Original article

The Effect of Alfalfa (*Medicago sativa*) extract onthe number of macrophage, fibroblast, and amount of collagen in rat wound healing

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Abstract

Background: Alfalfa extract has been shown to have anti-inflammatory activity in wound healing. ***Objectives***: This study aims to investigate the effect of Alfafa on the number of macrophages, fibroblast, and collagen in the rat wound healing. ***Method***: In this post-test-only control group design study, 50 Wistar rats were randomly divided into five groups. K0 (negative control) group was treated with ointment base. K1 (positive control) group was treated with gentamicin. P1, P2, and P3 groups were treated with 10%, 20%, and 30% alfalfa extract respectively. The termination of rats conducted on day 3 and day 7 post of first treatment (5 rats/group/period). On day 3, the number of macrophage and fibroblast were evaluated using Hematoxylin-Eosin staining. On day 7, the number of collagen was evaluated using Sirius-red staining. The data were analyzed using one way ANOVA followed by Tukey's post hoc test. ***Result***: The result, showed a significant difference in the number of macrophages between the negative control treated group and K1 groups. The number of fibroblasts was significantly different between the P3 group and the P1, K1 group. There was a significant difference in the number of collagens between negative control andtreated group, and between the positive control and P2, P3 group. ***Conclusion***: The administration of alfalfa extract at the dose of 20% can accelerate wound healing by increasing the number of fibroblasts and collagen fibers and decreasing number of macrophages.

Keywords: alfalfa; macrophage; fibroblast; collagen

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Introduction

Wounds are often caused by different factors including surgery, trauma, extrinsic factors (such as distress, burns, and cuts), and pathologic disorders such as diabetes or vascular disease. Wound healing is a dynamic and complex process that occurs in simultaneous and overlapping phases, with the appropriate stages of inflammation, proliferation, and remodeling happening at the appropriate time. The skin is the largest organ in the human body and is involved in a variety of functions including hydration, protection from toxins and pathogens, vitamin D production, excretion, and heat control. As a result, severe skin injury can be fatal. *Medicago sativa* (alfalfa), a natural source of bioactive compound, has been shown to be useful in the prevention and treatment of a variety of diseases. Isoflavonoids with estrogenic activity, flavonoids, saponins, sterols, phenolic acids, and fatty acids are the primary bioactive substances found in plant aerial sections[2]. The extracts of alfalfa aerial parts have been shown to have a a moderate anti-inflammatory effect[1].

The human body will often respond to an injury by initiating the wound healing process and producing scar tissue. Scars are excellent tissues for neo-formation. However, they do not replicate the physiological tissue properties and functions replaced by them. Neo-formation in humans repairing the inevitable damage replaces the missing tissue with an extracellular matrix composed primarily of

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fibronectin and collagen types I and III. Certain skin elements, subepidermal appendages, hair follicles, and glands may not recover after a severe injury. The scar tissue matrix, referred to as granulation tissue, is the final product characterized by a dense population of fibroblasts, granulocytes, macrophages, capillaries, and collagen fibers[2].

Inflammation lasts five days from the time of the injury.Neutrophil cells dominate this process, resist pathogens, and exclude debris from the cellular matrix and unidentified substances. The presence of chronic neutrophils exacerbates wound healing. It can lead to acute injuries, resulting in massive wounds[3]. If the wound is not infected, neutrophil amounts decrease, accompanied by phagocytosis and macrophage formation. Pro-inflammatory cytokines, anti-inflammatory cytokines, and growth factors such as transforming growth factor (TGF-β), tumor necrosis factor (TNF-α), interleukin 1 (IL-1), interleukin 6 (IL-6), and interleukin 8 (IL-8), as well as proteinase (Collagenase Enzyme), matrix metalloproteinase (MMPs), and prostaglandin E2, are all secreted by macrophages. Proliferation begins on day 3 and extends until day 21, where the platelets and granulation tissue (new blood vessel/angiogenesis, fibroblasts, and macrophages) replacethe provisional matrix. Angiogenesis is essential because it provides oxygen, nutrients, and a required mediator for the healing process to the wound site.

