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***RESEARCH ARTICLE***

# Chlorogenic Acid Fractionation in Robusta Green Bean Extract as a Combination Agent of Dental Pulp Stem Cells in Periodontal Tissue Engineering

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## ABSTRACT:

**Background:** Robusta coffee beans contain very high chlorogenic acid. In the last decade, chlorogenic acid was developed as an adjunct in stem cells to enhance the anti-inflammatory and antioxidant properties of stem cells when used as a therapeutic agent. Chlorogenic acid can increase proliferation and migration and inhibit the production of pro-inflammatory cytokines in stem cells thereby increasing the ability of stem cells to regenerate tissue. **Purpose:** To analyze the levels of chlorogenic acid in robusta coffee bean extract which can be used as a combination agent for Dental Pulp Stem Cells (DPSC) in periodontal tissue engineering therapy. **Materials and Methods:** Robusta coffee bean extract was obtained from the Coffee and Cocoa Research Center, Jember Regency which was processed and processed using the fractionation method. This study used DPSC with the extraction of premolar teeth of orthodontic patients. The toxicity test was performed on the coffee extract 0.0625%; 0.125%; 0.25%; 0.5% to determine the biocompatible concentration of DPSC. **Results:** The highest fractionation and measurement of chlorogenic acid content obtained was 30.49%. Robusta coffee bean extract with a chlorogenic acid content of 30.49% at concentrations of 0.125% and 0.0625% did not cause toxicity to DPSC. **Conclusion:** Chlorogenic acid in robusta coffee bean extract concentrations of 0.125% and 0.0625% are relatively biocompatible as a combination agent for DPSC in periodontal tissue engineering therapy.

**KEYWORDS:** Robusta coffee beans, DPSC, Periodontal engineering, Periodontitis, Regeneration.

## INTRODUCTION:

Robusta coffee beans contain several anti-inflammatory and antioxidant compounds whose benefits have been widely studied, including caffeine, caffeic acid, chlorogenic acid, and trigonelline. These chemical components are a good source of anti-inflammatory and antioxidants for the body.[1] However, of these compounds, chlorogenic acid is the highest content of polyphenolic compounds in coffee beans compared to other compounds.[2–4]

Chlorogenic acid is well known for its various biological properties such as anti-inflammatory, antioxidant, antimicrobial, antifungal, and antiviral activities.[5,6] In addition, several studies have revealed that chlorogenic acid is an effective anti-inflammatory, antipyretic, and analgesic agent in both in vitro and in vivo experiments.[7–10] The highest content of chlorogenic acid can be found in robusta coffee beans, which is 6.1-11.3%. This amount is the largest content compared to other coffee beans.[3]

In addition to the high anti-inflammatory content in robusta coffee beans, robusta coffee is the main and largest commodity in Indonesia. The production and export value of Indonesia's robusta coffee globally ranks second after Vietnam. High exports in several countries

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make coffee a relatively large foreign exchange contributor. Its availability is very abundant in Indonesia, making this type of coffee easy to obtain.[11] However, its use in the medical field is still not known.

Several researchers have proven the potential of chlorogenic acid as an additive in stem cells to increase the anti-inflammatory and antioxidant power of stem cells when used as a therapeutic agent. One of them is chlorogenic acid which can increase proliferation and migration and inhibit the production of pro-inflammatory cytokines in stem cells, thereby increasing the ability of stem cells to regenerate tissue.[9,12,13]

In the periodontal field, stem cells are used as materials for tissue engineering therapy. In vivo studies reported that Dental Pulp Stem Cells (DPSC) have the potential to repair periodontal bone defects by providing optimal results of bone repair, cementum formation, and periodontal ligament around the bone defect. DPSC is reported to be effective in increasing bone regeneration in the treatment of socket preservation and intrabony defects.[14,15] However, the presence of bacterial toxins, especially LPS P. gingivalis in the periodontal tissue can affect the success of DPSC in regenerating periodontal tissue. Several studies reported that P. gingivalis LPS can stimulate the secretion of proinflammatory cytokines such as IL-1β and TNF-α in excess, thereby inhibiting the proliferation and differentiation of osteogenic stem cells. This has an impact on delaying treatment success.[16,17]

