



## Protective Effect of Lemon (*Citrus limon L.*) Ethanol Extract Cream as an Antioxidant against Exposure to Ultraviolet B Rays in the Skin of Male Wistar (*Rattus norvegicus*) Rats

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### ABSTRACT

**Background:** Indonesia is an archipelagic tropical country that get sun exposure all the time. Exposure to radiation can cause acute effects in the form of erythema through the inflammatory process. Antioxidants are substances that can protect the body from damage caused by ROS. Natural antioxidants can be found in vegetables and fruits, one of which comes from lemon extract (*Citrus limon L.*). Lemon extract is known to have active compounds in the form of flavonoids and phenols which can act as antioxidants. The aim of this research was to determine the protective effect of lemon extract on UVB exposure in the skin of male wistar rats.

**Methods:** This research used the true experimental posttest only control group design method. Samples were divided into three treatment concentrations, namely ethanol extract of lemon 5%, 10%, and 20%.

**Result:** After testing for normality, the significance value was obtained ( $p < 0.05$ ). Based on the results of the normality and homogeneity test, the results of the data distribution are not normal and the homogeneous tests of the hypotheses used are the Kruskal-Wallis non-parametric test. Kruskal-Wallis non-parametric test results showed a significant difference with the significance value ( $p = 0.001$ ). The best degree of erythema score was found in the cream of 10% ethanol extract of lemon with an average of  $0.8 \pm 0.84$ .

**Conclusion :** The ethanol extract of lemon (*Citrus limon L.*) cream in a certain dosage has a significant effect on reducing the erythema degree score in the back skin of male Wistar rats (*Rattus norvegicus*) after exposure to UVB rays.

**Keywords:** antioxidants; erythema; Citrus limon L; ultraviolet B rays; wistar rats

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## Introduction

Indonesia is an archipelagic country and one of the tropical countries that get exposure by sun all the time. Exposure by sunlight produces ultraviolet (UV) radiation. Continuous exposure by ultraviolet radiation to the skin has a huge possibility on formation of reactive oxygen species (ROS) that can cause damage to the structure and function of the skin.<sup>1</sup> Exposure of ultraviolet radiation can cause an acute effects and chronic effects. The acute effects are effects that happen immediately after exposure have time from 30 minutes to several hours after exposure to erythema.<sup>2</sup>

Ultraviolet (UV) is electromagnetic radiation with a wavelength of 100 - 400 nm which is sourced from the radiant solar energy. UVB is very dangerous because it can affect cellular activity. Ultraviolet radiation can cause cellular homeostatic disruption which triggers the formation of ROS.<sup>3</sup> Free radicals are atoms or molecules that have unpaired electrons in their structure and are highly reactive.<sup>4</sup>

The light of ultraviolet B (UVB) plays an important role in causing short-term erythema with an interval of 6 to 24 hours after exposure. In the case of erythema, UVB light is 1000 times more dangerous than UVA.<sup>5</sup> UVB increases the secretion of Vascular Endothelial Growth Factor (VEGF) and Fibroblast Growth Factor 2 (FGF2) from keratinocytes. The occurrence of erythema can also be affected by the vasodilation of blood vessels after exposure to ultraviolet radiation because UVB exposure causes direct damage to DNA. As a result of DNA damage, many cytokines and inflammatory mediators accumulate in the skin.<sup>6</sup>

Antioxidants are substances that can eliminate oxidative damage to a particular target molecule, prevent, slow down and neutralize free radicals for example by breaking the chain reaction of free radicals.<sup>7</sup> Based on the source, antioxidants consist of natural and artificial antioxidants. Natural antioxidants are usually obtained from plants. Antioxidants can be obtained, one of which comes from lemon extract (*Citrus limon L.*). The phenol component in lemons has antioxidant properties such as lifting oxygen, breaking down peroxide and inhibiting free radicals. While the content of

flavonoids in lemons has benefits as anti-cancer, anti-viral and anti-inflammatory.<sup>8</sup>

## Methods

The lemon used as sample follows inclusion and exclusion criteria. Inclusion criteria consisted of: the lemon was taken from Banjar Sabang, Selulung Village, Kintamani District, Bangli Regency, ripe and ready to harvest, around 30-36 weeks old, had good quality without problems. Whereas unripe lemons, contains rotten part, or infected with the disease were not used in this study.

The tools used in this study were 50x40x20 cm Wistar rat cages equipped by sawdust with complete food and beverage containers, solar UVB simulators, shavers, digital scales, Buchi Rotavapor R-210, blenders (Philips), emulsifiers, stopwatches time, rulers, gloves, books and recording devices.

