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POTENTIAL ACCELERATING EFFECT OF *Ageratum conyzoides* L. LEAVES EXTRACT ON FIBROBLASTS DENSITY OF INCISION WOUND OF MALE WHITE MICE (*Mus musculus*)

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ABSTRACT

***Background**: Wound treatment using traditional medicine has been known widely in various countries in the world. Ageratum conyzoidesL. is commonly known by the ancient people to treat wound due to its potential anti-inflammatory effect. This study aims determine the effect of Ageratum conyzoidesL. leaves extract on fibroblast density of incision wound of male white mice (Mus musculus).*
***Methods**: This post-test only control group design experimental study used 35 male white mice which were randomly divided into five groups, i.e. negative control group K(-), positive control group K(+) (10% povidone iodine), and treatment group P1, P2, and P3 that were each given billy-goat weed leaf with increasing dose (15%, 30%, and 45% respectively). On each day, the length of the incision was measured by a ruler. After 7 days, the mice were terminated to obtain wound tissue which were used to prepare H&E stained histopathological sections to observe fibroblast density. Non-parametric analyses using Kruskall-Wallis and Mann Whitney test were used to compare the wound length and fibroblasts density.*
***Results**: Lengths of incision wound between all pairs of groups at the 7^{th} day are significantly different ($p< 0.05$) with group P3 showed the shortest one. Significant differences were also observed in fibroblasts density between group K(-) and K(+), K(-) and P1, K(-) and P2, K(-) and P3, K(+) and P3, P1 and P3, P2 and P3 ($p< 0.05$) with group P3 showed the highest density among all groups.*
***Conclusions**: Ageratum conyzoidesL. leaves extract 45% has more potential effect than povidone iodine 10% in accelerating healing process by enhancing fibroblasts density.*

***Keywords**: Ageratum conyzoides L., fibroblasts density, incision wound.*

INTRODUCTION

Wound is the loss or damage of a part of tissue. This condition can be caused by trauma of sharp or dull objects, temperature changes, chemical substances, explosion, electric shock, or animal bite. The process of wound healing is divided into 4 phases, i.e. inflammation, hemostasis, proliferation or granulation, and remodeling phase [1]. The target of biological process of the body in compensating wound is the components that act in wound healing phases. Fibroblast is one of the components of healing in fibroplasia process. Fibroplasia is a process of wound repair that involves connective tissue that has four components: formation of new blood vessels, migration and proliferation of fibroblasts, deposition of extracellular matrix (ECM), and maturation and organization of fibrous tissues (remodeling) [2,3].

Taqwim*et al*. stated that wound healing is a natural process of the body, although medicaments can be used to accelerate its process such as normal saline and povidone iodine. However, standard wound antiseptics such as povidone iodine is not always available in homes. Thus, Indonesian ancestors inherit

and frequently use traditional medicine, obtained from herbs or plants, especially in remote area [4,5]. One of the traditional herbs often used in wound healing is *Ageratum conyzoides* L. (billygoat-weed, chickweed, white weed) leaves [6]. *Ageratum conyzoides* L. is commonly used as a medicament for wounds and indigestion. It is believed that *Ageratum conyzoides* L. can stop bleeding and accelerate healing process [7]. Afrianti R et al. has shown that *Ageratum conyzoides* L.) leaves extract could improve healing percentage and the density of collagen fibers of hyperglycemic wound [8]. This study was conducted to analyze the potential activity of *Ageratum conyzoides* L. leaves extract on incision wound healing process of white male mice (*Mus musculus*) in term of fibroblast density as well as wound length and the effective dose.

MATERIALS AND METHODS

This was an experimental study with post-test only control group design using male white mice (*Mus musculus*) as research subjects. Ethical clearance approval No.112/EC/FK/XII/2017 was obtained from Research Ethics Committee of Faculty of Medicine, Universitas Swadaya Gunung Jati. The study was conducted in the Food and Nutrition PAU Laboratory and Pathology Anatomy Laboratory of Universitas Gadjah Mada, Yogyakarta, Indonesia.

