

Antimicrobial and Wound Healing Activity of *Ixora coccinea* Leaf Extract in Hydrogel Formulation

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ABSTRACT

Objectives. The purpose of this study was to evaluate the antimicrobial and wound healing property of the preformulated hydrogel containing the methanolic leaf extract of *Ixora coccinea*, as well as its acute dermal irritation using New Zealand rabbits.

Methods. Mature dried leaves of *I. coccinea* was subjected to extraction using maceration and was concentrated in vacuo. Sodium carboxymethylcellulose and gelatin were used to create hydrogel in which the crude extract was incorporated. Physicochemical properties of the extract and preformulated hydrogel were characterized, while its antimicrobial activities against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Staphylococcus epidermidis* were determined using the agar well method and compared to the standard drug 2% w/v mupirocin ointment. A wound excision model in rats was used to determine the wound healing property of the preformulated hydrogel against povidone-iodine ointment. Lastly, animal testing was performed following the OECD Guidelines and upon approval of the IACUC Committee.

Result. The preformulated hydrogel was effective against *S. aureus* (p-value ≤ 0.001) but resistant to *P. aeruginosa* and *S. epidermidis*. Furthermore, the wound contraction rate in groups treated with preformulated hydrogel (p-value = 0.006) is significantly higher than in groups treated with hydrogel base and povidone-iodine. Moreover, no dermal erythema and edema were observed with albino rabbits.

Conclusion. The preformulated hydrogel with *I. coccinea* methanolic leaf extract is non-irritating, effective against staphylococcal infections commonly found in wounds. Hence, it is a good substitute for povidone-iodine in wound treatment.

Key Words: antimicrobial, hydrogel, *Ixora coccinea*, skin irritation, wound healing

INTRODUCTION

Ixora coccinea L., locally known as Santan of the Family Rubiaceae, is a small-medium evergreen shrub widely cultivated throughout Southeast Asian regions and beneficial for treating various ailments such as chronic ulcer, hypertension, menstrual irregularities, sprain, and skin infections.¹ Its leaf extract is proven effective against *Staphylococcus aureus*² while its methanolic leaf extracts are effective antioxidants, anti-inflammatory agent, fibroblast proliferative, and stimulates Smad mediated collagen production at 2.5% with wound contraction rate greater than gentamicin sulfate (0.01% w/w) in both *in vitro* and *in vivo* studies. Phytochemical screening showed the presence of alkaloids, flavonoids, tannins, and triterpenes which possibly contribute to its pharmacologic effects.^{1,3}

Hydrogels are soft, elastic semisolid dosage forms with 70 – 90% water content, easier to be applied on

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wounds, and removed without any skin damages because it provides a moist environment that favors tissue epithelium and granulation which are important wound healing processes.^{4,5} It is ideal for biomedical applications such as the superabsorbent crosslinked hydrogels with higher carboxymethylcellulose (CMC) amounts which swells readily upon liquid contact and enables higher drug loading percentage due to the presence of carboxyl group.⁶ It is widely used in tissue engineering, plant breeding, drug delivery and wound dressing because of its biocompatibility, biodegradability, and wound healing promotion by regulating the transepidermal water movement.⁷⁻⁹

This study aimed to utilize 2.5% crude methanolic leaf extract of *I. coccinea* in preformulating a topical hydrogel and determine its antimicrobial, wound healing property, and acute dermal irritancy for possible wound treatment substitute.

MATERIALS AND METHODS

Materials

All materials and chemical reagents were supplied by the Pharmacy Laboratory, Adamson University - College of Pharmacy except for the microorganisms such as *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), and *Pseudomonas aeruginosa* (ATCC 27853) which were procured from the Department of Medical Microbiology, College of Public Health, University of the Philippines - Manila.

Collection and Preparation of Extract

The leaves of *I. coccinea* were harvested from Laguna during September 2018 in which some fresh plant samples were sent to the Bureau of Plant Industry (BPI) for authentication, while others were washed with tap water to remove dust or any substances that may contaminate the collected samples. It was then air dried, ground using a Holz portable mechanical grinder, macerated with methanol for three days, filtered, concentrated using IKA® rotary evaporator set at 70°C, and subjected to oven-drying at 45°C for 5 hours to evaporate any methanol present and remove moisture from the samples.

