Effect of Combination of Soybean and *Phaleria macrocarpa* Ethanol Extract on IL6, TNFα, VEGF and Fibroblasts in Mice Exposed to UVB

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ABSTRACT

UV exposure causes inflammation and the generation of reactive oxygen species, both of which contribute to skin aging. The purpose of this research was to determine how a combination of *Phaleria macrocarpa* extract and soybean extract affected the number of fibroblasts, VEGF, IL-6, and TNF alpha expression, and blood levels of IL-6 and TNF alpha in UV-B-exposed mice. In this study, mice were placed into four groups: one control group, three treatment groups, and a combination of *Phaleria macrocarpa*:soybeans at a 1:1 ratio (com group). The mice were euthanized on days 5 and 21 for histological preparations and then examined under a light microscope. Using an Olympus C-21 microscope with an Optilab Advances camera at 1000x magnification, the fibroblast was studied by counting the number of fibroblast cells per field of view. The immunohistochemical approach was performed to analyze the expression of VEGF, IL-6, and TNF- in skin tissue. The ELISA technique was used to quantify the levels of IL-6 and TNF-alpha. SPSS ver 21 was used to analyze the data. On days 5 and 21, the number of fibroblasts and expression of VEGF, IL-6, and TNF alpha were significantly higher in the combination group than in the control, *Phaleria macrocarpa*, and soybean treatment groups. However, there was no significant change in IL-6 and TNF alpha levels across groups on days 5 and 21 (p > 0.05). Finally, a 1:1 mixture of *Phaleria macrocarpa* and soybeans reduced the number of fibroblasts and the production of VEGF, IL-6, and TNF alpha on days 5 and 21, but not in serum levels.
Key words: Inflammation, UV B radiation, Skin wound.

INTRODUCTION

Indonesian biodiversity has the potential to lead to the discovery of novel chemicals for medicinal development. The utilization of plant-derived compounds is scientifically verified and extensively traded. As a result, it needs ongoing study. Soybean plants, for example, are high in protein (39.4%-44.4%), oil (14.0%-18.7%), free fatty acids (31-71 mg/100 g), and triglycerides (90.1-93.9 g/100 g).[1] Genistein and daidzein are the primary isoflavones found in soybeans. Isoflavones are known as phytoestrogens, which means estrogen obtained from plants, due to their comparable structure and action to estrogen.[2] Soybean isoflavones may also act as an antioxidant, preventing oxidation, which causes photoaging and skin cancer.[3] According to one research, soybean extract includes isoflavone active compounds that may prevent keratinocyte mortality caused by UV radiation as well as inhibit hydrogen peroxide in cells.[4] TGF-, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) activities are all stimulated by isoflavone treatment. VEGF levels may influence wound healing, re-epithelialization, angiogenesis, and granulation tissue production during the remodeling phase.[5] An extract of *Phaleria macrocarpa* (Thymelaceae family) includes active components such as alkaloids, saponins, flavonoids, and polyphenols.[6] A cytotoxic effect of *Phaleria macrocarpa* extract on MCF7 cells (breast cancer cells) has been shown.[6] Cell death is caused by the cytotoxic action in both aberrant and normal cells. Phaleria macrocarpa extract is selective against normal cells and acts as an immunostimulant to counteract the immunosuppressive impact. Given the plant's synergistic effects, a study to evaluating the efficacy of combining the two plants with the optimal dosage of each plant to enhance VEGF levels, increase the number of fibroblasts, reduce TNF-, and decrease IL-6 is required.