Ageratum conyzoides L. fresh leaves were obtained from *Ageratum conyzoides* L. plants in Cirebon City, West Java, Indonesia, confirmed by a taxonomist of Universitas Negeri Semarang, Central Java, Indonesia. The collected leaves were washed under running water, drained, cut into pieces, and dried under the sun until completely dry. Afterward, *Ageratum conyzoides* L. leaves were blended and weighed. Three kilograms of sample was put in a closed container and incubated for 3 days at room temperature shielded from light while stirring repeatedly. The sample was then filtered and squeezed. Ethanol 70% was added into the juice adequately and the pulp was re-macerated for 3 times. The macerated extract was collected and concentrated by using rotary vacuum evaporator and oven to obtain a viscous extract.

Thirty-five male white mice (*Mus musculus*) of 20-40 grams of weight and 8 weeks of age were randomly divided into 5 groups, i.e 2 control groups and 3 treatment groups. At first, all the mice were wounded by 2 cms length incision at the back of the mice which previously administered with 1 cc of ketamin anesthesia intraperitoneally. Control groups consist of negative control group K(-) which was without any treatment and positive control group K(+) treated with 10% povidone iodine topically. Meanwhile the 3 treatment groups consist of group P1 which was administered by 15% *Ageratum conyzoides* L. leaves extract topically, group P2 with 30% of the extract, and group P3 with 45% of the extract. Extract concentration given was determined according to the results obtained from previous study conducted by Afrianti R [9].

The treatment was done for 7 days (twice a day), and on each day, the length of incision wound was measured by a ruler and the healing process was analyzed visually. At the 7th day, all mice were terminated by cervical dislocation. The wound area was excised and fixated by formalin 10%. Hematoxilin and Eosin (H&E) stained sections were prepared for all mice groups according to the laboratory protocols. Histologic analysis was performed by using binocular light microscope Olympus CX23 with ocular lens 100x and 400x. Fibroblasts were counted in 5 viewing fields for each section slide of each group.

RESULTS

Macroscopic incision length analysis

All incision wounds were measured each day for 7 days and the lengths of incision at the 7th day from all groups were compared. Table 1 shows length comparison from each day of all groups.

Table 1. Incision Length Measured Each Day

Group	Day	Incision Length of Mouse (cm)							Mean Length (cm)
		1	2	3	4	5	6	7	
K(-)	0	2,00	2,00	2,00	2,00	2,00	2,00	2,00	2,00
	2	2,00	2,00	2,00	2,00	2,00	2,00	2,00	2,00
	4	2,00	2,00	2,00	2,00	2,00	2,00	2,00	2,00
	6	2,00	2,00	2,00	2,00	2,00	2,00	2,00	2,00
	7	1,98	2,00	2,00	2,00	1,99	2,00	2,00	1,995
K(+) Povidone Iodine	0	2,00	2,00	2,00	2,00	2,00	2,00	2,00	2,00
	2	1,98	1,94	1,97	1,99	1,98	1,98	1,98	1,947
	4	1,83	1,80	1,77	1,79	1,82	1,80	1,81	1,802
	6	1,43	1,38	1,40	1,37	1,33	1,37	1,35	1,376
	7	1,09	1,12	1,03	0,99	1,09	1,03	0,98	1,047
P1 (15% of extract)	0	2,00	2,00	2,00	2,00	2,00	2,00	2,00	2,00
	2	2,00	2,00	2,00	2,00	2,00	2,00	2,00	2,00
	4	1,98	1,98	1,99	1,94	1,97	1,90	1,98	1,963
	6	1,87	1,90	1,90	-	1,90	1,90	1,88	1,892
	7	1,80	1,83	1,87	-	1,84	1,86	1,82	1,837
P2 (30% of extract)	0	2,00	2,00	2,00	2,00	2,00	2,00	2,00	2,00
	2	2,00	2,00	2,00	2,00	2,00	2,00	2,00	2,00
	4	1,88	1,90	1,84	1,82	1,87	1,90	1,87	1,868
	6	1,58	1,62	1,76	1,70	1,75	-	1,80	1,702
	7	1,58	1,62	1,76	1,70	1,75	-	1,80	1,702
P3 (45% of extract)	0	2,00	2,00	2,00	2,00	2,00	2,00	2,00	2,00
	2	1,90	1,94	1,92	1,95	1,93	1,92	1,95	1,930
	4	1,77	1,79	1,80	1,81	1,81	1,79	1,80	1,795
	6	1,38	1,40	1,37	1,35	1,30	1,34	1,30	1,348
	7	0,98	1,00	0,95	0,92	0,89	0,90	0,90	0,934

Incision length observation on each day has been revealed that group K(+), P1, P2, and P3 showed a remarkable healing process compared to group K(-). At the 7th day, wound length in mice group P3 was the shortest among all groups (Fig. 1.).

