory activity of rose water to neutrophils in inflammatory reactions.

MATERIALS AND METHODS

Agents Rose water and rose oil were gifts from Bulgaria Rose Japan Ltd. (Tokyo, Japan, lot: 153 and 61, respectively). According to the company’s analytical certificates, the main constituents of rose oil are citronellol (31.47%), geraniol (19.10%), nerol (9.68%), and phenethyl alcohol (1.52%). The

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essential oil content in rose water was 0.095%, the main constituents of which were phenethyl alcohol (43.65%), citronellol (18.24%), and geraniol (14.8%). Human recombinant tumor necrosis factor alpha (TNF-α), Dextran 200000, sodium dodecyl sulfate (SDS), geraniol, and phenethyl alcohol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Citronellol was from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Roswell Park Memorial Institute medium (RPMI) 1640, lipopolysaccharide (LPS) (Escherichia coli Serotype 0127:B8), and N-formyl-Met-Leu-Phe (fMLP) were from Sigma-Aldrich Japan (Tokyo, Japan). Crystal violet (CV) was from Merck (Darmstadt, Germany). Fetal bovine serum (FBS) was from Thermo Trace (Melbourne, VIC, Australia). Mueller–Hinton broth was from Becton, Dickinson and Company (Sparks, MD, U.S.A.).

Measurement of Candida Mycelial Growth The mycelial growth of *C. albicans* was measured based on the method of Abe et al.17 The clinically isolated strain of *C. albicans*, TIMM1768, which was maintained in our laboratory and stored at −80°C, was used for the experiment. RPMI1640 containing 2% FBS was diluted 1:3 with distilled water (diluted RPMI medium). TIMM1768 was washed with diluted RPMI medium by centrifugation (1500 rpm, 3 min, 4°C) and suspended in the same medium to a concentration of 4.0 × 10^5 cells/mL. Rose water was diluted to 1.25–100% using diluted RPMI medium. Rose oil, citronellol, geraniol, and phenethyl alcohol were dissolved to 10% using dimethyl sulfoxide (DMSO) and further diluted using diluted RPMI medium to 0.000625–0.08%. Our preliminary experiments indicated that DMSO did not significantly affect growth of *Candida* at these concentrations (< 0.1%) (data not shown). Aliquots of 100 µL of rose water, rose oil, and its constituents were poured into the wells of 96-well flat-bottomed culture plates, followed by 100 µL of TIMM1768 (500 cells/well). Final concentrations of *C. albicans* cells and rose water were 2.5 × 10^5 cells/mL and 0.625–50%, respectively. The mixtures were then incubated for 16 h at 37°C in a 5% CO₂ incubator. After overnight culture, the supernatants were discarded to remove nonadherent yeast-form cells. The adherent mycelial cells were sterilized with 70% ethanol, washed three times with distilled water, and dried. They were then stained for 15 min at room temperature by the addition of 100 µL of 0.02% CV, washed three times with distilled water, and solubilized by addition of 150 µL of isopropanol containing 0.04 n HCl and 50 µL of 0.25% SDS. The mycelial growth of *C. albicans* was evaluated by measuring the absorbance of triplicate samples at 620 nm (OD620). The values of mycelial growth were expressed as the ratio relative to the control without oils.

Measurement of Viability of MRSA MRSA, *Staphylococcus aureus* 32CF, which was maintained in Teikyo University School of Medicine and stored at −80°C, was used for the experiment. MRSA was grown on Mueller–Hinton agar plates at 37°C for 18 h and diluted to McFarland 0.5 (ca. 1.5 × 10^8 colony forming units (CFU)/mL) using Mueller–Hinton broth.

The killing activity of rose water against MRSA was measured based on a modification of the method described previously by Abe et al.8) Heparinized venous blood obtained from healthy volunteers was mixed with 7% dextran solution and allowed to stand at room temperature for 1 h. The upper layer was collected to remove erythrocytes. The leukocyte-rich upper layer was centrifuged to collect the neutrophil-rich layer in polymorphrep (Axis-shield PoC AS, Oslo, Norway), which was washed with RPMI1640 containing 10% FBS, and suspended in the same medium to a concentration of 4.0 × 10^6 cells/mL.

