Protective 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-κB) 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-κB 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 problem[1]. Because of the increase in life expectancy and the current status of the demographics, this number is expected to triple by 2020[2]. 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 molecule mechanism of AMD is still ambiguous[3]. Light-induced retinal damage is a model that has essential characteristics of human AMD[4].

Propolis is a complex apicultural product collected by bees from tree buds and barks[5]. 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 countries[6]. Several species of bees produce propolis, such as honeybees of *Apis* spp and stingless bees of *Trigona* spp[7, 8]. The chemical composition of propolis depends on several factors such as region, plant sources, and bee species[8]. Propolis is composed of resin and balsam, wax, essential and aromatic oils, pollen, and other organic substances[9, 10]. Caffeic acid, one of the major compounds in bee products, is known to protect against retinal damage[11].

Stingless bees from *Melipona* spp are closely related to honey bees (*Apis* spp.), but they do not have stings and live in perennial eusocial colonies[12, 13]. Stingless bees are widespread over tropical and some subtropical regions of the world[14]. 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 antimicrobial[15], antitumor[16], and antioxidant[17] 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 activity[18]. The purpose of this study was to investigate whether stingless bee propolis from Indonesia has protective actions against light induced cell damage. In this

study, we explored the protective actions of *Trigona 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 sturdy 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 Tubingen; Edmun 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 U/mL penicillin, and 100 µg/ml streptomycin under a humidified atmosphere of 5% CO_2 at 37°C. The 661W cells were passaged (subcultured) by trypsinization every 3-4 days.

Stress-induced cell damage assay

The 661W cells (2×10^3 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, Wako, 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 H_2O_2 (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 staining was carried out following light exposure.

Nuclear 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-H$_2$DCFDA (Thermo Fisher Scientific, Waltham, MA, USA), an oxidative stress indicator. CM-H$_2$DCFDA following uptake within the cell is converted to dichlorodihydrofluorescin (DCFH) by an intracellular esterase. At the end of the light exposure period, CM-H$_2$DCFDA 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 cell[19].

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 procedures[20, 21]. DPPH radical scavenging activity was calculated from the integration values of the ESR spectra corrected with a Mn^{2+} 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 report[22]. 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 under14 h light: 10 h dark cycle. Light-induced retinal damage was performed as previously reported[23]. 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