According to the Indonesian health profile for 2018, the elderly population totaled 24,754,444 people. It is estimated that the elderly population in Indonesia will triple by 2050, compared to 2016. According to the 2015 Central Java Health Profile, it was 7.82, and in 2014 it was 7.63. According to the Central Java Statistics Agency, the elderly population has increased by 0.2%. Meanwhile, according to the health profile of Semarang in 2015, it was 7.82%, while in 2016, it was 8.02%, from 2015 to 2016, there was an increase of 0.20%. The majority of elderly women experience skin aging, such as wrinkles or fine lines that appear in the corners of the eyes, on the forehead, around the lips (facial wrinkles), and dryness of the skin. In Europe, an open multicenter study conducted a study on women with a maximum age of 65 years, with a total of 234 women who had used isoflavones for 3 years since menopause. The findings of the study showed that the use of isoflavones reduced facial wrinkles by 22% and skin looseness by 24%. Meanwhile, postmenopausal women who did not use isoflavones had an increase in skin dryness and roughness. Meanwhile, postmenopausal women who did not use isoflavones had increased skin dryness and roughness.
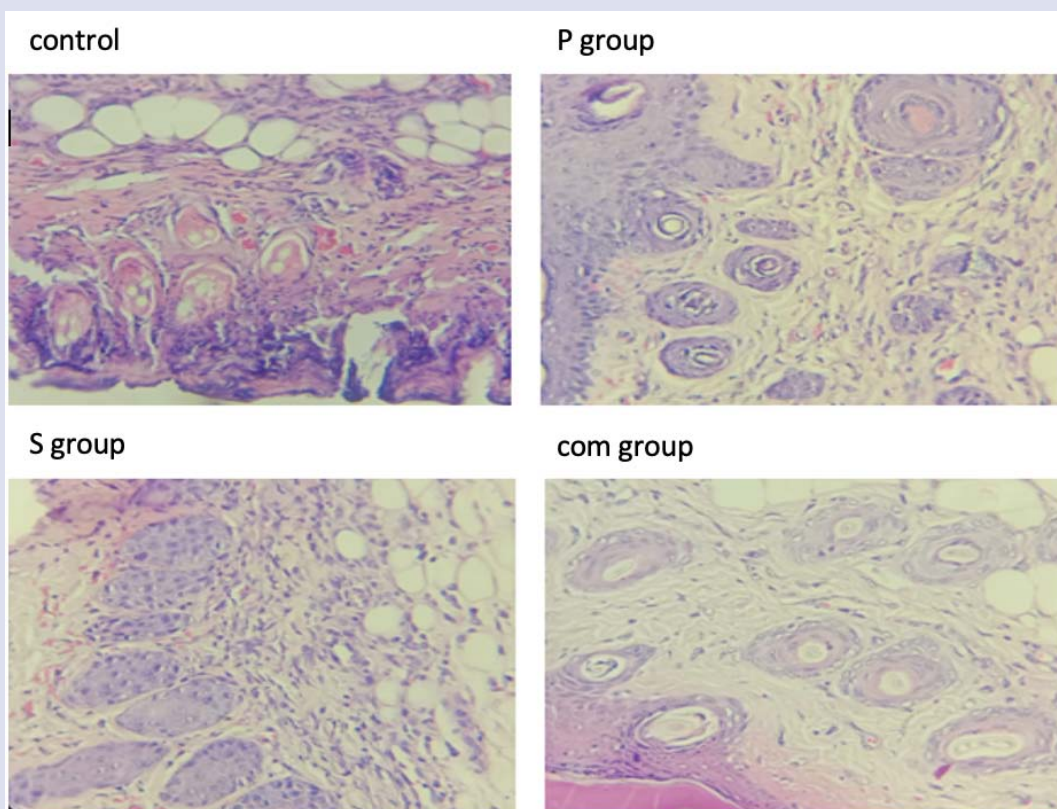
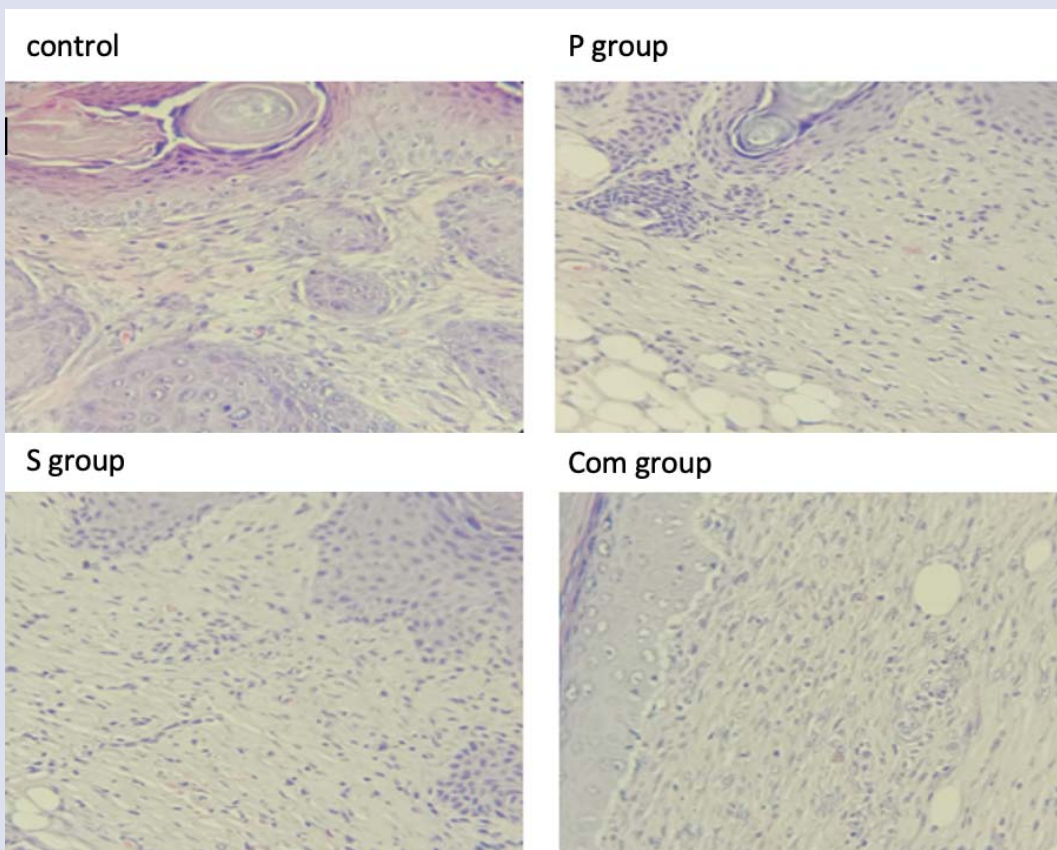**Figure 1:** Histopathology of skin on day 5 after treatment showing the number of fibroblast cells.