Besides the advantages of stem cells as a regenerative therapy for periodontal tissue, this therapy must also be able to control the possibility of inflammation that occurs and prevent disease progression. Because basically, stem cell therapy will be used in patients with periodontitis, which is a chronic inflammatory disease. Therefore, the administration of chlorogenic acid to increase the anti-inflammatory and antioxidant power of DPSC needs to be explored further so that it can increase the success of periodontal regeneration treatment.[18,19]

The utilization of robusta coffee bean extract to increase the anti-inflammatory and antioxidant power of DPSC itself is still not well understood until now. Therefore, this study aims to analyze the optimal levels of chlorogenic acid and the concentration of robusta coffee bean extract to be used as a combination of stem cells in periodontal tissue engineering therapy.

## MATERIALS AND METHODS:

### Materials:

Robusta coffee bean extract was obtained from the Coffee and Cocoa Research Center, Jember Regency which was processed and processed using the fractionation method so that it had a chlorogenic acid content of 30.49%. The processed coffee bean extract was then made at a concentration of 0.5%; 0.25%; 0.125%; 0.0625%. Dental pulp stem cells (DPSCs) were obtained from the pulp of the 1st or 2nd premolars that were extracted due to indications for orthodontic treatment. The criteria for teeth that can be used are healthy teeth, no caries, and perfectly closed tooth roots in young patient donors aged between 19-29 years, healthy, free from infectious and infectious diseases.

### Extraction and Isolation of Chlorogenic Acid in Robusta Coffee Beans:

Extraction and isolation of chlorogenic acid were carried out using the fractionation method. Fractionation was carried out using the elution liquid in a suitable TLC as the mobile phase and silica gel as the stationary phase. A total of 10 mg of robusta coffee bean extract was put into a glass column that already contained silica gel. The elution liquid was added in a gradient using n-hexane : ethyl acetate (10:1 - 1:1) followed by chloroform : methanol (5:1 - 1:1) and allowed to flow through the column. The presence of compounds in these fractions was detected by TLC, fractions having the same pattern were then combined into one so that fractions with almost the same properties were obtained.

### Analysis of Chlorogenic Acid Levels in Robusta Coffee Bean Extract:

Analysis of chlorogenic acid levels using the Thin Layer Chromatography (TLC) method. TLC was carried out to determine the chromatogram pattern resulting from the fractionation process of chlorogenic acid compounds in the sample. Determination of chlorogenic acid levels in robusta coffee bean extract begins with making a standard curve with a range according to linearity. Furthermore, the sample solution was prepared by weighing a certain number of samples of robusta coffee bean extract as much as 3 replications then dissolved in methanol. Then the standard solution and sample were put in a vial and spotted on a Silica Gel 60 F254 TLC plate to be analyzed under analytical conditions (eluent composition, wavelength, and test concentration) according to the optimization results. The spots formed are scanned at the optimized wavelength. Then do the calculation of % w/w levels.

### DPSC Culture and Isolation:

The pulp tissue was chopped and given 3 ml of collagenase type 1, then incubated in a 5% $CO^2$ incubator, at 37ºC for 30 minutes. After incubation, 3 ml of 25% MEM solution was added and incubated again. The pulp and media were transferred to a 15 ml conical tube, then centrifuged at 3500 rpm for 5 minutes at 28ºC. After being centrifuged, the supernatant was

discarded and the pellet was planted in a petri dish containing 3 ml of 25% MEM solution media. Incubate and observe cell growth periodically on day 3 and day 7 to ensure cells can grow and there is no contamination. If the cell culture is 80% confluent, the culture is harvested up to 2-3 times (passage). Cell culture used in the experiment was cell culture 4 times (passages).[20]

**DPSC characteristic analysis:**

DPSC characteristics test includes identification of morphology and cell surface proteins. Morphological identification of DPSC was carried out using an inverted light microscope to confirm the shape of the stem cells used in the study. While the identification of cell surface proteins using flow cytometry with antibody markers CD105, CD90, CD45, CD34.

