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Original Research Article

Antibacterial Activity of *Dipterocarpus alatus* Twig Extract against Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

Dipterocarpus alatus has been used for the treatment of external wounds and skin diseases in traditional medicine. *Staphylococcus aureus* is a major pathogen for human superficial skin infections. This study determined the antibacterial effects of *D. alatus* twig crude extracts against methicillin-sensitive/resistant *S. aureus* (MSSA and MRSA) *in vitro* and *in vivo*. Several organic solvents were used to extract *D. alatus* twig. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the *D. alatus* extracts for MSSA and MRSA were investigated using broth micro-dilution assays. The most potent extract was studied in a mouse superficial wound model with MRSA infection. The ethyl acetate-methanol extract of *D. alatus*, MeOH(EtOAc), showed the lowest MIC and MBC values (0.390 and 0.781 mg/mL, respectively) against both MSSA and MRSA compared to the other extracts. MRSA-infected mouse superficial wounds showed increased transepidermal water loss (TEWL), mast cell accumulation, and MRSA colony numbers. Treatment with MeOH(EtOAc) extract (20 mg/mL), or MeOH(EtOAc) emulgel (20 - 40 mg/mL), 100 µL applied daily for 10 days, reduced TEWL values, mast cell accumulation, and MRSA colony numbers in wounds compared to the non-treated group. The wounds of the MeOH(EtOAc) extract and MeOH(EtOAc) emulgel treatment groups were healed on day 8 while the wounds of the dipterocarpol and tetracycline treatment groups were not. Therefore, MeOH(EtOAc) extract or MeOH(EtOAc) emulgel are good candidates for a topical product to treat ulcerated wounds with MRSA infection.

Keywords: Dipterocarpol, Resin tree, MRSA, MSSA, Antibacterial activity, Wound healing.

Introduction

One major pathogen for sepsis and abscess formation in the skin and soft tissue is *Staphylococcus aureus*, an aerobic Gram-positive spherical bacteria.[1,2] The rates of resistance among *S. aureus* strains to typical antibiotics has been rising.[3] *S. aureus*, which cannot be killed by methicillin or oxacillin is called methicillin-resistant *S. aureus* (MRSA).[3] MRSA is a common hospital-acquired infection that is now appearing as a community-acquired infection.[4] Therefore, searching for new plants with activity against MRSA is required.

Dipterocarpus alatus Roxb. ex G. Don, common name: resin tree or Yang na in Thai, is a popular plant in Southeast Asia with multiple applications. The oleo-resin of *D. alata* is used in the production of paper, wax, dyes, and varnish oil because of its water-proofing properties.[5] The bark oil of *D. alatus* is traditionally used for the treatment of skin diseases such as ulcerated or abscessed wounds.[6] Due to a large number of bioactive constituents in *D. alatus*, including sesquiterpenes, triterpenes, and coumarin derivatives, antioxidant and cytotoxic effects have been reported.[7, 8] Oligostilbenoids from *D. alatus* have been reported to have acetylcholine esterase inhibitory effects.[9] Vaticaffinol, a resveratrol tetramer from ethanol *D. alatus* crude extract, was shown to inhibit xanthine dehydrogenase and xanthine oxidase activities in mouse livers.[10] Dipterocarpol derivatives, which are major constituents of *D. alatus*, have shown cytotoxic effects and antimicrobial activity against *S. aureus*, *Candida albicans*, and *Cryptococcus neoformans*.[11,12] In our recent report, crude methanol extracts from several parts (barks, leaves, oleo-resin, wood, and twigs) of the *D. alatus* tree were shown to have antibacterial effects against MRSA, and the twig extracts showed the highest potency against MRSA with associated wound-healing effects.[13] Therefore, in the current study, a range of organic solvents and methods were used to make *D. alatus* twig extracts to identify the optimal extracts inhibiting MRSA.