Figure 2: Histopathology of skin on day 21 after treatment showing the number of fibroblast cells.
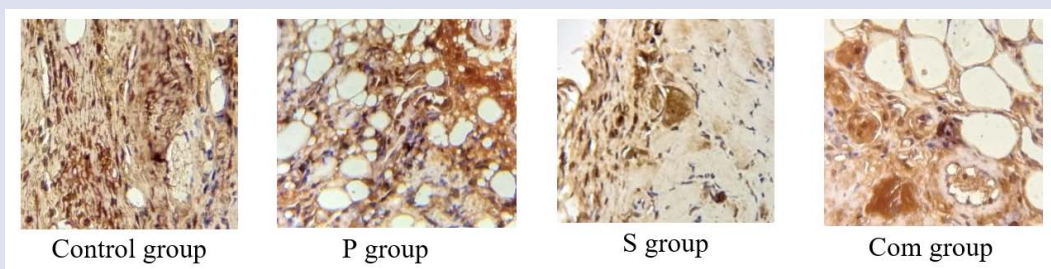
Figure 3: Expression of VEGF in skin tissue in all groups on day 5. The expression of VEGF shown purple color in the cytoplasm of dermis and epidermis cells.
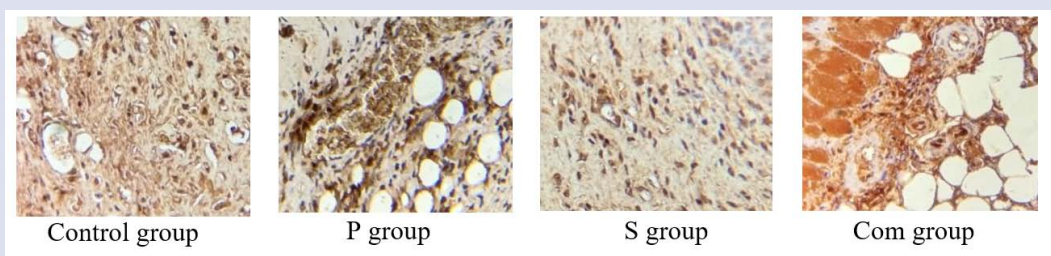

Figure 4: Expression of VEGF in skin tissue in all groups on day 21. The expression of VEGF shown purple color in the cytoplasm of dermis and epidermis cells.
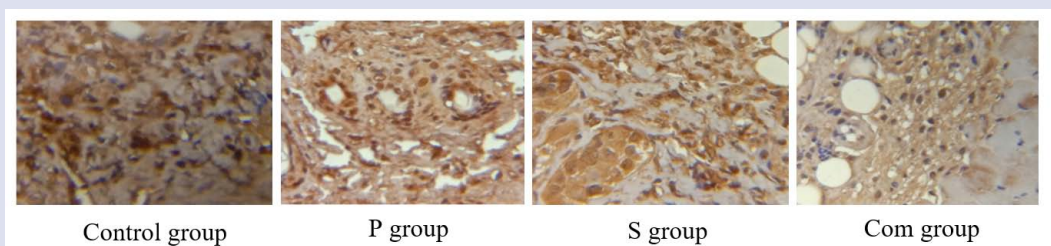

