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2,2-Dichloro Carboxymethyl Chitosan with Crosslinker Urea-Terephthalic acid as Wound Healing on Mice

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ABSTRACT

A syntactical research into 2.2-dichloro carboxyl methyl chitosan-urea-phthalic acid (DCMChi-UTER) was conducted. This chitosan derivate compound was a graft-crosslink polymer. Then pasta/cream was extracted from this compound by adding Vaseline and inserting it into a homogenizer, the process whose result is Vaseline-DCMChi-UTER paste. The paste concentration is 10% and 20%. From the wound healing test on mice given a 1 cm-long incision on the right back which was later treated with the paste once a day, a good condition results macroscopically and the histology shows that recovery takes place on the fourth day of the application, the wound nearly recovers (the wound nearly closes) totally on the seventh day, and the wound recovers (the wound totally closes) on the fourteenth day after the application. The histology analysis shows that when viewed from inflammatory cells, reepithelization, torn epidermal cells, keratin migration, scar tissue, fibro proliferative tissue, broken collagen tissue, hair follicle growth and the number of fibroblast cells, it turns out that this medicine is better than the positive control.

Keywords: Chitosan, Chitin, Inflammatory cells, Urea-terephthalic acid

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INTRODUCTION

It is known that chitosan and its derivatives have various uses and this is due to the unique nature of chitosan, such as the fact that chitosan is biodegradable, biocompatible in a normal body; non-toxic, attachable to mammalian and microbial cells, and can be regenerative to connected gum tissue [1]. If chitosan binds big or small substituents, the hydrogen bond becomes weak, and therefore in N-alkaline water the chitosan swells although the hydrophobicity is low and is able to form film [2,3]. From chitosan it is easier to make its derivatives by reacting a certain reagent to atom C number 6 and to atom N, and as a result, a substitution reaction takes place. On chitosan a cross-link reaction can be made by using cross-linker [4-10]. The chitin and chitosan derivatives can be made into film, gel, suspension, fiber and balls and therefore they can be used on a number of veterinary areas [11,12], due to the nature of chitosan: being biodegradable, biocompatible, mucoadhesive, anti-microbe, anti-viral, non-toxic, and adjuvant. As a result, chitin and chitosan are widely used in the areas of chemistry, biotechnology, farming, poultry, dentistry, food technology, environment protection, pharmacy, and medicine [13-15]. Chitosan in the field of health can serve as an anti-thrombogenic, cholesterol decrease, antitumor, and antibacterial as well as wound healer [1,16-18].

EXPERIMENTAL

Material

Chitosan commercial and chloroacetic acid, terephthalic acid, benzaldehyde, acetic acid glacial, urea, NaOH, ethanol, isopropanol, sulfuric acid, acetone, buffer phosphate (pa), ethyl chloride, ketamine, xylaxine and eosin.

Analytical instrument

FTIR (Shimadzu 8400 S) spectra were measured by on a shimadzu Fourier Transform Infrared Spectrophotometer, vaseline-DCMChi-UTER to make homogeny by Homogenizer Ace NS OSK 7313, while histologist analysis microscope (Olympus CH 20), camera (Sony 1CX 205 AK).

Synthesis of kitosan derivatives

Stage 1: Synthesis 2,2-dichloro carboxymethyl chitosan (DCMChi)

Carboxymethyl chitosan is synthesized with the modified method of Chen-Park [19]. As much as 10 g of kitosan is diluted in 100 ml acetic acid of 2% and added to 13.5 g of NaOH, then heated in a water bath at 50ºC and stirred using a magnetic stirrer for 1 h. Solution 15 g of tri chloroacetic acid in 20 ml of isopropanol is added drop by drop. The mixture is then refluxed at 50ºC for 4 h. The reaction is stopped by adding 200 ml ethanol of 70%. The resulting product is DKMKi analyzed with FTIR.

Stage 2: Synthesis DCMChi-UTER

Carboxymetil chitosan -urea terephthalic acid is synthesized with the method of Chen-Park [19]. 2 g of urea are diluted in 60 ml aquades and 4 g of terephthalic acid is added and refluxed for 3 h at 100^{o}C. As much as 2.72 g of DCMChi are added bit by bit into 60 ml aquades and slowly stirred for 4 h at 60^{o}C. The result of this reaction is DCMChi-UTER, which is later washed with NaOH 0.1 M, aquades and acetone, and finally dried. The result is characterized by FTIR.

