

Accelerated wound healing ability of *Jatropha sap* by iota carrageenan-poly (vinyl alcohol) hydrogel film

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ABSTRACT

Jatropha sap (JTS), an important fluid carried in xylem and phloem tubes of *Jatropha multifida* L. plant, has good wound healing property. However, physicochemical stability of JTS needs to be improved in order for it to be useful as a topical wound-healing agent. In this study, we developed an iota carrageenan-polyvinyl alcohol (IC-PVA) hydrogel film (HF) as a carrier of JTS and evaluated its wound-healing ability. The characterization of JTS secondary metabolites by ultraviolet-Vis spectrophotometry suggested presence of flavonoid, saponin, and alkaloids in the sap. We successfully extracted IC from *Euchima spinosum* using alkaline solvent at 80°C–90°C with calcium chloride as the precipitator. The result of computer simulation using Discovery Studio software and Autodock Tools showed the presence of hydrogen bonding interaction of IC-PVA. IC-PVA/JTS HF with excellent physical properties including high swelling ratio (246.32%) and high gel fraction (16.75%). In addition, irritation test in mice confirmed the absence of hypersensitivity reaction, redness, and allergic reactions. Interestingly, IC-PVA/JTS HF significantly accelerated wound healing when compared to the nontreated group/control with 98% wound closure by 10 days. These results suggest that IC-PVA HF has improves wound-healing ability of JTS.

Key words: Hydrogel film, iota carrageenan, *Jatropha sap*, polyvinyl alcohol, wound dressing

INTRODUCTION

A wound is an injury to the skin caused by friction with an external object that can be a primary problem for some people. Wound healing process occurs in several phases,

starting from hemostasis, inflammation, cell migration, fibroblast proliferation, angiogenesis, re-epithelization, and remodeling phase. Coagulation rate, leukocyte level, fibroblast proliferation, and infection at wound site can influence the wound healing process.^[1]

Nowadays, wound dressing is used as a standard treatment for wound healing. Wound dressings can be the traditional type of dressing (bandage, gauze, and sponge) or the modern type of dressing such as hydrocolloid, foam, hydrogel, or hydrogel film (HF).^[2] Among these dressing options, HF is preferred as it maintains wound moisture, reduces bacterial levels and chemical mediators, provides autolytic debridement of eschar, and permits transmission of water

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vapor.^[3] In addition, HF is constructed by polymerizing the matrix system which reduces drug administration frequency and absorbs the wound exudates.^[4]

Iota carrageenan (IC), a natural polysaccharide extracted from *Euchima spinosum*, forms a stronger gel in calcium solution and has greater stability compared to kappa and lambda carrageenan. However, the hydrogel phase system of carrageenan has poor mechanical properties. Therefore, a crosslinker needs to be added to improve the mechanical properties of hydrogel carrageenan. Commonly used crosslinker includes biopolymer such as cellulose groups, or semi synthetic/synthetic polymers such as poly-vinyl alcohol (PVA).^[5-7]

PVA is a nontoxic, biocompatible, and biodegradable synthetic polymer that is simple to prepare, has excellent mechanical strength and also thermo stable.^[8] Carrageenan modified with PVA has higher tensile strength,^[9] ideal swelling behavior, best scaffolding composition,^[10] excellent mechanical and biodegradable properties.^[11]

Jatropha sap (JTS), a fluid transported in xylem and phloem cells of *Jatropha multifida* L. plant, is empirically used as a natural remedy for wound healing. JTS has abilities to accelerate the coagulation time, affect in granulation process, has an astringent activity which affect in hemostasis phase, induce the re-epithelization process, and activate the transforming growth factor beta function in migration and proliferation phases.^[12-14]

In previous studies, JTS was shown to heal traumatic ulcers in rats (*Rattus norvegicus*).^[13] It also effective in stopping hemorrhage in normal rats and rats with homeostasis disorder.^[12] It also had some effects on homeostasis phase by its astringent activity to precipitate a protein and the accelerate the coagulation time by its effect in cascade reaction of coagulation in the wound-healing process.^[14]

In this study, we fabricated IC-PVA HF containing JTS (IC-PVA/JTS HF) and characterized its physicochemical properties. Then, we investigated the wound-healing ability of IC-PVA/JTS HF in mice.