Physicochemical Tests for Extract

- Physical Appearance** – The color and odor of concentrated methanolic leaf extracts of *I. coccinea* was assessed using visual-tactile method.
- pH** – Using a pH meter (Sartorius® Basic Meter PB-11), the pH of concentrated methanolic leaf extract of *I. coccinea* was measured at room temperature in which its reading was recorded after immersing the electrode in the solution.
- Solubility** – The solubility of concentrated methanolic leaf extracts of *I. coccinea* was determined by dissolving one gram of the extract in 10 milliliters (mL) of water, ether, and chloroform.

Hydrogel Preformulation

Formulation parameters of A. A Rescober (2019) was adapted with some modifications. Carboxymethylcellulose (CMC) was gradually dissolved in 20 mL deionized water and gelatin in 20 mL hot deionized water. Once dissolved, the gelatin solution was gradually added to CMC solution and continuously stirred until it became a homogeneous mixture. The methanolic leaf extracts of *I. coccinea* was dissolved in 20 mL deionized water then gradually added to the CMC-Gelatin solution with continuous stirring. A sufficient volume of solvent was added to the mixture to achieve the desired 100 mL volume. Table 1 below shows the percentage of each excipient and the corresponding use in the preformulation.

Table 1. Hydrogel preformulation

Ingredients	Concentration	Uses
<i>I. coccinea</i> methanolic leaf extracts	2.50%	Active ingredient
Carboxymethylcellulose	1.60%	Viscosity enhancer
Gelatin	0.40%	Viscosity enhancer
Deionized water	95.50%	Solvent

Quality Control Tests for Preformulated Hydrogel

- Physical appearance** – Color, odor, grittiness, and homogeneity of the preformulated hydrogel were visually analyzed and recorded.
- pH** – Using a pH meter (Sartorius® Basic Meter PB-11), the pH of preformulated hydrogel was measured at room temperature in which its reading was recorded after immersing the electrode.
- Viscosity** – SNB-2 Digital Viscometer with spindle No. 4 was used to determine the viscosity of the preformulated hydrogel.
- Spreadability** – The preformulated hydrogel was placed in between two glass slides and slipped off in which the spreadability (S) was calculated using the formula: $S = \frac{M \cdot L}{T}$ wherein M is the weight of substance placed on the top glass slide, L is the length traveled and T is the time taken to separate the two glass slides.
- Sterility Test** – Sodium Thioglycollate (FTM) and Soybean Casein Digest (SCD) media were prepared separately then two milliliters of preformulated hydrogel were transferred using a sterile syringe to each media followed by incubation for 14 days (30-35°C for FTM and 20-25°C for SCD) in which absence of turbidity indicates sterility of the preformulated hydrogel.

In-vitro Antimicrobial Activity

The Agar well method was used to determine the antimicrobial activities of preformulated hydrogel wherein an aliquot of 0.5 mL inoculums of *S. aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228) and *P. aeruginosa* (ATCC 27853) were spread into separate Petri dishes containing Mueller-Hinton Agar. It was left for several minutes inside the Class II Biosafety Cabinet (Telstar BIO II Advance 4), punched

aseptically using disposable 3 mL sterile syringe barrels, and filled with the following test groups: 100 µL hydrogel base (negative control), 100 µL Mupirocin Ointment 2% w/v (positive control), and 100 µL preformulated hydrogel. The plates were placed inside the incubator for 24 hours at 37°C wherein zones of inhibition were measured using a digital Vernier caliper (VINCA DCLA-0605). Results were interpreted based on the 2018 Guidelines of Clinical and Laboratory Standards Institute (CLSI)¹⁰ as shown in Table 2 below.

Table 2. Interpretative table of selected microorganism for susceptibility testing on Mupirocin based on zone diameter in millimeter (mm)

Microorganisms	Resistant	Intermediate	Susceptible
<i>Staphylococcus spp.</i>	≤ 28	—	≥ 29
<i>P. aeruginosa</i>	≤ 14	15 - 20	≥ 21

In vivo Wound Healing Activity and Acute Dermal Irritancy

Preparation of test animals

An approval from the Institutional Animal Care and Use Committee (IACUC) of Adamson University was obtained before the conduct of animal testing. Healthy male Sprague Dawley rats at 3 months old weighing 100 – 200 g and New Zealand rabbits at 1 – 2 kg were purchased from a BAI-Accredited animal breeder (LAF-0036). These test animals were housed inside the Animal House of Adamson University – College of Pharmacy with a maintained temperature of 25 ± 5°C and acclimatized for seven days with free access to food pellets and water ad libitum before the experimental procedures were carried out. Also, these test animals were randomly selected and assigned to different treatment groups to avoid bias.

