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The Protective Effect of Propolis Nanoemulsion (NEP) Against UVB Irradiation Inducing Photoaging in Human Dermal Fibroblast (HDF)

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Abstract

The skin damage induced by UV irradiation can cause photoaging as a consequence of reactive oxygen species (ROS) generation. Propolis is one of the most abundant natural product sources of polyphenols and derived from various plant resins collected by honeybees. This study aimed to determine the due administration of the propolis nanoemulsion (NEP) as an anti-photoaging by observing the concentration of intracellular ROS and lipid peroxides in human dermal fibroblast (HDF) by exposure to UVB. The cytotoxic of NEP was tested by the MTT assay, intracellular ROS was measured using the DCFDA assay, lipid peroxide products were analyzed by the TBARS assay and cell nuclei was observed by DAPI staining. PSA analysis revealed that the sizes of NEP were about 131.9 to 131 nm. The optimum concentration of NEP was 0,1µg / ml. ROS production in HDF treatment of NEP significantly decreases ($p < 0.05$). Lipid peroxide products showed the same pattern as ROS concentration. The lipid peroxide concentration in the treatment NEP significantly decreases ($p<0.05$). In conclusion, the propolis in NEP acts as an antioxidant and has the potential to reduce the production of ROS and lipid peroxides caused by exposure to UVB.

Keywords: HDF cells, nanoemulsion, propolis, photoaging, UVB, MTT, ROS, DCFDA, TBARS

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1. Introduction

Aging is a biological process that naturally happens in all living organisms. Aging process occurs in all organs of the body including skin as the outer organ which plays a role in the protection system [1]. Ultraviolet irradiation (UVR) can elevate the accumulation of free radicals within cells. The free radical is an unstable molecule that is highly reactive and seeks for its electron pair. Free radical molecule is also called reactive oxygen species (ROS) [2]. Direct and indirect UVR will activate various inductive ROS reactions and initiate the signal transduction pathway which causes skin damage and stimulate premature aging in skin due to UVR, a process called photoaging [3]. UVR, especially UVA and UVB, can penetrate through the dermal layer and cause photoaging [4].

The concept of Anti-Aging Medicine (AAM) considers aging as a prevented disease to increase human quality as people getting old [5]. Using the additive antioxidant is one of the solutions often applied to prevent or slow the aging process. One of the natural materials which contains a high antioxidant property is propolis. Propolis contains several complex natural compounds, which have potential antioxidant properties such as flavonoids and polyphenols [6]. Several studies have mentioned the role of propolis as an anti-microbe [7], anti-inflammation [8], antitumor [9, 10], and antioxidant [11]. Until now, there is no study that tests propolis as an anti-photoaging property in the human skin. In tropical country, high exposure of UVR throughout the year will increase photoaging risk in the skin.

In this study, propolis was applied in the form of propolis nanoemulsion (NEP) into human dermal fibroblast (HDF) cells as a photoaging model. The UVB irradiation treatment, which has fifth times photon energy greater than UVA, is assumed to be more harmful and can cause photoaging in skin. In this study, the role of NEP in inhibiting photoaging in HDF cells due to UVB exposure was done by ROS concentration analysis, lipid peroxide product concentration, and nucleus morphology. The objective of this research is to know the effect of nanoemulsion propolis (NEP) as an anti-photoaging compound by observing the concentration of intracellular ROS and lipid peroxide products in HDF cells after being irradiated using UVB.

2. Methods

2.1. Cell culture

Human dermal fibroblast (HDF) cells were obtained from 3 and 5 years old male's preputium tissue by a primary explant culture method. HDF cells cultured in the growth medium Dulbecco's Modified Essential Medium (DMEM, Biowest, cat. number: L0064-500) was added with 10% Fetal Bovine Serum (FBS) (Biowest, cat. number: S1810-500), 100 IU/ml penicillin-streptomycin (Gibco, cat. number: 15140148), 50µg/ml gentamycin (Gibco, cat. number: 15750060), and 2mM L-glutamine (Biowest, cat. number: X0550-100). HDF cells were grown in the 25 cm^2 flask (Iwaki) at 37 ^{0}C inside the incubator CO2 5% (Heraeus). The growth medium was replaced every 3 days. Cells, which were confluent, were subcultured into two 25 cm^2 flasks using 0, 02% EDTA, and 0, 25% trypsin (Sigma).

2.2. Propolis Extraction and Identification of Active Substance

Propolis was extracted using maceration method as has been done by Jun [6]. Extraction was done by using 70% ethanol as solvent. The solution was stirred steadily for seven days with 200 rpm speed in the dark room. Then, the solution was filtered and concentrated using rotary evaporator. The active substances in ethanolic extracts of propolis (EEP) was analyzed using Gas Chromatography-Mass Spectrometry (GCMS), and anti-oxidant activity of EEP was measured using 2, 2-diphenil 1-pichylhydazyl (DPPH assay).

