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Original Article

THE ROLE OF PROPOLIS *TETRAGONULA SP.* IN OXIDATIVE STRESS AND ITS PROTECTIVE EFFECT AGAINST UV RADIATION ON CELLS

SAFIRA CANDRA ASIH[1], DIAH KARTIKA PRATAMI[2], MASAFUHMI YOHDA[3], YOSUKE FUKUTAMI[3], AHMAD FARIED[4], MUHAMAD SAHLAN[1,5*]

[1]Department of Chemical Engineering, Faculty of Engineering, Universitas Indonesia; Depok, 16424, Indonesia, [2] Laboratory of Phytochemistry, Faculty of Pharmacy, Universitas Pancasila, Jakarta, 12640, Indonesia, [3]Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology; Tokyo, Japan, [4]Department of Neurosurgery and Oncology and Stem Cell Working Group, Faculty of Medicine, Universitas Padjadjaran; Bandung, Indonesia, [5]Research Center for Biomedical Engineering, Faculty of Engineering, Universitas Indonesia, Kampus UI, Depok, 16424, Indonesia

[*]Email: sahlan@che.ui.ac.id

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ABSTRACT

Objective: This report presents a study the anti-oxidant properties of propolis wax from *Tetragonula sp.* bees by evaluating the protection ability of propolis towards cells from ultraviolet (UV) exposure. The next evaluation was using a literature study and LC-MS (Liquid Chromatography-Mass Spectrometry) to find compounds in propolis that are responsible for anti-oxidative stress activity.

Methods: HEK 293T (Human Embryonic Kidney 293 T) and fibroblast cells line were used. Four tests were performed on the cells, namely: cell proliferation assay using water-soluble tetrazolium salt (WST-8); lactate dehydrogenase (LDH), and free radicals produced on cells test by measuring fluorescence intensity produced by dichlorofluorescein; cell viability through observation using fluorescence microscope on cells stained with Hoechst and propidium iodide (PI); and reactive oxygen assay (ROS) Assay. Before UV exposure, propolis wax was added to the cells in different concentrations. The authors also analyzed the component in propolis wax using LC-MS.

Results: Based on this assessment, it was found that propolis wax successfully protects the cells against UV-induced free radicals' formation by maintaining the cell proliferation rate, reducing the free radicals produced after UV exposure, and decreasing the number of cell death. Nevertheless, we found that a greater concentration of propolis wax tends to be toxic to the cells. While on the LC-MS results obtained about 83 compounds, in which 35 of them are flavonoid and polyphenols derived compounds with antioxidant properties.

Conclusion: Based on these findings, propolis wax produced *Tetragonula sp*. can be used as a potential alternative treatment of anti-oxidative stress and anti-free radicals.

Keywords: Propolis, Oxidative stress, Anti-oxidant, Free radicals

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INTRODUCTION

Oxidative stress is known to be involved in the pathogenesis of lifestyle-related illnesses, including atherosclerosis, hypertension, diabetes mellitus, ischemic diseases, and malignancy [1]. In addition, oxidative stress has been known to be harmful due to the oxygen-free radicals attacking the biological molecules in the body, such as lipids, proteins, and DNA; which then cause diseases and disrupt the body's metabolism. Exposure to oxidative stress to the body comes from both intrinsic and extrinsic factors. However, this intrinsic factor is not more significant than the extrinsic factor of oxidative stress called Reactive Oxygen Species (ROS) [2, 3].

On the other hand, it is found that antioxidant compounds can neutralize free radicals before they 'touch' the cells by donating their electrons so that free radicals are no longer become 'radical' and end their life cycle as free radicals [4]. That way, antioxidants reduce the risk of chronic disease caused by free radicals and ultraviolet (UV) radiation. Recently, research on identifying and isolating new antioxidants from natural ingredients has become one of the most widely conducted studies [5]. For example, Propolis is a honeybee product that is currently the trend [6]. Propolis is a mixture of resin complexes collected by honeybees from various plant sap. Once collected, the material is then enriched again by saliva and enzyme secretions from bees to be used to construct and protect honeycomb. Despite numerous studies reporting the benefits of propolis, therapeutic applications and utilization in the pharmaceutical industry are limited due to the variability in the chemical composition of propolis depending on the geographical and plant-dependent aspects that bees use to collect propolis. However, in general, propolis is consists of polyphenols (flavonoids, phenolic acids, and esters), terpenoids, steroids, and amino acids where these ingredients have antioxidant activity [7].

