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ORIGINAL ARTICLE

Wound Healing Effectivity of the Ethanolic Extracts of *Ageratum conyzoides* L. Leaf (White and Purple Flower Type) and *Centella asiatica* and Astaxanthin Combination Gel Preparation in Animal Model

Ageratum conyzoides L. Yaprağı (Beyaz ve Mor Çiçekli Tür) ve *Centella asiatica* Etanol ile Hazırlanmış Ekstreleri Astaksantin Kombinasyonunu İçeren Hazırlanmış Jelin Hayvan Modelinde Yara İyileştirici Etkisi

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ABSTRACT

Objectives: The study's objective was to determine the wound healing activity of the combination of ethanolic extracts of *Ageratum conyzoides* L. leaf (white and purple), *Centella asiatica,* and astaxanthin gel preparation.

Materials and Methods: For in-gel preparation, three different formulas of gelling agents, namely carbopol 934 (1%), hydroxypropyl methylcellulose (HPMC) (9%), and natirum-carboxymethylcellulose (Na-CMC) (4%), were employed. Then, the organoleptic, pH, spreadability, and viscosity of the formulas were evaluated. To determine wound healing activity, six treatments, including negative control (placebo), positive control (bioplacenton), BP5 (*A. conyzoides* L. leaf ethanolic extract of white flower type 5%, *C. asiatica* L. Urb leaf ethanolic extract 2.5%, astaxanthin 0.05%), BU5 (*A. conyzoides* L. leaf ethanolic extract of purple flower type 5%, *C. asiatica* L. Urb leaf ethanolic extract 2.5%, astaxanthin 0.05%), BU10 (*A. conyzoides* L. leaf ethanolic extract of purple flower type 10%, *C. asiatica* L. Urb leaf ethanolic extract 5%, and astaxanthin 0.1%), and BP10 (*A. conyzoides* L. leaf ethanolic extract of white flower type 10%, *C. asiatica* L. Urb leaf ethanolic extract 5%, and astaxanthin 0.1%) were evaluated. All treatments were applied to an incision wound (1.5 cm). Measurement of the wound length was conducted daily for 14 days.

Results: The results showed that the carbopol 934 (1%) gelling agent formula was better than HPMC and Na-CMC. Meanwhile, the percentages of wound healing activity for negative, positive, BP5, BU5, BU10, and BP10 groups were 72.51%, 69.36%, 70.14%, 81.70%, 86.54%, and 80.21%, respectively. The BU5 and BU10 showed significant activity ($p<0.05$) compared with positive and negative controls.

Conclusion: BU10 provided the best wound healing activity and can be developed as a commercial product.

Key words: *Ageratum conyzoides* L., astaxanthin, *Centella asiatica,* gel preparation, wound healing

ÖZ

Amaç: Çalışmanın amacı, *Ageratum conyzoides* L. yaprağı (beyaz ve mor), *Centella asiatica* ve astaksantin jel preparatının etanol ekstrelerinin kombinasyonunun yara iyileştirici aktivitesini belirlemekti.

Gereç ve Yöntemler: Jel hazırlamada, 3 farklı jelleştirme ajanı formülümüz vardı: Karbopol 934 (%1), hidroksipropil metilselüloz (HPMC) (%9) ve sodyum-karboksimetil selüloz (Na-CMC) (%4). Daha sonra bu formülleri organoleptik özellikleri, pH'leri, yayılabilirlikleri ve viskoziteleri dahil olmak üzere farklı parametreler ile değerlendirdik. Yara iyileştirme aktivitesini belirlemek için oluşturulan altı grup: Negatif kontrol (plasebo), pozitif kontrol (bioplasenton), BP5 (beyaz çiçekli *A. conyzoides* L. yaprağının etanol ekstresi %5, *C. asiatica* L. Urb yaprağı etanol ekstresi %2,5 ve astaksantin %0,05), BU5 (mor çiçekli *A. conyzoides* L. yaprağının etanol ekstresi %5, *C. asiatica* L. Urb yaprağı etanol ekstresi %2,5 ve astaksantin %0,05), BU10

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(mor çiçekli *A. conyzoides* L. yaprağının etanol ekstresi %10, *C. asiatica* L. Urb yaprağı etanol ekstresi %5 ve astaksantin %0,1), BP10 (beyaz çiçekli *A. conyzoides* L. yaprağının etanol ekstresi %10, *C. asiatica* L. Urb yaprağı etanol ekstresi %5 ve astaksantin %0,1) şeklindeydi. Tüm gruplar 1,5 cm uzunluğunda kesi ile tedavi edildi. Yara uzunluğunun ölçümü 14 gün boyunca günlük olarak gerçekleştirildi.

