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yuanita Tamara <tamara-y@fkg.unair.ac.id>

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Dear **Prof. Dr. Tamara Yuanita,**

It's a great pleasure for me to inform you that your manuscript which tittled " **Cytotoxicity Test of Cacao Pod Extract (Theobroma Cacao. L)in Human Periodontal Ligament Fibroblast Cells (HPdLF) as Root Canal Irrigation Material** " has been accepted and will be finalized for **issue 2022; volume 15 number 4** which will be released either late Desember 2022 or early January 2023.

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Cytotoxicity Test of Cacao Pod Extract *(Theobroma Cacao. L)* in Human Periodontal Ligament Fibroblast Cells (HPdLF) as Root Canal Irrigation Material

Tamara Yuanita[1]*, Nawira[1], Amanda Diah Prameswari H[1], Ari Subiyanto[1], Sukaton[1], Anuj Bhardwaj[2,3]

1.Dept. of Endodontic and Conservative Dentistry; Faculty of Dentistry, Universitas Airlangga, Surabaya, Indonesia
2.Dept. of Conservative Dentistry and Endodontics, College of Dental Sciences, Rau, Indore, MP, India.
3.Dept. of Conservative Dentistry, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.

Abstract

One of the requirements for material as root canal irrigants is having low toxicity level since irrigation solution have the possibility of extruding into periapical tissue. Ligament periodontal cells are the major cells that play a role when reaction occur in periapical tissues due to endodontic materials. Fibroblast cells are also known as cells that first react when irrigation solution extruded to periradicular. Cacao pod peel extract, on the other hand, is known to have antioxidant, antibacterial, and anti-inflammatory characteristic so that it can be used as an alternative irrigation solution derived from natural ingredients. Nevertheless, every new material must go through biocompatibility test first before it is applied to humans and made into a patent product. This research aims to analyzed the toxicity of cacao pod peel extract (Theobroma Cacao L.) on human periodontal ligament fibroblast (HPdLF) cells.

Primary cell cultures of periodontal ligament fibroblast were prepared from extracted premolar teeth for orthodontic treatment reason. They were exposed to 10 groups of concentrations of cacao pod peel extract range from 3000 µg/ml to 5,8593 µg/ml and tested for toxicity using MTT Assay, followed by ELISA Reader. Cacao pod peel extract at ≥ 750 µg/ml causes the death of > 50% cells, so it cannot be used as a root canal irrigant solution because it is toxic

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Introduction

Endodontic is one of the branches of Dentistry which deals with diagnosis and treatment of the pulps and periapical diseases. Root canal treatment has principle of care known as Triad Endodontic. The principles are cleaning and shaping, sterilization, and obturation.[1] The stage of cleaning and shaping is a combination process of mechanical instrumentation and irrigation with antibacterial material, so it can be considered as using chemo mechanical preparation. The main objective of chemo mechanical preparation is to eliminate the microorganisms in the root canal, remove pulp tissues which can cause microbial growth, and prevent the debris being pushed into the apical foramen which can cause inflammation.[2]

The ideal requirements of root canal irrigation solution are low toxicity level, broad spectrum of antibacterial properties, dissolving the remaining of necrotic pulp tissue, preventing the formation of smear layer during root canal preparation or being able to dissolve it immediately after it is formed.[1] The main irrigation solutions commonly used are sodium hypochlorite, chlorhexidine and EDTA. In addition of the effectiveness of these irrigation solutions, the toxicity they have is quite high when extruding into periapical tissues. Extrusion of irrigation solutions can occur when there are over-instrumentation and the use of irrigation needles in the root canal with excessive pressure during root canal treatment.[3] The condition of teeth with apical openings, the presence of external resorption, and perforation of the cavity wall can also cause extrusion of irrigation solutions to periradicular tissues.[4]

*Corresponding author:
Tamara Yuanita,
Faculty of Dental Medicine, Universitas Airlangga
St. Mayjen. Prof. Dr. Moestopo No. 47, Surabaya, Indonesia.
E-mail: tamara-y@fkg.unair.ac.id