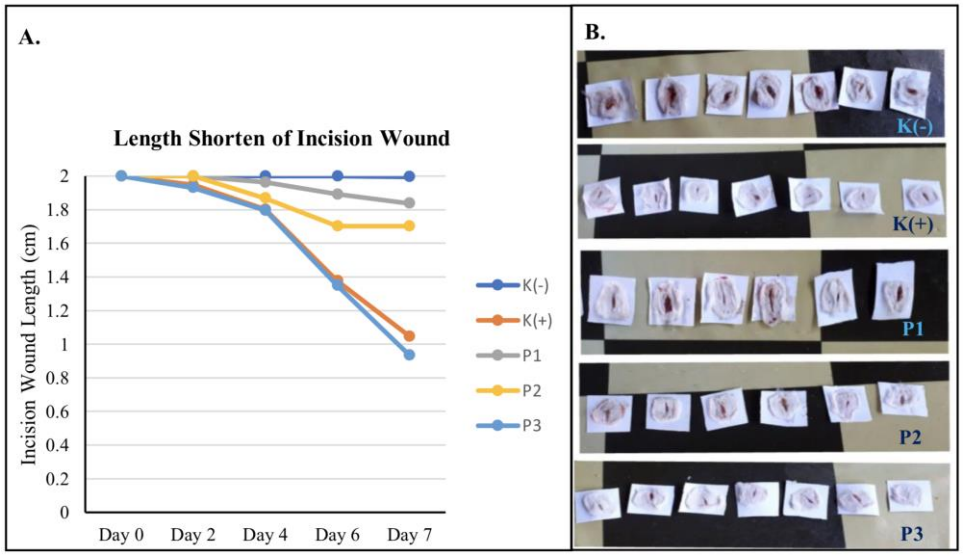


Figure 1. Macroscopic observation on incision wound. A. Graph showing length shorten of incision wound measured day by day using a ruler. Group P3 showed the fastest shorten of incision length among all groups, followed by group K(+). B. Incision wound of all groups taken after 7 days of treatment. Incision length in group P3 and K(+) reduced almost half of the initial length which can be seen from group K(-). K(-) is negative control group, K(+) is positive control group treated by povidone iodine

topically, P1 was treated by treatment group 1 with 15% *Ageratum conyzoides*L. leaves extract topically, P2 treated with 30% of the extract, and P3 treated with 45% of the extract.

Mean length of incision wound at the 7th day of all mice group were compared by using non-parametric Kruskall-Wallis analysis. With Confidence Interval (CI) 95%, *p* value = 0.000 has shown that there are at least 2 groups which have significant difference in the length of incision on day 7. Thus, post-hoc Mann-Whitney test was performed to see which pair groups have the significant difference (Table 2). The statistic analysis has shown that the lengths of incision wound between all pairs of groups at the 7th day are significantly different.