Determination of swelling degree

The degree of swelling is decided by Andreopoulos and Indushekar [20], weighing the dry DCMChi-UTER of 0.1 g and sinking it in 5 ml buffer phosphate at the acidity level of pH 3, 5, 7, 9 and 12 for 24 h. The water content is counted using the formula of the degree of swelling=[(Wb– Wd)/Wd] × 100%, Wb=substance weight after being sunk in the buffer; Wd=dry weight of the compound being tested.

Ethical test

As this test involves animal testing, an official letter of animal testing ethics should be acquired from an authorized institution (from the Faculty of Veterinary Studies of Airlangga University).

DCMChi-UTER toxicity test

A solution of DCMChi-UTER in acetic acid of 2% is made in order to produce the main solution with a concentration of 3,000 mg/liter. This main solution is diluted and results in concentrations of 5, 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 mg/kg BW. Each concentration is tested on 5 mice, and they are observed and the number of dead mice is recorded every 3, 6, 12, 24 and 48 h. The number of dead mice is counted and the LD_{50} is decided.

Production of medicine paste and test against open wounds

2.2-dichloro karboksimetil kitosan-Benzaldehid (DKMKi-B) and DKMKi-UTER in the form of gel is added to Vaseline additive, so paste is formed with concentrations of 10 and 20% and is used as an ointment for open wounds (wound healing). The test mice are divided into 5 groups, each of which is composed of 6 mice. Group K+ is called positive control group, Group K- is called negative control group (without medication); Group O1 is action group with medicine 1 (DCMChi-UTER 10%); and Group O2 is action group with medicine DCMChi-UTER 20%. The back of the mice is shaven, and sprayed with a local anesthetic. Then, a 1 cm long incision is made on the skin epidermis layer of the right back of the mice using a sterile scalpel. Next, the wound is treated with the medicine DCMChi-UTER. Medication is applied to the wound every day, while the condition of the wound in monitored on a daily basis and the length of the wound is measured on day 4,7 and 14. The histologist analysis is done on respective days. From each of the mice groups, 2 mice are taken and killed by injecting an overdose of ketamine and xylaxine. When the mice die, the epidermis skin tissue is taken and the histological preparation is made. The histologist analysis is used to see the scar tissue, fibro proliferative and reepithelization tissue, epidermis cells, number of inflammatory cells, keratinocytes migration, collagen, and hair follicles (day 4, 7,14). The recovery process of the wound is monitored macroscopically and histologically.

The making of histological preparate

The preparation for the histological test is made from the mice's skin epidermis layer (testing animal), using the method of paraffin. Buffer formalin is prepared, a sample (epidermis layer) is cut as needed and inserted into the buffer formalin, and the sample is sunk in the buffer formalin for 24 h. Then, the sample is inserted into the koset tissue; and the sample is washed with running water for 2 h or overnight. To dry the water content, the sample is dipped in alcohol for 30 min, starting from the lowest concentration to the highest 70% (4x), 80% (2x), and 100% (1X). After dehydration, the sample is washed with xylol 1 for 15 min, and xylol 2 for 1 night. Then, the sample is put into a mixture of xylol/paraffin (1:1) for 30 min. Later, it is put into 3 stages of liquid paraffin, each of which lasts for 1 h. A paraffin mold is made from waxed paper, then the box is filled with liquid paraffin, and the sample is put into the paraffin in a way that the position confirms the need and the paraffin is left to cool and harden overnight. The paraffin mold is opened; the paraffin block containing the sample is fastened to the surface of the holder while the sides are trimmed. The holder knife is adjusted so that the incision thickness confirms with the requirement (4-5 microns). The paraffin block is cut into ribbons and cut in preferred parts. The preferred sample is taken by using a hammer and a brush. The incision is put into a water bath (40–45-45^{o}C) so that the incision ribbons swell. The surface of the glass slide is covered with Mayer's albumin egg white chicken/glycerin (1:1). The sample incision is taken using a glass slide and dried before being put into an oven at 50^{o}C for at least 2 h. The glass slide containing the incision is put into xylol twice for 10 min.