Vascular Endothelial Growth Factor (VEGF) secretes growth factors such as fibroblast growth factor (FGF), TGF-, and Angiotensin during angiogenesis in endothelial cells. These growth factors promote angiogenesis in endothelial cells. After sufficient tissue development, endothelial cell migration and proliferation slow, and excess cells undergo apoptosis. Keratinocyte cells proliferate and migrate concurrently with angiogenesis, forming an epithelium to protect the damaged region. Macrophages release the growth factors PDGF, FGF, and TGF-, which stimulate fibroblasts to proliferate, migrate, and produce extracellular matrix during the proliferation process (collagen, elastin fibers, and reticular fibers). Collagen is the primary protein that makes up the extracellular matrix and is necessary for wound healing, scar formation. Collagen produced on day 3 of wound healing reaches a peak on day 7 and begins to degrade on the 15th day[4].The scar tissue matrix is the finished product with a dense mass of fibroblasts, granulocytes, macrophages, capillaries, and collagen fibers[5].

The antibiotic prophylaxis including topical application of gentamycine have been extensively used. Although it has been shown to improve the therapeutic effectiveness and reduce wound healing time,it is likely to contribute to the development of antibiotic resistance. It suggest an urgent need for a safe, practical alternative wound-healing options[6]. Our hypothesis was that alfafa use may accelerate the wound healing process. Thus, the purpose of this *in vivo* study was to evaluate topical effectiveness of cream containing alfafa extract on on the number of macrophage, fibroblast, and amount of collagen in rat wound healing.

Materials and Methods

Subjects

The study was carried out on 10-14 week-old healthy male Wistar rats, weighing 200-350g. All 50 rats were divided into five groups, with ten rats in each group. K0 group (negative control) was treated with ointment base, treatment groups of P1, P2, P3were treatedwith topical alfalfa extract at a different concentration 10%, 20%, 30%, respectively, and K1 group (control positive) was treated with gentamicin cream.

Alfalfa cream preparation

Alfalfa plants were dried at 500ºC for more than 8 hours. The dried plant was extracted using the maceration with 5000 ml solvent (96% ethanol). Alfalfa extract was formulated into a cream with a base mixture from vaseline, albumin, and adapalene ointment at different concentration (10%, 20%, and 30%) of alfalfa crude extract.

Table 1. Compositions of alfalfa cream formulation

Composition of alfalfa extract	Alfalfa extract (grams)	Vaseline albumin (grams)	Adapalene (grams)
10%	5	38.25	6.75
20%	10	34	6
30%	15	29.75	5.75

Pretreatment and treatment with alfalfa cream

All rats were anesthetized subcutaneously with 0.2 cc ketamine(Rottex medica, Germany). Insicinal skin was created in the the dorsal region with an scalpel device 3 cm in length and 0.5 m subcutaneous layerin depth. On days 3 and 7, rats were terminated, and skin tissue samples were fixed in 10% formalin. The termination was carried out at an animal laboratory in Universitas Wahid Hasyim's Faculty of Pharmacy. The cream was applied topically to the dorsal region

of 4 groups of rats at a dose of 0.5g twice daily for 7 days using an ivory spoon.

Histological preparation

Skin tissue samples were obtained and fixed in 10% formalin for 24 hours,. The fixation stage was used to prevent tissue destruction, interrupt metabolic processes, preserve cytological and histological components, and harden soft tissue in preparation for coloring. the dehydration stage was performed using an ethanol solution. At 4ºC, the dehydration mechanism was carried out under agitated conditions. Samples from each group were embedded in paraffin and then stored at temperature of 58-60ºC in an incubator. Then the tissue was sectioned with micrometo obtain 5µm-thick paraffin section . For 24 hours, the preparations were stored at 38-40ºC in an incubator[7].

Histopathological observation

Samples contain skin tissue biopsy was taken to the anatomy pathology laboratory of Sultan Agung Islamic Hospital for the evaluation of the number of macrophages and fibroblasts using hematoxylin-eosin staining using on day 3. On day 7 the sections were stained with Picrosirius red for observation of collagen fibers distribution. Olympus BX51 microscopy at 100x and 400x magnification was used to observe skin tissue biopsy from rats. Macrophage cells were visualized in violet color and then quantitatively counted in a 10x field of view. Fibroblast cells were visible in violet color surrounding the connective tissue fibers, then quantitatively counted at 10x the field of view using image roaster software. Collagen observations made at 10x and 40x magnification under an Olympus microscope ((BX-51; Olympus, Tokyo, Japan). Optical photographs were taken and