This study aims to evaluate the antioxidant properties of propolis wax from Tetragonula sp. bees. This study is also to clarify the protective effect of propolis wax from *Tetragonula sp* from UVA radiation and find the compounds in propolis Tetragonula sp related to this active compound's activity by using literature study and LC-MS.

MATERIALS AND METHODS

Materials

The medium used for Human Embryonic Kidney (HEK) 293T cells line was Minimum Essential Medium (MEM) bought from Sigma Aldrich (Tokyo, Japan), while the medium used for human normal skin fibroblast cells was Roswell Park Memorial Institute 1640 (RPMI-1640) was purchased from Fujifilm Wako Pure Chemical Corporation (Tokyo, Japan). Penicillin and streptomycin were purchased from Fujifilm Wako Pure Chemical Corporation (Tokyo, Japan). Fetal Bovine Serum (FBS) was purchased from GE Hyclone (Tokyo, Japan). Phosphate Buffer Saline (PBS) was purchased from Nakalai Tesque (Tokyo, Japan). Trypsin-EDTA was purchased from GE Hyclone (Tokyo, Japan). The cell counting kit (WST-8) and LDH Assay Kit-WST were purchased from Fujifilm Wako Pure Chemical Corporation (Tokyo, Japan). The cell staining: Hoechst 33342 was purchased from Kodak (Tokyo, Japan), while propidium iodide was purchased from Dojindo Laboratories (Tokyo, Japan). Dichlorodihydrofluorescein (DCFH-DA) was purchased from Sigma Aldrich (St. Louis, USA). The propolis wax used in this study came from the *Tetragonula sp* bee from Sulawesi, Indonesia. The propolis wax was purchased from PT. RinBiotek Indonesia. The propolis extraction uses the method described previously by Pratami *et al.* [8].

Cell cultures

The HEK 293T cell line was purchased from Dharmacon (Tokyo, Japan) and maintained in MEM containing 10% (FBS), 100 ug/ml penicillin, and 100 ug/ml streptomycin. The human fibroblast cell line was purchased from the JCRB Cell Bank (Tokyo, Japan). Cells were cultured in MEM containing 10% FBS, 100 ug/ml penicillin, and 100 ug/ml streptomycin. Both cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The HEK 293T cells were passaged every three days, while fibroblast cells were passaged every 5 or 6 d.

Exposure of HEK 293T and fibroblast cells to UVA radiation

The method was based on a technique Murase (2013) described with some changes in cell culture and UVA radiation conditions [9]. First, the HEK 293T and fibroblast cells were seeded into density 5000 cells/well into 96 well-plate, and then the cells were then incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C overnight. The entire medium was then replaced with phenol red-free MEM containing 10% FBS mixed with the propolis concentration. One hour after adding reagents, the cultures were exposed to 4 J/cm^2 of UV A light. The UV A light was above the 96-well plate at a fixed distance of 11.5 cm. The control cells were incubated under the same conditions as experimental cells but were not exposed to UV A because they were covered with aluminum foil.

Cell proliferation assay

This assay has the purpose of evaluating the cell survival after UV A radiation by examining the change of absorbance followed by the cellular reduction of WST-8 to formazan. The experiments were performed in phenol red-free MEM at 37 °C. The cell proliferation kit reagent was added to the well with a 5 ul/well concentration after the UV A radiation and then the cells were incubated for 4 h (h). The absorbance was measured after incubation using a microplate reader (Biorad, Hercules, USA) at 365 nm. After subtraction of background absorbance, this absorbance was expressed as a percentage in the control cells (not exposed by UV A and not added by propolis).

