Protestive Effects of Indonesian Propolis Against Light-Induced Retinal Damage

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ABSTRACT

The pharmacological and biological activities of Indonesian propolis are mostly unexplored except for its anticancer properties. This study was designed to explore the protective effects of the methanol extract of Indonesian propolis (MEP) obtained from the stingless bee (Trigona incisa) against light-induced retinal photoreceptor damage. Murine photoreceptor (661W) cells were cultured and treated with MEP. The cells were exposed to cellular stress inducers such as tunicamycin, hydrogen peroxide, and light irradiation. The protective effects of MEP were assessed by determining production of intracellular reactive oxygen species (ROS), analyzing changes in nuclear factor-kappa B (NF-kB) level, analyzing caspase-3/7 activation, and by using the zebrafish retinal degeneration model. MEP significantly reduced cell death induced by tunicamycin, hydrogen peroxide, and white light irradiation in a concentration-dependent manner. The phosphorylation of NF-kB decreased and light-induced activation of caspase-3/7 and intracellular accumulation of ROS were inhibited. The thickness of outer nuclear layer (ONL) in zebrafish retina significantly increased following MEP treatment. The MEP obtained from stingless bee propolis has a protective effect on photoreceptor retinal cells and has the potential to be developed as a supplement in prevention of retinal diseases.

Keywords: Indonesian propolis, oxidative stress, photoreceptor, retinal damage, zebrafish.

INTRODUCTION

The retina plays an important role in vision. The majority of people in developing countries have eye degeneration and vision problems1. Because of the increase in life expectancy and the current status of the demographics, this number is expected to triple by 20202. Our vision depends on absorption of light by photoreceptor cells of the retina. Exposure to light can cause cellular damage leading to lipid peroxidation and oxidative stress in the retina. Age-related macular degeneration (AMD) is a common and painless eye condition as well as a leading cause of vision loss in people older than 50 years, although the molecular mechanism of AMD is still ambiguous3. Light-induced retinal damage is a model that has essential characteristics of human AMD3.

Propolis is a complex apicultural product collected by bees from tree buds and barks3. Bees use propolis to protect the hive from microorganisms and fill the cracks in the hive. Propolis is used as an alternative medicine for the prevention of diseases worldwide. Since ancient times, it has been used as a traditional folk medicine in many countries3. Several species of bees produce propolis, such as honeybees of Apis spp and stingless bees of Trigona spp7, 8. The chemical composition of propolis depends on several factors such as region, plant sources, and bee species8. Propolis is composed of resin and balsam, wax, essential and aromatic oils, pollen, and other organic substances9, 10. Caffeic acid, one of the major compounds in bee products, is known to protect against retinal damage11. Stingless bees from Melipona spp are closely related to honey bees (Apis spp.), but they do not have stings and live in perennial eusocial colonies12, 13. Stingless bees are widespread over tropical and some subtropical regions of the world14. Complete information regarding the biological activities of bee products obtained from stingless bee in Indonesia is not yet available. In other countries, propolis obtained from stingless bees is well known for its therapeutic properties, including antimicrobial15, antitumor16, and antioxidant17 activities. Local people in East Kalimantan, Indonesia use propolis for several therapeutic purposes such as for toothache, wounded eye, gastritis, and cancer, although little information is known about the use of stingless bee propolis in Indonesia. The pharmacological properties of propolis from Indonesian stingless bee have not been explored. Previously we found that propolis produced by Trigona spp has anticancer activity18. The purpose of this study was to investigate whether stingless bee propolis from Indonesia has protective actions against light induced cell damage. In this

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study, we explored the protective actions of *Trigonza incisa* propolis native to East Kalimantan province, against light-induced cell damage.

