Membrane of Usnic Acid in Solid Dispersion and Effectiveness in Burn Healing

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ABSTRACT
Usnic acid, a yellow crystalline of secondary metabolite of Usnea sp., has been known for its tremendous pharmacological activities including antibacterial and anti-inflammatory activities. The aim of this study was to prepare membrane containing usnic acid and evaluate its effectiveness for burns healing. Prior to the preparation of membrane, usnic acid was modified into solid dispersion (SD) system by freeze drying method using PVP K-30 (ratio usnic acid- PVP: 1:2 w/w). Then, usnic acid in solid dispersion with equivalent concentration of usnic acid at 0.5% (F1), 1% (F2), and 2% (F3) were formulated into membrane using poly-vinyl alcohol (PVA), glycerin and distilled water. The membranes were evaluated for the physical and mechanical properties including: appearance, thickness, tensile strength, percent of elongation, and Young's modulus. The effectiveness of burn healing activity was done on male white rabbits by creating superficial burns and evaluated the diameter of wound for 21 days. The appearance of each membrane was transparent but the color was different due to the concentration of usnic acid. The thickness of each membrane was less than 0.1 mm. F2 had the highest tensile strength, percent of elongation, and Young's modulus. Meanwhile, F3 showed a better result in burn healing compared to F2 and F1. The higher concentration of usnic acid solid dispersion in membrane increased effectiveness of burn healing significantly ($p<0.05$).

Keywords: *usnic acid, solid dispersion, membrane, burn healing*

1. INTRODUCTION

Usnic acid, firstly isolated in 1844 from lichen genus Usnea, is a secondary metabolite that mostly produced in *Usnea longissima, U. articulate, U. complanata, U. meridionalis, U. barbata* [1,2]. The most reactive part of usnic acid (see Figure 1) is the triketone group of the polyketide which primarily responsible for the usnic acid activity [3]. Some of the pharmacological activities of usnic acid are antioxidants, antimicrobials, antiprotozoa, antiviral, anti-inflammatory and antitumor [4]. Both lichen and the extracts contained usnic acid have been used in alternative medicine and cosmetics [1]. However, the utilization of usnic acid is still limited because of low solubility in water, thus strategies for increasing solubility are needed to provide optimal effects. One of the methods to increase the solubility of usnic acid is preparation in solid dispersion system. The previous studies have shown the solubility of solid dispersion usnic acid-PVP K-30 which increased 20 times compared to intact usnic acid by freeze drying method [5] and has also been prepared into a hydrogel preparation [6].

The activities of usnic acid as anti-inflammatory and antibacterial are potential to be further developed in burn healing process. Previous studies have been carried out to achieve the optimal effectiveness of usnic acid in wound healing such as liposome formation, formulations in bioadhesive polymers and salt formation [7–10]. However, membrane preparation contained usnic acid has not been reported yet as an alternative for burn healing treatment that meet the characteristics of wound cover including safety and efficacy [11]. Membrane is

known to have several advantages: transparent, easy to apply, permeable to oxygen, water and carbon dioxide, so that skin tissue can still do respiration and prevent from secondary infection [12–14].

Therefore in this preliminary study, solid dispersion of usnic acid was prepared into membrane preparation in order to investigate the physical-mechanical properties and evaluate the effectiveness in burn healing process using male white rabbits.

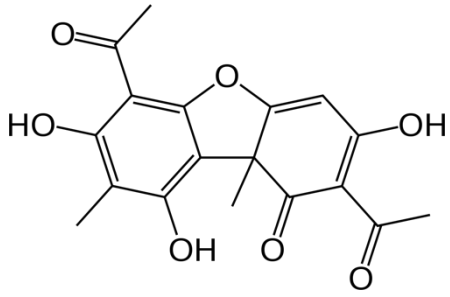

Figure 1. Chemical structure of usnic acid

2. METHODS

2.1. Materials

Usnic acid isolated from Usnea sp as described in previous work [20], polyvinylpyrrolidone K-30 (Shin-Etsu Chemical, Japan), polyvinyl alcohol (PT. Brataco, Indonesia, glycerin (PT. Brataco, Indonesia), a marketed gel (PT. Kalbe Farma, Indonesia) and distilled water.

