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Research Report

# Genotoxicity test of propolis extract, mineral trioksida aggregat, and calcium hydroxide on fibroblast BHK-21 cell cultures

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***ABSTRACT***

***Background:*** *Health industry has always used natural products as an alternative. Propolis, a natural antibiotic, is a resinous yellow brown or dark brown substance derived from honey bees (Apis mellifera). The main chemical compounds contained in propolis are flavonoids, phenolics and other various aromatic compounds. Flavonoids are well known plant compounds that have antibacterial, antifungal, antiviral, antioxidant and anti-inflammatory proprieties. Propolis is expected to be an alternative used for root canal treatment with lower toxicity compared to calcium hydroxide ($Ca(OH)_2$. Over the last decade, a new material, mineral trioxide aggregate (MTA) was developed, and has been used as the gold standard. All materials used in mouth should be biocompatible. The initial level of material biocompatibility evaluation involves toxicity and genotoxicity tests.* ***Purpose:*** *This research is aimed to conduct comparison test of genotoxicity effect of propolis extract, MTA and $Ca(OH)_2$ on fibroblast BHK-21 cell culture.* ***Methods:*** *This research was conducted with single-cell gel electrophoresis method.* ***Results:*** *The results indicate that propolis extract cannot cause DNA damage, while MTA can cause apoptosis and $Ca(OH)_2$ can cause neucrosis.* ***Conclusion:*** *It can be concluded that propolis extract has genotoxicity effect lower than MTA and $Ca(OH)_2$, but MTA has lower effect on fibroblast BHK-21 cell culture.*

***Keywords:*** *Propolis extract; mineral trioxide aggregate; $Ca(OH)_2$; genotoxicity; single-cell gel electrophoresis*

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## INTRODUCTION

Currently, calcium hydroxide ($Ca(OH)_2$) becomes the most common drug used in endodontics since its ability has been proven by many scientific researches. $Ca(OH)_2$ is an excellent therapeutic material widely used as a medicine in endodontic therapy. $Ca(OH)_2$ was used as a medicine in endodontic therapy for the first time in 1920. The drug is used in dental care, such as pulp capping, pulpotomy, apexification, root perforation, and internal or external resorption. $Ca(OH)_2$ does not cause DNA damage at a concentration of 20-100 µg/ ml based on the result of genotoxicity test.[1,2]

On the other hand, the biocompatibility of mineral trioxide aggregate (MTA) has been developed. Several in vivo and in vitro studies show that MTA has sealing ability and excellent biocompatibility. MTA is used in dentistry as a root canal filling material. It means that MTA can be considered as potential and ideal material for repairing perforation, apical barrier to teeth with an open apex, pulp capping and pulpotomy of young permanent teeth.[3] Many researches on genotoxicity of MTA show that MTA does not cause DNA damage at a concentration of 1-1000 µg/ ml.[1,4,5]

In the twelve century, propolis was used by Egyptians, Greeks, and Romans as a medicine to cure skin bruises and wounds, to regenerate tissue, to treat mouth and throath infections, as well as to cure dental caries since it has anti-inflammatory, antiseptic, and antimicotic effects.[7] There have been many studies on propolis showing that propolis has antioxidant, antibacterial, antifungal, antiviral and anti-inflammatory effects. Since propolis has anti-inflammatory

effect, it is known that propolis can inhibit prostaglandin synthesis, can increase both body's resistance to the presence of phagocytic activity and self-healing, and can also stimulate immune cells. Propolis also contains iron and zinc considered as essential elements for the synthesis of collagen.

Recently, many researches have studied on the use of propolis in dentistry, especially related with antimicrobial and antiinflammatory activities in kariology, oral surgery, pathology, periodontics and endodontics.[8] However propolis from Piracicaba, Brazil can be considered as toxic at a concentration of 2 mg/ mL on fibroblast cells. [9] Toxicity test on propolis extract, Apis mellifera L, applied on fibroblast BHK-21 cells at a concentration of 1.5 mg/ ml cannot be considered as toxic.[10] Furthermore, propolis has more advantages than $Ca(OH)_2$ used as pulp capping agent in vital pulp therapy. Thus, propolis is expected to be an alternative new compound used in root canal treatment since it has lower toxicity than $Ca(OH)_2$.[11]

Actually, all materials used in mouth should be biocompatible. Biocompatibility is an ability of a material to show a response to the host in particular. Evaluation of the biocompatibility of a material is the initial level of toxicity and genotoxicity tests. Toxicity test is conducted by simply placing the material to be tested directly on the membrane tissue or cell culture. Genotoxicity test is still required to see whether there is any change in DNA of either human or non-mammalian cells caused by certain materials.[12] Therefore, this research conducted a comparison test of genotoxicity effect on propolis extract, MTA and $Ca(OH)_2$ using cell culture with single-cell gel electrophoresis method. Finally, this research is aimed to evaluate the genotoxicity effects of propolis extract, MTA and CH in order to see fragmentation (damage) of DNA from fibroblasts, and to determine the genotoxicity ratio of propolis extract, MTA, and $Ca(OH)_2$.

