ORIGINAL ARTICLE

Antibacterial and Wound Healing Activity of 2% Formulation of 2-Medpy-3-CN on Infected Burn Wounded Animal Model

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ABSTRACT

Introduction: Humans have learned to recognize and process plants into medicinal forms through centuries. Burns can spread to other tissues, especially when infected with bacteria such as Methicillin-Resistant Staphylococcus aureus (MRSA). The study aimed to assess the in vivo antibacterial and wound healing activity of 2% formulation of 2-Medpy-3-CN on infected burn wounded animal model. Methods: In vitro antibacterial activity of the Alsti was done by broth dilution and disc diffusion methods. Alsti 2% ointment was prepared for the infected burn wound treatment. A total of 18 rats are grouped into A, B, C, and D, the first three groups (A-C) were injured thermally, and Group D was used as healthy controls. The three test Groups were exposed to MRSA ATCC 43300 at 105 CFU/mL. Group A was treated with 2% Alsti, Group B with Silver sulfadiazine 1% (SSD), and Group C was untreated. Wounds healing was assessed by the healed area and microscopic identification of hematoxylin and eosin (H&E)-stained skin tissue. Results: Wound healing progresses with application of Alsti 2% ointment as observed through wound diameter and histopathological changes of the skin. Wound diameter decreases with treatments, while the contrary was observed in the non-treated group. Microscopic observation of the stained skin showed that epidermal development, and collagen formation progress with treatment days. Untreated wounds showed marked inflammation, progressive ulceration, and necrosis. Conclusion: Alsti 2% formulation showed antibacterial and wound healing activities, hence, can be used as alternative in burn wound infections.

Keywords: Allium stipitatum, Burn wound, Antibacterial, MRSA, Silver sulfadiazine

INTRODUCTION

Methicillin-resistant Staphylococcus aureus (MRSA) infections result in substantial morbidity and mortality in the world, partly due to the challenge of therapeutics, which frequently involved resistance to several antibiotics (1, 2). The emergence of antibiotic resistance has become a significant global issue as there are a few, or occasionally less potent antimicrobial agents available against many bacteria, including MRSA. The increase in virulent strains of S. aureus, especially the MRSA is of great concern. The full spectra of antibiotics are always available, but more than 95% of MRSA do not respond to first-line antimicrobials because of artifices in bacterial genomes that confirm the law that simple genomes are produced more rapidly than complex genomes, especially in wound infections (3, 4).

Physical, chemical, thermal, microbial, or immunological assaults may result to wound, which disrupt the cellular, anatomical, and functional integrity of living tissue. In other words, wound is a rupture in the epithelial barrier that may extend into underlying structures and result in their functional impairment (5, 6). It may present as simple or severe skin disorders which can extend to other tissues and anatomical structures such as subcutaneous tissue, muscles, tendons, nerves, vessels, and even bones, with the colonization of microbial community (7). Even though some bacteria are part of intact skin microbiota and wounds, wound healing may be impeded by a critical threshold of established bacteria and by the formation of a biofilm. Because of these realities, bacterial infections are still recognized as one of the contributors to substantial morbidity and mortality, regardless of recent improvements in wound care. The dominant microbial strains in patients with colonized wounds are MRSA and Pseudomonas...
aeruginosa according to Singhal, (8). Efficient and targeted distinctive biological wound cures due to the environment and the highly complex mechanism of wound healing. Research works are inspired to discover more effective therapeutics for chronic and acute wounds, including those related to burns (9).

Among the most common and debilitating types of traumas is the one related to burns. Infected burns are among the prevalent causes of skin disorders and complications, owing to the multiple changes in anatomical characteristics specific to burn injuries and difficulties in the diagnosis and treatment (7, 10). Humans have learned to recognize and convert native plants from their local surroundings into medicine for centuries. From dawn time, medicinal plants were used for trauma, infection, sickness, and damage from various tissues (11). Topical antimicrobials such as silver nitrate, mafenide acetate, silver sulfadiazine, and silver-impregnated dressings were administered for prophylaxis and therapeutics in burn wound management (12, 13).

