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The Effect of Sapodilla Leaf Extract (*Manilkara Zapota L.P Royen)* on IL-1 and TNF-α Expression in Male White Rats Wistar Strain Induced with UVB Light

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ABSTRACT: Excessive UVB exposure causes health problems in humans, the effect on the skin is molecular and cellular changes. Sunburn is the most obvious acute clinical effect, resulting in the production of TNF-α by keratinocytes, IL-1 is a cytokine that influences TNF-α. Skin wounds release various damage associated molecular patterns (DAMPs) which will activate inflammatory signaling pathways such as NLRP3 inflammasomes and toll-like receptors (TLRs) which cause increased production of inflammatory cytokines. The aim of this study was to examine the anti-inflammatory effect of *Manilkara zapota* leaf extract which was assessed based on the expression of IL-1 and TNF-α in Wistar rats exposed to UVB. Post-test only control group design experimental research using rats (Rattus norvegicus) as research objects, divided into 4 groups, namely (K1) control rats without treatment, (K2) rats exposed to UVB light then given placebo cream, (K3) rat group exposed to UVB light then given 25% sapodilla leaf extract cream, and (K4) mice were exposed to UVB light then given 50% sapodilla leaf extract cream. The average expression of IL-1 using the One-Way ANOVA test was 0.197 ($p<0.05$), which means there was no significant difference in the average expression of IL-1 between groups. Meanwhile, the average TNF-α expression between groups was 0.036 ($p<0.05$), the results showed that there was a significant difference in the average TNF-α expression between groups, TNF-α expression using the Post Hoc LSD test of sapodilla leaf extract cream 50% dose (K4) reduced TNF-α expression to a lesser extent than K1 or K2. Administration of leaf extract cream (*Manilkara zapota*) affected the expression of TNF-α, but did not affect the expression of IL-1 in a mouse model experiencing sunburn.

KEYWORDS: sunburn, *Manilkara zapota* cream, IL-6, TNF-α

I. INTRODUCTION

Excessive exposure to solar radiation can cause health problems in humans. Solar radiation has many effects on human health, all of which are supported by molecular (e.g., DNA damage) and cellular (e.g., *Langerhans* and dermal dendritic cell migration) changes.[1] Sunburn (erythema) is the most obvious acute clinical effect and skin cancer is the most common chronic effect. The World Health Organization (WHO), estimated about 11 million people each year experience sunburn. [2]

Sunburn wound healing is a complex process consisting of inflammatory cell infiltration, cell proliferation, and tissue remodeling phases to restore skin integrity and function. Exposure to ultraviolet B results in the production of TNF-α by keratinocytes. TNF- α plays an important role in the inflammatory cascade. Interleukin-1 (IL-1) is a cytokine that affects TNF-α. Skin wounds releasing various *damage associated molecular patters* (DAMPs) activate inflammatory signaling pathways such as NLRP3 inflammasom and *toll-like receptors* (TLRs) that cause increased production of inflammatory cytokines.[3]

Sapodilla leaf extract (*M. Zapota*) has a very significant flavonoid content known to have anti-inflammatory effects. The health benefits of sapodilla have been reported to be that its leaves can exhibit anti-inflammatory and antipyretic activity. Previous data showed the antioxidant function of *Manilkara zapota* from polyphenols, especially gallocatechin or catechin, and also neo-bioactive polyphenols with methyl 4-O-galloylchlorogenate and 4-O-galloylchlorogenic acid in Methanol-extracted *Manilkara zapota*. Phytoconstituents such as flavonoids, saponins, tannins, glycosides shown by phytochemical screening in crude extract of *M. Zapota leaves* have anti-inflammatory effects. The mechanism of action of flavonoid compounds as anti-inflammatory can go

through several pathways by inhibiting cyclooxygenase (COX) and lipooxygenase activity, inhibition of leukocyte accumulation, inhibition of neutrophil degranulation, and histamine inhibition. *M. zapota has an* effect in inhibiting the activity of the enzyme α-glucosidase significantly in low concentrations (IC50 2.51 ± 0.15 µg/mL).[4]

