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WOUND HEALING *IN VIVO* AND *IN VITRO* STUDY OF BINAHONG LEAVES (*ANREDERA CORDIFOLIA* (TEN.) STEENIS) AND PEGAGAN (*CENTELLA ASIATICA* (L.) URBAN) ETHANOLIC EXTRACT

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Abstract

Anredera cordifolia and *Centella asiatica* are the traditional medicinal plant used to treat several diseases in Southeast Asia countries. The objectives of this research were to evaluate the activities of these two plants extracts-based ointment and its combination as a wound healing in diabetic-induced rabbits. This study was performed by *in vivo* method as wound healing in diabetic-induced rabbits. Morton-Malone's method was used to produce a wound in rabbit. The observation parameters was included decreasing in erythema scores, swelling score and wound diameter. The *in vitro* method was carried out using cell proliferation method. The *in vivo* study showed that binahong, pegagan extracts -based ointment and its combination had ability to decrease of erythema scores, swelling vast, and wound vast in diabetic-induced rabbits comparable to standard drug. *In vitro* study revealed that *Anredera cordifolia* and *Centella asiatica* increased the proliferation of fibroblast cell comparable to standard drug. *Anredera cordifolia*, *Centella asiatica* extract-based ointment and its combination had the ability to promote wound healing in diabetic-induced rabbits. The mechanism was by increasing the proliferation of fibroblast cell.

Keywords: *Anredera cordifolia, Centella asiatica, wound healing, diabetes mellitus, proliferation cell*

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Introduction

Diabetes mellitusis one of a serious problems in the world and has contribution against various complications. The number of the people in the world with diabetes mellitus has increased dramatically every year. Based on the reports, the number of diabetes mellitus patients in the worldwide would increases to 363 million in 2030 and mortality related to diabetes mellitus around 9%, especially type II diabetes mellitus [1]. Furthermore, the prevalence of diabetes in Indonesia (productive age urban Indonesian) was 4.6%. Patient with diabetes mellitus in Indonesia until 2014 around 9 million cases and the number of death in adults due to diabetes mellitus around 175.935.

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia as a result of diminished insulin secretion and insulin resistance [2]. Hyperglycemia condition has important role in pathogenesis of complications related to diabetes mellitus, such as retinopathy, neuropathy, nephropathy, cataract, and impaired wound healing [3].

Wound is defined as a physical damage which resulted in an opening of the skin area. Wound in diabetic condition have slow progress and impaired. It can persist for weeks, although receiving sufficient care [4]. Wound healing is a complex mechanism as a result from recovery of anatomy continuity and its function through several processes that basically classify into three phases, that are inflammatory phase, proliferative phase, and remodeling phase [5].

Anredera cordifolia and Centella asiatica are commonly used as a traditional medicine in Indonesia. Anredera cordifolia, belongs to Baselaceae family [6], known as binahong in Indonesia, has been evaluated for its analgesic activity [7], antihyperlipidemic activity [8], and in improving kidney failure [9]. Formulation for Anredera cordifolia as wound healing gel has been evaluated [10]. Whereas Centella asiatica, belongs to Apiaceae family, known as pegagan in Indonesia, traditionally used to heal wound and improve appetite [11]; it's also used for its activity as diuretics, hemostatic, and antiinfection [12]. This plant has been reported having several activity, including antioxidant, antimicrobial, cytotoxic, neuroprotective, anti inflammatory, anti diabetic, anti ulcer, and wound healing. The bioactive constituents of these plants are the triterpenoic acid (asiatic acid, madecassic acid), triterpenes saponin (madecassoside and asiaticosside), flavonoids (quercetin, kaempherol, catechin, rutin, apigenin and naringin), and other phenolic compounds [13,14].

Based on the traditional use and another studies, combination of these two plants was thought to be efective as an anti-inflammatory agent. Moreover, combination of these two plant extracts as a wound healing has never been investigated before. The aims of this study were to evaluate the wound healing activities of binahong and pegagan extracts-based ointment and its combination as a wound healing in diabetic-induced rabbits.

