

## Effects of Oral and Topical Application of *Centella Asiatica* Extracts on The UVB-Induced Photoaging of Hairless Rats

Yeny D. Rahmawati<sup>1\*</sup>, Aulanni'am Aulanni'am<sup>1,2\*</sup>, Sasangka Prasetyawan<sup>1</sup>

<sup>1</sup>Laboratory of Biochemistry, Chemistry Department, University of Brawijaya

<sup>2</sup>Faculty of Veterinary Medicine, University of Brawijaya

\*Corresponding email: aulani@ub.ac.id, yeny.dr@gmail.com

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### ABSTRACT

Chronic exposure to UVB irradiation can cause premature skin aging (photoaging), triggered the production of ROS and oxidative damage in cell components that affect the molecular pathway of dermal fibroblast activity. This paper presents the potential of oral and topical administration of *C.asiatica* extract on hairless rats skin tissue exposed to UVB based on the expression of TGF- $\beta$ 1 and MDA dermis. A total of 30 male-hairless Wistar rats were divided into 6 experimental groups, exposed by UVB with a total dose of 840 mJ/cm<sup>2</sup> for four weeks. Topical gel (2.5%, 5% and 10% extract) and Oral (250 mg/kgBW) therapy was administered once a day after irradiation. Expression of TGF- $\beta$ 1 and MDA dermis were observed by immunohistochemistry. Results of statistical analysis showed that oral and topical therapy of *C. asiatica* extracts are capable of inhibiting decrease of TGF- $\beta$ 1 (at 5% and 10% concentration) and increase of MDA (at 10% concentration) expression significantly ( $p < 0.05$ ) in the dermal fibroblast cells of Wistar hairless rat skin exposed to chronic UVB. Research results suggest that *C. asiatica* extracts have potential as natural skin protection against UVB damage.

Keyword: photoaging, *Centella asiatica*, UVB, TGF- $\beta$ 1, MDA, MMP-1

### INTRODUCTION

Skin aging is the complex biological process that is affected by a combination of endogenous factor such as genetic, hormone, racial, and also exogenous factor likely environment and lifestyle [1,2]. One of the environmental factors that play a pivotal role in skin aging is ultraviolet radiation exposed by the sunlight. This premature skin aging condition is known as photoaging [3].

UVB radiation is the most active component of the sunlight. It causes direct damage in the DNA level and protein, and also indirect damage in the macromolecules that can increase the ROS production and induce the decrease of enzymatic antioxidants [4]. UVB exposures in human skin mainly trigger the production of superoxide anion ( $O_2^{\cdot-}$ ) through NADPH oxidase activation and the respiratory chain reaction in the mitochondria, and further causing chain oxidative reactions [5]. The ROS also have a role in the molecular pathway for inducing the transcription factor AP-1 that stimulate the collagen breakdown through up-regulation of matrix metalloproteinase (MMP) enzyme [6]. On the other hand, UVB irradiation also inhibits the TGF- $\beta$  signal as the main regulator of type 1 procollagen synthesis [7]. The increase of collagen breakdown and the decrease of collagen synthesis causes photoaging.

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Repeated exposures of UV irradiation also actuate the formation of peroxy free radical that can produce MDA as secondary products [8]. Furthermore, it crosses linked and polymerizes the collagen that leads to decrease of skin elasticity and the ability to absorb water [9]. This phenomenon causes skin photoaging.

*Centella Asiatica* (L.) Urban is a perennial plant that has been used as a traditional herb medicine in Indonesia. In Ayurveda (an Indian system of medicine), *C. asiatica* is used for the management of central nervous system, skin and gastrointestinal disorders [10]. Originated from South-East Asia, South Africa, and Madagascar[11], *C. asiatica* has been reported to promote collagen synthesis [17,25], comprise high antioxidant activity [12-14] and UV protection [15].

Beside terpenoids, *C. asiatica* also contains high phenolic and flavonoids like quercetin, campherol, catechin, rutine, aphigenine, naringin, and volatile oils [10]. The pharmacological effect of this plant to the skin has a correlation with its bioactive compound of pentacyclic triterpenoids such as asiaticoside, madecassoside, asiatic acid and madecassic acid as the main components [16]. Those compounds have been reported to promote collagen synthesis [15-17], while the ability to promote the collagen synthesis is an important criterion in relation to anti-aging skin product. Aside from stimulating collagen synthesis, many studies also showed skin protection effects of *C. asiatica* against UV irradiation. *In vitro* research, in the normal human cell, dermal fibroblasts (NHDF) treated with titrated extract of *C. asiatica* (TECA) resulted in photo-protective effect through the change of miRNA [18]. The research about the photo-protective effect in animals has been conducted by Kwon et al. [19] using an aqueous extract of *C. asiatica* as a nanoencapsulated form within gelatin. The results showed that the extract was able to reduce MMP-1 expression as collagen breakdown efficiently in UV-irradiated rat skin.

