JURNAL KEDOKTERAN SYIAH KUALA
www.journal.usk.ac.id/jks
ORIGINAL ARTICLE
P-ISSN: 1412 – 1026
E-ISSN: 2550 - 0112

The effect of Kirinyuh leaves aqueous extract (Chromolaena odorata (L) R.M.King & H.Rob.) on phase 2 collagen density wound healing in mice (Mus musculus)

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ARTICLE INFO

Keywords:
Chromolaena odorata (L) R.M.King & H.Rob., collagen, Mus musculus, wound healing

Article History:
Received: 18/10/2023
Accepted 02/04/2024
Published Online 30/04/2024

ABSTRACT

Introduction: Various plants have been extensively researched for their ability to hasten wound healing. Kirinyuh plants (*Chromolaena odorata (L) R.M.King & H.Rob.*) includes several secondary metabolite chemicals that can help to accelerate wound healing.
Objective: To determine the effect of water extract of kirinyu leaves (*C. odorata*) on collagen density at the end of phase 2 wound healing in mice (*Mus musculus*) and the amount of concentration of water extract of kirinyu leaves (*C. odorata*) that is most effective in collagen formation.
Methods: A total of 28 male mice aged 2-3 months were given an incision wound on the back and separated into four treatment groups by giving kirinyuh leaf aqueous extract (*C. odorata*). Group I received no therapy (control), Group II received 2.5% extract administration, Group III received 5% extract administration, and Group IV received 7.5% extract administration. Wound healing was observed histologically by measuring the density of collagen using Mallory's phosphotungstic acid hematoxylin stain solution with aniline blue. The ANOVA test was used statistically to examine variations in collagen density across groups.
Results : The histology analysis revealed that the average quantity of collagen sequentially was 67.5% for group I, 59.5% for group II, 64.9% for group III, and 71.0% for group IV. Meanwhile, statistical tests revealed that there was no significant difference between the groups, with $p>0.05$ significance.
Conclusion: Administrating kirinyuh leaf aqueous extract (*C. odorata*) did not have a significant effect on the density of collagen in mice (*Mus musculus*).

INTRODUCTION

Wounds are described as the destruction or disruption of normal anatomical structures and functioning (Velnar et al., 2009). This damage might be modest, such as destroying the skin epithelium's integrity, or it can be severe, involving the subcutaneous tissue and underlying tissues such as tendons, muscles, blood vessels, nerves, parenchymal organs, or even bones (Enoch & Leaper, 2008; Velnar et al., 2009). Many factors influence wound healing, including

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https://doi.org/10.24815/jks.v24i1.34823

oxygenation, infection by bacteria or fungus, foreign body invasion, the presence of vascular problems, nutrition, and medicines (Guo & DiPietro, 2010).

Collagen is an extracellular matrix (ECM) that fibroblasts create. Myofibroblasts, which develop from fibroblasts, use the transmembrane to connect intracellular stress fibers to extracellular fibronectin fibrils. Contraction of these stress fibers condenses the ECM, making a chamber for new collagen synthesis. In the beginning, fibroblasts make type III collagen, which is then replaced by more mature type I collagen. This process is repeated until wound contraction occurs, which is the primary purpose of collagen (Cañedo-Dorantes & Cañedo-Ayala, 2019; Stunova & Vistejnova, 2018). After collagen is deposited in the wound, the process of re-epithelialization, or the migration of keratinocytes from the wound's margins, begins and finishes with a completely closed wound (Stunova & Vistejnova, 2018).

Many herbal plants have been shown to help with wound healing. *Chromolaena odorata (L) R.M.King & H.Rob*. is one of these plants that has been studied for its efficacy as an alternative treatment for wound healing (Anggraeni & Bratadiredja, 2018; Kumar et al., 2007). *Chromolaena odorata (L) R.M.King & H.Rob.* (*C. odorata*) was discovered to include a variety of secondary metabolites, primarily saponins and flavonoids (Ngozi et al., 2009; Vijayaraghavan, Rajkumar, Bukhari, et al., 2017). The saponins in *C. odorata* are thought to play a function in platelet aggregation and fibroblast migration to the wound site. Furthermore, saponins function as anti-inflammatory agents during the wound healing process (Owoyele et al., 2005; Vijayaraghavan, Rajkumar, Bukhari, et al., 2017). Flavonoids contained in *C. odorata* are also believed to act as antiseptics which can be used to sterilize wounds. *C. odorata* also has antimicrobial properties which are useful for keeping the wound condition free from bacterial or fungal invasion (Aro et al., 2009; Vital & Rivera, 2009).