Materials and methods

Preparation plant materials

Centella asiatica and Anredera cordifolia plants were obtained from Lembang-Bandung, West Java, Indonesia. Fresh plants were dried at 50-60°C and then grinded into small pieces. Identity authentication of plant was performed by Herbarium Bandungense, School of Life Science and Technology, Bandung Institute of Technology.

Animals

Male and female rabbit weighing 3-5.5 kg were used as an experimental animal. The animals were kept at standard laboratory conditions at 24-26 °C, humidity 70-75%, and 12 h light/dark cycle. Animals were fed with pet food standard and water ad libitum. The methods in this study were conducted in accordance with ethics and guide for animals care and used.

Preparation of ethanolic extract of Centella asiatica (CAE)

200 g dried herbs of Centella asiatica was macerated using 2 L ethanol [15]. The mixture was filtered using a filter paper and the filtrate was concentrated using rotary evaporator at 60°C.

Preparation of ethanolic extract of Anredera cordifolia (ACE)

200 g dried leaves of Anredera cordifolia was macerated using 2 L ethanol [10]. The mixture was filtered using a filter paper and the filtrate was concentrated using rotary evaporator at 60°C.

In vivo wound healing activity of Centella asiatica extract and Anredera cordifolia extract

The animals were devided into two groups, first group were male rabbits and the second were female rabbits, each group consisted of 3 rabbits. Diabetic condition (blood glucose level > 150 mg/dl) in rabbit was obtained by alloxan administration at dose of 250 mg/kg bw. The alloxan administration was started from 150 mg/kg bw [16], but did not give hyperglycemic condition. Therefore the dose was increased and hyperglycemic condition was obtained at dose of 250 mg/kg bw. After hyperglycemic condition was developed, the wound healing assay was carried out using Morton & Malone's method. The diabetic rabbit was shaved and then anesthetized. The wound was made by cutting out the rabbit's skin in circle with diameter 1 cm and 4 wounds were made on each rabbit [17]. The diabetic-wounded animal was treated 3 times a day for 14 days, using 2% CAE ointment or 2% ACE ointment or 2% combination of CAE-ACE ointment, and Sanoskin® ointment as a standard drug. The erythema score, swelling score, and wound diameter were measured as parameter during the experiment.

In vitro wound healing activity of Centella asiatica extract and Anredera cordifolia extract

The 3T3 fibroblasts were seeded into 96-well plate with a density 5000 cell/ well, then after 24 hours incubation, added by 2% CAE ointment or 2% ACE ointment or 2% combination of CAE – ACE ointment, and Sanoskin® ointment as a standard drug and incubated again for 24 hours. The last step was added by MTS {[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium} (1:5) to each well, and then incubated for 3 hours before absorbance measurement was measured at λ 490 nm [18].

Results and Discussion

In vivo wound healing activity of Centella asiatica extract and Anredera cordifolia extract

After administered with alloxan at dose of 250 mg/kg bw, the blood glucose level of rabbit was measured during the experiment (Table 1).
The wound healing study was performed in hyperglycemic rabbits, and the erythema score, swelling score, and wound diameter were measured as parameter during the experiment (Table 2, Table 3, and Table 4).
Based on wound severity degree demonstrated that the extract treated wound had comparable result to the standard drug. There was no significant difference among all treated groups (2% ACE ointment, 2% CAE ointment, and 2% combination of CAE-ACE ointment). This result indicated that both Centella asiatica and Anredera cordifolia extract had the ability to improve wound in hyperglycemic rabbit.

