Pharmaceutical and Clinical Journal of Nusantara (PCJN)
Volume 1, N0. 01 (2022), Page 44 - 55
https://nusantarascientificjournal.com/index.php/pcjn/article/view/5

Apis Mellifera Propolis Extract as A Stimulator of Brain Cell Development (Study on Experimental Animals)

Maslichah Mafruchati[1*], Jonathan Makuwira[2]
[1]Department of Veterinary Anatomy, Faculty of Veterinary Medicine (60115), Universitas Airlangga, Mulyorejo, C Campus, Surabaya, Indonesia
[2]Malawi University of Science and Technology, P.O Box 5196, Limbe, Malawi.
Coressponding authors: maslichah-m@fkh.unair.ac.id

Abstract

Apis mellifera could have potential as cell growth stimulator ability of the propolis. It also expected to increase the growth and proliferation of brain cells. This study aimed to analyze Apis mellifera Propolis Extract as a Stimulator of Brain Cell Development in Experimental Animals. This research is an experimental study using a completely randomized design (CRD) with 6 treatments and 4 replications. The research consisted of making Propolis Extract, Making Media (DMEM Stock Media, Washing Media, Culture and Treatment Media), Brain Cell Isolation and Culture, Observation of Culture Results (Cell Viability, Population Doubling Time, The results found that all treatments with propolis extract showed higher average viability than the control, which had average viability of 86.3%. Based on PDT (Population Doubling Time value), propolis extract has a good effect on brain cell growth, hence increasing cell proliferation. In addition, the confluent brain cells of rats given propolis extract had higher average confluency than those not given propolis extract. Propolis extract can protect neurons from damage by increasing the brain-derivated neurotrophic factor (BDNF) gene expression in cell line neuron cultures.

Keywords: *Apis mellifera, Brain Cell, Propolis Extract, Stimulator.*

Abstrak

Apis mellifera berpotensi sebagai stimulator pertumbuhan sel propolis. Selain itu juga diharapkan dapat meningkatkan pertumbuhan dan proliferasi sel-sel otak. Penelitian ini bertujuan untuk menganalisis Ekstrak Propolis Apis mellifera sebagai Stimulator Perkembangan Sel Otak pada Hewan Percobaan. Penelitian ini merupakan penelitian eksperimen dengan menggunakan Rancangan Acak Lengkap (RAL) dengan 6 perlakuan dan 4 ulangan. Penelitian terdiri dari pembuatan Ekstrak Propolis, Pembuatan Media (Media Stok DMEM, Media Pencucian, Kultur dan Media Perawatan), Isolasi dan Kultur Sel Otak, Pengamatan Hasil Kultur (Viabilitas Sel, Waktu Penggandaan Populasi, Hasil penelitian didapatkan bahwa semua perlakuan dengan propolis viabilitas rata-rata ekstrak propolis lebih tinggi dibandingkan dengan kontrol yang memiliki viabilitas rata-rata 86,3%. Berdasarkan nilai PDT (Population Doubling Time), ekstrak propolis berpengaruh baik terhadap pertumbuhan sel otak sehingga dapat meningkatkan proliferasi sel. Selain itu, sel otak konfluen tikus yang diberi ekstrak propolis memiliki rata-rata pertemuan yang lebih tinggi daripada yang tidak diberi ekstrak propolis.Ekstrak propolis dapat melindungi neuron dari kerusakan dengan meningkatkan ekspresi gen Brain Derivated Neurotrophic Factor (BDNF) pada kultur cell line neuron.