Figure 5: Expression of IL-6 in skin tissue in all groups on day 5. The expression of IL-6 shown purple color in the cytoplasm of dermis and epidermis cells.
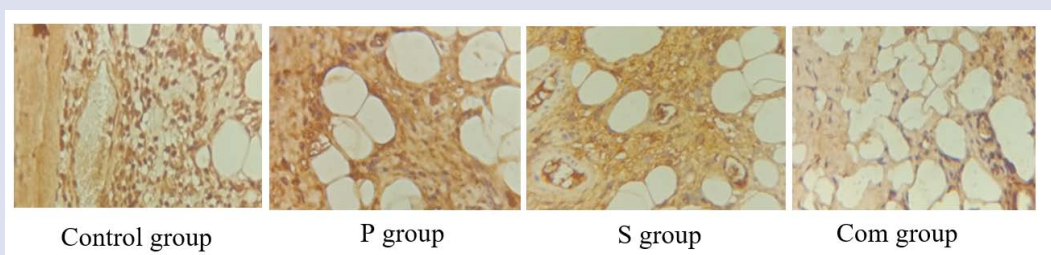
Figure 6: Expression of TNF alpha in skin tissue in all groups on day 5. The expression of TNF alpha shown purple color in the cytoplasm of dermis and epidermis cells.

Table 1: Mean number of fibroblasts on day 5 and 21.

Observation days	Mean ± SD			
	Control group (n=5)	P group (n=5)	S group (n=5)	Com group (n=5)
Day 5	17.60 ± 0.37*	14.60 ± 0.37**	14.48 ± 0.18**	13.08 ± 0.27***
Day 21	21.12 ± 0.39[a]	12.12 ± 0.23[b]	12.24 ± 0.22[b]	9.76 ± 0.33[c]

(*, **, ***) showed significant difference at 0.05 using least significant difference

(a,b,c) showed significant difference at 0.05 using mann whitney U

Table 2: Mean VEGF expression on day 5 and day 21.

| Observation days | Mean ± SD | | | |
	Control group (n=5)	P group (n=5)	S group (n=5)	Com group (n=5)
Day 5	39.80 ± 0.44*	28.8 ± 0.44**	30.4 ± 1.51***	22.6 ± 0.54****
Day 21	57.60 ± 2.38[a]	24.2 ± 1.30[b]	24.00 ± 0.00[b]	14.20 ± 0.44[c]

(*, **, ***, ****) showed significant difference at 0.05 using least significant difference

(a,b,c) showed significant difference at 0.05 using mann whitney U

Table 3: Results of the analysis of the Mann-Whitney U expression of VEGF on day 5 and 21.

Treatment groups	Significancy of VEGF expression on Day 5 (p value)	Significancy of VEGF expression on Day 21 (p value)
Control vs P group	0.000	0.000
Control vs S group	0.000	0.000
Control vs com group	0.000	0.000
P group vs S group	0.000	0.990
P group vs com group	0.000	0.000
S group vs com group	0.000	0.000

Table 4: Mean of IL-6 expression on day 5 and day 21.

| Observation days | Mean ± SD | | | |
	Control group (n=5)	P group (n=5)	S group (n=5)	Com group (n=5)
Day 5	8.60 ± 0.28*	6.88 ± 0.23**	7.00 ± 0.20**	4.36 ± 0.33***
Day 21	11.64 ± 0.62[a]	6.00 ± 0.14[b]	5.80 ± 0.24[b]	2.68 ± 0.23[c]

(*, **, ***) showed significant difference at 0.05 using least significant difference

(a,b,c) showed significant difference at 0.05 using mann whitney U

Table 5: Results of the analysis of the Mann-Whitney U on expression of IL-6 on day 5 and 21.

Treatment groups	Significancy of IL-6 expression on Day 5 (p value)	Significancy of IL-6 expression on Day 21 (p value)
Control vs P group	0.000	0.000
Control vs S group	0.000	0.000
Control vs com group	0.000	0.000
P group vs S group	0.888	0.816
P group vs com group	0.000	0.000
S group vs com group	0.000	0.000

Table 6: Mean of TNF alpha expression on day 5 and day 21.

| Observation days | Mean ± SD | | | |
	Control group (n=5)	P group (n=5)	S group (n=5)	Com group (n=5)
Day 5	7.48 ± 0.11*	4.76 ± 0.17**	4.76 ± 0.17**	4.64 ± 6.35***
Day 21	8.84 ± 0.43[a]	3.20 ± 0.40[b]	2.96 ± 0.32[b]	0.76 ± 0.22[c]

(*, **, ***) showed significant difference at 0.05 using least significant difference

(a,b,c) showed significant difference at 0.05 using mann whitney U

Table 7: Results of the analysis of the Mann-Whitney U on expression of TNF-α on day 5 and 21.