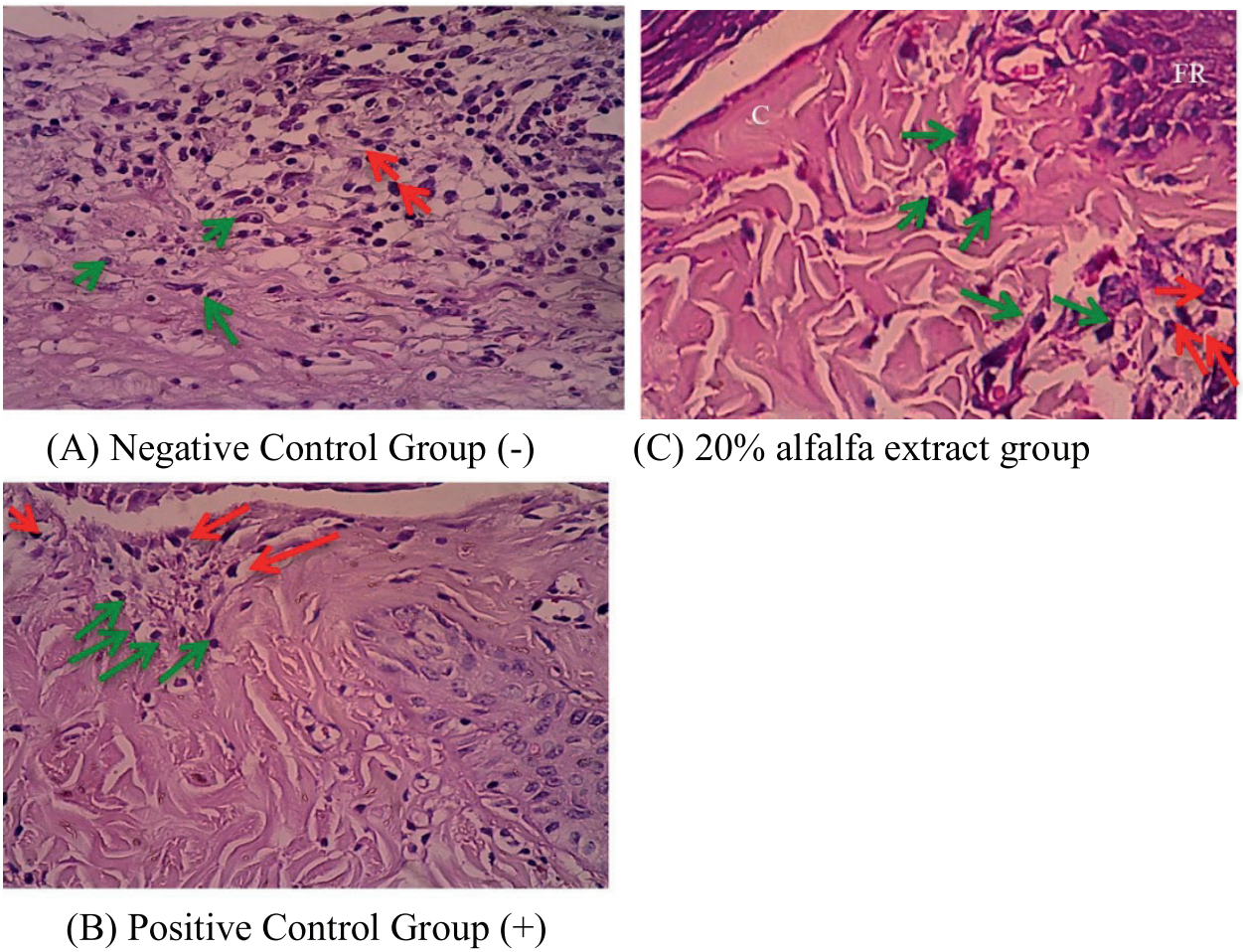

Figure 1. Hematoxylin-eosin staining of skin tissue showing the macrophage (red arrows) and fibroblast (green arrow) in groups Negative control group, Possitive Control group (B) Alfafa Group (C), (magnification: 400X)

