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 sodium-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

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

**Gereç ve Yöntemler:** Jel hazırlanması, 3 farklı jelleştirme ajani formüllümüz 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 değerlendirildik. Yara iyileştirme aktivitesini belirlemek için oluşturulan alt grub: Negatif kontrol (placebo), pozitif kontrol (bioplacenton), BP5 (beyaz çiçekli *A. conyzoides* L. yaprağının etanol ekstresi %5, *C. asiatica* L. Urb yaprağı etanol ekstresi %2,5, 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 (beyaz çiçekli *A. conyzoides* L. yaprağının etanol ekstresi %5, *C. asiatica* L. Urb yaprağı etanol ekstresi %2,5 ve astaksantin %0,05).
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 CD31.6

Ageratum conyzoides, Centella asiatica, and astaxanthin have been shown to have wound healing activity.3,6,7 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 ColIα1 and bFGF.12 ColIα1 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/kep-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 natrium-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. Therefore, these simplicia were qualified for further wound healing test activity. Phytochemical screening study was positive for flavonoid, alakoid, saponin, polyphenol, tannin, quinone, steroid-triterpenoid, and monoterpen-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).

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**Table 1. Organoleptic and microscopic data**

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

**Table 2. Non-specific parameters**

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

+: Positive results, -: Negative results

Figure 1. Organoleptic evaluation of three basis gel preformulation
HPMC: hydroxypropyl methylcellulose, Na-CMC: Na-triuron-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      |
| Triethanolamine             | -      | -      | 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|
The results of the evaluation showed that carbopol 1% gave the best formulas and fulfilled the criteria. Hence, carbopol 1% (F3b) was combined with *A. conyzoides* L. leaf ethanolic extract (white and purple flower type), *C. asiatica* L. leaf ethanolic extract, and astaxanthin.

**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 lasts 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.

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. The flavonoids in *A. conyzoides* L. leaf, such as kaempferol and quercetin, showed anti-inflammation, antioxidant, and immunomodulatory activity. 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.

**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.
## ACKNOWLEDGMENTS

The authors thank Yusuf Firmansyah for helping in animal care.

Conflicted of interest: No conflict of interest was declared by the authors. The authors are solely responsible for the content and writing of this paper.

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