## MATERIALS AND METHODS

Fibroblast BHK-21cell culture derived from fibroblasts of hamster's kidney was used in this research. Fibroblast BHK-21cell culture was prepared at the Central Laboratory of Veterinary Farma (PUSVETMA), while DNA extraction and electrophoresis were conducted in the Laboratory of Tissue Culture Tropical Desease Centre (TDC). This research can actually be considered as a laboratory experimental observational research with 30 samples for each treatment based on the formula. Fibroblast cells were then divided into five groups, namely group 1 as media control consisted of 3 wells; group 2 as cell control consisted of 3 wells; group 3 treated with propolis extract as much as 1.5 mg/ml; group 4 treated with MTA with the ratio of powder: liquid about 3: 1; and group 5 treated with 50% $CA(OH)_2$ as control group.

Genotoxicity test was conduced with DNA electrophoresis examination. DNA was extracted from cells with spin coloumn invitrogen method. The concentration of DNA was measured. Electrophoresis was conducted to see whether there was DNA cut or not. Propolis extract was prepared with 11 g/100 ml of phenol as well as organic solvent required to dissolve the phenol. Phenol should be gradually diluted with buffer solves solution (BSS). The propolis extract was applied into each sample, about 0.5 ml. In other words, the total of the propolis extract used for 30 samples with a concentration of 1.5 mg.was 2.1 ml comparable with 15 ml of propolis extract with a concentration of 1.5 mg. $Ca(OH)_2$ powder was prepared with a concentration of 50% (50 g/ 100 ml). It was then applied after it was dissolved in 100 ml of sterile distilled water again with a ratio of 1: 1. Fibroblast BHK-21 cel culture was prepared until the samples of BHK-21 in the form of frozen liquid nitrogen ($-80^0$ C) were taken and cashed 30 minutes with a stream of water, and then centrifuged at 500 rpm. Fibroblast cells were transferred into four small roux bottles each of which was filled with 10 ml eagle medium containing 10% bovine serum, and then covered with aluminum foil.

Bottle was put into an incubator at a temperature of $37^0$ C for 24 hours. Confluence cells were removed and washed with 15 ml of PBS solution for three times. One ml of 0.25% trypsin-versene was given, then shaken and sprayed into the wall of the bottle for 5-10 minutes until the cells were separated from the wall of the bottle. The rest of the cells in the roux was added with 10 ml of medium eagle and 10% bovine serum, and then shaken until all cells were separated from the wall of the bottle and also from the bonds between cells. The cells were moved into four microplates (24 wells), each of which was filled with 1 ml of medium and 0.5 ml of the cell. Those microplates were closed and incubated for 24 hours. Microplates were observed under a light microscope to see whether fibroblast cells that had been grown in each of the wells were enough or not for the research. Propolis extract, MTA and calcium hydroxide were prepared to be applied in each well, and then incubated again for 24 hours at a temperature of $37^0$ C. After that, the samples were taken, and the cells were washed with PBS three times. Trypsination was conducted by adding trypsineversene and 1% EDTA, and then waited for a while. Another eagle's medium was then given in order to obtain a cell suspension. The cells harvested were then packed into eppendorf tubes that had been sterilized twice and washed with PBS once. Each eppendorf tube was put based on treatment group for DNA electrophoresis after DNA extraction was conducted.

DNA extraction with spin coloumn invitrogen method was conducted. 200 ml of cell culture samples were put into eppendorf tube, and then added with 20 ml of Proteinase K and 20 ml of RNase A. The mixtures were divortexed and incubated for two minutes at room temperature, than were added with 200 ml of pure genomic link$^{TM}$ lysis/ binding buffer, and then vortexed. Those were incubated in water bath at a temperature of 55 ° water bath for ten minutes. 200 mL of 96-100% ethanol were added, and vortexed for

± five seconds. Those were put into coloumn spin, and then centrifuged at 10,000 g for 1 minute at room temperature. The coloumn spin was added with 500 ml of wash buffer I, and then centrifuged 10,000 g for 1 minute. The coloumn spin was added with 500 ml of wash buffer II, and then centrifuged at maximum speed for three minutes at room temperature. The coloumn spin was transferred to a new eppendorf, and then added with 25-200 ml of elution buffer. It was incubated for 1 minute, and then centrifuged at maximum speed.