The previous study showed that 5% of *C. odorata* extract, was able to accelerate the wound healing process at the end of phase 2 (Kumar et al., 2007; Vijayaraghavan, Rajkumar, Bukhari, et al., 2017). Unfortunately, the effect of *C. odorata* on collagen density at phase 2 of the wound healing process was not determined (Kumar et al., 2007). The aim of this study is to determine the impact of kirinyuh leaf (*C. odorata*) aqueous extract on collagen density in mice (*Mus musculus*) at the end of phase 2. This study will also establish the highest effective concentration of kirinyuh leaf (*C. odorata*) aqueous extract affecting collagen production at the end of phase 2 of wound healing in mice (*Mus musculus*).

METHODS & MATERIALS

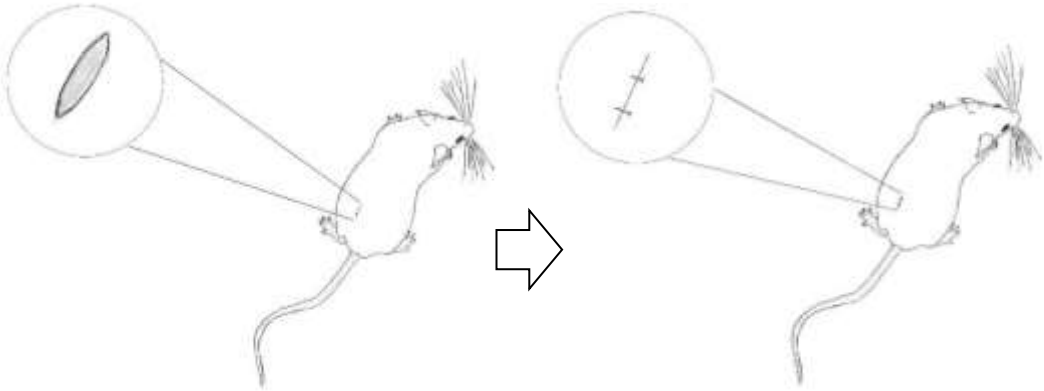
A Completely Randomized Design (CRD) with the Posttest Only Control Group Design approach was employed in laboratory experimental research. In this work, 28 male mice (*Mus musculus*) were employed as test animals. The experimental animals were put into four groups of seven mice each, with three groups receiving therapy and one being a control group.

The Ethics Committee of the Faculty of Veterinary Medicine, Universitas Syiah Kuala, with reference number 182/KEPH/XI/2022, has determined that this research meets the conditions for being conducted on experimental animals.

The experimental animals used as research samples were 28 male mice (*Mus musculus*). The inclusion criteria were male mice (*Mus musculus*), aged 2-3 months, body weight 20 to 30 grams, and in good health. Meanwhile, mice (*Mus musculus*) that are sick (marked by inactive movements), have anatomical abnormalities, and dead mice will be excluded.

Experimental animals were placed in cages that had previously been dried and covered with sawdust. Cages and sawdust mats are cleaned at least 3 times a day, food and drink are changed and cleaned at least once a day. The animal cages were placed in a room with good air ventilation and indirect sunlight. The size of the cage is 100 cm^2 with a minimum height of 13 cm for each mouse.

After one week of adaptation, the experimental animals were treated for damage to the left side of their back. This process involved shaving the dorsal left side of the mice in an area of 2 cm x 1 cm, followed by disinfection on the shaved left side of the back of the mice using 70% alcohol.



Figures 1. Design of incisions and after suturing in mice

Furthermore, experimental animals were given anaesthetic action using local anaesthesia with lidocaine. A 1.5 cm long incision is made on the back parallel to the vertebrae, 3-4 cm from the ear, up to the fascia. Then the wound was sutured using 5-0 polypropylene thread as many as 2 stitches with a distance of 5 mm. The action ends with the administration of aqueous extract *C. odorata*. Each treatment group received the following treatment:

Table 1. Sample group and treatment for each group

Sample Group	Treatment
Group 1	Control: Experimental animals were treated with a wound on the left dorsal side and left for 21 days
Group 2	Treatment 1: Experimental animals were treated with a wound on the left dorsal and given a drop of *C. odorata* aqueous extract with a concentration of 2.5% daily
Group 3	Treatment 2: Experimental animals were treated with a wound on the left dorsal and given a drop of C. odorata aqueous extract with a concentration of 5% daily
Group 4	Treatment 3: Experimental animals were treated with a wound on the left dorsal and given a drop of *C. odorata* aqueous extract with a concentration of 7.5% daily