Materials used in this study were lemons, ethanol 96%, Wistar rats (*Rattus norvegicus*) male aged 2.5 - 3 months with body weight 150-200 gram with animal feed, vaseline alba, paraffin liquid, stearate acid, nipasol, nipagin, TEA, aquades.

This research is purely experimental research to find out the effect of ethanol extract of lemon (*Citrus limon L.*) cream in reducing erythema degree score due to UVB exposure in vivo with the Post Test Only Control Group Design pattern in male Wistar (*Rattus norvegicus*) rats. This study was divided into two groups namely : the control group (K) and the treatment group (P). The control group was positive control that was only exposed to UVB while the treatment group was divided into five groups based on the dose of the use of ethanol extracts of lemon (*Citrus limon L.*) in each group containing five male Wistar rats (*Rattus norvegicus*) namely cream base (P2), the concentration of cream extract is 5% (P3), 10% (P4), and 20% (P5). The research was conducted at the Laboratory of Agricultural Technology, Laboratory Pharmacy of Mahasaraswati University and Laboratory of Pharmacology.

Furthermore, to find out the groups that show different erythema scores with the control group, further tests using the *Mann-Whitney* test

are needed. The homogeneity test was done by using Levene's Test.

This study has received permission from the Research Ethics Commission with its number 584 / UN14.2.2.VII.14 / LP / 2019.

This research was conducted in the following steps.

### Processing of Lemon Extract

Making ethanol extract of lemon by maceration method. Lemon fruit is washed with running water then dried for 30 minutes then 3 kg of lemon is cut into small pieces. Furthermore, drying can be done by leaving the room temperature or in an oven at 50<sup>0</sup>C. The dried lemon is then mashed by blending to obtain 200 gram of fine powder. Then 96% ethanol solvent is added until all ingredients were submerged in a ratio of 1:10 times simplicia. The maceration process was carried out for 1 x 24 hours while stirring occasionally or with the help of a shaker every 1 hour. After that maserat filtered using filter paper. The remaining deposits in the macerator were macerated for 24 hours. The first and second maceration resulted of the mixture are mixed and then concentrated by evaporating the solvent using vacuum rotary evaporator at low pressure at a temperature of 40<sup>0</sup>C -50<sup>0</sup>C at a speed of 100 rpm until a thick extract was obtained.

### Lemon Fruit Ethanol Extraction Cream

The extracted lemon fruit ethanol was then processed into two-phase preparation to form the lemon fruit ethanol extract. Ethanol extract of lemon fruit was made into O/W type cream. The oil phases (algae vases, paraffin liquid, stearate acid, and nipasol) were melted in a vapor cup at 700C - 750C. The phases of water (nitrogen, TEA, and water) were heated to the same temperature. Put the oil phase into the mortar kind of oil and then slowly add the water phase. Stir ingredients until homogenous and creamy. Subsequently, the extracts in the mortar were made in doses of 5%, 10%, and 20%.

### Erythema Test Procedure

Rats were divided into five test groups and shaving was carried out on the backs of rats with an area of 4x4 cm. Avoid the occurrence of cuts or blisters on the back, if this happened then wait until the back skin returns to normal. The sample was then given treatment according to the group. One by one the first group of rats was exposed with adjusted doses of preliminary test results then erythema observation, measured shortly and 24 hours after exposure and interpreted according to the erythema degree score table. The second group sample was smeared with extract base and then exposed one by one with the dose adjusted from the preliminary test results, The third group sample was smeared with 5% cream ethanol extract of lemon fruit and then exposed one by one with the dose adjusted from the preliminary test results. The fourth group sample was smeared with 10% cream of ethanol extract of lemon fruit and then exposed one by one with the dose adjusted from the preliminary test results. The fifth group sample was smeared with 20% cream of ethanol extract of lemon fruit and then exposed one by one with the dose adjusted from the preliminary test results, measured shortly and 24 hours after exposure and interpreted according to the erythema degree score table.

Table 1. Erythema Degree Score<sup>9</sup>

Skin Reaction	Score
Without erythema	0
Very little as erythema (almost invisible)	1
Erythema is clearly seen (diameter 25,1 – 30 mm)	2
Moderate erythema (diameter 30,1 – 35 mm)	3
Severe erythema (dark red by forming a scar, diameter > 35 mm)	4

**Result**

This study used five treatment groups with three variations of extract concentration. Based on a preliminary test of the UVB ray dose used in the main test that is 200 mJ / cm<sup>2</sup>. Measuring the degree of erythema score on the back skin of male Wistar rats (*Rattus norvegicus*) from each treatment group was carried out 24 hours.