LDH assay

This assay has the purpose of evaluating the cell death after UV A radiation by examining the change of absorbance followed by the cellular reduction of LDH reagent to formazan. The experiments were performed in phenol red-free MEM at 37 °C. First, the Cytotoxicity LDH Assay Kit-WST was added to the well that consisted of lysis buffer, working solution, and stop solution with concentrations of 50 ul/well, 100 ul/well, and 5 ul/well respectively, after the UV A radiation. Then, the absorbance was measured after incubation using a microplate reader (Biorad, Hercules, USA) at 490 nm. After subtraction of background absorbance, this absorbance was expressed as a percentage in the control cells (not exposed by UV A radiation and not added by propolis).

ROS assay

ROS assay was performed as described by Murase (2013) and Kim (2016) with some modifications [9, 10]. After the cells were incubated overnight with the density of 5000 cells/well, the cell medium was replaced with the propolis-containing medium and then incubated for one h. After that, the DCFH-DA probe was added with the concentration of 10 um and then incubated for 20 min. After incubation for 20 min, the medium was replaced with a propolis-containing medium to remove the extra probe. The cells were then exposed to the UV A radiation 4 J/cm2. The cells then were incubated for four h and the fluorescence intensity was measured using a microplate (Biorad, Hercules, USA) at an excitation/emission wavelength of 485/535 nm.

Liquid chromatography-mass spectrometry (LC-MS) conditions

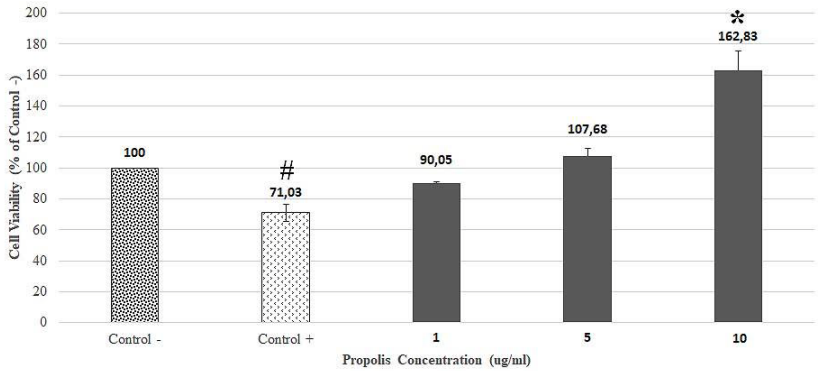
The LC-MS analysis was performed using ACQUITY UPLC™ I-Class connected with mass spectrometer Xevo G2-XS QTof (Waters, USA) in Waters Solution Centre, Singapore. To identify bioactive compounds in supplied propolis samples, the first thing to do is sample preparation with the following steps: dissolving 80% methanol in water used as an extraction compound, then 1 ml of solvent (MeOH/water, 80/20) added to 10 mg propolis followed by sonication for one h in a water bath of temperature 27 °C, soluble propolis solution centrifuged for 10 min at a speed of 12000 rpm, after centrifugation, the supernatant is taken and transferred for subsequent ready for injection.

The LC was using Acquity UPLC™ I-Class system with Flow-Through Needle with this condition: column temperature was set at 45 °C, the flow rate was 0.5 ml/min, mobile phases (A) Water add 0.1% Formic Acid and (B) Acetonitrile was added 0.1% Formic Acid, and 2.5 µl injection volume. Gradient solvent system consisting of A: B mobile phases as follows: t= 0 min 99% A; t= 2 min 65% A; t= 13 min 1% A; t= 17 min 1% A; t = 20 min 99% A.

Data acquisition was using MSE mode with MS parameters as follows: scan range was 50-1500 m/z (acquisition time 20 min), the capillary voltage was 1 kV (ESI Negative), source temperature was 100 °C, desolvation temperature was 550 °C, cone voltage 40 V, cone gas flow 50 L/hr, desolvation gas flow was 900 L/hr, scan times were 0.2 s (continue), and MSE condition was high energy ramping at 15-45 eV. Data acquisition and processing use Waters natural product application solution with UNIFI® Software.

Statistical analysis

All experimental data were running in triplicate. The data are presented as means±SEM Statistical comparisons were made using the student's t-test or Dunnett's test. A value of *P<0.005 was considered to indicate the statistical significance.



