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Research Report

Cytoxicity test of NaOCl and Mangosteen *(Garcinia Mangostin L.)* peel extract used as an irrigation solution in human periodontal ligament fibroblast cells (HPdLFc)

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ABSTRACT

Background: *Root canal irrigation is an important stage in root canal treatment as it is requires to eliminate necrotic and debris tissue as well as root canal wetting. Unfortunately, root canal irrigation can cause the material utilised to pass into the apical foramen leading to periapical complications. Consequently, the irrigation solution should have low toxicity. Sodium hypochlorite (NaOCl) is a commonly used irrigation solution since it has antibacterial properties. Moreover, NaOCl is also known to have the ability to dissolve necrotic tissue, vital pulp tissue and organic components of dentin and biofilms. Nevertheless, it can still cause damage when coming into contact with periapical tissues. On the other hand, Mangosteen peel extract (Garcinia mangostana L.), also has antibacterial activities. Hence, Mangosteen peel extract is assumed to be employable as an alternative irrigation solution.* ***Purpose:*** *This research aimed to reveal the toxicity levels of NaOCl and Mangosteen peel extract (Garcinia mangostin L.) used as irrigation solution in human periodontal ligament fibroblast cells (HPdLFc).* ***Methods:*** *HPdLFc were obtained from periapical tissues taken from one third of the first premolar teeth cultured. These cells were subsequently divided into several groups exposed to NaOCl and Mangosteen peel extract at certain concentrations. A toxicity test was then conducted using MTT assay. The results were analyzed with an Elisa reader. Cell deaths and LC_{50} were then calculated.* ***Results:*** *NaOCl became toxic at a concentration of 0.254 µl/ml or 0.025%, while Mangosteen peel extract became so at one of 2.099 ug/ml or 0.209%.* ***Conclusion:*** *NaOCl can be toxic at a concentration of 0.254 µl/ml or 0.025% and Mangosteen peel extract at one of 2.099 µg/ml or 0.209%.*

Keywords: *Cytotoxicity; NaOCl; Mangosteen peel extract; HPDLFc*

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INTRODUCTION

Root canal treatment is the most common form applied in the field of endodontics. Its main goal within endodontic therapy is to improve and regenerate periapical tissue. In achieving this, root canals must be biomechanically cleaned of bacteria and debris without causing irreversible damage to the surrounding periapical tissues.[1]

Root canal treatment can be divided into three stages: biomechanical preparation (cleaning and shaping), dressing and obturation. Biomechanical preparation is a process intended to remove not only all infected pulp tissues, both in vital and non-vital conditions, but also microorganisms from inside the root canal. However, root canal irrigation is still required to eliminate necrotic and debris tissue, as well as root canal wetting. The number of microorganisms in the root canals can, as a result, be reduced since unclean root canal walls can become a breeding ground for bacteria, reducematerial attachment, increase apical cleft and cause root canal blockage.[2]

The most commonly used irrigation solution is sodium hypochlorite (NaOCl) which has antibacterial abilities. Moreover, NaOCl also has the ability to dissolve necrotic tissue, vital pulp tissue and organic components of dentin and biofilms and is commonly used as an irrigation solution at concentrations of 0.5-5.25%.[3] Unfortunately, NaOCl has

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several disadvantages, such as increasing toxicity as its concentration increases, producing unpleasant odors and tastes and causing damage when coming into contact with periradicular tissue.[4] In addition, when NaOCl passes from the root canal into the periapical tissues it can also cause complications, one of which is chemical burns which can ultimately result in tissue necrosis. Inflammatory reactions then will occur with the potential to cause both swelling and pain in the surrounding mucosa.[5]

In recent years, there have been a number of reports on the activities and possible applications of natural ingredients as a form of root canal disinfection. Natural irrigation can be used as an alternative in order to avoid the toxic effects of chemical irrigation materials. For instance, Mangosteen (*Garcinia mangostana L.*) is a plant indigenous to Indonesia and several parts of Southeast Asia. Mangosteen peel contains active ingredients, including: xanthones, flavonoids, saponins, tannins, garcinon, gartanin, vitamins B1, B2, terpenes, anthocyanins, phenol and other bioactive substances.[6]