Crude methanol *extract of* M. Zapota *leaves is known to have antioxidant activity of 3,523 ± 0,382 mmol* trolox equivalent/gram. Photochemical analysis showed *M. Zapota* contains flavonoids, steroids, glycosides, anthraquinones, antrons, coumarins, phenols, and tannins. Phenols extracted from plants are antioxidants that can terminate free radicals.[5] M. *Zapota leaves* are known to have antioxidant, antimicrobial, anti-inflammatory, anti-tumor, anti-cancer, *antipyretic*, anti-diarrheal, anti-nociceptive, and ability to lower uric acid levels. Based on the analysis conducted, *M. Zapota* leaves have a total phenol capacity, flavonoid capacity, and antioxidant capacity that is higher than other plant parts. This can be due to high ultraviolet exposure to the leaves. A higher proportion of flavonoids are found in leaves in tropical regions compared to cold conditions due to the high UV radiation obtained.[6]

Currently, research on the effects of *M. Zapota* leaves is still limited. In vivo as well as in vitro studies have been conducted on this leaf extract. Recent research examined the effects of M. zapota bark in *vivo* on wistar rats. In the study, there was an increase in epithelial tissue, collagen proliferation, and keratinization in wound healing activities in experimental animals given *M. Zapota*.[7]

The goal of sunburn wound treatment is rapid wound healing to prevent infection and minimize complications. Even so, there are still no studies that examine the effect of giving *Manilkara zapota* leaf extract on the healing process of the skin in burns. This study will evaluate the anti-inflammatory effect of *Manilkara zapota* leaf extract assessed based on IL-1 and TNF-α expression in UVB-induced wistar rats.

II. MATERIAL AND METHOD

Study Design and Experimental Animals

This research is an experimental study *post-test only control group design*. The study subjects used 28 male rats of the Wistar strain (*Rattus norvegicus*) aged 2-3 months, with a body weight of 190-210 grams that met the inclusion and inclusion criteria, adapted for 7 days. The study subjects were randomly divided into 4 groups, namely the rat group as a control without treatment (healthy control) (K1), the rat group with UVB light for 3 days then given placebo cream for 3 days as much as 1.5 grams (K2), the rat group with UVB light for 3 days then given sapodilla leaf extract cream (*Manilkara zapota)* 25% for 3 days as much as 1.5 grams (K3), and the rat group with UVB light for 3 days then given 50% sapodilla leaf extract cream for 3 days as much as 1.5 grams (K4). After treatment for 3 days, Day 4 Wistar rat skin tissue samples were taken to analyze IL-1 and TNF-α expression using *the Enzyme-linked immunosorbent assay* (ELISA) method.

Research Materials

Research materials include ethanol extract of *Manilkara zapota* leaves, aquabides, ketamine, 70% alcohol, 80%, paraffin, Fine test ELISA kit Rat TNF-α, Fine test ELISA kit Rat IL-1.

Research Equipment

This study used several equipment, namely a cage measuring 55 x 22 x 22 cm equipped with a feed and drinking bin, UV-B lamps, digital scales, PVC pipes, 3 and 5 ml syringes, tools for making examination samples (scalpel knives), ethanol extraction tools *Manilkara zapota leaves:* ovens, maceration bottles, *rotary evaporators,* and supporting tools for making cream preparations: porcelain cups, water heater, mortar.

Making *Manilkara zapota* Leaf Extract Cream

Samples of fresh Sapodilla leaves as much as 1 kg, washed thoroughly using running water. The leaves are then dried in an oven at 40^{o}C. Simplisia is checked for moisture content with *a moisture balance* (Ministry of Health RI, 1985). The drying result of simplisia is considered good if the moisture content is below 10%. Simplisia is then carried out dry sorting to remove dirt that is still left during the drying process, cut into small pieces, and weighed then blended into powder. Leaf powder is sifted with a sieve the size of 20 mesh. 450 grams of leaf simplisia powder was extracted using maceration method with 96% ethanol solvent as much as 1,500 ml. Leaf simplisia powder is put into a dark-colored bottle separately. Then simplisia is soaked using ethanol solvent for 3 days and occasionally stirred 3 times a day, after 3 days then filtered and the sandpaper is re-macerated for 2 days with 96% ethanol as much as 1,500ml. Repetition is carried out twice. The collected filtrate is then thickened using a *rotary evaporator* at 40°C until a thick extract is obtained. The thick extract of sapodilla leaves obtained is calculated in yield, then made in the form of 25% and 50% cream.[8]