On the 21st day, wound collagen was examined by removing 1.5 x 1 cm of wound tissue for histological investigation. Zenker's solution was used to fix the preparations, which were subsequently sliced to a thickness of 6 microns (Isenhath et al., 2007) and stained with Mallory's Phosphotungstic Acid Hematoxylin Stain technique with Aniline Blue then the thickness of the

collagen connective tissue was observed under a light microscope with 40x magnification. Collagen is stained in blue. One histological preparation was observed in 1 field of view, then the amount of collagen was counted in that 1 field of view and the percentage was calculated using image processing software, namely ImageJ.

Data analysis used the One-Way ANOVA test to see whether or not there was a significant difference between the treatment groups. Based on the results from the One-Way ANOVA test data, a significant difference was found, then the data was tested with the LSD method difference test to see the most significant difference in dose.

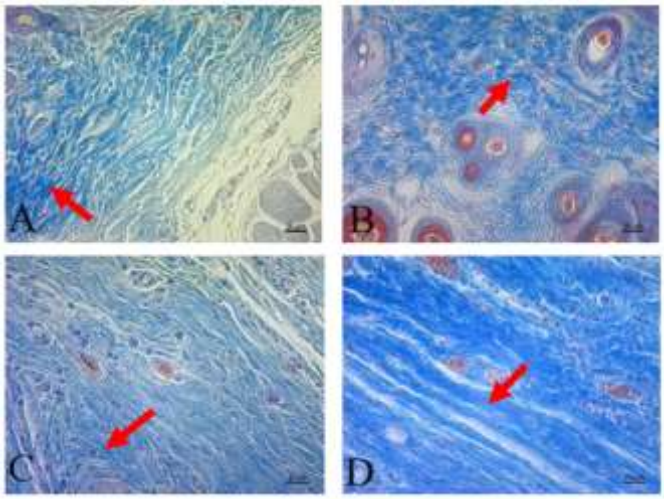
RESULTS

Based on table 2 below, the phytochemical screening test conducted in this study, the aqueous extract of kirinyu (*C. odorata*) leaves contains metabolites in the form of alkaloids, steroids, terpenoids, saponins, flavonoids, tannins, and phenolics.

Table 2. Results of phytochemical screening of aqueous extract of kirinyu leaves (*C. odorata*)

Metabolite Content	Results
Alkaloid	+
Steroid	+
Terpenoid	+
Saponin	+
Flavonoid	+
Tanin	+
Phenolic	+

The results of microscopic examination of collagen on day 21 using Mallory's Phosphotungstic Acid Hematoxylin Stain look as figure 2 follows:



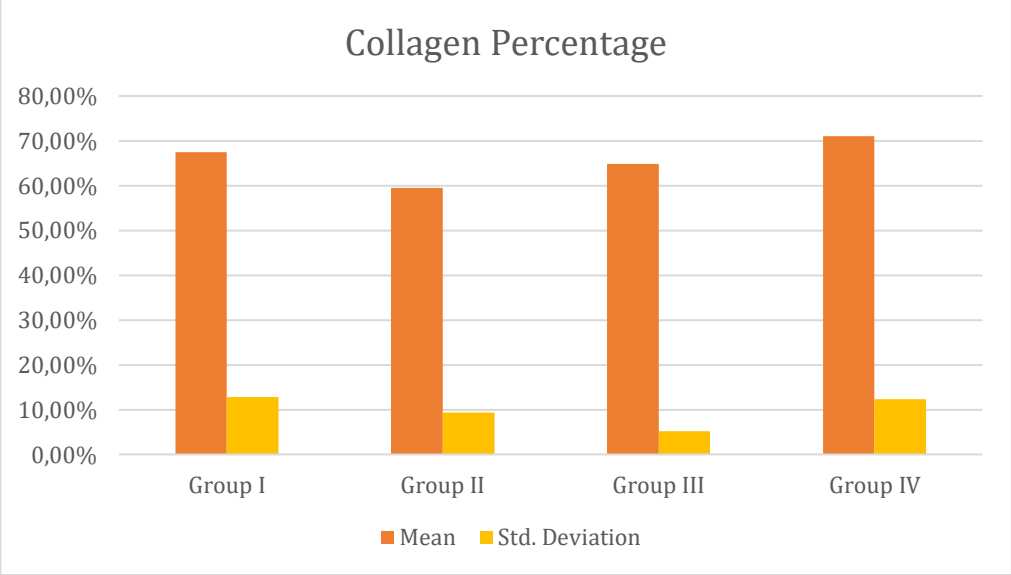
Figures 2. Histological picture of staining of wound tissue