MATERIALS AND METHODS

Materials

The IC standard was purchased from Karaindo (Central Java, Indonesia), while the sodium hydroxide (NaOH), calcium chloride (CaCl₂), hydrogen chloride (HCl), PVA, the dimethylol dimethyl hydantoin and the sodium metabisulfite (Na₂S₂O₅) were purchased from Brataco Chemical (Jakarta, Indonesia). The red algae (seaweed) 6–8 weeks old were purchased from local fishermen of the community of fishermen marine plant cultivation (North Gorontalo, Gorontalo, Indonesia). The *J. multifida* L. (*Euphorbiaceae*) Sap (JTS) was obtained from medicinal plant cultivation sold by the villagers of South

Molingkapoto (Kwandang, North Gorontalo, Gorontalo, Indonesia).

Collection and characterization of *Jatropha multifida* L. (*Euphorbiaceae*) sap by ultraviolet-vis spectrophotometry

The JTS was taken from the base of the leaves of *J. multifida* L plant. And prepared with the addition of antioxidants Na₂S₂O₅ 0.5% with ratio 5:1 (JTS: Na₂S₂O₅). The characterization of JTS performed using ultraviolet (UV)-Vis spectrophotometry Perkin Elmer, where the JTS prepared in 1000 ppm, 100 ppm, and 10 ppm into aqueous solution then analyzed at a wavelength of 200–700 (nm).^[15]

Extraction and characterization of iota carrageenan by liquid chromatography-mass spectra

IC was extracted from the red algae using alkaline solution NaOH 0.9 N with ratio 1:20 (sample: solvent) and extracted at temperature 80°C–90°C about 30 min. The filtrate and the residue were separated, and the filtrate was precipitated using CaCl₂ 3% w/v, neutralized using HCl to achieve pH 7–8 and evaporated at temperature 45°C–50°C.

The powder of the standard carrageenan and the extracted carrageenan was made into liquor to 10,000 ppm by dissolving 50 mg of each powder into 5 mL ultra water in different containers, and the solutions were diluted to 10 ppm, by taking 15 µL from the stock of each solution and had been sufficient to 15 mL. Then, the solution running on liquid chromatography-mass spectra (LC-MS) waters instrument.^[16]

Computer simulation

On interaction between IC and PVA, it was evaluated using E2-Toshiba AMD APU HD Graphics 1800 1.70 GHz devices (Toshiba Coporation, Japan), Discovery Studio v. 16.1.0 free trial (Dassault Systemes BIOVIA) and Autodock Tools (ADT) 1.5.6 software. The simulation was carried out by downloading the file two-dimensional (2D) IC and PVA from Pubchem.ncbi.nlm.gov. The 2D structure was prepare using Discovery Studio software v. 16.1.0 (Dassault Systemes BIOVIA), and interaction was observed using ADT, by ligand-ligand interaction method.^[17]

Preparation of hydrogel film

Preparation of the HF was carried out by combining the IC with various concentration of PVA (6%, 8%, and 10%). Each base was dissolved in water by heating and stirring with ultraturax for 15 min, then centrifuged at 250 rpm for 15 min, and then inserted into the mold, and evaporated at 45°C.^[18]

Evaluation of hydrogel film

Swelling ratio

The swelling ratio was evaluated by weighing the HFs mass at the break of every interval time in the immersion process and stopped when it reached the maximum point marked when the mass decreased.^[19] The percent of swelling ratio

determined by the equation:

$$\text{Swelling ratio (\%)} = \frac{\text{The maximum mass after Swelling} - \text{The mass before swelling}}{\text{The mass before swelling}} \times 100$$

Gel fraction

The amount of gel fraction was obtained by weighing the remaining of the HF after pass through the soaking and drying processes repeatedly,^[20] then the percent of the gel fraction determined by the equation:

$$\text{Gel fraction (\%)} = \frac{\text{The mass of gel fraction}}{\text{The mass hydrogel film}} \times 100$$