This research was studied using hairless rats as photoaging model exposed to chronic UVB (840 mJ/cm<sup>2</sup> in four weeks) [20] to investigate the potency of both oral and topical therapy of *C. asiatica* extract towards dermal fibroblast cells, where collagen as the major components of skin extracellular matrix mainly produced. Furthermore, the observation of TGF-β1 expression was conducted since it is a key regulator of procollagen synthesis, whereas MDA is a biomarker of oxidative damage of cell components.

## EXPERIMENT

### Materials

Materials used in this research included *Centellae herb* powder (Materia medica, Batu), ethanol (Bratachem), hydroxypropyl-methylcellulose (HPMC SH-60), aquadest, primary antibody: (1)Rb pAb to TGF-β1 (Santa Cruz) (2)Rb pAb to Malondialdehyde (AbCAM), Immunohistochemistry Kit (ScyTek), xylo, hydrogen peroxide, formalin, paraformaldehyde, bovine serum albumin, phosphate buffer saline, fetal bovine serum, and paraffin.

### Instrumentation

Instrumentation applied in this work such as LC-MS/MS spectrometry (Thermo, hypersil gold column 50 mm x 2.1 mm x 1.9 μm), solar stimulator UVB with built-in LCD timer (YONKER YK-6000BT), UV Light-meter (Lutron YK-35UV), microscope (Olympus BX53), Glassware, Refrigerator 4°C, Freezer -20°C, Microtome (Leica Jung), an incubator.

### Experimental animal

Male Wistar rats (*Rattus norvegicus*) age 8-10 weeks, weight 180-200 grams, were used as animal models. All rats were being adapted in Biosains Laboratory for a week, *ad libitum* fed with standard food, and shaved on the left abdomen (5x5 cm<sup>2</sup>) before UVB exposure[20]. The use of experimental animals has been approved by the animal care and use committee Universitas Brawijaya with ethical clearance No: 837-KEP-UB.

### Preparation of *C. Asiatica* Extract

*Centellae herba* powder (500 g) was macerated in ethanol (5 L, 96 %) for 3 days, while stirred occasionally using overhead stirrer (30 minutes, at 500 rpm). The macerates were then filtered using semipermeable paper (filter paper), and the filtrate was evaporated using rotary evaporator vacuum to get the viscous extract.

### Preparation of Oral and Topical Administration

Oral extract with dose 250 mg/kgBW was prepared as follow: viscous extract of *C. asiatica* (16.7 g) in a beaker glass was diluted with aquadest, it was then stirred while adding the aquadest until 1 L of the mixture was obtained.

Topical gel was prepared as quoted by Sujono's research with modification [21] : hydroxypropyl-methylcellulose (4 g) was dispersed into aquadest (30 mL, 80 – 90 °C) in a 50 mL of beaker glass until swelled, then stirred it in cold condition (ice cubes in the basin) until the gel formed. Viscous *C. asiatica* extract was then added into the mixture that already formed gel (@ 0 g, 1.25 g, 2.5 g and 5 g to make a concentration of 0%, 2.5%, 5%, and 10%) and stirred it while added aquadest until homogenous gel mixture weight 50 g was obtained. The topical gel was ready to store in a tube.

### Analysis of Bioactive Compound in *C. asiatica*

Bioactive compounds contained in *C. asiatica* were analyzed qualitatively to identify the existence of triterpenoid compound using LC-MS/MS. The mobile phase was composed of 0.1% aqueous formic acid (A) and acetonitrile formic acid solution (B) in gradient. The linear gradient was maintained at a rate of 300 µL/min. The sample injection volume was 2 µL at 160 °C. The column temperature was kept constantly at 300°C and autosampler compartment was kept at 160 °C. ESI ionization was directed to spray voltage at 3.0 kV, vapor temperature at 2500 °C, and capillary temperature at 300 °C. Nitrogen was applied as sheath gas pressure at 40 psi and argon was used as aux gas pressure at 10 psi.