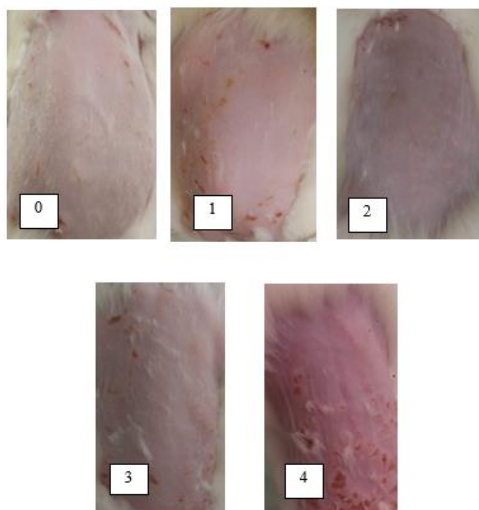


Figure 1. Criteria results for the degree of erythema score based on the results of the study

Data erythema score of the dorsal skin of male Wistar rats in each group were tested for normality using the *Shapiro-Wilk* test. The results show the data in P1, P2, and P3 groups were not normally distributed with p values <0.05, while the data in the P4 and P5 groups were normally distributed with p values > 0.05. Homogeneity test results in this study indicate all data are homogeneous with the results of p = 0.830 (p > 0.05). Based on the results of the normality test and homogeneity test shows the results of the distribution of abnormal and homogeneous data, the using of hypothesis test was a non-parametric test with the *Kruskal-Wallis* test.

The *Kruskal-Wallis* non-parametric test showed that the erythema score of the P1 (control) group was higher than that of the P2 group (cream base). In contrast to the treatment group, group P3 (cream 5%), group P4 (cream 10%) and group P5 (cream 20%) produced the

same median erythema score and were lower when compared to the negative and positive control groups. Analysis of significance with the *Kruskal-Wallis* test showed that the value of p = 0.001. This shows that the erythema score of the dorsal skin of male Wistar rats after being treated was significantly different (p <0.05).

Tables 1. Erythema degree score in each treatment group

Treatment Group	Erythema Degree Score					Average ± STD
	I	II	III	IV	V	
P1 : Negative Control	3	3	4	4	3	3.4 ± 0.55*
P2 : Cream Basis	3	2	3	2	2	2.4 ± 0.55*
P3 : Cream 5%	1	2	2	1	1	1.4 ± 0.55*
P4 : Cream 10%	1	2	0	0	1	0.8 ± 0.84*
P5 : Cream 20%	2	1	1	0	1	1.0 ± 0.71*

Note: \* in the same column shows significant differences (p <0.05)

Based on the measurement results erythema degree score has a variation of scores on each repetition and shows that there are significant differences between treatment groups. There were no significant differences in the control group and the cream base group. So it can be concluded, administration of standard cream did not affect the formation of erythema in the cream base group when compared with the control group. All treatment groups (P3, P4, and P5) showed significant differences when compared to the control group and cream base. However, no significant differences were found between the treatment groups with the control group and cream base. This shows the administration of lemon ethanol extract in the form of cream can reduce the number of erythema based on the erythema score significantly to the control group and cream base with a minimum effective dose of 5%. The average score of the lowest erythema degree was found in the cream of 10% ethanol extract

of lemon which is equal to  $0.8 \pm 0.84$  furthermore it can be said that the lowest degree of erythema score was found in the cream of 10% ethanol extract of the lemon. The results of the average change in erythema degree score in each treatment group can be described in the diagram as follows.

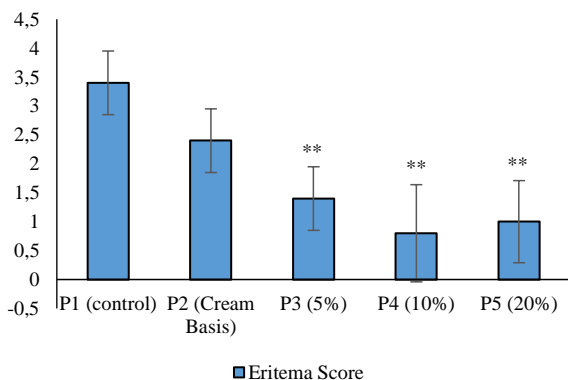


Figure 2. Erythema Score of Back Skin of Male Wistar Rats

Based on the diagram of the results of the study in Figure 2 shows that the concentration of ethanol extract of lemon cream is the most effective in reducing the erythema degree score at a concentration of 10% while at a concentration of 20% there is a slight increase in the erythema degree score compared to 10% of ethanol extract. An increase in erythema degree score at a concentration of 20% indicates the possibility of pro-oxidants if the dose is increased. It can be said that there is a relationship between erythema and the ethanol extract concentration of lemon (*Citrus limon L.*) with the average result of erythema degree score in the skin of male Wistar rats (*Rattus norvegicus*) which have been exposed to UVB rays.