An *in vitro* study conducted by Mooduto et al proves that sodium hypochlorite at 0.25 µl/ml can kill 52,17% of human periodontal ligament fibroblast cells.[2] Similarly, research conducted by Yuanita et al in toxicity test of sodium hypochlorite (NaOCl) irrigation argues that the NaOCl irrigation solution at 0.254 µl/ml can kill 50% of fibroblast cells in human periodontal ligament.[5] Chlorhexidine irrigation solution, furthermore, is also known to carry a toxic effect on human periodontal ligament fibroblast cells due to its ability to inhibit protein synthesis.[6] Research on the cytotoxicity test of Qmix (a combination of 17% EDTA and 2% CHX) on human periodontal ligament fibroblast cells found that Qmix at a concentration of 0.363 µl / ml can kill 50% of those cells.[7] Due to the weakness of irrigation solution, such as the presence of toxic effects on healthy tissue, natural materials which have almost the same effectiveness, but low toxicity are now being developed.

Cacao pod peel has natural antibacterial properties, antibacterial, and anti-inflammation. Cacao pod is the biggest part of the fruit itself (75.52% of fresh cacao fruit). The production of cacao beans increases every year, therefore it increases the amount of discarded beans.[8] Cacao pod is potential as a natural antibacterial because it contains polyphenol compounds. According to research about the phytochemical screening in cacao pod peel, there are groups of alkaloids, flavonoid, tannin, polyphenol, saponin, quinone, and sesquiterpenoid compounds contained in it.[9]

Cacao pod peel extract is also known to inhibit the formation of E. Faecalis biofilm with a Minimum Biofilm Inhibitory Concentration (MBIC) value of 3.12%.[10] Also followed by research about the minimum inhibitory concentration of cacao pod husk extract is at concentration 6.25% that can effectively reduce the thickness of E. Faecalis extra polymeric substance biofilm.[11] Cacao pod peel extract also can effectively be used as irrigation solution to clean the root canal wall at concentration of 6.25%.[12] Hence, this research used cacao pod peel extract as a new alternative agent of irrigation solution for root canal treatment.

However, every new material derived from the test results that have been carried out *in vivo* on experimental animals as well as on bacterial culture media must go through biocompatibility tests before being applied to humans and made as a patent product. Biocompatibility is generally determined by certain tests using toxicological principles that provide information about the potential for material toxicity in clinical applications.[13] To investigate the biocompatibility of a material, a cytotoxicity test was conducted through *in vitro* testing using cell cultures. The indicator for determining toxicity to culture cells is the LC_{50} (Lethal Concentration). LC_{50} is a concentration of material that can kill 50% of the number of cell cultures.[14]

The main cells of periodontal ligament are fibroblast, they have the speciality to produce collagen at very high levels. At the time of its formation, the ligament space is occupied by loose connective tissue that connects the root to the supporting bone. Then, fibroblast produce collagen bonds that make strong tooth anchors. Ligament fibroblasts are unique cells, which are able to simultaneously synthesize and degrade collagen.[15] To obtain a representative research result on the condition of the human oral cavity, the fibroblasts used in this research were human primary fibroblast taken from periodontal ligament since they are believed to be the first cells to contact when the irrigation solutions is extruded into periradicular tissues.[6]

Materials and methods

Research Sampels

Before conducting the research, ethical approval has been obtained from the Research Ethics Committee of the Faculty of Dentistry Faculty, Universitas Airlangga with the certificate number 197 / HRECC. FODM / IX / 2019. This research sample used in this study was cocoa pod peel extract made at Faculty of Pharmacy in Widya Mandala University. First, fresh forastero cacao fruit was taken from plantations in Banyuwangi region, and then extracted by maceration method using 70% ethanol solvent until thick extracts was obtained. Second, the thick extracts were diluted with 5% FBS (Fetal Bovine Serum) solution until it had concentrations of 3000; 1500; 750; 375; 187,5; 93,75; 46,875; 23,4375; 11,7187; 5,8593 (µg/ml).