### UVB Exposure and Therapy

A 30 hairless-male Wistar rats were divided into 6 experimental groups: KN=negative control group (without UVB exposure and therapy); KP=the gelling agent base (HPMC) positive control group (without *C. asiatica* extract); P=*parasol*<sup>®</sup> group (brand anti-UV in the market, contained octyl methoxycinnamate); T1=topical 2.5% extract + 2 mL oral extract 250 mg/kgBW; T2=topical 5% extract + 2 mL oral extract 250 mg/kgBW; T3=topical 10% extract + 2 mL oral extract 250 mg/kgBW. A rat was inserted into a special wooden box, then irradiated on the left abdomen (hairless) using solar stimulator. Irradiation was held every Monday, Wednesday, and Friday for four weeks. The total dose of UVB radiation was 840 mJ/cm<sup>2</sup>: week I @50 mJ/cm<sup>2</sup>, week II @70 mJ/cm<sup>2</sup>, week III and IV @80mJ/cm<sup>2</sup>. Topical gel therapy applied 4 hours after irradiation. The topical gel remains applied on days without irradiation once a day for four weeks. The oral extract administered once every day after irradiation for four weeks.

## Immunohistochemistry and Microscopic Observation

Prior to immunohistochemical staining, slides were deparaffinized and rehydrated using xylol solution, graded alcohol (absolute, 96%, 80%, 70%, 50% dan 30%), and distilled water for 3x5 min. Immunohistochemical staining was started with endogenous peroxidase blocking. Slides were dropped using hydrogen peroxide in the deionized water for 20 minutes. And then serum blocking was conducted by dropping the slides using BSA 1% solution in FBS at room temperature. Afterward, slides were dropped by primary antibody anti-TGF- $\beta$ 1, anti-MMP-1 and anti-MDA which diluted in 1% BSA in FBS solution in a ratio of 1: 200. And then, slides were dropped using a secondary antibody with biotin-labeled and kept overnight at 4°C. Afterward, slides were dropped using SA-HRP and kept for 120 minutes at room temperature, and then was dropped by chromagen DAB and incubated for 20 minutes, washed by distilled water, and then dried in the oven. After finishing one step, washing was conducted using PBS at pH 7.4 for 3x5 minutes. Finally, slides were covered by cover-glass, kept overnight and ready to be observed under a microscope.

MMP-1 and MDA expression were observed at least 3 viewings with magnification 400 $\times$  using Microscope Olympus BX53 equipped with an optilab pro camera. Microphotograph results were analyzed using software Image Raster 2.1.

## Statistical Analysis

Data are expressed as mean  $\pm$  the standard of deviation (SD). For quantitative analysis of multiple comparisons using a one-way ANOVA followed by posthoc's test for normal and homogenous data. A *p*-value of less than 0.05 considered statistically significant. Statistical analysis was performed using SPSS version 16 software.

## RESULT AND DISCUSSION

### Bioactive Compounds of *C. Asiatica* Extract

Based on the LC-MS/MS chromatogram results (data not shown) represented in Table 1, there are 3 triterpenoid compounds identified in the *C. Asiatica* extract. Moreover, LC-MS/MS chromatogram showed the highest peak at 2.18 minutes with molecular weight 976 *m/z*. This result was alleged as ion-molecule of chemical formula  $C_{48}H_{78}O_{20}$  that corresponded to madecassoside (MS). The other peaks corresponded to asiaticoside (AS) and madecassic acid (MA) [22]. These compounds are triterpenoid compounds that play pivotal roles as skin protective and anti-aging ingredients addressing skin damage in *C. asiatica* extract [16].

