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Combinatory effects of *Dipterocarpus alatus* twig emulgel: Wound-restoring, antibacterial, and anti-inflammatory activities against methicillin-resistant *Staphylococcus aureus*-infected mouse superficial wounds

Waranya Chatuphonprasert [a,b], Nitima Tatiya-aphiradee [b], Khaetthareeya Sutthanut [c], Sutthiwan Thammawat [a], Ploenthip Puthongking [c], Naroeporn Nopwinyoowong [b], Kanokwan Jarukamjorn [b,c,*]

[a] *Division of Pre-clinic, Faculty of Medicine, Mahasarakham University, Maha Sarakham, 44000, Thailand*
[b] *Research Group for Pharmaceutical Activities of Natural Products Using Pharmaceutical Biotechnology (PANPB), Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, 40002, Thailand*
[c] *Division of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002 Thailand*

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ABSTRACT

Dipterocarpus alatus has been used for the treatment of infectious skin diseases and ulcerative wounds in Thai traditional medicine. A major pathogen in human superficial skin infections is methicillin-resistant *Staphylococcus aureus* (MRSA). This study determined the wound healing, antibacterial, and anti-inflammatory activities of *D. alatus* twig emulgel against MRSA-infected mouse superficial skin wounds. Ethyl acetate-methanol crude extract of *D. alatus* twig was incorporated into emulgel at concentrations of 20 and 40 mg/g (D20 and D40) and its activity was compared to tetracycline emulgel (160 μg/g, Tetra). MRSA-infected superficial wounds demonstrated decreased skin barrier strength, increased transepidermal water loss (TEWL), and mast cell accumulation. Expression of toll-like receptor 2 (TLR-2), NF-κβ, TNFα, IL-1β, IL-6 and IL-10 genes were induced after MRSA infection. Daily application of 100 μL of D20 or D40 for 9 days restored skin barrier strength and TEWL while reducing mast cell and MRSA numbers compared to the non-treated group (MRSA-NT). The wounds treated with D20 and D40 were entirely healed on day 9. Expression of TLR-2 and cytokine-related genes NF-κβ, TNFα, IL-1β, IL-6 and IL-10 were normalized by treatment with either D20 or D40. Therefore, emulgel containing 20 to 40 mg/g ethyl acetate-methanol crude *D. alatus* twig extract is a good candidate for development as a topical formulation for MRSA-infected ulcerated wounds.

1. Introduction

Staphylococcus aureus, an aerobic Gram-positive spherical bacterium, is a major pathogen for ulcerative skin and soft tissue infection with abscess or furuncle [1–3]. *S. aureus* isolates which are resistant to methicillin or oxacillin are classified as

* Corresponding author. Faculty of Pharmaceutical Sciences, Khon Kaen University, 123 Mitrapharb Road, Khon Kaen 40002 Thailand.
E-mail address: kanok_ja@kku.ac.th (K. Jarukamjorn).

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methicillin-resistant *S. aureus* (MRSA) [4] and the rate of resistance to empiric antibiotics, including methicillin, in *S. aureus* isolates is rising [5]. Therefore, the identification of novel alternative treatments for MRSA infection is required.

The resin tree (Yang-na in Thai), *Dipterocarpus alatus* Roxb. ex G. Don, is a perennial plant in the Dipterocarpaceae family that can grow up to 40 m tall. It is native to both evergreen and deciduous forests and is commonly found in Cambodia, Laos, Myanmar, the Philippines, and Viet Nam, as well as in Thailand. *D. alatus* is one of the most important timber species, after teak, in Thailand [6]. Among skin diseases, ulcerated wounds are traditionally treated with bark oil extract of *D. alatus* [7]. Several bioactive constituents from *D. alatus.* including sesquiterpenes, triterpenes, and coumarin derivatives, have antioxidative and cytotoxic properties [8,9] and oligostilbenoids from *D. alatus* stem wood demonstrated acetylcholine esterase inhibition [10]. Moreover, a resveratrol tetramer from *D. alatus* (vaticaffinol) was shown to possess inhibitory activity against xanthine dehydrogenase and xanthine oxidase in mouse livers [11]. For antimicrobial activity, dipterocarpol derivatives, which are major constituents of *D. alatus,* inhibited the growth of *Candida albicans*, *Cryptococcus neoformans*, and *S. aureus* [12,13]. A previous study reported that extracts prepared from *D. alatus* twig showed anti-MRSA effects [14].