Research Methods

The making of Human Periodontal Ligament Fibroblast cells (HPdLF) was done by taking the periodontal ligament of 1/3 premolar root extracted from the orthodontic treatment. The making of cell culture then was performed at LPPT (*Laboratorium Penelitian dan Pengujian Terpadu*) at Gajah Mada University, Yogyakarta.

Subsequently, the extracted premolar teeth were inserted into a 10 ml tube DMEM (Dulbecco's Modified Eagle Medium) medium included with 100 µg/ml of fungizone. 100 µg/ml of penicillin, and 100 µg/ml of streptomycin. The periodontal ligament then was taken using a scalpel on apical area, then placed on small petri dish and covered by sterile plate. After that, it was added a complete medium 3 ml of DMEM 10% and then incubated at a 5% CO_2 incubator. Every 4 day the media were changed until the cells became 80% confluent. Then continued by making a secondary culture.

Cells that have become 80% confluent were measured and divided into 96 plates wells. Each well was filled with 100 µl of cell with a density of 2 x 10^4 / 20,000 cells / well, and leave for 1-2 hours. Three wells were not filled with any cells in order to be used as media control. After that, 100 µl of cacao pod peel extract with various concentrations was added and incubated in a CO_2 incubator for at least 24 hours (5% CO_2, 37^O temperature, 98% humidity) so that the cell would attach again after being harvested. After 24 hours, observation was carried out under a microscope and then they were photographed. Afterwards, 100 µl of PBS was added to the entire wells filled with the cells. Next, PBS (Phosphate Buffer Saline) was discarded by turning the plate over the tissue paper. Then 100 µl of MTT (5 mg of MTT, 1 ml of PBS, 9 ml of DMEM medium/ growing medium) then was added to each well and incubated until formazan was formed around 4 hours.

Afterwards, stopper solution of 100 µl SDS (Sodium Dodecyl Sulfate) 10% was added to 0.01 N HCl in each well, then incubated overnight. The cells in each well then were calculated by ELISA Reader (Bio-Rad® Model 680 XR Micro Plate Reader) at a wavelength of 550 nm. Fibroblast cells that are still alive will turn blue after formazan was given, because viable cells with active metabolism convert MTT into a purple-colored formazan product with an absorbance maximum near 570 nm. While the dead cells did not turn blue, as the dead cells lose the ability to reduce tetrazolium salts into colored formazan products. Thus, the intensity of the colored product is directly proportional to the number of viable cells present in the culture. The cell death was then counted using Meyer formula.

After the results of the study were obtained, the toxicity test results of the cacao pod peel extract (*Theobroma cacao. L*) on human periodontal ligament fibroblast (HPdLF) were analyzed. The first data analysis was aiming to look for data normality using Kolmogorov-Smirnov Test. Then the homogeneity test was conducted with Levene Test. Based on the results of the data analysis above, it is known that the data was normal and homogeneous, so the next statistical test used parametric tests. The parametric test used was One Way ANNOVA test and continued with the Tukey HSD test to determine the differences between the experimental groups.

Results

The test result on the eleven treatment groups were listed below on figure 1 and 2.

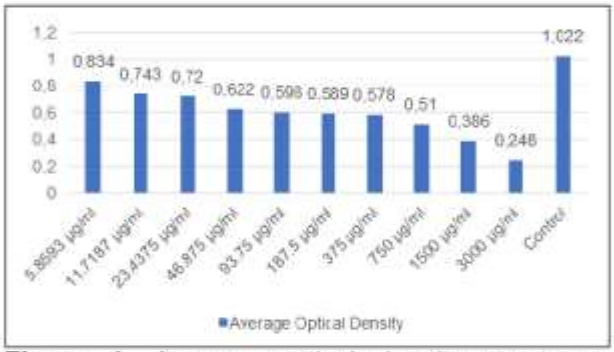

Figure 1. Average optical density on every concentration of cacao pod extract.