**MATERIALS AND METHODS**

**Preparation of methanol extract of propolis (MEP)**

The propolis used in this study is produced by the stingless bee (*T. incisa*), native to Indonesia. The plant origin of the propolis was Mango tree (*Mangifera indica*). This sample was collected from the Mulawarman university botanical garden, Samarinda, East Kalimantan province, Indonesia after the honey harvesting season on February, 2013. Propolis was then separated from debris, wood, and other impurities. It was then stored at -20 °C until used. Propolis sample (500 g) was extracted with 800 ml of 96% (v/v) methanol (MeOH) for 24 h at RT following centrifugation at 100 rpm (7400 Tungden; Edmund Buchler, Germany). This process was repeated thrice. Then, the extracts were collected and filtered using Whatman filter paper No.2 (Sigma-Aldrich, St. Louis, MO, USA). The filtrate was evaporated at 40 °C to get the methanol extract of propolis (MEP).

**Cell culture**

Murine retinal corn cell-derived 661W cells were a kind gift from Dr. Muayyad R. Al-Ubaidi (University of Houston, Houston, TX, USA). The cells were maintained in DMEM media containing 10% fetal bovine serum (FBS), 100 μg/mL penicillin, and 100 μg/ml streptomycin under a humidified atmosphere of 5% CO2 at 37°C. The 661W cells were passaged (subcultured) by trypsinization every 3-4 days.

**Stress-induced cell damage assay**

The 661W cells (2×10⁴ cells/100 μl) were seeded into a 96-well and incubated for 24 h. The medium was replaced with 1% FBS-DMEM containing MEP at three different concentrations, such as 1, 3, and 5 μg/mL, or with 1 mM (final concentration) tauroursodeoxycholic acid (TUDCA) or 1 mM of N-acetyl-L-cysteine (NAC, Wakó, Osaka, Japan) as positive control and cultured at 37°C for 1 h. After preincubation, tunicamycin at a final concentration of 2 μg/mL or H₂O₂ (Wako) at a final concentration of 0.3 mM was added to the cells. Nuclear staining assays were carried out after 24 h or 27 h, respectively. Light-induced cell damage was caused to expose to 2,500 lux of white fluorescent light (Nikon, Tokyo, Japan) for 24 h at 37°C. Nuclear damage was carried out following light exposure.

**Cell staining**

Following stress-induced cell damage assays, Hoechst 33342 (excitation, 360 nm; emission, 490 nm) and propidium iodide (PI) were added to the culture medium (final concentrations of 8.1 and 1.5 μM, respectively) and incubated for 15 min. Microscopic images through fluorescence filters for Hoechst 33342 and PI were captured by Olympus Camera (Tokyo, Japan).

**Measurement of intracellular ROS production**

Intracellular radical activation within 661W cells was determined using CM-H₂DCFDA (Thermo Fisher Scientific, Waltham, MA, USA), an oxidative stress indicator. CM-H₂DCFDA following uptake within the cell is converted to dichlorodihydrofluorescein (DCFH) by an intracellular esterase. At the end of the light exposure period, CM-H₂DCFDA was added to the culture medium and incubated at 37°C for 1 h at a final concentration of 10 μM. Fluorescent signals were measured using a Varioskan Flash 2.4 microplate reader (Thermo Fisher Scientific) at 485 nm (excitation) and 535 nm (emission). Then ROS oxidize a non-fluorescent DCFH to fluorescent DCFH. The number of cells was determined by Hoechst 33342 staining and used to calculate ROS production per cell¹⁹.

**Electro Spin Resonance (ESR) Assay**

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich. This assay was carried out following the steps of previous procedures²⁰, ²¹. DPPH radical scavenging activity was calculated from the integration values of the ESR spectra corrected with a Mn²⁺ marker. The DPPH (200 μM) and various concentrations of MEP or Trolox were dissolved in ethanol. The DPPH solution (100 μl) and sample solution (100 μl) were mixed and transferred to an ESR spectrometry cell. The ESR spectrum of DPPH radical was measured after 2 min of mixing. The measurement condition for ESR (JES-FA 200, JEOL, Tokyo, Japan) was as follows: center field 330 mT, sweep width 1.5 × 10 mT, sweep time 2 min, field modulation width 2 × 0.1 mT, amplitude 4 × 100, and microwave power 2.0 mW.