2.3. Formulation and Characterization of Nanoemulsion Propolis (NEP)

Nanoemulsion propolis (NEP) formulation was done using sonication method described by Mauludin et al. [12] by adding colliphor RH40 as a surfactant, glycerin as co-surfactant, and VCO as oil phase. Surfactant and co-surfactant were stirred using a magnetic stirrer, then the oil phase and propolis were added. The next step for facilitating nanoemulsion formation was sonication using the water bath sonicator. The NEP was added with deionized water gradually and homogenized until the mixture was clear, stable, and the particle size was under 200 nm. The diameter of NEP globule was observed using Particle Size Analyzer (PSA, Delsa Nano C particle analyzer Beckmann Coulter). The existence of active substances in NEP was analyzed using Spectrophotometer Fourier Transform Infrared (FTIR) according to Butnariu & Giuchici [13].

2.4. Cytotoxic Assay

The determination of NEP concentration was done using cell viability evaluation. The cells was treated with NEP in series of concentration (0 µg/ml, 0.1 µg/ml , 0.5 µg/ml, 1 µg/ml, 10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml) with different incubation time. HDF cells were seeded in 96 well plates, with the total cell number was 5.000 cells/well and

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incubated with complete growth medium containing NEP for 24 hours, 8 hours, and 1 hour. After incubation, cells were washed with Phosphate Buffer Saline (PBS) and incubated in growth medium for 24 hours. Cell viability evaluation was done using the MTT assay. The growth medium was discarded and then added with 100µl/well complete growth medium and added with 10µl MTT (12mM). Cells were incubated for 4 hours. After 4 hours, the medium was discarded and 50µl DMSO/well was added. The solution was resuspended and incubated for 10 minutes at 37ºC. The absorbance was analyzed with ELISA microplate reader (Bio-Rad) at 550nm wavelength.

2.5. Reactive Oxygen Species (ROS) Assay

HDF cells, which were confluent, were trypsinized and seeded in the well plate. After 24 hours, cells were treated with NEP and then incubated for one hour. Cells were washed with PBS and irradiated using UVB 200mJ/cm2 (312 nm, Philips 9W/12) in PBS.

Intracellular Reactive Oxygen Species (ROS) in HDF cell culture was measured using 2', 7'–dichlorofluorescin diacetate (DCFDA kit, Abcam). HDF cells were seeded in 96-wellplates with 10.000 cells/well. Cells were added with 100µl DCFDA 20µM and incubated 37 ^{0}C for 45 minutes. Then, cells were treated with 100µl NEP 0, 1µg/ml and incubated 37 ^{0}C for one hour. After incubation, cells were irradiated with UVB 200mJ/cm2. Fluorescence intensity formed was read immediately using the fluorescence microplate reader (Thermo scientific) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.6. TBARS Assay

Lipid oxidation by ROS was measured using Thiobarbituric Acid Reactive Substances (TBARS) assay. HDF cells were seeded in 6-wellplates at the total cell number of 500.000 cells/well. Cells were treated with NEP 0, 1µg/ml and incubated 37 ^{0}C for one hour. After that, cells were irradiated with UVB 200mJ/cm2 in PBS and incubated in complete growth medium for 24 hours. MDA measurement was done with obtaining the cells using 500µl TCA 2, 5%. The cells were centrifuged for 2 minutes with 13.000 g speed. 250µl supernatant of cell lysate was added with 200µl TCA 15% and 400µl (TBA 0, 67% and 0, 01% BHT). The mixture solution was heated for 20 minutes in a water bath at 95ºC. After cooled down at the room temperature, the solution was added with 750µl buthanol and homogenized. 200µl upper phase of this solution was MDA-TBA adduct, which seemed in pink color, was transferred into 96-wellplate and the absorbance was read using a spectrophotometer at 532 nm wavelength. MDA concentration was obtained by interpolating MDA standard curve, which was resulted from linear regression, and normalized with the total amount of protein.

2.7. DAPI Staining

Nucleus morphology was observed using DAPI staining. In principle, DAPI can make a mark on DNA with binding with nitrogen base A-T in DNA. If any, changes in nucleus morphology condition indicated by an unstained region in the nucleus means that there is a damage in DNA. HDF cells were seeded in the culture dish d=3cm (Iwaki). After UVB irradiation and NEP treatment, cells were fixed using 4% paraformaldehyde (Sigma) for 15 minutes. Cells were washed using PBS. Then, the cell membrane was permeabilized with 0, 5% Triton X-100 (Sigma) for 10 minutes. Blocking was done by incubating cells in 1% Bovine Serum Albumin (BSA). Nucleus was stained by incubating cells in DAPI 1µg/ml for 40 minutes. Cells were washed using PBS and all the samples were dripped with glycerol-PBS (2:1) and closed using cover glass. Cell morphology was observed using a laser scan confocal microscope (Olympus).

3. Results and discussion

3.1. Cell Culture

HDF was isolated from preputium tissue and it was used as a research model to study photoaging. The morphology of HDF under the microscope was fibroblastic-like and adhere (Figure 1).