**Table 1.** Interpretation of LC-MS/MS results for *C. asiatica* extract

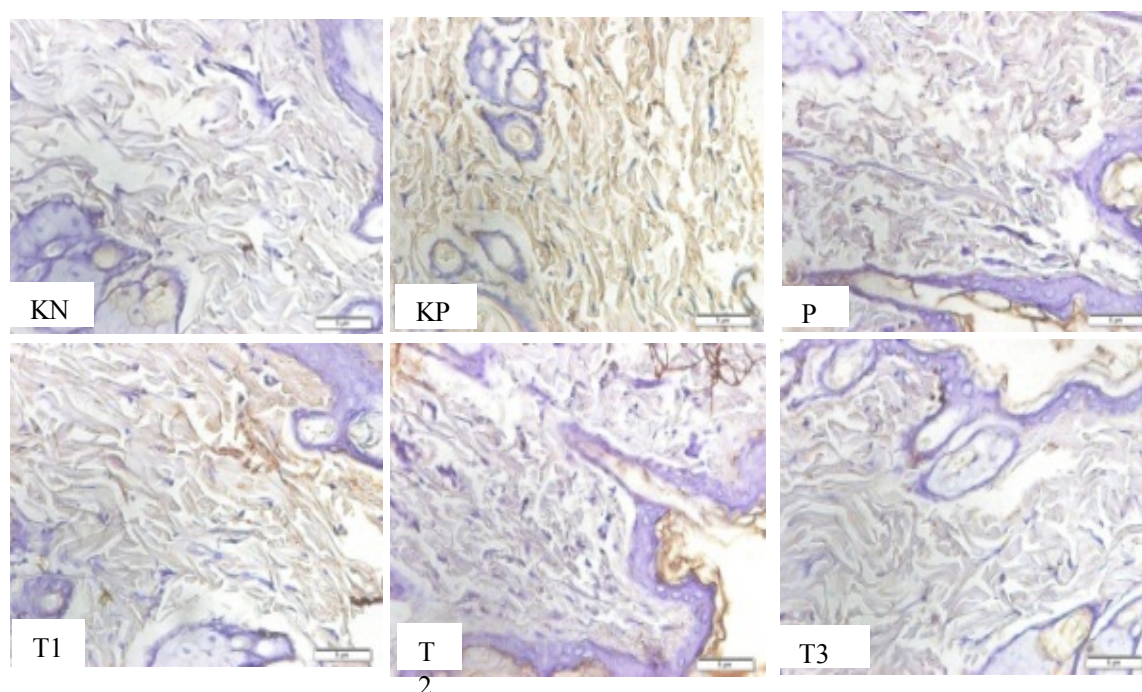
Peak	Fragment Ions (m/z)	[M] <sup>+</sup> (m/z)	Standard Fragment Ions (m/z)	Prediction of triterpenoid compounds
2.18	452.50-453.50	976	487.3	MS
3.79	450.50-451.50	522	453.4	AS
5.27	452.50-453.50	506	451.2	MA

### Expression of TGF- $\beta$ 1 and MDA of Dermal Fibroblast

Based on these data (Figure 1 to 4), it is clear that chronic UVB light with a total dose of 840 mJ/cm<sup>2</sup> for four weeks has a negative impact on dermal fibroblast cells. UVB rays are able to penetrate the dermis layer and interact with the intracellular chromophors thus affecting fibroblast and extracellular matrix activity [23]. The increase of MDA levels in the