In vivo wound healing studies

The animals were divided into several groups consisting of nontreated group, control negative group (IC-PVA) of HF, control positive group of *J. multifida* L. sap (JTS), and group test (IC-PVA/JTS). Each group had been treated except nontreated group and observed for 21 days and followed by calculating the percent of wound closure for each group treatment.^[4] All the animal on this study were approved by the Ethics Committee Research Tasikmalaya Health Polytechnic of Ministry of Health (Reg. number: 3278012P).

$$\text{Wound closure (\%)} = \frac{\text{Wound diameter at first day} - \text{Wound diameter at day time (n)}}{\text{Wound diameter at first day}} \times 100$$

Irritation test

Irritation test was conducted in an animal group and observed for 24, 48, and 72 h after treatment the formula. Parameter observations were seen by the level of erythema (redness reaction) and the degree of edema (inflammation) that appeared.^[21]

Data analysis

The standard error of the mean \pm (SEM) was expressed for the quantitative data. The statistical analysis for the HF evaluation and the *in vivo* wound healing study was carried out using one-way ANOVA method.

RESULTS

Ultraviolet-vis spectrophotometry analysis of *Jatropha multifida* L

Judging by the responses of specific wavelengths from UV-Vis spectrum, these results indicate the presence of flavonoids, saponins, alkaloids, tannins, and polyphenols.

Liquid chromatography-mass spectra characterization of iota carrageenan

LC-MS analysis characterization showed that there were 5

specific peaks of molecular weight which similar between carrageenan extracted and standard carrageenan, (157 m/z, 309 m/z, 425 m/z, 639 m/z and 803 m/z) [Figure 1]. Based on this result iota-carrageenan was successfully extracted from *Euchima spinosum* using alkaline solvent under temperature 80-90°C with CaCl₂ as a precipitator.

Computer simulation

Ligand-ligand interaction analysis between IC with PVA showed the presence of hydrogen bonding interaction on sulfate group from IC with hydroxyl group from PVA, with the binding energy -0.074 Kcal/mol.

Evaluation of hydrogel film

Based on swelling ratio and gel fraction studies, the results thereby indicating that the concentration of PVA was statistically significant ($P < 0.01$) can improve the value of swelling ratio, yet insignificantly for the value of gel fraction.

In vivo wound healing studies

The results showed that the IC-PVA/JTS hydrogel film was significantly ($P < 0.01$) can accelerate wound healing effect compared with other groups. These results also describes that JTS has good activity in the healing process compared to the IC-PVA group and the non-treated group. Impressively, the IC-PVA hydrogel film base has a better ability compared to the non-treated group.

Irritation test

Based on irritation test, the IC-PVA/JTS has no irritant effect judging by the the absence of hypersensitivity reaction, redness or allergic reactions [Figure 2].

DISCUSSION

Ultraviolet-vis spectrophotometry analysis of *Jatropha multifida* L

The preparation of JTS was added with the antioxidant agent intended to improve the stability of secondary metabolites contained in the JTS.^[22] Based on the orientation result, the JTS was very susceptible to photo degradation. Therefore, the addition of antioxidant and appropriate containers had to be considered in order to maintain the

Table 1: Phytochemical screening of the *Jatropha multifida* L. (Euphorbiaceae) Sap

Secondary Metabolites	<i>Jatropha multifida</i> L. Sap
Glycosides	-
Alkaloids	+
Flavonoids	+
Phenol and tannins	+
Saponins	+
Steroids	-
Carbohydrate	-

stability of the JTS. The qualitative analysis to determine the content of secondary metabolites presented in the JTS was performed by spectrophotometry UV-Vis method, and the result can be seen in Table 1.