Mangosteen (*Garcinia mangostana L.*) peel extract also performs antioxidant, anti-inflammatory and antibacterial biological functions. Xanthone, an active ingredient of α-mangostain derivates, even has the ability to protect against increased oxidative stress and antioxidant deficiency. Meanwhile, γ-mangosten inhibits COX-1 and COX-2 activities at concentrations of 0.8 and 2 μM.[7] Matsuo[8] argues that flavonoids can act as antioxidants, protect against oxidative stress, while also inhibiting endothelial human umbilical vein cells (HUVE) and PC12 cells from lipid peroxidase. Nevertheless, at high concentrations, Mangosteen peel extract can also cause mitochondrial cell disorders, an increase in reactive oxidation stress (ROS) and loss of potential mitochondrial membranes, leading to cell death.[9]

Irrigation solutions can come into contact with pulp and periapical tissues. Blood and irrigation solutions that are expelled from the apical foramen can then lead to periapical complications. Cytotoxic effects of materials used in endodontic treatment are, consequently, considered to be of particular concern since they can cause damage and irritation leading to periapical tissue degeneration and wound healing delay.[1] Since irrigation solutions must also be biocompatible, cytoxicity tests can be used as an initial biocompatibility assessment of the irrigation solutions used against certain organisms. One cytoxicity test method is the enzymatic test using (3- (4.5-dimethylthiaziol-2yl) -2.5-diphenyl-tetrazolium bromide (MTT) assay. The basis of the MTT test is to measure the ability of living cells based on the mitochondrial activities of cell cultures. By using the cytoxicity test, an irrigation solution can be confirmed as toxic-based according to the toxicity parameters of median lethal concentration (LC50), indicating the ability of the material to cause 50% death of cell cultures.[10] In other words, an irrigation solution is considered to be toxic if the post-exposure percentage of living cells is below 50%.[11]

Other previous research conducted by Karkehabadi[1] on the cytoxicity test of NaOCl irrigation solution in human periodontal ligament fibroblast cells (HPdLFc) found that NaOCl became toxic at a concentration of 0.025%, while at one of 0.4%, it caused the death of all cells. Unfortunately, the cytoxicity test of Mangosteen peel extract in human periodontal ligament fibroblast cells (HPdLFc) has yet to be conducted. According to Freshney,[12] the initial assessment of biocompatibility and toxic effects of a material are supposed to be directly performed on cell culture.

The cytotoxicity of NaOCl and Mangosteen peel extract (*Garcinia mangostana Linn.*) used as irrigation solutions for human periodontal ligament fibroblast cells (HPdLFc) needs to be established since they have an important role in the development, function, and regeneration of periodontal tissues. Furthermore, HPdLFc are considered the first cells to come into contact with the irrigation solutions emerging from the root canal. Consequently, these cells, commonly studied for periodontal regeneration, are also recognised as the main cells reacting to endodontic material in the periapic tissues.[13,14]

MATERIALS AND METHODS

This research was a laboratory experimental study with post-test only control group design. The materials used in this research were sodium hypochlorite (NaOCl), mangosteen peel extract, the first premolar teeth, Dulbecco's Modified Eagle Medium culture media (DMEM), 10% Fetal Bovine Serum (FBS), MTT reagent [2- (4,5-Dimethylthiazol- 2-yl) -2, 5-diphenyl tetrazolium bromide], Phosphate Buffer Saline (PBS), 70% ethanol and sterile distilled water. The research was conducted at the Integrated Research and Testing Laboratory, Universitas Gajah Mada, Yogyakarta.

Mangosteen peel extract was produced and identified by Materia Medika, Batu, Malang. Mangosteen peels were extracted and dried by maceration method using 70% ethanol solvent. The internal and external surfaces of the mangosteen peels were washed with running water to remove any dirt, before being drained and dried in an oven at 50° for 24 hours. The peels were milled, sieved, weighed and divided into 200gm batches and then immersed in 70% ethanol solvent for 24 hours, agitated in a digital shaker at a speed of 50 rpm, filtered and placed in a rotary evaporator for three hours. The extract was dissolved in DMEM medium in a series of doses at concentrations of 4μg/ml, 2μg/ml, 1μg/ml, 0.5μg/ml, 0.25μg/ml and 0.125μg/ml.

Human periodontal ligament fibroblasts cells (HPdLFc) were obtained by culturing the first premolar teeth extracted during orthodontic treatment. Immediately after extraction, each tooth was placed in a 10ml tube containing DMEM medium to which fungizone and penstrep had been added. The periodontal ligament of each tooth was then carefully removed from one third of the periapical with a scalpel. The resulting fragment was placed in DMEM with a combination of 10% serum fetal bovine serum (FBS) and antibiotics before being cultured at 37°C with an

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atmospheric humidity of 5% CO2 and 95% water. Once every four days, the medium was discarded and replaced. The cells were observed until the level of confluence reached 90% .