The glass slide is then put into alcohol with grading concentrations (100%, 96%, 80%, and 70%) for 5 min each. Later, the slide is put into Hew dyes such as hematoxylin-eosin. Hematoxylin (EtOH 70%) for 10 min, washed with running tap water, before being put into ethanol-acid. It is then put into Eosin (EtOH 70%) for 5 min and washed with aquades. After that, it is put into alcohol with grading concentration (70%, 80%, 96%, and 100%) for 5 min every stage and washed (clearing) with xylol twice for 10 min. The glass cover is attached to a mounting media such as entellan.

RESULTS AND DISCUSSION

Synthesis and characterization

The synthesis of 2.2-dichloro carboxyl methyl chitosan (DCMChi) by using the DCMChi compound method [2,3], which is characterized by FTIR (Figure 1) and absorption peak is acquired at the wave number υ (cm^{-1}) 3435 (-OH); 1658 (-N-H); 1149-1035 (C-O-C); 1658 (COOH); 746 (-C-Cl) [21,22], the existence of groups of which shows that DCMChi has been formed. To make sure that this compound has been formed, the spectra are compared to the chitosan spectra in Figure 1. The reaction result of DCMChi with urea-terephthalic acid is 2.2-dikloro carboxymethyl-urea- terephthalic acid (DCMChi-UTER). This compound is characterized by the FTIR tool.. The peak of absorption is with wave number υ (cm^{-1}) 1641, 3435 (O=C-N-H); 1035 (- C-N urea); 2889 (-COOH), 1641(NCON), 1149-1035(C-O-C), 3435(-OH), 1425 with 2 peaks (showing the existence of aromatic rings) and 746 (-C-Cl) [22] The presence of those groups shows that DCMChi -UTER has been formed. To ensure that DCMChi-UTER has been formed, it needs to be compared with spectra FTIR of combined compound DCMChi, urea and terephthalic acid (Figure 2). The profiles of spectra of both pictures are different, which shows that DCMChi-UTER has successfully been synthesized.

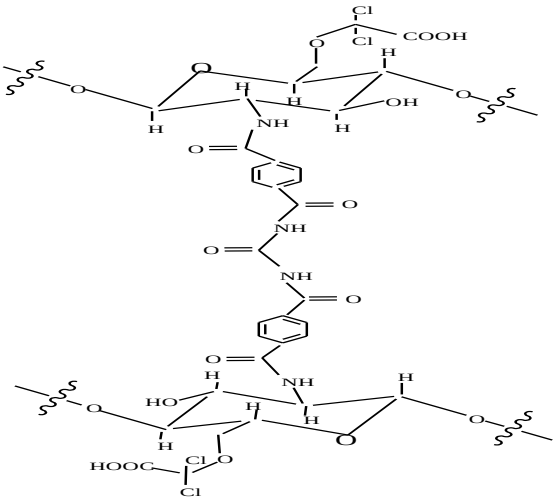

Figure 1: The structure molecule of DCMChi-UTER

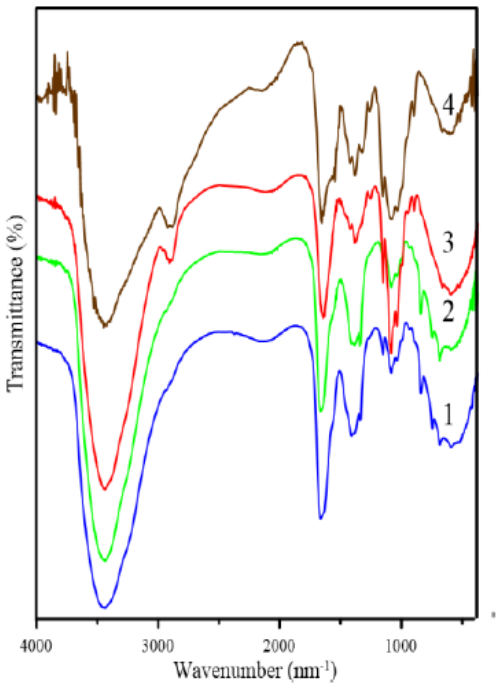

Figure 2: Spectra of mixture DCMChi-UTER+urea+terephthlalic acid (1), DCMChi (2), DCMChi-UTER (3) and Chitosan (4)

In the DCMChi –UTER toxicity test on 10 groups of mice (each group containing 5 mice) is given medicine: group I with the dosage of 5 mg/mg/kg Bw kg BB, group II 10 mg/kg BW, group III 20 mg/kg BW, group IV 40 mg/kg BW, group V 80 mg/kg BW, group VI 160 mg/kg BW, group VII 320 mg/kg BW, group VIII 640 mg/kg BW, and group × 2560 mg/kg BW. The result of toxicity test on the observation at the 3rd, 6th, 12th, 24th, and 48th h shows that not one of the mice dies. This proves that the paste/cream of DCMChi-UTER is non-toxic and safe to use as a wound healer.