Emulgel is a hybrid pharmaceutical formulation of lotion (emulsion) and gel for topical drug delivery. This combined formulation has advantages in the versatility of drug loading and high loading capacities for both polar and non-polar compounds, as well as greaseless, spreadable, and emollient physical properties and a long shelf life. The beneficial features of an emulgel system for wound healing were demonstrated to relate to a three-dimensional hydrogel matrix formation at applied sites which facilitated formation of an occlusive film, drug release to target cells, and absorbance of wound-exudate [15,16]. This makes emulgel a preferable drug delivery system for analgesics, anti-inflammatory, anti-acne, and anti-fungal drugs and herbal medicinal extracts [17–19]. Therefore, emulgel has potential for the topical application of complex chemical composition extracts, including *D. alatus* twig extract.

Herewith, a topical emulgel formulation containing *D. alatus* twig extract was developed and its antibacterial, anti-inflammatory, and would healing activities were assessed in an MRSA-infected mouse superficial skin wound model.

2. Materials and methods

2.1. D. alatus twig emulgel formulation

D. Alatus twigs (voucher specimen No. PSKKF03682) were procured in Khon Kaen Province, Thailand (January 2020). The plant was identified by a verified botanist and the voucher specimen has been retained in Division of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University (KKU), Thailand.

MeOH(EtOAc) extract was chosen in this study because a previous report revealed that MeOH(EtOAc) extract of *D. alatus* twigs had the highest anti-bacterial activity (with the lowest MIC and MBC values) among various extracts [20]. In brief, twigs of *D. alatus* were washed with water, cut, and dried at 45–50 °C in a hot air oven, before mashing and macerating with ethyl acetate (EtOAc) for 24 h (repeated 3 times). The organic solvent was filtered using Whatman® No.1 and the rest was dried and kept in a freezer at −20 °C for further study as the EtOAc extract. The residue from the previous step was further macerated with methanol (MeOH) overnight 3 times. The MeOH layer was filtered and the rest was dried over a rotary evaporator to yield the MeOH(EtOAc) extract for preparation of *D. alatus* twig emulgel.

Emulgel base was formulated from a mixture of lipophilic and hydrophilic compounds in a ratio of 1:5.7 (w/w), comprising of glycerin, polysorbate 80, niacin, triethanolamine, phenoxyethanol of coconut oil, shea butter, tocopheryl acetate, sorbitan monooleate, and carbomer. In optimal designated concentrations of each component, three different phases were separately prepared based on their physicochemical properties, including aqueous phase, oil phase, and thickening agent phase. The emulsion was constituted by mixing between the 75–80 °C pre-heated aqueous and oil phases with continuous stirring until obtained a milky homogeneous emulsion. Then, the thickening agent phase was added to the emulsion followed with continuous stirring until a homogenous thickening mixture, emulgel base (Base), was obtained. The Base was further used in formulating of tetracycline emulgel (160 μg/g; Tetra) and *D. alatus* twig emulgel (20 and 40 mg/g; D20 and D40).

Tetracycline emulgel at 160 μg/g (Tetra) was formulated by well mixing in a 1:100 vol to mass ratio of tetracycline HCl solution (concentration 17.40 mg/mL prepared in deionized water) with emulgel base. *D. alatus* twig emulgel at concentrations of 20 and 40 mg/g were separately formulated. The *D. alatus* twig extract solution at concentrations of 0.175 and 0.35 g/mL were separately prepared in 35% aqueous ethanol and used to formulate the *D. alatus* twig emulgel preparations. With a 1:25 vol to mass ratio of the *D. alatus* twig extract solution and the emulgel base, the *D. alatus* twig emulgel at 20 and 40 mg/g were prepared using the *D. alatus* twig extract solution at concentrations of 0.175 and 0.35 g/mL, respectively.