Control – : Cell without UV Exposure and propolis
Control + : UV-Exposed Cell without propolis

*: significant compare to control + (p<0,05)
: significant compare to control – (p<0,05)

Fig. 1: The cell proliferation assay result on HEK 293T cell line

RESULTS AND DISCUSSION

Cell proliferation assay

The first test was a proliferation test that aimed to evaluate cell endurance. Proliferation itself has a meaning that is a growth caused by active cell division and not caused due to the increase of cell size. In this test, chemical reagents are used WST-8, which will then be reduced to a formazan dye with a yellowish color as it interacts with the dehydrogenase activity occurring in the cell. WST-8 has a very high solubility rate in water and can also dissolve in mammalian cell growth medium. The amount of formed formazan dye shows how much dehydrogenase activity occurs in the cell so that it is proportional to the number of living cells. In other words, the darker or intense yellow color produced from the formazan dyes that react with the cell means the number of living cells contained therein is even higher [11].

The differential sensitivity of the HEK 293T cell lines exposed with UV A to dose-dependent-propolis was observed in this study. We examined the differences in the viability of these cells to UV A radiation that is commonly used as stimuli of oxidative stress. Treatment with propolis (1, 5, and 10 ug/ml) produced a dose-dependent induction in cell growth (fig. 1). Cells treated with 10 ug/ml propolis were more highly protected cell from UV (162.83% vs control positive; significant with $p<0.05$), whereas cells treated with 1 ug/ml propolis slightly protected (90.05% vs control positive).

Propolis has a good on the protection of cells from UV radiation. Therefore, propolis can behave as an antioxidant agent preventing or alleviating harmful oxidative processes caused by various factors, like trichlorfon, tebuconazole, paracetamol, methylmercury, or UV irradiation [12].

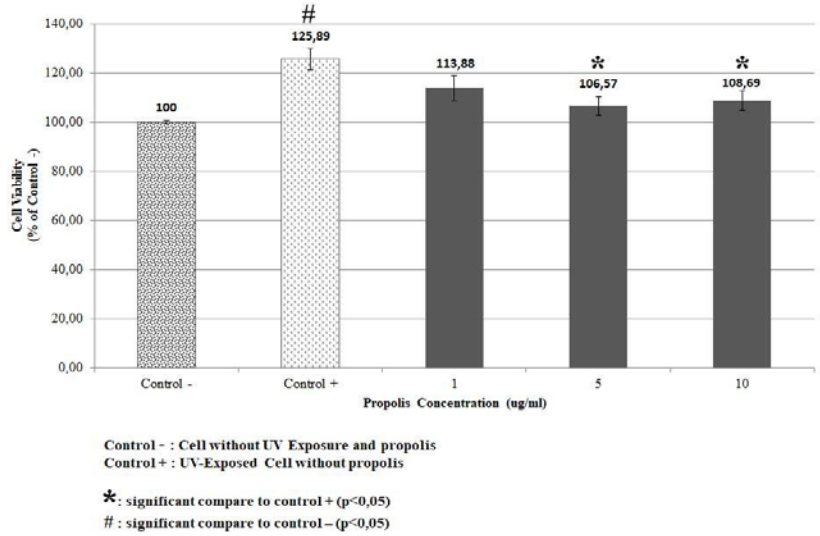


Fig. 2: The LDH assay result on HEK 293T cell line

LDH assay

The second test was the LDH test. The main reagent used for the toxicity test is WST-8 with the addition of several other reagents, which will detect dead cells by the fluorescent/color they emit so that cell absorbance can be calculated. The WST reagent will interact with damaged cells or dead cells. In the dead cells, the lactate dehydrogenase enzyme (LDH) oxidizes lactate to produce NADH that will react with WST-8 and transform into a yellowish-colored formazan or yellowish-orange compound [13].

The resulting intensity or color depth is proportional to the amount of WST-8 transformed into formazan resulting in a high absorbance value. This means that the absorbance value is proportional to the number of dead or damaged cells. We use WST-8 for the LDH test compared to other tests since WST has very stable properties. LDH activity can easily be quantified using a spectrophotometer and microplate reader on OD 450 nm [13, 14].