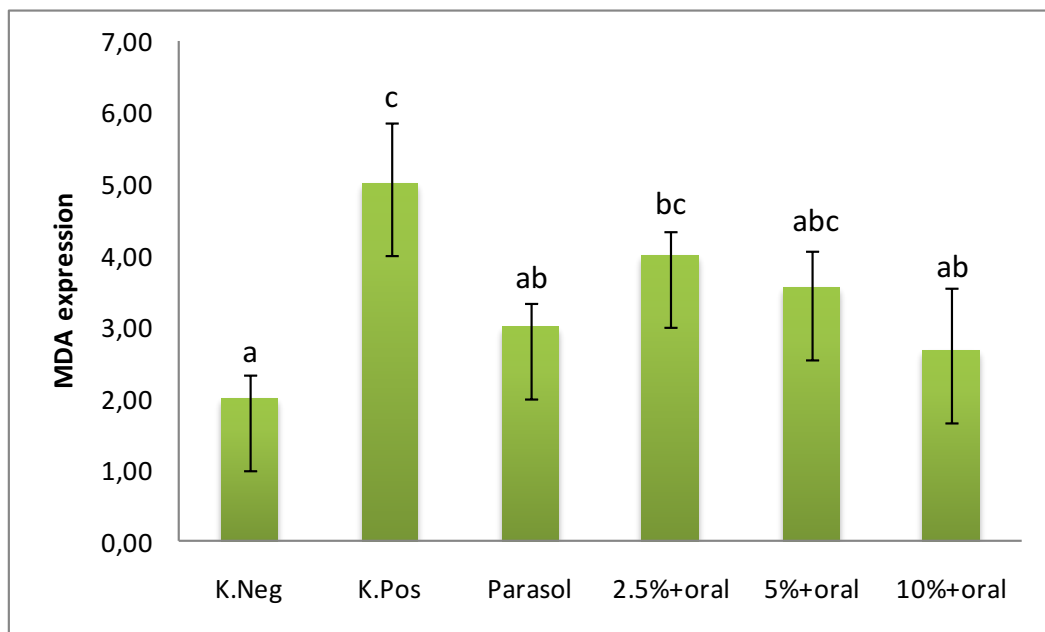


positive control group (KP) compared to a negative control group (KN) characterized higher oxidative damage induced by the UVB. UVB exposure with a total dose of 840 mJ/cm<sup>2</sup> was probably sufficient of forming ROS in the skin surface. The formation of ROS in the skin surface triggers oxidative damage toward cell components attributed by the increase of MDA level as biomarker occurrence of lipid peroxidation [8]. The accumulation of ROS in the skin surface would also affect the molecular pathway. The ROS would activate transcription factor, AP-1 which will inhibit the TGF- $\beta$  signal and activate the MMP(s) enzymes [6]. This molecular pathway explaining the decrease of TGF- $\beta$ 1 expression in the positive control group (KP) compared to a negative control group (KN) as resulted from the experiment.

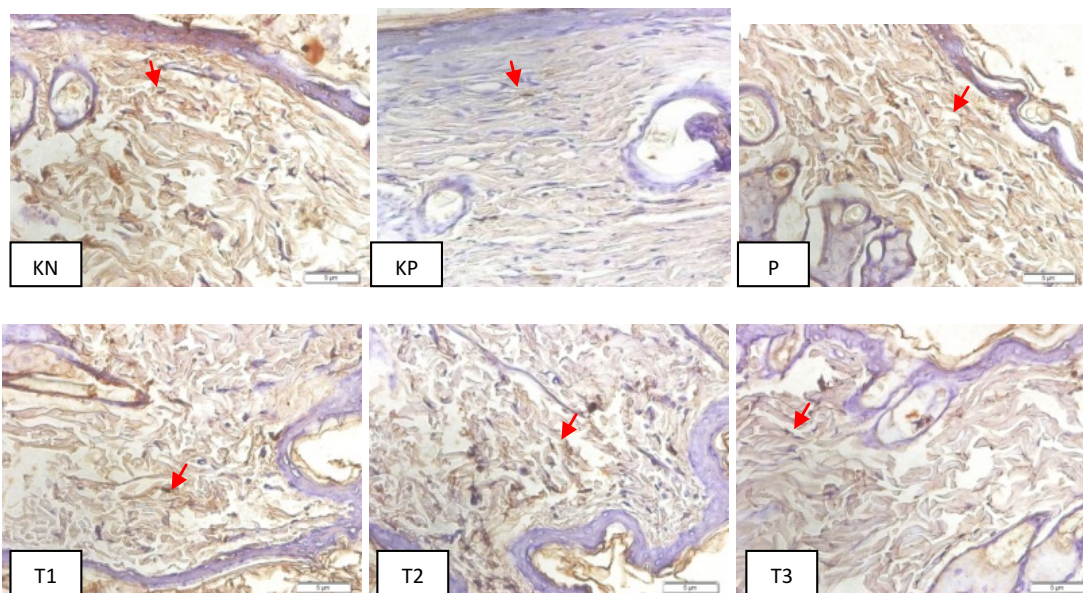


**Figure 1.** Expression of MDA dermal fibroblast cells by using IHC method. The observation performed using an Olympus BX53 microscope with built in Optilab pro camera and 400 $\times$  magnification. The brown colored cells indicated expression of MDA. Description: KN= negative control (without UVB and therapy); KP= positive control (HPMC); P=parasol; T1=2.5% topical extract + 2mL oral 250mg/kgBW; T2=5.0% topical extract + 2mL oral 250mg/kgBW; T3=10% topical extract + 2mL oral 250mg/kgBW