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Materials and Methods

Dipterocarpus alatus crude extract preparation

The twigs of *D. alatus* were collected in Khon Kaen, Thailand in January 2020. *D. alatus* was identified by Suppachai Tiyaworanant, Division of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand. A voucher specimen was deposited in the Division of Pharmacognosy and Toxicology, Faculty of Pharmaceutical Sciences, Khon Kaen University, under the registration NO. PSKKF03682.

The twigs of *D. alatus* were dried at 50°C, mashed using a herb grinder and macerated with either methanol (MeOH), ethyl acetate

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(EtOAc), dichloromethane (CH_2Cl_2), hexane (Hex), MeOH:EtOAc (1:1), or MeOH:CH_2Cl_2 (1:1). The residues from the EtOAc, CH_2Cl_2, and hexane extractions were extracted again with MeOH to make MeOH(EtOAc), MeOH(CH_2Cl_2), and MeOH(Hex) extracts, respectively (Figure 1). After that, evaporation was performed to eliminate organic solvents from all extracts. Dipterocarpol contents in each extract were determined using HPLC analysis as previously described (Table 1).[14]

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC was determined using a serial-dilution method in Mueller Hinton broth (MHB) as previously reported.[15] Inocula (2×10^6 CFU/mL of either MSSA DMST-2933 or MRSA DMST-20651) were prepared by resuspending isolated bacterial colonies in sterile 0.9% NaCl and adjusting the turbidity to match a McFarland standard solution. The inocula were incubated in MHB and serial-dilutions of each of the *D. alatus* extracts (0.195 – 100 mg/mL), or diptrocarpol (0.125 – 32 µg/mL), or tetracycline (0.125 – 32 µg/mL) at 37°C for 18-24 h. The live bacteria were quantified by the degree of resazurin-colour change compared to control medium without bacterial inoculum. The minimum concentration of each of the test compounds that inhibited bacterial growth was the MIC value. For MBC determination, all the media in the wells with a higher concentration than the MIC value were pipetted and spread on Mueller Hinton agar, then incubated at 37°C for 18-24 h. The lowest concentration of each of the test compounds with no colonies of MSSA or MRSA was the MBC.[15] MIC and MBC were determined from two-independent experiments (n = 4-5).

The tape stripping mouse model

Seven-week-old male Institute of Cancer Research (ICR) mice were provided by the Northeast Laboratory Animal Center, Khon Kaen University. All mouse protocols were approved by the Institutional Animal Ethic Committee for Use and Care of laboratory animals (Approval no. IACUC-KKU-92/2562). Briefly, mice were anaesthetized using 50 mg/kg of pentobarbital sodium and the back hair was completely shaved for a 2×2 cm^2 area. Pieces of sticky bandage were quickly stuck to and pulled off the shaved-back skin 15-25 times until the skin became inflamed and red, without bleeding.

The skin damage of all wounds was standardized to a trans-epidermal water loss (TEWL) value of 70-80 g/m^2h before 10 µL of a 1×10^8 CFU/mL MRSA inoculum was applied to the damaged skin.[15]

Mice were divided into 7 groups (n = 9-10, Table 2). An hour later, a 100-µL aliquot of each of the *D. alatus* extracts (20 - 40 mg/mL), dipterocarpol (400 µg/mL), or tetracycline (160 µg/mL) in emulgel base was applied to the wound surface and this was repeated daily for ten days. The emulgel consisted of a mixture of cream and gel bases. For cream base preparation, an oil phase was incorporated into a water phase using non-ionic surfactant to stabilize the formulation. While the gel base was prepared by dissolving carbomer 934 in slightly alkaline conditions. All phases were mixed using a homogenizer (Wiggens GmbH, Straubenhardt, Germany) to obtain emulgel. The TEWL was measured on days 1, 4, 6, and 8. MRSA colonies from the wounds were aseptically swabbed for culturing on 6 µg/mL oxacillin-contained mannitol salt agar on days 1, 3, 5, 7, and 9.[16]