2.2. Preparation of solid dispersion

Solid dispersion of usnic acid was prepared using PVP K-30 at ratio 1:2 w/w by freeze drying method as described in previous works [5,15].

2.3. Preparation of membrane

Membrane of usnic acid was prepared through precipitation by solvent evaporation method. Polyvinyl alcohol was dispersed in distilled water on a water bath for 30 minutes, then glycerin was added as a plasticizer. Usnic acid solid dispersion (UA-SD) was added with various concentrations as equivalent to 0.5%, 1% and 2% of usnic acid (Table 1) and then homogenized using magnetic stirrer. The pH mixture was determined using a pH meter (Metter Toledo, the USA) prior to membrane formation. The mixture was then dropped into a glass dish and dried in oven (Memmert, Germany) at temperature 40°C. After drying process, membrane was peeled off and stored in a sealed container.

Table 1. The formula of usnic acid – solid dispersion membrane

Materials	Formula 0 (F0)	Formula 1 (F1)	Formula 2 (F2)	Formula 3 (F3)
UA SD (%)	0	0.5	1	2
PVA (%)	10	10	10	10
Glycerin (%)	5	5	5	5
Distilled water (ad %)	10 0	10 0	10 0	100

2.4. Membrane appearance

Examination of membrane appearance was conducted visually including color, transparency, and presence or absence of air bubbles.

2.5. Membrane thickness

The thickness of membrane was measured by using a micrometer (Digimatic micrometer, Japan). The thickness of each membrane was measured at five different locations (1 center and 4 circle edges). Membrane thickness was calculated as the average value of calculated locations.

2.6. Mechanical properties

The mechanical properties of membrane was evaluated using a texture analyzer (TA.XT2, Stable Micro System, the UK). Membrane size 10 mm x 300 mm was measured the tensile strength, % elongation and Modulus Young's according to the following equations. The measurements were conducted in triplicate.

$$\text{Tensile strength } \left(\frac{N}{mm2}\right) = \frac{\text{Force (F)}}{\text{Surface Area (A)}}$$

$$\%\ \text{Elongation } (\%) = \frac{\text{L}-\text{Lo}}{\text{Lo}} \text{x } 100\%$$

$$\textit{Modulus Young's } (\text{N/mm}^2) = \frac{\text{Tensile strenght}}{\%\ \text{Elongation}}$$

Note: L_0 = length before measurement, and L = length after measurement

2.7. In vivo burns healing test

Six groups of four white male rabbits (*Oryctolagus cuniculus*), age 4 -5 months and weight 2–2.5 kg, were acclimated a week prior to the experiments and the ethical clearance has been approved by Ethical Committee of Andalas University (No.

089/KEP/FK/2018). Each group consisted of four rabbits and had each treatment as seen in Table 2. The fur around the rabbit's back was shaved cleaned with 70% alcohol. Each rabbit was then injected ketamine-xylazine (25mg/kg – 1mg/kg) intramuscularly. The burn wounds were done by contacted 1 cm standard-sized square-shaped copper plate xinto the back skin for 20 s until second degree of superficial wound was formed which characterized by blistered, dry skin and a pale red wound as seen in Figure 2.