3.2. Propolis Extraction and Identification of Active Substance

Propolis was obtained as one of the bee (*Trigona sp*.) products that were bred in the heterogenous forest in Subang, Jawa Barat. In this study, crude propolis can be extracted to obtain extract ethanol propolis (EEP). Based on GC-MS result (Supplementary 1), EEP contains 30 different kinds of chemical compounds. Generally, active substances contained in EEP were $\pm$ 55% polyphenol and ester which has a role as an antioxidant, and the others were $\pm$ 12% essential oil, and 33% other organic compounds. The active substances which had a role in antioxidant activity were found as phenolic acid, polyphenol, and catechol. This result was supported by the study of Kurek-G´orecka *et al*. [14] which mentioned that ± 50% propolis composition was flavonoid, phenolic acid, and ester derivation.

From the DPPH result, IC_{50} score for EEP was 127.759 µg/ml and IC_{50} score for ascorbic acid was 82.443 µg/ml (Supplementary 2). With comparing this IC_{50} score, it was

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known that EEP had relative anti-oxidant potency 0,65 times greater than ascorbic acid. elative anti-oxidant potency of EEP in this research was better than Talla's research [15] that stated EEP had relative anti-oxidant potency only 0,13 times greater than ascorbic acid.

3.3. *Formulation and Characterization of Nanoemulsion Propolis (NEP)*

The optimization result showed that NEP formed from 5% VCO, 40% surfactant (Kolliphor RH40 : glycerin = 3:1), 3% EEP, and 52% aquabidest has a dark red color. Droplet NEP was measured and had a globule size in 131nm, in pH 6,8 (Figure 2).

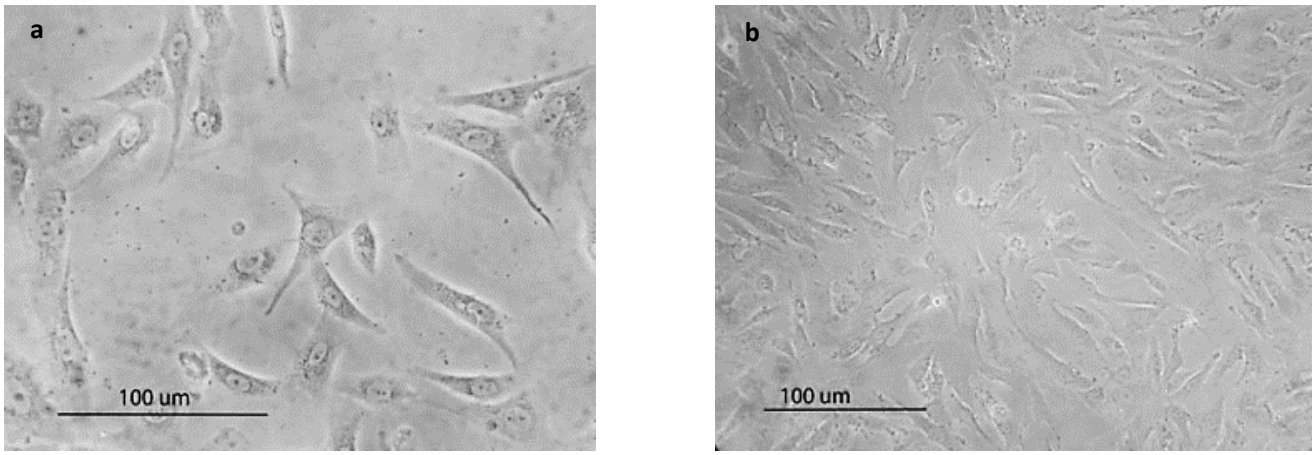

Figure 1 HDF cells from primary culture. (a) Morphology of HDF cells (magnification 400x), (b) HDF cells reached 70% *confluent* (magnification 200x).