Extraction and characterization of iota carrageenan by liquid chromatography-mass spectra

Carrageenan is a biopolymer that is composed of a long polysaccharide chain divided into three types: kappa carrageenan, IC, and lambda carrageenan.^[3,7] IC is a type that can be founded in *E. spinosum* which consist of monomer D-galactose-4 sulfate, 3,6 anhydro D-galactose-2 sulfate.^[23]

The use of alkaline solution will decrease the cell permeability and makes the extraction process more easy than without using alkaline solution. Na⁺ ions form NaOH molecule, which improves the stability of the carrageenan by replacing the unstable sulfate groups and forms more stable carrageenan. In addition, the strength of carrageenan was affected by the equilibrium between sulfate groups and 3,6 anhydro D-galactose groups. The strength of the gel is inversely with the amount sulfate groups and directly proportional with 3,6 anhydro D-galactose groups.^[3,7]

High temperature range (80°C–90°C) was used in order to accelerate the extraction process, which is high temperatures could lose the cell membrane composition, as the results it was easy to be entered by the solvent during the extraction process. The filtrate was neutralized using HCl until it reached pH 7–8 for maintaining the stability of carrageenan.^[7] Carrageenan extract was precipitated by CaCl₂ to provide a strong gel of carrageenan replace the unstable sulfate groups.

Based on the results of the LC-MS analysis, there were five specific peaks of molecular weight form carrageenan extracted

which similar with the standard carrageenan, (157 m/z, 309 m/z, 425 m/z, 639 m/z and 803 m/z) [Figure 1]. Based on these data, the results of fragmentation were interpreted as a fragment of 3,6 anhydro-D-galactose (157), D-galactose, 3,6 anhydro-D-galactose (309), two groups of 3,6 anhydro-D-galactose and 1 galactose cluster (425), D-galactose 2 Sulfate, 3,6 Anhydro-D-galactose (639) and D-galactose 2 sulfate, 3,6 anhydro-D-galactose 1 sulfate (803). As we known that the basic structure of IC contain structure core of D-Galactose and 3,6 anhydro-D-Galactose and also contain of sulfate groups on each core molecule of IC, in which lambda-carrageenan does not contain of sulfate groups and the kappa-carrageenan only have one sulfate group. The results of this analysis showed that IC was successfully extracted from *E. spinosum* using alkaline solvent under temperature 80°C–90°C with CaCl₂ as a precipitator.

Computer simulation

The results of computer simulations using Discovery Studio software and ADT showed the presence of hydrogen bond interaction between sulfate group from IC and hydroxyl group from PVA, with the binding energy –0.074 kcal/mol [Figure 3a and b]. The interaction between IC and PVA allowed the formation of crosslinking in the HF which that has an important role for maintaining the consistency of the HF.^[24] Based on the computer simulation data, it showed that IC could be combined with PVA as a crosslinker to form crosslinking through hydrogen bonding interactions.

Preparation and evaluation of hydrogel film

The preparation of HFs was carried out by varying the PVA concentration of 6%, 8%, and 10%, the concentration had chosen by considering from basic orientation of

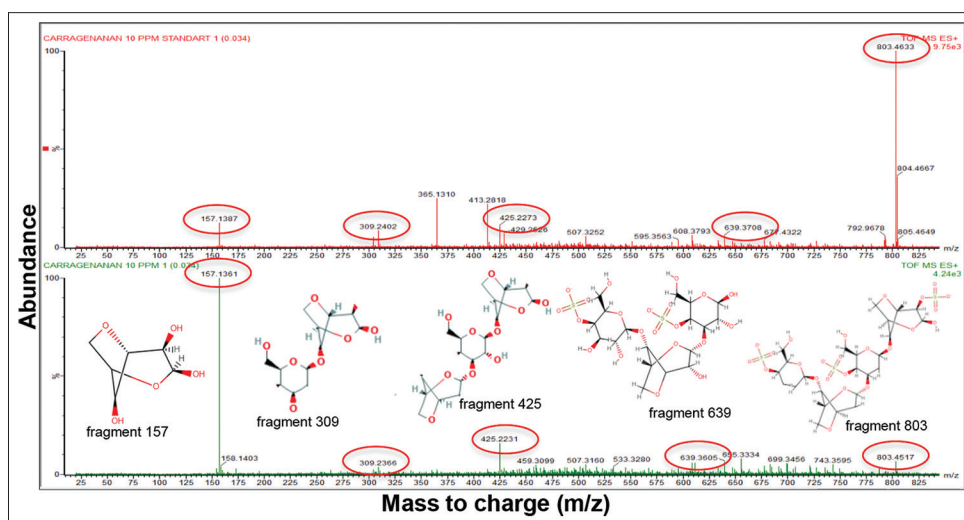


Figure 1: Liquid chromatography-mass spectra characterization of iota-carrageenan: iota-carrageenan extract and top: carrageenan standard. The liquid chromatography-mass spectra results represent by fragmentation structures of iota-carrageenan extract compared to the fragmentation structure of iota carrageenan standard