Bulgular: Karbopol 934 (%1) jelleştirici madde formülü, değerlendirme testine göre HPMC ve Na-CMC'den daha iyiydi. Negatif, pozitif, BP5, BU 5, BU10 ve BP10 grupları için yara iyileştirme aktivitesi yüzdesi sırasıyla; %72,51, %69,36, %70,14, %81,70, %86,54 ve %80,21 olarak bulundu. BU5 ve BU10, pozitif ve negatif gruplara kıyasla anlamlı aktivite gösterdi ($p<0,05$).

Sonuç: BU10, en iyi yara iyileştirme aktivitesi gösteren formül olarak ticari olarak geliştirilme potansiyeli olan formülasyon olarak belirlendi.

Anahtar kelimeler: *Ageratum conyzoides* L, astaksantin, *Centella asiatica*, jel hazırlama, yara iyileşmesi

INTRODUCTION

A wound is defined as a physical, chemical, or thermal injury or insult that results in an opening or breaking in the integrity of the skin or disruption of anatomical and functional integrity of living tissues.[1] Global wound prevalence has reached ~8.2 million people, and medical care costs range from $28.1 to $96.8 billion.[2] Many wound healing products are available in the market. To date, there is no standard topical treatment for wound healing. Bioplacenton is a topical preparation that is available in the market. This product is commonly used for wound healing treatment by Indonesians.[3] The ingredients of bioplacenton include neomycin sulfate 0.5% and placenta extract 10%.[4] Placenta extract accelerates the healing of the wound size, followed by reduction of transforming growth factor and elevation of vascular endothelial growth factor and $CD31^+$.[5]

Ageratum conyzoides, *Centella asiatica*, and astaxanthin have been shown to have wound healing activity.[6,7] Ethanolic extract of *A. conyzoides* exhibits a 40% increase in tissue tensile strength and a 33% decrease in re-epithelialization time, high collagen, and cellular infiltration.[8] Different extractions of *C. asiatica* (hexane, ethyl acetate, methanol, and water extract) show tensile strength and develop epithelization and keratinization of the wounds.[9] Asiaticoside and madecassoside from *C. asiatica* play an essential role in this wound healing activity.[10] Astaxanthin is a powerful antioxidant, which is isolated from a lobster.[11] Besides that, astaxanthin provides wound healing activity by reducing iNOS and increasing Col1A1 and bFGF.[12] Col1A1 provides instructions for making collagen, which supports many tissues, including the skin. Meanwhile, bFGF regulates many biological functions, including tissue repair.[13,14] However, the wound healing activity of these combinations is still unknown. Therefore, this study aimed to evaluate the wound healing activity of *A. conyzoides, C. asiatica,* and astaxanthin combination gel preparation.

MATERIALS AND METHODS

Ethical clearance

All the procedures were performed according to the Guide for the Care and Use of Laboratory Animals and approved by Bakti Tunas Husada Health Sciences College Ethical Committee (no: 03/kepk-bth/04/20).

Plant materials and extract preparation

A. conyzoides and *C. asiatica* leaves were collected from the Galunggung Mountain area, Tasikmalaya, West Java. The plants were authenticated by the School of Life Science and Technology, Institut Teknologi Bandung. Astaxanthin was obtained from Sigma Aldrich. The leaves were shade-dried and coarsely powdered by a grinder and stored in an airtight container at room temperature. The dried leaves of *A. conyzoides* L. (1000 g, purple flower type and 1000 g white flower type) and *C. asiatica* (2000 g) were used for maceration by ethanol 96% for 24 h, and this process was repeated thrice. The extract was filtered and concentrated using a rotary evaporator at 60°C. The percentage yield was calculated, and the extract was preserved in a refrigerator at 4°C until further use.

Standardization of simplicia

Simplicia was standardized using organoleptic, microscopic, and secondary metabolite analysis. The secondary metabolites, including alkaloid, flavonoid, polyphenol, quinone, tannin, monoterpenes-sesquiterpenes, triterpenoid, and steroid, were determined according to Fransworth's methods.[15]

Preformulation of gel preparation

The objective of the gel preformulation was to determine the best gel formula from three bases, including carbopol 934 1%, hydroxypropyl methylcellulose (HPMC) 9%, and natirum-carboxymethylcellulose (Na-CMC) 4%. The preformulation was checked for organoleptic, pH, homogeneity, viscosity, and spreadability.