Visualization of DNA was conducted with electrophoresis method by making 12.5% acrylamide gel consisted of bis-acrylamid, 10% *APS*, 0.5 X TBE and Temed. Main gel was poured until the upper limit of main gel line. The stacking gel was poured on the top of maingel. Wells were molded in the stacking gel, and DNA samples were prepared to be electrophased. Loading dye was added into the DNA samples with a ratio of DNA samples and dye loading, about 5 : 2. The gel was put into the buffer, and then the electrophoresis tools were set. DNA samples were put into those electrophoresis wells. The electrophoresis was conducted at 120 V for 90 minutes. The gel was removed, and then staining was conducted by putting the gel into the drying solution for 5 minutes, and into fixer solution for 15 minutes. $AgNO_3$ staining was conducted for 1 hour. The gel was washed three times, and then added with develop solution until the band appeared. Finally, the results were read with the help of three skilled staff.

## RESULTS

This research was conducted to determine genotoxicity effect leading to cell degeneration through DNA fragmentation (DNA damage) in fibroblast BHK-21 cell culture due to the apllications of propolis extract, MTA, and $CA(OH)_2$. Based on the reading results of DNA electrophoresis visualization in media control and cell control, it is known that there was no result in media control because there was no cell in media control. Meanwhile, there were two cells fragmented in cell control, both at 130 bp (Figure 1). Moreover, based on the visualization of DNA electrophoresis, it is known that in the applicaton of MTA there was non-random DNA fragmentation, namely DNA ladder called as apoptosis (Figure 2), while in the application of $CA(OH)_2$, there was DNA smear/necrosis (Figure 3). It is also known that in the application of propolis extract, there was no fragmentation (Figure 4).

Based on the results of Kruskal-Wallis test, moreover, it is known that there was significant difference in those three samples in each group, namely fragmentation, apoptosis, and necrosis ($p < 0.05$). Kruskal-Wallis test is a

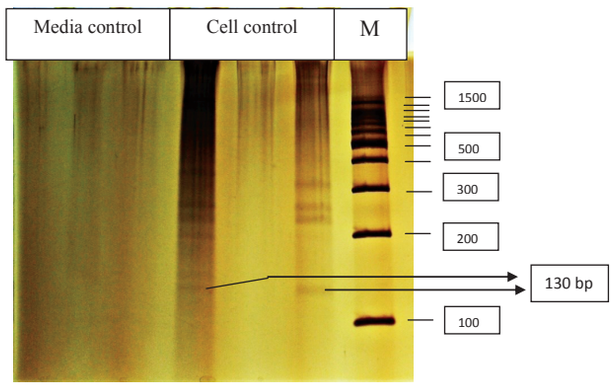


**Figure 1.** DNA electrophoresis visualization in media control and cell control.
Note: M = marker (100-1500bp).

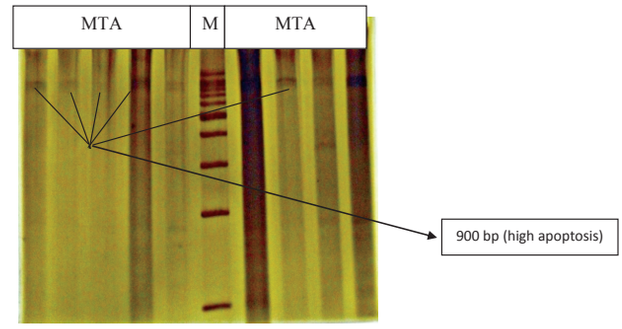


**Figure 2.** DNA electrophoresis visualization in MTA (high apoptosis).
Note: M = marker (100-1500bp); MTA= samples of mineral trioxide aggregat application.

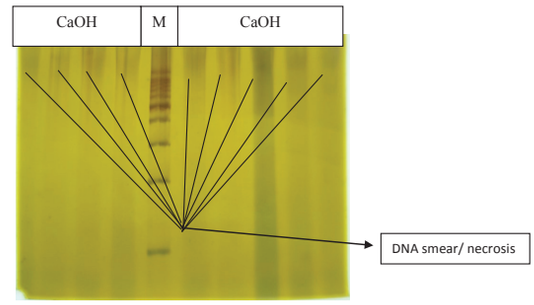


**Figure 3.** DNA electrophoresis visualization in $CA(OH)_2$ (necrosis).
Note: M = marker (100-1500bp); $CA(OH)_2$ = samples of $CA(OH)_2$ application.