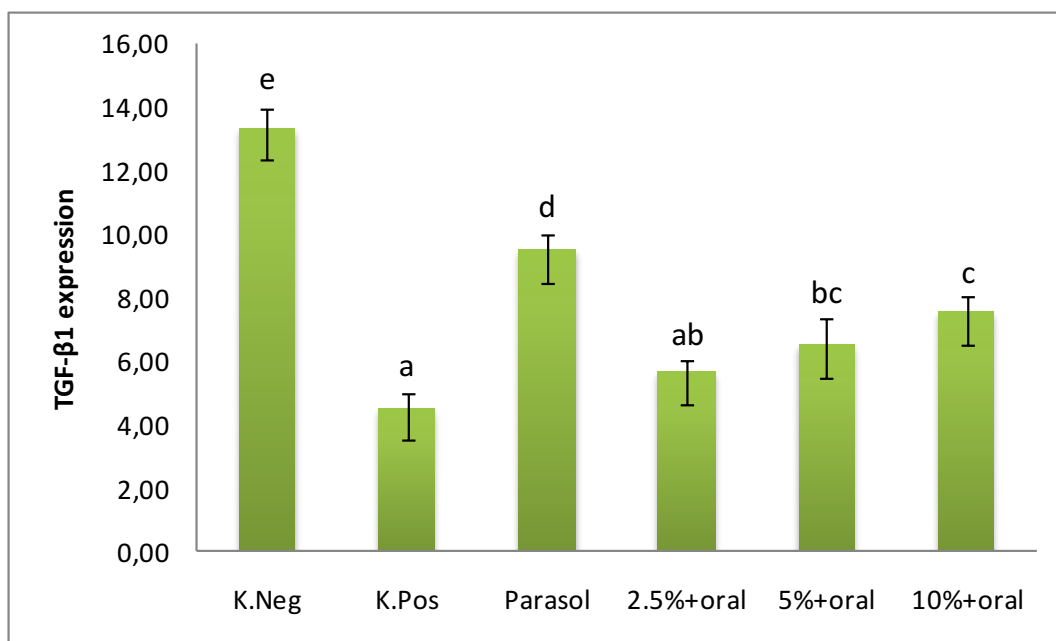
In this case, to demonstrate the UV-protection action of *C. asiatica* extract in protecting skin from premature-skin aging (photoaging) due to chronic UVB exposure, the hairless skin rats were treated with *C. asiatica* extract both oral (250 mg/kgBW, 2mL) and topical gel with variation concentration of 2.5%, 5%, and 10% extract. Statistical analysis results (Figure 2 and Figure 4) showed that oral and topical therapy of *C. asiatica* extracts is capable of inhibiting decrease of TGF- $\beta$ 1 (at 5% and 10% concentration) and increase of MDA expression (at 10% concentration) significantly ( $p < 0.05$ ) compared to the positive control. Furthermore, these data also described that the inhibition was dose dependent manner wherein the higher concentration of *C. asiatica* extract, the greater also the capability of inhibiting decrease of TGF- $\beta$ 1 and increase of MDA.



**Figure 2.** Percentage of MDA expression in dermal fibroblast cells.



**Figure 3.** Expression of TGF- $\beta$ 1 dermal fibroblast cells by using IHC method. The observation performed using an Olympus BX53 microscope with built in Optilab pro camera and 400 $\times$  magnification. The brown colored cells indicated expression of TGF- $\beta$ 1. Description: KN= negative control (without UVB and therapy); KP= positive control (HPMC); P=parazol; T1=2.5% topical extract + 2mL oral 250mg/kgBW; T2=5.0% topical extract + 2mL oral 250 mg/kgBW; T3=10% topical extract + 2mL oral 250 mg/kgBW



**Figure 4.** Percentage of TGF-β1 expression in dermal fibroblast cells.

Skin aging is associated with increased AP-1 activity, impaired in TGF-β signaling and increased MMP(s) expression as a response to UV-irradiation [24] forming ROS in the skin surface [5]. This experiment showed that the UV-protection effect of *C. asiatica* extracts against chronic UVB exposure towards dermal fibroblast cells of hairless rats skin supposed to be a synergistic effect of triterpenoid saponin of madecassoside, asiaticoside and madecassic acid by stimulating procollagen synthesis via a cellular mechanism, with phenolic compounds of flavonoid acting as an antioxidant. As an antioxidant, bioactive components in *C. asiatica* extract might counteract free radicals by scavenging mechanism. Thus it prevents the oxidative chain reaction and downregulation of extracellular matrix in dermal fibroblast cells.

## CONCLUSION

Oral and topical therapy of *C. asiatica* extract was capable of inhibiting decrease of TGF-β1 and increase of MDA expression significantly ( $p < 0.05$ ) in dermal fibroblast cells of hairless Wistar rat skin exposed by UVB. Therefore, the oral and topical therapy of *C. asiatica* is suggested to be potential natural skin protection against UVB damage. This protective activity is might be due to the synergistic effect of its bioactive components from triterpene as madecassoside, asiaticoside and madecassic acid with the phenolic component of flavonoid. Further experiments in the future are expected to find out the inhibition pathway and optimum concentration of *C. asiatica* extract.

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