Treatment of Experimental Animals

Rats that have been adapted for 7 days are anesthetized with a mixture of ketamine (60 mg / kgbb) and xylazine (20mg / bb), then the hair on the dorsal part of the rat is shaved clean with a size of 5x5 cm. The rats' backs were exposed to UV light (broadband with peak emission 302 nm) with a minimum dose of erythema 160 mJ/cm2/day for 3 days. The rats were then given treatment according to their group. Topical treatment is given once a day for 3 days after UV irradiation B.

III. RESULT

Flavonoid and phenol content of sapodilla leaf extract (*Manilkara zapota*) and validation results of HE staining in UV-B exposed rats

Qualitatively, sapodilla leaves contain phenol and flavonoid compounds.[9] Quantitative analysis of the content of sapodilla leaf extract obtained phenol levels with 3 repetitions obtained an average result of 170.1 mg / ml, while the results of flavonoid levels analyzed with 3 repetitions obtained an average result of 56.8 mg / ml.

The study subjects were exposed to UV light (broadband with peak emission 302 nm) with a minimum dose of erythema 160 mJ/cm2/day for 3 days. Day 4 skin tissue samples were taken to make anatomical histopathology preparations with HE staining, the results of observations were carried out by anatomical pathology experts.

The results of anatomical pathology examination showed that the black arrow of the HE preparation in the treatment group found sunburn cells while in the control group no sunburn cells were found, observations were made with a microscope at 40x magnification.