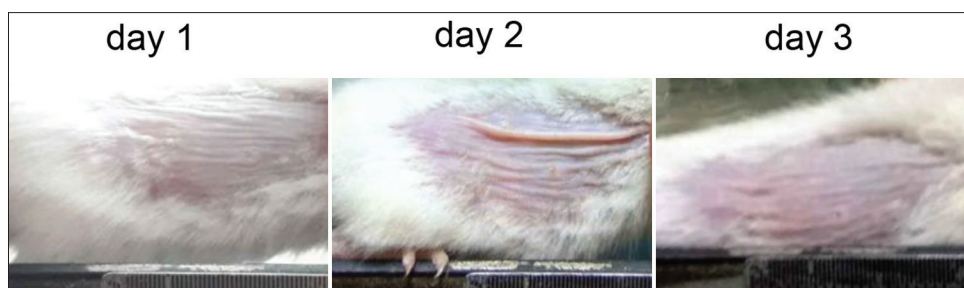


Figure 2: Irritation test. The irritation test was performed for 24, 48, and 72 h, by assessing the irritant reaction (edema and erythema) on the animal skin

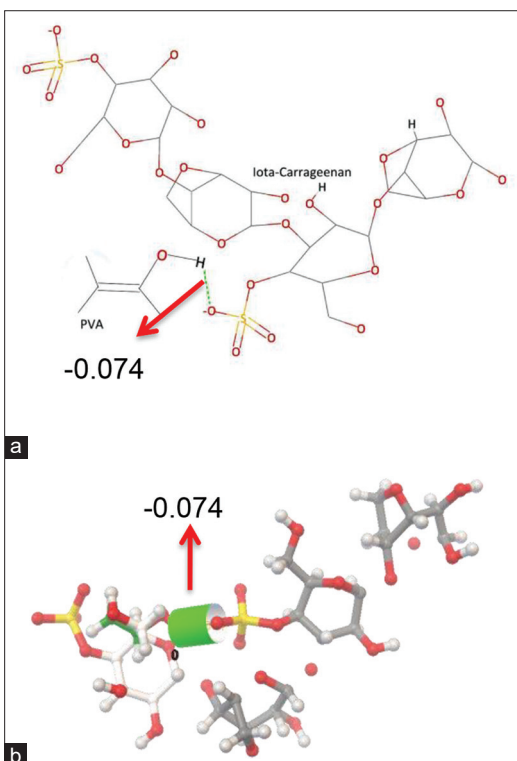


Figure 3: Computer simulation. (a) The two-dimensional Visualization of hydrogen bonding interactions (green line) between iota carrageenan with polyvinyl alcohol; (b) The three-dimensional visualization

PVA capability to swell in water where it was equal to the range of 5%–10%. The heating and ultraturax mixing processes were conducted to provide a fast dissolution and homogenous gel phase system, and centrifugation was conducted to remove the trapped bubbles in the gel. On the last part, the solvent evaporation process will evaporate the number of water molecules in the gel phase system then form the HF.

Organoleptic test

All of the formulas were able to form the HFs in a high consistency, yet the IC-PVA of 6% and IC-PVA 8% were damaged, the surfaces of the films were uneven, and the film structures were thin. Besides, The IC-PVA 10% HF formula has an excellent physical property in terms of organoleptic,

transparent and has a good film surface [Figure 4a]. PVA concentration has a great affect of the film formation. According to previous study,^[25] an appropriate of equimolar ratio and a high concentration of PVA will form film structure of with excellent consistency and transparency.