Table 2. Treatment groups for burn healing activity

Groups	Treatments
I	Negative control (not given a membrane)
II	Membrane without usnic acid (F0)
III	Membrane with 0.5% usnic acid (F1)
IV	Membrane with 1% usnic acid (F2)
V	Membrane with 2% usnic acid (F3)
VI	Positive control (a marketed gel)

The average diameter of the wound was measured vertically, horizontally, and diagonally for 21 days of observation and the percentage of burn healing was calculated using this equation:

$$\text{Burn healing } (\%) = \frac{d1^2 - d2^2}{d1^2} \text{x } 100\%$$

Note : d1 = the diameter on day after burn wound (mm)
d2 = the diameter on observation day (mm)

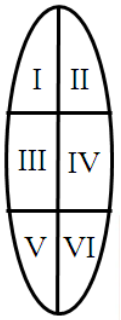

Figure 2. Location for burns on rabbit's back skin

3. RESULTS AND DISCUSSION

3.1. RESULTS

Usnic acid-solid dispersion (UA-SD) membranes were prepared in several concentrations that equivalent to concentration of usnic acid 0.5% (F1), 1% (F2) and 2% (F3). The different concentrations were designed to find out the optimum concentration effect both for physical and mechanical characteristic, and burn healing activity. In general, marketed burn healing preparations contain 1% of active ingredient. Appearance test was done visually and the result is shown in Figure 3. All membranes were transparent, the membrane without usnic acid (F0) has a clear color, while the membrane UA-SD has a yellowish color which became more concentrated in accordance with the increasing concentration of usnic acid in solid dispersion system due to the yellow color of usnic acid. Moreover, all membranes were flexible, had a smooth surface, free of particles and air bubbles.

2.8. Data analysis

The percentage of burn healing was analyzed using two-way ANOVA (Analysis of Variance) method and followed by Duncan's post hoc test to determine the effect of membrane preparation in percentage of burn healing in each group. Results shows significantly different if p values is <0.05.

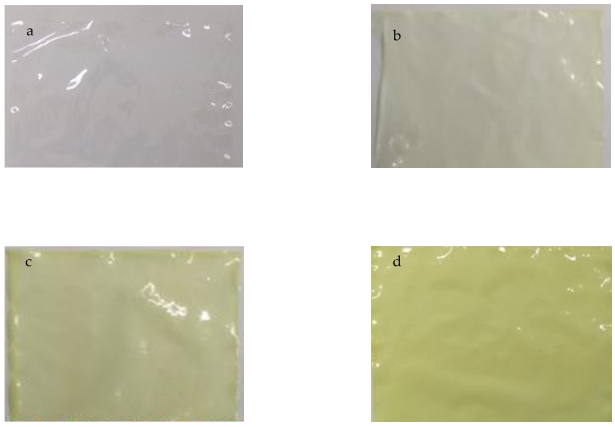

Figure 3. Membrane appearance (a) F0, (b) F1, (c) F2, and (d) F3

The pH of membranes preparation, as shown in Table 3, was in accordance with the pH of skin 4-7 [16]. For topical preparation, the pH should not be too acidic which can cause skin irritation, and also it should not be too alkaline to prevent scaly skin. The highest pH of the membrane preparation was F0 (without usnic acid), and the lowest one is 2% UA-SD membrane (F3). This indicated that pH value was influenced by the amount of usnic acid contained in the membrane formula, where the

greater the concentration usnic acid, the lower the pH value. This result was due to usnic acid as weak acid that has pH 3-5.5 [17]. The average thickness of the membrane were 0.079 - 0.098 mm, as seen in Table 3. At the same membrane volume, the thickness value was greater with increasing concentration of solid dispersion. This result was due to the greater amount of solute in the solid dispersion that incorporated into the membrane.

Table 3. The pH and thickness of membrane

Formula	pH	Membrane thickness (mm ± SD)
F0	4.8 ± 0.02	0.079 ± 0.004
F1	4.5 ± 0.01	0.084 ± 0.003
F2	4.4 ± 0.01	0.090 ± 0.002
F3	4.2 ± 0.01	0.098 ± 0.004

