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Original Research Article

Therapeutic Effects of *Dipterocarpus alatus* Roxb. Ex G. Don Ointment on Methicillin-resistant *Staphylococcus aureus*-Infected Skin Abrasion Wounds in Mice

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ABSTRACT

Skin abrasion wounds, especially those infected with methicillin-resistant *Staphylococcus aureus* (MRSA), pose a risk of developing into severe lesions. MRSA is resistant to typical antibiotics, which necessitate intravenous antibiotic and/or surgical interventions. A Yang-Na (*Dipterocarpus alatus* Roxb. Ex G. Don) twig extract has been shown to exhibit antibacterial and wound healing properties in mice with MRSA-infected wounds. This study aimed to examine the efficacy of an ointment containing Yang-Na twig extract against MRSA-infected abrasion wounds in mice. Skin abrasion wounds induced on mice were infected with MRSA (n=10) and left untreated or treated daily with an ointment base, tetracycline ointment (160 µg/g), or Yang-Na twig ointments (20 and 40 mg/g) for 9 days, alongside a non-infected control group. MRSA infection significantly compromised skin integrity, as evidenced by weakened skin barrier strength and enhanced transepidermal water loss. The infected wounds showed signs of deterioration and substantial numbers of MRSA colonies, along with mast cell infiltration and increased mRNA expression of inflammatory-related genes (TLR-2, NF-κB, TNF-α, IL-1β, IL-6, and IL-10). Treatment with Yang-Na twig ointments restored skin integrity and improved wound appearance within a week. Mast cell infiltration and expression of inflammatory-related genes were normalized and no MRSA colonies were observed in the wounds treated with Yang-Na twig ointments. The other treatments did not achieve the same results. These findings highlight the therapeutic effects of the Yang-Na twig ointment as an antibacterial and wound healing remedy with anti-inflammatory properties.

Keywords: *Dipterocarpus alatus*, Resin tree, MRSA, Skin integrity, Inflammation, Wound healing.

Introduction

Infection is a significant trigger for illnesses and can have a substantial impact on the economy.[1] Skin abrasions, characterized by bruising or scratching of the skin, are often contaminated with foreign materials and dirt leading to increased risk of infection and potential development of more severe lesions. Despite advances in infection prevention and treatment, the number of antibiotic-resistant strains of *Staphylococcus aureus* is on the rise globally.[2] Methicillin-resistant *S. aureus* (MRSA), which is resistant to all typical antibiotics, has become a serious concern both in hospitals and the general population.[3] MRSA-induced infection in skin abrasion wounds often necessitates intravenous antibiotics and/or surgical interventions, which occasionally prove ineffective.[4] In addition, community-associated MRSA strains cause a significant number of infections in soft tissue and skin, varying by country.[5]

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Consequently, the development of a new effective antibacterial agent against MRSA infections is imperative.

Yang-Na or *Dipterocarpus alatus* Roxb. ex G. Don, colloquially known as the resin tree and belonging to the Dipterocarpaceae family, is a perennial plant found in tropical countries, including Thailand. *Dipterocarpus* genus has been previously documented for health benefits.[6] In Ayurvedic medicine, Yang-Na bark oil has been used for external wound healing, while its oleo-resin has shown efficacy in treating skin ulcers.[7,8] Additionally, various extracts from different parts of Yang-Na have demonstrated antioxidant and cytotoxic properties.[9] Recently, an extract derived from Yang-Na twigs was shown to exhibit both antibacterial and wound healing properties in mice with MRSA-infected wounds.[10] The rationale of the study is to develop an ointment containing Yang-Na twig extract, followed by an investigation into its therapeutic effects on MRSA-infected skin abrasion wounds in mice.

Materials and Methods

Chemicals and reagents

Mannitol salt agar and toluidine blue O were products of Himedia (Maharashtra, India) and Sigma-Aldrich (Saint Louis, MO, USA), respectively. Tetracycline hydrochloride and oxacillin sodium were supplied by Merck Ltd. (Feltham, United Kingdom). Zoletil®100 (a cocktail of tiletamine-zolazepam) and Thiopental VÚAB (thiopental sodium) were products of Virbac (Westlake, TX, USA) and the Research Institute of Antibiotics and Biotransformations (Praha, Czech Republic), respectively. ReverTraAce® and Thunderbird™ qPCR Mix were products of Toyobo (Osaka, Japan). TaqMan™ Gene Expression Master Mix for toll-like receptor 2 (TLR-2, Mm01213946_g1), nuclear

factor-kappa B (NF-κB, Mm00456853_m1), tumor necrosis factor alpha (TNF-*α*, Mm00443258_m1), interleukin (IL) 1 beta (IL-1*β*, Mm00434228_m1), IL-6 (Mm00446190_m1), IL-10 (Mm01288386_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Mm99999915_g1) were products of Applied Biosystems (Waltham, MA, USA). All other chemicals were obtained from premium-quality suppliers.