Evaluation

Swelling ratio

Swelling ratio evaluation intended to see how better the ability of HFs to swell and absorb the liquid.^[26] In this case, the property could be representative for describing the ability of the film to absorb wound exudates which known that may affect of the wound healing acceleration. The results shows that the concentration of PVA was statistically significant ($P < 0.01$) in affecting the value of swelling capability. The IC-PVA 6% HF has swelling ratio of 66.41%, IC-PVA 8% HF of 98.10%, and IC-PVA 10% HF of 246.32% [Figure 4b]. The results describes that PVA concentration was directly proportional with the percent of swelling ratio. The results are in line with the earlier research where the PVA could increase up to 10 times leading to a significant swelling capability.

Gel fraction

The gel fraction is a parameter used to assess the ability of a gel to maintain consistency. It forms as a value to indicate the number of crosslinking formed in the gel.^[27] Based on the evaluation of the gel fraction, IC-PVA 6% HF has 9.74%, IC-PVA 8% HF has 15.13% and the IC-PVA 10% HF has 16.75% [Figure 4c]. From these data, the concentration of PVA was directly proportional with the percent of the gel fraction, but the correlation is not statistically significant ($P > 0.01$). It was assumed that the ratio of IC-PVA concentration had been greater than or equal to the equimolar point resulting an insignificant changes of the gel fraction, especially on the PVA concentration of 8% and 10%. From the results of the organoleptic evaluation, swelling ratio and gel fraction, we took the IC-PVA 10% as the best HF formula and formulated with the JTS and tested the wound healing activity and its irritant effect.

In vivo wound healing studies

In vivo wound healing study results, the IC-PVA/JTS was statistically significant ($P < 0.01$) in providing accelerated

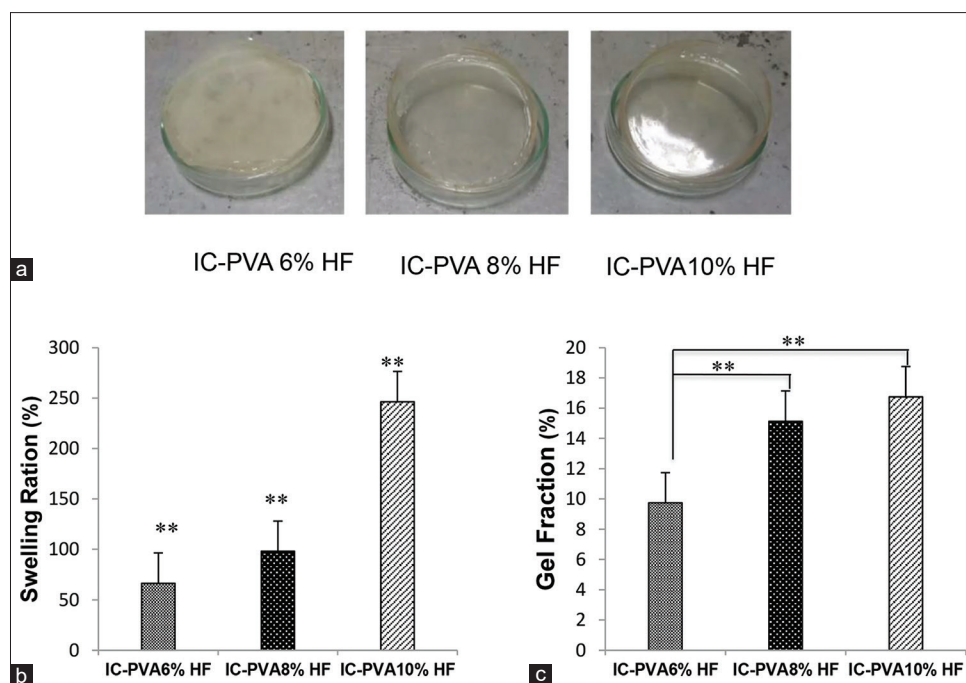


Figure 4: Hydrogel film evaluation. (a) The macroscopic visualization results of hydrogel film; (b) Results of swelling ratio. The iota carrageenan-polyvinyl alcohol hydrogel films (1 cm × 2 cm) were weighed in advance and weighed again at the interval time during the immersion process; C. Results of gel fraction. The iota carrageenan-polyvinyl alcohol hydrogel films were weighed and passing through soaking and drying processes repeatedly. Each value represented the mean ± standard error of the mean. * $P < 0.01$ compared to the contro