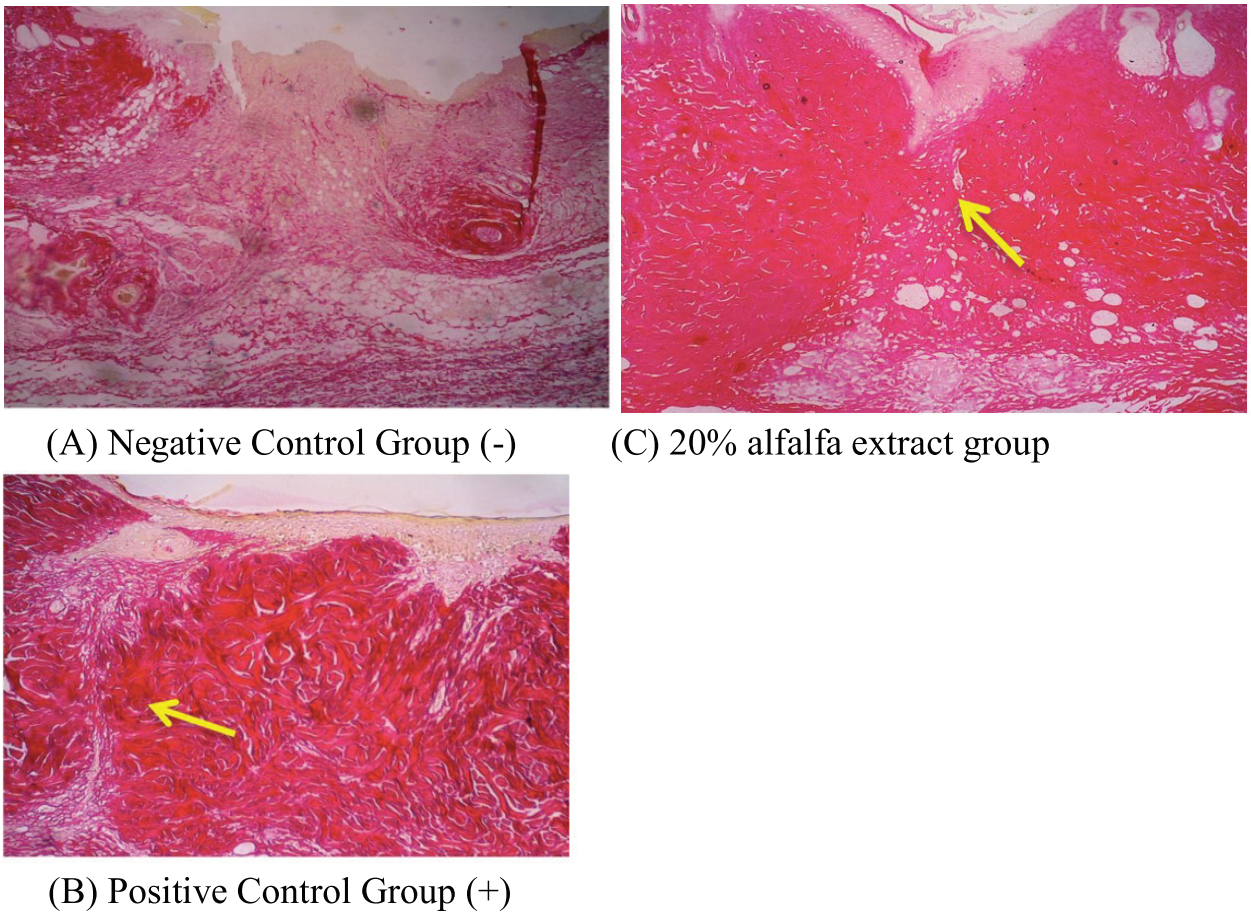

Figure 2. Histopathological section of skin stained with sirius red dye showing collagen density (yellow arrow) in Negative control group (A), Possitive Control group (B) Alfafa Group (C) on day 7 (10x magnification : 10x).

analyzed using Adobe Photoshop CS3 and ImageJ to calculate amount of collagen expression

Data analysis

Data were expressed as Mean ± Standard deviation (SD). All data were tested for normality using a Shapiro-Wilk's test and Levene's test to examine homogeneity of variance. Fibroblast data were analyzed using one-way analysis of variance (ANOVA)followed by Tukey's post-hoc test. Differences between groups were assessed using Kruskal Wallis and the Mann-Whitney U test. The statistical analyses were performed using the Statistical Package for Social Sciences version 11.0 (SPSS Inc., Chicago, IL, USA).A difference in the mean values of $p < 0.05$ was considered to be statistically significant.