Toluidine blue O staining

Mice were sacrificed using pentobarbital sodium at a dose of 200 mg/kg. Mouse wound skin was immediately cut using a surgical blade and washed in cold 1×phosphate buffered saline (pH 7.4) before fixing in a 10% neutral buffer formalin solution (pH 7.4, 10-fold volume) overnight.[13] After fixing, samples were slowly dehydrated in 50 to 100% ethanol, then embedded in paraffin blocks. The paraffin embedded-skins were cut into 5 µm sections and fixed on glass slides. The slides were dewaxed in xylene and gradually rehydrated from 100 to 70% ethanol. Skin samples (on days 3, 7, and 10) were stained with 0.1% toluidine blue O solution for 3-5 min. The slides were dehydrated again with absolute ethanol (5 min) before mounting with cover slips.[16] The skin histomorphology images were collected at 200-fold magnification under a light microscope and analyzed by Motic image plus 3.0 software.

Statistical analysis

TEWL values and colony numbers are expressed as mean ± standard deviation of 6-9 replicates and were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test (version 23; SPSS Inc., Chicago, IL, USA). $p \leq 0.05$ was considered as statistically significant.

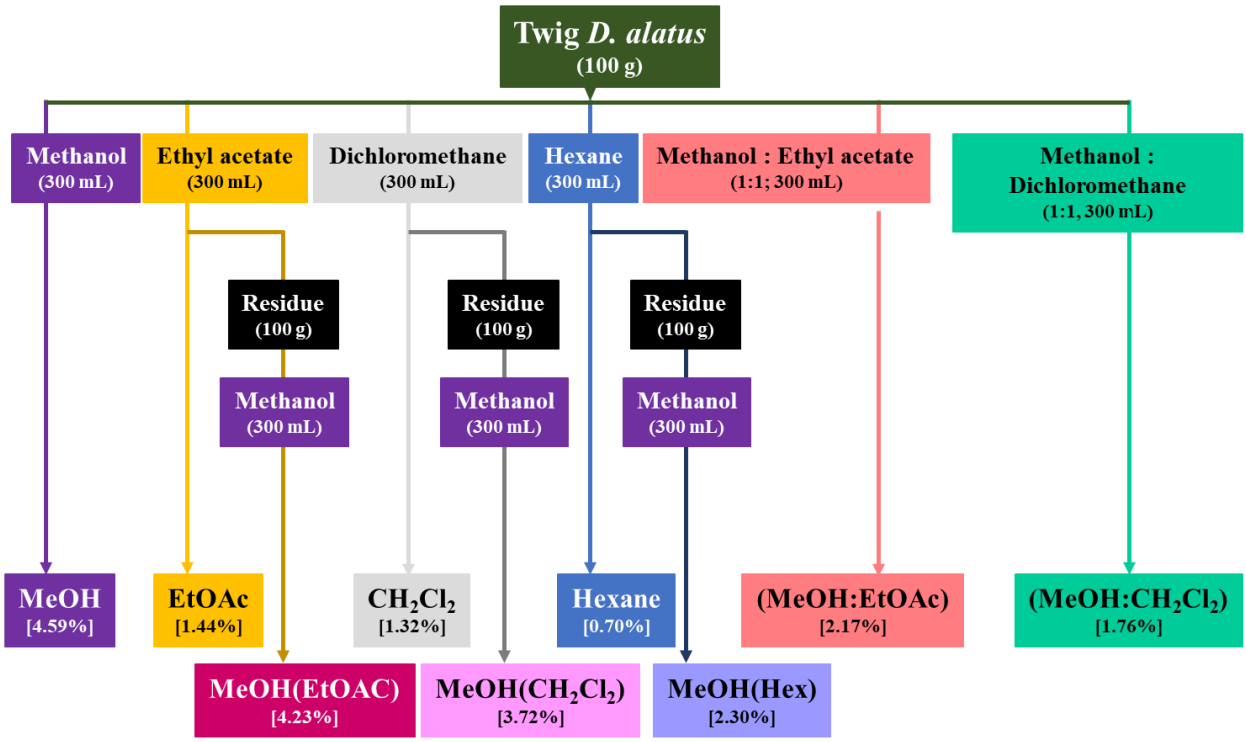

Figure 1: Flow chart for preparation of *D. alatus* extracts

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Results and Discussion

Antibacterial activity of D. alatus twig extracts in vitro

Community-acquired MRSA infection is increasing and MRSA is a major pathogen for soft tissue and superficial skin infections.[17] A number of studies have searched for new agents or antibiotics from natural sources including *D. alatus*, which is one candidate with reported antibacterial activity.