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RESEARCH ARTICLE

Soursop (Annona Muricata, Linn) Leaf Ethanol Extract Cream Application Affected the Expression of TNF-α and VEGF on Balb/C Mice Skin Exposed To Acute UVB

Agus Susanto[1*], Taufiqurrachman Nasihun[2], Atina Hussaana[3]
1Magister of Biomedicine, Faculty of Medicine, Sultan Agung Islamic University
2Department of Biochemistry, Faculty of Medicine, Sultan Agung Islamic University
3Department of Pharmacology Faculty of Medicine, Sultan Agung Islamic University
*Corresponding author email: agussusantomd@gmail.com

ABSTRACT
Background*: Healing has been associated with TNF-α and VEGF expression. However, the effect of the soursop leaf ethanol extract cream on the expression of TNF-α and VEGF in burn healing has not been established.* ***Objective:*** *To prove the soursop leaf ethanol extract cream can reduce TNF-α expression and increase VEGF expression on BALB /c mice skin exposed to acute UVB.*
Methods: *This study was an experimental methods with a post-test only control group design. 48 mice were divided into 4 groups: control (CG) (0.3 g base cream) and 3 study groups (treated with 2.5% (ANL2.5-G), 5% (ANL5-G), 10% (ANL10-G) soursop extract. The treatment of 0.3 g cream was given for 5 days, three times/day. Skin tissue specimen was taken on day 6 for a TNF-α expression examination, and on day 8 for VEGF expression examination. The tissue sample were prepared using the IHC method.*
Results: *The Kruskal Wallis test showed that there was a significant difference in TNF-α and VEGF expression among all 4 groups, p <0.01. The Mann Whitney test showed differences in TNF-α and VEGF expression between the two groups p <0.05. 10% cream of soursop leaf ethanol extract significantly affected the expression of TNF-α and VEGF.*
Conclusion: *The cream of soursop leaf ethanol extract decreased TNF-α expression and increased VEGF expression*

Key words: *Soursop leaf ethanol extract cream, TNF-α expression, VEGF expression*

ABSTRAK
Latar belakang: Penyembuhan luka bakar berhubungan dengan ekspresi TNF-α dan VEGF. Namun belum diketahui pengaruh krim ekstrak etanol daun sirsak terhadap ekspresi TNF-α dan VEGF pada penyembuhan luka bakar. **Tujuan:** Membuktikan bahwa krim ekstrak etanol daun sirsak dapat menurunkan ekspresi TNF-α dan meningkatan ekspresi VEGF pada kulit mencit BALB/c yang dipapar UVB akut.
Metode: Penelitian ini mengunakan metode eksperimental dengan posttest only control group design. Subyek penelitian berjumlah 48 ekor mencit, yang dibagi menjadi 4 kelompok Kelompok Kontrol (C-G) hanya dioleskan base krim 0,3 g, kelompok daun sirsak 2.5% (ANL2.5-G), 5% (ANL5-G), 10% (ANL10-G) masing-masing dioleskan 0,3 g krim ekstrak etanol daun sirsak 2.5%, 5%, 10%. Pemberian perlakuan tiga kali sehari selama 5 hari. pengambilan jaringan kulit dilakukan pada hari ke 6 untuk pemeriksaan ekspresi TNF-α dan pada hari ke-8 untuk menilai ekspresi VEGF. Pembuatan preparat menggunakan metode IHC.
Hasil: Uji Kruskal Wallis menunjukkan bahwaterjadi perbedaan ekspresi TNF-α dan VEGF pada ke-4 kelompok secara bermakna, p < 0.01. Uji Mann Whitney menunjukkan ada perbedaan ekspresi TNF-α dan VEGF antar dua kelompok dengan nilai p<0,05. PIII dengan dosis 10% krim ekstrak etanol daun sirsak berpengaruh secara signifikan terhadap ekspresi TNF-α dan VEGF.
Kesimpulan: Krim ekstrak etanol daun sirsak berpengaruh menurunkan ekspresi TNF-α dan meningkatkan ekspresi VEGF