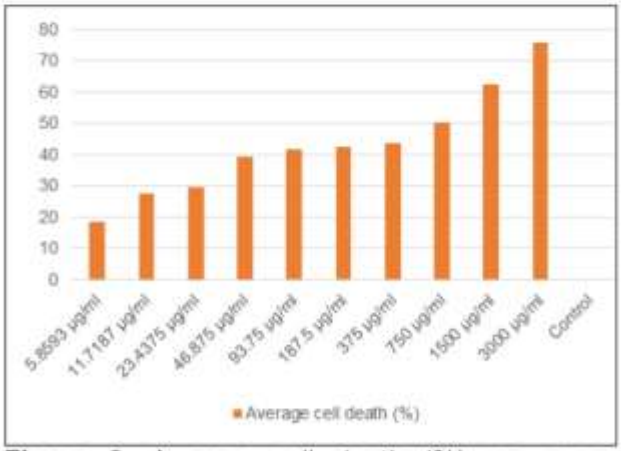

Figure 2. Average cell death (%) on every concentration of cacao pod extract.

Based on the figures above, the higher the concentration, the lower the optical density value

will be. It means that the lower optical density indicates higher cell death. This matter shows that the concentration directly proportional with the toxicity value. In other words, the concentration of cacao pod peel extract causing the death of human periodontal ligament fibroblast cells as much as > 50% (LC_{50}), which is 750 µg / ml. It indicates that the concentration of ≥ 750 µg / ml is a toxic concentration.

Group (µg/ml)	N	Subset for alpha = .05					
	1	2	3	4	5	6	1
3000	8	.24850					
1500	8		.38600				
750	8			.51000			
375	8				.57850		
187.5	8				.59850		
93.75	8				.58925		
46.875	8				.62250		
23.4375	8					.72050	
11.7187	8					.74300	
5.8593	8						.83425
Sig.		1.000	1.000	1.000	.142	.883	1.000
Means for groups in homogenous subsets are displayed.							
a Uses Harmonic Mean Sample Size = 8.000.							

Table 1. Tukey HSD Significance Test.

The result of the Tukey HSD test showed whether there was a significant difference between the experimental groups or not. A significant difference can be indicated if each average value of each concentration is in a different subset column.

Moreover, the results in table 1 above show that there was no significant difference between the concentrations of 375 µg / ml; 93.75 µg / ml; 187.5 µg / ml; 46.875 µg / ml as well as between concentrations of 23.4375 µg / ml; 11.7187 µg / ml (the average values were in the same subset column). However, there were significant differences between the concentration of 750 µg / ml and the concentration of 3000 µg / ml; 1500 µg / ml; and other concentrations below it (the average values were in different subset columns). In other words, there was a significant difference in the concentration of 750 µg / ml. Consequently, it can be concluded that the LC_{50} value of cocoa pod peel extract can be obtained at that concentration.

Discussion

Every interaction of a material with biological cells will cause an effect. One of the objectives of the toxicity test is to determine or to detect when the toxic effect appears depending on several factors, such as the dose of material, the intrinsic potential of toxicity, and also by the contact period of xenobiotics with the biological system organisms.[16] In this research, the cytotoxicity tests of cocoa pod peel extract (*Theobroma Cacao. L*) was carried out on Human Periodontal Ligament Fibroblast (HPdLF) cells. This research aims to determine the toxicity level of materials in culture cells. The cytotoxicity tests, thus, was conducted to determine the value of the LC_{50} (Lethal Concentration). LC_{50} is a concentration of the materials which can kill 50% of the number of cell cultures so that it can be understood that the minimum concentration of cacao pod peel extract may safely use as a material for root canal irrigation solution.