A

Light exposure 24 h

Control　　　Vehicle　　　5 µg/ml MEP　　　NAC
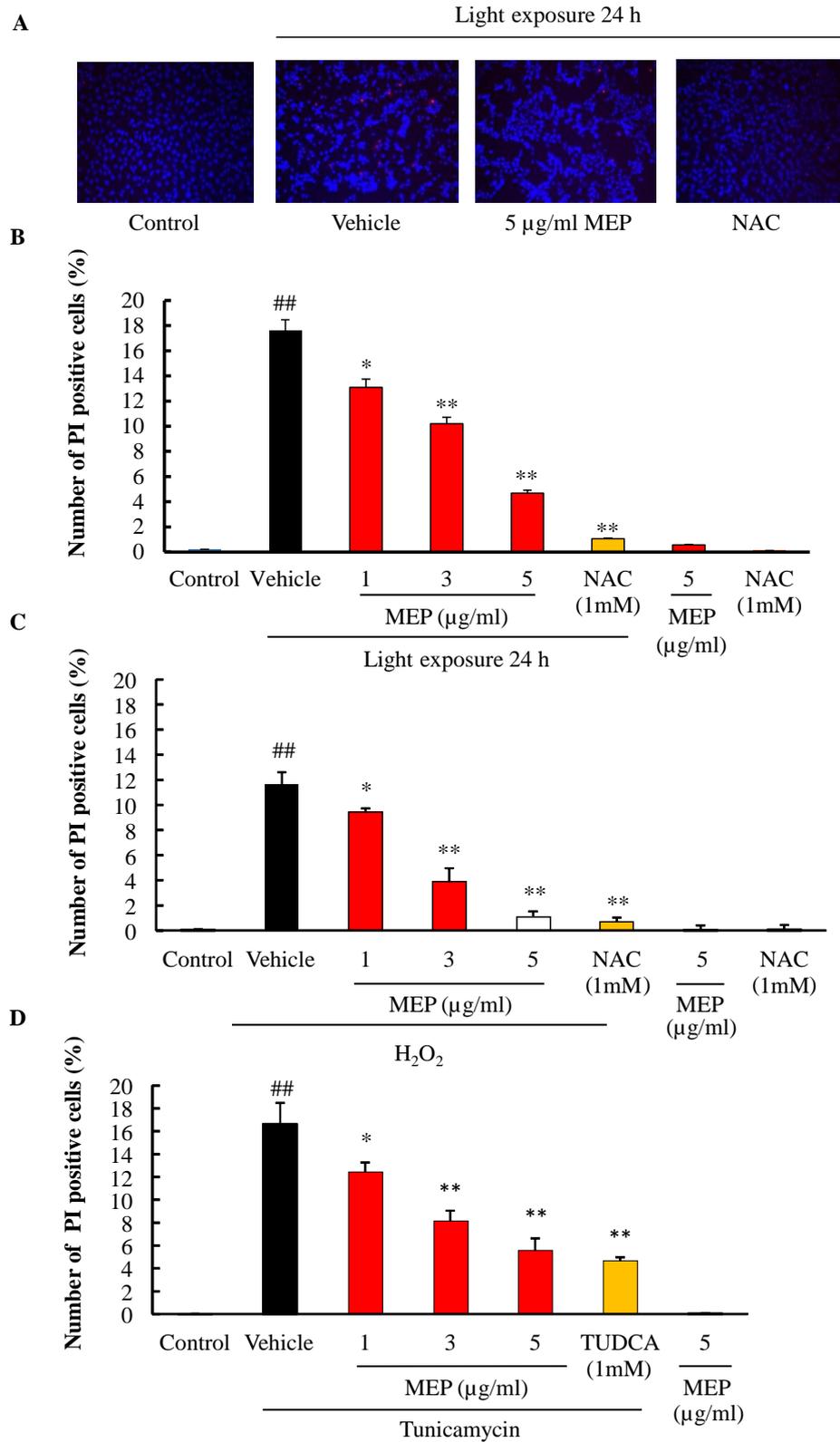
B

C

D

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_2O_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[23].
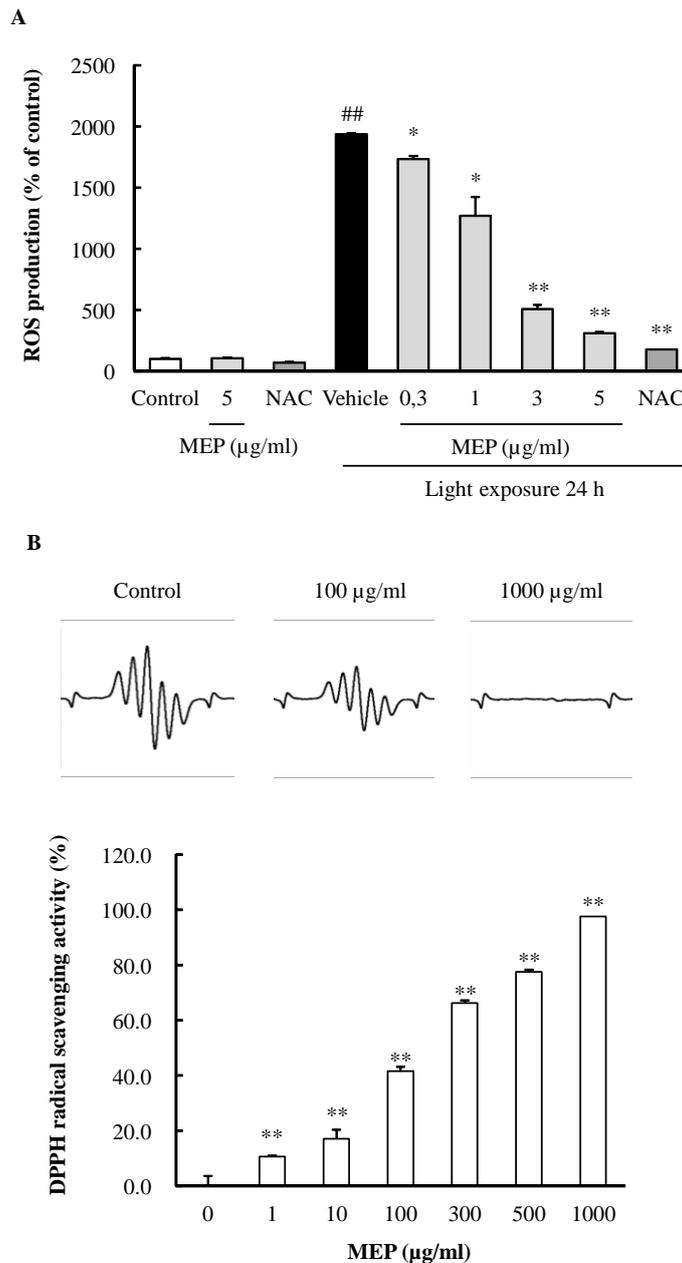
Statistical analysis