Kata kunci: Krim ekstrak etanol daun sirsak, ekspresi TNF-α, ekspresi VEGF

INTRODUCTION

Wound healing process involves factors such as Tumor Necrosis Factor (TNF-α) as a pro inflammatory agent and the Vascular Endothelial Growth Factor (VEGF) as a growth factor for a physiological and faster healing. Soursop (Annona muricata, Linn) leaves containing chemicals including alkaloids, essential oils, acetogenin, antioxidant and anti-inflammatory flavonoids (Foong and Hamid, 2012) are expected to accelerate the burn healing mediated by changes in levels of TNF-α and VEGF. The effect of the soursop leaf ethanolic extract cream application with a different concentrations on TNF-α and VEGF expression in BALB/c mice exposed to acute UVB requires further studies.

The findings of the National Health Surveys

showed a high prevalence of sunburn among adults. In the United States, 18.5% of adults have sunburns at least once a year and 9.7% have two sunburns a year and 8.0% experience have three or more burns a year. The high prevalence has been associated with sunbathing in summer (Haase et al., 2008). Skin reactions due to acute UVB exposure includes erythema, reddish skin to brown due to dermal vasodilation and leukocyte infiltration (Rhodes et al., 2009). Acute exposure to UVB causes the release of TNF-α, IL-1α, and IL-6 activating cytokines in the epidermis, followed by migration and capture of macrophage cells leading to formation of capillaries by endothelial cells (Elias et al., 2008). Soursop leaf ethanolic extract containing flavonoids, as anti-inflammatory and antioxidants has been reported to increase the number of non-active macrophage cells into active macrophages followed by migration to erythema areas (Acar T et al., 2012). This macrophages serve functions in the cleansing of bacteria and debris from the wound area, and stimulate growth factors such as PDGF, TGF, EGF, FGF needed for proliferation of fibroblast and stimulate the migration of fibroblasts to the injured area. Fibroblasts trigger VEGF expression affecting vascular permeability, re-epithelization, and angiogenesis leading to the formation of capillaries (Barrientos et al., 2008). In addition, as an antioxidant, flavonoids can neutralize various ROS products by donating hydrogen ions to prevent photo oxidation due to excessive release of ROS from inflammatory processes and cell metabolism in wounds (Elias et al., 2008).These stimulate VEGF binds to VEGF receptors on endothelial cells, triggering Tyrosine Kinase Pathway towards angiogenesis (Pakorny et al., 2011).

The purpose of this study was to determine the effect of soursop leaf ethanol cream on TNF-α and VEGF expression in mice exposed to acute UVB.

METHODS

This was an experimental method with a Post Test Only Control Group Design. The research subjects were 48 mice randomly divided into 4 groups per study. Control Group (CG) was given 0.3 g base cream. The study groups were treated with 0.3 g of soursop leaf ethanol extract at a different concentrations: 2.5% (ANL25-G), 5% (ANL5-G), and 10% (ANL10-G) on the backs of mice with erythema. The treatments was given three times a day for 5 days and 7 days. The skin tissue sample of all groups was taken for TNF-α expression examination on day 6 and VEGF expression evaluation on day 8. TNF-α and VEGF examination was carried using Imunohistochemistry method. The study was conducted after obtaining approval from the ethics committee of Faculty of Medicine, Sultan Agung Islamic University, Semarang.

UVB Exposure to Mouse Back Skin

Before UVB exposure, the back skin of BALB/c mice were shaved 2x3 cm in size. The shaved mice were then placed in a closed container in a way that the back skin of the mice were exposed to UVB emitted from Philip Narrowband TL-F72-100W/12 doses of one half DEM (irradiation for 15 minutes at a distance of 50 cm) per day for 3 days (Hussaana, 2009).