Cytotoxicity is generally evaluated by quantifying the amount of damaged cells' plasma membrane. On the other hand, the LDH is a stable enzyme and is found in all cell types. Therefore, the LDH enzyme will rapidly escape to the cell culture medium during damage to the plasma membrane. In the LDH method, we calculated the number of cells that die after exposure to UV A radiation; then, the more absorbance value means the more cells that die due to exposure.

The decrease in the number of HEK 293T cells dead exposed with UV A to dose-dependent-propolis was also observed in this study. We examined the differences in the cell death of these cells to UV A radiation that is commonly used as stimuli of oxidative stress. Treatment with propolis (1, 5, and 10 ug/ml) produced a dose-dependent decrease in the number of dead cells (fig. 2). Cells treated with 5 and 10 ug/ml propolis were highly protected cells from death by UV A (106.57% and 108.69 vs control positive; significant with $p<0,05$) whereas cells treated with 1 ug/ml propolis slightly protected the cells (133,88% vs control positive).

That result shows that propolis has a good effect on protecting HEK 293T cell death caused by UV A radiation. In other research, Saito *et al.* (2013) findings indicated that water extract of green Brazilian Propolis acts as an early inducer of HO-1 and a rapid activator of Nrf2 to protect against UVA-induced oxidative stress [15].

Cell death assay

We also performed the apoptosis test; with Hoechst 33342 and PI staining. Apoptosis is a physiological mechanism of the body in which cell death responds to the presence of cytotoxic agents and UV radiation is one of the triggers of apoptosis in cells [16]. This test also differentiated between without and by using exposure to UV radiation to observe how the effect of propolis protection against cells exposed to UV radiation can be observed.

This test used two kinds of cell staining that is Hoechst 33342 and propidium iodide (PI). Hoechst 33342 is a cell that will dye the nucleic acids, and the color it emits is blue fluorescence when binding to dsDNA. Hoechst 33342 has an excitation per emission wave at 350/461 nm; therefore, Hoechst 33342 will color all cell types, either living or dead cells [17, 18]. Then the second type of cell staining used is PI (propidium iodide). Just like Hoechst 33342,

propidium iodide will also color cell DNA. The difference lies in the type of cell to be colored, where PI will only color the dead cells; this is because the PI dye can not pass through the dead cell membrane, so it can be used to detect cells that have apoptosis [19].

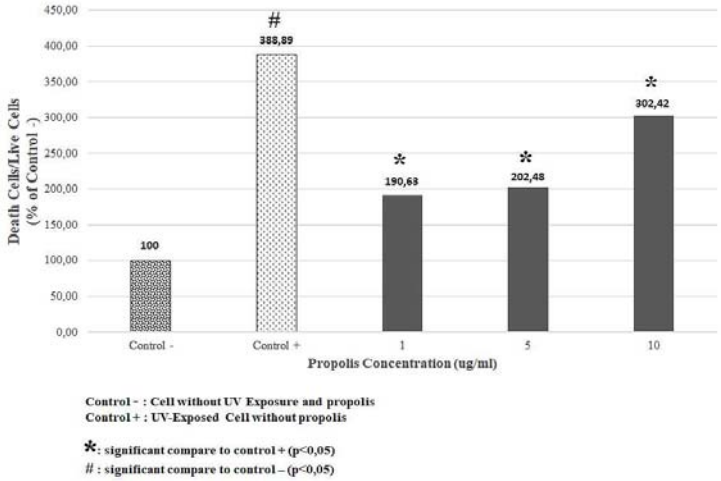


Fig. 3: The apoptosis assay result on HEK 293T cell line

The decrease in the number of HEK 293T cells apoptosis exposed UV A to dose-dependent-propolis was also observed in Hoechst 33342 and PI staining assay. We examined the differences in the apoptosis of these cells to UV A radiation, and treatment with propolis (1, 5, and 10 ug/ml) produced a dose-dependent decrease in the number of dead cells per live cell (fig. 2). Cells treated with 1, 5 and 10 ug/ml propolis were shown protected cell from death by UV A (190.63%, 202.48%, 302,42% vs control positive, respectively; significant with $p<0.05$). From that result, propolis has a good effect on protecting against cell death caused by UV A radiation. The effect of propolis protection increases with decreasing apoptosis cells in a dose-dependent manner. Many studies have demonstrated that the mechanisms of action of bioactive compounds of propolis involve the scavenging of free radicals and cell apoptosis. Czyżewska *et al.*'s (2016) report suggest that polyphenols' synergistic effects in propolis are responsible for their potential on decreasing apoptosis cells [20].