Treatment groups	Significancy of TNF-α expression on Day 5 (p value)	Significancy of TNF-α expression on Day 21 (p value)
Control vs P group	0.000	0.000
Control vs S group	0.000	0.000
Control vs com group	0.000	0.000
P group vs S group	0.525	0.975
P group vs com group	0.000	0.000
S group vs com group	0.000	0.000

Table 8: Mean of serum level of TNF alpha and IL-6 TNF on day 21.

| Observation days | Mean ± SD | | | |
	Control group (n=5)	P group (n=5)	S group (n=5)	Com group (n=5)
IL -6 (pg/mL)	62.00 ± 13.32*	64.40 ± 9.29*	37.20 ± 6.53*	57.80 ± 22.47*
TNF-α(pg/mL)	154.60 ± 8.68[a]	159 ± 5.07[a]	136 ± 20,24[a]	153.00 ± 46.21[a]

(*) showed no significant difference at 0.05 using ANOVA

(a) showed no significant difference at 0.05 using ANOVA

There have been extensive studies on these two plants. However, few studies have been conducted on the combination of extracts from the two plants with a single dose of 10 mg of soybean plant extract given orally based on the study by Ernawati (2019) and extracts of *Phaleria macrocarpa at* a dose of 0.146 mg/day.[7] with the hope that the two plants can have a synergistic effect, considering that both of them contain active substances that function as antioxidants to prevent ROS. The production of reactive oxygen species (ROS) includes superoxide ions, hydrogen peroxide, hydroxyl radicals, and peroxyl radicals. The energy of UV-B rays with free radical reactions can damage molecules in the epidermis layer and a little in the dermis layer, especially DNA and cell membranes, causing skin blood vessels to dilate and attract inflammatory cells.[8] ROS can activate cell surface receptors, such as epidermal growth factor (EGF), interleukin 1 (IL-1) and tumor necrosis factor (TNF), to induce intracellular signals and activate activator protein-1, which is made up of two subunits, c-Jun and c-Fos. Excess c-Jun in AP-1 can inhibit fibroblast proliferation, causing matrix metalloproteinase (MMP) activity to be damaged and collagen synthesis to be reduced.[9,10]

MATERIALS AND METHODS

This was an experimental laboratory with *posttest only control group design* using BALB/c mice exposed to UVB as experimental animals. The research sites are at the Central Laboratory of the UNISSULA Medical Faculty, the Anatomical Pathology Laboratory of RSI Sultan Agung and the Chemistry Laboratory of the Faculty of Medicine, UNISSULA.

In this study, the research group was randomly divided into 4 treatment groups for 5 days (1 control group and 3 treatment groups). The treatment group was exposed to UV-B rays by giving the *Phaleria macrocarpa* extract at the dose of 0.14 mg/day orally (P group), the soybean extract group at the dose of 10 mg orally (S group), the combination group of *Phaleria macrocarpa* extract and soybean extract (1:1) orally (com group). All groups were observed on day 5 and 21.

Independent variable

Soybean Extract: The soybean extract from local soybean seeds was then extracted by fractionation method. Soybean extract was given orally at a dose of 10 mg/day for 5 days to 21 days after exposure to UV-B. The dose of extract of *Phaleria macrocarpa* derived from the dried pulp of *Phaleria macrocarpa*, which was extracted with 96% ethanol solvent using the soxhletation method, a single dose of 0.14 mg/day was given. The combination of *Phaleria macrocarpa* and soybean extract (1:1)

Dependent variable

Number of fibroblast cells, expression of IL6, VEGF expression, and TNF-α in skin tissue on day 5 and 21 using immunohistochemical methods and serum level of IL6 and TNF-α on day 21 using ELISA methods.