**Robusta Coffee Bean Extract Toxicity Analysis on DPSC:**

Cells were cultured on 96 well plates and incubated for 24 hours. After 24 hours, all well plates were given robusta coffee bean extract in various concentrations. Giving Robusta coffee bean extract each concentration of 0.5%; 0.25%; 0.125%; 0.0625% as much as 200 µl/well plate. Each well plate was incubated for 24, 48, and 72 hours. After incubation, 25 µl of MTT (3-4-5-dimetihylthiazol-2YL-2,5-dibromurodiphenyltetrazolium) was added to each well. Then incubated for 4 hours at 37°C. The color change was observed with Elisa Reader at a wavelength of 595 nm.[21]

## RESULT:

**Analysis of Chlorogenic Acid Levels in Robusta Coffee Bean Extract:**

The results of the standard calculation of chlorogenic acid obtained a calibration curve of chlorogenic acid levels with absorbance obtained with a linearity value of $R^2 = 0.9992$. The calibration curve is a graph that forms a straight line (linear) which states the relationship between the concentration of the working solution and the proportional response of the instrument used. Measurements at the lowest level of 15 µg/ml and the highest level of 90 µg/ml with each concentration were repeated 3 times to calculate the standard deviation. The results showed a concentration of 90 µg/ml was the peak of a linear calibration curve (Figure 1).

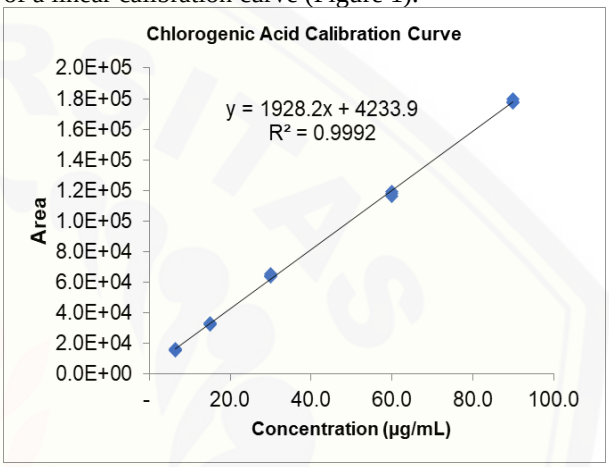


**Figure 1. Chlorogenic acid calibration curve**

Fractionation of chlorogenic acid levels in robusta coffee bean extract obtained the highest chlorogenic acid fraction content of 30.49%. This concentration is the largest concentration that has been obtained from 13.5 mg of robusta coffee bean extract. From the sample weight, the chlorogenic acid content of 4.1 mg of chlorogenic acid was calculated (Table 1, Figure 2).

**Table 1. Table of recapitulation of chlorogenic acid sample calculations. Sample P2 can be fractionated with the highest chlorogenic acid content.**