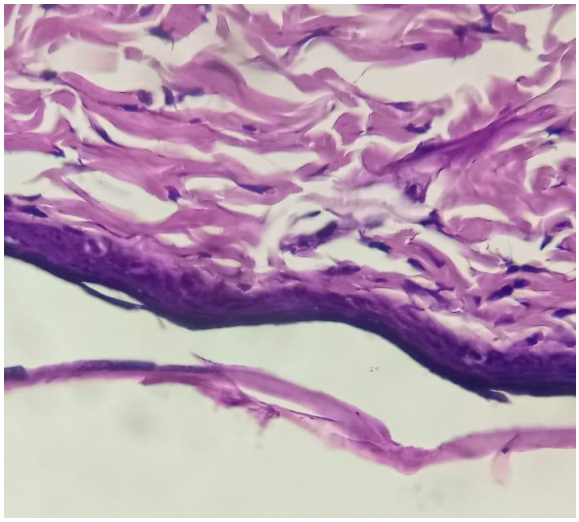

Figure 1. Control group HE staining results at 40x magnification

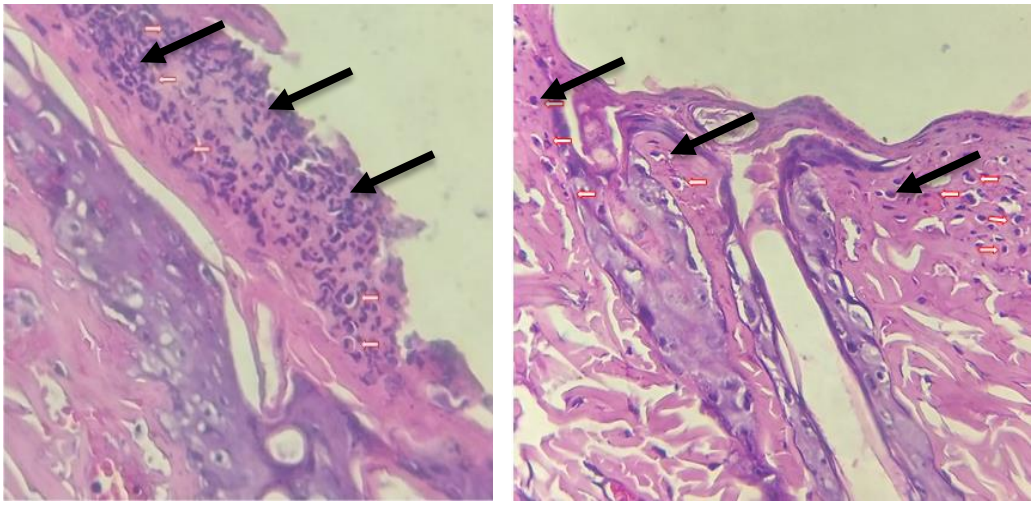

Figure 2. Results of HE staining UV-B exposed mice in the treatment group at 40x magnification

Analysis of IL-1 expression in UV-B exposed rats by administering sapodilla leaf extract cream

The results of descriptive analysis of IL-1 expression in each group of research subjects are shown in table 1 as follows:

Table 1. Results of descriptive IL-1 expression test and *One way anova test* for each group of rats exposed to UV-B by giving sapodilla leaf extract cream.

Group	K1 (Control)	K2 (Placebo)	K3 Dosage 25%	K4 (Dose 50%)	*P value*
IL-1 expression (pg/mL)					
Rat 1	89.51	84.15	101.21	79.31	
Rat 2	65.07	63.29	74.85	82.59	
Rat 3	92.49	71.50	62.51	72.96	
Rat 4	82.08	59.00	98.38	97.17	
Rat 5	99.49	65.75	95.24	99.47	
Rat 6	79.51	72.28	92.85	54.27	
Mean±SD	84.69±12.01	69.32±8.82	88.82±26.06	82.59±17.62	
Shapiro-Wilk	0,878*	0,722*	0,163*	0,929*	
Leuvene Test					0,084*
One way Anova					0,197

Description: * *Shapiro-Wilk* = Normal (p>0.05)
* *Leuvene Test* = Homogen (p>0.05)
* *One way Anova* = Signifikan (p<0,05)

The mean expression of IL-1 in the positive control (K1) was 84.69 pg / mL, the average expression of IL-1 was lowest in the placebo cream group (K2) 69.32 pg / mL, while the average expression of IL-1 was highest in the treatment group of 25% (K3) dose of sapodilla leaf extract cream was 88.82 pg / mL and the average expression of IL-1 in the 50% (K4) dose of sapodilla leaf extract cream treatment group was 82.59 pg / mL.

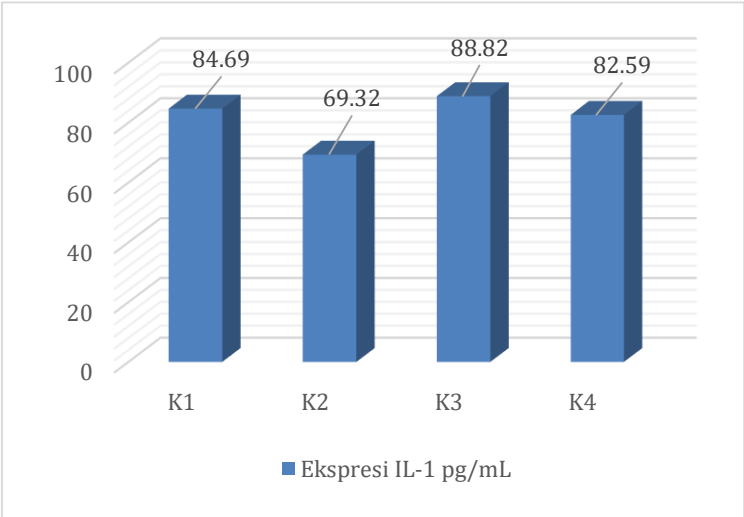

Figure 1. Graph of average IL-1 expression between groups of UV-B exposed rats with sapodilla leaf extract cream

Table 1 shows that IL-1 expression data are normally distributed (p>0.05), and have homogeneous data variations with results of 0.084 (p>0.05). The results of the data were normally distributed and homogeneous, eligible for parametric statistical tests, to compare the average IL-1 expression between groups, carried out *One way anova* test and Posh Hoc LSD *test* to see which dose was most influential.