[11,13] The results of the *in vitro* MIC and MBC testing of the various *D. alatus* extracts, dipterocarpol, and tetracycline are presented in Table 1. Comparing the activity of all extracts, the MeOH(EtOAc) extract showed the lowest MIC and MBC values against MSSA and MRSA with values of 0.391 and 0.781, respectively, Dipterocarpol showed an antibacterial effect against MRSA, corresponding with a previous report.[13] However, the content of dipterocarpol in each solvent extract was not related to the antibacterial effect (Table 1). Hence, it is likely that there are other active constituents in *D. alatus* with antibacterial effects. Therefore, for the *in vivo* study in infected mouse wounds, we investigated the antibacterial activity of MeOH(EtOAc) extract as it had the highest efficacy against MSSA and MRSA. Dipterocarpol was chosen as a chemical marker.

Wound healing and antibacterial activities of D. alatus twig extract in mouse skin

We employed a tape stripping technique combined with MRSA-induced superficial infection in mice as a model for testing antimicrobial and wound healing effects *in vivo* (Figure 2). In this study, the wounds appeared to be healed 6 days after the tape stripping in the control (non-infected) group. In contrast, wounds in the MRSA-infected emulgel base treatment group appeared ulcerated at the 8th day. Mice treated with either the MeOH(EtOAc) extract or the MeOH(EtOAc) extract in emulgel base showed wounds recovered at day 8 whereas the dipterocarpol and tetracycline treated groups showed incompletely healed wounds (Figure 2).

TEWL values representing the degree of mouse skin damage[13,15] are presented in Table 3. TEWL was similar for all groups the first day after tape-stripping. For the control (non-infected) group, TEWL was very low on day 8, indicating complete wound healing. The MeOH(EtOAc) extract and MeOH(EtOAc) emulgel treatment groups showed slightly decreased TEWL values, while the tetracycline and dipterocarpol treatment groups showed higher TEWL values until day 8.