Wound healing activity test

The treatments were

(i) Negative control (placebo),

(ii) Positive control (Bioplacenton),

(iii) BP5 (*A. conyzoides* L. of white flower 5%, *C. asiatica* L. Urb 2.5%, astaxanthin 0.05%),

(iv) BU5 (*A. conyzoides* L. of purple flower 5%, *C. asiatica* L. Urb 2.5%, astaxanthin 0.05%),

(v) BU10 (*A. conyzoides* L. of purple flower 10%, *C. asiatica* L. Urb 5%, and astaxanthin 0.1%), and

(vi) BP10 (*A. conyzoides* L. of white flower 10%, *C. asiatica* L. Urb 5%, and astaxanthin 0.1%).

All treatments were applied to an incision wound of 1.5 cm. The wound healing capacity was determined by daily measurement of the wound length using calipers for 14 days.

Statistical analysis

The obtained data were analyzed using analysis of variance, followed by posthoc test of least significant difference. The data were considered significant if the p value was ⟨0.05. All statistical analyses were performed using SPSS 16.00.

RESULTS AND DISCUSSION

Standardization of simplicia

The standard was evaluated based on organoleptic, microscopic, and non-specific parameters as well as phytochemical screening. The results of the organoleptic and microscopic parameters (Table 1), and non-specific parameters such as water content, ash content, dry shrinkage, and yields (Table 2) fulfilled the Indonesia Materia Medica Standard and Indonesia Herbal Pharmacopeia criteria.[16,17] Therefore, these simplicia were qualified for further wound healing test activity. Phytochemical screening study was positive for flavonoid, alkaloid, saponin, polyphenol, tannin, quinone, steroid-triterpenoid, and monoterpene-sesquiterpene, but negative for tannin (Table 3).

Evaluation of the gel preparation

In the organoleptic evaluation, carbopol 934 gel preparation gave the best texture and color compared with Na-CMC and HPMC (Table 4, Figure 1). Thus, carbopol 934 bases in three concentrations (0.5%, 1%, and 1.5%) were used for further gel preparation formula evaluation (Table 5). The parameters, including stability, organoleptic, pH, viscosity, and spreadability for three cycles at two temperatures, 2°C and 40°C (Table 6).

Table 1. Organoleptic and microscopic data

Simplicia	Organoleptic	Microscopic
Centella asiatica L. Urb	Form: Powder Color: Green Odor: Aromatic typical Taste: Bitter	Stomata, hair cover, oxalic acid, sklerenkim, epidermis, wooden vessel
Ageratum conyzoides L. leaf (white flower type)	Form: Powder Color: Pale green Odor: Aromatic typical Taste: Bitter	Stomata, hair cover, secretion cells and essential oil, stomata
Ageratum conyzoides L. leaf (purple flower type)	Form: Powder Color: Green Odor: Aromatic typical Taste: Bitter	Stomata, hair cover, secretion cells, epidermis, wooden vessels

Table 2. Non-specific parameters

No.	Parameters	Results (%)	Standard (%)
1	Water content a. *Ageratum conyzoides* L. leaf (white flower type) b. *Ageratum conyzoides* L. leaf (purple flower type) c. *Centella asiatica* L. Urb	 5.33 5.33 6.67	 ⟨10
2	Ash content a. *Ageratum conyzoides* L. leaf (white flower type) b. *Ageratum conyzoides* L. leaf (purple flower type) c. *Centella asiatica* L. Urb	 11.84 10.57 10.60	 ⟨13 ⟨13 ⟨18.05
3	Dry shrinkage a. *Ageratum conyzoides* L. leaf (white flower type) b. *Ageratum conyzoides* L. leaf (purple flower type) c. *Centella asiatica* L. Urb	 8.7 9.91 9.68	 ⟨10 ⟨10 ⟨11
4	Yields a. *Ageratum conyzoides* L. leaf (white flower type) b. *Ageratum conyzoides* L. leaf (purple flower type) c. *Centella asiatica* L. Urb	 20.16 13.87 11.74	 ⟩7.2