2.2. The tape stripping mouse model

The male ICR mice (6–7 weeks old, weight 30–35 g) were provided by Nomura Siam International, Bangkok, Thailand. All mice were acclimated for a week in NELAC, KKU. Animal handlings and mouse experiments were authorized by Institutional Animal Ethic Committee for Use and Care of KKU (Approval No. IACUC-KKU-5/2565). The mice were randomly divided into six groups (n = 9–10 per group).

Briefly, the mouse back hair was entirely shaved for an area of 2 × 2 cm^2 under anesthesia with 30–50 mg/kg of Zoletil®100, a mixture of tiletamine and zolazepam. The ulcerative wounds were induced with quickly sticking and pulling off the shaved skin for 20–25 times pieces of sticky bandage until the skin became reddish and inflamed, without bleeding at the same level of a *trans*-epidermal water loss (TEWL) value [20].

The damage of wounds was standardized to a *trans*-epidermal water loss (TEWL) value of 70–80 g/m^{2}h, and then a 10 μL-aliquot of 1×10^8 CFU/mL MRSA inoculum (in sterile 0.9% sodium chloride) was applied to the wound [20]. At the first hour after the MRSA inoculation, a 100 μL-aliquot of each emulgel formulations, namely Base, Tetra, D20, and D40, were daily applied to the wounds for 9 consecutive days (Day 1–9).

On days 1, 3, 5, 7, and 9, MRSA colonies from the mouse skin were swabbed under the safety laminar cabinet (animal biosafety level 3) for culturing on oxacillin (6 μg/mL)-contained mannitol salt agar. The MRSA was incubated for overnight before colony counting. The skin barrier strength and TEWL were measured on days 1, 2, 4, 6, and 8 using GPSkin device (Gpower Inc, Seoul, South Korea) [21]. Photographs of the wounds were collected daily. After 24 h of the last treatments, the wounds were collected for RNA preparation and tissue processing.

2.3. Toluidine blue O staining

After euthanizing mice with thiopental (200 mg/kg), the wounds (1×1 cm^2 each) were immediately collected and immersed in ice-cold phosphate-buffered saline (PBS) before embedding in 4% paraformaldehyde (PFA) in PBS (10-fold volume) for 18–24 h. Then, skin samples were progressively dehydrated in ethanol (50 to 100%). After dehydration, skin tissues were soaked in xylene for 1 h, xylene: paraffin (1:1) at 55–60 °C for 1 h before embedding in paraffin. The paraffin-embedded skins (days 2, 4, 6, and 8) were cut into 5 μm-sections and heat-fixed on glass slides (55–60 °C). The slides were dewaxed in xylene (3–5 min for 2 times) and gradually rehydrated in ethanol (100 to 70%), followed by staining with 0.1% toluidine blue O solution (pH 2.0–2.5) for 5 min. The slides were washed with distilled water and ethanol, before mounting (Permount™) with coverslips. Wound histological images were collected at 100- and 400-fold magnification using C×23-microscope and Olympus EP50 (Olympus, Tokyo, Japan). Mast cells were independently counted by two researchers.

2.4. Quantitative qPCR analysis

Total RNA of the wound was extracted with acid guanidinium thiocyanate-phenol-chloroform, then reverse-transcribed to cDNA using ReverTraAce® kit (Toyobo, Osaka, Japan). Expression level of interleukin 1beta (IL-1β, Mm00434228_m1), IL-6 (Mm00446190_m1), IL-10 (Mm01288386_m1), nuclear factor kappa B (NF-κβ, Mm00456853_m1), toll-loke receptor 2 (TLR-2, Mm01213946_g1), tumor necrosis factor alpha (TNF-α, Mm00443258_m1), and a reference gene GAPDH (Mm99999915_g1) mRNAs was determined using TaqMan™ Gene Expression Master Mix (Applied Biosystems, Waltham, MA, USA) with THUNDERBIRD™ qPCR Mix (Toyobo, Osaka, Japan). The fold changes of mRNA expressions were calculated using ΔΔCt method.