## Discussion

This research used lemon ethanol extract cream which was known to have antioxidant content. This study did not measure the antioxidant ability of ethanol extracts of lemon but measured the ability of the erythema score of male Wistar rats exposed to UVB at concentrations of 5%, 10%, and 20%. The

cream base has no antioxidant activity. Base cream used types O/W that functions in aesthetic and cosmetic use.<sup>10</sup>

Exposure to UV-B light absorbed causes changes in cellular immunology. Activation of NFκB in fibroblasts induces transcription of pro-inflammatory cytokines (IL-1, IL-6, CEGF, TNF-α) causing inflammatory cell infiltration. Inflammatory mediators stimulate neutrophils to release neutrophil collagenase and stimulate inflammatory cells to release the enzyme NADPH oxidase (NOx) causing superoxide anion formation which causes an increase in ROS production. Activation of endothelial cells nitric oxide synthase (eNOS) and Xanthine Oxidase (XO) causes increased release of Nitric Oxide (NO) and superoxide anions. In addition to vasodilation of blood vessels, NO can react with free radicals.<sup>11</sup>

Phytochemical analysis of lemon fruits and seeds based on research by Mather et al. Lemon contains flavonoids and phenols which act as antioxidants.<sup>12</sup> The presence of the most powerful chemical compounds found in lemons is the content of flavonoids and phenols. Flavonoids are often associated with health products because they have antioxidant, anti-inflammatory and anti-cancer benefits. Besides flavonoids can inhibit several enzymes such as Xanthine Oxidase (XO), Cyclo-Oxygenase (COX), Lipoxygenase, and Phosphoinositide 3-kinase. Flavanones are a type of flavonoid found in all citrus gologans such as oranges, lemons, and grapes.<sup>13</sup> A study using the in silico method in the binding mode of flavonoids with COX-2 shows that some flavonoids and flavones containing 2,3 double bonds can act as COX-2 preferential inhibitors.<sup>14</sup>

This study shows that the administration of lemon ethanol extract cream at a concentration of 5%, 10%, or 20% can inhibit the acute effects of UV-B exposure. This is following several studies both in animals and humans that prove that polyphenol compounds (flavonoid compounds) can act as photoprotective substances against skin inflammation due to UV exposure, oxidative stress, DNA damage, and all that three.<sup>15</sup> The study of Aquino et al topical quercetin treatment showed the protective effect of ROS induced by UVB exposure in rat skin. In

this study also showed a topical application of *Culcitium reflexum* H.B.K leaves containing isorhamnetin, quercetin, and kaempferol significantly reduced erythema after UVB exposure.<sup>16</sup>

In this study there was a slight increase in the degree of erythema score at a concentration of 20%, this is presumably due to the effect of the dose given on the treatment. The content of active compounds found in lemons that act as antioxidants, namely flavonoids and phenols, is thought to have a pro-oxidant effect at certain doses. Previous studies have reported that dosage problems and toxicological risks can arise when the dosage of compounds increases beyond a certain threshold.<sup>17</sup> Small polyphenol compounds can show prooxidant activity because they are easily oxidized.<sup>18</sup>

In this study there are still some limitations such as giving erythema scores in this study are subjective therefore the results can be different if the same test is done by researchers in the future. In this study, phytochemical tests were not carried out compounds contained in lemon extract therefore researchers suspected related compounds that affect the erythema degree score in the treatment group and the control group. Also, the selection of cream doses applied to experimental animals is based on the wishes of researchers so that they are not based on special calculations or preliminary tests and shaving done on experimental animals can cause injury to the back skin of experimental animals so that it can affect the grading of erythema degrees.

## Conclusion

This study shows that the administration of lemon ethanol extract good cream 5%, 10%, or 20% cream can inhibit the acute effects of UV-B exposure. The results of this study indicate that there is no significant difference in the reduction in the erythema degree score in the groups given the cream 5%, 10%, and 20%. The best results were obtained at a cream concentration of 10% ethanol extract of lemon.