Ethical clearance

All the study protocols involving animals were approved by *KomisiEtikPenelitianKedokteran dan Kesehatan* (Medical and Health Research Ethics Committee) Faculty Medicine of Universitas Islam Sultan Agung.

Results

The negative control group contained well-characterized macrophage cells but no visible fibroblasts, whereas the positive control group contained abundant fibroblasts and only a few macrophage cells. The other group almost all showed fibroblasts and a few macrophages due to differentiation. After the day 7 the rats were terminated to get skin tissue slices for histopathological examination with Sirius red staining, observed under a microscope with a at10x magnification the calculated amount of collagen. Collagen accumulation has begun to appear in all groups, notably the 20% and 30% alfalfa extract groups. It appears that the collagen density was getting more pronounced. Collagen formation was not apparent in the negative control group.

Table 2. The number of macrophages, fibroblasts, and amount of collagen

Variable	group					p-value
	K0	P1	P2	P3	K1	
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	
Numberof Macrophage	315.2±204.3	75.8±51	47.2±26.2	105.2±63.6	141.6±67.9	
Shapiro-Wilk	0.199	0.134	0.679	0.052	0.782	>0.05*
Levene						0.000**
Kruskal Wallis						0.011****
Number of Fibroblast	86.6±32	43.6±19.2	65±40.4	106.8±36.6	39±16.17	
Shapiro-Wilk	0.411	0.699	0.438	0.105	0.055	>0.05*
Levene						0.128**
Anova						0.010***
Amount of Collagen	24.21±5.2	46.09±5.6	46.9±4.8	44.7±11.2	38.37±8.2	
Shapiro-Wilk	0.003	0.813	0.317	0.960	0.003	</>0.05*
Levene						0.227**
Kruskal Wallis						0.000****

Discussion

This research aims to demonstrate the efficacy of cream containingalfalfa extract on the site of the incision. Since the negative control group had no anti-inflammatory, the number of macrophages in KO remains very high compared to other groups. In comparison to alfalfa extract as an anti-inflammatory, it contributes to infection prevention by reducing the role of macrophages. There was a significantdifferencein number of macrophagebetween the negative control and treatment groups.A study showed a similar finding that flavonoidsfrom ginger extract can inhibit macrophage cell growth. As three days of red ginger extract administration suppressed the inflammatory mechanism in the injury, the inflammatory period ended[8]. There was a difference between K1 and P2, but not between K1 and P3.A higher concentration of aromatic ginger (*Kaempferia galanga*) essential oil has been shown to suppress phagocytic activity. This is because the more significant the concentration of aromatic ginger essential oil, the more active compounds it contains, the more disruptive the receptors on the surface of macrophage cells become, reducing phagocytosis. The study showed that 10% aromatic ginger essential oil (moderate dose) significantly decreased the number of macrophages[9].

Macrophages induce fibroblasts into the proliferation phase, fibroblasts responsible for producing connective tissue-forming fibers such as elastin, collagen, and reticular fibers used during the tissue reconstruction process. Epithelialization occurs due to the expression of TGF-β, which has occurred since the beginning of tissue damage. Macrophages and vascular endothelial cells constitute the formation of granulation. As a result, the formation of new blood vessels with the help of FGF and VEGF. At the end of the fibroblast phase, the skin tissue decreases in the number of fibroblast cells. Skin tissue of the negative control group (K0) still had very high fibroblasts. These findings are in agreement with that of previouse study showing that negative control group had higher fibroblast than the group given *Apium graveolens* Linn therapy[10]. This finding suggests that the proliferation phase of the negative control group has not entirely been completed. The different test results found there was differences in P3 with P1 and K1.

There was a statistically significant difference in amount of collagen between the treatment and gentamicin cream, indicating that the treatment group using alfalfa extract cream could replace gentamicin cream in treating wounds. There is a difference between K1 and P3 because K1 is a treatment with antibiotics as an antibacterial, whereas P3 treatment with alfalfa extract has an active compound with more than antibacterial properties. The absence of statistically significant differences in collagen density between P2 and P3illustrates that both these

treatments give results comparable to the wound healing process in terms of collagen density.Since small doses have the same effect as large doses, it is preferable to receive small doses. This finding is similar to that of the recent study on the effect of *Apium graveolens* Linn on fibroblast and collagen density in incision wound healing. There were significant differences in the fibroblast density area between the *Apium graveolens* Linn extract 50% group (70%)and the control group[10].