Neutrophil adhesion test was performed as described by Yakuwa et al.16) Rose water containing 10% FBS was diluted to 5–80% with distilled water containing 10% FBS. Rose oil, citronellol, geraniol, and phenethyl alcohol were dissolved to 10% using DMSO and further diluted to 0.4% using RPMI1640 containing 10% FBS. The adherent neutrophils were collected using RPMI containing 1% CV and fMLP solution were used instead of LPS solution and fMLP solution were used instead of LPS solution. TNF-α was diluted to 2 × 10^{-3} µg/mL (>20 units/mL) using RPMI1640 containing 10% FBS. fMLP solution was diluted in DMSO (1 × 10^{-3} M) and diluted to 1 × 10^{-3} M with saline and further diluted to 1 × 10^{-5} M with RPMI1640 containing 10% FBS. All experiments were performed using neutrophils from different volunteers at least three times.

Measurement of Expression Level of Neutrophil Membrane Antigen Cluster of Differentiation (CD) 11b The expression level of the neutrophil membrane antigen, CD 11b, was measured using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, U.S.A.) with fluorescence-labeled monoclonal antibodies, according to Tansho-
Nagakawa et al. \(^{19}\) Neutrophil suspension (final concentration: 2×10^6 cells/mL), LPS (final concentration: 1 µg/mL), and rose water (final concentration: 0–20%) in RPMI containing 10% FBS were added to 1.5-mL tubes and incubated for 1 h at 37°C in a 5% CO₂ incubator. In another experiment, TNF-α (final concentration: 2.5×10^-8 µg/mL) was used instead of LPS. A fluorescence-labeled antibody was added to the collected neutrophil suspension and allowed to react on ice for 30 min. Cell Wash (Becton Dickinson) was added to remove the floating fluorescence-labeled antibody. Anti-CD11b (Becton Dickinson) antibody was used as fluorescence-labeled antibody to analyze neutrophil membrane antigen. On FACS analysis, fluorescence-labeled antibodies bound to neutrophil membrane antigens were fixed using Cell Fix (Becton Dickinson). For quantitative analysis of fluorescence, 10,000 cells were collected and analyzed using Cell Quest software (Becton Dickinson). The amount of fluorescent dye molecules bound to the cells indicates the absolute amount of fluorescence-labeled antibodies bound to the cells. The fluorescence intensity of the cells was measured with a flow cytometer, and the data are presented as mean fluorescence intensity (MFI).

**Measurement of the Chemotaxis of Neutrophils by Chemotactants** Chemotaxis assay was performed according to Kanegasaki et al.\(^{20}\) and Nitta et al.\(^{21}\) Time-lapse images of neutrophils during chemotaxis were obtained using TAXIScan (Effect Cell Institute, Tokyo, Japan) equipped with a 12-channel chamber. The chamber was filled with RPMI-N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES) medium containing 1% FBS and 0.05% HSA, and maintained at 37°C during the experiments. The surface of the glass plate, where the cells migrate, was pretreated with RPMI-HEPES medium containing 10% FBS. Aliquots of 1 µL of neutrophil suspension (5×10^6 cells/mL) were injected into compartments through a hole connected to the compartment. Aliquots of 1 µL of chemoattractants, interleukin (IL)-8, and fMLP (final concentration: 10^-8, 10^-7 M, respectively), were then injected into the other compartment to initiate chemotaxis. Images of the channels were recorded channel-by-channel digitally at 1-min intervals. Migration velocity was calculated from the migratory pathway data using TAXIScan Analyzer 2 (Effect Cell Institute).

**Statistical Analysis** The results are expressed as the mean±standard deviation. The data were compared using Student’s t-test.