Kata kunci: Apis Mellifera, ekstrak propolis, sel otak, stimulator

Received: *November 2022* ***Accepted:*** *November 2022*

Pharmaceutical and Clinical Journal of Nusantara (PCJN)
Volume 1, N0. 01 (2022), Page 44 - 55
https://nusantarascientificjournal.com/index.php/pcjn/article/view/5

INTRODUCTION

In the 1970s, initiated by the Apiary Scout Center, modern honey bee cultivation began to be developed using European bee species (Apis mellifera) imported from Australia. Starting from 20 stup (bee boxes), Apis mellifera as a gift from President Suharto's visit to Australia in 1974, given to the Scout Movement [1]. It has grown to tens of thousands of colonies in a few years and involves hundreds of breeders. In 2006, the Directorate General of Land Rehabilitation and Social Forestry (RLPS) of the Ministry of Forestry documented at least 33,000 Apismellifera colonies (Ditjen RLPS, 2006). Apis mellifera cultivation is essential to Indonesia's beekeeping and honey production. Kuntadi (2008), citing data from the Directorate General of RLPS, said that Apis mellifera accounts for about 25% of Indonesia's total honey production, which averages 4,000 tons per year.

The priority area for developing European bee cultivation is Java. Until now, the production and grazing bases of Apis mellifera bee are mainly around the north coast of Central Java, East Java, and West Java [2]. It is related to the availability of good bee-feeding plants in the area and road infrastructure that reaches remote areas following the presence of the food source plant itself [3].

Propolis is a substance created by bees in hive defense. In addition, it can also strengthen structural stability, prevent parasites and diseases from entering, prevent decay, and reduce vibrations from outside the nest, as well as close the cracks of damaged nests (Siregar, 2011). The function of propolis is not only for the benefit of bees but is also widely used by humans as an alternative medicine to cure various diseases. According to [4], propolis is called bee glue because the material is sticky like glue. Bees produce propolis by collecting resin or sap from various plants mixed with saliva and various enzymes present in bees.

Propolis contains active compounds including vitamins, minerals, enzymes, flavonoids, terpenoids, steroids, amino acids, caffeic acid and its derivatives, caffeic acid phenyl ester-caffeic acid phenylethyl ester (CAPE) (Lawal et al., 2015). These active compounds give propolis a variety of biological and pharmacological properties, including anti-cancer, anti-bacterial, anti-inflammatory, anti-microbial, anti-fungal, tumoricidal, immunomodulator, antioxidant, anti-bacterial, parasitic, anti-diabetic, anti-histamine, antacid, anti-protozoal, anti-Helicobacter pylori (ulceration treatment), as a therapeutic agent, cell stimulator, and the ability to neuroprotective [5].

Propolis extract, which has potential as a neuroprotective, is expected to protect brain cells from damage. The brain is an organ composed of many neurons and is very sensitive to free radical induction. It

causes the brain to have high polyunsaturated fatty acids and low antioxidant molecules compared to other organs [6]. This condition causes a low rate of cell proliferation in it. Moreover, to increase the proliferation of brain cells, it is necessary to induce compounds that can increase the number of cells due to cell growth and division so that new cells are available to replace damaged cells [7].

Propolis extract could stimulate growth and increase the number of cells in rat spleen cells, human lymphocytes, rat kidney cells, and rat liver cells in vitro. Based on the results of the MTT test in 1 mg/mL of propolis, the maximum yield of cell growth was shown by spleen cells as much as 48%, and the minimum results found in liver cells by 18%. The number of rat spleen cells, human lymphocytes, rat kidney cells, and rat liver cells increased by 65%, 55%, 35%, and 25%, respectively, at a dosage of 2 mg/mL. Direct observations indicated that all normal cells induced by propolis had a longer life span than cells that were not induced by propolis extract (control). It shows that propolis extract can increase cell viability and work as a stimulator of cell growth.

The cell growth stimulator ability of the propolis extract is also expected to increase the growth and proliferation of brain cells. Proliferation is the initial part of forming neurons. With the proliferation process, brain cells will increase and replace damaged cells to maintain the integrity between neurons. In vitro, the rate of cell proliferation can be determined from the value of Population Doubling Time (PDT). The PDT value is when it takes for the cell population to double its number [8]. If the PDT value is low, cell proliferation is fast, and vice versa [9].

Propolis extract has compounds that can help the growth of neurons in vitro, such as polyphenols and terpenoids that can protect neurons from damage and tannins that can maintain the integrity of cell membranes so that they can increase cell survival, growth, and proliferation. With this ability, propolis extract can protect cells from damage, increase cell viability, and speed up the time for proliferation [10]. This study aimed to analyze Apis Mellifera Propolis Extract as a Stimulator of Brain Cell Development in Experimental Animals.