Table 2. Mann Whitney test of 7th day incision length

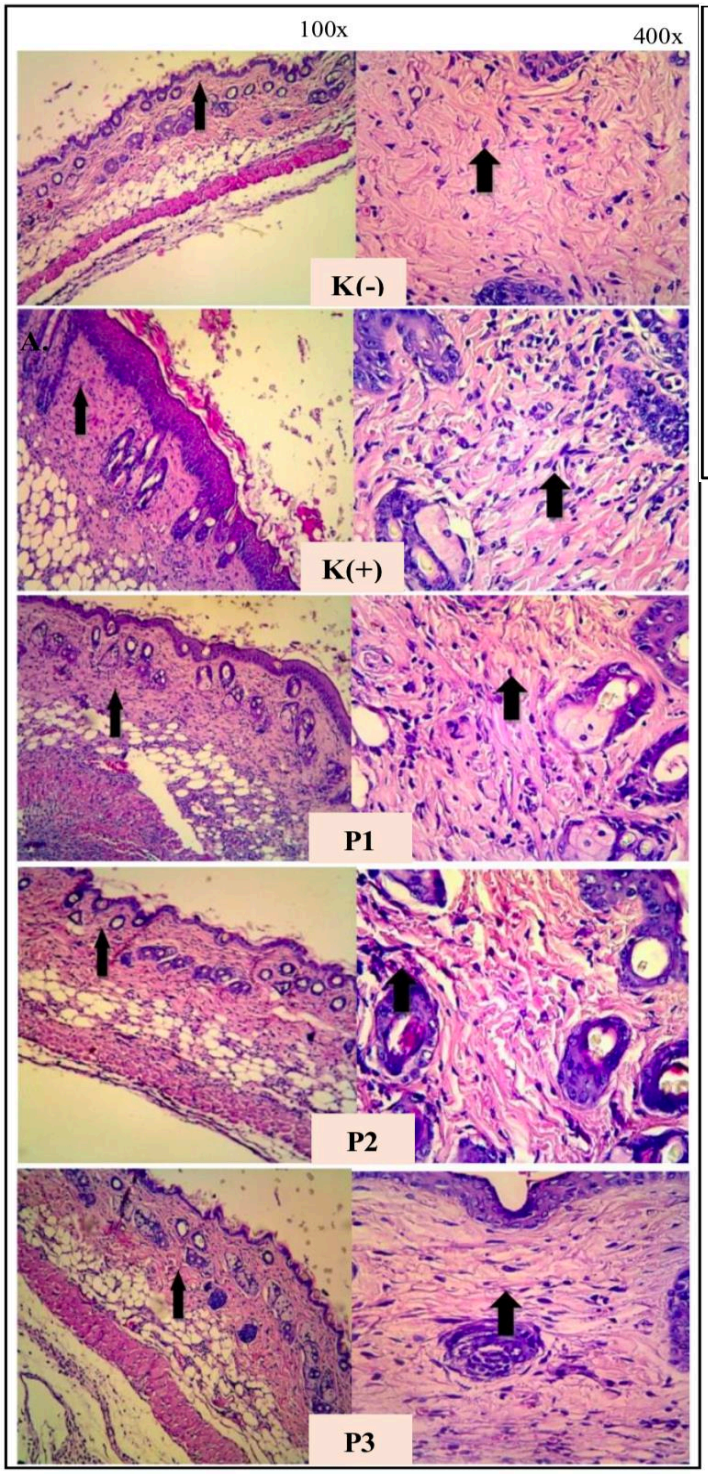
	K (-)	K (+)	P1	P2	P3
K (-)	#				
K (+)	0,001*	#			
P1	0,001*	0,001*	#		
P2	0,001*	0,001*	0,002*	#	
P3	0,001*	0,002*	0,001*	0,001*	#

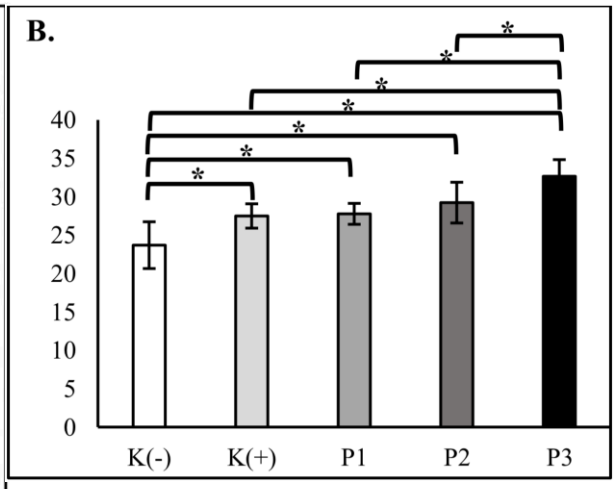
* shows significant difference ($p < 0.05$)

Microscopic analysis of fibroblasts density

Microscopic analysis was done by counting fibroblasts density in 5 viewing field of each H&E stained sections prepared from all groups (Fig. 2.). The fibroblasts observed in all groups were counted and compared by using statistical analysis.