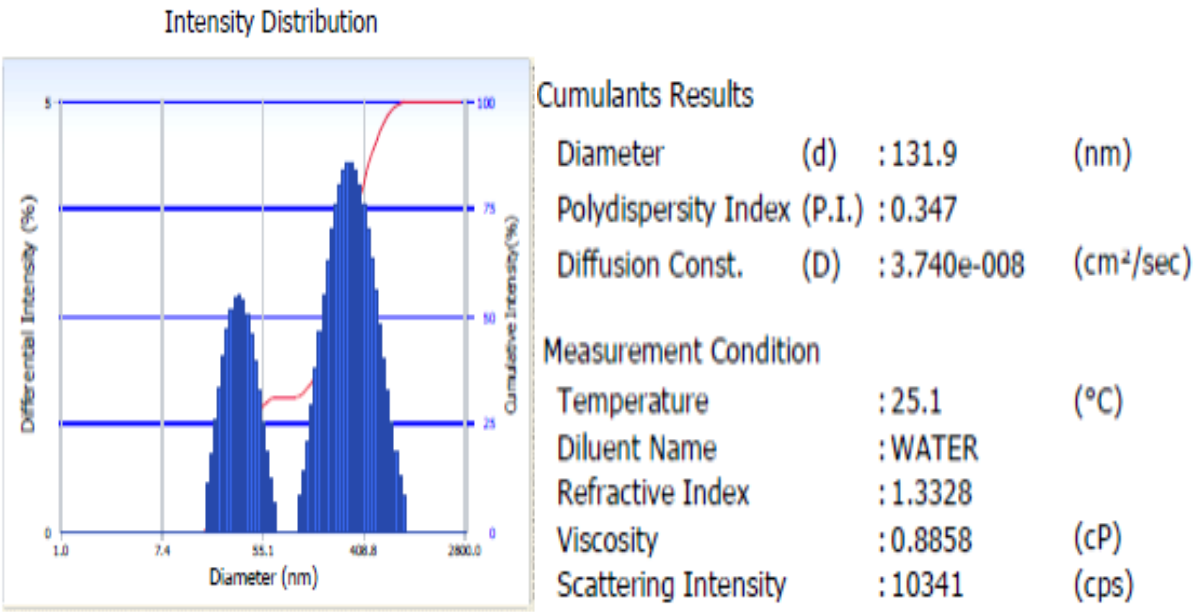

Figure 2 NEP Characterization using Particle Size Analizer

Composition analysis using FTIR (Figure 3) proved that the peak of NEP was the same as the peak of EEP. The peak of NEP and EEP at the frequency ± 2930 and ± 3300 cm^{-1} was known as the O-H group that indicates alcohol and phenol compounds. Besides that, there was also peak frequency at 1730 cm^{-1} that identified as C-O and C=O group which are ester compound and carboxilate acid. In addition, vibration in the peak of frequency ± 1300, ± 1400 cm^{-1} were

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identified as the C-H group which are alkene compounds. Another alkene compound was also found at frequency ± 1630 cm^{-1}. These findings were similar to Butnariu *et al*. [13] study which showed that NEP had frequency at 1450 and 1460 cm^{-1} that confirmed the existence of the C-H group and frequency at 1700 cm^{-1} showed C=O group as characteristic of NEP. Thus, this study confirms that the characteristic of propolis did not change while developing NEP since the chemical groups in both types of propolis were the same as shown by the FTIR analysis.

3.4. *Cytotoxicity of NEP*

Cytotoxicity of NEP was determined by measuring the cell viability with an MTT assay. NEP at 0,1-1µg/ml concentration did not cause any significant decline to cell viability ($p<0,05$). NEP concentration above 10µg/ml was toxic, therefore cell viability fell into under 50%. This phenomenon was analyzed using the toxicity parameter [16].

The incubation time after cells were exposed with NEP at these three concentrations (i.e., 0.1 µg/ml, 0.5 µg/ml, 1 µg/ml) were 1, 6, and 24 hours before the UVB irradiation. There is no significant difference among the nine groups. NEP treatment at 0,1µg/ml concentration, which was incubated for one hour (*), showed the optimal concentration and significant difference in comparison to UVB exposure without the NEP treatment group (**) ($p<0,05$) (Figure 4).

Cells incubated in 0,1µg/ml NEP for 1 hour showed a significant increase (i.e., 12%) compared to cells in UVB irradiation without NEP treatment (Figure 5). The increase of cell viability after NEP treatment was assumed due to the inhibition of damage and cell death mechanism which affected by high ROS production within the cells. This inhibition was occurred due to the presence of polyphenol in NEP (Figure 6).

3.5. *NEP Potency as Anti-photoaging Agent*

In this study, HDF cells exposed to UVB in 200mJ/cm^2 dosage showed an increase of fluorescence intensity. This fluorescence showed the existence of ROS which is high after UVB irradiation.

Also, NEP treatment at 0,1µg/ml concentration decreased ROS production in HDF cells after being exposed by UVB irradiation. The fluorescence intensity in this treatment decreased with a score of 20%. This score showed a significant difference ($p<0,05$) compared to UVB irradiation without any NEP treatment (Figure 6). The decline of ROS production showed the NEP effect as an anti-photoaging agent in HDF cells. Active substances in EEP, i.e., polyphenol and catechin, act as an antioxidant. This antioxidant property was assumed to decrease the intracellular ROS through scavenger activity of ROS and increasing intracellular antioxidant, e.g., SOD, GPx, CAT, and other compounds. A study of Sarma et al. [17] showed a similar result. Polyphenol, which has an aromatic ring with –OH or $–OCH_3$ groups, acts as a radical scavenger by giving the electron or hydrogen atom directly to ROS [17]. The increase of antioxidant enzyme in observational groups occurred because of polyphenol and catechin activity. Lopes *et al*. [18] also mentioned that propolis can increase the antioxidant enzyme activity (e.g., SOD, GPx, and CAT) in mice's lungs which were exposed to cigarette's smoke. Thus, it can be concluded that propolis can decrease ROS production.