Wound healing activity

Acclimatized rats were anesthetized with 10mg/kg ketamine intraperitoneally, back hairs were shaved using a razor blade, and the surgical site was disinfected with 70% alcohol.¹¹ The dorsal skin was folded, raised cranially, and caudally at the midline to form a sandwiched skinfold. Then, test animals were placed in a lateral position followed by pressing down with 4-mm diameter sterile biopsy punch to completely removed the two skin layers to create a symmetrical full-thickness excisional wound.¹² Homeostasis in the wounded area was achieved by blotting the wound with a cotton swab soaked in normal saline solution. As presented in Table 3, test animals were randomly divided into four groups namely the untreated (Group I), treated with hydrogel base (Group II), treated with preformulated hydrogel (Group III), and treated with the standard drug Povidone-Iodine (Group IV). Wounds were left uncovered and rats were then caged individually.¹¹

Table 3. Animal Groupings for Wound Excision Model

Treatment Groups	Description	No. of Rats Used
1	Untreated	5
2	Hydrogel base	5
3	Preformulated hydrogel of <i>I. coccinea</i>	5
4	Povidone Iodine	5

Wound Area Measurement

The wound reduction area was monitored on predetermined days (2nd, 4th, 8th, 12th, and 16th day) and measured using a Vernier caliper.¹³

Wound Contraction Rate

Wound Contraction Rate (WCR) was calculated using the formula provided wherein it is the percentage of the reduction in original wound area size as measured using a Vernier caliper in millimeters.¹³

$$WCR = \left(\frac{\text{Initial wound area} - N^{\text{th}} \text{ day wound area}}{\text{Initial wound area}} \right) (100)$$

Acute Dermal Irritancy Test

OECD Guidelines were followed wherein furs in the dorsal area trunk of the New Zealand rabbits were shaved using a disposable and sanitized safety razor. A single dose of 0.5 mL preformulated hydrogel was applied to the shaved and intact skin using a 1 mL syringe, while the untreated skin areas served as the positive control. Treated areas were then covered with 6 cm² sterile gauze pads and 1-inch micropore to hold the gauze pad in place. Three test patches were applied sequentially at different skin areas wherein patches were removed after three minutes, an hour, and 4 hours to observe and grade for any presence of severe skin reactions.

Statistical Analysis

Data collected was analyzed using One-Way Analysis of Variance (ANOVA) in Stata v.13 in which a p-value of less than 0.05 is considered significant.

RESULTS

Extraction Yield

The obtained dried methanolic leaf extract was 55.2 g (6.691%) from macerated 825 g of *I. coccinea* dried leaves.

Physicochemical Tests of the Extract

Methanolic leaf extracts of *I. coccinea* was dark green with a leafy odor. It was slightly acidic with an average pH of 4.58, soluble in water, sparingly soluble in ether, and practically insoluble with chloroform.

Characterization of Preformulated Hydrogel

As shown in Figure 1, the hydrogel base was transparent in appearance, smooth and homogenous in texture which