Non-parametric statistical analysis (Kruskall-Wallis test) was used to see whether there is significant different in fibroblasts density among five groups. With Confidence Interval (CI) 95%, *p* value = 0.000 has shown that there are at least 2 groups which have significant difference in fibroblasts density. Thus, post-hoc Mann-Whitney test was performed to see which pair groups have the significant difference. Mann-Whitney analysis of fibroblast density has shown that there is significant difference in fibroblasts density between group K(-) and K(+), K(-) and P1, K(-) and P2, K(-) and P3, K(+) and P3, P1 and P3, P2 and P3 (*p* value < 0.05).


100x
400x
A.
K(-)
K(+)
P1
P2
P3


B.
40
35
30
25
20
15
10
5
0
K(-)
K(+)
P1
P2
P3

DISCUSSION

Shortening healing process by Ageratum conyzoides L. application on incision wound of male white mice (Mus musculus)

The results of this study showed wound healing acceleration in group with povidone iodine and 15%, 30%, and 45% billy-goat weed leaves extract showed significant differences compared to negative control group. Wound healing duration difference was more significant in 45% billy-goat weed group compared to those that were given 15% and 30% billy-goat weed leaves extract. Billy-goat weed leaf extract with 45% dose had better effect from positive control group of povidone iodine 10% as positive control. This means that the length of incision wound in group P3 showed the fastest healing process compared to all other groups. Billy-goat weed leaves extract contains alkaloid, flavonoid, saponin, *p*-hydroquinone, terpenoid, steroid, beta-sitosterol, saponin, tannin, and coumarin, which were effective in affecting wound healing duration by increasing cell proliferation, collagen synthesis, and epithelialization in wounds [9-11]. This study is in concordance with other previous study by Agyare*et al.* which revealed that *Ageratum conyzoides* L. leaves extract healed wound faster than other plant extracts.

Enhancing effect of Ageratum conyzoides L. topical application on fibroblasts density of incision wound of male white mice (Mus musculus)

This study has shown that fibroblast density mean in negative control group was lower compared to povidone iodine positive control group with mean difference of 3.80 cells. Even though povidone iodine can increase fibroblast density, previous study conducted by Bigliardi showed that excessive use of povidone iodine can increase allergic reaction and, in the study conducted by Nurdiantini, povidone iodine is irritative and toxic if they went through blood vessels, and excessive use can inhibit wound granulation [12-14]. Mean fibroblast density of negative control group was lower compared to treatment group P1, P2, and P3.

Previous study by Anggraeni R showed that the administration of billy-goat weed (*Ageratum conyzoides*L.) with 2% and 4% concentration on Day 3 showed that billy-goat weed leaf gel extract significantly affect the increased number of fibroblasts post-extraction in wistar rats [15]. Kaur R and Dogra NK stated that billy-goat weed leaves extract contained alkaloid, flavonoid, saponin, *p*-hydroquinone, terpenoid, steroid, beta-sitosterol, saponin, tannin, and coumarin, which were effective in affecting wound healing duration by increasing cell proliferation, collagen synthesis, and epithelialization in wounds [10,11].

Flavonoid can prevent or prolong the onset of cell death, especially fibroblast, in line with vascularization increase in wound, thus reducing lipid peroxidation. When fibroblast is protected, then it can migrate to wound area, thus increasing fibroblast density [16]. Saponin can increase monocytes proliferation, therefore it can increase the number of macrophages. Macrophages will secrete growth factors such as platelet derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF-β) which can attract more fibroblasts to wound area, thus increasing fibroblast density [17]. The same goes for billy-goat weed leaves extract that contains tannin, saponin, and flavonoid which can increase fibroblast density.