3.6. *The Effect of NEP on Lipid Peroxidation*

The lipid membrane component in the cell is vulnerable to damage, mainly by irradiation [19]. This study showed that UVB irradiation to HDF cells increased the lipid peroxide product and NEP decreased lipid peroxide product that has been formed (Figure 7).

Polyunsaturated fatty acid (PUFA), which was contained in the cell membrane, is very sensitive to ROS. Hydroxyl radical (·OH) compound could cause peroxidation in PUFA. The lipid peroxidation process has been mentioned by Sharma *et al*. [17] and such mechanism was also assumed to happen in this research. Lipid peroxidation was started from initiation reaction by ROS activation. This ROS react with methylene group from PUFA formed lipid peroxy radicals and hydroxyperoxide. Malonialdehyde (MDA) is the final product from fatty acid peroxidation in phospholipid membrane and can potentially damage the cell membrane. In this research, the formed MDA can be measured as a lipid peroxide, which was formed due to UVB irradiation.

The concentration of lipid peroxide in NEP treatment reached a score of 18%, showing a significant difference ($p<0,05$) compared to UVB treatment only. The fall of lipid peroxide product was assumed due to the existence of active substances in EEP such as polyphenol and catechin which have a role in initiating process by inhibiting ROS production, therefore it affected the following reaction in lipid peroxide formation. It was also supported by the statement from Sharma *et al*. [17] that polyphenol inhibited ROS and lipid peroxide by blocking radical lipid alkoxyl. In this study, propolis has a role in inhibiting ROS and made lipid peroxide concentration dropped. This was supported by Silva *et al*. [20] who stated that propolis contained polyphenol which is very effective in inhibiting lipid oxidation. This indicated that polyphenol compound in propolis has a role as lipid protection of cell membrane. Thus, EEP formulated as NEP has potential as a lipid peroxidation inhibitor by scavenging ROS in initiating the process of lipid peroxidation due to UVB irradiation.

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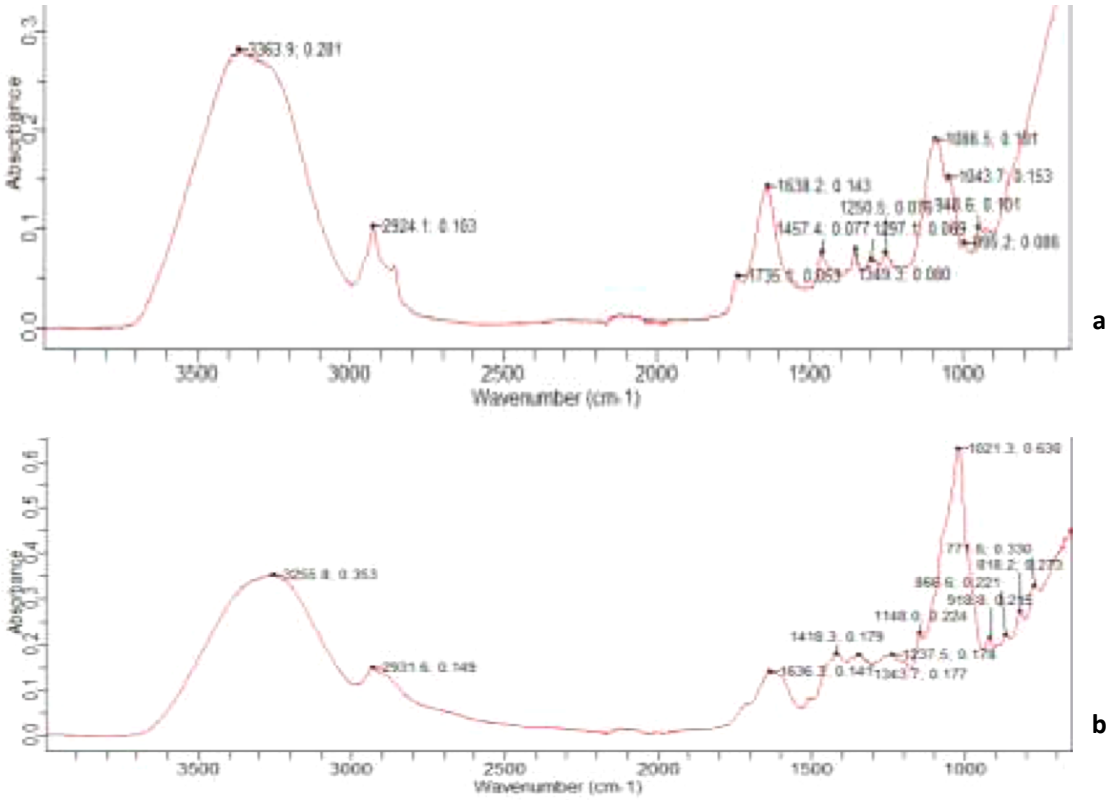

Figure 3 FTIR Chart that showed composition of chemical groups at (a) NEP and (b) EEP