The average result of IL-1 expression between groups as evidenced by the *One way anova Test* to determine the presence or absence of meaningful differences between groups. *The One way anova* test obtained a p value of 0.197 (p > 0.05) which means there is no significant difference in the average IL-1 expression between groups, so there is no need to continue the *Posh Hoc LSD test*. It can be concluded that the administration of sapodilla leaf extract cream was not significantly different in reducing IL-1 expression compared to the positive group without treatment.

Analysis of TNF-α expression in UV-B exposed rats with sapodilla leaf extract cream

The results of descriptive analysis of TNF-α expression in each group of study subjects are shown in table 2 as follows:

Table 2. The results of the descriptive test of TNF-α expression and *One way anova test* for each group of rats exposed to UV-B by giving sapodilla leaf extract cream

Group	K1 (Control)	K2 (Placebo)	K3 Dosage 25%	K4 (Dose 50%)	*P value*
Expresi TNF-α (ng/L)					
Rat 1	294.07	304.73	304.73	301.39	
Rat 2	309.45	282.37	282.37	316.81	
Rat 3	271.46	317.18	317.18	284.22	
Rat 4	250.45	262.94	262.94	290.22	
Rat 5	326.4	335.72	335.72	276.01	
Rat 6	300.75	363.91	363.91	260.11	
Mean±SD	292.09±27.26	311.14±36.37	336.99±26.06	288.12±19.74	
Shapiro-Wilk	0,901*	0,992*	0,094*	1,000*	
Leuvene Test					0,527*
One way Anova					0,036*

Description: * *Shapiro-Wilk* = Normal (p>0.05)

* *Leuvene Test* = Homogen (p>0.05)

* *One way Anova* = Signifikan (p<0,05)

The average expression of TNF-α was lowest in the 50% (K4) treatment group of sapodilla leaf extract cream at 288.12 ng / L, compared to the positive control group (K1) at 292.09 ng / L, while the average expression of IL-1 was highest in the 25% dose of sapodilla leaf extract cream group at 336.99 ng / L, and the average expression of IL-1 in the placebo cream group (K2) was 311.14 ng / L.

Table 2 shows that TNF-α expression data are normally distributed (p>0.05), and have homogeneous data variation (p>0.05). The results of normally distributed and homogeneous data are eligible for parametric statistical tests, to compare the average TNF-α expression between groups, One-Way ANOVA test and Posh Hoc LSD test to see which dose has the most effect.

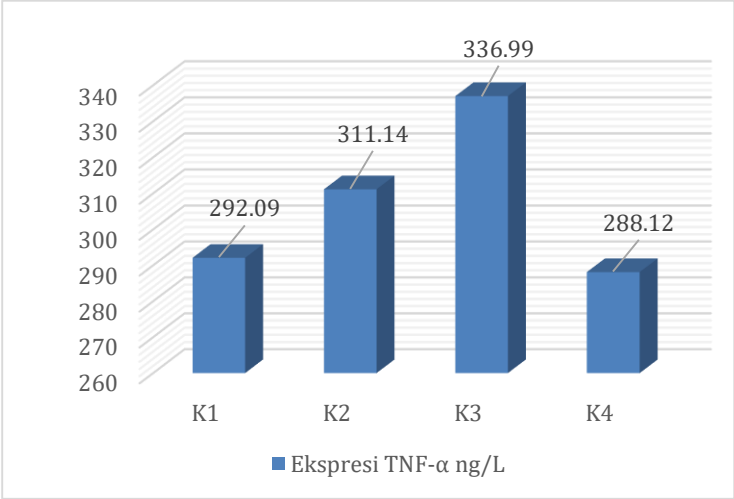

Figure 2. Average expression of TNF-α between groups of UV-B exposed rats with sapodilla leaf extract cream