Collection and extraction of Yang-Na twig ointment

Yang-Na twigs were harvested in January 2021 in Khon Kaen, Thailand, and a voucher specimen (PSKKF03682) was deposited in Division of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University. Methanol-ethyl acetate extraction was performed to prepare Yang-Na twig ointment.[9,11] Yang-Na twigs were cleansed with water, dried at 50°C, and ground to a powder using mill machinery. The Yang-Na twig powder (100 g) was macerated with ethyl acetate (600 mL) at room temperature overnight three repetitions. The residue was then re-extracted with methanol (600 mL) to obtain the Yang-Na twig extract.[9,11]

Preparation of Yang-Na twig Ointment

Yang-Na twig ointment is a topical preparation containing hard paraffin, wool fat, white soft paraffin, and cetostearyl alcohol. To prepare the ointment base (B), these ingredients were melted in a warm water bath at 65 to 70°C. Once the initial mixture was melted, it was cooled down to 40 to 45°C before adding clove oil, almond oil, and propylene glycol. Then, either Yang-Na twig extract or tetracycline was incorporated as the final ingredient. The mixture was gently stirred using a magnetic stirrer while cooling until a homogeneous mass was formed, resulting in Yang-Na twig ointment 20 mg/mL (YN20) and 40 mg/mL (YN40) and tetracycline ointment (TT). This manufacturing process ensured thorough mixing of the ingredients and consistency of the ointment.

Microorganism

Methicillin-resistant *S. aureus* (MRSA; DMSC20651) was obtained from the Ministry of Public Health (Nonthaburi, Thailand).

Induction of skin abrasion wounds and infection

Sixty 6-week-old male ICR mice (weight ~30-35 grams) were supplied by Nomura Siam International, Thailand. Mice were allocated into six groups (n = 10 each group) at random. All mice had a weeklong acclimation period in the NELAC, Khon Kaen University. The IACUC of Khon Kaen University certified for animal handling and experimental studies [Approval number: IACUC-KKU-5/2565].

A 2 × 2 cm^2 area of the mouse's rear fur was thoroughly shaved after being anesthetized with Zoletil®100. To create abrasion wounds, pieces of adhesive bandage were rapidly adhered and removed from the shaved skin for 20 to 25 repetitions. This process was repeated until there was no observable bleeding and the skin was flamed and reddened, equivalent to a transepidermal water loss (TWL) value normalized to 40 to 60 g/m^2h, which was determined using a GPSkin (Gpower Inc., Seoul, South Korea).[12] Following this, the wound was inoculated with 1×10^6 CFU of MRSA in sterile 0.9% NaCl. For nine consecutive days, the ointment formulations, i.e., B, TT, YN20, and YN40, were applied to the wounds.

The wounds were swabbed on every other day (days 1, 3, 5, 7, and 9) in an animal biological safety cabinet level 3 and cultured on mannitol salt agar containing oxacillin (6 g/mL). Plates were incubated overnight prior to colony counting. During day 0 to 8, a GPSkin was employed to measure the TWL value and skin barrier strength of the wounds. The wounds were photographed daily and tissue processing and total RNA preparation were performed 24 hours after the final treatment.[13]

Histology of the skin abrasion wounds

Following euthanasia by Thiopental VÚAB (200 mg/kg), the wound tissues were collected, stored in ice-cold phosphate-buffered saline (PBS), and fixed in PBS with 4% paraformaldehyde for 24 hours. Then the wound samples were dehydrated using a gradual ethanol solution (50 to 100%) before soaking in xylene for 1 hour at 55 to 60°C, followed embedding in paraffin for 1 hour. The embedded tissues from days 2 to 8 were sliced into 5 μm-slices and heated for fixing on glass slides. The slides were then slowly refreshed in ethanol (100 to 70%) after being dewaxed in xylene twice (for 3 to 5 minutes each time). The tissue slices were coloured with 0.1% toluidine blue O solution (pH 2.0 to 2.5) for 5 minutes prior to rinsing with ethanol and distilled water and mounting with Permount™ ~~and.~~ Images were captured at 400× magnification using an Olympus CX23 microscope coupled with an Olympus EP50 (Tokyo, Japan), and two examiners separately counted the mast cells.