Fibroblast cells are known as the dominant cells found in connective tissue and they function to secrete collagen and extracellular substances.[17] Cells that often used in *in vitro* cytotoxic tests are fibroblast cells taken from gingiva and human periodontal ligaments.[18] In addition, the cells in the periodontal ligament are the main cells that play an important role when a reaction occurs in periapical tissues due to endodontic materials and fibroblast cells themselves which becoming the first cells to react when extrusion of irrigation solutions into periradicular tissue.[6] To obtain the result conditions of research representative of the human oral cavity (*in vivo*), the fibroblasts used in this research were human primary fibroblasts derived from the periodontal ligament. Therefore, in this research the toxicity test of fibroblast cells in human periodontal ligaments was carried out.

The toxicity test method used was the MTT Assay method. This method is commonly used to determine the number of cultures because it has a simple, fast, sensitive, accurate, and used method of testing from plant extracts.[19] The principle of the MTT method is the reduction of MTT tetrazolium yellow salt by the system reductase. Tetrazolium succinate which is included in the respiratory chain in the mitochondria of living cells forms purple formazan crystals and is not water soluble. Addition of stopper reagent (detergent) will dissolve this colored crystal which is then measured by its absorbance (Optical Density) using Microplate Reader. The purple intensity that is formed is proportional to the number of living cells. So if the intensity of the purple color gets bigger, then it

means that the number of living cells will increase.[20]

In this research, the forastero-type of cocoa pod peel extract (*Theobroma Cacao. L*) from Banyuwangi was made at the Pharmacy Laboratory, Faculty of Pharmacy, Widya Mandala University, Surabaya. The extraction method used was maceration method with 70% ethanol. The maceration method was chosen because *Theobroma Cacao L.* is known to have a fairly high phenolic content that has anti-oxidants, therefore this method is considered the best one for extracting polyphenol content, while the ethanol solvent was chosen because the solvent has a hydroxyl group which can bind polar compounds such as flavonoids and alkaloids.[19]

The results of the cytotoxicity test of cocoa pod peel extract on human periodontal ligament cells in the concentrations of 3000 µg / ml; 1500 µg / ml; 750 µg / ml; 375 µg / ml; 187.5 µg / ml; 93.75 µg / ml; 46.875 µg / ml; 23.4375 µg / ml; 11.7187 µg / ml; 5.8593 µg / ml show cell death percentages of 75.684%; 62.255%; 50.130%; 43.415%; 42.372%; 41.460%; 39.113%; 29.530%; 27.346%; 18.415%. It can be concluded that the higher the concentration used, the higher the percent of human periodontal ligament cell fibroblast death rate will be.

This condition is known caused by the toxicity of a material that is influenced by the content of active substances in the material used. Not all concentrations can cause toxicity to periodontal ligament fibroblast cells. An injury to cells is influenced by the severity of the stimulus that affects the cell.[21] The concentration of a material can affects the weight and lightness of the stimulus. At concentrations of 750, 1500, 3000 µg / ml, cell death occurs more than 50% since the compounds contained in these concentrations have more toxic activity compared to concentrations below 375 µg / ml. At low concentrations, the active compounds in cocoa pod peel extract are non-toxic to cells so that cell death occurs in a lower number.

Taxonomy identification of the plant and the analysis of the phytochemical substances were done at Industrial Research and Consultation Hall of Surabaya. The results of phytochemical analysis obtained are shown in the following results: Tannin (2.11%), Alkaloids (2.89%), Terpenoids (0.98%), Flavonoids (1.08%), Saponins (3.01%), and Theobromine (2.82%). The highest content of cacao pod extract in this study was saponins, followed by alkaloids, theobromine, tannins, flavonoids, and terpenoids.