Analysis of the difference in the average expression of TNF-α between groups with *the One way anova* Test obtained a *p value* of 0.036 (p < 0.05). The results showed a significant difference in the average expression of TNF-α between groups. Furthermore, *a post hoc* LSD test was carried out to determine the most influential dose of sapodilla leaf extract cream, shown in the following table 3:

Table 3. Test results of *Post Hoc LSD* TNF-α TNF- rats exposed to UV-B by giving sapodilla leaf extract cream

Group	K1	K2	K3	K4
K1(Control)		0,276	0,016*	0,818
K2(Placebo)			0,144	0,191
K3(Dosis 25%)				0,009*

Description: * Means p<0.05

The results of *the Post Hoc LSD* test showed that the difference in the average expression of TNF-α between groups, giving 50% (K4) dose of sapodilla leaf extract cream decreased TNF-α expression lower than the positive group (K1) without treatment or the group with placebo cream treatment, but in the group giving 25% dose of sapodilla leaf extract cream did not experience a decrease in TNF-α expression with the highest results between treatment groups.

IV. DISCUSSION

This study aims to observe the effect of giving leaf extract cream (*Manilkara zapota*) in repairing skin damage due to dermatoheliosis (*Sunburn*) caused by UVB exposure. UV radiation induces proinflammatory cytokines such as the production of TNF-α and IL-1 by keratinocytes, dermal fibroblasts and other inflammatory cells. TNF-α stimulates the release of many cytokines and chemokines.[10] Previous research in mice with administration of etanercept (TNF blocker, 4 mg/kg/hr) blocked TNFα inhibiting UVB-induced macrophages, mast cells, and neutrophils. The skin of UVB-irradiated mice contained more mature collagen than mice treated with etanercept and UVB+etanercept.[10] Thus, this study tries to contribute and provide an alternative with the use of natural herbal antioxidants in the form of sapodilla leaf extract cream formulations to repair skin damage due to UVB exposure. Excessive UVB exposure causes sunburn characterized by abnormal keratinocytes that occur prematurely and vacuoles known as sunburn cells, mild epidermal spongiosis, thinning of Langerhans cells, enlargement of endothelial cells, and dermal infiltration of neutrophils. Induces proinflammatory cytokines including IL-1 and TNF-α in the skin. TNF-α stimulates chemotaxis of inflammatory cells in the skin. These cells secrete metalloproteinases (MMPs) and other enzymes that damage the skin matrix.[10]

Continuous exposure of the skin to radiation causes topical skin damage and causes an inflammatory response, resulting in premature skin aging, defined as "photoaging", which is characterized by wrinkles, weakness, dryness, roughness, and pigmentation.[11] UVB radiation can rapidly induce TNF-α expression in keratinocytes (KCs), dermal fibroblasts and mast cells, causing an inflammatory cascade of skin.[10]

In this study showed a decrease in TNF-α expression with the administration of 50% dose of sapodilla leaf extract cream compared to the positive group and placebo cream group. *Manilkara zapota* leaf extract has anti-inflammatory activity that inhibits the release of TNF-α which suppresses inflammation and tissue damage. At a dose of 25% sapodilla leaf extract cream did not experience a decrease in TNF-α expression, the antioxidant content in the preparation was still smaller than free radicals so that it could not reduce TNF-α expression, the increasing trend was thought to be due to the immune response, the high level of cell apoptosis in the inflammatory environment, in addition to the absence of pre-test examination of TNF-α expression so that it could not distinguish TNF-α expression before and after treatment.