Determination of mRNA expression

Total RNA was prepared from the wounds using the acid guanidinium thiocyanate-phenol-chloroform method. ReverTraAce® was used to reverse-transcribe total RNA into cDNA. Expression of the TLR-2, NF-κB, TNF-*α,* IL-1*β*, IL-6, and IL-10 target genes and a reference gene (GAPDH) were quantitatively determined by the CFX96 Touch (Bio-Rad Laboratories Inc., Hercules, California, USA) coupled with TaqMan™ Gene Expression Master Mix and Thunderbird™ qPCR Mix. The $^{\Delta\Delta}$Ct method was used to determine fold changes in mRNA expression.[14]

Statistical analysis

The means and standard deviations of all results are shown (n = 6-10). The study employed ANOVA coupled with a Tukey's *post hoc* test (SPSS® ver. 26, IBM®, Armonk, NY, USA). $p<0.05$ was used to determine significance.

Results and Discussion

The appearance of the abrasion wounds and the MRSA colony count

Figure 1 depicts mouse rear skin after fur shaving at before and after the induction of the abrasion wounds and on days 6 and 8 of the treatments. After the induction of the abrasion wounds, the appearance and integrity of the wounds were similar across all groups as they were normalized for TWL values between 40 and 60 g/m^2h. On day 8, wounds treated with Yang-Na twig ointments at both concentrations (20 and 40 mg/g) and the non-infected control wounds had healed while the untreated wounds (MRSA-NT) and wounds treated with ointment base (MRSA-B) or tetracycline ointment (MRSA-TT) had not. Reduced numbers of MRSA colonies recovered from the wounds (Table 1) corresponded well with improvements in the wound's appearance and skin integrity. No MRSA colonies were observed on the plates from the non-infected control wounds throughout the 9-day-observation period. Wounds with MRSA infection showed the largest number of colonies on day 1 and these numbers decreased by varying amounts in the following days, depending on the treatment. The MRSA colony count in untreated abrasion wounds (Non treatment) and those treated with ointment base or tetracycline ointment remained high until day 5 before substantially dropping on day 7, although small numbers of colonies were remained on day 9. Interestingly, the wounds treated with Yang-Na twig ointments showed a sharp decline in the MRSA colony count recovered starting on day 3 with little or no colonies recovered on day 7. The infected skin abrasion wounds treated with the Yang-Na twig ointments showed a sharp decline in the viable MRSA colony count recovered from the wounds on day 3 of treatment, which agrees with a previous report on the antimicrobial, antioxidant, anti-inflammatory, and wound-healing effects of a Yang-Na extract and its phenolic, flavonoid, tannin, and alkaloid constituents.[10]

Skin integrity of the abrasion wounds

After the induction of abrasion wounds, all mice demonstrated a significant decrease in skin barrier strength (Figure 2A) and an increase in TWL (Figure 2B) with or without MRSA infection. The skin barrier strength of untreated infected abrasion wounds (MRSA-NT) and infected wounds treated with either ointment base (MRSA-B) or tetracycline ointment (MRSA-TT) remained relatively low until day 8. In contrast, the wounds treated with the 20 and 40 mg/mL Yang-Na ointments (MRSA-YN20 and MRSA-YN40) showed progressive increases in skin barrier strength to a level near that of the control (non-infected abrasion wound; CT). The TWL values of the MRSA-YN20 and MRSA-YN40 were restored to the level seen before abrasion wound induction on day 2 (Figure 1B), while the increased TWL values

of the uninfected control (CT) persisted on day 2 and normalized on day 8.