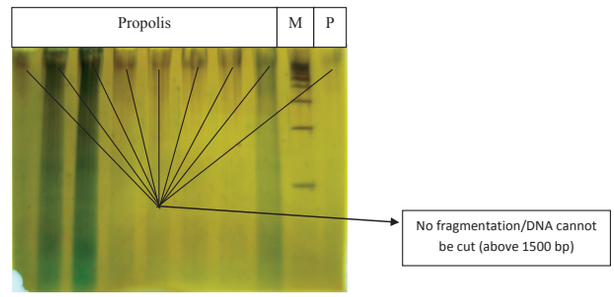


**Figure 4.** DNA electrophoresis visualization in propolis extract (good DNA).
Note: M = marker (100-1500bp); P= samples of propolis extract application.

**Table 1.** Kruskal-Wallis test on the fragmentation, necrosis, and apoptosis of propolis extract, MTA, and $CA(OH)_2$ applied in fibroblast BHK-21 cell culture

| | Kruskal-Wallis Propolis Extract-MTA-$CA(OH)_2$ |
|---|---|
| Significance of fragmentation | p=0.000* |
| Significance of necrosis | p=0.000* |
| Significance of apoptosis | p=0.000* |

Note: *p<0.05 = There was significant difference.

**Table 2.** Mann-Whitney test on the fragmentation, necrosis, and apoptosis of propolis extract, MTA, and CA(OH)2 applied in fibroblast BHK-21 cell culture

| | Propolis | $CA(OH)_2$ | MTA |
|---|---|---|---|
| Propolis | - | F: p=0.000*<br>A: p=0.000* | F: p=0.000*<br>N: p=0.317<br>A: p=0.094 |
| $CA(OH)_2$ | N: p=0.000* | - | F: p=0.000*<br>N: p=0.000*<br>A: p=0.000* |

Note: F = fragmentation; N = necrosis; A = apoptosis; *p<0.05 = There was significant difference.

one-way variance analysis with a non-parametric method used to test whether the samples come from the same distribution. In other words, it is used to compare more than two independent or unrelated samples. When Kruskal-Wallis test leads to significant results, Mann-Whitney test then must be conducted with three or more groups. Mann-Whitney will help analyze specific sample pair for a significant difference. The difference between two samples in each group can be considered significant if p<0.05.

The result analysis of Kruskal-Wallis test on the occurrence of fragmentation in cultured BHK-21 cells with the application of propolis extract, MTA and $CA(OH)_2$ showed that there were significant difference within them (significant, p = 0.000) (Table 1). The result analysis of Mann-Whitney test showed that there was significant diference between propolis and MTA with p = 0.000. There was also significant diference between propolis and $CA(OH)_2$ with p = 0.000 and between MTA and $CA(OH)_2$ with p = 0.000 (Table 2). Second, the result analysis of Kruskal-Wallis test on the occurrence of necrosis in cultured BHK-21 cells with the application of propolis extract, MTA and $CA(OH)_2$ showed that there were significant difference within them (significant, p = 0.000) (Table 1). Then the result analysis of Mann-Whitney test showed that there was no significant diference between propolis and MTA with p = 0.317. But, there was significant diference between propolis and $CA(OH)_2$ with p = 0.000, and between MTA and $CA(OH)_2$ with p = 0.000 ( Table 2). Finally, the result analysis of Kruskal-Wallis test on the occurrence of apoptosis in cultured BHK-21 cells with the application of propolis extract, MTA and $CA(OH)_2$ showed that there were significant difference within them (significant, p = 0.0005) (Table 1). Then the result analysis of Mann-Whitney test showed that there was no significant diference between propolis and MTA with p = 0094. There was significant diference between propolis and $(CAOH)_2$ with p = 0.000, and between MTA and $CA(OH)_2$ with p = 0.000 (Table 2).

## DISCUSSION

One of the requirements of dental materials that can be applied in oral cavity is that it must be biocompatible and does not contain toxic, irritation, inflammation, allergy, genotoxic, or carcinogenic substances.[12,15] Based on the ISO-1099315, there are three kinds of genotoxicity testing, ie gene mutations, chromosomal aberrations, and DNA effects. DNA effect test is used to detect the presence of damaged cells. There are actually several methods for detecting DNA damage, such as single-cell electrophoresis gel. If these materials are genotoxic, there will be both non-random DNA fragmentation as DNA ladder, commonly called apoptosis appears, and random DNA fragmentation that is not clearly visible spread throughout DNA smear, commonly called necrosis.