The antimicrobial activity of Allium species has been recognized since ancient times. The first citation dated to the fifteenth century BC in Egypt when garlic was used as a treatment for microbial infections in folk medicine. In 1858, an experimental exploration into garlic began with the work of Pasteur, who first discovered the antibacterial effects of garlic extracts (14). The extract of Allium stipitatum exhibited antibacterial activity against many species of bacteria (15), including Acinetobacter spp, Escherichia coli, and Stenotrophomonas maltophilia, Gram-positive MSSA (methicillin-sensitive S. aureus) and MRSA in vitro (16). The study aimed to determine the in vivo antibacterial and wound healing activity of 2% formulation of synthesized bioactive 2-Medpy-3-CN on infected burn wounded animal model.

MATERIALS AND METHODS

The microbial study was carried out at the Faculty of Medicine and Health Sciences Universiti Putra Malaysia (UPM). The animal study was performed at the animal house, in the Faculty of Veterinary Medicine UPM. Ethical clearance numbered UPM/IACUC/AUP-R003/2019 was obtained from the Institutional Animal Care and Use Committee, UPM.

Test compound

The purification of the test compound from the bulb of A. stipitatum was fully discussed in the earlier study (17). Briefly, A. stipitatum bulbs were collected from Iran, identified, and authorized by a plant taxonomist. The plant material was washed with clean water, sliced into small pieces, and dried under shade. Five kilograms of the plant material was macerated into powdered form and then soaked into dichloromethane for 72 hours, the extract was then filtered with No. 1 Whatman’s paper (11 µm pore) to remove solid particles, and the filtrate was dried at 40°C. The crude product was further processed by column chromatography (CC) and thin-layer chromatography (TLC) using dichloromethane (CH2Cl2) as the mobile phase to produce pure 2-(methylthio)pyridine-3-carbonitrile (2-Medpy-3-CN). In this study, the test compound was commercially synthesized by Prestwick Chemical Inc, Illkirch-Graffenstaden, France. It was named Alsti as it was purified from A. stipitatum and will be addressed as such.

In vitro antibacterial activity

The antibacterial activity of Alsti was evaluated by the disc diffusion method as done by Karunanidhi et al. (17). At a concentration of 0.5 McFarland (1 x 10⁶ CFU/mL), 10 µL of overnight culture of MRSA ATCC 43300 was dispersed uniformly on Mueller-Hinton agar (MHA) plates. Sterile filter paper discs 6 mm in diameter were impregnated with Alsti powder at 10 mg/mL of DMSO 10%. The discs were placed on the MHA containing MRSA. Vancomycin disc was used as a positive control, the plates were incubated at 37°C for 24 hours, then the inhibition zones were assessed.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the method employed by Parvekar et al. (18). Different concentrations of Alsti (4 - 16 ug/mL) were introduced into a test tube containing sterile Mueller-Hinton broth (MHB). Then 10 µL of the MRSA suspensions (0.5 McFarland standard) were inoculated into the test tubes. Test tubes containing broth without MRSA served as controls. The test tubes were then incubated at 37°C for 24 hours. The differences in turbidity before and after incubation was observed with the naked eye. The lowest concentration that no visible turbidity was observed was considered MIC. Then aliquot of 10 µL of the test tubes that show no visible turbidity were inoculated on agar plates containing MHA and incubated at 37°C for 24 hours and observed for growth. Any concentration from the inoculated broth that shows remarkable zone of inhibition was considered MBC.

Preparation of test compound

Simple ointment base (SOB; BP:1980) was used to prepare 2% Alsti for topical application as described by Goldsmith, (19) and Demilew et al. (20). Fifty (50) grams of the SOB was placed in a beaker in a water bath at 65°C, then, Alsti powder (1g) was added into the ointment base. It was subjected to constant stirring and homogenization and then spun at 1500 rpm for 10 minutes to produce a final concentration.