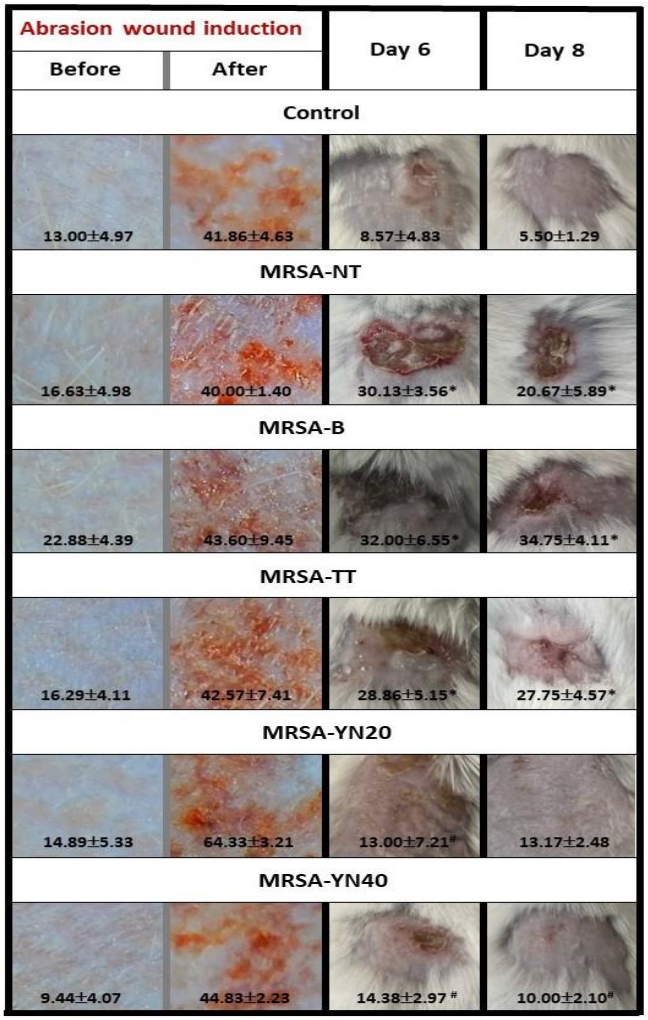

Figure 1: Abrasion wound appearance.
MRSA, methicillin-resistant *Staphylococcus aureus*; NT, non-treatment; B, ointment base; TT, tetracycline ointment 160 µg/g; YN20 and YN40, Yang-Na twig ointments 20 and 40 mg/g. Each wound photograph with a number of TWL (mean ± SD) is shown. **p*<0.05 VS control and #*p*<0.05 VS MRSA-NT on the same day.

The MRSA-NT, MRSA-B, and MRSA-TT groups showed a gradual decrease in TWL from day 2, but the values remained noticeably greater than the control on the last day of observation (day 8). Wound healing is a process that ensures the restoration of skin integrity. In this process, inflammation is the first phase after injury.[15] Abrasion wounds are a common type of skin injury that can occur due to mechanical trauma, such as friction, scratching, or scraping.[16] These wounds are characterized by damage to the skin's outermost layer, the stratum corneum, which can compromise skin barrier function and increase the rate of TWL. In addition, infections of skin abrasion wounds are often superficial and restricted to epidermis and upper dermis.[16] As a result, reducing the rate of TWL is an important aspect of wound healing and can aid in prevention of infection, inflammation, and further damage.[17] In the context of wound management, reducing the rate of TWL can be achieved through various approaches, including the use of occlusive dressings, moisturizers, and barrier ointments. These interventions aim to enhance skin hydration and prevent excessive water loss, thereby improving skin barrier function and supporting wound healing.[18] In this study, the mice with abrasion wounds showed lower skin barrier strength and higher TWL values, particularly those with MRSA infection, which was consistent with previous findings.[10,19,20] The Yang-Na twig ointments were able to increase skin barrier strength and reduce TWL to improve skin integrity revealing their potential as a natural alternative treatment for infected abrasion wounds, especially those caused by MRSA.