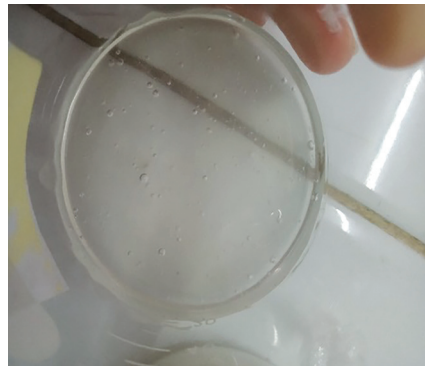
Table 3. Phytochemical screening

Secondary metabolite	*Ageratum conyzoides* L. leaf		*Centella asiatica* L. Urb	
	Simplicia	Extract	Simplicia	Extract
Flavonoid	+	+	+	+
Alkaloid	+	+	+	+
Saponin	+	+	+	+
Polyphenol	+	+	+	+
Tannin	-	-	-	-
Quinone	+	+	+	+
Steroid/triterpenoid	+	+	+	+
Monoterpene/sesquiterpene	+	+	+	+

+: Positive results, -: Negative results

HPMC
Texture: Bubbly and liquid
Colour: Yellowish

Na CMC
Texture: Liquid
Colour: Clear



Carbopol 934
Texture: Viscous
Colour: Clear

Figure 1. Organoleptic evaluation of three basis gel preformulation
HPMC: hydroxypropyl methylcellulose, Na-CMC: Natirum-carboxymethylcellulose

Table 4. Preformulation of gel preparation

Substances	F1 (%)	F2 (%)	F3 (%)
Carbopol 934	-	-	1
Hydroxypropyl methylcellulose	9	-	-
Na-carboxymethylcellulose	-	4	-
Propylenglycol	15	2.5	2
Triethinolamine	-	-	qs
Propyl paraben	0.15	0.2	-
Methyl paraben	0.18	0.18	-
Tween 80	-	2	-
Aquades	Ad 20 g	Ad 20 g	Ad 20 g

Table 5. Formulation of gel preparation

Substances	F3a (%)	F3b (%)	F3c (%)
Carbopol 934	0.5	1	1.5
Propylenglycol	2	2	2
Triethanolamine	qs	qs	qs
DMDM hyndantoin	0.5	0.5	0.5
Aquades	Ad 15 g	Ad 15 g	Ad 15 g

Table 6. Evaluation of gel preparation

Parameter	Hasil			Standard
	F3a (%)	F3b (%)	F3c (%)	
Organoleptic	Yellowish	Clear	Clear	Clear
Homogenity	Homogen	Homogen	Homogen	Homogen
Consistency	Viscous	Viscous	Viscous	Viscous
pH	7	6.25	6	4-6.5
Spreadability	7.2 cm	5.8 cm	4.8 cm	4-6 cm

The results of the evaluation showed that carbopol 1% gave the best formulas and fulfilled the criteria.[18-20] Hence, carbopol 1% (F3b) was combined with *A. conyzoides* L. leaf ethanolic extract (white and purple flower type), *C. asiatica* leaf ethanolic extract, and astaxanthin.

The determination of wound healing activity

Wound healing is comprised of three phases: Inflammation, proliferation, and remodeling. The first phase involves polymorphonuclear and macrophage inflammation, which last 3-5 days. The second phase is marked with a new tissue formation, fibroblast, endotel, and collagen formation. The third phase is the maturation phase that provides tensile strength, epithelium, and new tissue growth.[21-23]

The BU10 treatment showed the best wound healing activity compared with other groups ($p<0.05$) (negative and positive controls, BP5, and BP10), but not superior ($p>0.05$) than BU5 (Figure 2, 3). The wound healing percentage of BU10 was 86.54%, with complete remission time on the 8th day (Figure 2). Meanwhile, the positive control (bioplacenton) showed no difference from the negative control ($p>0.05$). Currently, we could not confirm this phenomenon.

The wound healing activity of BU10 may be due to the secondary metabolite composition in *A. conyzoides* L. leaf (purple flower type), *C. asiatica*, and the antioxidant activity of astaxanthin.[13-14,24-29] The flavonoids in *A. conyzoides* L. leaf, such as kaempferol and quercetin, showed anti-inflammation, antioxidant, and immunomodulatory activity.[24,25] Alkaloid and saponin composition of *A. conyzoides* L. leaf also has a role in wound healing activity through fibroblast initiation, anti-inflammation, cell repairing, and strength of the skin cells.[26,27]