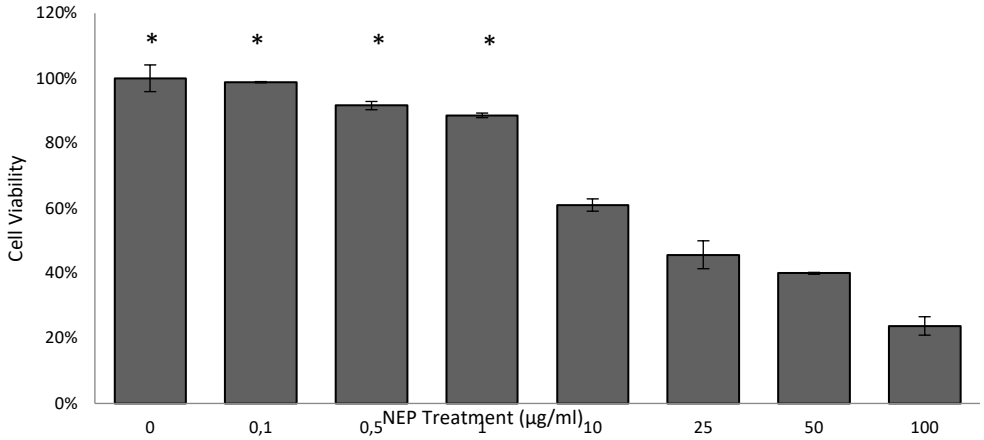

Figure 4 HDF cell viability percentage after NEP treatment in different concentration for 24 hours. NEP exposed in 0,1-1µg/ml (*) did not cause any significant effect in compare to control group (HDF cells without any treament(*)).

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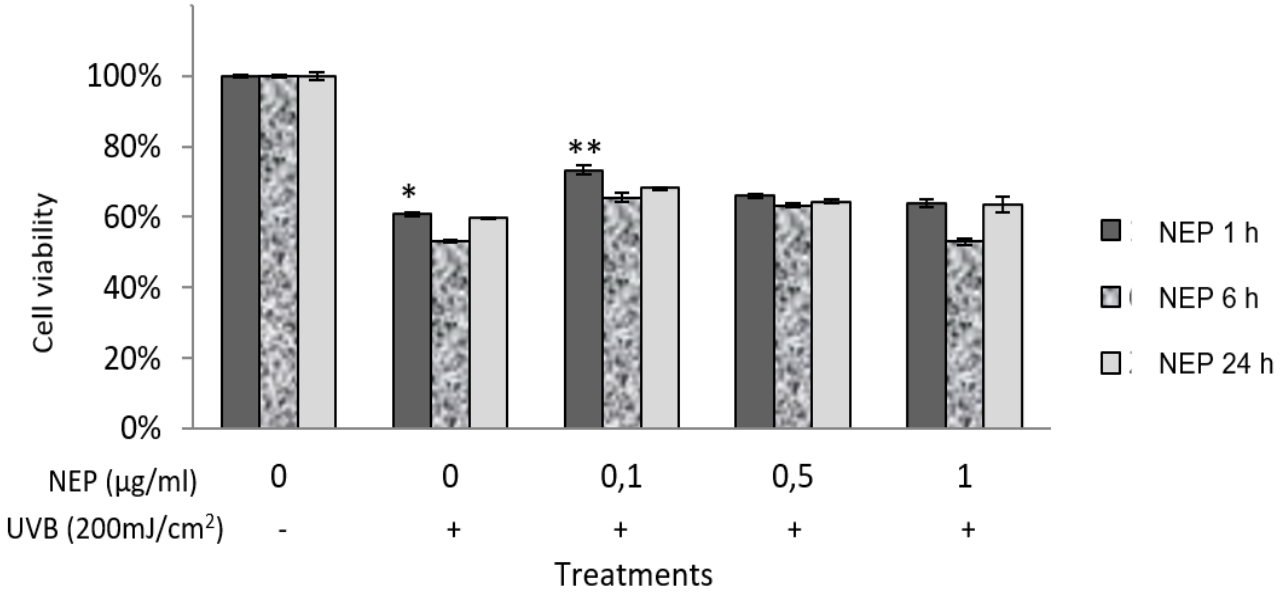

Figure 5 Percentage of cell viability after NEP treatment in different concentration (0,1-1µg/ml) each group for 1, 6, and 24 hours. NEP treatment in 0,1µg/ml concentration for 1 hour (**) showed a significant difference ($p<0,05$) in compare to HDF cells after UVB exposure group (*).

