

Cytotoxicity test of apple cider vinegar as a root canal irrigant against fibroblast cells

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ABSTRACT

Background: Apple cider vinegar potentially can be used as an alternative to irrigation solutions because of its antibacterial compounds that can inhibit *Enterococcus faecalis*, a pioneer bacteria that cause root canal treatment failure. One of the ideal irrigation solution requirements is that it isn't toxic to oral cavity tissues, so it's necessary to run a cytotoxicity test on apple vinegar solution. Cytotoxicity test is the initial part of the evaluation of a dental material before it can be used by humans. Cytotoxicity test was performed on fibroblast cells because the irrigation solution can contact with fibroblast, which are the main cells in the periodontal ligament around the apical. The purpose of this study was to analyze the *in vitro* cytotoxicity effect of ACV on fibroblast cells.

Method: Apple vinegar with concentrations of 0.31%, 0.63%, 1.25%, 2.5%, and 5% was tested using the MTS assay method.

Result: The results showed that there was a cytotoxicity effect of apple vinegar solution as a root canal irrigation agent against fibroblasts cell. Apple cider vinegar with concentrations of 1.25%, 2.5%, and 5% are potentially toxic because the percentage of cell viability is less than 70%.

Conclusion: There is a cytotoxicity effect of apple cider vinegar solution as a root canal irrigant on fibroblast cells.

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INTRODUCTION

Root canal irrigation is one of the keys to successful endodontic treatment. Root canal irrigation agents are made from synthetic chemicals as well as natural ingredients.¹The most common irrigation solutions are NaOCl or sodium hypochlorite. NaOCl can clean debris and organic material in root canals, it can also dissolve necrotic tissue. Irrigation solutions may come into contact with the pulp and periapical tissues. Pulp is a tissue that fills the pulp chamber and root canal which consists of cell components, including fibroblast cells. The apical foramen connects the dental pulp and the periapical tissue consisting of cementum, alveolar bone, and the periodontal ligament.² Fibroblast cells are the main cells in the periodontal ligament, so the irrigation solution that comes out of the apical foramen will cause a cytotoxic effect on fibroblast cells and complications in the periapical tissue.^{3,4}

The cytotoxic effects of irrigants can cause periapical tissue degeneration and delayed wound healing due to irritation and tissue damage.³ Research conducted by Karkehabadi on the cytotoxicity test of NaOCl irrigating solution on human periodontal ligament fibroblast cells (HPdLFc) found that NaOCl was toxic at a concentration of 0.025%, while at a concentration of 0.4% it caused the death of all cells.⁵ The entry of NaOCl into the periapical tissue can occur because of the wide apical foramen, lack of apical constriction, and high pressure during root canal irrigation.⁶

NaOCl with a concentration of 5.25% can cause damage to the periapical tissue because it can cause pain, periapical bleeding, and swelling.⁷ Some natural ingredients are known to have antibacterial potential, so they can be used as irrigation agents with minimal cytotoxic effects, one

of which is apple cider vinegar solution. Apple cider vinegar has an antimicrobial role by eliminating cellular integrity, because it contains a number of biologically active components such as flavonoids, acetic acid, polyphenols, amino acids, vitamins, and minerals.⁸

In previous studies, it was found that apple cider vinegar has the potential to be used as an alternative irrigation solution because of its ability to inhibit *Enterococcus faecalis* which is the main bacterium that causes endodontic treatment failure.⁹ One of the requirements for an ideal irrigation solution is that it is not toxic to tissues in the oral cavity, so the researcher intends to conduct a cytotoxicity test of apple cider vinegar solution against fibroblast cells so that the data obtained from this study are expected to be used as a basis for using apple cider vinegar solution as an alternative material for root canal irrigation.¹⁰

This study aims to determine the cytotoxicity effect of apple cider vinegar solution on fibroblast cells in vitro.

METHOD

Type of this research is an experimental laboratory research with a post-test only control group design. The research was conducted at Aretha Medika Utama Biomolecular and Biomedical Research Center, Bandung. Apple cider vinegar solution using brand B (0301120-C054) and 3T3 BALB/C (ATCC® CCL-163) fibroblast cells were obtained from Aretha Medika Utama Biomolecular Laboratory and Biomedical Research Center.