**RESULTS**

**Inhibitory Effects of Rose Water and Rose Oil on C. albicans Mycelial Growth** Rose water inhibited C. albicans mycelial growth in a dose-dependent manner (Fig. 1A) with the 50% inhibitory concentration (IC₅₀) of ca. 2.2%. Figure 1B shows the microscopic features of C. albicans cells cultured in medium with or without rose water. These observations indicated that most C. albicans cells proliferated as the mycelial form in the absence of rose water. Rose water at concentrations <10% dose-dependently reduced the number of mycelial-form cells, but increased the number of yeast-form cells. However, at 50%, rose water clearly inhibited yeast-form growth. These results indicated that rose water has inhibitory activity against Candida mycelial growth at low concentrations and against yeast growth at relatively high concentrations. Rose oil and its components (citronellol, geraniol, and phenethyl alcohol) were also examined for their activity against C. albicans mycelial growth. Figure 2 shows that the inhibitory activities of citronellol and geraniol were similar to those of rose oil, but that of phenethyl alcohol was much weaker than rose oil—the IC₅₀ values were 0.0008% for rose oil, 0.00045% for geraniol, 0.001% for citronellol, and 0.016% for phenethyl alcohol.

**Killing Activity of Rose Water against MRSA** Rose water reduced the viable MRSA cell number time-dependently from 25 to 100% (data not shown). When measured at 30-min intervals, 100% rose water was shown to reduce the viable cell number to 1/100th at 30 min and to about 1/10000th at 1 h (Fig. 3). These observations indicated that rose water killed about 99.99% of MRSA within 1 h. Furthermore, 75% rose water also reduced the cell number to less than 1/100th at 1.5 h, indicating that 75% rose water killed more than 99% of MRSA within 1.5 h. These results indicate that rose water has bactericidal activity against MRSA and acts within a short time.

**Effects of Rose Water and Rose Oils on Neutrophil Adhesion** Rose water was shown to have antimicrobial activities against both Candida and MRSA, both of which cause cutaneous infection. As inflammatory symptoms accompanying infections are among the main clinical complaints, we evaluated the antiinflammatory effects of rose water, specifically the effects on neutrophils that play a major regulatory role in inflammation. Rose water dose-dependently inhibited LPS-induced neutrophil adhesion (Fig. 4A). Figure 4B shows a typical photograph of adhering neutrophils stained with CV. The results clearly indicated that rose water dose-dependently suppressed adhesion of neutrophils stimulated by LPS with an IC₅₀ of 6–15%.

Rose oil, citronellol, geraniol, and phenethyl alcohol dose-dependently inhibited LPS-induced neutrophil adhesion (Fig. 5). The activities of citronellol and geraniol were similar to that of rose oil, while that of phenethyl alcohol was weaker; the IC₅₀ values of rose oil, citronellol, geraniol, and phenethyl alcohol were ca. ≤0.003–0.0095, ≤0.003, ≤0.003%, and 0.006–0.16%, respectively.

Rose water also dose-dependently inhibited TNF-α, and fMLP-induced neutrophil adhesion with IC₅₀ values of 5–12 and 3–6%, respectively (data not shown).

These results indicated that rose water has similar inhibitory effects against neutrophil adhesion induced by these stimuli.

**Effects of Rose Water on Expression Level of the Neutrophil Membrane Antigen, CD11b, Induced by LPS and TNF-α** It was well known that membrane adhesion molecules of human neutrophils are increased by stimulants, such as bacterial LPS. In a preliminary study, we examined whether LPS induced cell-surface expression of CD11b (leukocyte functional antigen (LFA)-1), CD11b (Mac-1), CD128 (IL-8R), CD120 (TNF-R), and CD88 (C5a-R). As our preliminary experiment clearly showed that CD11b expression was increased when neutrophils were incubated with bacterial LPS, we evaluated the effects of rose water on LPS-induced cell-membrane expression of CD11b. As shown in Fig. 6, 20% rose water suppressed the LPS-induced expression of CD11b to the control level. We confirmed that 20% rose water itself did not affect the level of CD11b expression (data not shown).
TNF-α also increased the expression of CD11b. Rose water suppressed the induced expression in a dose-dependent manner (Fig. 7).

