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Ethanol Extract of *Chromolaena odorata* Leaves in Ointment Form: Evaluating Its Efficacy in Healing Excisional Wounds in Male White Rats

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ABSTRACT

The leaves of *Chromolaena odorata* (*C. odorata*), which have been traditionally employed by the coastal communities of Aceh for the treatment of small wounds, have significant concentrations of flavonoids and phenolics. The objective of this study was to evaluate the efficacy of an ointment made from ethanol extract of *C. odorata* leaves in boosting wound healing and increasing the growth and expression of fibroblasts and Platelet Derived Growth Factor (PDGF-BB) in excision wounds of male white rats. On the 15th day, there was a decrease in the size of the wound as follows: The closure rates for Betadine® were measured at 96.07%, whereas the control group had a cure rate of 38.12%. The ointments containing *C. odorata* extract at concentrations of 4%, 6%, and 8% showed closure rates of 80.93%, 95.38%, and 95.75% respectively. The statistical analysis showed that there was no significant distinction between the 6% and 8% concentrations of the extract and Betadine®. On the seventh day, the number of fibroblast cells per field was 70.60 for Betadine®, 69.20 for the 6% extract, and 48.00 for the control. There were no significant differences between Betadine® and the 6% extract. On day 14, the expression of PDGF-BB was observed. The number of cells per field was 125.20 for Betadine®, 106.40 for the control, and 123.80 for the 6% extract. The results of the extract were similar to those of Betadine®. The results of this study demonstrate that the ointment made from the ethanol extract of *C. odorata* leaves effectively improves the healing of wounds, stimulates the activity of fibroblasts, and increases the production of PDGF-BB. These effects are comparable to the effectiveness of Betadine® in treating excisional wounds.

Keywords: Chromolaena odorata, excision wounds, fibroblasts, PDGF BB expression

INTRODUCTION

A wound is a condition characterized by the loss or damage of human tissue, typically resulting from the opening or destruction of the skin. This leads to a disruption of normal skin function and anatomy [1]. The process of mending such wounds is a complex and detailed one, which involves the regeneration of tissue layers and cells that have lost their ability to function. This process begins with an intricate series of biochemical processes that start to mend the damage. These activities include angiogenesis (the creation of new blood vessels), collagen deposition (the primary structural protein in skin and other connective tissues), and the formation

of granulation tissue, which is a new connective tissue and tiny blood vessels that develop on the surface of a wound during the healing process [2].

The process of wound healing is carefully coordinated in a model that includes four consecutive phases: Hemostasis, Inflammation, Proliferation, and Remodeling [3]. During the process of Hemostasis, the body promptly responds to prevent bleeding by forming a blood clot. The Inflammatory phase ensues, with the objective of eradicating bacteria and eliminating debris, so priming the wound bed for the proliferation of fresh tissue. Following then, the Proliferation phase entails the construction of fresh tissue and a transient matrix that occupies the wound defect. Ultimately, in the Remodeling phase, the wound undergoes maturation and the temporary matrix is substituted with the permanent matrix, which imparts durability and adaptability to the healed skin.

Due to the increase in antibiotic resistance and the negative consequences linked to synthetic medications, there is a renewed emphasis on traditional wound care procedures, which are highly regarded for their limited side effects. In tropical locations, a common traditional cure includes using a poultice made from the leaves of *C. odorata*, a plant belonging to the Asteraceae family. This plant is well-known in traditional medicine for its ability to stop bleeding. The plant's wound healing properties are attributed to the secondary metabolites obtained from the leaves through the process of soaking, pressing, and crushing. *C. odorata* contains a high concentration of sesquiterpene lactone and diterpene lactone, as well as other advantageous components such flavonoids, terpenoids, and sterols [5]. Research, including tests conducted by Owolabi et al. (2010), has identified various chemical components in it, such as α-pinene and β-germacrene D, that have shown antibacterial properties against pathogens including Bacillus cereus and Aspergillus niger. In addition, the leaves contain terpenoid chemicals that are recognized for their antibacterial effects [6]. Additional research conducted by Tran et al. (2011) indicates that the leaf extract of *C. odorata*, which consists of a combination of flavonoids, phenolics, alkaloids, terpenoids, and essential oils, also has antibacterial activities that could enhance the process of wound healing [7].