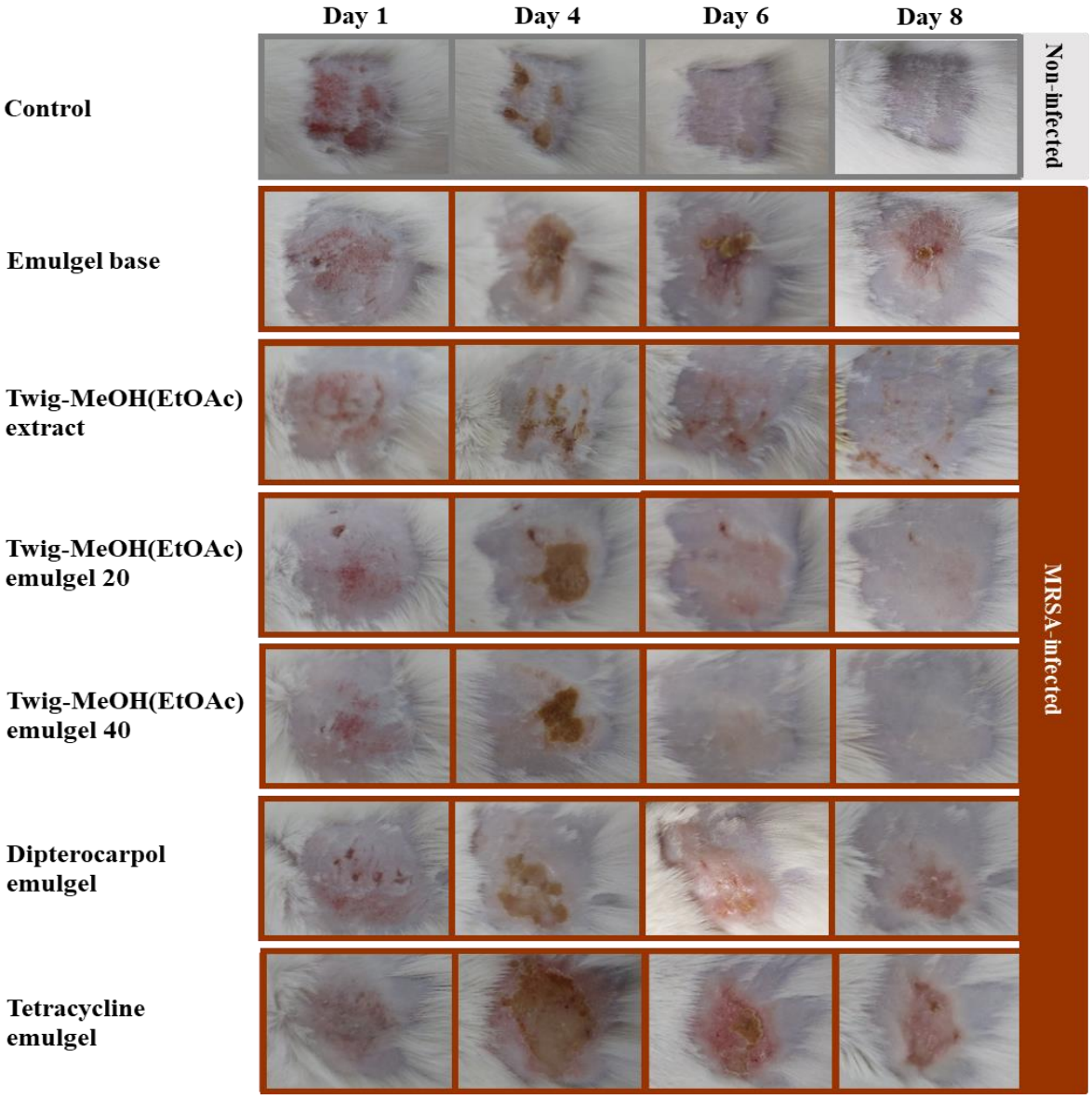

Figure 2: Effects of *D. alatus* extracts on the appearance of superficial wounds after tape-stripping and MRSA-infection. Images represent the wound appearance for each treatment group on days 1, 4, 6, and 8.

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Interestingly, MeOH(EtOAc) extract significantly reduced TEWL values on day 6 and 8 compared to the emulgel base. However, both emulgel base and MeOH(EtOAc) emulgel showed similar TEWL levels, which were higher than the control group. In our previous report, 20 mg/mL of methanol *D. alatus* twig extract in 10% ethanol in propylene glycol improved wound appearance and decreased TEWL to the same level as the control group.[13] In this study, we examined MeOH(EtOAc) extract and MeOH(EtOAc) emulgel. Emulgel or other gellified emulsions can be administered by various routes such as topical application, oral administration, or injection.[18] Emulsions are primarily formulated to increase solubility of poorly water soluble compounds, stabilize antimicrobial agents, or enhance therapeutic efficacy.[19] Herein, two MeOH(EtOAc) emulgel dosages (20 and 40 mg/mL) were evaluated, but there were no differences in wound appearance, TEWL, or MRSA colony number between the MeOH(EtOAc) extract and the MeOH(EtOAc) emulgel. Therefore, the formulation of emulgel should be developed in further studies to improve activity. Figure 3 presents the toluidine blue O-stained mouse skin sections. Mast cells (black arrows) appear as red-purple or dark blue spots.[17,20] Mast cells are responsible for allergic and anaphylaxis reactions, but they are also important effectors in the immune responses against bacterial pathogens, especially for skin infections.[20] On day 3, all infected groups showed mast cell accumulation in the skin sections, while the control (non-infected) group did not. On day 7 and 10, the MeOH(EtOAc) extract and MeOH(EtOAc) emulgel treatment groups showed a marked reduction in the number of mast cells, while the emulgel base treatment group did not. Moreover, mast cells were detected in the skin of the dipterocarpol and tetracycline treatment groups until day 10. These results indicated that the superficial MRSA infection was reduced by MeOH(EtOAc) extract and MeOH(EtOAc) emulgel, in agreement with the results of the wound appearance and TEWL.