Immunohistochemical methods

The tissue was embedded in preparation (paraffin block) with xylene 3 times for 3 minutes each. The preparations were rehydrated using 100%

ethanol, 95% ethanol and 70% ethanol for two minutes, two minutes, one minute and finally with water for one minute. Soak in peroxidase blocking solution at room temperature for 10 minutes. 4. Incubate preparations in prediluted blocking serum at 25°C for 10 minutes. The preparation was soaked in 25°C anti-p53 monoclonal antibody for 10 minutes. After washed Phosphate Buffer Saline (PBS) for 5 minutes, the preparation was incubated with secondary antibody (conjugated to horse radish peroxidase) 25°C for 10 minutes. The slide was washed with PBS for 5 minutes. The preparation was incubated with peroxidase 25°C for 10 minutes. The slide was washed with PBS for 5 minutes. The preparation was incubated the preparations with the chromogen DAB (Diaminobenzinidine) 25°C for 10 minutes. preparations were incubated with Hematoxylin Eosin for 3 minutes and washed with running water. The preparation and drip with mounting media. The preparation was covered with a coverslip. The expression of VFGF (purple in the cytoplasm) in epidermal and dermis cells was observed using a light microscope (Olympus CX 21) with 1000 x magnification connected to the Optilab pro software in 5 fields of view.

Serum level of IL-6 and TNF-α

The blood sample of mice were taken (about 1 cc) from the orbital sinus area and then centrifuged to obtain the serum. The serum level of IL-6 and TNF- was obtained using ELISA method and measured with ELISA reader at 450 nm and present as absorbance. Level of serum IL-6 and TNF-α was determined with linearity equation from standard curve.

Precondition variable

A UV-B ray device with the Kernel brand type KN-4003 PHILIP was used for irradiation which was given once a day for 5 days for 3 weeks at the dose of 1 DEM for each exposure for 50 seconds with an irradiation distance of 12 cm.

Statistical analysis

The Data were collected and presented descriptively then analyzed for normality with the Shapiro Wilk test, homogeneity test of variance between groups using Levene's t test. In this study, the mean fibroblast parameters were compared. The data were normal and homogeneous in distribution, so the oneway ANOVA parametric statistical test was performed. The results of $p < 0.05$ on day 5 day of observation, it means that there was a difference. It was followed by the Post Hoc LSD test. Meanwhile, on day 21 observation, the data were not normal, so the Kruskal Whallis test was applied.

RESULTS AND DISCUSSION

RESULTS

On day 5 and 21, the mean number of fibroblasts in combination group was significantly increased than those of control group (p=0.000). However, there was no significant difference in number of fibroblasts between P and S groups (table 1).

The results of the above data visually showed that the number of fibroblasts on day 5 in the control group compared to P, S and com

group have a high number while on day 21 also showed an increase in the number of fibroblasts in control group. The data for each group were tested for normality and homogeneity using the Shapiro Wilk test and homogeneity with the Levene test showed that all groups had p value > 0.05, meaning that the data were normal and homogeneous. Then the Parametric Statistical test was carried out, namely One Way ANOVA on day 5 of observation, while the fibroblast data on day 21 the data was not normally distributed so that a non-parametric test was carried out, namely the Kruskal Wallis test. Based on the results of the Post Hoc, it can be concluded that the number of fibroblasts in P group and S group showed no significant difference because of the value (p 0.05) but if both were compared with the control group, all of them showed a significant difference with p value = 0.000.

There was no significant difference in the number of fibroblast cells between P and S group. Based on the results of the Mann Whitney test on day 21, the number of fibroblasts between the control group and all treatments showed a significant difference.

VEGF expression

On day 5, the expression of VEGF in combination group was significantly increased than those of control group (p=0.000). On day 21 the expression of VEGF in skin tissue showed a significant difference among groups (p<0.000). However, there was no significant difference between P dan between P and S groups (p=0.99). In combination group, the expression of VEGF in skin tissue was decreased significantly compared to control group. P group, and S group (p<0.0001) (table 2).

The results showed that on day 5 the combination treatment group showed the lowest IL-6 level, this also happened on day 21, when viewed in terms of the ratio of the control group on day 5 and day 21, the IL-6 level value was very high compared to the control group.

On day 5, control group had the highest VEGF expression, followed by the P and S groups, while the lowest was seen in the com group.

The statistical analysis of normality and homogeneity tests using the Shapiro-Wilk test and homogeneity with the Levene test showed that all treatment groups had normal data, p value > 0.05 but the control group p < 0.05, meaning that the data was not normal while the results of the Levene test were in the control group. as well as treatment p value > 0.05 then the data is homogeneous. Then a non-parametric statistical test was carried out, namely the Kruskal Wallis test on day 5 of observation while the VEGF data on the 21st day the data was not normally distributed so that a non-parametric test was carried out namely the Kruskal Wallis test showing p value = 0.000 so that between groups there was a significant difference and followed by Mann Whitney test on day 5

Interleukin 6 (IL-6)

The level of IL6 levels both on day 5 and day 21 shows the lowest value in the combination treatment group while the highest value in the control group.