METHODOLOGY

This research is an experimental study using a completely randomized design (CRD) with 6 treatments and 4 replications. The treatments used were control (without treatment) and rat brains given propolis with 5 different concentrations. The distribution of the treatment is as follows:

- P1 (treatment 1): brain cell culture without propolis extract (control)
- P2 (treatment 2): brain cell culture with propolis extract 20 g/mL
- P3 (treatment 3): brain cell culture with propolis extract 40 g/mL

- P4 (treatment 4): brain cell culture with propolis extract 60 g/mL
- P5 (treatment 5): brain cell culture with propolis extract 80 g/mL
- P6 (treatment 6): cultured brain cells with propolis extract 100 g/mL.

Propolis Extract Manufacturing

300 grams of raw propolis was obtained from beekeeping in East Java which was then extracted using the maceration method with 1200 ml of 96% ethanol solvent. The maceration results were then evaporated using a rotary evaporator for 1 hour.

Media Creation

DMEM Stock Media

DMEM stock media was made in 100 ml with a composition of 1.35 g DMEM, 0.37 g NaHCO3, 0.238 g HEPES, 0.006 g penicillin, 0.01 g streptomycin, and 100 ml sterile DI. These materials were dissolved until homogeneous, then filtered using a 0.22 m Millipore membrane. The media stock was added with 10% FBS as a culture medium.

Washing Media

The washing medium was made of 0.9% NaCl and antibiotics (penicillin and streptomycin), 4 ml of DMEM 0% media, and 1 ml of 10% DMEM media. The washing medium was made directly during cell implantation.

Culture Media and Treatment

Culture media were prepared from DMEM and 10% FBS added with propolis with different concentrations (20 g/mL, 40 g/mL, 60 g/mL, 80 g/mL, and 100 g/mL). The media was then put into a TC dish of 3 ml and incubated in an incubator for 60 minutes at 37oC and 5% CO2. The treatment used propolis which was diluted using DMSO first. A 10% DMSO stock solution was made by dissolving 1 ml of DMSO into 10 ml of sterile DI. Then a stock extract solution was made by dissolving 10 mg of propolis extract into 10 ml of 10% DMSO to obtain a stock solution of propolis extract with a concentration of 1 mg/ml or 1000 ug/ml. Furthermore, propolis extract was made with specific concentrations from the solution, namely 20 g/mL, 40 g/mL, 60 g/mL, 80 g/mL, and 100 g/mL.

Brain Cell Isolation and Culture

Neonatal rats aged 3-4 days were dislocated, the head was dissected, and the whole brain was taken. Then washed with 0.9% NaCl + antibiotics 3 times. After that, it was chopped into pieces and homogenized using a 3 ml syringe. Then put it into a 10 ml centrifugation tube. Centrifugation was carried out at a speed of 3500 rpm for 10 minutes and 3 times. The first centrifugation results from homogenization using a syringe, and the pellet is taken.

The second centrifugation consisted of pellets from the results of the first centrifugation, which added 2 ml of 0% DMEM media, and the pellets were taken. The third centrifugation was pelleting from the results of the

Pharmaceutical and Clinical Journal of Nusantara (PCJN)
Volume 1, N0. 01 (2022), Page 44 - 55
https://nusantarascientificjournal.com/index.php/pcjn/article/view/5

second centrifugation, which was added with 1 ml of 10% DMEM. The result of the last centrifugation has discarded the supernatant and left a little with the pellet, which was then homogenized. 50 l of suspension was taken and put into a TC dish containing each treatment. Incubated, and the media was changed every 3 days [11].

Observation of Culture Results
Cell Viability

The first step before observing cell viability is that the cell culture media is removed first. Then the cells in the media were washed twice using PBS, then 500 l of trypsin-EDTA 0.25% was added and homogenized. After being homogeneous, the cells were incubated for 3 minutes at 37°C and 5% CO2. The result of trypsinization was taken as much as 10 l to be stained with 10 l 0.4% trypan blue. Cells added with trypan blue were observed under a microscope for viability.