In this research, to test the genotoxicity effect of propolis extract, MTA and $CA(OH)_2$, fibroblast BHK-21 cells were used. This is because fibroblast cells are important cells in dental pulp, periodontal ligament and gingiva.[17] BHK-21 cell culture, derived from fibroblasts of hamsters' kidney, has been chosen to be used to biocompatibility test because their passage can do more than 50-70 times with high-speed cell growth (2 x 105 cells/$cm^3$ of the surface of the culture), and can also maintain cell integrity, as well as because the cells are able to divide themselves and multiply in suspense, thus increasing the efficiency of cell culture.[18]

Based on the test results on genotoxicity effect, it is known that there was significant difference of the fragmentation of fibroblast BHK-21 cell culture whithin the applications of propolis extract, MTA and $CA(OH)_2$. It also known that the application of propolis extract in fibroblast BHK-21 cell culture did not cause DNA damage as shown in the visualization of DNA electrophoresis, DNA found was not cut cut and above 1500 bp. Propolis extract used in this research is propolis extract from Lawang, East Java, where the largest composition of propolis extract in Lawang is phenylic acid, which has the basic element of flavonoid. Flavonoid is a class of compounds that has antibacterial, antifungal, antiviral, antioxidant and anti-inflammatory properties. Propolis, furthermore, is found to be highly effective against gram-possitive bacteria,[19] especially against *Staphylococcus aureus*,[20] and against gram-negative bacteria, especially against *Salmonella*.[21] Flavonoids and caffeic acids in propolis are known to play an important role in reducing inflammatory response of lipoxygenase by inhibiting working mechanism of arachidonic acid.

Flavonoids and caffeic acid also helps immune system to promote phagocytic activity, and stimulate cellular immunity.

Propolis helps the process of hard tissue formation, the stimulation of various enzyme systems and cell metabolism, as well as the circulation and. formation of collagen that helps in wound healing. This effect has been caused by arginine, vitamin C, provitamin A, B complex and minerals, such as copper, iron, zinc and bioflavonoids contained in propolis.

Propolis is a good antimicrobial agent because it prevents bacterial cell division as well as breaks down the cell walls of bacteria and sitoplasma.[22] Moreover, the results of a research on the antibacterial effectiveness of the three intracanal materials commonly used against *Enterococcus faecalis* (*E. faecalis*) show that in vitro propolis has antibacterial activity against *E. faecalis* in the root canal, so propolis can be used as an alternative intercanal material.[23] Another in vivo research on the effectiveness of propolis and $Ca(OH)_2$ used as a short term intracanal medication against *E. faecalis* shows that propolis is more effective as an intracanal medication than calcium hydroxide.[24] Propolis compared with other experimental materials is the most irritant material as well as one of the valuable alternative endodontic materials.[21]

Based on the results of this research, it is known that there was significant difference of the occurrence of necrosis whithin the application of propolis extract, MTA and $CA(OH)_2$. It is known that the application of $CA(OH)_2$ caused necrosis more than propolis and MTA. Moreover, it is known that the application of propolis extract did not cause any damage to DNA because the DNA found was not cut and was above 1500 bp. In addition, it is also known that the application of MTA did not cause necrosis, but caused apoptosis with a low level of damage. Thus, it can be said that the application of MTA caused apoptosis more than the application of propolis extract and $CA(OH)_2$, but the occurrence of apoptosis between in the application of propolis and in the application of MTA did not differ significantly since some of the applications of propolis extract can cause apoptosis at the level of damage that is not much different from MTA. MTA is a mixture of smooth Portland cement and bismuth oxide. It is also known that MTA contains a number of $SiO_2$, CaO, MgO, $K_2SO_4$ and $Na_2SO_4$. Portland cement mainly contains a mixture of dicalcium silicate, tricalcium silicate, tricalcium aluminate, gypsum, and tetracalcium aluminoferrite. It is known MTA has biocompatibility, excellent sealing, as well as antibacterial and low cytotoxic effect. MTA does not cause any sitomorphology change in fibroblast cells and osteoblastic cells. It also known that MTA has an ability to induce the release of bioactive dentine matrix protein. Therefore, the initiation of hard tissue bridge (in coronal or apical) can stimulate cell proliferation and cell migration, then differentiating, as a result stimulating the hard tissue and periodontal tissue regeneration.[17,26]

It can be concluded that propolis extract has lower genotoxicity effect than MTA and $CA(OH)_2$. MTA has lower effect than $CA(OH)_2$ on fibroblast BHK-21 cell culture. It is suggested for further research that the genotoxicity effect of propolis extract is needed to be analysed on fibroblast cells in vivo in order to know the appropriate dose and shape of propolis extract used in dental therapies, especially in the field of endodontics.

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