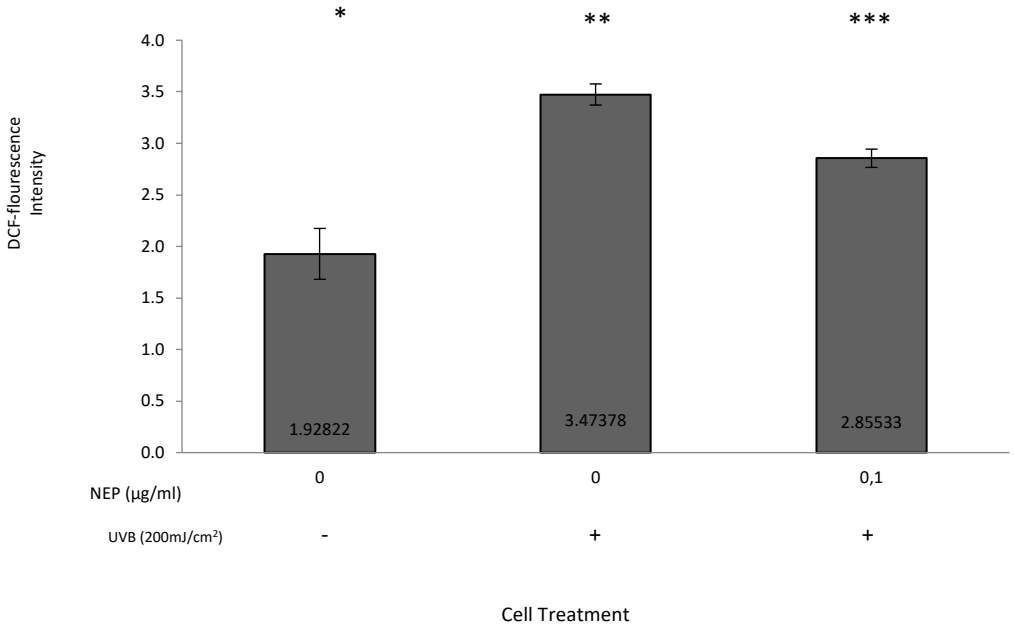

Figure 6 Fluorescence intensity within HDF cells that represented intracellular ROS after NEP 0,1µg/ml treatment for 1 hour. NEP exposure affected the descline of ROS products within HDF cells (***) in comparison with HDF cells which irradiated with UVB without NEP treatment(**).

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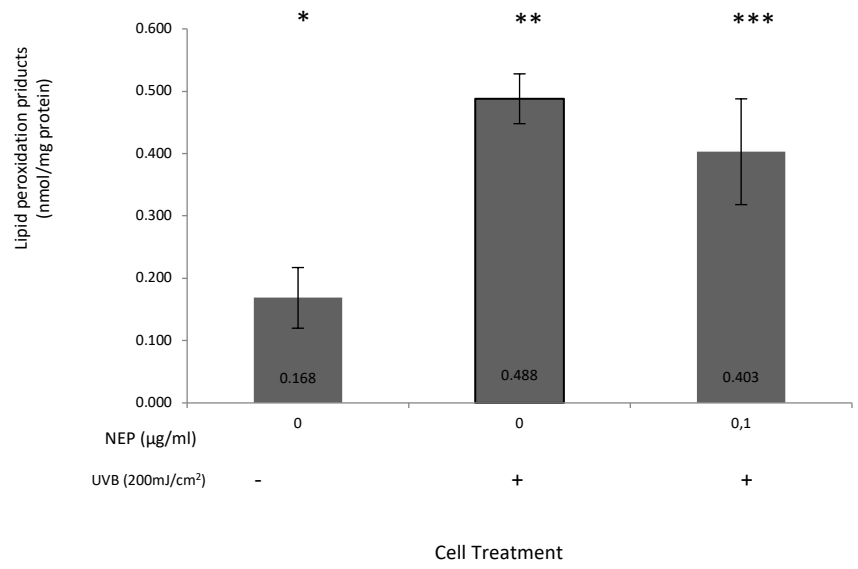

Figure 7 Concentration of lipid peroxide product in HDF cells after NEP 0,1µg/ml treatment for 1 hour. NEP treatment affected the decline of lipid peroxide product in HDF cells (***) in compares to HDF cells were only treated by UVB irradiation (**).

3.7. The Effect of NEP on HDF Nucleus Damaged by UVB Irradiation

The change of nucleus morphology was indicated by the unstained region after DAPI staining, marking the damage in the DNA. In this research, UVB exposure with 200mJ/cm^2 dosage can change the nucleus morphology (Figure 8). DNA damage due to UVB irradiation has been well-known because of the photon energy from UVB that can bind directly with DNA and produce dipyrimidine photoproduct, such as cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) photoproducts (6-4PPs). Dipyrimidine photoproduct is known for its ability to change DNA conformation by forming kink because of the existence of lesion in DNA structure which cause irregular DNA structure [21]. Dipyrimidine photoproduct occurs because of the unexpected binding between nitrogen base in DNA, especially thymine and adenine. Thymine does not bind the adenine, therefore DAPI in the observational groups can not bind A-T bindings in DNA. In this study, there was an unstained region by DAPI (Figure 8.b). However, this study did not analyze DNA damage using a specific marker to evaluate the damage directly. Straface *et al*. [22] mentioned that UVB irradiation with 200mJ/cm^2 dosage into fibroblast cell culture caused DNA damage. DAPI staining in Straface study showed a similar result with this study, which was the presence of an unstained region by DAPI inside the nucleus after UVB irradiation. In addition, Straface *et al*. [22] evaluated the same phenomenon by doing a spesific Hoechst stain which also showed an unstained region. Those were showed as fluorescent spots in the nucleus. Straface *et al*. [22] also stated that this condition was correlated with the unique reorganization activity after cells being exposed by UVB irradiation and indicated the cellular senescence.