Generally, good membrane has requirements including convenience, easy to use and has elasticity [12,18]. The tensile strength, the percent elongation, and Modulus Young's are the general parameters to determine the mechanical properties of a membrane. The results of membrane mechanical test can be seen in Table 4. F0 had the greatest tensile strength and percent elongation, while F2 had the highest Modulus Young's. This phenomenon indicated the interaction between PVA and PVP-K30 in membrane likely influenced the mechanical properties of the membrane. PVP is known as an amorphous and rigid polymer which has high glass transition temperature (Tg), while PVA is a semi crystalline polymer that has hydroxyl groups that form hydrogen bonds [19]. These polymer that blends in hydrogel created intermolecular hydrogen bond [20]. Therefore, membranes containing UA-SD had lower percent elongation and tensile strength compared to membrane without solid dispersion (F0). However, the decrease in percent elongation was not influenced by the concentration of UA-SD. As shown in Table 4, F2 membrane had the highest percent elongation and tensile strength compared to F1 and F3. This was likely due to that 1% of usnic acid in solid dispersion generated an interaction between PVP and PVA under optimal conditions.

Table 4. The mechanical properties of membrane

Formula	Tensile strength (N ± D)	% Elongation (% ± SD)	Modulus Young's (N ± D)
F0	22.72 ± 1.51	622.9 ± 23.01	3.65 ± 0.12
F1	16.48 ± 1.76	487.6 ± 21.78	3.38 ± 0.25
F2	20.34 ± 1.88	530.0 ± 17.47	3.82 ± 0.25
F3	17.74 ± 2.55	494.7 ± 34.38	3.57 ± 0.28

The effectiveness of membrane in burn activity was carried out using white male rabbits (*Oryctolagus cuniculus*). Rabbits are benign and non-aggressive experimental animals, and have large back area so that one rabbit would have 1-6 wounds so that it can be easily observed [21]. The membrane, size 2.5 cm x 2.5 cm, was changed every 3 days with consideration on prior test result. The membrane began to dry and difficult to be removed from the wound surface on the 4th day, resulting damage to form new fibroblast tissues. Based on the observation, it was known that the animals suffered superficial degree burns with an average burn diameter 17.65 mm. As positive control, a marketed gel was chosen and given once a day. Burn area observation for all test groups on days 0 to 21 showed changes in wound size and shape, as can be seen in Figure 4.

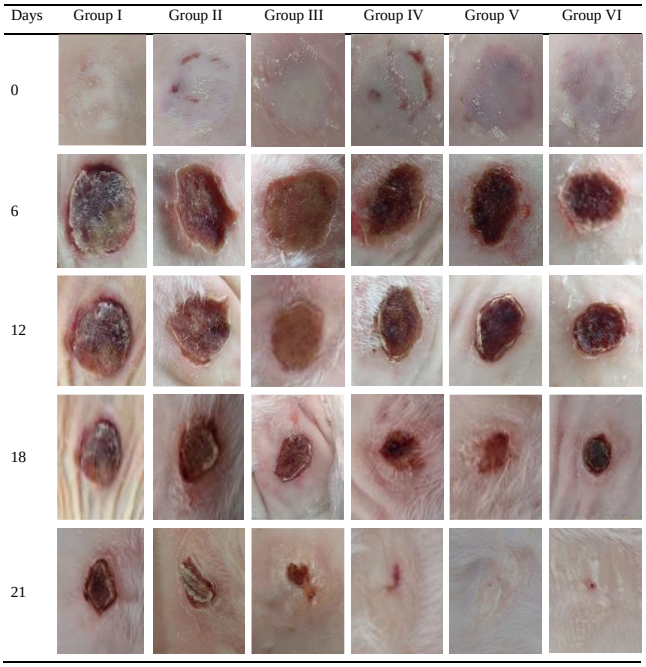

Figure 4. The burns healing observation in each treatment group

The calculation of the average percentage of wounds from day 0 to day 21 is shown in Table 5. The results of two-way ANOVA test of all test groups on the percentage of burn healing showed a significant difference between the treatment groups and healing time on the percentage of burns healing. Moreover, the ANOVA result using Duncan's post-hoc showed between groups had significant differences, except F3 and positive control groups. According to this result, F3 was the most effective of all test groups.