A

B

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-H$_2$DCF) 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

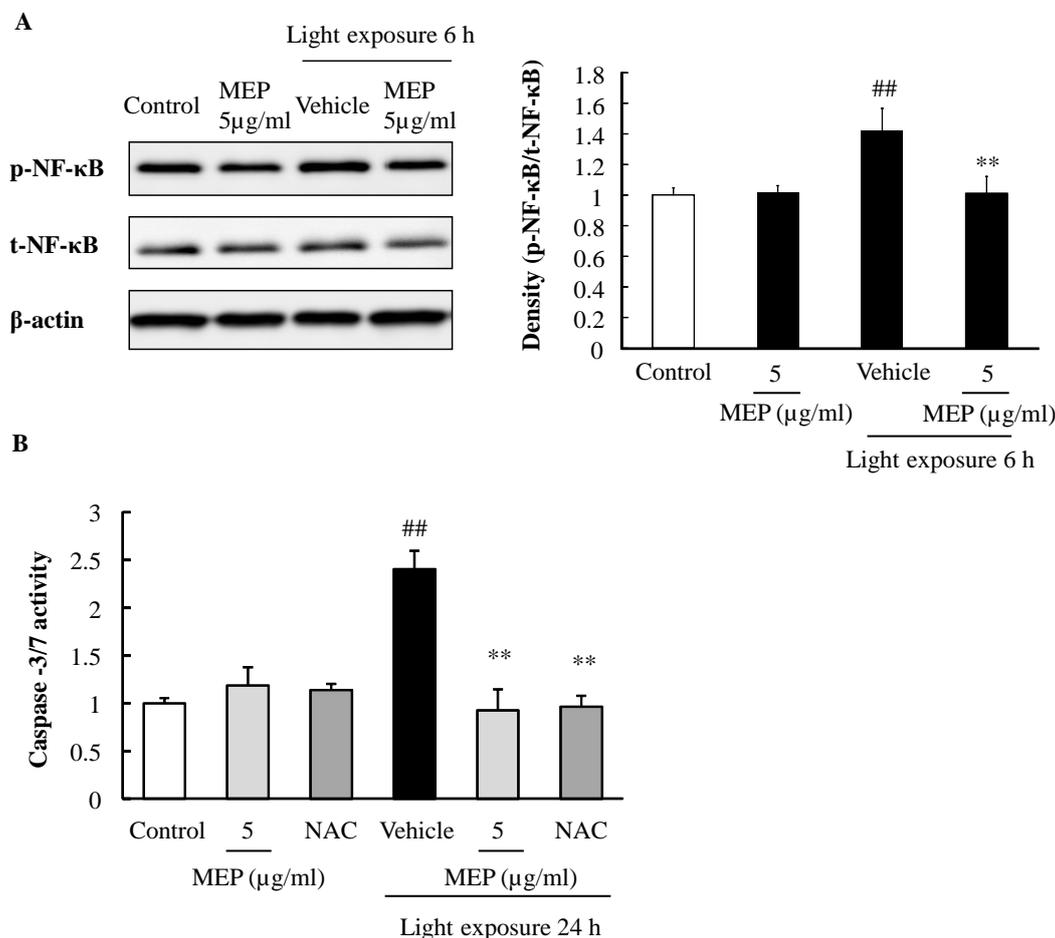Figure 3: (A) Western blot showing the effects of MEP (5 µg/ml) on phosphorylation of NF-κB in light-induced cell damage. Cells were treated with MEP for 1 h, then exposed to 2,500 lux white light for 6 h. The cells were collected and the lysate was analyzed by western blot. Quantitative analysis of 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 represented as mean ± SEM (n=3)

A

Light exposure 24 h

Control   Vehicle   1.0 µg/eye   5.0 µg/eye

ONL
INL
GCL

Zpr-1 Hoechst33342

B

ONL thickness (µm)

Control   Vehicle   1.0   5.0

MEP (µg/eye)

Light exposure 24 h

C

ONL cell number

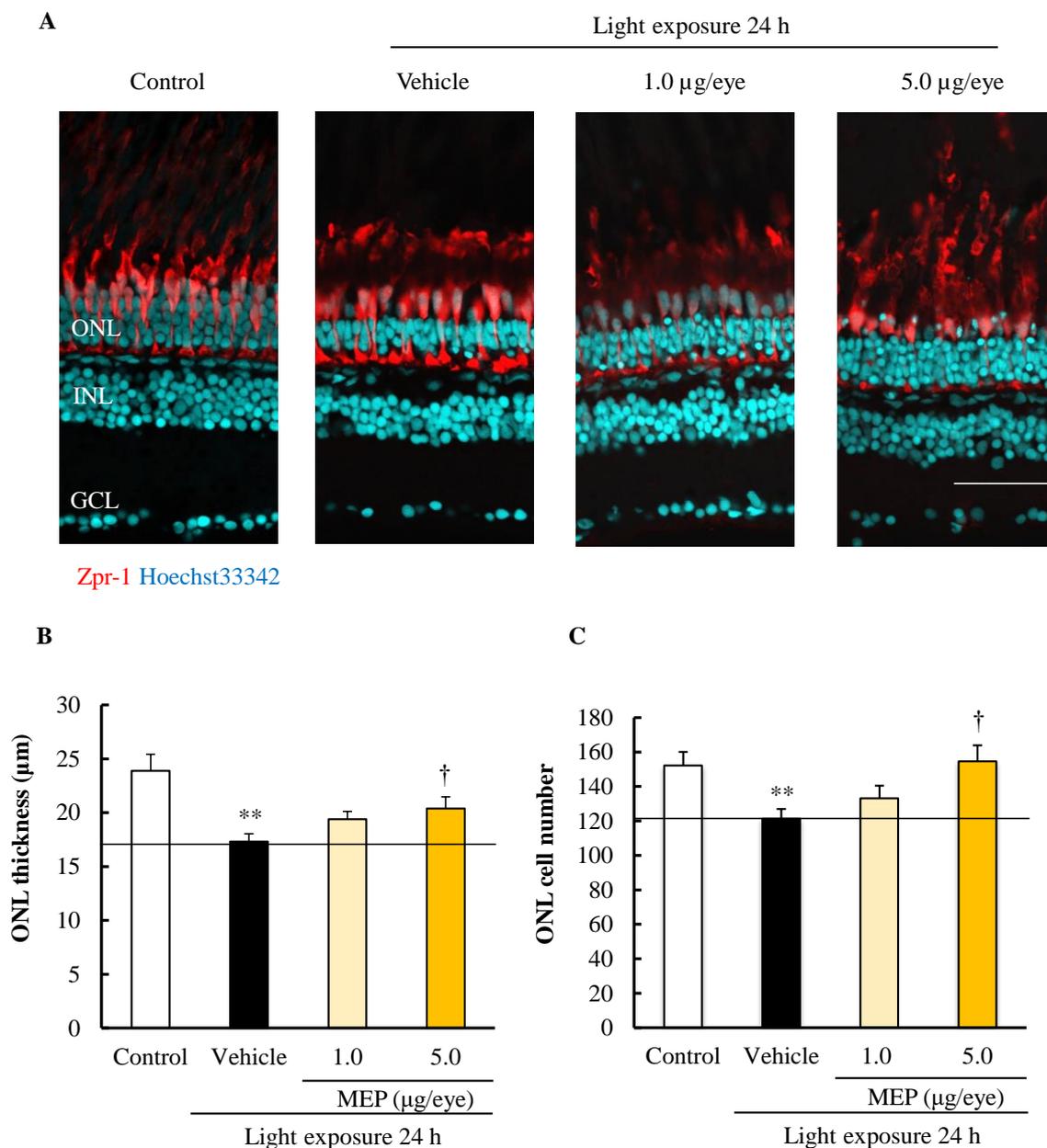Control   Vehicle   1.0   5.0

MEP (µg/eye)

Light exposure 24 h

Figure 4 : Retina sections were stained for cone cell (Zpr-1 antibody, red) and nuclei (Hoechst33342, blue) (Figure A). The number of nuclei (blue) in ONL was counted, and the thickness of ONL was measured (Figure B, C). The scale bar represents 50 µm. Data are expressed as mean ± SEM (n=5-6) **$P<0.01$ vs. Control (Student's *t*-test), †$P<0.05$ vs. Vehicle (Dunnett's test) INL; inner nuclear layer, ONL; outer nuclear layer, GCL; ganglion cell layer.

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 exposure[23]. In recent studies, light exposure can cause degeneration of retinal function, and light irradiation can induce photoreceptor cell death by mechanisms such as oxidative stress[23] and ER stress[22, 24]. These stresses are associated with NF-κB activation[25]. 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 apoptosis[26]. 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-κB[27]. 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 irradiation[23]. 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 glaucoma[28]. The methanol extract of *Crocus sativus* reinforces cellular antioxidant defenses against oxidative stress and mitochondrial dysfunction in a fly model[29]. 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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REFERENCES

1. Cheung LK, Eaton A: Age-related macular degeneration. *Pharmacotherapy* 2013;33:838-855.
2. Yoon SM, Lee BL, Guo YR, Choung SY: Preventive effect of Vaccinium uliginosum L. extract and its fractions on age-related macular degeneration and its action mechanisms. *Arch Pharm Res* 2016;39:21-32.
3. Marc RE, Jones BW, Watt CB, Vazquez-Chona F, Vaughan DK, Organisciak DT: Extreme retinal remodeling triggered by light damage: implications for age related macular degeneration. *Mol Vis* 2008;14:782-806.
4. Maeda A, Palczewska G, Golczak M, *et al.*: Two-photon microscopy reveals early rod photoreceptor cell damage in light-exposed mutant mice. *Proc Natl Acad Sci USA* 2014;111:E1428-1437.
5. Sforcin JM, Bankova V: Propolis: is there a potential for the development of new drugs? *J Ethnopharmacol* 2011;133:253-260.

6. Castaldo S, Capasso F: Propolis, an old remedy used in modern medicine. *Fitoterapia* 2002;73:S1-S6.

7. Alvarez PLJ, Cruz MB, Micor JRL, Fajardo Jr AC, Cervancia CR, Hizon-Fradejas AB: Identification of flavonoids and phenolic compounds in propolis from stingless bee (*Tetragonula biroi* friese) nests and extracts from five tree sources using tandem liquid chromatography-mass spectrometry. *The Philippine Entomologist* 2014;27.

8. Bankova V, Popova M, Trusheva B: Propolis volatile compounds: chemical diversity and biological activity: a review. *Chem Cent J* 2014;8:28.

9. Silici S, Kutluca S: Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region. *J Ethnopharmacol* 2005;99:69-73.

10. Wagh VD: Propolis: a wonder bees product and its pharmacological potentials. *Adv Pharmacol Sci* 2013;2013:308249.

11. Akyol S, Ugurcu V, Balci M, *et al.*: Caffeic acid phenethyl ester: its protective role against certain major eye diseases. *J Ocul Pharmacol Ther* 2014;30:700-708.

12. Michener CD: The Meliponini. In: Vit P, Pedro SRM, Roubik DW, eds. *Pot-Honey: A Legacy of Stingless Bees*: Springer 2013:3-17.

13. Roubik DW: Stingless bee nesting biology. *Apidologie* 2006;37:124-143.

14. Rasmussen C, Cameron SA: Global stingless bee phylogeny supports ancient divergence, vicariance, and long distance dispersal. *Biol J Linn Soc* 2010;99:206-232.

15. Varthamanan J, Jayanthi B: Evaluation of antioxidant and antimicrobial activity of stingless bee propolis (*Tetragonula iridipennis*) of Tamilnadu, India. *Int J Pharm Pharm Sci* 2014;6:8.

16. Choudhari MK, Punekar SA, Ranade RV, Paknikar KM: Antimicrobial activity of stingless bee (Trigona sp.) propolis used in the folk medicine of Western Maharashtra, India. *J Ethnopharmacol* 2012;141:363-367.

17. Campos JF, Dos Santos UP, da Rocha Pdos S, *et al.*: Antimicrobial, Antioxidant, Anti-Inflammatory, and Cytotoxic Activities of Propolis from the Stingless Bee Tetragonisca fiebrigi (Jatai). *Evid Based Complement AlternatMed* 2015;2015:296186.

18. Kustiawan PM, Puthong S, Arung ET, Chanchao C: In vitro cytotoxicity of Indonesian stingless bee products against human cancer cell lines. *Asian Pac J Trop Biomed* 2014;4:549-556.

19. Matsunaga N, Imai S, Inokuchi Y, *et al.*: Bilberry and its main constituents have neuroprotective effects against retinal neuronal damage in vitro and in vivo. *Mol Nutr Food Res* 2009;53:869-877.

20. Izuta H, Narahara Y, Shimazawa M, Mishima S, Kondo S, Hara H: 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity of bee products and their constituents determined by ESR. *Biol Pharm Bull* 2009;32:1947-1951.

21. Ogawa K, Oyagi A, Tanaka J, Kobayashi S, Hara H: The protective effect and action mechanism of Vaccinium myrtillus L. on gastric ulcer in mice. *Phytother Res* 2011;25:1160-1165.

22. Nakanishi T, Shimazawa M, Sugitani S, *et al.*: Role of endoplasmic reticulum stress in light-induced photoreceptor degeneration in mice. *J Neurochem* 2013;125:111-124.

23. Saito Y, Tsuruma K, Shimazawa M, Nishimura Y, Tanaka T, Hara H: Establishment of a drug evaluation model against light-induced retinal degeneration using adult pigmented zebrafish. *J Pharmacol Sci* 2016;131:215-218.

24. Yamauchi M, Tsuruma K, Imai S, *et al.*: Crocetin prevents retinal degeneration induced by oxidative and endoplasmic reticulum stresses via inhibition of caspase activity. *Eur J Pharmacol* 2011;650:110-119.

25. Nakajima S, Kitamura M: Bidirectional regulation of NF-kappaB by reactive oxygen species: a role of unfolded protein response. *Free Radic Biol Med* 2013;65:162-174.

26. Shukla A, Timblin C, BeruBe K, *et al.*: Inhaled particulate matter causes expression of nuclear factor (NF)-kappaB-related genes and oxidant-dependent NF-kappaB activation in vitro. *Am. J. Respir. Cell Mol Biol* 2000;23:182-187.

27. Hayden MS, Ghosh S: Regulation of NF-kappaB by TNF family cytokines. *Semin Immunol* 2014;26:253-266.

28. Mi XS, Feng Q, Lo AC, *et al.*: Protection of retinal ganglion cells and retinal vasculature by Lycium barbarum polysaccharides in a mouse model of acute ocular hypertension. *PLoS One* 2012;7:e45469.

29. Rao SV, Muralidhara, Yenisetti SC, Rajini PS: Evidence of neuroprotective effects of saffron and crocin in a Drosophila model of parkinsonism. *Neurotoxicology* 2016;52:230-242.