Procedures Thawing Cells 3T3 BALB/C were as follows: Cells 3T3 BALB/C were taken from a liquid nitrogen tank (temperature -196°C), then thawed in an ultrasonic cleaner (Digital Pro, JP-020S) at 37°C for 2 minutes. 3T3 BALB/C cells were put into a 15ml centrifuge tube (Corning, 430791) containing 4 ml of culture medium Dulbecco's

modified Eagle's medium-High Glucose (Biowest, L0103-500). 3T3 BALB/C fibroblast cell culture medium was prepared by mixing 10% Fetal bovine serum Premium/FBS (Biowest, S181B-500), 1% Antibiotic-Antimycotic (ABAM) (Biowest, L0010-100), MEM Vitamins 100x (Biowest, X0556-100), 1% Amphotericin B (Biowest, L0009-050), 1% Nanomycopulitine (Biowest, LX16-100), and 0.01% Gentamicin (Gibco, 15750060) up to 100% of the total volume in a 50 mL tube (Corning, 430824). 3T3 BALB/C cells were centrifuged (MWP 260r) at 1600 rpm for 5 minutes.

The supernatant was discarded and the 3T3 BALB/C cell pellet was resuspended with 5 ml of culture medium. The 3T3 BALB/C cell suspension was put into flask T25 (Corning, 430168). The 3T3 BALB/C cells were incubated in the incubator at 37°C and 5% CO₂. Cells that have grown in a T25 flask (Corning, 430168) are observed under an inverted microscope (Olympus CKX41-F32FL) until they are about 70-80% confluent.

The subcultures of 3T3 BALB/C cells were as follows: 3T3 BALB/C cells that had grown in T25 flasks (Corning, 430168) were observed under an inverted microscope (Olympus CKX41-F32FL) to a confluent level of about 70-80%. The culture medium in the T25 flask (Corning, 430168) was discarded, and then rinsed with PBS (Biowest, X0515-500) 1 time as much as 2 ml to remove the remaining medium. Adhered cells were removed with the help of 2 ml Trypsin 0.25% - EDTA in HBSS (Biowest, L0931-500), and then incubated (Thermo IH3543) for 3 minutes at 37°C. Make sure the cells are completely detached from the bottom of the flask by checking using an inverted microscope. After all the cells have been removed, add 4 ml of growth medium to stop the trypsin action.

The cells are then put into a 15 ml centrifuge tube (Corning, 430791), and centrifuged (MWP

260r) at 1600 rpm, 5 min. Discard the supernatant and resuspend the pellet with 2 ml of culture medium. The cell suspension was divided into 2 T25 flasks (Corning, 430168) and then the cells were incubated (Thermo IH3543). Change the growth medium or add once every 2-3 days during the cell treatment procedure.

Sample preparation was carried out to obtain the final concentration of apple cider vinegar used in the cytotoxicity test. The ratio between the number of cell medium and the concentration of the sample used in the cytotoxicity test is 10:1, so apple cider vinegar solutions brand B (0301120-C054) with concentrations of 0.312%, 0.625%, 1.25%, 2.5%, and 5% are needed.

The sample preparation procedure was as follows: (1) Preparation of Stock Solution: 1 ml of apple cider vinegar solution was dissolved in 9 ml of 10% DMSO (Merck, 1029521000), resulting in 10 ml of stock solution of apple cider vinegar brand B (0301120-C054) with a concentration of 10%; (2) Preparation of Working Solution (WS) Series: CA 5% : 500 µl stock solution + 500 µl DMSO 10% (Solution A), CA 2.5% : 500 µl solution A + 500 µl DMSO 10% (Solution B), CA 1.25 % : 500 µl solution B + 500 µl DMSO 10% (Solution C), CA 0.625% : 500 µl solution C + 500 µl DMSO 10% (Solution D), and CA 0.313% : 500 µl solution D + 500 µl DMSO 10 % (Solution E); (3) Control (-) using cells and medium; (4) Control DMSO using cells, medium, and DMSO 10% (Merck, 1029521000); (5) Control (+) using NaOCl 0,25% (Proclin). (6) Sample filtration: The working solution was filtered using a 0.22 µm syringe filter tissue culture (Sartorius, 17845), so that a sterile sample was obtained.