Cell viability can be seen from the number of dead and living cells. Live cells will not be stained, while dead cells will be stained with trypan blue. Observation of cell viability using cell countess by taking pellets as much as 5 l and trypan blue as much as 5 l then homogenized. Take 5 l of pellet that has been mixed with trypan blue and put it into the chamber. The cell count will appear on the cell countess screen with green color for the percentage of live cells and red for the percentage of dead cells.

Population Doubling Time

Population Doubling Time is the time it takes for the cell population to double its original number. Population Doubling Time (days) is calculated using the formula: 1 per ((log of final cell count minus log of initial cell count) x3.32) per culture time.

Cell Confluence

Cell confluence was observed under an inverted microscope after incubation. Determination of cell confluence was carried out using ImageJ Fiji software. The software can be used for the calculation of confluence in cell growth. The analysis result is the area fraction, representing the total surface area covered by cells in the photographed area. These results will show the value of cell confluence on the TC dish as a whole.

RESULT AND DISCUSSION

Cell Viability

Cell viability can be measured using a stain containing 0.4% trypan blue. The general protocol used to observe cell viability is to clarify the plasma membrane by staining. Damaged or dead cell membranes will bind the dye so that the cells are stained, while regular or living cell membranes will not be stained because the cell membrane is impermeable to dyes, as shown in Figure 1.

Pharmaceutical and Clinical Journal of Nusantara (PCJN)
Volume 1, N0. 01 (2022), Page 44 - 55
https://nusantarascientificjournal.com/index.php/pcjn/article/view/5

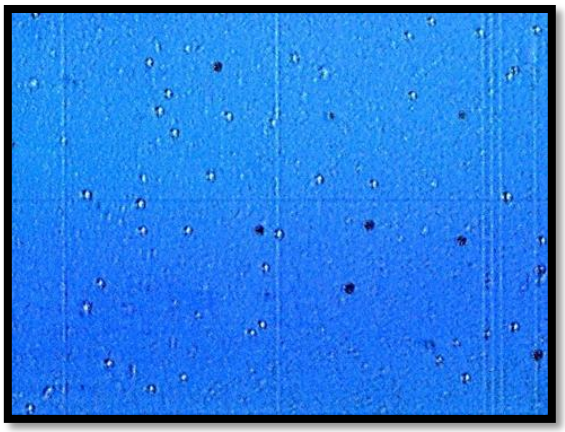

Figure 1. Cell viability with trypan blue 0.4%
staining under an inverted microscope with 200x magnification; black arrow: colored cells (dead); white arrows: cells that are not stained or clear (live).

After comparing the number of living cells and the number of dead cells, the data was obtained from each treatment's percentage of live cells. Based on table 1, it can be seen that the culture of rat brain cells (Rattusnorvegicus) in 10% DMEM media supplemented with propolis extract at doses of 20 g/mL, 40 g/mL, 60 g/ml, and 80 g/mL showed average results. The average viability increased successively, namely 86.3%, 88.0%, 89.5%, 89.8%, and 90.3%. Meanwhile, at a 100 g/mL concentration, the average viability decreased to 87.5%. All treatments with propolis extract showed higher average viability than the control, which had average viability of 86.3%. The difference in the viability results of brain cells cultured with propolis extract showed that in propolis extract, there were compounds that could help cells increase their survival ability; therefore, the viability of brain cells cultured with propolis extract was higher than brain cells cultured without extract propolis.