Mallory's Phosphotungstic Acid Hematoxylin Stain with Aniline Blue to assess collagen fiber indicated by red arrows (Figure A: group I, Figure B: group II with 2.5% extract administration, Figure C: group III with 5% extract administration, Figure D: Group IV treated with 7.5% extract.

Table 3. Descriptive statistical test results for collagen density in each group

	N	Range	Minimum	Maximum	Mean	Std. Deviation
Group I	7	36.08	49.37	85.45	67.5020	12.85765
Group II	7	26.43	47.74	74.17	59.4943	9.39791
Group III	7	15.33	60.01	75.34	64.8843	5.23404
Group IV	7	35.87	48.05	83.92	71.0486	12.28164
Valid N (listwise)	7					

Based on the results of the description of Table 3 above, it can be seen the difference in the mean collagen formed in each group. The increase in collagen was most seen in the administration of 7.5% aqueous extract of Kirinyuh (*C. odorata*) leaves, with an average collagen formed of 71% visual field as shown in the bar chart below.



Figures 3. The mean and standard deviation percentage of collagen for each group

Table 4. Analysis of differences in collagen between groups

	Mean ± SD	**F**	***P* value***
Group 1	67.5 ± 12.8	1.535	.231
Group 2	59.5 ± 9.4		
Group 3	64.8 ± 5.2		
Group 4	71.0 ± 12.2		

*ANOVA test

Based on the results of the ANOVA test above, with a p value>0.05, it can be concluded that there was no significant difference between the control group, the group treated with 2.5%, 5, and 7,5% aqueous extract of Kirinyu (*C. odorata*) leaves.

According to the LSD post hoc test table, there was a significant difference between the groups treated with 2.5% Kirinyu leaf aqueous extract (*C. odorata*) and 7.5% Kirinyu leaf aqueous extract (*C. odorata*).

Table 5. The results of the LSD post hoc test

(I) Experimental Group	(J) Experimental Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Group I	Group II	8.00771	5.55411	.162	-3.4554	19.4708
	Group III	2.61771	5.55411	.642	-8.8454	14.0808
	Group IV	-3.54657	5.55411	.529	-15.0097	7.9165
Group II	Group I	-8.00771	5.55411	.162	-19.4708	3.4554
	Group III	-5.39000	5.55411	.342	-16.8531	6.0731
	Group IV	-11.55429*	5.55411	.048	-23.0174	-.0912
Group III	Group I	-2.61771	5.55411	.642	-14.0808	8.8454
	Group II	5.39000	5.55411	.342	-6.0731	16.8531
	Group IV	-6.16429	5.55411	.278	-17.6274	5.2988
Group IV	Group I	3.54657	5.55411	.529	-7.9165	15.0097
	Group II	11.55429*	5.55411	.048	.0912	23.0174
	Group III	6.16429	5.55411	.278	-5.2988	17.6274

*. The mean difference is significant at the 0.05 level.

In this study, because there was no significant effect between the administration of Kirinyuh (*C. odorata*) leaf extract and the control group, further assessment to determine which treatment group has the most effect cannot be conducted.

DISCUSSION

According to the results of the phytochemical testing, the aqueous extract of Kirinyuh (*C. odorata*) leaves includes a lot of secondary metabolites that function as an antioxidant and to speed up wound healing. This is consistent with prior research, which found that aqueous and ethanol extracts of Kirinyuh (*C. odorata*) leaves contain a variety of active compounds, including alkaloids, flavonoids, tannins, saponins, and terpenoids (Gogoi et al., 2021; Ngozi et al., 2009; Sirinthipaporn & Jiraungkoorskul, 2017; Vijayaraghavan, Rajkumar, & Seyed, 2017). Antioxidants act as an antidote to free radicals like reactive oxygen species (ROS), which hinder wound healing (Vijayaraghavan, Rajkumar, Bukhari, et al., 2017). *C. odorata* leaf extract possesses antimicrobial properties in addition to the potential to heal wounds. The ethanol extract of *C. odorata* inhibited the development of both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria. Furthermore, the ethanol extract of *C. odorata* demonstrated the potential to prevent fungal growth (Stanley et al., 2014).