Based on the test of swelling degree with the method of Andreopoulos, the results are as seen in Table 1.

Table 1: Swelling Degree of DCMChi-UTER

pH	Dry Weight (g)	Weight after being sunk for 24 h in the buffer (g)	Swelling Degree (%)
3	0.2097	0.5497	61.85
5	0.1503	0.3933	61.78
7	0.1270	0.4959	74.38
9	0.1367	0.3677	62.82
12	0.1139	0.3993	69.89

The highest swelling degree 74.38% at neutral pH.

Based on the macroscopic and histological analysis of the wound at the epidermis layer on day 4, 7 and 14, it reveals that the wound starts to recover on day 4 after the medication; the wound nearly recovers (the wound is nearly closed) on day 7 after the medication, and the wound recovers completely (the wound is totally covered) on day 14 after the medication, as seen in the Figures 3 and 4.

(a) (b) (c)
(b)

Figure 3: Macroscopic observation on the wound condition on day 4 (a), on day 7 (b), on day 14 (c)

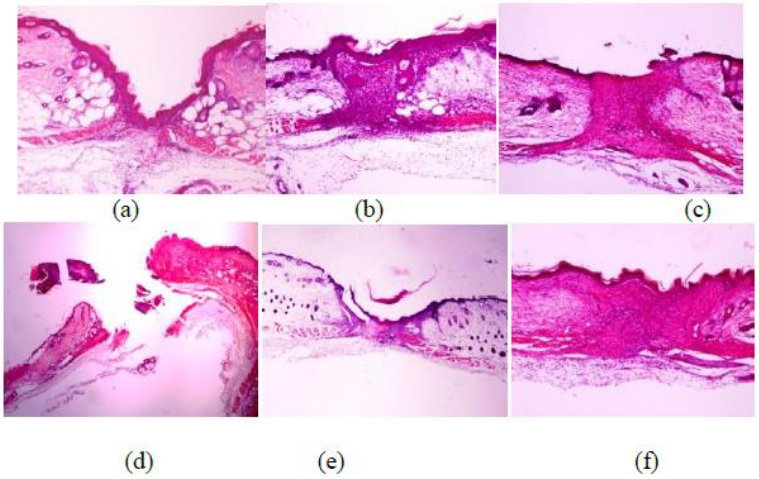

Figure 4: Microscopic observation on the wound condition on day 4 (a), on day 7 (b), on day 14 (c) with the DCMChi-UTER concentration of 10% and the condition of positive control wound on day 4 (d), on day 7 (e) and on day 14 (f)

Chitin, chitosan, and their oligomer can speed the recovery particularly in the phase of proliferation and matrix formation [23]. In this research, it is hoped that the DCMChi-UTER can undergo a depolymerization in order to produce a derivate of N-acetylglucosamine (chito-oligomer), which in turn comes into the metabolic mechanism and merges with glycoprotein. Chito-oligomer plays an important role in synthesis hyaluronic which is essential to wound recovery [24-26]. From microscopic observation with the parameter of reepithelization, damaged epidermis cells, keratin migration, number of inflammatory cells, scar tissue, fibro proliferative tissue, damaged collagen cells, growth of hair follicles and number of fibroblast, it turns out that the paste/cream medicine of Vaseline-DCMChi-UTER with 10% and 20% concentration has a better histologist parameter than the positive and negative control, as shown in the following Figures 5-9. During the process of reepithelization, the keratinocyte at the edge of the wound moves through the scar tissue in order to undergo a differentiation in order to form a new outer layer on the skin, and the wound recovery finally reaches the phase of maturity [24]. On day 7, the wound undergoes reepithelization and the scar tissue is replaced with fiber, and hair follicle starts to grow [25]. The picture shows that the length/width of the wound treated with the Vaseline-DCMChi-UTER paste is the narrowest (closed), meanwhile the positive and negative control of the wound width is bigger, which means that the Vaseline-DCMChi-UTER medicine has better healing power than the positive control. When observed from the scar tissue (granulation), the Vaseline- DCMChi-UTER medicine is better than the positive control.