Preparation of Annona muricata Linn cream

Extraction of Annona muricata is carried out by maceration method. The soursop leaves were washed with running water, air dried for ± 1 week. The dried samples were cut into small pieces, crushed to powder and sifted, weighed 100 grams and mounted into an erlenmeyer flask, macerated using 96% ethanol solvent to 1 liter and shaken for 3x24 hours. After that, the soaked powder of soursop leaves and ethanol was kept in a tightly closed container and protected from sunlight, left to stand for one day until it settled. The maceration results were filtered using filter paper, then re-maserated to get a clear greenish liquid, and then stirred in the rotary evaporator. The solvent from the extract was evaporated using a water bath with a temperature of 40^0C until the extract thickened. After weighed, the extract was re-evaporated with the oven to remove the remaining ethanol. Evaporation with an oven at 40^0C, every 15 minutes the extract was weighted to get a comparable weight. The extract was mounted into the mortar and added with tween while stirred to obtain an even and homogeneous results, then kept in a different pot with a different concentration (2.5%, 5%, 10%)

Identification of TNF-α and VEGF in Immunohistochemical Preparations

The evaluation of TNF-α expression and VEGF was performed on skin in paraffin block with immunohistochemical preparations. TNF-α expression and VEGF were observed under a light microscope at 400x magnification with 5 microscopic field view for each group. TNF-α expression was characterized and quantified based on the number of large nucleated macrophage cells and brownish cytoplasm per field view. Whereas, VEGF expression was characterized and calculated based on the number of capillary vascular endothelial cells with cell nuclei and brownish red cytoplasm for each group with Axio Vision Software.

Table 1. Mean Expression (± SD) of TNF-α and VEGF

Variables	C-G	ANL2.5-G	ANL5-G	ANL10-G	P Value (*Kruskal Wallis*)
TNF-α expression (%)	6,97±0,94	5,57±0,27	3,97±0,20	1,73±0,84	0.001
VEGF expression (%)	3,37±0,29	6,20±0,22	7,97±0,20	9,80±0,31	0.001

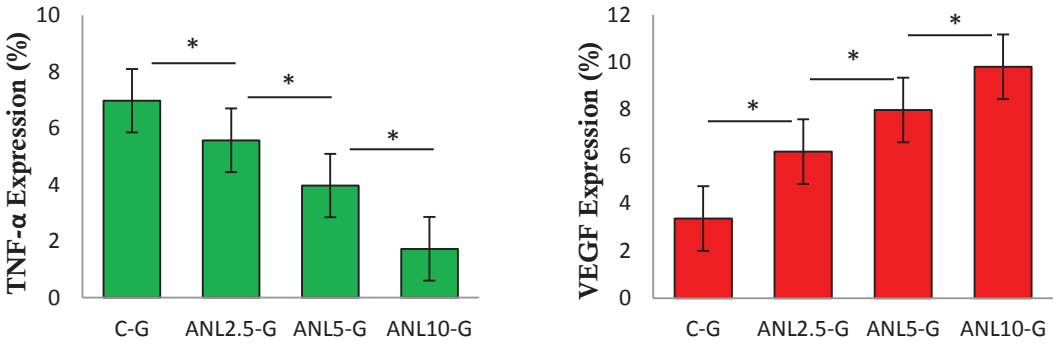

Figure 2. Mann Whitney analysis on TNF-α and VEGF Expression in four Groups. Note: * p < 0.05

STATISTICAL ANALYSIS

The statistical analysis used was Kruskal Wallis followed by the Mann Whitney test, considering that the data obtained were not normally distributed and not homogeneous. The results of this study were considered significant if the value of p <0.05.

RESULTS

The results of the effect of cream containing soursop leaf ethanol extract on TNF-α expression and VEGF were characterized by macrophage cells with a large cell nuclei and brownish cytoplasm can be seen in Figure 1 as follows:

After observation and quantification with a light microscope at 400x magnification on macrophage cells (TNF-α) and endothelial cells (VEGF) in each group, the mean expression of TNF-α and VEGF are shown in table 1.