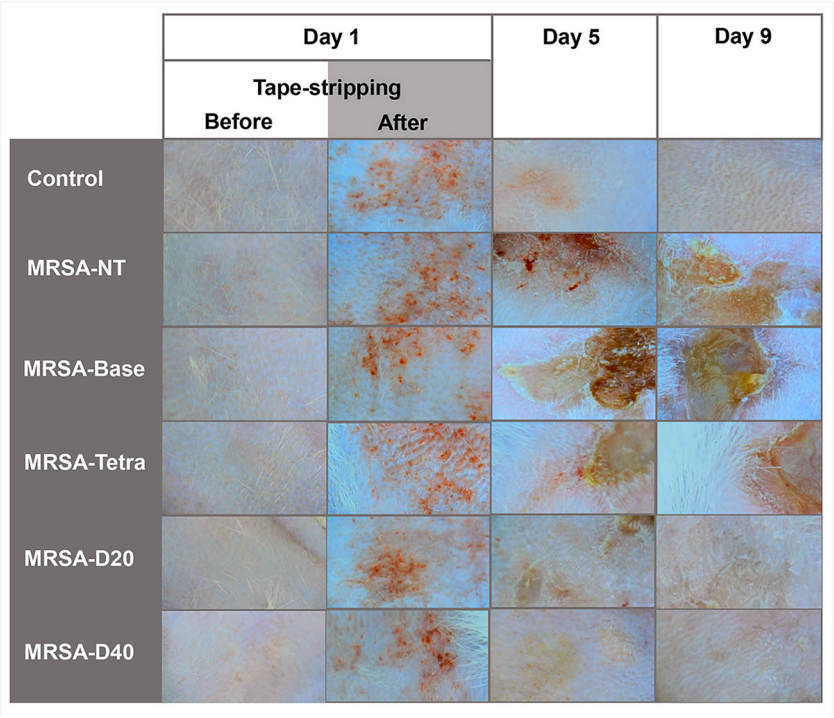


Fig. 1. The wound appearance on the mouse skin.

2.5. Statistical analysis

All results are presented as mean ± standard deviation (n = 6–9). One-way analysis of variance couple with Tukey's post hoc test was carried out (IBM® SPSS® software ver. 26, Armonk, NY). A significance was considered at $p < 0.05$.

3. Results

3.1. Wound appearance, skin barrier strength, TEWL, and MRSA

After the induction of superficial wounds by the tape stripping technique, non-infected wounds (control) appeared healed within 5 days (Fig. 1). MRSA-infected wounds with non-treatment (MRSA-NT), Base (MRSA-Base), and Tetra (MRSA-Tetra) appeared ulcerous on day 5 and this was maintained until day 9, whilst MRSA-infected wounds treated with either D20 or D40 demonstrated no ulcers and closure of the wounds on day 5 (Fig. 1). The skin barrier strength and TEWL values corresponded with wound appearance. The skin barrier strength values of the MRSA-D20 and MRSA-D40 groups were comparable to the control from day 3 onwards, whereas those of the MRSA-NT, MRSA-Base, and MRSA-Tetra groups were significantly less than the control until day 9 (Fig. 2A). TEWL values (Fig. 2B) represent the passive loss of water through the skin because of damage to the epidermis after tape stripping. On day 1 after tape stripping, the TEWL values were comparable among all treatment groups. On the last day of observation (day 9), the TEWL values of the non-infected control and the MRSA-D20 and MRSA-D40 treatment groups were low, indicating the completion of wound healing. The TEWL values of the MRSA-Base and MRSA-Tetra treatment groups remained high until day 9, indicating that the wounds did not heal.

The number of MRSA recovered from wounds was the same for all treatment groups on the first day of infection (>500 colonies, Table 1). MRSA colony numbers decreased for all treatment groups over the time course, but the D20, D40, and Tetra treatment groups showed significantly less MRSA colonies than the NT and Base treatment groups on days 3, 5, and 7, and no viable MRSA were recovered from the D20, D40 and Tetra treatment groups on day 9.