Another crucial element in the process of wound healing is the Platelet-Derived Growth Factor (PDGF), which plays a vital role in stopping bleeding by promoting the movement of cells like neutrophils, monocytes, and fibroblasts into the skin at the site of the wound. PDGF stimulates both fibroblast proliferation and the synthesis of the extracellular matrix, which is essential for wound healing. PDGF plays a vital role in the wound healing process by stimulating fibroblasts to generate a collagen matrix and facilitating the transition to a myofibroblast phenotype. This change is essential for the contraction phase of wound healing. Therefore, PDGF is a critical promoter in the cascade of events involved in wound healing. The healing process encompasses three main activities: reepithelialization (rebuilding of the outer layer of the skin), angiogenesis (formation of new blood vessels), and extracellular matrix deposition (formation of a supportive structure outside of cells). The efficacy of PDGF in wound healing is substantiated by methods such as its in vitro effects on pivotal wound-healing cells, the examination of PDGF and its receptors' expression during the healing process, and the observed effects resulting from its topical treatment on wounds [9].

Despite the progress in understanding wound healing mechanisms and the extensive use of both synthetic drugs and traditional remedies, there remain significant gaps in integrating these approaches into a universally accepted clinical framework. Current research often focuses on either pharmaceutical treatments or traditional methods. There is a considerable lack of comprehensive studies that bridge the gap between modern medical science and traditional medicine, particularly in

quantifying the efficacy and mechanistic basis of plant-based treatments like Chromolaena odorata in wound care. Additionally, while the role of growth factors such as PDGF in wound healing is well-documented, the specific interactions between such growth factors and plant-based treatments remain underexplored.

MATERIALS & METHODS

Materials

The plant material utilized in this study consisted of leaves from the *C. odorata* plant. The chemicals employed include chloral hydrate, iron (III) chloride, lead (II) acetate, concentrated sulfuric acid, concentrated hydrochloric acid, methanol, chloroform-isopropanol, acetic acid anhydride, toluene, and 96% ethanol. The substances listed are Meyer's reagent, Bouchardat's reagent, Dragendorff reagent, Liebermann-Burchard reagent, Molis reagent, ketamine HCl, adeps lanae, vaseline album, Aquades, 70% alcohol, Betadine ointment, xylene, Mayer's Haematoxyllin solution, lithium carbonate solution, eosin solution, and Entellan ®.

Tools

The tools include laboratory glassware, blender, electric balance, drying cabinet, rotary evaporator, electric oven, furnace, pH meter, ointment bottle, hair shaver, ruler, petri dish, mortar, scalpel, gloves, and punch biopsy.

Plant Identification

The process of identifying plants was conducted at the "Herbarium Medanense" plant laboratory, which is located in the Faculty of Mathematics and Natural Sciences, Department of Biology, at the University of Sumatera Utara in Medan. Approval number: 1511/MEDA/2023

Preparation of Dried *C. odorata* Leaf Samples

The leaves of *C. odorata* were gathered and rinsed extensively with flowing water, then dried and measured for their wet weight. Subsequently, the material is subjected to a drying process in a specialized cabinet at a controlled temperature of 40°C. Once completely dried, the product is then measured for its dry weight and subsequently pulverized using a blender [10].

Dried Samples Characterization

The analysis of dried powder involves assessing the water content, water soluble content, ethanol soluble content, total ash content, and acid insoluble ash content [11], [12].

Extraction of *C. odorata* Leaves

The maceration process was employed to produce an ethanol extract of *C. odorata* leaves, at a ratio of 1:10. A portion of *C. odorata* leaf simplicia powder was placed in a container and mixed with 10 parts of 96% ethanol solvent (pa). Immerse for a duration of 6 hours, intermittently agitating, followed by a subsequent period of 18 hours of inactivity. Subsequently, it was filtered. The filtration procedure was repeated once, and then all the macerate was gathered and subjected to evaporation using a rotary evaporator until a concentrated extract was obtained [13], [14].