The mean TNF-expression in the C-G group was found to be the highest, followed by the ANL2.5-G, ANL5-G, and ANL10-G group. On the contrary, the mean VEGF expression in the C-G group is the lowest, followed by ANL2.5-G, ANL5-G, and ANL10-G is the highest. The results of the Kruskal Wallis statistical analysis showed a significant difference in TNF-α and VEGF expression between groups (p <0.01). To see groups that were significantly different, a Mann Withney test was performed as described below.

Expression of TNF-α

The results of the Mann Whitney analysis showed that the mean expression of TNF-α levels in the three ANL groups (2.5%, 5%, and 10%) was lower than that of the control group, p <0.05. TNF-α expression in the ANL5-G and ANL10-G groups was also significantly lower than the ANL2.5-G group. The similar result was also found in ANL10-G when compared with the ANL5-G group (p <0.05) (figure 2)

VEGF Expression

The results of the Mann Whitney analysis showed that mean VEGF expression in the three ANL groups (2.5%, 5%, and 10%) was higher than that of the control group (p <0.05). VEGF expression in the ANL5-G and ANL10-G groups were also significantly higher than that of the ANL2.5-G group. The same results were found between ANL10-G when compared with the ANL5-G group, p <0.05 (figure 2).

DISCUSSION

This study showed that acute UVB exposure increase TNF-α expression. This finding supports that of a study conducted by (Elias et al., 2008) showing that in vivo acute UVB exposure increased levels of TNF-α. Increased expression of TNF-α due to acute UVB irradiation has been reported by (Rhodes et al., 2009) where the skin has reddish to brownish erythema. Erythema increases vascular permeability causing vascular vasodilation in the cutaneous layer and increase in proinflammatory cytokines, including TNF-α (Clydesdale et al., 2011). Circulating TNF-α binds to TNFRI (p55) and TNFRII (p75) induces macrophages to produce PDGF, TGF, EGF, FGF needed for proliferation and migration of fibroblast cells to the injured area. Fibroblasts trigger VEGF in the proliferation phase to affect vascular permeability,

re-epithelialization, and angiogenesis (Nissen et al., 2008).

The results of this study indicate that administration of soursop leaf cream 2.5 - 10% was shown to reduce TNF-α expression and increase VEGF expression compared to the control group. This illustrates that the creams of soursop leaf ethanol extract had an effect on TNF-α expression and increase VEGF expression in mice exposed to acute UVB rays.

The administration of soursop leaf extract at the dose of 10% resulted in the highest decrease in the expression of TNF-α, followed by the dose of 5%, and 2.5%. This illustrates that the decrease in TNF-α expression in accordance with the cream dose of soursop leaf ethanol extract given. The higher the dose of soursop leaf cream extract given, the lower the expression of TNF-α. This could have been due to flavonoids in leaf ethanol extract cream having anti-inflammatory activity that accelerate healing by increasing the number of neutrophyl cells and then monocytes that immediately differentiate into macrophages. Neutrophyl produces a higher number of reactive oxygen species (ROS), proteases, and proinflammatory cytokines to clean wounds. After serving its function, netrophyls undergo apoptosis, then phagocitos by macrophages (Markham K.R, 2008). Macrophage will also phagocytes bacteria and debris to clean wounds. Furthermore, macrophages also secrete various cytokine molecules which then bind to the targettic cell receptors so that they can call other phagocytes around the erythema to accelerate the rate of the inflammatory phase leading to the proliferation phase (Falanga, 2014). The proliferation phase takes place after suppression of TNF-α activity which is characterized by granulation tissue formation, which is a combination of cellular elements including fibroblasts and inflammatory cells, which together result in new capillaries in extra cellular loose connective tissue from the collagen, fibronectin and hyaluronic acid matrix (Cornelissen, 2014).

CONCLUSION

The topical application of cream containing soursop leaf ethanol extract decreased TNF-α expression and increased VEGF expression on the erythema skin of BALB/c mice with UVB-induced.

CONFLICT OF INTEREST

There is no conlict of interest.

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