Table 1. Average viability (%) of rat brain cells (Rattusnorvegicus) in vitro on day 10 with and without propolis extract

Treatment	Average Viability (%)
P1 (0 µg/mL)	86.3
P2 (20 µg/mL)	88.0
P3 (40 µg/mL)	89.5
P4 (60 µg/mL)	89.8
P5 (80 µg/mL)	90.3
P6 (100 µg/mL)	87.5

Pharmaceutical and Clinical Journal of Nusantara (PCJN)
Volume 1, N0. 01 (2022), Page 44 - 55
https://nusantarascientificjournal.com/index.php/pcjn/article/view/5

***Population Doubling Time* (PDT)**

Another parameter in this study is the value of Population Doubling Time (PDT) to determine the level of proliferation of brain cells. The faster the cell proliferation process, the faster the PDT value is achieved. Determine the value of PDT and it can be done by counting the number of cells before and after being cultured. The higher the PDT value, the lower the rate of cell proliferation, and vice versa.

Based on table 2, it can be seen that the administration of propolis extract in 10% DMEM media to brain cell culture can accelerate the PDT value or time for cell proliferation as the concentration of the extract increases with the average PDT value of 5.03 days (P2), 3.69 days (P2) P3), 3.41 days (P4), and 2.88 days (P5). The fastest PDT value was achieved by P5, which was 2.88 days, which means that in the P5 treatment, brain cells could double their original number in 2.88 days or about three days. While at P6, the PDT value was higher than in the previous treatment, which was 4.65 days, which means the proliferation rate decreased compared to the previous treatment but was still higher than P1 or control. It indicates that the treatment of propolis extract has a beneficial effect on the growth of brain cells to increase cell proliferation.

Table 2. Average PDT values (days) of rat brain cells (Rattusnorvegicus) on day 10 in vitro with and without propolis extract

Treatment	Average PDT Value (days)
P1 (0 µg/mL)	5.03
P2 (20 µg/mL)	4.55
P3 (40 µg/mL)	3.69
P4 (60 µg/mL)	3.41
P5 (80 µg/mL)	2.88
P6 (100 µg/mL)	4.65

Confluency

Confluency is the constant growth of cells to cover the culture dish. The cells have reached confluent, then the cells in the culture medium have used the substrate, and the cells are interconnected with one another. Table 3 shows the average confluency with and without in vitro administration of propolis extract in rat brain cells (Rattusnovergicus).

Pharmaceutical and Clinical Journal of Nusantara (PCJN)
Volume 1, N0. 01 (2022), Page 44 - 55
https://nusantarascientificjournal.com/index.php/pcjn/article/view/5

Table 3. The results of the average confluency (%) of rat brain cells

Treatment	Average confluency(%)
P1 (0 µg/mL)	7.68
P2 (20 µg/mL)	10.11
P3 (40 µg/mL)	9.80
P4 (60 µg/mL)	12.23
P5 (80 µg/mL)	11.48
P6 (100 µg/mL)	13.58

Table 3 shows that the brain cell confluency of mice not given propolis extract (P1) had an average of 7.68 %. The concentration of propolis extract, which had the highest confluent value of rat brain cells, was P4 12.23 %, P5 and P6 13.58 %, P2 10.11%, and P3 9.80 %. Based on these results, it can be seen that the confluent of rat brain cells that were given propolis extract had a higher average confluent than those that were not given propolis extract. It can be seen that propolis extract can accelerate the confluent process of rat brain cells in vitro. Propolis can accelerate brain cell confluent because it contains compounds that help cell growth.

Cell culture is the culture of cells derived from organs or tissues that have been decomposed into cell suspensions. This cell suspension is then cultured in vitro in a container such as a vial, tube, or cup or into a cell suspension in a growth medium [12]. In vitro technique is used to maintain or reproduce parts of living tissue grown on a medium adapted to the physiological conditions of these living things [13]. Primary cell cultures can be obtained by growing cells from disaggregating tissue fragments using enzymes or obtained mechanically. In primary explants, there will be a selection based on the ability of cells to migrate from explants and grow into primary cell cultures [7].

Primary culture denotes the culturing stage in which tissue-isolated cells are grown until they occupy all accessible substrates under proper and controlled circumstances (reach confluent). At this point, the cells must be subcultured by transferring them to a fresh substrate and growth medium to allow for continued expansion [14]. Primary neuronal cultures from mice have been widely used to study neuronal properties such as axon elongation, synaptic transmission, and excitotoxicity (neuronal death due to excess glutamate) [15].