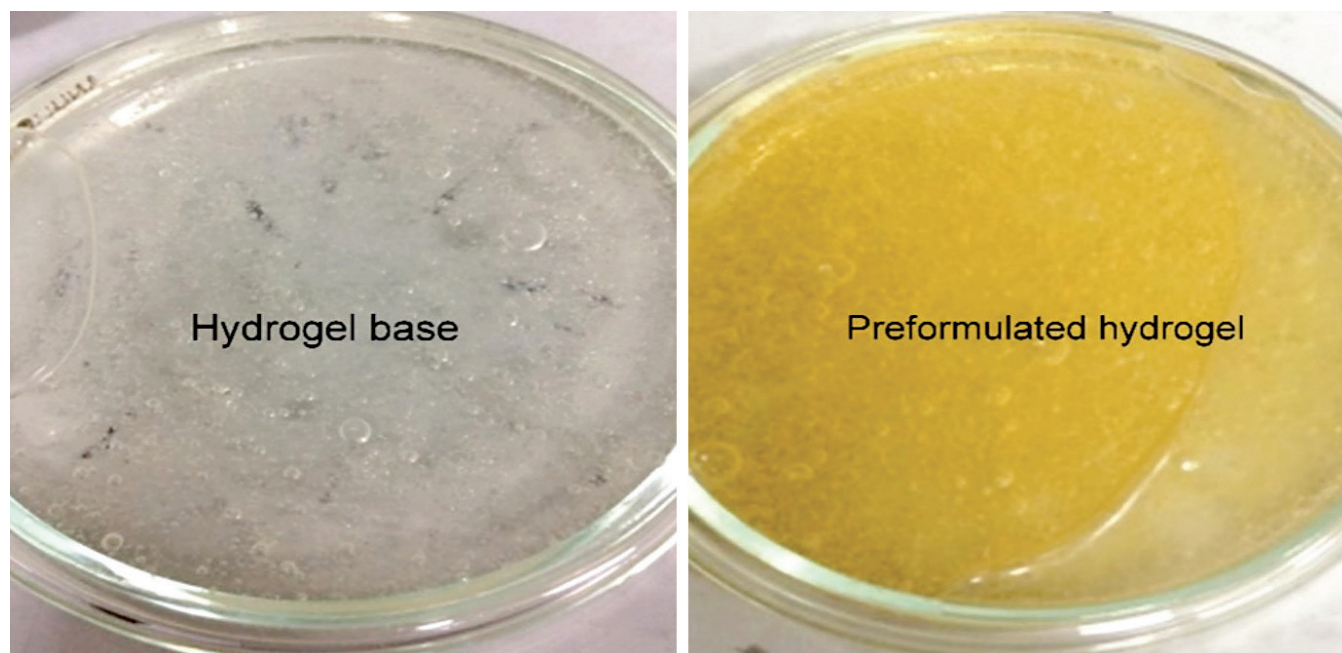


Figure 1. Hydrogel base appearance upon incorporation of *I. coccinea* leaf extract.

turned honey-colored with a sweet odor upon the addition of *I. coccinea* extract. Its average pH was 3.13, viscosity 55.335 poise, and spreadability rate of 5.789. Also, it was sterile since turbidity was not observed on FTM and SCD after 14 days.

In-vitro Antimicrobial Activity

After a 24-hour incubation period, the antimicrobial activities of 2% mupirocin ointment (positive control), 2.5% preformulated hydrogel, and hydrogel base (negative control) were determined by measuring the inhibitory diameter zones against *S. aureus*, *S. epidermidis* and *P. aeruginosa* using a digital Vernier caliper. Based on the results shown in Table 4 below, the preformulated hydrogel had an average inhibitory diameter of 29.21 mm for *S. aureus*. Also, both preformulated hydrogel and hydrogel base were not effective against *S. epidermidis* and *P. aeruginosa*, unlike with 2% mupirocin ointment with average inhibitory diameters of 34.72 mm, 32.91 mm, and 22.50 mm on *S. aureus*, *S. epidermidis* and *P. aeruginosa* respectively. Results were statistically analyzed using One-way ANOVA wherein the p-values acquired

were < 0.05 indicating a significant difference in the *in-vitro* antimicrobial activity against selected microorganisms.

Pharmacologic Activity

Wound Healing Activity

Wound contraction rates (WCR) of each treatment group were observed after 2nd, 4th, 8th, 12th and 18th day wherein the untreated group was the least on the 2nd day (\bar{x} = 2.1), although it progressed after the 4th day (\bar{x} = 19.73) and until the 12th day of treatment (\bar{x} = 97.12). These results were contrary to the group treated with preformulated hydrogel with WCR of 20.50%, 37.11% and 52.68% for 2nd, 4th, and 8th day respectively, wherein complete healing were observed as early as 12th day compared to the standard drug povidone-iodine with WCR of 4.93%, 19.27%, 31.86%, 83.82% and 100% on the 16th day. The group treated with hydrogel base also showed promising wound healing activity of 10.20%, 25.21%, 41.89%, and 100% for 2nd, 4th, 8th, and 12th day respectively.