Cytotoxicity test of Apple Cider Vinegar Solution was carried out as follows: Cells were harvested and counted using a hemocytometer (Neubauer). Cells were grown at a density of 5000

(5×10^3) cells/well in a 96-wellplate. Cells were incubated for 24 hours in a 5% CO₂ incubator at 37°C. After being incubated (Thermo IH3543) for 24 hours, the old medium was discarded and then replaced with a new culture medium with a different sample concentration in each well, then incubated in a 5% CO₂ incubator at 37°C. After 24 hours of treatment, 20 µl of MTS reagent (Promega, G5430) was added to each well, and then incubated for 20 minutes in an incubator at 37°C and 5% CO₂. The absorbance was measured using a spectrophotometer (Multiskan GO Thermo Scientific 51119300) with a wavelength of 490 nm.

How to calculate the percentage of viability and IC₅₀ is as follows:

$$\%Viability = \frac{Absorbance\ sample}{Absorbance\ control} \times 100\%$$

$$IC_{50} = \frac{Absorbance\ control - Absorbance\ sample}{Absorbance\ control} \times 100\%$$

The data analysis test was carried out using the Kolmogorov-Smirnov test to see normally

distributed data and homogeneity test with Levene Statistic, where previously the measurement results were tabulated according to each group, after that the Dunnet T3 Post Hoc test was carried out.

RESULTS

Cytotoxicity test of apple cider vinegar was carried out by MTS test on 3T3 BALB/C fibroblast cells. 3T3 BALB/C fibroblast cells were treated with five concentrations of apple cider vinegar, namely 5%, 2.5%, 1.25%, 0.63%, and 0.31%, then the absorbance was measured using spectrophotometry which was read at a wavelength 490nm. The results of the cytotoxicity test can be seen based on the number of living cells, the percentage of cell viability, and the percentage of inhibition. The results of the apple cider vinegar cytotoxicity test on 3T3 BALB/C fibroblast cells can be seen in table 1.

Table 1. Cytotoxicity Test Results of Apple Cider Vinegar Solution on 3T3 BALB/C cells

Sample	Number of Cells	Cell Viability (%)	Inhibition (%)
K DMSO	22169 ± 262 ^c	90.62 ± 1.07 ^c	9.38 ± 1.07 ^c
K-	24464 ± 1598 ^c	100.00 ± 6.53 ^c	0.00 ± 6.53 ^c
K+	1058 ± 143 ^a	4.33 ± 0.58 ^a	95.67 ± 0.58 ^a
CA 5%	8780 ± 757 ^b	35.89 ± 3.10 ^b	64.11 ± 3.10 ^b
CA 2,5%	10688 ± 387 ^b	43.69 ± 1.58 ^b	56.31 ± 1.58 ^b
CA 1,25%	11723 ± 464 ^b	47.92 ± 1.90 ^b	52.08 ± 1.90 ^b
CA 0,63%	18797 ± 763 ^c	76.83 ± 3.12 ^c	23.17 ± 3.12 ^c
CA 0,31%	22254 ± 224 ^c	90.96 ± 0.91 ^c	9.04 ± 0.91 ^c

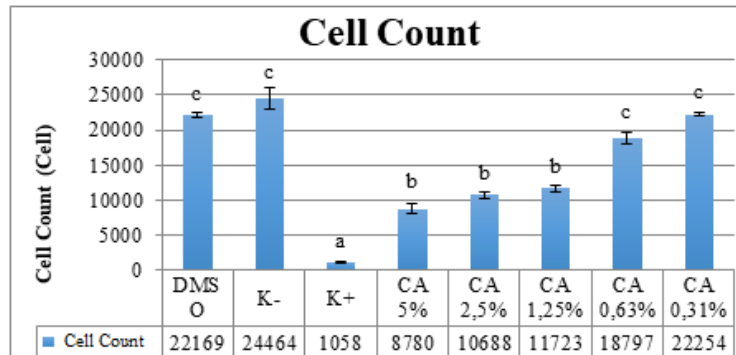
Note: Data is presented in mean ±SD. Different superscript marks (a,b,c) showed a significant difference ($p < 0.05$) based on Dunnet T3 post hoc test.

The results in table 1 show that the greater the number of cells that survive after being given treatment, the greater the percentage of cell viability and the lower the percentage of inhibition. The percentage of cell viability indicates the average number of cells that survive, while the percentage of cell inhibition is the inhibitory effect of apple cider vinegar on cell growth. The percentage of cell viability can be obtained from the result of reducing

the percentage of live cells before being treated with the percentage of cell inhibition.