Collagen staining with Mallory's Phosphotungstic acid hematoxylin stain solution with aniline blue can keep a unique blue color in collagen tissue without fading the stain (Suvarna et al., 2018). Using this technique, bones and their components are stained orange to red, muscle fibers are stained red, and collagen fibers are stained blue (Sterehi & Keefer, 1998). Therefore, researchers recommend this examination to assess collagen density in tissue preparations in future studies.

In this study, the highest density of collagen was in the administration of 7.5% aqueous extract of Kirinyuh (*C. odorata*) leaves. This is slightly different from previous research by Vijayagrahan which showed that the most collagen was formed in the administration of 5% aqueous extract of Kirinyu (*C. odorata*) leaves (Vijayaraghavan, Rajkumar, & Seyed, 2017; Vijayaraghavan, Rajkumar,

Bukhari, et al., 2017). This can be influenced by the day of sampling, as in this study the sample was collected on the 21st day, while the sample was obtained on the 16th day in the prior study. The 21st day was chosen for this investigation since it was the last day of the proliferative phase before the remodelling phase began. On the 21st day, there was an increase in collagen diameter, collagen maturation, and no additional collagen production (Enoch & Leaper, 2008; Velnar et al., 2009). Yudika conducted another study that assessed collagen in wounds using Kirinyuh (*C. odorata*) leaf extract and found significant variations on the 7th and 14th day of evaluation (Yudhika, 2020).

Another study from Thang et. al. also showed that the administration of Kirinyuh (*C. odorata*) leaf extract 10 – 100 µg/ml in cell culture increased fibroblast proliferation and stimulated migration of keratinocytes with its antioxidant content (Thang et al., 2001). Its antioxidants protect fibroblast cells and keratinocytes from the effects of free radicals such as ROS. Differences in research Thang et al.'s contribution to this study is the use of methanol as a solvent. In comparison to water, methanol and ethanol are good polar molecules for extracting chemicals from plants. However, water-based compounds are superior to other solvents for moisturizing wounds (Thang et al., 2001). Pandit et al. demonstrated that the application of Kirinyuh leaf extract (*C. odorata*) in cell culture also increased fibroblasts (Pandith et al., 2013).

Several studies have revealed that changes in plant development sites alter phytochemical composition. According to Lavola et al., increasing the amount of carbon, UVB, and temperature affects the concentration of secondary metabolites in plants (Lavola et al., 2013). Gwynn et al. found similar results, specifically those that increased UVB and carbon produced changes in the number of secondary metabolites in plants (Gwynn-Jones et al., 2012). Heinäaho et al. discovered that environmental variables and soil conditions influenced the concentration of secondary metabolites in plants (Heinäaho et al., 2006). Some of these factors may cause the extracted content in Kirinyuh *(C. odorata)* leaves to differ from prior studies, making the influence of secondary metabolite content less influential on collagen and lead to no significant difference in colagen results.

Secondary metabolite content differed depending on the age of the leaves, as observed in studies that investigated differences in secondary metabolite content between sprouts and old leaves. Secondary metabolites are more abundant in old leaves than in sprouts (Rao et al., 2021). This could also have an impact on the study's findings. This study did not consider the age of the leaves when extracting them, which resulted in non-uniform secondary metabolite content in the extracts.

The researchers suggested that future research should explore the effects of administering the aqueous extract of Kirinyuh (*C. odorata*) leaves on other cellular parameters. Research needs to be done for other types of wounds such as excision wounds, pressure sores, or diabetic wounds to explore more potential from Kirinyuh leaves (*C. odorata*). For further research that wants to use aqueous extracts, you can increase the concentration of the extract which may increase collagen. It is also necessary to conduct research using other parts of the plant such as stems and roots.

CONCLUSION

According to the findings of this investigation, the administration of Kirinyuh (*C. odorata*) leaf aqueous extract did not substantially impact the density of collagen in incision wounds inflicted on mice (*Mus musculus*) compared to the control group.

ACKNOWLEDGEMENTS

The authors would like to thank the Division of Plastic Reconstructive and Aesthetic Surgery, Department of Surgery, Faculty of Medicine, Syiah Kuala University / Dr. Zainoel Abidin Regional General Hospital Banda Aceh, supervisors, and academic examiners in this study that the research can be completed properly.

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