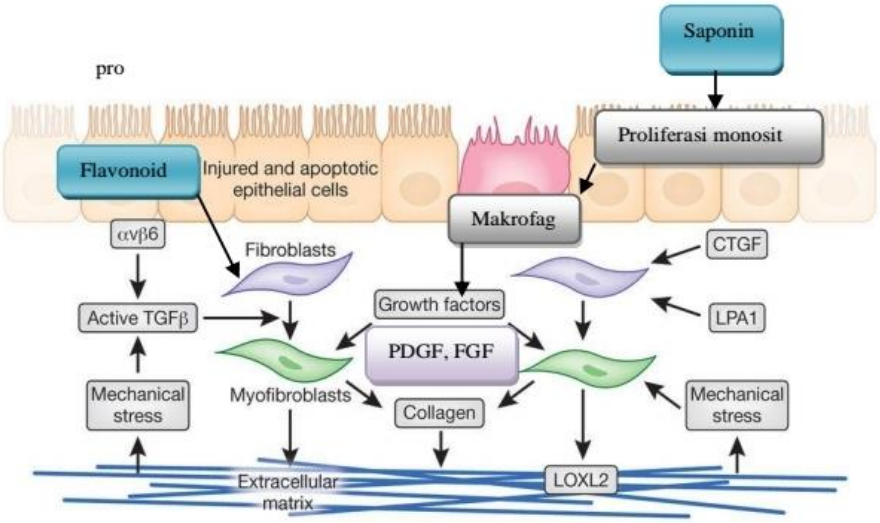


Figure 3. The role of fibroblast on wound healing [16]

The role of fibroblast in this study is undergoing repairing phase, in which fibroblast is responsible in the preparation of protein structure product which will be used during tissue reconstruction process. Fibroblast in normal state, as in this study, will perform fibroblast division which is rarely seen. However, during cell wound, fibroblast is more active in producing extracellular matrices. Fibroblast proliferation in wound healing process will naturally stimulated by interleukin-Ib (IL-Ib), platelet derived growth factor (PDGF), and fibroblast growth factor (FGF). Furthermore, Kanzaki *et al*. revealed that fibroblast migration in wound area is stimulated by transforming growth factor (TGF), which is growth factor produced by granulation tissues formed during inflammatory process [18]. Wound healing process in this study was highly affected by the role of fibroblast migration and proliferation in wound area. Besides fibroblast, there were also collagens which served more specifically in producing connective tissue matrix and with the release of substrates by fibroblast, it gives sign that macrophages, new blood vessels and fibroblasts in unity can enter wound area [18-20].

This study did not use single active ingredient, therefore other active ingredients contained in billy-goat weed (*Ageratum conyzoides* L.) leaves extract can affect the results of this study. There was no test on active ingredients, thus the amount of active ingredients that give favorable effect was not known. Toxicity level in billy-goat weed (*Ageratum conyzoides* L.) leaves extract was also not analyzed. It is recommended to analysis which active compounds favor the healing process for further analysis.

CONCLUSION

Ageratum conyzoides L. leaves extract 45% is more effective in accelerating the healing process compared to povidone iodine. The extract enhances the fibroblasts density microscopically and wound closure macroscopically which was observed from wound length.

CONFLICT OF INTEREST DECLARATION

The authors declare that there is no conflict of interest regarding the publication of this paper.