Brain cells develop from undifferentiated progenitor cells.

Neuron progenitor cells in in vitro culture have a spherical morphology, spherical with short extensions and elongated spindle-shaped (Riyacumala, 2010). In contrast, neuroglia cells will be shaped like multipolar fibroblasts before confluent and in the form of polygonal single-layered epithelial cells with regular sizes when confluent [16]. The primary brain cell cultures in this study were obtained from the brains of postnatal rats. According to Puspitasari (2013), the network is a source of several neurons with multipotent characters and many neuronal progenitor cells. To determine with certainty, the type of cells that develop in the primary culture of neurons can be confirmed by immunohistochemical assays.

The brain is the organ most susceptible to free radical attack because of its high-fat content (about 80%); thus, the risk of oxidative damage is very high (Aksenova, 2005). In addition, damaged brain nerve tissue cannot regenerate, and it can cause disease (Horner, 2005). In vitro, the problem of brain nerve cells is also not far from the in vivo state. Primary brain nerve cell culture is notoriously difficult. It is because the brain nerve cell culture has a short survival. Therefore, additional materials are needed in culture media that can increase survival and increase the proliferation of brain nerve cells. It can also be helpful for efforts to repair brain nerve cells so that diseases originating from oxidative damage can be avoided [17].

In this study, the additive used was propolis extract. According to Ni (2017), propolis extract can protect neurons from damage by increasing the brain-derivatedneurotrophic factor (BDNF) gene expression in cell line neuron cultures. The mechanism of propolis extract in increasing BDNF gene expression in cells occurs because propolis extract contains Caffeic acid phenylethyl ester (CAPE) compounds which can increase extracellular signal-regulated kinase (ERK) activation. ERK activation then alters the localization and phosphorylation of different target molecules, including transcriptional regulators (Moosavi, 2015). ERK activation is essential in proliferation because ERK translocation can trigger cells to enter the cell cycle through growth factors, namely BDNF [18].

BDNF will eventually result in phosphorylation of the CREB protein. Target gene transcription is enhanced by phosphorylation of CREB binding to CREB-binding protein. These genes are involved in cell survival, differentiation, growth, synaptic plasticity, and long-term memory activities (Moosavi, 2015). Brain-derived neurotrophic factor is one of CREB's target genes. BDNF belongs to the family of neurotrophins. With a structure similar to growth factors and plays a role in the proliferation, differentiation, and survival of nerve cells (Juananda, 2015). With the increased expression of this BDNF

Pharmaceutical and Clinical Journal of Nusantara (PCJN)
Volume 1, N0. 01 (2022), Page 44 - 55
https://nusantarascientificjournal.com/index.php/pcjn/article/view/5

gene, cells will enter the cell cycle and carry out mitosis; therefore, proliferation increases.

Another compound in propolis extract that has a role in brain cell culture is polyphenols. According to Farooqui (2012), propolis is one of the most abundant sources of polyphenols (50-55%) and mainly contains flavonoids, phenolic acids, and their esters. Moosavi (2015) added that polyphenol compounds have potent antioxidant and neuroprotective activities. Polyphenols can protect neurons from damage by involving neurotrophic effects so that they can increase the survival, growth, proliferation, and differentiation of neurons.

Polyphenols are not only known as antioxidants but also as regulators of cell metabolism. It can interact with the cell surface and then penetrate through the plasma membrane into the cytoplasm. Moreover, polyphenols can affect lipid membranes' physical properties, including diffusion, solubility, osmotic stability, permeability to water-soluble compounds, and can interact with cell membranes and fusion. One group of polyphenols, such as flavonoids, also have a role in brain cell culture. Its antioxidant ability can protect cells from oxidative damage caused by reactive oxygen species (ROS) [19].