On the other hand, the P3 group still showed a high number of fibroblasts, which could occur because, in the previous phase, the number of macrophages was still high, so that P3 had not yet completed the fibroblast phase. The faster the completion of the fibroblast phase, the faster the formation of collagen produced by fibroblasts.Collagen plays a role in the maturation phase. Collagen is synthesized oxidatively through the hydroxylase. K0 showed differences compared to P1, P2, P3, and there was difference between K1 and P2 and P3. Similar results were reported with increase number of collagen in the treatment group compared to the control group due to tannins, steroids, flavonoids, saponins, terpenoids, phenolic compounds, and anthraquinone in the wildflower extract of *Hageniaabyssinica* (Bruce) J.F. Gmel. The active compound in the *Hageniaabyssinica*is an antibody serving as antibiotics and can stimulate the growth of new cells in wounds showing fast wound contraction, and reduced epithelization period after skin incision[11].

An incisised wound causes tissue damage that stimulates vascular response and cellular activity in the body. Alfalfa has an anti-inflammatory and antimicrobial activity for wound healing, has active compounds such as flavonoids, saponins, and tannins, which play a role in wound healing[12]. Flavonoids' lipophilic composition disrupts the microbial membrane in the wound, thereby protecting it from infection[13].The active compounds in alfalfa can increase IFN-γ proliferation and stimulate macrophage phagocytosis activity, lysosomal enzyme activity, and TNF-α release and modulate the release of nitric oxide by macrophages. Activated macrophages will increase their efficiency as APC cells. APC will be given priority to lymphocytes, so this process is necessary to initiate the immune response. Macrophages release cytokines and chemokines in response to bacterial components that initiate the inflammation[14].Flavonoids can accelerate wound healing by migrating epithelial cells to the core of the wound as it expands, increasing myofibroblast activity and further epithelialization[1]. Saponins contribute to granulation and collagen tissue formation by activating the cytokine TGF-β, which helps in wound contraction[15].

The active alfalfa compound functions to accelerate wound healing. Flavonoids convert procollagen into collagen, thereby accelerating collagen biosynthesis. The stimulation of collagen biosynthesis is thought to be due to inhibition of the metalloproteinase enzyme, which plays a role in accelerating the breakdown of collagen molecules[16]. This enzyme activity is slightly inhibited from increasing the amount of collagen that occurs as needed by the skin. Tannins play a role in stabilizing collagen so that wounds heal quickly[17].

After a statistical test of fibroblast density, there was a significant difference in the between group using P1, P2, and P3 treatment cream of the control group in the wound healing process. According to the theory, alfalfa contains various active ingredientsincluding flavonoids, namely apigenin, which can be anti-inflammatory.

This study has several limitation. First, This potential for anti-inflammatory and antioxidant causes in the treatment group found a smaller area of fibroblast density than the control group[18]. The researchers examined the number of macrophages and fibroblasts only on day 3 which is too early. The fibroblasts begin entering the wound site two to five days after woundingand their numbers peak at one to twoweeks post-wounding[19]**.** In addition, the evaluation of amount of collagen was not optimumbecause it wastoo early.The inflammatory step of the wound healing process should be observed on day 5[20].

Conclusion

The present study demonstrated that alfafa showed wound healing activity in rats which may be attributed specifically to their ability to increase the number of fibroblast and collagen and decrease the number of macrophage. Alfalfa extract had the most significant potential for wound healing at a concentration of 20%. Hence, the study scientifically

provide supports for the potential of alfafa in wound healing.

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Conflict of interest

The investigators declare that they have no conflict of interests.

Authors' Contributions

Data gathering and idea owner of this study: Yustiana Arie Suwanto, Atina Hussaana

Study design: Atina Hussaana, Chodidjah

Data gathering: Atina Hussaana, Chodidjah, Yustiana Arie Suwanto

Writing and submitting manuscript: Yustiana Arie Suwanto, Atina Hussaana, Chodidjah

Editing and approval of final draft: Atina Hussaana

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