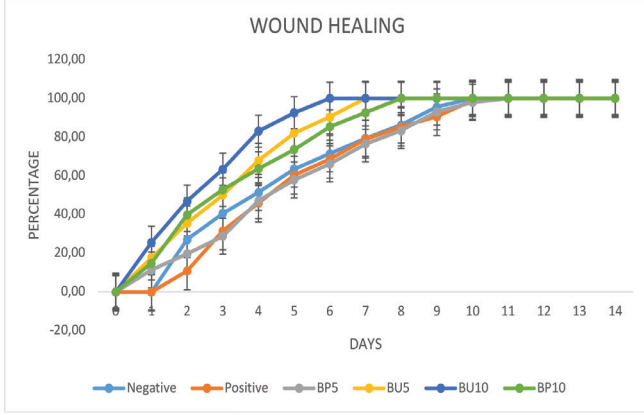


Figure 2. Wound healing activity test

Besides, *C. asiatica* secondary metabolites such as asiaticoside and madecassoside play an important role in wound healing activity although madecassoside is more effective than asiaticoside.[10,28] Asiaticoside stimulates collagen, epidermis formation, antioxidant activity, and anti-inflammation activity, resulting in the inhibition of scar formation.[28,29]

CONCLUSION

The combination of *A. conyzoides* L. leaf ethanolic extract (purple flower type) 10%, *C. asiatica* L. Urb leaf ethanolic extract 5%, and astaxanthin 0.1% showed the best wound healing activity and can be developed as a commercial product. Future studies are required to determine the relationships between antioxidants and wound healing activities.

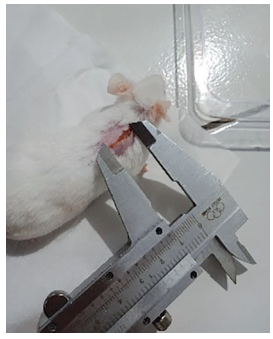
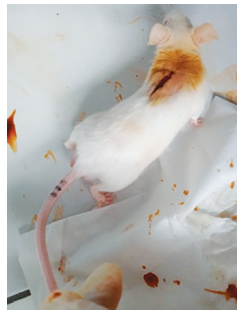
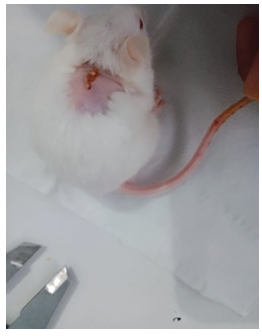
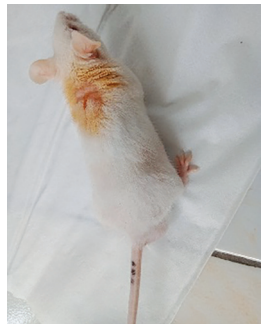
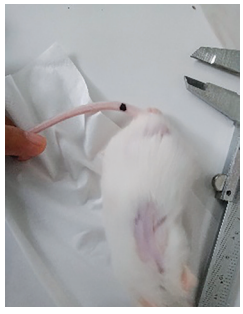
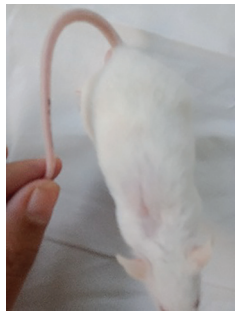
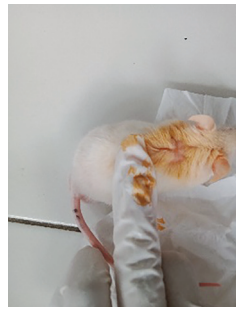
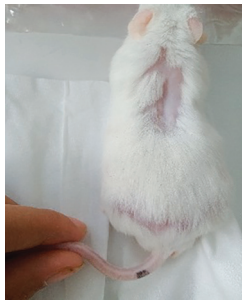
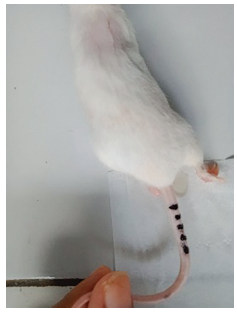
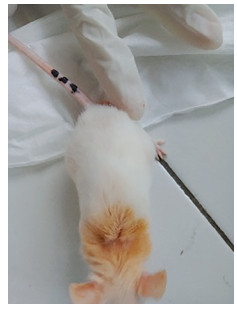
Time (days)	Negative	Groups positive	BU10
1			
6			
10			
14			

Figure 3. Wound healing comparison between negative control vs. positive control vs. BU10

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