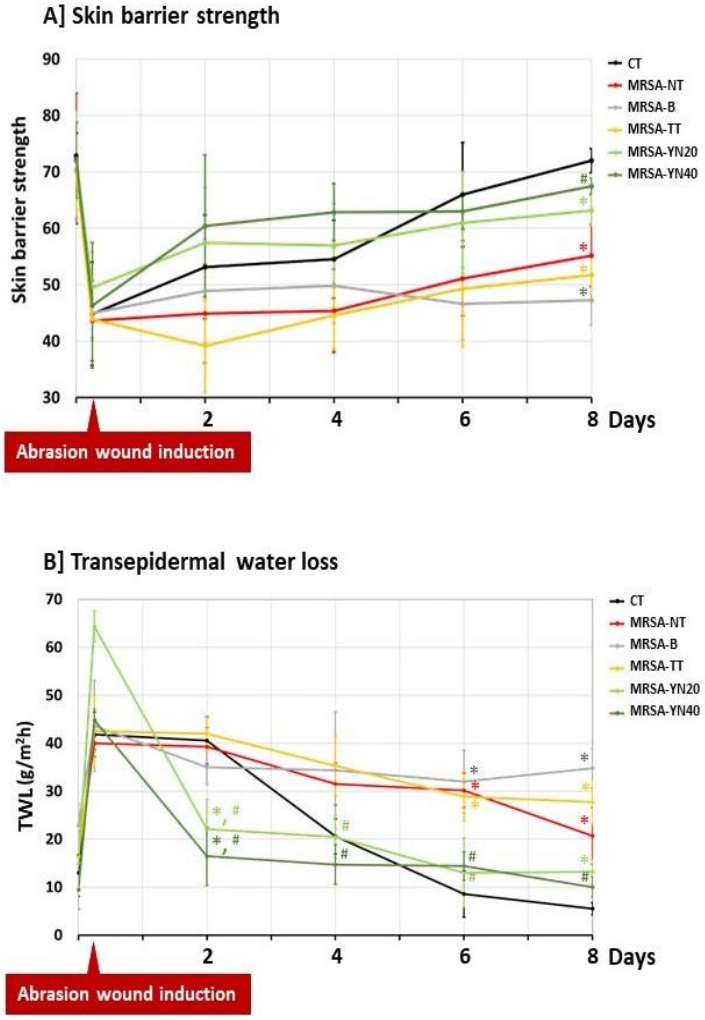

Figure 2: Skin integrity of abrasion wounds.
TWL, transepidermal water loss; CT, non-infected control; MRSA, methicillin-resistant *Staphylococcus aureus*; NT, non-treatment; B, ointment base; TT, tetracycline ointment 160 µg/g; YN20 and YN40, Yang-Na twig ointments 20 and 40 mg/g. **p*<0.05 VS CT and #*p*<0.05 VS MRSA-NT on the same day.

These improvements in skin barrier strength and skin integrity might be attributed to the presence of various antioxidant, anti-inflammatory, antimicrobial, and cytotoxic bioactive compounds in the Yang-Na twig extract.[9,10]

Mast cell infiltration of the abrasion wounds
Sections from the skin abrasion wounds were stained with toluidine blue O and mast cell counts per high power field were determined. Mast cell (red arrows) infiltration and mast cell numbers (mean ± SD) are presented in Figure 3. Increased mast cell infiltration correlated well with reduced skin integrity (Figure 2) and increased MRSA colony count (Table 1). The number of mast cells present in the wound sections was relatively high in the untreated abrasion wounds (NT) and the wounds treated with either B or TT compared to the uninfected control and infected wounds treated with YN20 and YN40. These findings demonstrated that the degree of mast cell infiltration in abrasion wounds was associated with both skin integrity and MRSA colonization.[21] The number of mast cells in the wounds was counted every other day with toluidine blue O-staining,[22] and the results showed that the untreated infected wounds and those treated with B or TT had higher numbers of mast cells compared to the uninfected control and the infected wounds

treated with YN20 and YN40. These findings were consistent with a previous study that showed mast cell infiltration was increased in wounds with delayed healing and chronic infections.[23]