Table 4. Zone of Inhibition of Treatment Groups

Treatment Groups	<i>S. aureus</i> ^a (mm)	<i>S. epidermidis</i> ^b (mm)	<i>P. aeruginosa</i> ^c (mm)
2% Mupirocin ointment (+) control	34.72 ± 2.13 Susceptible	32.91 ± 0.153 Susceptible	22.50 ± 0.435 Susceptible
Preformulated hydrogel	29.22 ± 0.229 Susceptible	0 Resistant	0 Resistant
Hydrogel base (-) control	0 Resistant	0 Resistant	0 Resistant

Nb. n = 3 x 3; p-value^a = 0.0273; p-value^{b,c} = 0.0221

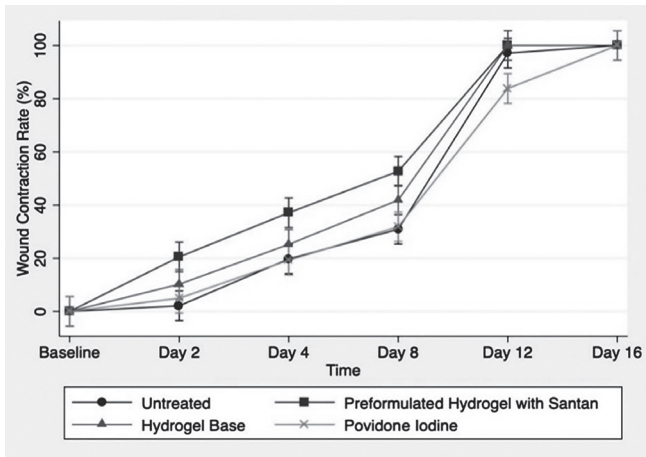


Figure 2. Mean % wound contraction rate of the preformulated hydrogel and controls at different time periods.

As observed in Figure 2, there is no sufficient evidence to say that there is a significant difference in the mean wound contraction rate between untreated and povidone-iodine group (p -value = 0.730). This indicates that the povidone-iodine did not exert any wound healing property since its level of healing is similar to the untreated group. There is, however, a significant difference in the wound contraction rate between the untreated and povidone-iodine group and the preformulated hydrogel and hydrogel base. This indicates that both groups exerted wound healing property but the preformulated hydrogel is significantly higher than that of the hydrogel base (p -value=0.006). The wound contraction rate significantly increases with time except from Day 12 to Day 16 (p -value=0.165) which showed

no significant difference because most of the wounds have already healed on Day 12.

Acute Dermal Irritancy Test

As observed in Figure 3, the preformulated hydrogel topically applied to the test animals showed no signs of erythema, eschar, and edema. A confirmatory test was performed using a second rabbit (OECD, 2002) and still showed the same results wherein the skin was still intact when patches were removed indicating the non-irritancy of preformulated hydrogel.

DISCUSSION

The preformulated hydrogel containing 2.5% of methanolic leaf extracts of *I. coccinea* was honey-colored, sweet in odor, smooth, homogenous in texture, and acidic in nature with an average pH of 3.13. According to Nagoba et. al, wound healing progression decreases when pH is elevated to an alkaline condition. Also, an acidic environment helps in wound healing by controlling wound infection, altering protease activity, releasing oxygen, and enhancing epithelization and angiogenesis.¹⁴

Its viscosity and spreadability were 55.335 poise and 5.7887 $\frac{g \cdot cm}{s}$ respectively in which gels containing CMC were noted for maximum spreadability range of 12.8 to 15 indicating greater spreadability of preformulated hydrogel.¹⁵ It was also sterile with no presence of turbidity after 14 days and upon comparison to FTM and SCD medias with inoculums of *Bacillus subtilis* and *Candida albicans* indicating good preformulation process. Since it was applied to wounded skins of test animals, sterile wound preparation