A cytotoxicity test was subsequently carried out using MTT Assay. There were 12 groups, namely: a 1µl/ml NaOCl treatment group, a 0.5µl/ml NaOCl treatment group, a 0.25µl/ml NaOCl treatment group, a 0.125µl/ml NaOCl treatment group, a 0.0625µl/ml NaOCl treatment group, a 0.03125µl/ml NaOCl treatment group, a 4µg/ml Mangosteen peel extract treatment group, a 2µg/ml Mangosteen peel extract treatment group, a 1µg/ml Mangosteen peel extract treatment group, a 0.5µg/ml Mangosteen peel extract treatment group, a 0.25µg/ml Mangosteen peel extract treatment group and a 0.125µg/ml Mangosteen peel extract treatment group. Each group consisted of three samples. The fibroblast cells were then divided according to the criterion of a cell density of 2 x 10^4/ 20,000 cells/ well in 96 well plates. Thereafter, 100ul NaOCl and 100ul Mangosteen peel extract were added at various concentrations, with the plates being incubated in 5% CO2 at 37° C for 24 hours. Following the incubation period, the cell medium was discarded and a maximum of 10µl. 100ul MTT was added to each well. Stop Solution was also introduced into each of the wells and incubated overnight. The Optical Density values of the Formazan crystalline formed were read by spectrophotometry using an ELISA reader at a wavelength of 550 nm. The percentage of cell deaths was then calculated using the following formula:

$$\% \text{ Cell Death} = \frac{\text{OD Control} - \text{OD Sample}}{\text{OD Control}}$$

RESULTS

The research findings were presented in the form of reading results produced by an ELISA Reader and revealed the absorbance levels or Optical Density values quantifying both the living cells and cell death percentages. At this point in the procedure, the observation and reading results of the absorbance values of the toxicity tests on the NaOCl and Mangosteen peel extract in human periodontal ligament fibroblasts cells (HPdLFc) were divided into 12 groups (each undergoing the treatment on three occasions) accompanied by the controls depicted in Figures 1 and 2.

According to the content of Figure 1, the higher the concentration, the higher the cell death rate. The highest percentage recorded, namely 87.195%, was that at a concentration of 1µl/ml, while the lowest occurred at one of 0.0625µl/ml. Based on the statistics in Figure 2, the higher the concentration of mangosteen peel extract, the greater the number of cells which died. The highest percentage of cell death, in this case 57.17%, was at a concentration of 4mg/ml, while the lowest was recorded at one of 0.125µg/ml.

The results of a Shapiro-Wilk test, conducted to analyze the normality of the data distrbution, indicated that it was normal. Moreover, those of a Levene test confirmed that the data was homogeneous. A one-way Anova test was then carried out to identify any differences between the groups (Table 1).

Probit analysis was subsequently performed to determine the LC_{50} value of each irrigation solution based on its concentration and cell death percentage. The LC_{50} value of NaOCl, based on the probit analysis, was obtained at a concentration of 0.25µl/ml (as illustrated in Figure 3). On the other hand, the LC_{50} value of Mangosteen peel extract, was obtained at one of 2.09µg/ml (as demonstrated in Figure 4).

Table 1. The one-way Anova test

	Sum of squares	df	Mean square	F	Sig.
Between groups	1729.925	6	288.321	22.149	0.000
Within groups	182.244	14	13.017		
Total	1912.169	20			

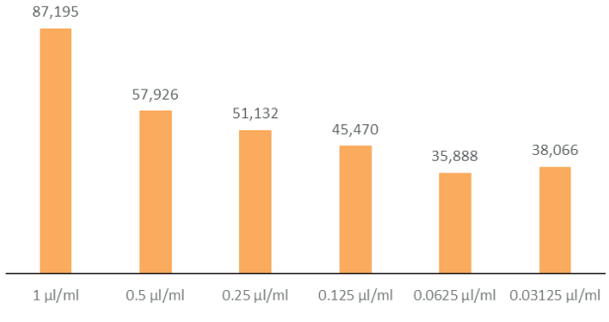

Figure 1. The mean percentage of the cell death of human periodontal ligament fibroblasts cells (HPdLFc) exposed to NaOCl.