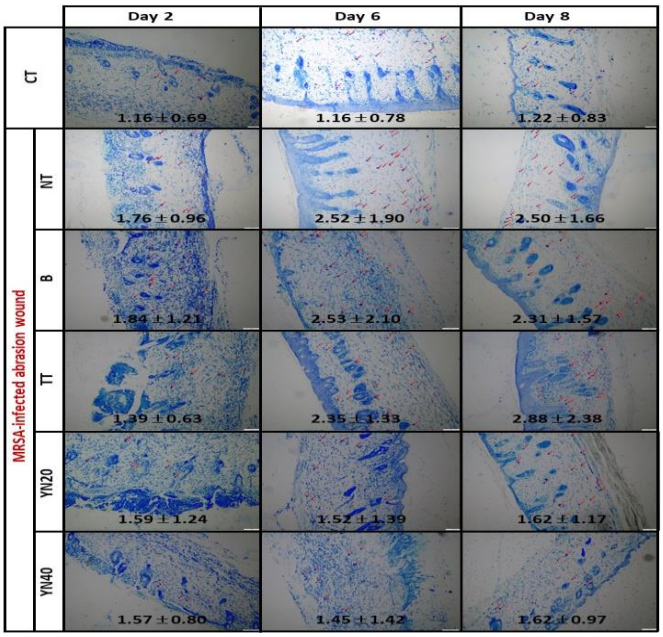

Figure 3: Histology of toluidine blue O-stained abrasion wounds.
Mast cells were counted per high power field and presenting by mean ± SD. CT, non-infected control; MRSA, methicillin-resistant *Staphylococcus aureus*; NT, non-treatment; B, ointment base; TT, tetracycline ointment 160 µg/g; YN20 and YN40, Yang-Na twig ointments 20 and 40 mg/g. Red arrows indicate mast cells.

Upon activation following injury, mast cells play a crucial role in wound healing by releasing cytokines, growth factors, and other mediators that regulate inflammatory response and mediate tissue repair.[24] Excessive activation of mast cells can lead to chronic inflammation and impaired healing.[25] Hence, inhibiting mast cell activation can alleviate production of inflammatory cytokines and lead to smaller lesions with better-organized collagen fibers.[26] Abrasion wound histology analysis revealed that mast cell infiltration correlated well with skin integrity and appearance, as well as the MRSA colony count. Yang-Na twig ointments reduced mast cell infiltration in MRSA-infected abrasion wounds, which is consistent with previous studies of the anti-inflammatory and wound healing properties of Yang-Na twig extracts.[10,19]

Expression of inflammatory-related genes in infected skin abrasion wounds

Infected abrasion wounds showed significantly increased expression levels for the inflammatory-related genes, TLR-2, NF-κB, TNF-α, IL-1β, IL-6, and IL-10, which were gradually suppressed each day with treatment (Figure 4). On day 8, YN20 and YN40 treatments had significantly down-regulated TLR-2 expression compared to the control (Figure 4A), whereas the other treatments did not. Expression levels of NF-κB were high until day 8 in the MRSA-NT, MRSA-B, and MRSA-TT groups.

In contrast, the expression of NF-κB was the same as the uninfected control on day 8 for the MRSA-YN20 and MRSA-YN 40 (Figure 4B). Expression of TNF-α, IL-1β, and IL-6 mRNA was significantly high in the MRSA-NT group throughout the observation period, while the other treatments restored expression of these genes to control levels on the last day of observation (Figure 4C – 4E). The increased IL-10 expression seen after induction of the abrasion wounds declined over the observation period, though it remained higher than the uninfected control on the final day of observation (Figure 4F). Expression of TLR-2 is up-regulated in the early phase of wound healing and skin wound healing is essentially deferred in TLR-2 deficient mice.[26]

TLR-2 is known to be expressed on keratinocytes and is capable of identifying certain components of *S. aureus*,[27] which are a fundamental constituent of the epidermis and play a role in wound healing as a trigger for the generation of IL-1β, IL-6, IL-10, and TNF-α cytokines.[26,28,29] In the present study, the induction of TLR-2, NF-κB, TNF-α, IL-1β, IL-6, and IL-10 in the infected skin abrasion wounds was suppressed with treatment, consistent with previous reports relating the role of these inflammatory markers in wounds.[19,30]

Table 1: MRSA colony count on skin abrasion wounds

	The MRSA Colony Count[†]				
	Day 1	**Day 3**	**Day 5**	**Day 7**	**Day 9**
Non-infected wound	0	0	0	0	0
MRSA-infected abrasion wound					
Non treatment	>500	236 (199)	203 (211)	11 (12)	5 (7)
Ointment base	>500	293 (197)	233 (144)	18 (5)	10 (0)
Tetracycline ointment 160 µg/g	>500	250 (141)	303 (228)	13 (4)	5 (7)
Yang-Na twig ointment 20 mg/g	>500	8 (13)*	6 (13)*	3 (5)	0
Yang-Na twig ointment 40 mg/g	>500	5 (9)*	13 (10)*	0	0

Note. [†]The MRSA colony count is shown as a mean (SD). *p <0.05 VS Non treatment.