3.2. Effect of D. alatus twig emulgel on mast cell infiltration in MRSA superficial infections

Mast cells are seen as dark blue or red-purple spots by toluidine blue O staining (Fig. 3B, red arrows). The number of mast cells (Fig. 3A) correlated to the wound's appearance, and the skin barrier strength and TEWL values. Mast cells were observed in the wounds of all groups from day 1 (after tape stripping), with numbers peaking on day 4 in the MRSA-NT and MRSA-Base treatment groups (Fig. 3A). Treatment with either D20, D40, or Tetra significantly reduced the number of mast cells.

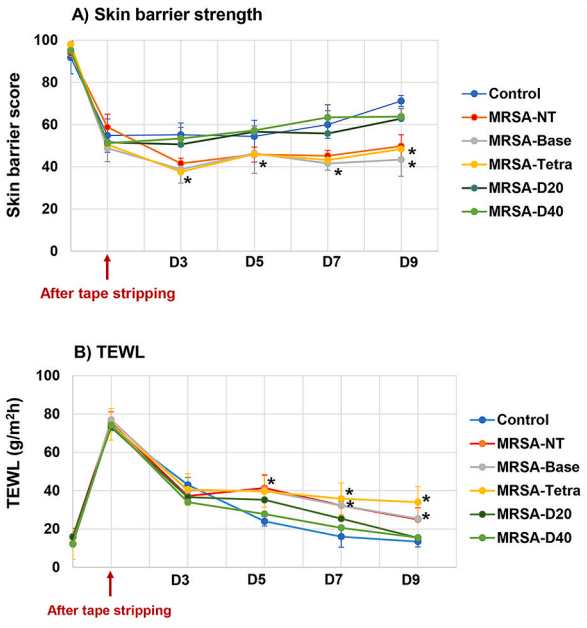
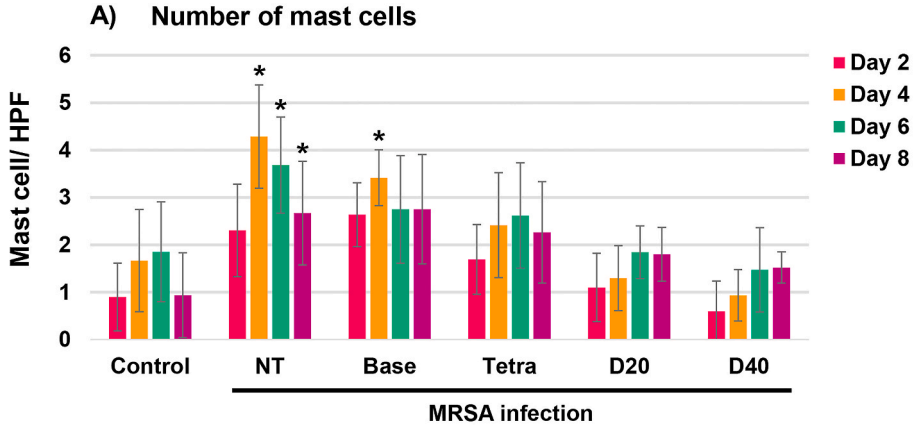


Fig. 2. The skin barrier strength and transepidermal water loss (TEWL) value of the wounds.
*$p < 0.05$ VS Control on the same day (n = 5).

Table 1
The number of MRSA colonies.

	Number of MRSA colonies (CFU)				
	Day 1	Day 3	Day 5	Day 7	Day 9
Control	0	0	0	0	0
MRSA-NT	>500	363 ± 199	268 ± 161	28 ± 18	10 ± 5
MRSA-Base	>500	357 ± 198	233 ± 131	20 ± 14	14 ± 10
MRSA-Tetra	>500	128 ± 67*	133 ± 32*	8 ± 6*	0
MRSA-D20	>500	148 ± 40*	130 ± 26*	10 ± 5*	0
MRSA-D40	>500	117 ± 42*	93 ± 27*	5 ± 3*	0

Note. CFU, colony forming unit; Control, non-infected mice; MRSA, MRSA-induced superficial infection; Base, emulgel base; Tetra, tetracycline emulgel (160 μg/g); D20 and D40, *D. alatus* twig emulgels (20 and 40 mg/g, respectively). *$p < 0.05$ VS MRSA-NT.