Effects of Rose Water on IL-8 and fMLP-Induced Neutrophil Migration in Vitro Although the chemoattractants, IL-8 and fMLP, increased the migratory capacity of neutrophils, 20% rose water had no effect on migratory capacity with or without chemoattractant (data not shown).

DISCUSSION

Here, we showed that rose water inhibited C. albicans mycelial growth at a low concentration, and had bactericidal activity against MRSA that were effective within a short time. Rose water also inhibited functional changes in neutrophils induced by bacterial LPS, the inflammatory cytokine, TNF-α, and fMLP at a low concentration. To our knowledge, this is the first report providing evidence for the suppressive activity of rose water on neutrophil stimulation.

These findings provide basic scientific information regarding the efficacy of rose water, which has traditionally been ap-
plied topically to skin and mucosa. Especially, we would like to emphasize the suppressive activity of rose water against neutrophil function induced by stimulation, because inflammatory symptoms of skin such as edema and pain could be enhanced by local stimulated response of neutrophils.

In this study, rose water showed similar suppressive effects against neutrophil activation induced by three different stimulants at 3–15%. These observations suggest that rose water may alleviate inflammation through suppression of neutrophil function excessively stimulated by various causes.

Rose water contains small amounts of essential oil components. The concentrations of citronellol, geraniol, and phenethyl alcohol in 10% rose water (IC_{50}) are calculated to be ca. 0.0017, 0.0014, and 0.0041%, respectively. As the IC_{50} values of citronellol and geraniol were <0.003%, we speculated that these components may contribute to the activity of rose water. This speculation was supported by our observation that rose oil, which consists mainly of citronellol and geraniol, can suppress neutrophil adhesion at very low concentrations. However, none of the components inhibited neutrophil adhesion by more than 50%, indicating the involvement of other components, such as water-soluble constituents, which remain to be characterized.

Neutrophil activation is known to occur through two steps, priming and triggering.\textsuperscript{15) Specific stimulants, such as TNF-\(\alpha\), IL-8, LPS, fMLP, and phorbol 12-myristate 13-acetate (PMA), prime neutrophils and induce the adhesion reaction, migration, activation of nicotinamide adenine dinucleotide

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**Fig. 3.** Cell Killing Effects of Rose Water against MRSA

MRSA was incubated with different concentrations of rose water in 37°C water bath with shaking. At 30-min intervals, culture fluid was diluted, incubated on Mueller-Hinton agar plates for 25 h at 37°C, and the colony forming units (CFU) were counted.

**Fig. 4.** Effects of Rose Water on LPS-Induced Neutrophil Adhesion

Human neutrophils were incubated in medium containing 1 \(\mu\)g/mL LPS and different concentrations of rose water for 1 h at 37°C in a 5% CO\(_2\) incubator. Neutrophils adhering to the plastic wells were estimated photometrically by crystal violet staining at 620 nm (OD\(_{620}\)). (A) Relative adhesion of neutrophils. (B) Adherent cells in the presence or absence of rose water.
phosphate (NADPH) oxidase, expression of surface proteins, such as CD11b, and expression of receptors for the triggering substances. Neutrophils in the priming state are further triggered by interaction with stimulants and microbes, which enhance the release of reactive oxygen species, such as O₂⁻.

LPS activates neutrophils through LPS receptors, such as CD14. TNF-α and fMLP also prime neutrophils through specific receptors. The priming of neutrophils by these factors is known to initiate the signal via p38 mitogen activated protein kinase (MAPK). In the present study, rose water showed similar inhibitory effects on neutrophil adhesion stimulated by LPS, TNF-α, and fMLP. Therefore, we speculated that rose water may suppress neutrophil adhesion through signal transduction below the interaction of the receptor with ligands in the membrane. Previously, we reported that essential oils suppressed the LPS- and TNF-α-induced neutrophil adhesion at low concentrations, but not PMA-induced adhesion. PMA activates protein kinase C in the cell membrane directly without the involvement of MAPK. These observations support the suggestion that rose water may suppress neutrophil activation perhaps through interactions with signal transduction via p38 MAPK.