Table 5. The average percentage of burn healing

Day	% of burn healing					
	Group I	Group II	Group III	Group IV	Group V	Group VI
3	5.24 ± 1.24	11.66 ± 3.63	11.97 ± 0.98	19.55 ± 1.47	18.61 ± 3.21	16.78 ± 3.3(
6	19.24 ± 1.90	25.17± 3.06	31.70 ± 1.97	36.08 ± 0.81	36.09 ± 5.18	32.43 ± 4.9]
9	32.27 ± 2.00	40.37 ± 2.36	49.35 ± 1.60	48.88 ± 4.56	50.16 ± 5.31	47.62 ± 1.3(
12	48.65 ± 0.93	52.33 ± 6.48	57.95 ± 1.84	63.01 ± 3.89	64.54 ± 5.66	63.32 ± 1.7(
15	58.12 ± 0.97	64.08 ± 1.77	71.25 ± 1.56	74.41 ± 4.10	76.92 ± 5.45	74.57 ± 3.5-
18	71.05 ± 1.76	73.78 ± 1.73	83.67 ± 1.86	87.60 ± 3,97	92.20 ± 2.25	90.54 ± 2.1]
21	80.27 ± 1.47	81.94 ± 1.45	91.69 ± 1.90	96.47 ± 4.20	100.00 ± 0.00	100.00 ± 0.0
$\overline{X}$	44.98^{a} ± 1.47	49.94^{b} ± 2.93	56.80^{c} ± 3.28	60.86^{d} ± 3.28	62.65^{e} ± 3.87	60.75^{d} ± 2.-

Means within column with different letter are significantly different (P<0.05)

3.2. DISCUSSION

Research on usnic acid has been extensively conducted due to its considerable pharmacological activities. However, the low solubility of usnic acid in water has been a challenge to prepare into pharmaceutical dosage forms. The preparation of usnic acid in solid dispersion is one of strategies to improve saturated concentration of usnic acid that likely increase the activities [5].

The F3 membrane and marketed gel showed complete healing process on the 21st day. The process of healing burns is a series including inflammatory phase, proliferation and maturation phase [22]. From day 0 to 6, there is an initial inflammatory phase that characterized by releasing various inflammatory mediators such as leukotrienes, prostaglandins and histamine as a response and reaction from the body. As the results, vasoconstriction of large blood vessels in the injured area, retraction of blood vessels, fibrin deposits, and formation of blood clots in the wound area occurred in order to keep hemostasis and prevent contamination from microorganisms [23]. The observations show changes in color and extent of the wound in the test animal. When the proliferation phase occurs on 6th day until the 21st day, the cells produce Fibroblast Growth Factor (FGF) and angiogenic factors to repair injured tissue. In addition, collagen and proteoglycans are synthesized which will form new polymeric tissue into wound area [24]. Thus, the wound is filled with inflammatory cells, fibroplasia and forms a reddish tissue with a smooth surface. The experiment result showed that there was the formation of fine collagen fibers which thicken align with length of day. This indicated that the process of collagen and tissue formation continue to strengthen new strong tissues. The final phase of the wound healing process is the maturation phase which starts from the 21st day and ends 1-2 years [22,23,25,26].

The amount of usnic acid influenced on the acceleration of burn healing. The presence of anti-inflammatory and antibacterial activities from usnic acid is known to play role in burn healing. Usnic acid has anti-inflammatory activity by inhibiting the secretion of pro-inflammatory cytokines and mediators such as TNF-α, IL-6, IL-1b, iNOS and COX-2, as well as increasing the release of anti-inflammatory molecules such as IL-10 and HO-1, and minimizing the excessive inflammatory reaction that can damage the tissue around the wound [27]. In addition, usnic acid shows antibacterial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Enterococcus faceum* through inhibition of RNA synthesis and direct mechanisms including disrupting DNA replication [28–30].

4. CONCLUSION

In conclusion, usnic acid solid dispersion membrane had proper physical and mechanical properties. The burn healing experiments have shown that application of usnic acid solid dispersion membrane with 2% concentration of usnic acid was the most effective among all test groups.

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