Binding results in the recruitment of several factors including TRADD, RIP1, TRAF2, and cIAP 1 and 2 resulting in the formation of complex I signaling via the NF-κB or MAPK pathway to activate p65 or AP1. Signaling complex I results in transcription of inflammatory genes (chemokines, cytokines) and catabolic matrix (MMPs, ADAMTSs) as well as pro-survival genes (cIAP1 and 2, cFLIP, TRAF1, TRAF2). The mechanism of TNFR1 bound to sTNF-α can be internalized and initiate the formation of Complex II or DISC leading to division of procaspase 8 and eventually apoptosis of cells.[12]

Cell death caused by injury causes the release of IL-1α which signals through IL-1R to induce an inflammatory response. IL-1R is constitutively expressed by various cell types and activation of NF-κB and MAPK downstream of IL-1R induces the production of proinflammatory mediators such as cyclooxygenase type-2 (COX-2), IL-6, TNF-α. TNF further increases the production of IL-1α and IL-1β, amplifying the inflammatory stimulation provided by the initial release of IL-1α.[13]

UVB radiation activates MAPK signals such as ERK1, JNK and p38, enhancing inflammatory markers such as TNF-α, IL-6 and COX-2.[14] Excess UVB exposure disrupted epidermal barrier function in mice in a dose-dependent manner that caused an acute inflammatory response or exacerbation of chronic inflammatory skin disease. The dominant source of cytokines in the epidermis is keratinocytes and most of the cytokines secreted from keratinocytes in UVR irradiation are interleukins (IL-1, IL-3, IL-6, IL-8, IL-33). UV-damaged keratinocytes secrete noncoding RNA that can activate TLR3 and induce inflammatory responses, such as an increase in IL-1.[14]

The results of IL-1 expression examination did not occur significant changes because IL-1 transcriptional stimulation occurred

within 30 minutes, and could be maintained for hours.[15] IL-1 plays a role in determining cell fate with respect to modulation of apoptotic pathways, cell proliferation and differentiation.[16] After UV exposure, keratinocytes secrete elevated levels of activated IL-1 family proteins that depend on inflammatory activation.[17] However, in the skin of mice, IL-1 production could not be detected in keratinocytes after UVR exposure or other inflammatory stimuli.[15]

In general, IL-1 cannot be detected or expressed in pro form at very low levels, in tissues and resting cells. The secretion of its active form can be induced in a wide variety of cells including human (but not mice) keratinocytes and a subset of myeloid cells (macrophages, monocytes, and dendritic cells) in response to infection, physical injury, or sun damage.[16] Irreversible damage to sunlight keratinocytes undergo apoptosis to remove damaged or mutated cells. UVR-induced apoptosis can be triggered by ROS formation or CD95 receptor activation. However, elevated CD95 regulation alone does not account for UV-induced apoptosis, which suggests there are other factors involved. In vitro studies on cultured keratinocytes show that IL-1 may protect against TNF-α and CD95-mediated apoptosis, but not against UV-induced cell death.[15]

Longer UV exposure with larger doses is necessary. The duration of UV exposure further increases IL-1 levels, the release of IL-1α from dead cells is a major driver of many inflammatory processes. IL-1α is referred to as "alarmin" and is a critical hazard-related molecular pattern (DAMP) during sterile injury.[13] The TNF-α pathway in complex I is tasked with repairing cell damage faster, while in complex II where cell apoptosis occurs, IL-1 plays a role in inflammatory conditions, IL-1 persists during the apoptosis process so that high IL-1 expression is often detected in chronic inflammatory conditions.

This study proved that the cream formulation of *Manilkara zapota* leaf extract affected TNF-α expression and did not affect IL-1 in rat models experiencing *sunburn*. However, studies still need to continue in animal models of UVB exposure in the early stages of IL-1 activation a few hours after UV exposure and observe the effect of IL-1 expression on chronic exposure longer than 6 days. Analysis of IL-1 and TNF-α pre-test also needs to be done to determine differences in expression before and after treatment to observe genetic and environmental factors that affect IL-1 and TNF-α expression.

V. CONCLUSION

1. Administration of leaf extract cream (*Manilkara zapota*) had no effect on IL-1 expression in UVB-induced wistar rats.
2. Giving leaf extract cream (*Manilkara zapota*) affects TNF-α expression in UVB-induced wistar rats.

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