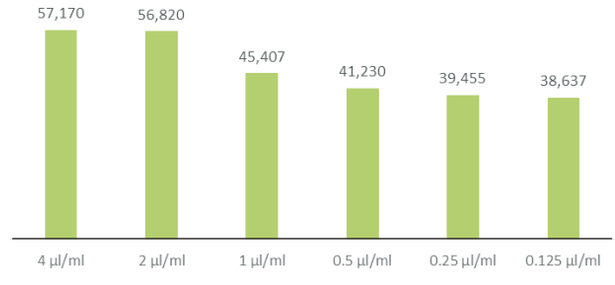

Figure 2. The mean percentage of the cell death of human periodontal ligament fibroblasts cells (HPdLFc) exposed to mangosteen peel extract.

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DISCUSSION

In this research, the cytoxicity tests of the NaOCl and mangosteen peel extract (*Garcinia Mangostin L.*) used as irrigation solutions were carried out by MTT Assay method to measure the biocompatibility of these solutions before they could be applied clinically. The optical density value results were then read by a spectrophotometer with the data being calculated to reveal the percentage of cell death. Probit analysis was then performed to determine the values of a lethal concentration of 50% (LC_{50}).

Sodium hypochlorite (NaOCl) is one of the most widely known and used endodontic irrigation solutions due to its antimicrobial activity and ability to dissolve organic and necrotic tissue remnants. Unfortunately, NaOCl can become toxic when exposed to cells and tissues around the root canal.

The death of periodontal ligament fibroblast cells is likely to be blocked by the presence of ROS in oxidative stress conditions. Reactive oxygen compounds, including hydroxyl groups, are strong oxidants. Negative effects on cells can arise due to reactivation of these compounds which can also damage cell components important to maintaining the integrity of cell life. Reactive oxygen species (ROS) are reactive oxygen compounds, well-known as free radicals which can be derived from the mitochondrial respiration chain and reactive chemical auto-oxidation.[15]

NaOCl, when in contact with the tissue, will produce hydroxyl ions and hypochlorous acid ($HOCl^-$). The NaOCl-produced hydroxyl ions will then react with oxygen produced by mitochondria in human periodontal ligament fibrous cells to form hydroxyl radicals through a process of autoxidation. If ROS production exceeds the capture capacity of antioxidants, it will lead to a condition called oxidative stress.[14]

The presence of oxidative stress will cause lipid peroxidation reactions in the plasma membrane and organelles. Thereafter, the unstable bond of fatty acids with free radicals will form lipid radicals that react with oxygen to form peroxyl radical lipids. These then react with other lipid radicals to become lipid peroxide, resulting in more severe membrane damage. ROS can also cause oxidation of amino acid chains, formation of covalent protein bonds and oxidation of proteins, leading to damage to the protein structure and an increase in proteasomal protein degradation. In addition, ROS can cause DNA damage and cross-linking DNA chains. This mechanism is what causes cell death indicated by high levels of NaOCl cytotoxicity.[14]

In this research, a cytoxicity test was also conducted on Mangosteen peel extract obtained by means of a maceration method at UPT Materia Medica, Batu, Malang. Based on probit analysis, the LC_{50} value of Mangosteen peel extract was found at a concentration of 2.099µg/ml. In other words, the concentration of Mangosteen peel extract killing 50% of periodontal ligament fibroblasts cells was 2.099µg/ml or 0.209%. This means that mangosteen peel extract at this concentration can become toxic.

The results showed that mangosteen peel extract must be more concentrated to cause 50% of cell death. Several active compounds contained in Mangosteen peel were reported to be responsible for several pharmacological activities. The active compounds identified at the Industrial Research and Consultation Center in Surabaya included: xanthones (4.01%), flavonoids (2.38%), tannin (12.05%) and saponin (4.38%).