Saponin is the highest content of compounds found in cacao pod peel extract, which plays a major role in the cause of cell death due to its toxicity. Saponins are triterpenoid and sterol glycosides. Saponins are derived from the Latin "sapo" which means soap, and were given such names because they resemble soap. At high concentrations, saponins along with flavonoids will have toxic effects on cells. Both of these compounds can inhibit cell proliferation and cause mitochondrial damage due to an increase in Ca^{2+} in the mitochondria resulting in mitochondrial permeability transition pore.[22] Increased Ca^{2+} in mitochondria occurs due to inhibition of Ca^{2+} ATPase. The formation of mitochondrial permeability transition pore, will result in oxidative phosphorylation and inhibited ATP formation. The inhibition of oxidative phosphorylation can cause cell death, while reduced ATP production happens through several processes before cell death occurs. Reduced production of ATP will result in active transport of Na^+, K^+ is disturbed so that influx of Ca^{2+}, H_2O, Na^+ and Eflux of K^+ occur, then cell swelling and endoplasmic reticulum may occur, and microvilli blebs disappear. This results in the breakdown of plasma membranes, organelles, and nuclei, and the release of compositions inside the cell.[23]

Saponins in improper concentrations can also result in excess free radicals called oxidative stress causing cell death. The high free radicals or Reactive Oxygen Species (ROS) may cause mitochondrial damage which triggers failure of oxidative phosphorylation and reduced ATP needed in the process of cell synthesis, resulting in cell death.[24] The free radicals themselves also result in lipid peroxidase resulting in cell membrane damage resulting on changes in osmotic pressure in the cell. These changes trigger cells swelling and cell death.[25]

In addition, alkaloids contained in the cacao pod peel also have a role to induce cell death. Alkaloids are the most secondary metabolic substances which have nitrogen atom and are mostly coming from plants, especially angiosperms.[26] Alkaloids have negative effects against cells because of the lipid membrane changes which are known to help the entrance of calcium ion inside the cell, increase the calcium ion which pass through the membrane causing the stimulation of membrane bond in intercellular

cAMP membrane bonds. This condition is caused by an increase in Ca^{2+} in the mitochondria resulting in an increase in phospholipid degradation which can cause lipid breakdown products. Because of the damage to the lipid layer in the plasma membrane, the plasma membrane is damaged so that the cell loses osmotic balance, extracellular and ionic influx may occur, and cellular component loss occur that may result in the cell to die or necrosis.[23]

Tannins, which are contained in a big amount inside of cocoa pod, are one of the chemical substances derived from polyphenols. In a high concentration, tannins have a high affinity level against the protein; thus, tannins may destroy the structure and the permeability of the cell membrane due to the formation of complex substances with protein through hydrogen bond. These cause a disruption to the protein structure and it may not function anymore and then cause protein denaturation. Denaturation disturbs the metabolism and physiological functions of cells, resulting in changes in permeability in the cell wall which results in cells undergoing lysis and eventually cell death.[26] Active tannin compounds and flavonoid groups are known to have antioxidant properties that can reduce the oxidative effects of ROS on cells, so that both of these compounds can increase cell viability. While tannins which are included in the polyphenol group, can reduce free radical oxidation with the ability to bind metal ions and free radicals that can trigger the emergence of ROS.[27] Flavonoids have the ability to stabilize ROS by reacting to reactive components of free radical substances. The hydroxyl groups contained in flavonoids make free radicals inactive due to reactions that bind to superoxide and peroxynitrite.[25] Moreover, that substance may inhibit the Xanthine Oxidase (XO), an enzyme which can trigger the escape of reactive oxygen such as superoxide radical and hydrogen peroxide.[28] Flavonoids which interact with hydrophobic layers can prevent the entrance of oxidant and protect their own structure and functions.

Conclusions

According to the result of this research, the value obtain for LC_{50} (Lethal Concentration) was at a concentration of 750 µg/ml. This indicates that the concentration can caused the death of fibroblast cells as much as 50.13% (more than 50%). Hence, cacao pod peel extract at concentration higher than or equal to 750 µg/ml cannot be used as irrigation solution because it is toxic. Meanwhile, cacao pod peel extract at concentration below 750 µg/ml is considered as safe to be used as root canal irrigation solution.

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Declaration of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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