Phytochemical Screening

Phytochemical screening was conducted on dried leaves and ethanol extracts of *C. odorata* leaves, specifically analyzing alkaloids, glycosides, saponins, flavonoids, tannins, and steroids/triterpenoids [15,16].

Animal Preparation

A total of 25 male rats were divided into 5 treatment groups, with each group consisting of 5 male rats. The Completely Randomized Design (CRD) is a research design in which treatments are assigned to experimental units completely at random. The rats were acclimatized for a period of 7 days. Subsequently, they were sedated using ketamine HCl. The hair on the back of the mouse, where the wound would be created, was shaved until it was completely smooth. Following this, the area was cleaned using cotton wool soaked in 70% alcohol. A 2 cm

punch biopsy was used to create an incision on the rat's back. The biopsy punch is applied to the skin and then spun while exerting pressure and pushing upwards until the tissue is severed [17]. The male rats will be categorized into five groups. Specifically, group 1 will get an ointment basis, whereas group 2 will be administered Betadine ® ointment 3. Group 3 is administered a 4% ointment made from ethanol extract of *C. odorata* leaves. Group 4 is administered a 6% ointment made from ethanol extract of *C. odorata* leaves. Group 5 is administered an 8% ointment made from ethanol extract of *C. odorata* leaves.

Wound Reduction Observations

Observe wound reduction by calculating the percentage of wound reduction using the formula:

$$P\% = \frac{d0 - dx}{d0} \text{x } 100\%$$

Information d0 = diameter on day 0
dx = wound diameter on the day of observation

Fibroblasts Examination

In this study, 15 male rats were divided into three groups of five, where group 1 received an ointment base, group 2 was treated with povidone iodine ointment as a post control, and group 3 was administered a 6% *C. odorata* leaf ethanol extract ointment. The fibroblasts were analyzed on days 3, 7, and 14 after the male rats were euthanized using chloroform. Subsequent skin tissue samples were prepared in paraffin, stained with Hematoxylin Eosin, and examined under a microscope at 400x magnification across three fields of view. The histology preparations involved several steps: the tissue embedded in paraffin was placed in a water bath and then retrieved with a glass object before incubation at 37°C for 24 hours. It was then deparaffinized using xylene, rehydrated in graded ethanol, and rinsed under running water. The samples were stained with Mayer's Haematoxyllin, washed, and dipped in lithium carbonate solution, followed by eosin. After several washes, the preparations were dipped in ethanol and xylene, sealed with Canada balm adhesive, covered with a glass cover, and observed with a light microscope [18].

PDGF-BB Immunohistochemical Examination

The PDGF-BB expression test, performed at the Histology Laboratory of the Faculty of Medicine, University of North Sumatra, Medan, used the mice that were previously used in the fibroblast count investigation. This experiment utilized the immunohistochemistry (IHC) technique to visually and numerically analyze the expression of PDGF BB in excisional skin samples on days 3, 7, and 14 using microscopy [19]. The immunohistochemistry smear process for PDGF BB involved the following steps: removing the paraffin from the slides using xylene, restoring their moisture by gradually exposing them to different concentrations of ethanol, subjecting them to heat in a microwave, allowing them to cool, and finally rinsing them with water. Next, the slides were subjected to peroxidase block reagent treatment, followed by washing and protein blocking. The PDGF BB primary antibody was administered, followed by post-primary therapy and subsequent application of a compact polymer. The visualization process involved the use of chromogen. After that, the slides were rinsed and stained with Haematoxylin. Subsequently, they were dehydrated using ethanol and cleared using xylene. Finally, the slides were mounted with Entellan under a cover slide.

Statistical analysis

The in vivo results were analyzed using ANOVA with Tukey's Multiple Comparison Test. P-values for significance were set at $P < 0.05$. Values for all measurements are expressed as mean ± SD.