The lower the percentage of cell viability, the higher the cytotoxicity of a material. Apple cider vinegar with a concentration of 5% had the lowest percentage of viability and apple cider vinegar with a concentration of 0.31% had the highest percentage of viability. This shows that 5% apple cider vinegar is more cytotoxic than apple cider

vinegar with other concentrations and the lowest cytotoxic apple cider vinegar is apple cider vinegar with a concentration of 0.31%.

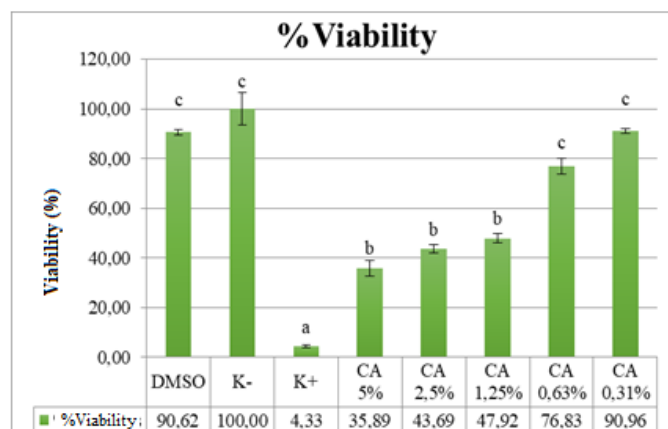


Graph 1. Number of Cells 3T3 BALB/C after being given Apple Cider Vinegar Solution at Various Concentrations

Graph 1 shows that the lower the concentration of apple cider vinegar, the more the number of cells that can survive. The highest number of living cells was found at a concentration of 0.31% apple cider vinegar. The number of surviving cells can be determined from absorbance measurements using spectrophotometry which is

read at a wavelength of 490 nm. The darker the visible color, the higher the absorbance value and the greater the number of live fibroblasts. The number of living cells can be calculated using the formula:

$$Living\ cell = 5000\ cell \times viability\ value(\%)$$



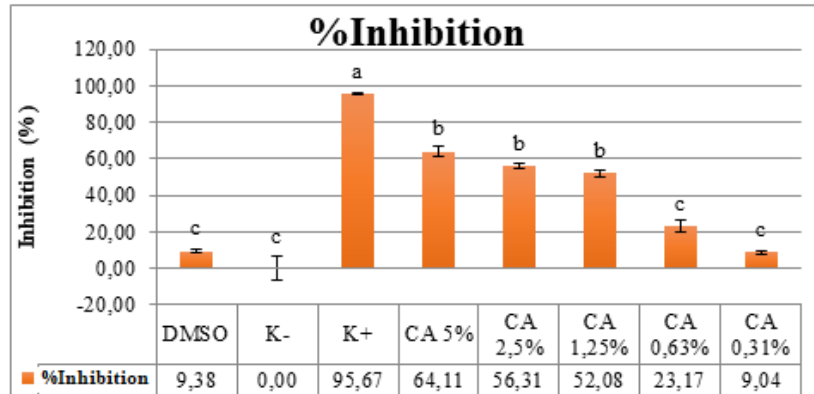
Graph 2. Viability of 3T3 BALB/C cells after being given apple cider vinegar solution at various concentrations.

Cell viability is the possibility of cells to survive after exposure to a material. Graph 2 shows the comparison of the percentage of viability of fibroblast cells after being treated with apple cider vinegar with different concentrations. Fibroblast

cells treated with 0.31% apple cider vinegar showed the highest cell viability, while the lowest was 5% apple cider vinegar. The percentage of cell viability increased with the lower concentration of apple cider vinegar.

According to ISO 10993-5: Biological Evaluation of Medical Devices on in vitro cytotoxicity tests, a compound is considered toxic if it has a cell viability of less than 70%. The lower the percentage of cell viability, the higher the potential for cytotoxicity of the compound. The results in

graph 2 show that apple cider vinegar with a concentration of 0.63% and 0.31% is not toxic to fibroblast cells. Apple cider vinegar with concentrations of 5%, 2.5%, and 1.25% was toxic to fibroblast cells because the percentage of cell viability at these concentrations was less than 70%.



Graph 3. Inhibition of 3T3 BALB/C cells after being given apple cider vinegar solution at various concentrations.