**Assay of caspase-3/7 activities**

Caspase-3/7 activity in the 661W cells was measured after 24 h of light exposure, using Caspase-Glo® 3/7 Assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions. After the light exposure, caspase-Glo 3/7 Reagent was added at a 1:1 ratio of reagent volume to sample volume, and then the cells were incubated for 1 h at 37°C. The lysate samples were then transferred to a white wall well plate. The luminescence of each sample was measured using Skan it RE for Varioskan Flash 2.4 (Thermo Fisher Scientific).

**Western blot assay**

The 661W cells were used for western blot analysis. The samples were prepared as described in a previous report²². For immunoblotting, the following primary antibodies were used: rabbit anti phospho-NF-κB receptor antibody, rabbit anti total-NF-κB receptor antibody (1:1,000; Cell Signaling), and β-actin mouse monoclonal antibody (1:2,000; Sigma-Aldrich). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific) and HRP-conjugated goat anti-mouse antibody (Thermo Fisher Scientific) were used as secondary antibodies. Immunoreactive bands were visualized using Immunostar-LD (Wako) and a LAS-4000 luminescent image analyzer (Fuji Film Co., Ltd., Tokyo, Japan).

**Zebrafish assay**

Adult zebrafish (*Danio rerio*) (6-month-old), were raised at 28.5°C under 14 h light: 10 h dark cycle. Light-induced retinal damage was performed as previously reported²³. Before starting the light irradiation, adult zebrafish were dark adapted for 14 days. MEP was injected intravitreally to zebrafish, the zebrafish were then transferred to a clear
Figure 1: (A) Effects of methanol extract of *T. incisa* propolis (MEP) on 661W cell damage induced by added stress inducer. Cells were treated with increasing concentrations of MEP following 24 h light exposure and then stained with Hoechst 33342 and PI complex. (B) Effects of MEP on 661W cell damage induced by light exposure. MEP and NAC significantly inhibited the light-induced retinal cell damage. (C) Effect of MEP on 661W cell damage induced by hydrogen peroxide (H$_2$O$_2$; 0.3 mM). Cells were treated with increasing concentrations of MEP after 24 h treatment with hydrogen peroxide. (D) Cells were treated with increasing concentrations (1, 3, and 5 µg/ml) of MEP after 27 h treatment with tunicamycin (2 µg/ml). *P<0.05, **P<0.01 vs. vehicle; ##P<0.01 vs. control (Student’s t-test and Dunnett’s test). Data are presented as mean ± SEM (n=6).
plastic tank, and exposed to light (~16,000 lx) from two halogen lamps for 24 h, with water temperature maintained at 30~34°C. There were two fans on both sides of the tank. To distinguish between the two groups, zebrafish fins were cut to several types and a compartment was placed in the middle of the tank. One air bubbler was introduced into the tank. All the experiments were performed in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Immunohistochemistry

The enucleated eyes were fixed in 4 % paraformaldehyde overnight at 4°C, soaked with 5% sucrose for 30 min at room temperature, with 20% sucrose for 5 h at 4°C, with mixed liquor containing optimum cutting temperature (OCT) compound (Sakura Fine technical, Co., Ltd., Tokyo, Japan), and with 20 % sucrose at a ratio of 1:2 for 30 min at room temperature. After that, the tissues were embedded in OCT compound. These tissues were immediately frozen with liquid nitrogen and stored at -80°C. Serial transverse sections were cut using a cryostat to a thickness 12 µm and placed on slides (MASCOAT; Matsunami Glass Ind. Ltd., Osaka, Japan) for immunohistochemistry. Immunohistochemical staining was performed as previously reported.