The data continued with statistical analysis to see the distribution of the data to see normality and homogeneity. With the Shapiro test the results showed that only in the combined group the P value < 0.05 so that the data was not normally distributed but the Levene test results showed a p value > 0.05, meaning that the data was homogeneous. Furthermore, a non-parametric difference test was carried out using the Kruskal Wallis test showing the value of P = 0.001 meaning that there was a significant difference between all groups, while on day 21 the data was normally distributed and homogeneous (p> 0.05), then the A test was carried out with the result p = 0.000 so that the bivariate test is continued, this can be seen in the table below.

The results of non-parametric analysis for IL6 observations on day 5 and parametric statistical analysis for day 21 showed that there was

a significant difference (p=0.000) between the control group and the treatment group. However, there was no significant difference between P and S groups (P>0.05). This means that the administration of soybean extract compared to the administration of *Phaleria macrocarpa* extract did not show any significant difference.

Tumor necrosis factor alpha (TNF- α)

The results above visually showed that the level of TNF-α on day 5 in the control group showed the highest value compared to P, S, and com groups while on day 21 the control group only showed an increase in the level of TNF-α on the contrary in the treatment group. decreased for all treatment groups. The data for each group were tested for normality and homogeneity using the Shapiro test Wilk and homogeneity with Levene test showed that all groups p value <0.05 means that the data was not normal and not homogeneous. Then the non-parametric statistical test, namely the Kruskal Wallis test, on day 5 of observation showed p = 0.02 while the TNF-α data on the 21st day the data was not normally distributed so that a non-parametric test was carried out, namely the Kruskal Wallis test. The results of the analysis can be seen in the table below.

On day 5 and 21 observations, the results of the bivariate analysis of all control groups compared to the treatment group showed a significant difference (p value = 0.05). there was no significant difference between *Phaleria macrocarpa* compared to the soybean group.

Level of IL-6 and TNF alpha in blood serum

The above data visually showed fluctuates among the groups. It was necessary to test for normality using the Shapiro Wilk and test for homogeneity using the Levene test. there was no significant difference among the groups (p value > 0.05).

DISCUSSION

The results of the study showed that the number of fibroblasts for all groups showed a significant difference when compared to the control group, but when compared with the group receiving soybean extract or the group that was given the *Phaleria macrocarpa* extract alone, it was proven that the two groups had no significant difference. However, if given a combination of soybean extract and *Phaleria macrocarpa* extract, there was a significant difference in all groups on the number of fibroblasts. The results of the paired T test for the difference in mean in the groups observed on day 5 and day 21 on the number of fibroblasts. Statistical analysis showed that there was no difference because p > 0.05. Fibroblasts contribute to wound healing during the proliferation stage. The participation of fibroblasts in the healing process is critical since they are responsible for creating protein structural products that will be utilized throughout the tissue regeneration process.[11] Normal fibroblast division activity is limited, but when there is damage, these cells seem to be more active in creating extracellular matrix. Interleukin-Ib (IL-Ib), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF). Keratinocytes along the wound edges exhibit morphological alterations after the occurrence of the wound. The epidermis thickens as well as the marginal basal cells expand and migrate to meet the gaps in the wound in injured skin followed by the fibroplasia process, which involves fibroblast proliferation, fibrin clot migration to the injured area, and the formation of new collagen and other proteins matrices involved in the formation of granulation tissue.

The statistical analysis in this study showed that the combination group on the 5 showed more fibroblasts compared with day 21. This was associated with cytokine levels and growth factor. Fibroblast cells are involved in producing growth factors that affect growth and differentiation. The wound healing process, especially in the proliferative phase, fibroblasts will enter the wound from the edges and migrate to the center in response to cytokines and growth factors.

Fibroblasts also synthesize proteins, such as collagen which forms collagen fibers, proteoglycans, glycosaminoglycans, fibronectin, reticulin, elastin and glycoproteins from the extracellular matrix. The expression of VEGF in the soybean group (S group) was higher than that of *Phaleria macrocarpa* group (P group) and the combination group. It might be due to imbalance in ROS production after UVB exposure and neutralization of the antioxidant system. an external stressor, which is played by mesenchymal stem cells in the deep dermis and stem cells in the epidermis decreased VEGF expression,

In the combination group *Phaleria macrocarpa* and Soybean VEGF expression was found lowest and different from all these groups. It was likely that there was a synergism of the content of *Phaleria macrocarpa* and soybean in protecting UVB exposure, DNA repair and also the involvement of the immune system, there was a decrease in inflammation leading to decreased expression of VEGF keratinocytes.[12] On day 21, the expression of VEGF in the control group was the highest, followed by the S group, P group and com group, respectively. Based on statistical analysis. The control group is different from the treatment P, S, and com groups but in the S group was no significant difference compare to P group, it is possible that the active substance content in soybean and MD as exogenous antioxidants and is supported by an endogenous antioxidant system so that it can reduce the expression VEGF was statistically the same, but clinically the expression of VEGF in the MD group was lower.