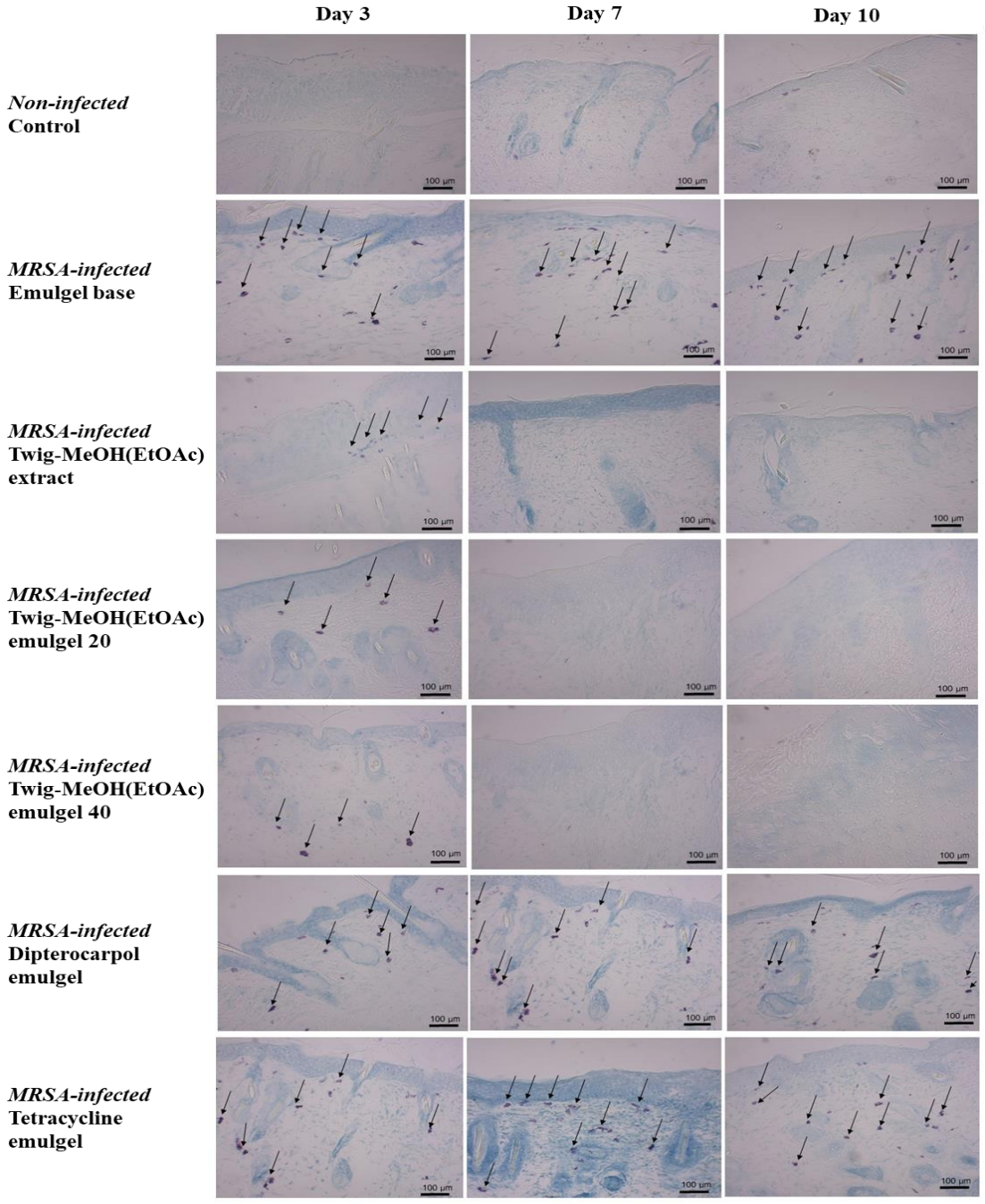

Figure 3: Effects of *D. alatus* extracts on histology of the superficial wounds with toluidine blue O staining. Black arrows indicate mast cells. Black bars represent 100 µm. Skin samples were photographed at 200-fold magnification.