Statistical analysis

Figure 2: (A) Effects of MEP on light-induced ROS production in 661 W cells. CM-H$_2$DCFDA probe used to measure the fluorescence intensity. Light exposure increase ROS production, which decreased following treatment with MEP and NAC. *P<0.05, **P<0.01 vs. vehicle; ##P<0.01 vs. control (Student’s t-test and Dunnett’s test). Data are presented as mean ± SEM (n=6).(B) Effects of MEP on radical scavenging activity. **P<0.01 vs. control (Student’s t-test and Dunnett’s test). Data are presented as mean ± SEM (n=3)
Data are presented as mean ± SEM. Statistical comparisons were made using a Student’s t-test or a one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. P value <0.05 was considered to be statistically significant.

RESULTS

Protective effects of MEP on stress-induced cell damage
We first investigated whether MEP from T. incisa species prevented murine-derived retinal corn (661W) cell death induced by light exposure, tunicamycin, and hydrogen peroxide. Cell death was detected by Hoechst 33342 and PI staining agents. Hoechst 33342 stains all cells (living and dead cells) whereas PI stains only dead cells. MEP showed protective effects against light-induced cell damage in comparison to vehicle-treated group (Figure 1A). The MEP of T. incisa reduced light-induced cell death in a concentration-dependent manner (Figure 1B). The effect was significant at both 3 and 5 µg/ml (p < 0.01). The MEP also decreased hydrogen peroxide-induced cell damage in a concentration-dependent manner (Figure 1C). The MEP of T. incisa also reduced damage from tunicamycin, an ER stress inducer (Figure 1D).

Effects of MEP on intracellular ROS production in retinal corn cells following light exposure
Intracellular ROS production is measured by conversion of CM-H2DCF to DCFH upon exposure to ROS. Production of DCFH was increased by light exposure whereas 5 µg/ml of MEP and 1 mM NAC significantly reduced light-induced ROS production in 661W cells (Figure 2A). Treatment with MEP at 1, 3, and 5 µg/ml significantly reduced light-induced ROS production in 661W cells in a concentration-dependent manner. The inhibitory effects against DPPH scavenging activity increased in a concentration-dependent manner (1-1,000 µg/ml). The effect was significant at 1 µg/ml concentration of MEP in comparison to control (Figure 2B) group.

Effects of MEP on phosphorylation of NF-κB and activation of caspase in retinal corn cells following light exposure

Figure 3: (A) Western blot showing the effects of MEP (5 µg/ml) on phosphorylation of NF-κB in light-induced cell damage. Cells were treated with MEP for 1 h, then exposed to 2,500 lux white light for 6 h. The cells were collected and the lysate was analyzed by western blot. Quantitative analysis of band density. ##P<0.01 vs. control; **P<0.01 vs. the vehicle-treated group (Student’s t-test) Data are presented as mean ± SEM (n=3)

(B) Effects of MEP and NAC on caspase-3/7 activity. #P<0.01 vs. control; **P<0.01 vs. the vehicle-treated group (Student’s t-test) Data are presented as mean ± SEM (n=3)
We investigated whether the MEP of *T. incisa* propolis affects the phosphorylation of NF-κB induced by light irradiation. The phosphorylation of NF-κB increased after 6 h light exposure and MEP treatment inhibited the up-regulation of NF-κB phosphorylation (Figure 3A). We also analyzed caspase-3/7 activation using a caspase-3/7 assay kit after 24 h of light exposure. We measured the luminescence and fluorescence of cells cultured in a 96-well plate using a spectrophotometer and then calculated the caspase-3/7 activity per cell. Caspase-3/7 activity was increased by light exposure, and treatments with 5 μg/ml MEP and 1 mM NAC significantly inhibited the activation of caspase-3/7 (Figure 3B).