REFERENCES

1. Rachman UA, Salikunna NA. Pengaruh Lendir Bekicot (*Achatina fulica*) Terhadap Waktu Penutupan Luka Sayat (Vulnus scissum) Pada Mencit *(Mus musculus*) (Effect of snail (*Achatina fulica*) slime on incision wound closure (Vulnus scissum) of mice *(Mus musculus*)). Kedokteran JI, Program M, Pendidikan S, Tadulako U, Anatomi BP. 2015;2(1).
2. Oky M. Pengaruh Cairan Cultur Filtrate Fibroblast (CFF) Terhadap Penyembuhan Luka; Penelitian eksperimental pada Rattus Norvegicus Galur Wistar (Effect of Culture Filtrate Fibroblas (CFF) fluid on wound healing; experimental study on Rattus Norvegicus Wistar strain). Kesehat Andalas. 2012:122-127.
3. Wilson LM. Patofisiologi Konsep Klinis Proses-Proses Penyakit (Patophysiology of clinical concept of diseases). Vol 1. 6th ed. (SA Price LW, ed.). Jakarta: EGC; 2013.
4. Dewi, Prastiana S. Perbedaan Efek Pemberian Lendir Bekicot (*Achatina fulica*) Dan Gel Bioplacenton^{TM} Terhadap Penyembuhan Luka (Effect comparison of snail (*Achatina fulica*) slime and Bioplacenton^{TM} gel on wound healing). Fakultas Kedokteran Universitas Sebelas Maret. Surakarta.2010.
5. Sinaga M. Penggunaan Bahan pada Perawatan Luka di RSUD Dr.Djasamen Saragih Pematang Siantar. J Keperawatan Klin. 2012.
6. Dinkes RI. Kebijakan Obat Tradisional Nasional Tahun 2007. Kesehatan D, Indonesia R. 2007.
7. Sina I, Sugara TH, Irawadi TT, Suprapto IH, Hanafi M. Uji Aktivitas Antibakteri Fraksi Etil Asetat Daun Tanaman Bandotan (Ageratum conyzoides L) (Anti Bacteria Activity of Ethyl Acetate Fraction Bandotan leaf (Agerantum conyzoides L)). Ilmiah J. 2016;1(1):88-96.
8. Afrianti R, Nofiandi D, Ulfa W. Luka Diabetes Melitus Yang Diberikan Sediaan Krim Ekstrak Daun Bandotan (Diabetes Mellitus wound treated by billy weed extract cream). Scientia. 2016;6(1):50-58
9. Afrianti R, Yenti R, Monica H. Pengamatan Serabut Kolagen Pada Proses Penyembuhan luka Dalam Sediaan Krim Ekstrak Etanol Daun Bandotan (*Ageratum conyzoides* L.) (Observation of collagen tissue of wound healing process of *Ageratum conyzoides* L. ethanol extract cream). Scientia.2014
10. Dalimartha S (dr). Atlas Tumbuhan Obat Indonesia. Jilid 2. jakarta: Trubus Agriwidya; 2006.
11. Kaur R, Dogra NK. A Review on Traditional Uses , Chemical Constituents and Pharmacology of Ageratum conyzoides L . (Asteraceae). Int J Phaemaceutical Biol Arch. 2014;5(5):33-45.
12. Agyare C. Medicinal Plants and Natural Products with Demonstrated Wound Healing Properties. 2016. doi:http://dx.doi.org/10.5772/46845
13. Bigliardi PL, Alsagoff SAL, El-Kafrawi HY, Pyon JK, Wa CTC, Villa MA. Povidone iodine in wound healing: A review of current concepts and practices. Int J Surg. 2017;44:260-268. doi:10.1016/j.ijsu.2017.06.073
14. Nurdiantini I. Perbedaan Efek Penggunaan Povidine Iodine 10% Dengan Minyak Zaitun Terhadap Penyembuhan Luka Robek (Laceratum Wound) (Effect difference between povidone iodine 10% use and olive oil on laceration wound healing). 2017;1:223-233.
15. Anggraeni R. Pengaruh Pemberian Topikal Ekstrak Daun Bandotan (*Ageratum conyzoides*) Terhadap Peningkatan Jumlah Sel Fibroblas Pada Soket Pasca Pencabutan Gigi Tikus Wistar (Effect of topical use of billy weed (*Ageratum conyzoides*) leaves extract on fibroblasts count increase of socket pasca teeth extraction of Wistar Rats). 2013:5.
16. Vicari K. Wound Healing. Nat Biotechnol. 2013.
17. Jatana Samreen DLA. Understanding Engineered Nanomaterial Skin Interactions and the Modulatory Effects of Ultraviolet Radiation Skin Exposure. 2014.
18. Sumbayak EM. Fibroblas : Struktur dan Peranannya dalam Penyembuhan Luka. (6).
19. Fawcett DW. Buku Ajar Histologi. 12th ed. (Tambayong ABJ, ed.). Jakarta: EGC; 2002.
20. M.S Y. Efektivitas Penggunaan Jintan Hitam (Nigella Sativa) dalam Proses Percepatan Penyembuhan Luka Setelah Pencabutan Gigi. Bagian Ilmu Bedah Mulut Fak Kedokt Gigi Univ Hasanudin Makassar. 2014.