RESULT

Dried Samples Characterization and Phytochemical Screening

Examination of the quality characteristics of dried sample fulfills the requirements for its use as a medicinal ingredient and determines the values for various product parameters. The dried parameters used in this research have met the requirements stated in the official monograph of the Indonesian Herbal Pharmacopoeia Edition II [20]. The results of characterization of *C. odorata* leaf showed in table 1. Furthermore, the results of the phytochemical screening of simplicia powder and ethanol extract contained secondary metabolite compounds in the alkaloids, flavonoids, glycosides, saponins, tannins and steroids/triterpenoids. The results of phytochemical screening can be seen in Table 2.

Table 1. Results of characterization of *C. odorata* leaf simplicia

Parameter	Results (%)
Water content	9.31
Water soluble essence content	26.16
Solubility of essence in ethanol	13.20
Total ash content	7.74
Acid insoluble ash content	0.48

Table 2. Secondary metabolite compounds found in simplicia and ethanol extract of *C. odorata* leaves

Phytochemical Constituent	Dried Leaves	Extract
Alkaloids	+	+
Flavonoids	+	+
Glycosides	+	+
Saponins	+	+
Tannin	+	+
Steroids / Triterpenoids	+	+

Excision Wounds Healing and Fibroblasts Evaluation

The healing progress of excision wounds was monitored by assessing the reduction in wound size over a 15-day treatment period. On day three, the treatments showed varying degrees of effectiveness: Betadine® ointment reduced wound size by 9.80%, the control ointment base by 3.46%, and *C. Odorata* Extract Ointments at different concentrations showed reductions of 6.11% for 4%, 7.37% for 6%, and 7.56% for 8%. Statistical analyses highlighted significant differences in the healing effects of the *C. Odorata* Extract Ointments at concentrations of 4%, 6%, and 8% when compared to Betadine® on day three. Data can be seen in Figure 1.

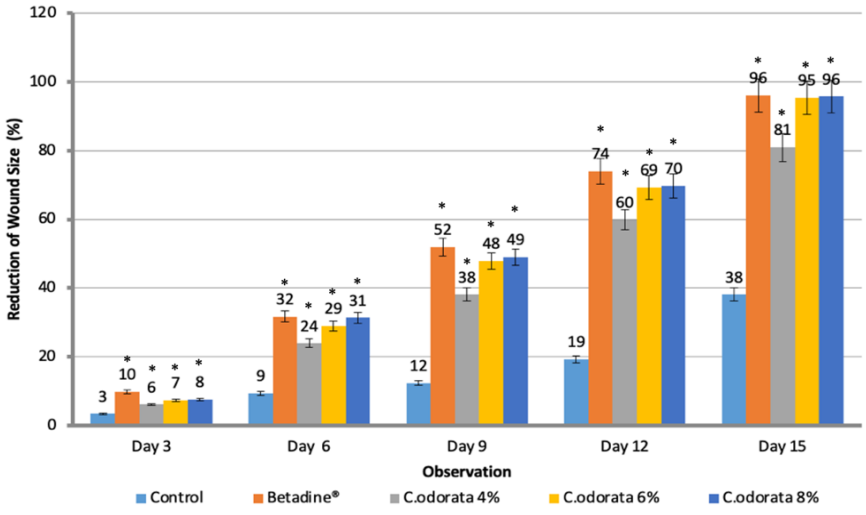

Figure 1. Percentage Reduction in Diameter of Rat Excision Wounds on Day 1, Day 5, Day 9, Day 12 and Day 15. *($p<0.05$) shows significant difference with the negative control group.

On the other hand, Figure 2 shows the histological analysis of fibroblasts, which indicates differences in cell numbers among various treatments. After three days, the application of Betadine® resulted in an average of 43.20 fibroblasts per field, while the control group had an average of 25.20 fibroblasts and the group treated with *C.*

Odorata Extract Ointment 6% had an average of 42.80 fibroblasts. The findings indicates that Betadine® demonstrates a minor superiority over the 6% *C. Odorata* ointment and a significant advantage over the control in terms of fibroblast proliferation. On the seventh day, the control group had an average of 48.00 fibroblasts per field. In comparison, the groups treated with Betadine® and *C. Odorata* Extract Ointment 6% had averages of 70.60 and 69.20 fibroblasts per field, respectively. Betadine® had the highest fibroblast count, while it was nearly equivalent to that of the *C. Odorata* Extract Ointment 6%.