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## References

1. Forman, H.J., Augusto, O., Brigelius-Flohe, R., Dennery, P. A., Kalyanaraman, B., Ischiropoulos, H., Mann, G. E., Radi, R., Ii, L. J. R., Vina, J., dan Davies, K. J. A. 2015. A Suggested Guide to Free Radical Research Terminology and Methodology. *Free Radical Biology and Medicine*. Elsevier.78: 233-235.
2. Heinrich, U., Gartner, C., Wiebusch, M., Eichler, O., Sies, H., Tronnier, H., Stahl, W. 2003. Supplementation with beta-carotene or a similar amount of mixed carotenoids protects humans from UV-induced erythema. *J.Nutr.* 133: 98-101.
3. Sharma, P., Jha, A. B., Dubey, R. S., and Pessarakli, M. 2012. Reactive Oxygen Species, Oxidative Damage, and Antioxidative Defense Mechanism in Plants under Stressful Conditions. *Journal of Botany*. 2012: 1-26.
4. Danusantoso, H. 2003. Peran radikal bebas terhadap beberapa penyakit paru. *Jurnal Kedokteran Trisakti*. 22(1): 31-36.
5. Birch-Marchin, M. A., Russell, E. V., and Latimer, J. A. 2013. Mitochondrial DNA damage as a biomarker for ultraviolet radiation exposure and oxidative stress. *British Journal of Dermatology*. 169(2): 9-14.
6. Mancebo, S. E. and Wang, S. Q. 2014. Skin cancer: role of ultraviolet radiation in carcinogenesis. *Rev Environ Health*. 29(3): 265-273.
7. Halliwell, B. 2012. Free radicals and antioxidants: updating a personal view. *Nutrition Reviews*. 70(5): 257-265.

8. Jonathan, H. 2013. Crane: Lemon Growing in the Florida Home Landscape. *US Florida*. 1153: 1095-1100.
9. Dewantara, I. G. N., Prasetia, I. G., Jemmy, A., Putri, N.N.T.A., Arsana, D. A.M.I.P., dan Prabayanti, N. P. 2015. Uji Eritema dan Edema Secara In Vivo pada Natrium Lauril Sulfat 10%. *4 (2) : 1-4*.
10. Juwita, A P, Yamlean, P V Y, and Edy H J. 2013. Formulasi Krim Ekstrak Etanol Daun Limun (*Syringodium isoetifolium*). *Jurnal Ilmiah Farmasi*. 2(2) : 8-12.
11. Kammeyer A. and Luiten R.M. 2015. Oxidation events and skin aging. *Ageing Research Reviews*. 21 : 16-29.
12. Mathew, B. B., Jatawa, S., and Tiwari, A. 2011. Phytochemical analysis of *Citrus Limonum* Pulp and Peel. *Int J Pharm Pharm Sci*. 4(2): 269-371.
13. Panche, A.N., Diwan, A.D., and Chandra, S.R. 2016. Flavonoids: an overview. *Journal of Nutritional Science*. 5(47) : 1-15.
14. D'Mello P, Gadhwal M, Joshi U, Shetgiri, P. 2011. Modeling of COX-2 inhibitory activity of flavonoids. *Int J Pharm Sci* 3. 3(4) : 33-40.
15. Poljsak, B. and Fink, R. 2014. The Protective Role of Antioxidants in the Defence against ROS / RNS-Mediated Environmental Pollution. *Oxidative Medicine and Cellular Longevity*. 2014: 1-22.
16. Aquino, R., Morelli, S., Tomaino, A., Pellegrino, M., Saija, A., Grumetto, L., Puglia, C., Ventura, D., Bonina, F. 2002. Antioxidant and photoprotective activity of a crude extract of *Culcitium reflexum* H.B.K leaves and their major flavonoids. *J. Ethnopharmacol*. 79(2) : 183-191.
17. Rietjens, I M C M, Boersma, M G, Haan L D., Spenkelink B., Hanem, M., Awad., Cnubben, N H P., Zanden, J J V., Woude, H V D., Alink, G M., Koeman, J H. 2016. The pro-oxidant chemistry of the natural antioxidants vitamin C, vitamin E, carotenoids and flavonoids. *Elsevier*. 11(3): 321-333.
18. Eghbaliferiz, S. and Iranshahi, M. 2016. Prooxidant Activity of Polyphenols, Flavonoids, Anthocyanins, and Carotenoids: Updated Review of Mechanism and Catalyzing Metals. *Wiley Online Library*. 30: 1379-1391.