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After the mouse skins were inoculated with MRSA, the number of MRSA colonies recovered from the wounds is shown in Table 4. The highest MRSA colony counts were observed on the first day of infection for all inoculated treatments, while the control (non-infected) group had no MRSA colonies. Treatment with MeOH(EtOAc) extract or MeOH(EtOAc) emulgel significantly reduced the number of MRSA colonies compared to the emulgel base group on the first day. On days 3 to day 5, treatment with MeOH(EtOAc) extract and MeOH(EtOAc) emulgel markedly suppressed the number of MRSA colonies compared with the emulgel base group and, on day 7, MRSA were completely eradicated in the MeOH(EtOAc) extract and MeOH(EtOAc) emulgel groups. Dipterocarpol and tetracycline slowly reduced the number of MRSA colonies, and completely exterminated MRSA on day 9. The emulgel base did not completely eradicate MRSA until the last day of the experiment. Tetracycline is a broad-spectrum antibiotic that has been used against Gram-positive, Gram-negative, and anaerobic bacteria in human and animals.[21,22] It is also available in many commercial topical formulations for skin infection. However, the incidence of *S. aureus* resistant to tetracycline has been increasing in patients with infected wounds.[23] Our recent study showed dipterocarpol (1.1 mg/mL) had anti-MRSA and wound healing effects.[13] However, in this study, we employed a decreased dose of dipterocarpol (400 µg/mL) that was equivalent to the dipterocarpol content in the MeOH(EtOAc) extract, and this lower dose did not have equivalent anti-MRSA and wound healing effects. Therefore, the wound healing and antibacterial effects of the *D. alatus* MeOH(EtOAc) extract appear to be dipterocarpol-independent. Therefore, further studies to elucidate the active constituents in the MeOH(EtOAc) *D. alatus* twig extract are required.

Table 1: *In vitro* MIC and MBC values of *D. alatus* twig extracts against *Staphylococcus aureus*.

Test compounds	Dipterocarpol content* (mg/g extract)	MSSA[1]		MRSA[2]	
		MIC	MBC	MIC	MBC
D. alatus extracts (mg/mL)					
MeOH	43.53 ± 0.24	0.781	1.560	0.781	1.560
EtOAc	122.89 ± 9.07	3.120	25.000	12.500	50.000
MeOH(EtOAc)	19.61 ± 0.15	0.390	0.781	0.390	0.781
CH_2Cl_2	163.27 ± 3.54	6.250	12.500	6.250	25.000
MeOH(CH_2Cl_2)	15.06 ± 2.28	0.781	0.781	0.781	0.781
Hexane	200.92 ± 2.16	12.50	25.000	12.500	50.000
MeOH(Hexane)	29.27 ± 1.25	0.781	0.781	0.781	0.781
MeOH:EtOAc	35.26 ± 1.02	0.781	0.781	0.781	0.781
MeOH:CH_2Cl_2	62.38 ± 0.70	3.120	6.250	3.120	12.50
Dipterocarpol (µg/mL)	-	0.250	0.500	0.250	0.500
Tetracycline (µg/mL)	-	0.250	0.250	32.000	32.000

Note: [1]MSSA, methicillin-sensitive *S. aureus*; [2]MRSA, methicillin-resistant *S. aureus*, MIC and MBC were determined from two-independent experiments (n = 4-5), *Dipterocarpol contents (n=3) were determined as described.[14]

Table 2: Design for treatments of superficial wounds.