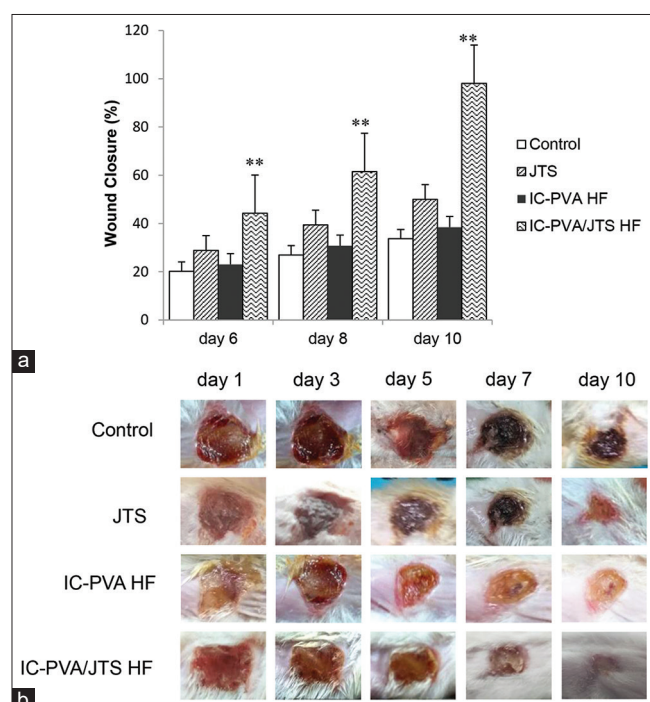


Figure 5: (a) The percent of wound closure at day 6, 8, and 10; (b) Macroscopic visualization of wound healing process at day 1, 3, 5, 7, and 10. The in vivo wound closure study was performed in full-thickness excisional wounds of mice on the 1st-10th days. Each value represented the mean ± S.E.M. * $P < 0.01$ compared with non-treated group

wound healing effect compared other groups [Figure 5a]. This indicated that the JTS which formulated with IC-PVA HF could accelerate the wound healing through the synergistic mechanism of actions.

These results, can be seen that JTS has good activity in the healing process compared to the IC-PVA group and the nontreated group. This finding result is in line with the earlier studies stated that JTS had wound healing ability through multiple mechanisms. Besides having antioxidant activity, the flavonoid also had an astringent property that was able to precipitate the protein that handles the vasoconstriction process and being one of the parameters of hemostasis phase. The saponin also contributed in collagen fiber formation by preventing elastin degradation and improving vascularization^[13] [Figure 5a and b].

Herein, the IC-PVA HF has a better ability compared to the nontreated group. This proved that the HF also had an important role in the healing process which in line with the previous studies stated that HF could provide a suitable environment in wounds, and it has the ability to control or manage the moisture and played a role in the absorption of exudate that can promote fibroblast proliferation in the wound healing process.^[4,28]

Irritation test

Irritation test was intended to guarantee the basic quality

requirements of the safe dosage properties. This evaluation was done to see the irritant effect of the active substance.^[29] The result showed that the absence of hypersensitivity reaction, redness or allergic reactions [Figure 2]. These results correlated with the previous studies which the *J. multifida* L. the JTS has no irritating effect,^[14] and also the biopolymer of the IC and the PVA had been reported have no instances of toxicity.

CONCLUSION

This research succeeded in accelerating the ability of JTS in the wound healing due to the IC-PVA formulation. When it was tested by in-vivo, it showed a significant effect ($P < 0.01$) of IC-PVA/JTS HF in providing accelerated wound healing effect with the wound closure of 98% on the 10th days compared with the other groups.

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Conflicts of interest

There are no conflicts of interest.

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