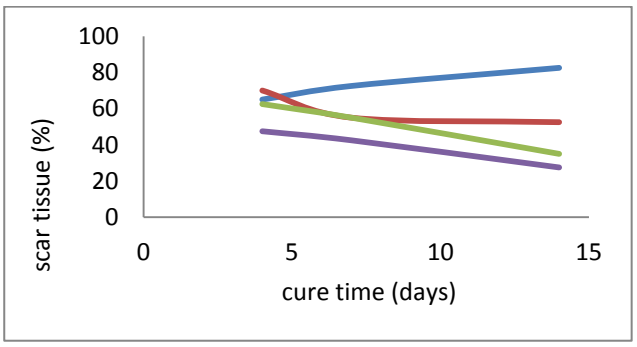

Figure 5: Scar Tissue Curve (%) vs. Cure Time (days), control negative (blue), control positive (red), medicine 1(green), medicine 2(violet)

The proliferation phase takes place on day 3-14 and is marked with the formation of scar tissue/granulation, scar tissue is a combination of cellular elements including fibroblast and inflammation cells [27]. Scar tissue is part of the wound recovery, a treatment using ointment/cream to prevent the formation of scar tissue after an operation [28]. With the medicine Vaseline-DCMChi-UTER, scar tissue formed is less than that in the positive control.

Collagen is a kind of fiber-filled protein whose function is to connect and support other body cells such as skin, bones, tendon muscles and cartilage [29]. Collagen in the form of triple-chain glycoprotein which is the main element of extracellular matrix serves to support scar tissue [27]. During the phase of maturity, the collagen fiber is initially distributed randomly and forms crisscrossing and finally mixes and forms a bunch of fibrils which slowly recovers the tissue and, in the end, rigidity and strength of tension improves [27]. Based on the histology analysis theory, on the wound in the skin bandaged with kitosan the collagen fiber becomes more delicate in the wound, and becomes mature on day 7 of control after the incision and also has a similar structure with normal skin; meanwhile, the pull strength is better than the control [24]. In this research, when seen from the broken collagen-tissue, the wound treated with Vaseline-DCMChi-UTER is better healed/has almost the same recovery as the positive control, as seen in the Figure 6.

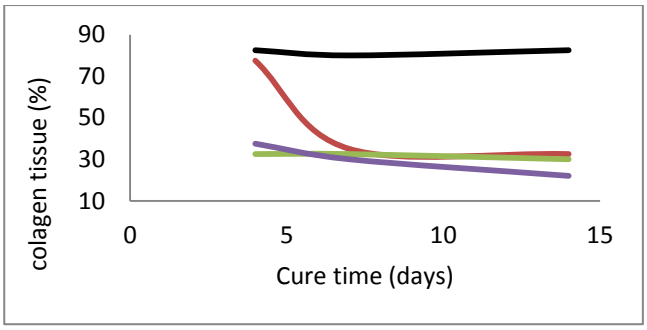

Figure 6: Curve Collagen tissue (%) vs. cure time (days) control negative (black), control positive (red), medicine 1(green), medicine 2(violet)

Inflammation is the local protection mechanism triggered by an injury or cell damage, to reduce, destroy or cover the injury agent as well as the damaged tissue [30].

When observed based on the number of wound inflammatory cells treated with Vaseline-DCMChi-UTER, the number is smaller than that in the positive control, which shows that the inflammation on the wound treated with Vaseline-DCMChi-UTER is lower than that in the positive control. This shows that the medicine is more effective than the result in the positive control.

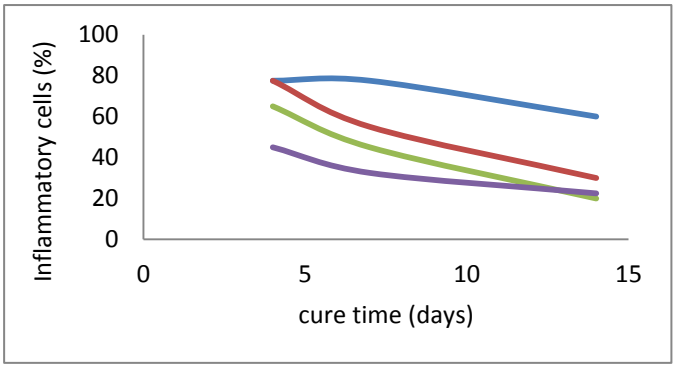

Figure 7: Curve inflammatory cells (%) vs. cure time (days) control negative (blue), control positive (red), medicine 1(green), medicine 2(violet)

As keratinocyte cells are a skin development agent, keratinocyte is a strong bound to stitch skin nerves and the tissue under the epidermis [31].