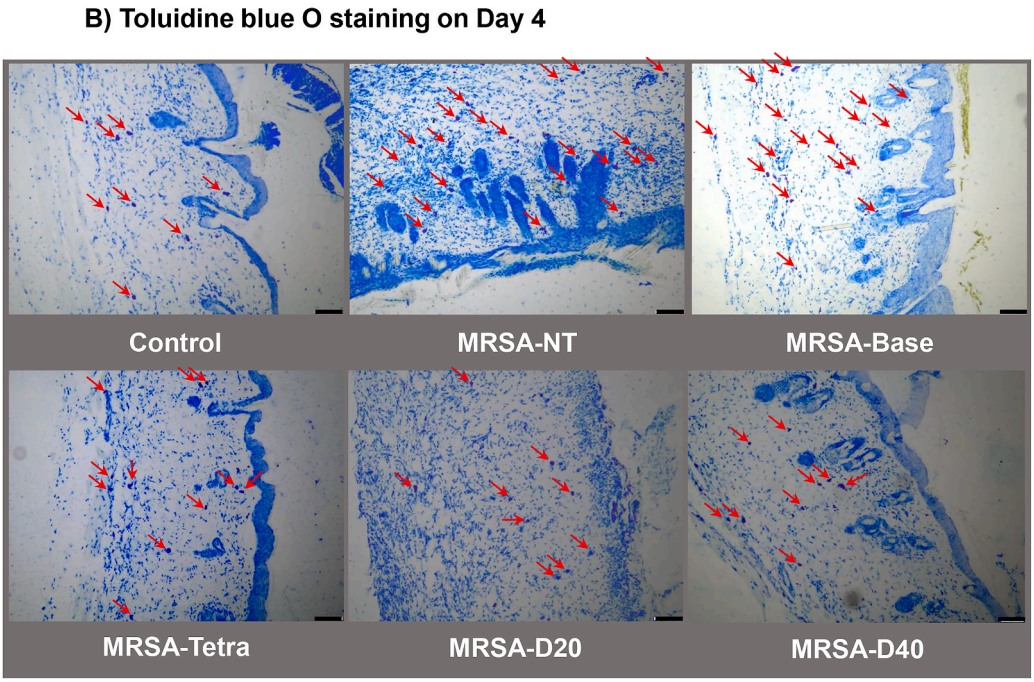


Fig. 3. The number of mast cells and toluidine blue O staining histology of the wounds.
A) *$p < 0.05$ VS Control on the same day (n = 5). B) The red arrows indicate mast cells (violet dot) and the scale bar is 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.3. Effect of D. alatus twig emulgel on the expression of TLR-2 and pro-inflammatory cytokine mRNAs in MRSA superficial infections

In general, MRSA infection induced expression of TLR-2 mRNA, with levels reaching their highest on day 6 before returning to normal on day 8 (Fig. 4A). D20 and D40 treatment suppressed TLR-2 expression compared to MRSA-NT from day 4, while Tetra treatment did not. In the NT and Base groups, expression of NK-κβ mRNA was significantly elevated on day 2 before returning to normal on day 4, but this was prevented by treatment with D20, D40, and Tetra (Fig. 4B). Expression of TNF-α mRNA was induced by MRSA infection from day 2 to 8 and this was markedly suppressed by treatment with D20 and D40 (Fig. 4C). Likewise, the interleukins IL-1β, IL-6 and IL-10 were induced by MRSA infection (Fig. 4D-F) and restored to normal levels by D20 and D40 treatments. Therefore, in addition to its antibacterial activity, the D20-and D40-emulgel formulations showed promising anti-inflammatory potential.