The death of human periodontal ligament firoblast cells due to the application of mangosteen peel extract in this research may also have been caused by an increase in ROS which causes an imbalance in oxidant and antioxidant status as well as a proloferation of pro-oxidants, referred to as oxidative stress.[13]

Xanthones are categorized into a polyphenolic class. The most important xanthone derivative in mangosteen peel extract is α-mangostin. When reacting with oxygen produced by mitochondria in human periodontal ligament fibroblast cells through an auto-oxidation process, the hydroxyl group of α-mangostin will result in the formation of free radicals/ ROS/semiquinone radicals. As a result, oxidative stress can compromise mitochondrial integrity. The active ingredient of flavonoids which plays a role is Quercetin. The hydroxyl groups of Quercethane then interact with oxygen through the autoxidation process also producing ROS/free radicals,

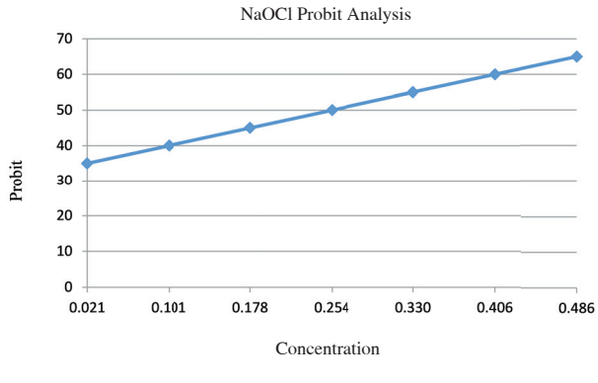

Figure 3. Determination of LC_{50} value of NaOCl.

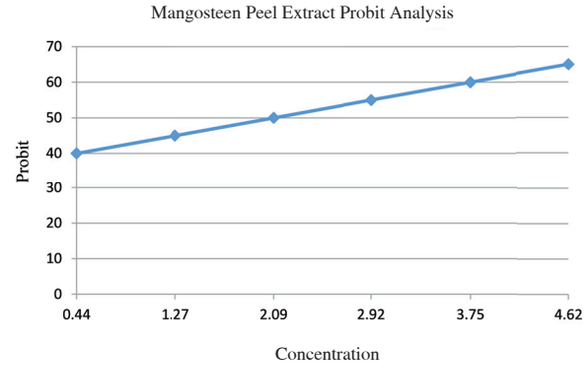

Figure 4. Determination of LC_{50} value of mangosteen skin extract.

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namely o-quiones. The overload of ROS will cause the release of Ca^{2+} due to the opening of mitochondrial pores (mPTP).[9] Excessive Ca^{2+} will, in turn, lead to distubances in membrane permeability as well as excessive reaction to the synthesis of the tricarboxylic acid chain. It can increase electron flow, resulting in oxidative phosphorillation failure and ATP depletion, while stimulating a reaction to lipid peroxidation in plasma membranes and organelles. Bonding fatty acids with unstable free radicals can then cause more damage to the membrane.[16]

Like xanthones, tannins and saponins at inappropriate concentrations can also interfere with the permeability of fibroblast cell membranes. Tannins have a high affinity for proteins that can form complex bonds with them, causing cytoplasmic disturbances (Moosophin et al., 2010). Meanwhile, saponins can break down the lipids in cell membranes, leading to disruptions to membrane permeability resulting in an imbalance of influx and exflux of ions (Ca^{2+}, Na^{2+}, K^{+}). Those reactions then cause cell death.[16]

Mangosteen peel extract at high concentrations has antioxidant ability[9] due to the xanthone, flavonoids and tannin it contains. Its hydrogen chain can release and combine with $O_2\bullet^-$, leading to the stable formation of phenoxil radicals. Besides inhibiting the occurrence of lipid peroxidation, it can prevent changes in the Globally Harmonized System (GHS) and Superoxide dismutase (SOD) and also return to near normal levels. Hence, mangosteen peel extract can prevent SOD depletion, in this case, glutathione (GSH), the best antioxidants playing an important role as antioxidant enzymes.[9] In other words, mangosteen peel extract is very effective at reducing the formation of reactive oxygen species (ROS), resulting in reduced cell death. Its antioxidant ability causes mangosteen peel extract to demonstrate lower cytotoxicity.

Previous research conducted by Kumar[17] also found that the anti-inflammatory effect of Garcinia mangostana L extract occurs through the down regulation of nitric oxide (NO) production. At concentrations of 0.976µg/ml to 15.625µg/ml, it can cause a down regulation in NO production. Its cytotoxic potency measured during this research using MTT assay was at the same concentration, i.e. at concentrations of 0.906µg/ml to 15.625µg/ml.

In conclusion, NaOCl irrigation solution can become toxic at a concentration of 0.254µl/ml or 0.025%, while mangosteen peel extract becomes toxic at one of 2.099µg/ml or 0.209%.

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