CONCLUSION

Combination of *Phaleria macrocarpa* and soybeans extracts reduced the number of fibroblasts and the production of VEGF, IL-6, and TNF alpha on days 5 and 21, but not in serum levels.

ACKNOWLEDGEMENT

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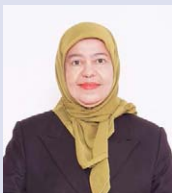
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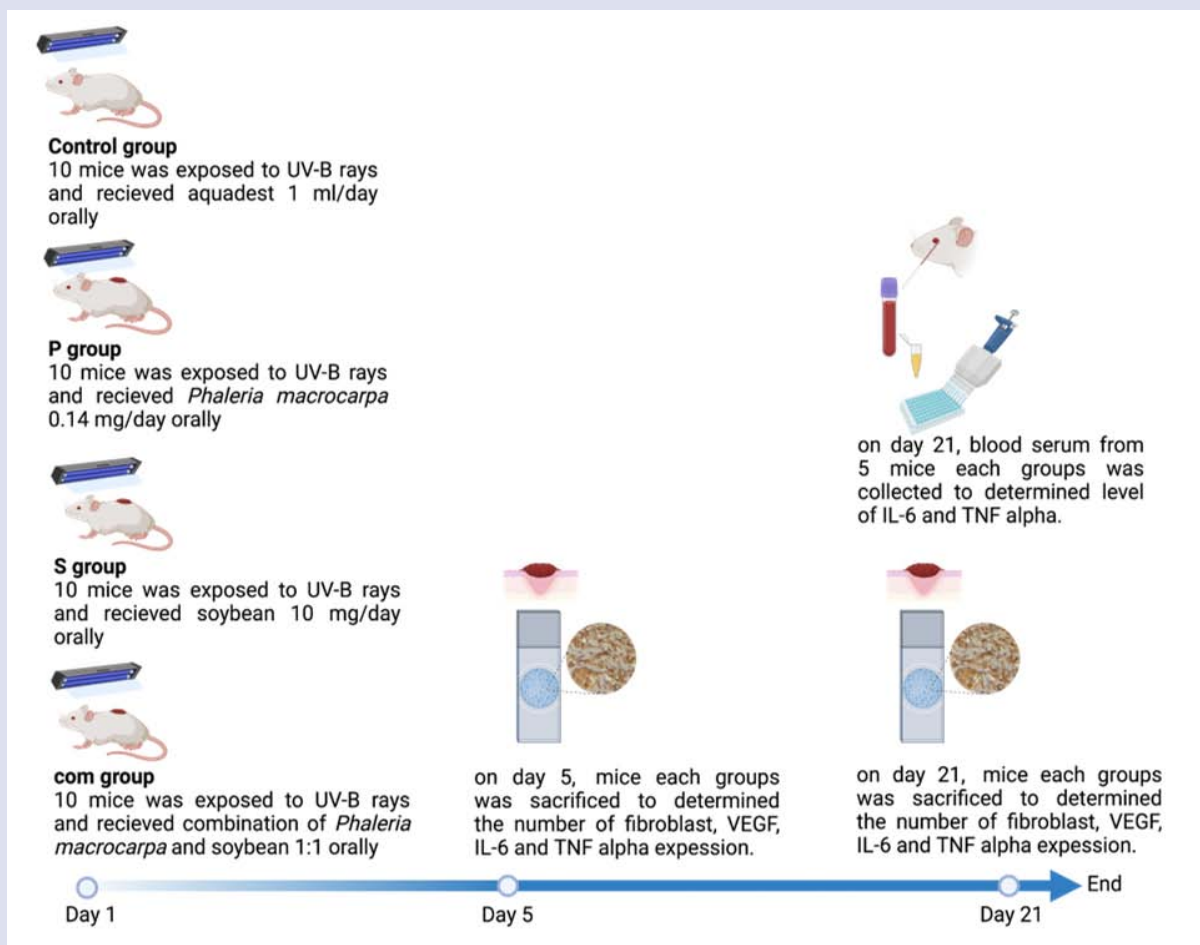
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