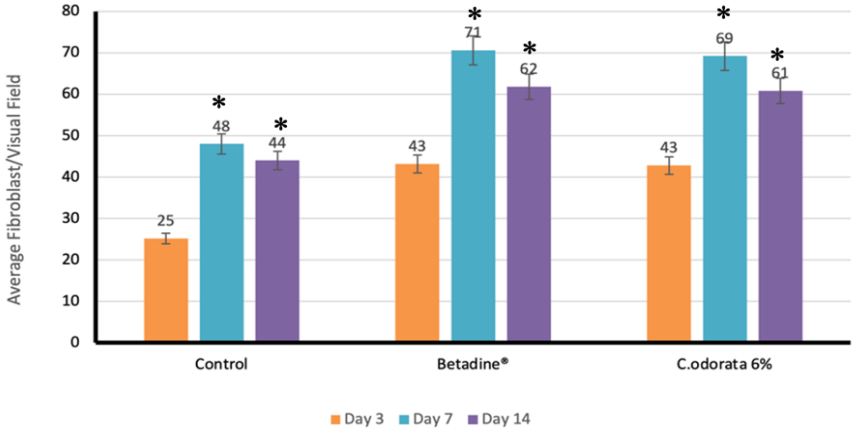

Figure 2. The average number of fibroblasts in excision wounds on the 3rd, 7th, and 14th day, *($p<0.05$) shows significant difference with the negative control group.

PDGF BB Immunohistochemistry Examination

On day 3, the average PDGF BB expression score in Betadine® treatment was 99.20 cells per field, compared to 84.40 cells per field in the control and 97.40 cells per field in the *C. Odorata* Extract Ointment 6% treatment. By day 7, there was an increase in the PDGF BB expression scores across all treatments: Betadine® rose to 121.20 cells per field, the control to 88.20 cells per field, and *C. Odorata* Extract Ointment 6% closely followed Betadine® with 119.60 cells per field. Data can be seen in figure 3.

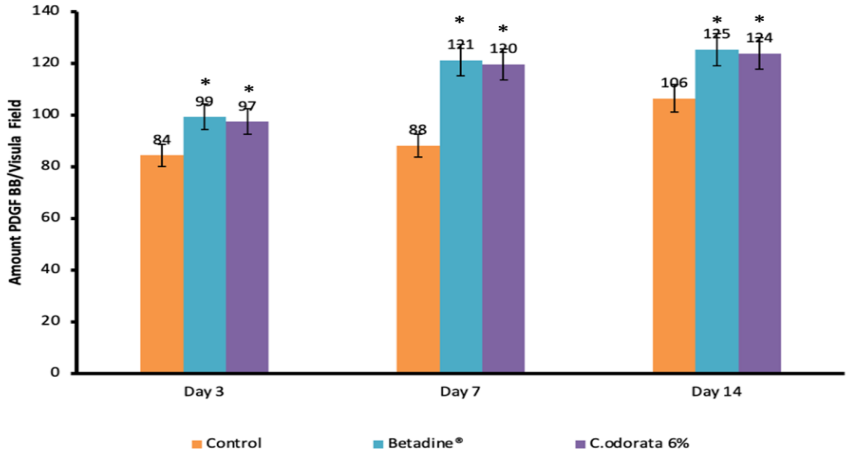

Figure 3. The average score of PDGF BB expression on Rat skin on the 3rd, 7th, and 14th day, *($p<0.05$) shows significant difference with the negative control group.