The Yang-Na twig ointments exhibited their anti-inflammatory activity via down-regulation of TLR-2. When *S. aureus* lipoproteins bind to TLR2 homo- or hetero-dimers, a series of signaling events occur that are transmitted through the intracellular adapter MyD88, leading to NF-κB signaling activation and releasing proinflammatory cytokines, IL-1β, IL-6, and TNF-α.[31,32] Overexpression of NF-κB can lead to impaired wound healing and prolonged inflammation.[33] In one study, *S. aureus* infection of superficial skin wounds led to a significant induction of IL-1β, IL-6, and TNF-α from days 1 to 6.[34] In another study, innate cells (particularly monocytes and macrophages) and T cells produced increased amounts of IL-10, anti-inflammatory cytokine, in response to *S. aureus* infection.[35] Therefore, the Yang-Na twig ointments showed significant anti-inflammatory potential.

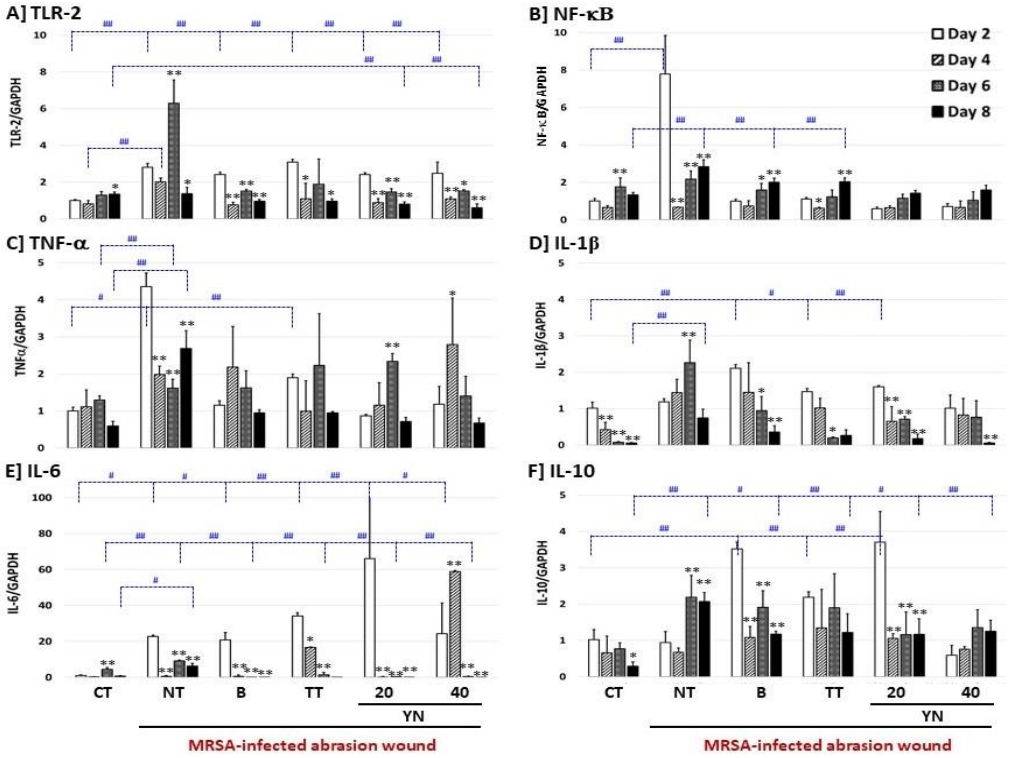

Figure 4: Expression of inflammatory related genes in abrasion wounds.
A) TLR-2, B) NF-κB, C) TNF-α, D) IL-1β, E) IL-6, and F) IL-10 mRNA. CT, non-infected control; MRSA, methicillin-resistant *Staphylococcus aureus*; NT, non-treatment; B, ointment base; TT, tetracycline ointment 160 μg/g; YN20 and YN40, Yang-Na twig ointments 20 and 40 mg/g. *, **p<0.05, 0.01 VS Day 2 within the same treatment; #,##p<0.05, 0.01 VS CT on the same day.

Conclusion

Overall, the results provide promising evidence for the use of Yang-Na twig ointments in the treatment of infected abrasion wounds, particularly those caused by MRSA. Further investigation should be explored to better understand the underlying mechanisms of action and to assess clinical efficacy of the Yang-Na twig ointments will be worthwhile.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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