Graph 3 shows that the lower the concentration of apple cider vinegar, the lower the percentage of inhibition. The lower the percentage of cell inhibition, the more cells that survive and the higher the percentage of cell viability. In addition to the calculation of the number of living cells, the percentage of cell viability, and the percentage of cell inhibition, IC_{50} is calculated which is the half-maximum inhibition concentration.

IC_{50} (Half-Maximal Inhibitory Concentration) is defined as the concentration required to inhibit cell proliferation by 50%. IC_{50} shows the percentage inhibition value of 50%, so at that concentration, the percentage of cell viability is 50%. Based on the results of the probit regression analysis, the estimated IC_{50} value was 3.096%, so that at that concentration, there was an inhibition of cell proliferation or inhibition of 50% and the percentage value of cell viability was 50%.

The data obtained were then tabulated and tested for normality and homogeneity test. The normality test was conducted to determine whether the data obtained were normally distributed. Based on the Kolmogorov-Smirnov normality test, $p > 0.05$ was obtained, so the data were normally distributed. The results of the homogeneity test with the Levene statistic in Appendix 4 show a significance value of 0.003, so it can be stated that the variance between groups was not homogeneous because $p < 0.05$.

The research data were normally distributed, but not homogeneous, so the data analysis was carried out using the Post Hoc test using the Dunnett T3 method to determine the significant differences between the treatment groups. Data from the Post Hoc Dunnett T3 test. The difference in the mean values presented in the mean \pm SD can be seen in table 1. Different superscript signs (a,b,c) show significant differences ($p < 0.05$) based on the Post test. Hoc Dunnett T3.

DISCUSSION

The results of the cytotoxicity test of apple cider vinegar solution on fibroblast cells showed that apple cider vinegar with concentrations of 0.31%, 0.63%, 1.25%, 2.5%, and 5% had a cytotoxic effect on fibroblast cells. Apple cider vinegar with concentrations of 1.25%, 2.5%, and 5% is potentially toxic because the percentage of cell viability is less than 70%. The percentage of cell viability decreased with increasing concentration of apple cider vinegar. This is because the principle of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) test based on the formation of colored formazan due to the conversion of tetrazolium salt to the mitochondrial activity of living cells, so the number of living cells will affect the amount of formazan produced. The number of living cells will be directly proportional to the absorbance measured using spectrophotometry at a wavelength of 490nm.¹¹

A substance is classified as slightly toxic if its mean cell viability is between 60% and 90%.¹² Apple cider vinegar has a fairly high cytotoxicity effect. Research conducted by Gopal, et al. (2015) stated that apple cider vinegar has potential toxicity even at a concentration of 0.7%. Factors that play a role in the cytotoxicity of a material include the dose of a cytotoxic agent, duration of exposure to a compound, and the mechanism of compound cytotoxicity.¹³ Cell death can be caused by depletion of ATP levels and defects in cell membranes.¹⁴ Cell viability and cytotoxicity tests are based on various cell functions, such as enzyme activity, cell membrane permeability, ATP production, nucleotide uptake activity, and co-enzyme production.¹⁵

In this study, it was found that the addition of apple cider vinegar concentration was inversely proportional to the number of cells that could

survive, so the higher the concentration of apple cider vinegar, the more dead 3T3 BALB/C fibroblast cells cause cell damage and death. Damage occurs due to injury that causes damage to cell membranes, mitochondria, and disruption of cell endogenous substrates due to stimulation of toxic substances.¹⁶

Fibroblast cell death can be caused by the content of active substances in a material that has a toxic effect. The biologically active ingredients in apple cider vinegar that can increase the percentage of 3T3 BALB/C fibroblast cell death are organic acids and phenolic compounds and their derivatives. Research by Zubaidah (2015) states that apple cider vinegar contains 135.38mg/l of phenol.¹⁷ Phenol compounds and their derivatives can cause changes in cell permeability by denaturing proteins in cell membranes. Changes in cell permeability cause the components in the cell cannot be maintained, so that it can result in cell death.¹⁶

The largest group of derivatives of phenolic compounds in apple cider vinegar are flavonoids.⁹ Flavonoids are prooxidants at high concentrations because they can trigger the formation of ROS (Reactive Oxygen Species). ROS have an important role in the occurrence of cell damage and are one type of oxygen derived from free radicals. ROS are produced in normal mitochondrial respiration and will be degraded by the immune system.^{1,18}