Nucleus morphology in HDF cells after NEP 0,1µg/ml treatment also showed an unstained region (Figure 8.c). In this study, there was no quantified calculation. However, the unstained region in NEP treatment seemed fewer than in UVB irradiation treatment. It was assumed because NEP had a role in inhibiting nucleus damage indirectly. Polyphenol and catechin in NEP have a role as anti-oxidant that can inhibit ROS which can impair DNA indirectly. The research of Stojilijkovic *et al*. [23] also stated that nucleus damage due to UVB irradiation by an indirect pathway through ROS formation can damage the nucleus.

ROS was inhibited by the anti-oxidant properties in NEP. In addition, it was occurred because EEP, as one of the components of NEP, had absorbance spectrum activity to UV. The main substance of EEP is polyphenol, and the biggest part of natural polyphenol is known as a pigment that can absorb UV irradiation. Propolis has absorbance spectrum activity to UV with maximum absorbance at 290 nm wavelength that includes UVB wavelength (290-320nm) [24]. In this study, NEP was assumed as a photoprotective agent in reducing DNA damage in the nucleus due to UVB irradiation.

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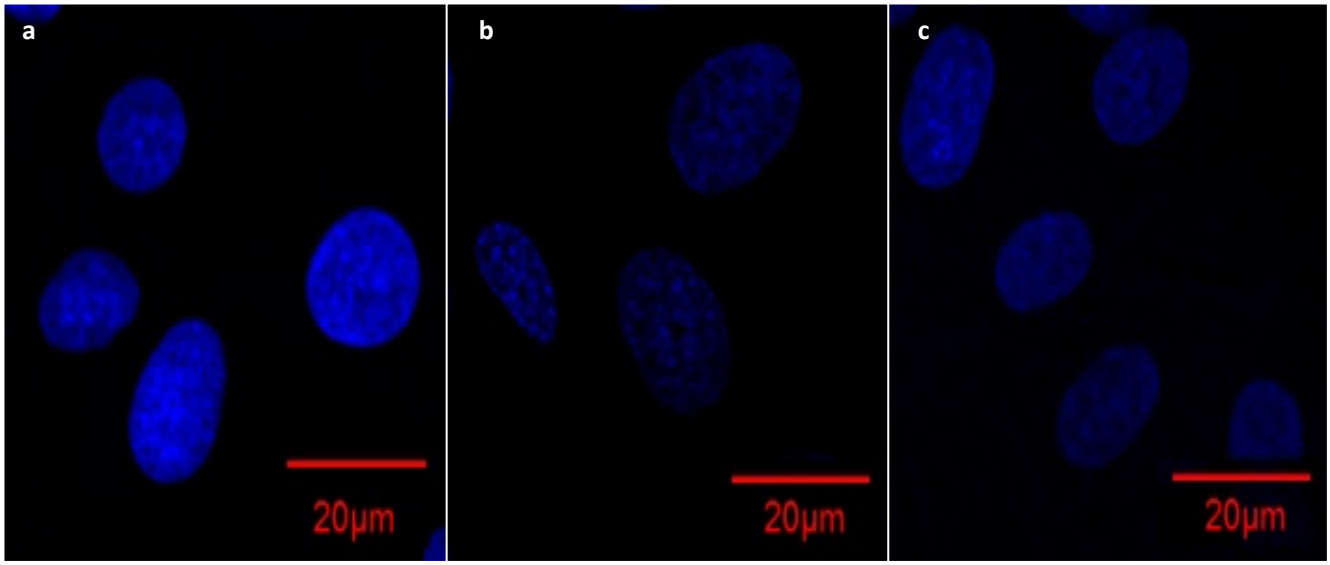

Figure 8 Nucleus morphology of HDF cells which was observed by DAPI staining. (a) normal control; (b) 200mJ/cm^2 UVB treatment; (c) NEP 0,1µg/ml treatment followed by 200mJ/cm^2 UVB irradiation.

4. Conclusion

UVB irradiation at 200mJ/cm^2 can stimulate stress condition to HDF cells, causing an increase of ROS production. NEP exposed pre-treatment can reduce stress condition due to UVB. There was a decline in ROS production until 20%. UVB irradiation at 200mJ/cm^2 can rise the lipid peroxide in HDF cells. NEP exposed pre-treatment can reduce lipid peroxide until 18% due to UVB. Polyphenol and catechin contained in propolis have a role as antioxidants which was given in NEP formed in reducing stress due to UVB irradiation.

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