Reactive oxygen species (ROS) assay

Furthermore, the free radical activity test was performed by calculating the ROS produced by the cell. X-ray radiation and ultraviolet light are essential sources of ROS formation since both rays can lyse water into radicals OH [21]. Free radical itself becomes the main trigger factor of oxidative stress. There is three damage target by ROS, DNA, RNA, proteins, and lipids because they attack important biomolecules in the body that play a role in metabolic processes, so this free radical ROS can disrupt the course of metabolism, which then causes oxidative stress [22].

The close connection between free radical ROS and oxidative stress makes ROS a marker in testing a substance's effect on oxidative stress. Furthermore, they were coupled with the fact that the primary source of the main ROS is UV solar radiation. Therefore, the authors use ultraviolet light radiation to trigger ROS production to test further how the effect of adding the main ingredient in this experiment is propolis wax to the amount of ROS produced [23].

The reagents used in this species' reactive oxygen counts test are 2,7-dichlorofluorescein diacetate (DCFDA) or 2,7-dichlorodihydrofluorescein diacetate (DCFHDA). This reagent is a reagent widely used in cell-based ROS detection [24]. DCFDA is a fluorescent probe sensitive to redox reactions and is cell-permeable or can penetrate cell membranes. DCFDA will oxidize ROS and RNS (reactive nitrogen species) present in the cell then transform into a product with high fluorescent content. DCFHDA is a stable dye substance and can diffuse into cells. DCFHDA will then be hydrolyzed by intracellular esterase resulting in DCFH being trapped in the cell. The ROS produced by the cell will then oxidize DCFH to a fluorescent compound of 2,7-dichlorofluorescein (DCF). Hence the intensity of the resulting fluorescence will be proportional to the amount of ROS produced by the cell.

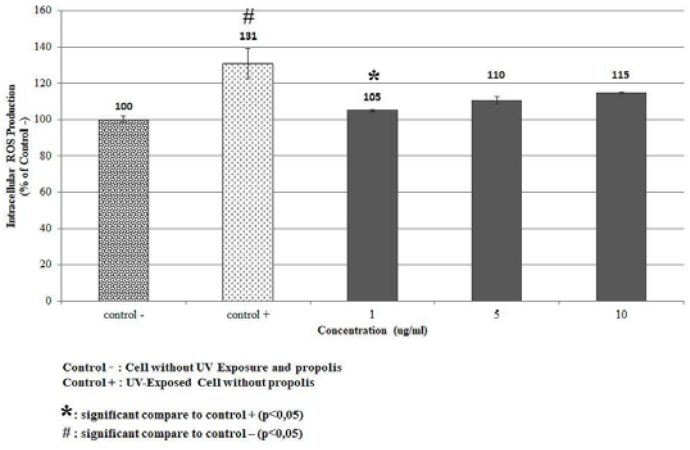


Fig. 4: The reactive oxygen species (ROS) assay result on HEK 293T cell line

Fig. 4 shows the results of the free radical number test on HEK 293T cells. The y-axis shows ROS production as the value of % control-while the x-axis shows the concentration of propolis. As explained previously, the intensity of fluorescence is proportional to the number of free radicals produced by the cell. Therefore, there can be a decrease in fluorescence power, which means a reduction in the number of free radicals produced by cells in cells treated with propolis compared with the control. Furthermore, propolis reduces the number of free radicals produced with a tendency the greater the concentration of propolis is given then the lower the number of free radicals produced by the cell.