Excessive free radicals known as oxidative stress are usually involved in cell damage. The presence of free radicals causes lipid peroxidation reactions in plasma membranes and organelles. The unstable bonds between fatty acids and free radicals can cause more severe membrane damage. Free radicals can also cause oxidation of amino acid chains, formation of protein covalent bonds, and protein oxidation. This will cause

damage to protein structure and increase proteasome protein degradation. In addition, free radicals can cause DNA damage and cross-linked DNA chains. This mechanism causes cell death that occurs due to the cytotoxicity of a material.^{1,19}

The results of research by Hung, et al (2015) stated that apple polyphenols contain procyanidins, flavonoids, epicatechins, and catechins which significantly suppress colon cancer cells.²⁰ Kao, et al (2015) in their research stated that the polyphenols present in apple cider vinegar have a cytotoxic effect on human bladder cancer cells (TSGH-8301), which are associated with apoptosis and oxidative stress.²¹ Another content of polyphenols in apple cider vinegar is tannins. This compound can cause genotoxicity at high concentrations. Initially, there will be disturbances in cell permeability which causes cells to become necrotic. This occurs due to the accumulation of compounds and the breakdown of fat due to the bond between cell lipoproteins and polar compounds in tannins.¹⁸

The normal functioning of enzymes and protein-based mechanisms in cells operate over a very narrow pH range of around 7.0.²² Apple cider vinegar solution has a fairly high acidity with a pH of around 2.0-3.0. This allows damage or cell death due to the organic acid components in apple cider vinegar. The most dominant organic acids in apple cider vinegar are acetic acid and lactic acid.²³ Cell damage due to organic acids can occur because the internal pH of the cell (usually around 7.6) is higher than the pH of organic acids outside the cell, so acetic acid will dissociate and acidify the cytoplasm, which can cause acid-induced protein disassembly, membrane, and DNA damage²⁴

Acetic acid can cause cell damage. In addition to its acidity, acetic acid can cause oxidative stress to cells. Acetic acid enters the cell via a membrane transporter, monocarboxylic

transporter (MCT), then becomes a substrate for acetyl-CoA, and is used in the tricarboxylic acid (TCA) cycle. Spesies oksigen reaktif (ROS) diproduksi melalui siklus TCA, seperti radikal superoksida, dan ROS akan menginduksi apoptosis dalam sel. ROS can cause adverse effects on cells, especially when intrinsic antioxidant defense mechanisms are reduced.^{25,26}

The results of this study showed that NaOCl as a positive control was more cytotoxic than apple cider vinegar. This is because NaOCl is a solution with a high pH which can disrupt the integrity of the cytoplasm. Sodium hypochlorite solution will release chlorine ions and hydroxyl ions when used as an irrigating solution. Therefore, there will be an increase in the formation of free radicals which will increase ROS (Reactive Oxygen Species) due to the release of chlorine ions. Furthermore, the release of hydroxyl ions can reduce ATP and increase ROS directly which will cause cell death.¹

Apple cider vinegar solution is known to have many benefits for the body. Apple cider vinegar solution also has the ability to inhibit the *Enterococcus faecalis* bacteria. However, the use of apple cider vinegar solution as an alternative to root canal irrigation should be considered and tested further because apple cider vinegar solution has a cytotoxic effect even at low concentrations.²⁷

Apple cider vinegar with concentrations of 5%, 2.5%, and 1.25% is called toxic because according to ISO 10993-5: Biological Evaluation of Medical Devices on cytotoxicity tests in vitro, a compound is considered toxic if it has a cell viability of less than 70%. The lower the concentration of apple cider vinegar, the lower the cytotoxicity. This is because the dilution process reduces the bioactive content in apple cider vinegar and reduces its acidity, thereby reducing the potential for cell damage. For further research, cytotoxicity test of apple cider vinegar solution can be carried out using

different methods, such as MTT assay and exclusion staining method with PrestoBlue. A cytotoxicity test of apple cider vinegar solution can also be performed on other cells in the oral cavity, such as human periodontal ligament cells (hPDL), human periodontal ligament fibroblast cells (HPDLFc), human gingival fibroblast cells (HGF), and other cells potentially exposed to the irrigation solution.^{28,29}

CONCLUSION

Based on the results of this study, it can be concluded that there is a cytotoxicity effect of apple cider vinegar solution as a root canal irrigant on fibroblast cells.

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