Flavonoids can protect cell line neurons from damage caused by hydrogen peroxide (H2O2) induction. They can increase cell viability at concentrations of 5 M, 10 M, 25 M, 50 M, 100 M, and 250 M (Dajas et al., 2003). According to Widayati (2019), flavonoids can stabilize ROS by reacting with free molecules, forming relatively stable radicals and lasting a long time until they react with non-radical products. In addition to polyphenols, propolis extract also contains terpenoids and tannins. Terpenoids are dehydrogenating and oxygenating derivatives of terpene compounds that can protect neurons against -amyloid peptides, glutamate, NO, oxygen, glucose loss, and other toxic stimuli by preventing apoptosis by targeting multiple kinases, increasing ROS clearance, and protecting mitochondrial integrity [20].

Meanwhile, tannins are a group of complex phenolic compounds, polyphenols, and flavonoids that can maintain cell membrane integrity because they have sufficient gallic acid residue to interact with all lipid head groups and cover the surface of the bilayer. If the integrity of the cell membrane is maintained, the permeability of the membrane can function correctly [21]. It causes material transportation in and out of the cell to run smoothly so that cell biological activities such as cell growth and development can run optimally. The compounds contained in the propolis extract can ultimately protect and support the survival of brain cells, thereby increasing cell viability. If the cell viability increases, the time used to proliferate will be faster [22].

Pharmaceutical and Clinical Journal of Nusantara (PCJN)
Volume 1, N0. 01 (2022), Page 44 - 55
https://nusantarascientificjournal.com/index.php/pcjn/article/view/5

CONCLUSION

Propolis extract can protect neurons from injury by enhancing the brain-derived neurotrophic factor (BDNF) gene expression in neuron cultures obtained from cell lines. The mechanism of propolis extract in increasing BDNF gene expression in cells occurs because propolis extract contains Caffeic acid phenylethyl ester (CAPE) compounds which can increase extracellular signal-regulated kinase (ERK) activation. Another compound in propolis extract that has a role in brain cell culture is polyphenols. Polyphenols are not only known as antioxidants but also as regulators of cell metabolism. In addition to polyphenols, propolis extract also contains terpenoids and tannins. Terpenoids are dehydrogenated and oxygenated derivatives of terpene compounds that can protect neurons against -amyloid peptide, glutamate, NO, oxygen, glucose loss, and other toxic stimuli by preventing apoptosis by targeting multiple kinases, increasing ROS clearance, and protecting mitochondrial integrity. Propolis from Apismellifera can be used as a stimulator of brain cell development, and further studies are needed using other experimental animals, including non-human primates.

REFERENCES

1. Douglas, M. R., Sponsler, D. B., Lonsdorf, E. V & Grozinger, C. M. County-level analysis reveals a rapidly shifting landscape of insecticide hazard to honey bees (Apis mellifera) on US farmland. *Sci. Rep.* **10**, 1–11 (2020).
2. Febriyanti, A. R., Ratnasari, R. T. & Wardhana, A. K. The Effect of Economic Growth, Agricultural Land, and Trade Openness Moderated By Population Density on Deforestation in OIC Countries. *Quant. Econ. Manag. Stud.* **3**, (2022).
3. Barkah, T., Rusgianto, S. & Wardhana, A. Impact of Agricultural Land and the Output of Agricultural Products Moderated with Internet Users toward the Total export of Agricultural Product in Three Islamic South East Asian Countries. *Media Agribisnis* **6**, (2022).
4. Himawan, R. G., Primarizky, H., Mafruchati, M., Triakoso, N. & Maslachah, L. Green Tea Leaves Extract Effect on Histopathology of Mercury Chloride Induced Rat's Liver. *Pollut. Res.* **40**, 326–329 (2021).
5. El-Seedi, H. R. *et al.* A Spotlight on the Egyptian Honeybee (Apis mellifera lamarckii). *Animals* **12**, 2749 (2022).
6. Yudaniayanti, I. S., Primarizky, H. & Nangoi, L. The effects of honey (Apis dorsata) supplements on increased bone strength in ovariectomized rat as animal model of osteoporosis. in *AIP Conference Proceedings* vol. 1945 20004 (AIP Publishing, 2018).
7. Mafruchati, M. Identification of Moniezia, Sp in goat intestines in Indonesia which can impede goat productivity. *Syst. Rev. Pharm.* **11**, 1341–1345 (2020).
8. Ghabanchi, J., Bazargani, A., Afkar, M. D., Foroshan, S. B. & Ayeen, S. D. In vitro assessment of anti-Streptococcus mutans potential of honey. *Iran. Red Crescent Med. J.* **12**, 61 (2010).