In vivo wound healing and antibacterial activity

Experimental animals and burn injury

A total of eighteen BALB/C female mice of about 21g and 7 weeks old were allowed to acclimatize for ten days, having free access to food and water. The animals were classified into four groups: Three groups, A, B, and
C consisting of five rats each, and D with three rats (Fig. 1). The experimental animals in groups A, B, and C were injured thermally in accordance with Balaji et al. (21). The hairs of the rats were trimmed using a hair clipper at the dorsum region of the animal making an area of about 4x4 cm two days before the commencement of the procedure. The rats were anesthetized with Xylazine (10 mg/kg) and Ketamine (80 mg/kg) intraperitoneally. A wooden-handled metal plate (3x3 cm) was heated to 300°C on the burner flames and then placed on the shaved area for five seconds with little pressure to produce a third-degree burn. Group D animals were left uninjured as healthy controls.

The wound was cleaned with 0.85% NaCl solution using sterile cotton wool before the wound infection procedures. Methicillin-resistant S. aureus ATCC 43300 suspensions at 10⁵ CFU/mL were prepared from nutrient agar grown overnight at 37°C. A volume of 10 µL of the bacterial suspension was evenly dispersed on the wound surface 30 minutes after the burn and allowed to stabilize before the animals were returned to their cages as done by Kumari et al. (22). The same was done for the five rats in each of the Groups (A, B, and C). After 24 hours of infection, the prepared 2% Alsti ointment was applied twice daily topically at the site of the wound for Group A. Group B was treated with 1% Silver sulfadiazine (SSD) ointment as a positive control, and Group C was left untreated as illustrated in Figure 1. The animals were observed on days 1, 5, 10, 15, and 20 and the diameter of the healed area was recorded as done by Farahpour et al. (23).

Among the groups, one rat was sacrificed on days 1, 5, 10, 15, and 20, and one from Group D each on days 5, 10, and 20 (Fig. 1). Small portions of the skin of the freshly euthanized animals were cut from the wounded area, then placed in 10% formalin until use. The skin was dehydrated with alcohol and then embedded in paraffin wax to form a block. The fixed skin was then sectioned 5µm thick and was hematoxylin and eosin (H&E) stained for histopathological examination. Skin from the unwounded rats was processed the same way and served as negative control. The prepared slides were examined using Zeiss microscope (AxioLab 5) with X5 and X10 objectives for histopathological changes such as edema, ulceration, necrosis, epidermal development, and collagen. The wound healing stages for each group were observed in 1 unit of microscopic field (2 x 2 mm) of the tissue as described by Kumari et al. (22), Church et al. (24), and Karunanidhi et al. (25), and scored as -, +1, +2, +3, +4 respectively to represent no significant, mild, moderate, high, and very high changes.

Statistical analysis

One-way ANOVA using Graphical prism software version 9.0 was conducted to compare means between the treated and non-treated groups, p<0.05 was considered statistically significant.

RESULTS

Drug-resistant pathogenic microbes contaminate wounds and impede the processes of wound healing. Hence, prompt medical care with a potent antimicrobial agent is needed. In some burn units, microbial infections from burn wounds have often been documented (26, 27). Antibacterial and wound healing activity of 2% Alsti was studied on 15 infected models using microscopic examination of their H&E-stained skin tissues of the euthanized animals. Alsti powder showed activity on MRSA with a marked mean diameter of the zone of inhibition. It was found to have MIC and MBC of 4 µg/mL and 8µg/mL respectively. The 2% ointment of Alsti; the test compound that was commercially synthesized from A. stipitatum by Prestwick Chemical Inc, Illkirch-Graffenstaden, France, was found to have a significant effect in the treatment of the wound infection including those infected with MRSA.