In vitro wound healing activity of Centella asiatica extract and Anredera cordifolia extract

From the in vitro study, the fibroblast cell was evaluated as the parameter (Table 5).
Data in the Table 5 showed that amount of fibroblast cell which was treated by 2% C. asiatica and 2% A. cordifolia were significantly different compared to treated using combination of C. asiatica and A. cordifolia ointment, 1% DMSO and the standard drug. The 2% combination of CAE-ACE ointment had the best activity in increasing the proliferation of fibroblast cell compared to C. asiatica and A. cordifolia as a single active ingredient, and it showed higher amount of the fibroblast cell compared to standard drug. Fibroblast is a major skin type that responsible in secreting a series of growth factor such as VEGF, interleukin, and TGF-β which is important for wound healing process and can be found in the granulation of injured tissue [19,20]. By increasing in proliferation in fibroblast, the wound healing process can be promoted.
Centella asiatica has been used as a traditional medicine for wound [11]. The traditional application has been proved by several scientific studies [21, 22]. The mechanism of Centella asiatica in healing wound was by inhibiting inflammation, increasing the collagen production, promoting angiogenesis, inducing vasodilatation, and acting as an antioxidant in the wound lesion. C. asiatica also affected the cellular growth and proliferation process in injured tissues [21]. The major and the most important constituent in C. asiatica was triterpenoid group, mainly pentacyclic triterpenic and their glycoside, included asiaticoside, asiatic acid, madecassoside, madecasic acid, brahmoside, brahmic acid, brahminoside, thankuniside, isothankuniside, centelloside, madasiatic acid, centic acid, cenellic acid, betulinic acid, indocentic acid [23]. The asiatic acid had ability to remodel and modulate inflammation [24] and increase collagen synthesis [25,26]. Asiaticoside was act by promoting type I collagen synthesis in

human dermal fibroblast [27] and increasing the antioxidant level [28]. Madecassoside was proved could promote burn wound healing in mice [29].

Anredera cordifolia or binahong has been commonly used as a traditional medicine for healing wound in Indonesia. The leaves was crushed and then put on the wound. Several scientific studies has been performed and similar result was showed in the present study. Anredera cordifolia had the activity for healing wound [30,31]. Moreover, Anredera cordifolia contain oleanolic acid which has antiinflammatory activity that can reduce pain in burns [32].

From this study, it was showed that as a single active ingredient C. asiatica and A. cordifolia had the ability to promote wound healing in diabetic condition. When the two plants were combined, the wound healing effect increased, although statistically was not significant, and the effect of the extract-based ointment were similar to standard drug.

Conclusion

Anredera cordifolia, Centella asiatica extract-based ointment and its combination had the ability to promote wound healing in diabetic-induced rabbits. The mechanism was by increasing the proliferation of fibroblast cell.

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Table 1. The blood glucose level after alloxan administration

No	Group	Body Weight (Kg) at Day-		Blood Glucose Level (mg/dl) at Day-		
		1	16	1	4	16
1	Rabbit 1	2.7	2.7	79	156	162
2	Rabbit 2	2.5	2.5	67	150	158
3	Rabbit 3	2.5	2.6	70	150	154
4	Rabbit 4	2.6	2.6	77	159	170

Table 2. Erythema score after extract administration

Group	Mean±SD
Sanoskin® ointment	0.81±1.06
2% ACE ointment	0.83±1.06
2% CAE ointment	0.81±1.04
2% combination of CAE – ACE ointment	0.78±1.07

n=36; F table=0.017; Sig=0.997

Table 3. Swollen score after extract administration

Group	Mean±SD (mm^2)
Sanoskin® ointment	42.53±69.61
2% ACE ointment	49.97±74.26
2% CAE ointment	50.61±76.03
2% combination of CAE – ACE ointment	46.78±68.02

n=36; F table=0.095; Sig=0.963

Table 4. Wound diameter after extract administration

Group	Mean±SD (mm^2)
Sanoskin® ointment	96.33±83.13
2% ACE ointment	99.61±84.99
2% CAE ointment	100.36±84.80
2% combination of CAE – ACE ointment	96.83±83.22

n=36; F table=0.02; Sig=0.996

Table 5. The amount of fibroblast cell

Group	Mean±SD (mm^2)
1% DMSO	6285.00±11.00
Sanoskin® ointment	6021.67±19.70
2% ACE ointment	4045.00±13.90
2% CAE ointment	4402.67±21.90
2% combination of CAE – ACE ointment	7806.33±27.36

n=3; F table=6.062; Sig=0.1