When observed based on the keratin migration on day 4, 7 and 14, the speed of keratin migration is faster in the wound treated with Vaseline-DCMChi-UTER than that in the positive control, which shows that the development of skin in the wound treated with this medicine is faster than the positive control.

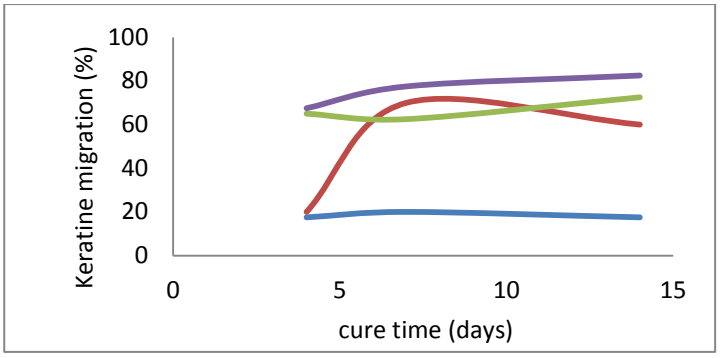

Figure 8: Curve keratin migration (%) vs. cure time (days) control negative (blue), control positive (red), medicine 1(green), medicine 2(violet)

Wound recovery has three stages: inflammation, proliferation, and maturity [24,27]. The proliferation stage takes place on day 3-14; fibroblast appears on day 3 and reaches its peak on day 7 [27]. In this research, the medicine 2 (DCMChi-UTER 20%) confirms the theory. Fibroblast is the main element in the formation of structural protein and collagen production [27]. The maturity stage takes place on day 7 to 1 year. During this stage substratum cell migration and inward cell growth, collagen and fibroblast piles up [27].
As seen in the Figure 9. Hair follicle growth, it turns out that the wound treated with medicine 1 and medicine 2 undergoes faster hair follicle growth than that in the positive control.

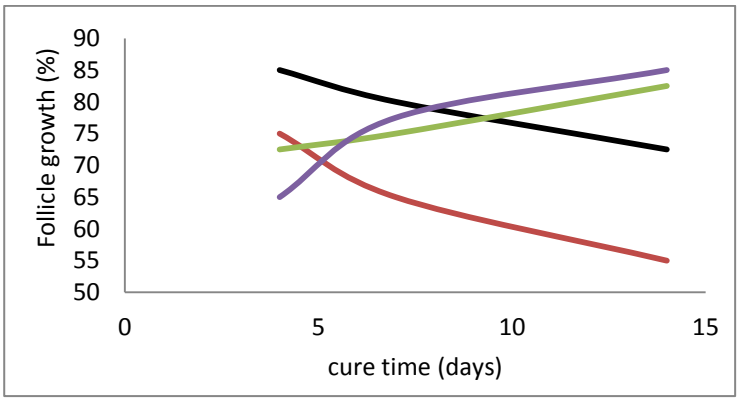

Figure 9: Curve Follicle growth (%) vs. cure time (days) control negative (black), control positive (red), medicine 1(green), medicine 2(violet)

CONCLUSION

The compound resulting from the synthesis 2.2-dichloro carboxyl methyl chitosan-urea-terephthalic acid combined with Vaseline produces cream/paste with a concentration of 10% and 20%. The macroscopic and histological analysis results show that the compound resulting from this synthesis can be used as a wound healing medicine for mice. The toxicity test results show that the compound is not toxic and is safe to use. The wound recovery starts on day 4, the wound nearly recovers (the wound is nearly covered) completely on day 7, and the wound is recovered (fully covered) on day 14 after the treatment. When observed based on inflammatory cells, reepithelization, damaged epidermal cells, keratin migration, scar tissue, fibro proliferative tissue, damaged collagen cells, hair follicle growth and fibroblast cell number, this compound heals faster than both positive and negative controls.

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