The unhealed wound diameter of the treated and non-treated wounds was measured in cm and is shown in Table I. The wounded diameter decreases with daily treatment which indicates the effectiveness of the ointments. Alsti 2% had a decrease in wound diameter of 0.5 cm between day 10 and day 15 with complete healing at day 20, non-treated wound (NT) became

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Mean wound diameter (cm)</th>
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<tbody>
<tr>
<td></td>
<td>Day 10</td>
</tr>
<tr>
<td>NT</td>
<td>2.5±0.08</td>
</tr>
<tr>
<td>Alsti 2%</td>
<td>2.5±0.08</td>
</tr>
<tr>
<td>SSD 1%</td>
<td>2.8±0.08</td>
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Key: NT = Not treated, SSD = Silver sulfadiazine

Figure 1: This represents daily utilization of the experimental animals at days 1 to 20. One animal was sacrificed per day for each group, making a total of five, three animals were sacrificed on days five, ten and twenty, which make a total of 18 rats.
septic.

Table II illustrates the histopathological structures observed microscopically from H&E-stained skin tissues. The degree of histopathological changes is represented by plus (+) signs, wound healing progress with days in the case of Alsti and SSD treated skin while it worsened in the case of non-treated skin.

Table II: Histopathological findings of the H&E-stained skin of the experimental animal

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Days</th>
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<th>E</th>
<th>U</th>
<th>N</th>
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<tbody>
<tr>
<td>NT</td>
<td>5</td>
<td>2+</td>
<td>2+</td>
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<tr>
<td>Alsti 2%</td>
<td>15</td>
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<td>SSD</td>
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</tbody>
</table>

Keys: C= Collagen; E= Edema; EP=Epidermis; N= Necrosis; NT =Not treated; U= Ulceration
Grading: - No significant changes
+ Mild
2+ Moderate
3+ High
4+ Very high

The photomicrograph of the treated and non-treated H&E-stained infected burn wounded skin tissues on days 5, 10, 15, and 20 are presented in Figure 2 to Figure 5. Non-treated infected burn wounds (Fig. 2) showed an increase in severity of tissue edema, ulceration, and necrosis by the number of days. No significant epidermal tissue development and collagen was observed. On the other hand, Alsti 2% and SSD treated infected burn wounded skin (Fig. 3 and Fig. 4) showed a decrease in tissue edema, ulceration, and necrosis. Formation of new epidermal tissue, collagen formation, and general healing progression with treatment days. These findings are compared to the control sample in Figure 5.

**DISCUSSION**

Wound healing involves a series of interconnected and interdependent physiological processes (28, 29). In less than four weeks, most wounds heal without complications and recover homeostasis, skin barrier function, pliability, and physiological functioning. Wound that is difficult to repair become chronic due to underlying conditions such as bacterial infections that may result in biofilms, necrosis or other complications (30, 31). This research was conducted to determine the antibacterial and wound healing effect of Alsti 2%, the synthesized herbal ointment. The compound exhibits wound healing and antibacterial activities against MRSA on burned wounded animals. The incidence of wound infection with MRSA is increasing (32, 33). Staphylococcus species are among the most frequently detected pathogens in community-acquired superficial wounds (34). Silver sulfadiazine 1% is an effective topical antimicrobial activity and has been applied topically as...
an alternative to systemic antibiotics in wound infection as affirmed by Blanchard et al. (35) and Schiavo et al. (36). It has activity against MRSA, and has been used where there is no evidence of systemic disruption in localized wound infections (37, 38), therefore used in this study as a positive control.