4. Discussion

Our previous study reported that *D. alatus* twig extract possessed MRSA inhibitory activity *in vitro*, which was greater than its phytochemical constituents, dipterocarpol and gurjunene [14]. Tetracycline is a broad-spectrum antibiotic with activity against

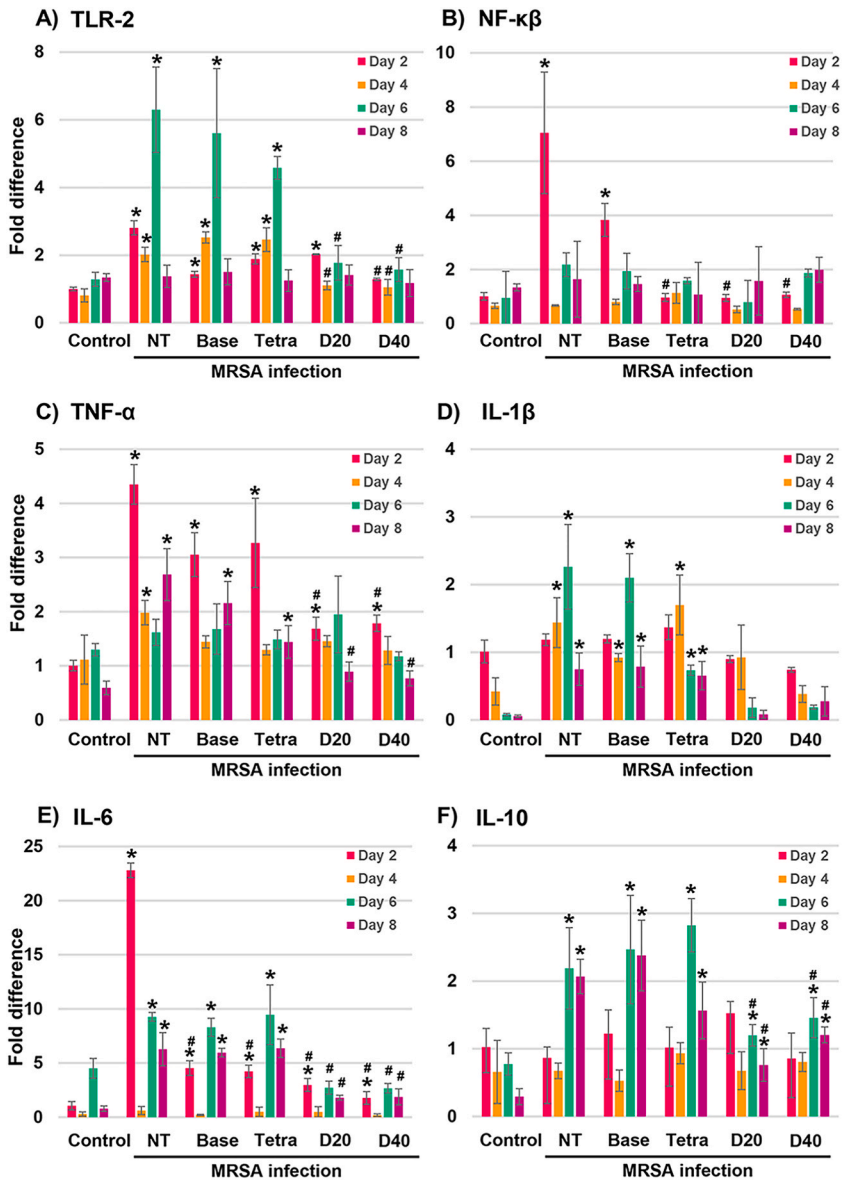


Fig. 4. The effects of *D. alatus* twig emulgel on the mRNA expression of A) toll-like receptor 2 (TLR-2) and pro-inflammatory cytokines: B) NF-κB, C) TNF-α, D) IL-1β, E) IL-6, and F) IL-10.
*$p < 0.05$ VS Control on the same day (n = 5); $^{\#}p < 0.05$ VS MRSA-NT on the same day (n = 5).

Gram-positive and Gram-negative aerobic and anaerobic bacteria that is usually prescribed for bacterial skin infection [22]. A topical tetracycline ointment was superior to a povidone-iodine gel in terms of inhibitory effects towards MRSA [23]. Hence, *D. alatus* twig extract (with concentrations of 20 and 40 mg/g; D20 and D40) and tetracycline (Tetra) emulgel formulations were prepared for comparison in the current study. The doses of D20, D40, and Tetra were selected based on the inhibitory concentrations of *D. alatus* twig extract against MSSA and MRSA *in vivo* [14,20].