9. Mafruchati, M., Wardhana, A. K. & Ismail, W. I. W. Disease and viruses as negative factor prohibiting the growth of broiler chicken embryo as research topic trend: a bibliometric review. *F1000Research* **11**, 1124 (2022).

10. Bicudo de Almeida-Muradian, L. *et al.* Standard methods for Apis mellifera honey research. *J. Apic. Res.* **59**, 1–62 (2020).

11. Nur, M. O. *et al.* Profil bakteri non spesifik dalam lendir serviks sapi perah pada fase folikuler dan fase luteal. *Ovozoa J. Anim. Reprod.* **9**, 17–22 (2020).

12. Luqman, E. M., Mafruchati, M., Rahardjo, B. P. S. & Hestianah, E. P. Crosstalk between Necrosis and Apoptosis of Embryonal Cerebral Cortex Neuron Mice (Mus musculus) Caused by Carbofuran Exposure. *Res. J. Pharm. Technol.* **12**, 5492–5498 (2019).

13. Yahya Al-ma'ruf, A. *et al.* Morphology and histology of paryphasmata and hemibaculum of Varanus salvator based on sexual maturity. *Open Vet. J.* **11**, 330–336 (2021).

14. Yip, C. W., Hon, C. C., Zeng, F. & Leung, F. C. C. Cell culture-adapted IBDV uses endocytosis for entry in DF-1 chicken embryonic fibroblasts. *Virus Res.* **165**, 9–16 (2012).

15. Mahaputra, L., Mafruchati, M., Triakoso, N. & Aries, R. D. Pemisahan Spermatozoa Sapi Limousin yang Memiliki Kromosom X dan Y dengan Percoll dan Putih Telur Ayam. *J. Bina Praja* **14**, 172–175 (2012).

16. Sunariani, J., Khoswanto, C. & Irmalia, W. R. Difference of brain-derived neurotrophic factor expression and pyramid cell count during mastication of food with varying hardness. *J. Appl. Oral Sci.* **27**, (2019).

17. Mafruchati, M. Duck meat, delicious yet low in unsaturated fat; comparative study between duck meat consumption in asean toward the number of covid cases. *Syst. Rev. Pharm.* **11**, (2020).

18. Murtaza, G. *et al.* Caffeic acid phenethyl ester and therapeutic potentials. *BioMed Res. Int.* **2014**, (2014).

19. Aengwanich, W. & Suttajit, M. Effect of polyphenols extracted from Tamarind (Tamarindus indica L.) seed coat on physiological changes, heterophil/lymphocyte ratio, oxidative stress and body weight of broilers (Gallus domesticus) under chronic heat stress. *Anim. Sci. J.* **81**, 264–270 (2010).

20. Dewi, S. K. *et al.* PENGARUH PEMBERIAN EKSTRAK KULIT PISANG KEPOK (Musa acuminata) TERHADAP PENURUNAN KADAR MALONDIALDEHID (MDA) PARU MENCIT JANTAN (Mus musculus) YANG DIPAPAR ASAP ROKOK. *J. Basic Med. Vet.* **8**, 123–130 (2019).

21. Mafruchati, M. Curcumin vs Chloroquine in Coronavirus Global Pandemic: Trend analysis Study in Google. *Indian J. Forensic Med. Toxicol.* **14**, 3138–3143 (2020).

22. Yunus, M. & Mafruchati, M. Eimeria tenella Pathogenicity Attenuation Through The Serial Passage of Precocious Lines Toward Number of Schizont and Cecal Histopathology In Broiler. *J. Poult. Sci.* **4**, 41–43 (2011).