DISCUSSION

The characteristics of dried sample must fulfill the requirements for its use as a medicinal purpose. Table 1 indicated that the water content should generally not exceed 10% according to specified requirements, the

water content measured was 9.31% [20]. If the water content during testing exceeds 10%, it can foster an environment conducive to microorganism growth, particularly fungi, which may reduce the shelf life and quality of the simplicia, potentially causing damage [21]. The determination of water soluble content levels is conducted to identify polar chemical compounds in simplicia such as flavonoids, glycosides, and saponins [22], with the results showing 26.16% water soluble essence. In contrast, the aim of measuring ethanol soluble essence levels is to identify polar, semipolar, and non-polar chemical compounds, with 13.20% ethanol soluble essence obtained. The variability in results between these tests highlights differences in the solubility of compounds in each solvent, suggesting a higher solubility for polar compounds [16]. Moreover, the ash content test provides insights into the mineral content of simplicia, derived from the initial processing of raw materials [23]. The total ash content test, which measures inorganic compound levels such as the metals K, Ca, Na, Pb, and Hg, indicated a low ash content of 1.69%, suggesting minimal internal mineral content in *C. odorata* leaves [23]. Acid insoluble ash content, measuring at 0.69%, indicates contamination with minerals or metals not soluble in acid, such as silicates from soil or sand [24]. This content helps identify the presence of external mineral impurities.

Additionally, Table 2 revealed positive results for alkaloids in *C. odorata* leaves, indicated by precipitates forming with Mayer, Bouchardat, and Dragendorff reagents [25]. Tests with FeCl3 on simplicia and ethanol extracts resulted in significant color changes, confirming the presence of tannins [26]. Saponin presence was confirmed through stable foam formation after treatment with hot water and 2N HCl [27]. Flavonoids were identified by a color shift to reddish-black in tests [28], and the Lieberman-Bouchard reagent indicated positive results for triterpenoids, showing a red-purple color [16], [29]. Tests also confirmed the presence of steroids.

Excision wounds healing evaluation that showed in figure 1 indicated that on day 6, wound reduction percentages were recorded as follows: Betadine® at 31.71%, control at 9.40%, 4% *C. Odorata* Extract Ointment at 23.96%, 6% *C. Odorata* Extract Ointment at 28.99%, and 8% *C. Odorata* Extract Ointment at 31.36%. By day 9, these values changed to 51.96% for Betadine®, 12.37% for the control, 38.14% for 4% ointment, 47.91% for 6% ointment, and 49.02% for 8% ointment). On day 12, the reductions were as follows: 74.01% for Betadine®, 19.30% for the control, 59.90% for 4% ointment, 69.23% for 6% ointment, and 69.75% for 8% ointment. By day 15, the final day of the observation period, Betadine® achieved a wound reduction of 96.07%, the control 38.12%, 4% ointment 80.93%, 6% ointment 95.38%, and 8% ointment 95.75% (Figure 1). Statistical analysis showed no significant difference in wound reduction between the 6% and 8% ointments compared to Betadine®. However, the wound reduction of 4% ointment was significantly different on day 15. R. Sowbarnika's study [2] noted that mice treated with *C. odorata* leaf extract achieved complete wound healing by day 16, while control mice treated with saline took 20 days.

On day 14, the average count of fibroblasts for Betadine® was 61.80 cells/field, for the control 44.00 cells/field, and for the 6% ointment, it was 60.80 cells/field. Statistical tests indicated that Betadine® was significantly different from the control but not from the 6% ointment treatment. The number of fibroblasts decreased by day 14 as collagen production peaked. New tissue formation on day 7 can stimulate surrounding cells to produce collagen, which ceases as the granulation phase concludes and is replaced by acellular scar tissue, reducing fibroblast numbers post-day 7 [30]. Furthermore, the PDGF BB score on day 14 was 125.20 cells/field for Betadine®, 106.40 cells/field for the control, and 123.80 cells/field for the 6% ointment. Betadine® showed a significant difference from the control but not from the 6% ointment. From day 3 to day

14, PDGF BB scores for the 6% ointment and Betadine® were higher compared to controls, illustrating the role of PDGF BB in the wound healing process by promoting cell migration and proliferation [31].

CONCLUSION

The application of an ointment made from the ethanol extract of *C. odorata* leaves has been found to enhance the healing process of excision wounds in male white rats. This ointment promotes wound closure and increases the presence of fibroblasts and PDGF BB expression in the wounds.

Declaration by Authors

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