Alsti 2% and SSD 1% treated and untreated skins show the mean diameters of unhealed wounded areas with respect to treatment days (Table I). At days 10 and 15 Alsti 2% showed the mean unhealed wound diameter of 2.5 cm and 2.6 cm whereas SSD 1% was 2.8 cm and 2.0 cm, respectively. When compared with the non-treated group, no significant difference was found (P>0.05). The wound healing activity may not be predicted using the diameter of the healed area because the infection may spread in the deeper tissue and cannot be measured precisely. The untreated wounds showed no significant healed areas and were proved septic. However, it was known that SSD has moderate activity on MRSA (38, 39). The inhibitory concentration of crude extracts of A. stipitatum varies from 50 to 1000 µg/mL (40). The activity of Alsti by disc diffusion technique have a mean inhibitory diameter of 28 mm. The compound had MIC and MBC of 4 µg/mL and 8µg/mL against the organism. However, the crude dichloromethane (DCM) extract of A. stipitatum was sensitive to MRSA at the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of 32 µg/mL and 128 µg/mL respectively according to Karunamidhi et al. (17). The difference in the values could be due to the use of crude extract of the active compound. Moreover, it may be because Alsti is the purest and synthesized form of the extract.

Reliable data on burn-associated pathophysiological responses constitutes a better understanding of injured skin and the stages of its development (41). The histopathological findings of the burn wound are characterized by inflammatory reactions, leading to rapid edema formation, due to increased microvascular permeability, vasodilation, and increased extravascular osmotic activity. This is followed by a healing process characterized by proliferation and remodelling (42). Figure 2 shows the photomicrograph of the H&E-stained treated and non-treated skin sections. Non-treated infected skins show marked edema, ulceration, and necrosis of the burned area, which is in line with the finding of Alturkistani et al. (43), and Neilson et al. (44).

In addition, the conditions are the same for all the treated and non-treated wounds at days 1 to 5, which signify the initial inflammatory phase with marked similarities that diminishes with the progression of treatment. The microscopic findings of the H&E-stained treated and non-treated skin of the experimental animal are scored in Table II. The determinants of inflammation including edema, ulceration, and necrosis, have reduced with days of treatment. This concords with the finding of Neilson et al. (44). Wound healing determinants including, improved epidermal development, and collagen deposition were found to have improved with treatment days from day 10 to 20. This is in line with the finding of Nielson et al. (44), and Rowan et al. (45). On day 10 both 2%, Alsti and 1% SSD treated samples show significant changes; progressively decreased ulceration, necrosis, and edema, and improved epidermal and collagen developments indicating a progressive wound healing process. There was also reduced or no edema, and no tissue necrosis which may be due to the antibacterial and healing effect of the ointments on day 20. This agrees with the finding of Mohammadi-rika et al. (46). The histopathological findings in this study are also in line with those of Wigger-Alberti et al. (47) and Kuhlmann et al. (48). They found marked progress of wound healing in their study after five days of treatment with a wound healing ointment containing Allium species, they also found that more than 75% of the wounds have healed after days 10 of treatment, with mild healing status in non-treated and non-infected wound commences around day 8 of observation. The slight differences may be due to local factors in the wound at a micro-environmental level after the inflammation phase, such as the bacterial strain used, and the concentration of the inoculum. Decrease in oxygen tension, decrease in pH, and increase in lactate, which initiates the release of certain growth factors contribute to neovascularization or vascular angiogenesis. Macrophages secrete angiogenic growth factors such as vascular endothelium growth factor (VEGF) and basic fibroblast growth factor (bFGF). The Epithelialization process is also stimulated by epidermal growth factor (EGF) and transforming growth factor (TGF-β) produced by macrophages and keratinocytes at the wound site (49, 50). This coupled with the antibacterial activity of the ointments potentiates wound healing during treatment with antimicrobials including Alsti 2%.

**CONCLUSION**

Burn wound is one of the debilitating injuries, especially when secondary bacterial colonization is involved. Thermal injuries need to be treated as soon as possible because of direct their exposure to microbial infection. They could become complicated, which may lead to severe tissue damage, necrosis, and septicemia when infected by opportunistic bacteria such as the Multi-Drug Resistant S. aureus (MRSA). Alsti 2% was found...
to exhibit wound-healing and antibacterial activity and therefore can be used as therapeutic formulation for infected burn wound. Further research is recommended to develop more treatment regimen for burn wound infections in order to prevent antibacterial resistance.

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