MRSA	Test compounds	Concentrations	Administration
–	Sterile water (control)	-	
+	Emulgel base	-	
+	MeOH(EtOAc) extract	20 mg/mL	
+	MeOH(EtOAc) emulgel 20	20 mg/mL	100 µL/day, 10 days
+	MeOH(EtOAc) emulgel 40	40 mg/mL	
+	Dipterocarpol emulgel	400 µg/mL	
+	Tetracycline emulgel	160 µg/mL	

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Table 3: Effects of *D. alatus* twig extracts on the TEWL value.

MRSA	Test compounds	TEWL (g/m^2h)			
		Day 1	Day 4	Day 6	Day 8
– (control)	Sterile water	74.08 ± 5.41	23.84 ± 5.93	11.39 ± 1.04	7.02 ± 0.31
+	Emulgel base	73.10 ± 14.32	54.42 ± 15.30*	54.70 ± 12.17*	48.76 ± 8.22*
+	MeOH(EtOAc) extract	78.59 ± 3.36	53.05 ± 10.95*	28.01 ± 17.59*,#	18.83 ± 12.02*,#
+	MeOH(EtOAc) emulgel 20	74.66 ± 14.04	61.97 ± 16.26*	54.61 ± 18.67*	45.74 ± 20.56*
+	MeOH(EtOAc) emulgel 40	76.61 ± 4.15	61.99 ± 18.02*	54.39 ± 21.58*	43.41 ± 17.91*
+	Dipterocarpol emulgel	75.35 ± 8.40	61.60 ± 10.37*	72.45 ± 11.33*	62.43 ± 16.81*
+	Tetracycline emulgel	75.00 ± 5.40	75.73 ± 12.61*	62.72 ± 15.63*	60.71 ± 5.88*

Note. Values are expressed as mean ± standard deviation of 9 replicates. *$p < 0.001$ VS control ~~at~~ on the same day; #$p < 0.05$ VS Emulgel base on the same day using one-way ANOVA with Tukey's *post hoc* test.

Table 4: Effects of MeOH(EtOAc) *D. alatus* twig extracts on MRSA colony numbers.

MRSA	Test compounds	Colony number (per plate)				
		Day 1	Day 3	Day 5	Day 7	Day 9
– (control)	Sterile water	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
+	Emulgel base	>500 ± 0.00*	16.50 ± 16.16*	13.33 ± 0.82*	4.80 ± 2.17*	3.00 ± 1.41*
+	MeOH(EtOAc) extract	346.10 ± 114.04*,#	5.50 ± 5.82*,#	1.11 ± 1.69#	0.00 ± 0.00#	0.00 ± 0.00#
+	MeOH(EtOAc) emulgel 20	375.40 ± 86.86*,#	6.60 ± 2.34*,#	1.22 ± 0.97#	0.00 ± 0.00#	0.00 ± 0.00#
+	MeOH(EtOAc) emulgel 40	329.00 ± 105.99*,#	3.20 ± 2.57*,#	0.10 ± 0.32#	0.00 ± 0.00#	0.00 ± 0.00#
+	Dipterocarpol emulgel	>500 ± 0.00*	11.80 ± 7.41*	11.20 ± 11.56*	3.63 ± 2.00*	0.00 ± 0.00
+	Tetracycline emulgel	>500 ± 0.00*	43.80 ± 34.97*,#	7.89 ± 3.41*	3.88 ± 2.03*	0.00 ± 0.00

Note: Values are expressed as mean ± standard deviation of 6-9 replicates. *$p < 0.05$ VS control on the same day; #$p < 0.05$ VS Emulgel base on the same day using one-way ANOVA with Tukey's *post hoc* test.

Conclusion

Ethyl acetate-methanol MeOH(EtOAc) extract of *D. alatus* twig showed the highest potency against MSSA and MRSA *in vitro*. In mouse skin, MeOH(EtOAc) extract and MeOH(EtOAc) extract in emulgel base showed similar effects for wound healing and in reducing MRSA numbers in superficial infections. Therefore, MeOH(EtOAc) extract in emulgel is a potential candidate for a novel topical product to treat ulcerated wounds with MRSA infection.

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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