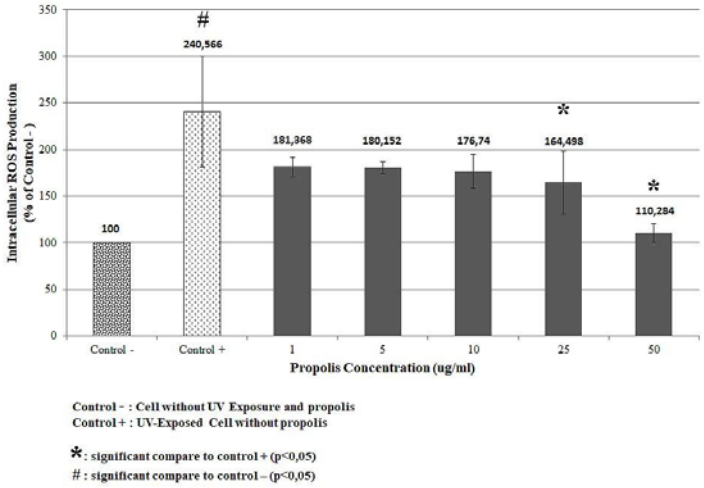


Fig. 5: The reactive oxygen species (ROS) assay result on fibroblast cell line

We examined the deference's in the ROS production in cells to UV A radiation, and treatment with propolis (1, 5, and 10 ug/ml) produced a dose-dependent decrease in the number of ROS production (fig. 4). Cells treated with 1 ug/ml propolis were shown decreasing of ROS by UV A in HEK 293T cells (105 ug/ml vs control positive; significant with $p<0.05$); with 25-30 ug/ml propolis in human fibroblast cells (164,498 and 110,284 ug/ml vs control positive, respectively; significant with $p<0.05$). The effect of propolis protection increases by decreasing the number of free radicals in a dose-dependent manner.

Overall, in general, the addition of propolis has been shown to affect the decrease in the number of free radicals produced by cells. Zhang's (2017) research that identifies free radical scavengers from Brazilian green propolis using off-line HPLC-DPPH assay and LC-MS reported that 9 compounds were discovered with free radical-scavenging activities [25, 26].

Table 1: Compounds found in propolis tetragonula sp related to ROS-prevention activity

No.	Group	Compound
1	Xanthone	1,7-Dimethoxy-2,3-methylenedioxyxanthone
2	Flavonoid	3,4',5-Trihydroxy-7-methoxy-8-isopentenylflavone
3	Flavonoid	3,5-Dihydroxy-3, 4',7-trimethoxy flavone
4	Flavonoid	3′,5-Dihydroxy-7,4'-dimethoxy flavone
5	Fenol	4-Hydroxyacetophenone
6	Flavonoid	4'-O-Methylbrazilin
7	Flavonoid	5,7,4′-Trihydroxy-8,3′-diprenylflavone
8	Chalcone	Bakuchalcone
9	Flavonoid	Flavenochromane B
10	Flavonoid	Galangin (Norizalphin)
11	Flavonoid	Ginkgetin
12	Flavonoid	Glabrol
13	Flavonoid	Irilone
14	Flavonoid	Isoxanthohumol
15	Flavonoid	Kuraninone
16	Flavonoid	Kushenol F
17	Flavonoid	Kushenol A
18	Flavonoid	Kushenol B
19	Flavonoid	Kushenol C
20	Flavonoid	Kushenol E
21	Flavonoid	Kushenol I
22	Flavonoid	Kushenol N
23	Flavonoid	Kushenol S
24	Flavonoid	Kushenol U
25	Flavonoid	Kushenol W
26	Flavonoid	Kushenol X
27	Flavonoid	Kuwanon C
28	Flavonoid	Kuwanon E
29	Flavonoid	Methyl kushenol C
30	Fenol	Moracin H
31	Isoflavon	Psoralenol

32	Isoflavon	Rhamnetin
33	Isoflavon	Scutellarein
34	Flavonoid	Sophoradichromane B
35	Flavonoid	Sophoradichromane D

CONCLUSION

Provision of propolis wax before cells exposed to UV radiation show a good effect against oxidative stress by looking at several parameters: increasing or maintaining cell viability rates after UV radiation exposure; reducing cell death-apoptosis rate after UV radiation exposure and decreasing the number of free radicals produced by cells after exposure to UV A radiation. Also, Based on the analysis performed using UPLC-MS, there were 35 compounds related to the anti-oxidative properties of propolis. These 35 compounds are essentially derivatives of polyphenols and flavonoids that are antioxidants.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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