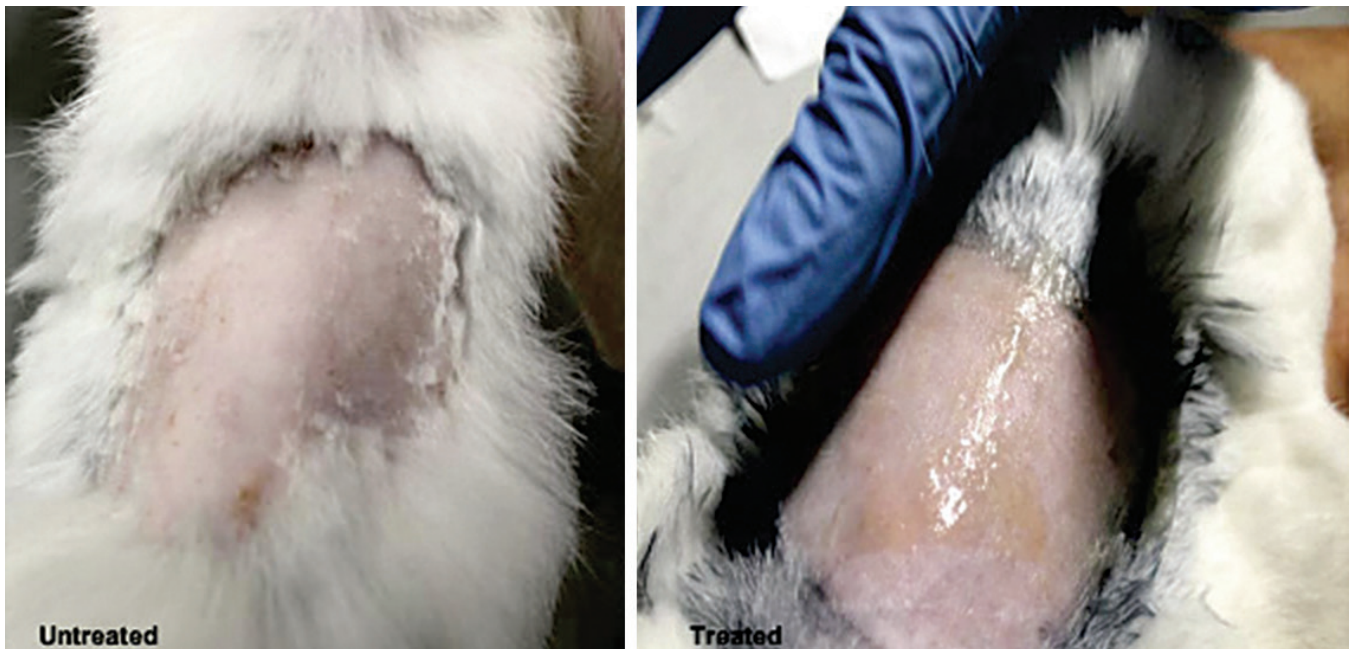


Figure 3. Comparison of untreated and treated patch testing.

prevents further microbial contaminations that may impair the wound healing process. Also, the preformulated hydrogel was effective only to *S. aureus* with an average inhibitory zone of 29.22 mm and significantly lower to the standard drug 2% mupirocin ointment. According to Marimuthu et. al, the antimicrobial activity of *I. coccinea* was due to its active constituents terpenoid, flavonoid, coumarin, alkaloid, and phenolic groups.¹⁶

Among the treatment groups, the preformulated hydrogel exhibited higher wound contraction rates which supported the previous studies of Chattopadhyay et. al on wound healing properties of the methanolic leaf extract with 96.78% WCR higher than gentamicin sulfate (89.59%) and non-treated control group.¹ Hydrogel base alone exhibited wound healing activity thus supporting Shah and Amini-Nik (2017) findings that hydrogel dressings are superior to conventional dressings as they can maintain a moist environment that enhances wound healing.⁴ The speedy recovery of wounds observed in Group II and III can be due to CMC as a hydrogel base ingredient since it favored neutrophil accumulation at the wound site. Upon the addition of methanolic leaf extract, its wound healing activity was increased and made it more effective than the standard drug povidone-iodine 2.5%. Also, the preformulated hydrogel was non-irritating with a Dermal Irritation Score of "0" as no presence of erythema, eschar, and edema were observed upon application. Histopathological observation and stability testing were delimited in this study and recommended to be determined in future research to fully assess its wound healing property and shelf-life.

CONCLUSION

The preformulated hydrogel containing 2.5% methanolic leaf extracts of *I. coccinea* was non-irritating, effective against *S. aureus*, has a better wound contraction rate, and a good substitute for povidone-iodine in wound treatment.

Statement of Authorship

All authors participated in data collection, analysis, and approved the final revision submitted.

Author Disclosure

All authors declared no conflicts of interest.

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