Neutrophil adhesion to a plastic plate is recognized as a parameter representing the priming state of neutrophils in inflammatory responses, which is mediated by major adhesion molecules, CD11b/CD18. In the present study, rose water decreased the expression levels of neutrophil membrane antigen CD11b induced by LPS and TNF-α. CD11b (Mac-1), which is induced by various cytokines and chemokines, binds to endothelial cell surface molecules, such as intercellular adhesion molecule (ICAM)1. This binding is known to be important for adhesion, transmigration, and emigration of neutrophils. In contrast, rose water did not affect neutrophil chemotaxis with or without stimulation by chemoattractant. Therefore, rose water was suggested to inhibit neutrophil infiltration into lesional areas through suppression of its binding to endothelial cells in inflammation.

Although there are many different causes of inflammation, microbial infection is one of the major causes. We reported previously that essential oils suppressed Candida growth and improved the symptoms in a mouse model of mucosal candidiasis. Essential oils also reduced the accumulation of inflammatory cells around the lesional area. Therefore, we evaluated the effects of rose water on both of which cause skin and mucosal infections.

Rose water suppressed mycelial growth of Candida at a concentration of ca. 2.2%, and inhibited yeast growth at 50%. Candida is commensal and known to change its growth form depending on its environmental conditions. Candida usually exists in the form of yeast with little virulence on the mucosa and superficial skin of a healthy host. In the case of tissue invasion, however, it often changes to the mycelial form, which accelerates adhesion and tissue penetration. By microscopic test for diagnosis, both yeast and mycelial forms are reported to be detected from lesional areas. Therefore, the inhibition of Candida mycelial growth means the suppression of Candida invasion and penetration into skin.

The concentrations of citronellol, geraniol, and phenethyl alcohol in 2.2% rose water (IC₅₀) were calculated to be 0.00038, 0.00031, and 0.00091%, respectively. The IC₅₀ value of geraniol was ca. 0.00045, and 0.0006% citronellol inhibited
mycelial growth by about 50%. Therefore, we speculated that geraniol, and possibly citronellol, may have contributed to the observed activity of rose water. Inouye et al.\textsuperscript{9)} reported that rose water did not inhibit \textit{C. albicans} mycelial growth. Although this seems contradictory to our observations, this discrepancy may have been related to differences in the chemical contents of rose water and experimental conditions used in the two studies.

Rose water reduced MRSA by about 99.99% within 1h, indicating that it showed bactericidal activity within a short time. Even in 50% solution, viable MRSA was decreased by 90 and 99% after 2- and 4-h incubation, respectively (data not shown). Therefore, cutaneous application of rose water may be effective to reduce MRSA, which would improve skin condition and prevent hospital infection.

Rose water is popular due to its pleasant scent, and has long been used for daily skin care and various skin troubles based on anecdotal evidence. We showed that rose water suppressed neutrophil activation induced by stimulants at 3–15%, inhibited mycelial and yeast growth of \textit{C. albicans} at ca. 2.2 and 50%, respectively, and >50% rose water killed MRSA within a short time. As rose water is usually used without dilution, these results suggest that its cutaneous application may inhibit the growth of microbes on the skin surface. Although we did not evaluate the cutaneous absorption of rose water, essential oils and their components are known to penetrate easily through the skin. Therefore, it is possible that the local concentrations of rose water components may be sufficient to suppress neutrophil activation and suppress invasion and penetration of microbes in the skin following cutaneous application. For quick relief from skin inflammation, both inhibition of its cause and suppression of inflammatory process are essential. In this study, we focused on microbes as the cause and neutrophils which played an important part to inflammation at a post-cell surface receptor level. Enhancement of the regulatory activity of farnesol against oral candidiasis in mice.

Further studies are required to determine whether rose water applied to the skin as a lotion shows antimicrobial and antiinflammatory effects in human subjects.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES


