The Effect of Indonesian Royal Jelly Supplementation on the Growth of Hybridoma and Its Monoclonal Antibody Production

Febby N. Ningsih[1(✉)], Tika Widayanti[1], Muhamad Sahlan[2], Andri P. Pramono[3], and Sabar Pambudi[1]

[1] Research Center for Vaccine and Drug, National Research and Innovation Agency, South Tangerang, Indonesia
febb001@brin.go.id
[2] Faculty of Medicine, Universitas Pembangunan Nasional Veteran Jakarta, Jl. RS Fatmawati Pondok Labu, South Jakarta 16514, Indonesia
[3] Department of Chemical Engineering, Faculty of Engineering, University of Indonesia, Depok 16424, Indonesia

Abstract. Monoclonal antibody (mAb) is the material that is used widely for biological experiments, therapeutic, and diagnosis. The production of monoclonal antibodies by *in vitro* method is still challenging, and it takes a high cost due to the use of animal-derived serum as the main growth-promoting factor. The previous study reported royal jelly (RJ) from *Apis mellifera* as a potential proliferation factor in mammalian cell culture. Hence, this study was performed to evaluate the effect of Indonesian RJ supplementation in culture medium on hybridoma growth and its MAb production. The research was conducted by true experimental methods using hybridoma clone SF2 and RF10. These clones were cultured in RPMI-1640 medium supplemented with 3% FBS and 1% Penicillin-Streptomycin as the initial culture medium. The number of viable cells was counted by haemocytometer on day 8 after gradual treatment with or without the addition of 1% RJ. MTT assay was performed to evaluate the cell viability. In addition, the level of secreted MAb in the culture medium was measured by indirect enzyme-linked immunosorbent assay (ELISA). All results were analyzed statistically. The results show that the addition of 1% RJ into the culture medium has decreased the cell viability compared to the control. Thus, cell viability reduction may influence the level of MAb produced by hybridoma clone SF2 and RF10. The negative result given in this experiment may have been caused by an unsuitable concentration of RJ used in this experiment. Also, a substance included in the crude RJ probably causes cytotoxicity in the cells.

Keywords: culture medium · hybridoma · monoclonal antibody · proliferation · royal jelly

1 Introduction

Monoclonal antibody (MAb) is a biological material widely applied for therapeutic, immuno-based assay and well-known as the primary raw material for diagnostic kit

I. Nurlaila et al. (Eds.): ICHR 2022, AHSR 56, pp. 210–219, 2023.
https://doi.org/10.2991/978-94-6463-112-8_21

development. According to the development process, the MAb is described as an antibody that specifically recognizes the epitope of foreign protein or antigens produced from B cells by various platform technology [1]. The hybridoma technique has been mainly used since introduced by Köhler and Milstein in 1975 due to its simplicity and efficiency [2, 3]. This technique is performed by fusion of B-lymphocyte isolated from mice that have been immunized by specific antigen into myeloma cells [3].

The large-scale production of MAbs is commonly performed using *in vitro* method by cultivating the hybridoma cells in a culture medium supplemented with animal derived-serum such as fetal bovine serum (FBS) or fetal calf serum (FCS) inside the bioreactor or fermenter, which is maintained under optimum condition for the cell to grow [4]. The serum contains various amino acids, growth factors, hormones, and nutrition supporting cell growth [5]. As growth and proliferating factors, the use of serum in culture media remains unavoidable. Meanwhile, using serum as supplementation of culture medium has several advantages and disadvantages [6]. The process of obtaining the serum from a calf or bovine may have an ethical issue related to animal welfare [6, 7]. Also, it has been reported that animal-derived serum has a large variability of content due to batch, source, and environment, which can ultimately lead to reproducibility problems in the experiment. Since the environment may have an influence on serum content, the risk of pathogens contamination must be considered too. Moreover, serum supplementation in a culture medium during the production of MAb gives impurities and causes difficulties in the purification process [5–10].

The problems that have been mentioned above encourage researchers to find a substitution of serum as a growth-promoting factor for cell culture. Several synthetic growth factors, amino acids, chemical compounds, also trace elements such as transferrin, selenium, and ascorbic acids were used to develop a new formula for serum-free medium [6, 11]. For example, the combination of insulin, transferrin, and selenium, or ITS, has recently been distributed and used as a growth-promoting factor in cell culture medium [12].

Indonesia as a mega-biodiversity country, is known for its various natural resources. Royal jelly (RJ) is secreted product from the hypopharyngeal and mandibular glands of the worker bees (*Apis mellifera L.*), which has functioned as a nutrition source for the larvae, also a development factor for the bees by regulating the metabolism and genes that involved in the growth process [13, 14]. Generally, RJ contains 50–60% water, 18% substances with a nitrogen structure, 15% glucose, 3–6% fats, and 1.5% trace elements [14, 15]. A study performed by Jiang *et al.* [16] shows that Major Royal jelly Protein (MRJP) which is included in RJ has an anti-senescence activity on human embryonic lung fibroblast (HFL1) cell lines. This protein can stimulate synthesize of DNA, albumin and enhance cell proliferation of the hepatocytes [17]. Moreover, it also increases the lifespan and reproductivity of Drosophila [18]. Therefore, RJ is expected to be a potential supplement in the culture medium for mammalian cell culture, especially on hybridoma. In this study, we investigated the effect of RJ supplementation in the culture medium and its influence on MAb produced by the hybridoma.

2 Materials and Methods

2.1 Design of Experiment

This study is a true experimental and was performed by a completely randomized trial method, with three replications for each control and treatment group (n = 3).

2.2 Preparation of Royal Jelly Powder

The Royal Jelly was collected from *Apis mellifera*, which was domesticated in Indonesia, then formed into powder by freeze-dry method according to the previous study by Fachrani *et al*. [19].

2.3 Medium Culture

There are two types of medium formula used in this experiment, RPMI-1640 supplemented with 3% FBS and 100 U/mL Gibco™ Penicillin-Streptomycin (Thermo Scientific, USA), with an additional 1% RJ as the treatment group and without RJ as control. MTT assay was carried out using an initial medium as a control and two treatment groups that consist of an initial medium supplemented with two concentrations of RJ, 1% and 0.5%, respectively.

2.4 Hybridoma Cell Lines

Hybridoma cell clones SF2 and RF10 were developed at Research Center for Vaccine and Drug, National Research and Innovation Agency, Indonesia. These hybridomas were adapted in growth medium RPMI with 3% FBS and 1% Penicillin-Streptomycin. Both clones produce Mouse IgG anti-Spike Protein of SARS-CoV-2 (Unpublished data).

2.5 Cell Culture Treatment and Monoclonal Antibody Production

On day 0, the hybridoma cells are seeded at a density of 1 x 10^5 viable cells per well (using a 12-well plate) with the initial medium at least for two days under condition temperature of 37 °C and supplied by 5% CO_2. The cells were maintained every two days by reducing and adding new media treatment in a ratio of 50:50 until day 7. On day 8, the number of viable cells per well is counted by hemocytometer and using trypan blue staining to differentiate viable cells from dead cells. Then, the culture medium of each treatment group was collected and centrifuged to remove the cells. The supernatant obtained was stored at 4 °C until applied.

2.6 MTT Assay

In a parallel experiment, the cell viability of each treatment group was evaluated by MTT assay. Initially, the cells were cultivated approximately at a density of 10^4/well in a 96-well plate with an initial culture medium. After the confluence of cells reaches approximately 70–80%, the cells are maintained by reducing and adding a new treatment

medium for 3 days. MTT reagent was dissolved in RPMI basal medium, then added into each well and incubated for 4 h under dark condition in an incubator supplied with 5% CO_2 at the temperature of 37 °C. The formation of formazan crystal was observed under the microscope, then the reaction was stopped using the mixture of DMSO and SDS lysis solution with a ratio of 3:2 [20]. The optical density at 550 nm (OD_{550}) was measured using Synergy HTX Multi-Mode Reader (Agilent Technologies, USA).

2.7 Measurement of Monoclonal Antibody Production by Indirect ELISA

The Nunc MaxiSorp™ 96-well high-binding microplate (Thermo Scientific, USA) was coated with 50 ng/well of antigen SARS-CoV-2 Spike Protein (Invitrogen, USA) and incubated for 2 h, then blocked for 2h with 5% of Bovine Serum Albumin (Sigma, USA) in Phosphate-Buffered Saline containing 0.05% Tween-20 (PBS-T 0.05%). One hundred fifty microliters of a supernatant sample was added into well as a primary antibody. A monoclonal antibody against SARS-CoV-2 Spike protein (Invitrogen, USA) was used as a positive control and a complete medium as a negative control. After overnight incubation at 4 °C, samples were washed with PBS-T 0.05% for 5 times before incubated for 2 h with a secondary antibody against mouse conjugated stabilized peroxidase (Invitrogen, USA) for supernatant samples and negative control, also anti-rabbit IgG conjugated HRP (GeneTex, USA) for positive control. Furthermore, the plate was washed for 5 times, then 50 ⌈L/well of 1-Step™ Ultra TMB-ELISA (Thermo Scientific, USA) was added to the well-plate and incubated under dark condition for 10 min. After incubation, 50 ⌈L/well of 0.5 N H_2SO_4 was added to stop the reaction. Lastly, the optical density at 450 nm (OD_{450}) was measured using the same multi-plate reader instrument that was used for the MTT assay.

2.8 Data Analysis

All results in this study represent at least two independent experiments that yielded similar findings. The statistical analysis was performed using GraphPad™ Prism 9. The distribution of % cell viability data was tested with the normality test Shapiro-Wilk, then the difference was analyzed using One Way ANOVA analysis followed with Tukey's test. Meanwhile, a descriptive statistic was used to analyze the number of cells/mL and MAbs production by comparing the mean and standard deviation (SD). All values are presented as the mean ± SD. The significance was set with P value < 0.05 and considered statistically significant.

3 Results

3.1 Cell Proliferation in Culture Medium Supplemented with Royal Jelly

The number of viable cells after being cultivated and adapted gradually in a medium containing 1% RJ is shown in Fig. 1. On day-0, cells were seeded at the same number of viable cells. After eight days was adapted and maintained gradually in a medium treated with 1% RJ, the number of viable cells on both clones increased almost three

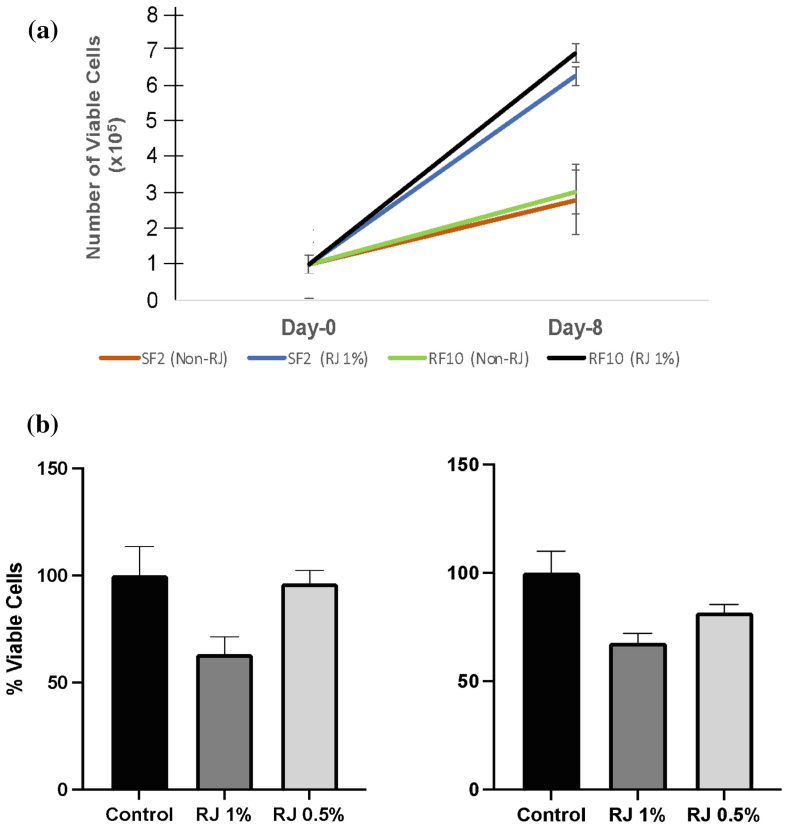

Fig. 1. Supplementation of RJ into the culture medium reduces the cell proliferation on both SF2 and RF10. **A)** The number of viable cells after eight days adapted in medium treatment. **B)** A comparison of % viable cells between control, medium treated 1% RJ, and 0.5% RJ by MTT assay. The values are the mean ± standard deviation (SD) (n = 3). *p < 0.05 and ** p < 0.01 versus control alone.

times from the initial number, approximately 2.8 x 10^5/mL for clone SF2 and 3 x 10^5/mL for clone RF10 (Fig. 1A). However, these numbers are still less than the number of the cells cultivated with the initial medium (without RJ supplementation). As presented in Fig. 1A, on day 8, the number of cells clone SF2 and RF10 were 6.2 x 10^5/mL and 6.8 x 10^5/mL, respectively.

In addition, the MTT assay result shows that the % cell viability on both clones was significantly decreased after adding 1% RJ in the culture medium. Meanwhile, the lower concentration of RJ added into the medium did not affect the % cell viability significantly (Fig. 1B). It is also supported by microscopy observation shown in Fig. 2. The confluence of both clones from day 6 adapted in the medium supplemented with 1% RJ was starting to decrease. On day 8, the cell confluency of both clones that were treated with 1% RJ was significantly lower than the control (Fig. 2).

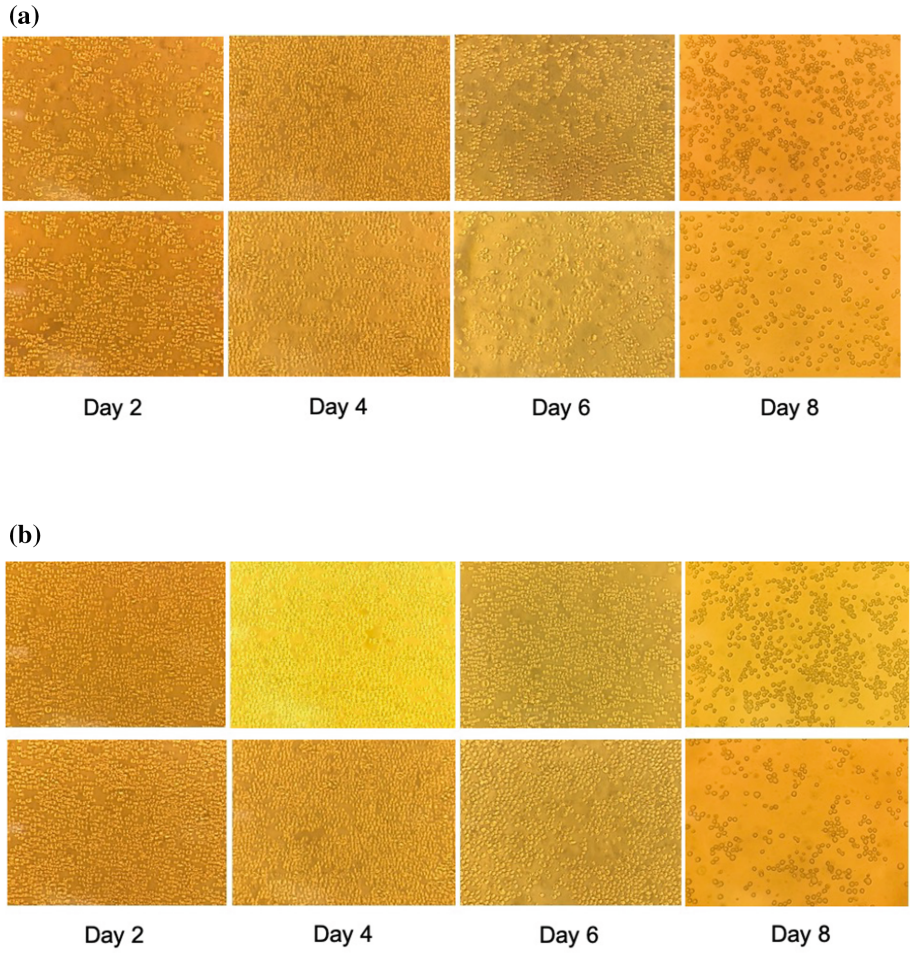

Fig. 2. The cell condition and confluency under the microscope (M = 200) on day 2, 4, 6, and 8 during adaptation in media supplemented with 1% RJ and control media. **A)** Clone SF2. **B)** Clone RF10.

3.2 Additional of Royal Jelly Reduces the Level of a Monoclonal Antibody Produced by Hybridoma

Further, the level of a monoclonal antibody that was released into the culture medium by both of clone SF2 and RF10 was measured by ELISA. In this experiment, the commercial MAb was used as positive control and the initial medium culture as a negative control. The commercial MAb gave a high signal with a value up to 1.5 at wavelength 450 nm. It shows that commercial MAb was reactive to antigen-coated on 96-well-plate. Meanwhile, the initial medium culture containing 3% FBS gave a low signal almost as the baseline. Therefore, both positive and negative controls show that the ELISA system during experiment was working well (Fig. 3).

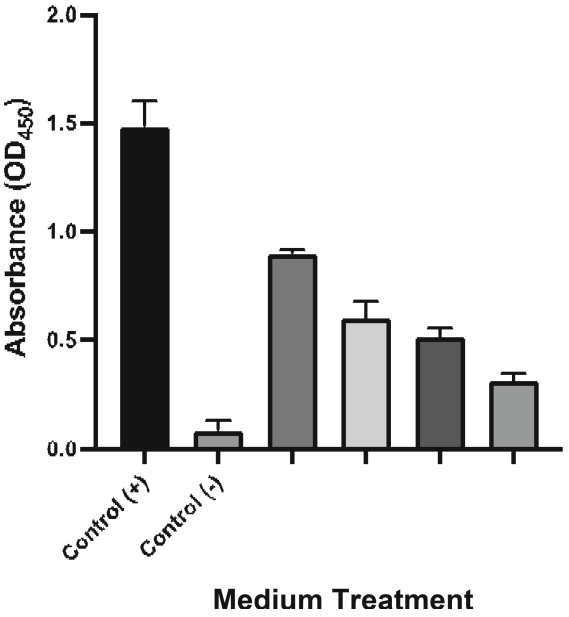

Fig. 3. The level of a monoclonal antibody produced by clone SF2 and RF10 that were cultivated in the medium with or without supplementation of RJ. The optical density was measured at 450 nm. The values are the mean ± standard deviation (SD) (n = 3).

Based on Fig. 3, the absorbance of the supernatant sample from clone SF2 that was treated with RJ is lower than the sample which was untreated by RJ. A similar data trend is also observed on the absorbance of samples from clone RF10. The monoclonal antibody that was released into the medium from clone RF10 treated with RJ was significantly decreased compared to the untreated sample.

4 Discussion

The results presented in this study show clearly that the addition of 1% RJ in the culture medium decreases the cell viability of both clones of hybridoma used in the experiment. The decreasing viable cell number has affected the reduction of the monoclonal antibody that is released into the culture medium. According to Gregory *et al.* [21], death cells affect the neighbour viable cells since lipid moieties from the death cells membrane, proteolytic enzymes, and immunomodulatory molecules released into the medium can suppress cell growth. Thus, the cells cannot reach the exponential number which is needed to maximize antibody production during the decline phase [21, 22]. The similar findings are also presented in the previous study by Fahrani *et al.* [19]. According to a previous study, an additional 1% royal jelly and honey in DMEM medium cannot promote proliferation in the human fibroblast cells [19, 23].

In addition, by comparing the cell number of hybridoma treated with RJ and without RJ, there is a significant decrease in cell number that indicates the cytotoxicity effect of

RJ. Royal jelly is a crude material that contains various substances, including a small number of polyphenols and enzymes, since it is derived from mandibular glands. A study reported that RJ has several bioactivities such as anti-inflammatory, anti-cancer, and antioxidant [24–26]. There is a possibility that some substances included in crude RJ may induce cell apoptosis on immortalized cells. As we know, the hybridoma was generated from the fusion between B-lymphocytes and myeloma cells, which means the hybridoma cell carried a part of myeloma character as immortal cells.

In conclusion, the supplementation of 1% RJ in a culture medium with 3% FBS is insufficient to promote cell proliferation and viability. It may cause the reduction of a monoclonal antibody produced by hybridoma. However, there are limitations in this study, such as cell cycle analysis and limited variant of RJ concentration tested in this study. Hence, the suitable concentration of RJ should be optimized in the future experiment. The fractionation of crude RJ must be performed to obtain MRJP and remove unnecessary substances since it may reduce the cytotoxicity on hybridoma and gives a clear explanation about the mechanism of action specifically.

Acknowledgments. The authors gratefully acknowledge Ms. Khayu Wahyunita for technical support and Faculty of Medicine, *Universitas Pembangunan Nasional Veteran* for providing the material.

References

1. Lu RM, Hwang YC, Liu IJ, Lee C-C, Tsai HZ, Li HJ, *et al.* Development of therapeutic antibodies for the treatment of diseases. J Biomed Sci. 2020 Jan;27(1):1. https://doi.org/10.1186/s12929-019-0592-z.
2. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 1975 Aug;256(5517):495–7. https://doi.org/10.1038/256495a0.
3. Liu JKH. The history of monoclonal antibody development - Progress, remaining challenges and future innovations. Ann Med Surg (Lon). 2014 Sep;3(4):113-6. https://doi.org/10.1016/j.amsu.2014.09.001.
4. Jyothilekshmi I, Jayaprakash NS. Trends in Monoclonal Antibody Production using Various Bioreactor Systems. J Microbiol Biotechnol. 2021 Mar;31(3):349-357. https://doi.org/10.4014/jmb.1911.11066
5. Brunner D, Frank J, Appl H, Schöffl H, Pfaller W, Gstraunthaler, G. Serum-free cell culture: The serum-free media interactive online database. ALTEX. 2010 Feb;27(1):53–62. https://doi.org/10.14573/altex.2010.1.53.
6. van der Valk J, Bieback K, Buta C, Cochrane B, Dirks WG, Fu J, *et al.* Fetal Bovine Serum (FBS): Past - Present - Future. ALTEX. 2018 Jan;35(1):99-118. https://doi.org/10.14573/altex.1705101.
7. Jochems CE, van der Valk JB, Stafleu FR, Baumans V. The use of fetal bovine serum: ethical or scientific problem? Altern Lab Anim. 2002 Mar-Apr;30(2):219–27. https://doi.org/10.1177/026119290203000208.
8. Even MS, Sandusky CB, Barnard ND. Serum-free hybridoma culture: ethical, scientific and safety considerations. Trends Biotechnol. 2006 Mar;24(3):105-8. https://doi.org/10.1016/j.tibtech.2006.01.001.
9. Manna L, Di Febo T, Armillotta G, Luciani M, Ciarelli A, Salini R, *et al*. Production of Monoclonal Antibodies in Serum-free Media. Monoclon Antib Immunodiagn Immunother. 2015 Aug;34(4):278-88. https://doi.org/10.1089/mab.2015.0004.

10. Baker M. Reproducibility: Respect your cells!. Nature. 2016 Sep;537(7620):433-435. https://doi.org/10.1038/537433a
11. Devireddy LR, Myers M, Screven R, Liu Z, Boxer L. A serum-free medium formulation efficiently supports isolation and propagation of canine adipose-derived mesenchymal stem/stromal cells. Plos One. 2019 Feb;14(2):e0210250. https://doi.org/10.1371/journal.pone.0210250.
12. Liu X, Zhang T, Wang R, Shi P, Pan B, Pang X. Insulin-Transferrin-Selenium as a Novel Serum-free Media Supplement for the Culture of Human Amnion Mesenchymal Stem Cells. Ann Clin Lab Sci. 2019 Jan;49(1): 63-71.
13. Buttstedt A, Moritz RFA, Erler S. Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the yellow gene family. Biol Rev Camb Philos Soc. 2014 May;89(2):255–69. https://doi.org/10.1111/brv.12052.
14. Gismondi A, Trionfera E, Canuti L, Di Marco G, Canini A. Royal jelly lipophilic fraction induces antiproliferative effects on SH-SY5Y human neuroblastoma cells. Oncol Rep. 2017 Sep;38(3):1833-1844. https://doi.org/10.3892/or.2017.5851.
15. Nagai T, Inoue R. Preparation and functional properties of water and alkaline extract of royal jelly. Food Chem. 2004 Feb;84(2):181–186. https://doi.org/10.1016/S0308-8146(03)00198-5.
16. Jiang CM, Liu X, Li CX, Qian HC, Chen D, Lai CQ, *et al.* Anti-senescence effect and molecular mechanism of the major royal jelly proteins on human embryonic lung fibroblast (HFL-I) cell line. J Zhejiang Univ Sci B (Biomed & Biotechnol). 2018 Dec;19(12):960-972. https://doi.org/10.1631/jzus.B1800257.
17. Kamakura M, Suenobu N, Fukushima M. Fifty-seven kDa protein in royal jelly enhances proliferation of primary cultured rat hepatocytes and increases albumin production in the absence of serum. Biochem Biophys Res Commun. 2001 Apr;282(4):865-874. https://doi.org/10.1006/bbrc.2001.4656.
18. Xin XX, Chen Y, Chen D, Xiao F, Parnell LD, Zhao J, *et al*. Supplementation with Major Royal-Jelly Proteins Increases Lifespan, Feeding, and Fecundity in Drosophila. J Agr Food Chem. 2016 Jul;64(29):5803–12. https://doi.org/10.1021/acs.jafc.6b00514.
19. Fachrani QS, Dhifanra MA, Nugraha Y, Yulianti R, Sahlan M, Pambudi S, *et al*. The effect of Indonesian honey Tetragonula sp. and Indonesian royal jelly *Apis mellifera* (*Ceiba pentandra*) to human preputium cell proliferation in serum-free DMEM. IOP Conf Ser: Earth Environ Sci [internet]. 2021;755:012043. Available from: https://iopscience.iop.org/article/https://doi.org/10.1088/1755-1315/755/1/012043.
20. Nga NTH, Ngoc TTB, Trinh NTM, Thuoc TL, Thao DTP. Optimization and application of MTT assay in determining density of suspension cells. Anal Biochem. 2020 Dec;610:113937. https://doi.org/10.1016/j.ab.2020.113937.
21. Gregory CD, Pound JD, Devitt A, Wilson-Jones M, Ray P, Murray RJ. Inhibitory effects of persistent apoptotic cells on monoclonal antibody production in vitro: simple removal of non-viable cells improves antibody productivity by hybridoma cells in culture. MAbs. 2009 Jul-Aug;1(4):370–6. https://doi.org/10.4161/mabs.1.4.9124.
22. Mochida H, Wang PC, Nayve Fr, Sato R, Harigae M, Nomura N, *et al*. Effects of High Cell Density on Growth-Associated Monoclonal Antibody Production by Hybridoma T0405 Cells Immobilized in Macroporous Cellulose Carriers. Biotechnol Bioprocess Eng. 2000 Apr;5(2):110–117. https://doi.org/10.1007/BF02931881.
23. Pramono A, Bustamam N, Amalia M, Sahlan M. Immense addition of royal jelly *Apis mellifera* (*Ceiba pentandra*) insufficient to increase fibroblast preputium proliferation. IOP Conf Ser Mater Sci Eng. 2019;508:1–6. Available from: https://iopscience.iop.org/article/https://doi.org/10.1088/1757-899X/508/1/012145/pdf.

24. Miyata Y, Araki K, Ohba K, Mastuo T, Nakamura Y, Yuno T, *et al*. Oral intake of royal jelly improves anticancer effects and suppresses adverse events of molecular targeted therapy by regulating TNFα and TGFβ in renal cell carcinoma: A preliminary study based on a randomized doubleblind clinical trial. Mol Clin Oncol. 2020 Oct;13(4):29. https://doi.org/10.3892/mco.2020.2099.
25. Almeer RS, Soliman D, Kassab RB, AlBasher GI, Alarifi S, Alkahtani S, *et al*. Royal jelly abrogates cadmium-induced oxidative challenge in mouse testes: Involvement of the Nrf2 pathway. Int J Mol Sci. 2018 Dec;19(12):3979. https://doi.org/10.3390/ijms19123979.
26. Petelin A, Kenig S, Kopinč R, Deželak M, Bizjak MC, Pražnikar ZJ. Effects of royal jelly administration on lipid profile, satiety, inflammation, and antioxidant capacity in asymptomatic overweight adults. Evid Based Complement Alternat Med. 2019 Jun;2019:4969720. https://doi.org/10.1155/2019/4969720.