**Effects of MEP on outer nuclear layer (ONL) morphology of zebrafish retina after light exposure**

In order to validate the protective role of MEP against light-induced retinal damage, we investigated the effects on zebrafish retinal morphology. Zebrafish were treated with retinal injection of MEP (1 μg/ml and 5 μg/ml). An hour later, the zebrafish were subjected to light exposure, and final analysis was performed after 24 h. The thickness of the ONL in an optic section significantly increased...
following treatment with 5 µg/ml MEP in comparison to vehicle (Figure 4).

DISCUSSION
The protective effects of MEP prepared from Indonesian stingless bee propolis, on light-induced retinal damage have not been reported. In this study, MEP protected photoreceptor cells against light-induced damage and damage caused by other stress inducers, such as hydrogen peroxide and tunicamycin. An antioxidant status was induced by MEP, which is attributable to increased DPPH radical scavenging activity and decrease in ROS production in photoreceptor cells after light exposure. ROS cause oxidative stress, which is one of the major factors of retinal cell death after light exposure23. In recent studies, light exposure can cause degeneration of retinal function, and light irradiation can induce photoreceptor cell death by mechanisms such as oxidative stress23 and ER stress24-25. These stresses are associated with NF-κB activation23. To further investigate the protective effects of MEP, we examined NF-κB activity. Light exposure also causes oxidative stress through NF-κB activation related to inflammation, and cell apoptosis26. NF-κB is considered as a critical key target for suppression of inflammation and a series of molecular cascades that can be induced by modulation of NF-κB27. MEP treatment did not affect the normal state of NF-κB phosphorylation; however, it reduced light-induced NF-κB phosphorylation. Thus, the target of MEP may be some stress-activated proteins, which are upstream of the NF-κB signaling pathway. In line with these reports and our results, the protective effects of MEP are mostly likely exerted by direct reduction of these stress factors or by inhibition of common pathways upstream of the NF-κB signaling pathway. The data of caspase-3/7 activity (Figure 3B) also indicates that MEP reduced apoptotic signaling. Moreover, light irradiation-induced caspase-3/7 activity was completely abolished by MEP and NAC, an antioxidant. Thus, oxidative stress may be important in light-induced 661W cell death.

Retinal morphology of zebrafish revealed increase in the thickness of ONL. MEP could prevent light-induced damage by increasing the thickness of ONL (Figure 4). Immunolabeling was significantly lower in MEP-treated zebrafish in comparison to that in the control group zebrafish measured 24 h after light exposure. These findings indicate that oxidative stress was reduced by MEP in light-damaged eyes. Our previous study revealed that NAC protected photoreceptor degeneration and apoptosis induced by light irradiation23. Cells exposed to radiant energy may be damaged because of heating and/or owing to generation of ROS. An antioxidant is inhibits oxidation of cell membranes. ROS damage mitochondria and lipids, leading to modification of retinal function. To the best of our knowledge, this is the first study to explore the association of propolis of T. incisa from East Kalimantan with prevention of light-induced retinal cell damage. Even at crude level, the MEP of T. incisa propolis could reduce damage to photoreceptor cells. Propolis obtained from another country in crude level also showed protective effects. The aqueous extract of Lycium barbarum Lynn protects retinal ganglion cells in an ocular hypertension model of glaucoma28. The methanol extract of Crocus sativus reinforces cellular antioxidant defenses against oxidative stress and mitochondrial dysfunction in a fly model29. We propose that the consumption of propolis is likely to offer traditional therapeutic advantage among humans to prevent or delay the progression of oxidative stress against eye damage. In this study, MEP decreased tunicamycin-induced cell death, indicating that constituents attributable for decreasing ER stress might be different from those responsible for the antioxidant activity. However, our study evaluated the protective ability of MEP against light-induced retinal damage. For future research, we will search for the active compound of MEP that is responsible for protection against light-induced retinal damage.

Our investigations indicated the protective activity of propolis obtained from stingless bee, abundant in the forest in East Kalimantan, Indonesia and commercially used by beekeepers.

CONFLICT OF INTEREST
The authors declare that there is no conflict of interest regarding the publication of this paper.

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