A tape-stripping and MRSA-induced superficial wound infection model in mice was employed for examining antimicrobial and wound healing actions [20,24]. Tape stripping disrupts the epidermal layer and reduces skin barrier strength. Skin barrier strength and TEWL values can be used to represent the overall skin health, the degree of skin damage, and the skin barrier integrity [21]. The application of MRSA to the wounds enabled the pathogens to colonize the wound and elicited a profound inflammatory response. This model represents the common clinical condition of an ulcerative wound with a staphylococcal infection that can be encountered in daily life [24]. In the present study, the wounds of normal mice (non-infected control) were self-healing within 9 days. MRSA infection prolonged wound healing with low skin barrier strength and high TEWL. Treatment of MRSA-infected wounds with D20 and D40 promoted wound healing, but treatment with Tetra did not.

Mast cells play key roles not only in allergic and anaphylactic reactions, but also in the immune response against bacterial pathogens, including in skin infections [25]. Mast cells release several pro-inflammatory and chemotactic mediators upon contact with pathogens [26] and IgE-activated mast cells have been shown to interfere with *S. aureus* growth and toxicity [27]. In the current study, the MRSA-NT group had the highest number of mast cells infiltrating wounds, reaching a peak on day 4 before numbers subsided on days 6 and 8. This recruitment of mast cells is mediated via tryptase, which contributes to inflammation in skin abscesses induced by *S. aureus* infection [28]. Accordingly, the D20, D40, and Tetra emulgel formulations may have reduced the number of mast cells in part due to their ability to reduce the number of MRSA present in the wounds.

TLR-2 is a membrane protein receptor expressed on epithelial cells. TLR-2 recognizes foreign substances including *S. aureus* and passes on appropriate signals to effector cells of the immune system. After *S. aureus* enters the body via exposed epithelial surfaces it initializes TLR-2-mediated local production of cytokines, chemokines, and antimicrobial peptides [29]. A previous study showed that TLR-2 was induced by MRSA infection on day 5 to day 8 [24], corresponding to the observations in the current study. Co-incubation of TLR-2 with virulent *S. aureus* leads to translocation of NF-κB, followed by enhanced transcription of NF-κB-controlled genes and the generation of proinflammatory mediators in primary human keratinocytes [30]. Induction of the NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways then leads to increase transcription of pro-inflammatory genes such as those encoding TNFα and IL-6 [31], which concurs with the present findings that MRSA infection induced expression of TLR-2, leading to up-regulation of NF-κB, TNFα, and IL-6. During localized subcutaneous infection, IL-10 production can play a detrimental role to facilitate bacterial persistence by controlling proinflammatory T cell responses [32]. *D. alatus* extract was reported to exert anti-inflammatory activity via nitric oxide, prostaglandin E2, IL-1β, and TNFα generation in lipopolysaccharide-activated RAW 264.7 cells [33]. This is the first time to report anti-inflammatory activity of *D. alatus* twig emulgel in MRSA-induced superficial infection in mice. This study examined the effects of *D. alatus* twig emulgel on MRSA, the most common drug-resistant skin pathogen; nevertheless, daily exposure to skin pathogens is not limited to MRSA. Therefore, clinical studies on the effect of this formulation are necessary and worthwhile.

5. Conclusion

The present findings reveal that *D. alatus* twig emulgel possesses antibacterial activity against MRSA and supports the wound healing process by dramatically down-regulating the accumulation of mast cells and the expression of inflammatory cytokines, namely TNF-α, IL-1β, IL-6, and IL-10 via TLR-2 and NF-κB pathways. Therefore, *D. alatus* twig emulgel is a promising antibacterial, wound healing, and anti-inflammatory candidate for MRSA-induced superficial skin infection.

Author contribution statement

Waranya Chatuphonprasert: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Nitima Tatiya-aphiradee: Performed the experiments.

Khaetthareeya Sutthanut: Performed the experiments.

Sutthiwan Thammawat: Performed the experiments.

Ploenthip Puthongking: Performed the experiments.

Kanokwan Jarukamjorn: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data will be made available on request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e17483.

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