| Sample | Sample weight (g) | Area | Measured concentration (µg/ml) | Dilution factor | Weirght (µg) | Calculated concentration (µg/g) | Level (%) |
|---|---|---|---|---|---|---|---|
| P1 | 0,0135 | 393.209,99 | 201,73 | 20 | 4.034,60 | 298.859,49 | 29,89 |
| P2 | 0,0135 | 401.065,93 | 205,80 | 20 | 4.116,09 | 304.895,40 | 30,49 |
| P3 | 0,0135 | 396.455,22 | 203,41 | 20 | 4.068,26 | 301.352,88 | 30,14 |
| C1 | 0,0189 | 385.278,35 | 197,62 | 20 | 3.952,33 | 209.118,16 | 20,91 |
| C2 | 0,0189 | 414.555,50 | 212,80 | 20 | 4.256,01 | 225.185,54 | 22,52 |
| C3 | 0,0189 | 411.400,59 | 211,16 | 20 | 4.223,28 | 223.454,12 | 22,35 |
| A1 | 0,0137 | 165.694,43 | 83,74 | 20 | 1.674,73 | 122.242,93 | 12,22 |
| A2 | 0,0137 | 159.062,51 | 80,30 | 20 | 1.605,94 | 117.221,85 | 11,72 |
| A3 | 0,0137 | 167.342,96 | 84,59 | 20 | 1.691,83 | 123.491,04 | 12,35 |

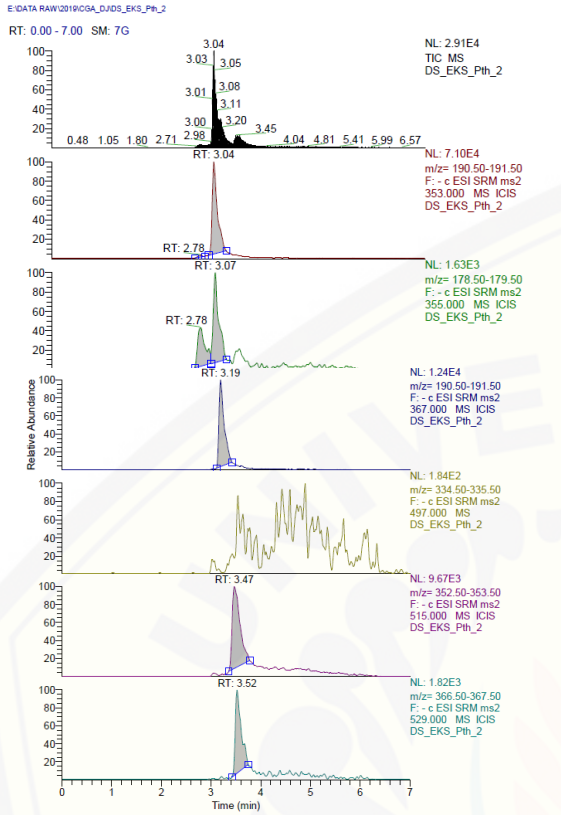


**Figure 2. Chromatogram of sample P2 with a concentration of chlorogenic acid found at 30.49%**

## DPSC Characteristics Analysis

On observation, the morphology of DPSC was fusiform in shape with a tapered tip, a cell nucleus in the middle, and a large cytoplasm, which can be seen in Figure 3 below.

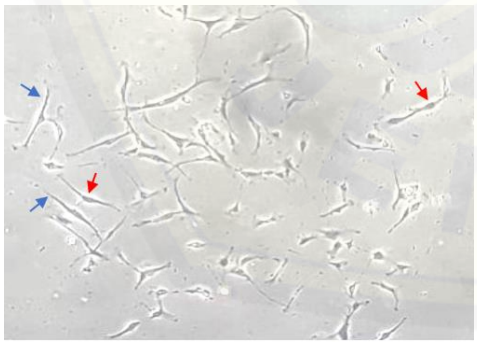

**Figure 3. Morphology of DPSC (section 1). A fibroblast-like morphology appears, which is a fusiform shape with a tapered tip (blue arrow), a cell nucleus in the middle, and a large cytoplasm (red arrow). Observations under a microscope with a magnification of 100x.**

Observation of DPSC surface expression markers was carried out using a flow cytometry (FCM) test to measure and analyze the surface characteristics of stem cells used in this study. The markers used as positive controls were CD90 and CD105 as markers of mesenchymal stem cell lineage. Meanwhile, the markers used as negative controls were CD34 and CD45, which are markers of hematopoietic stem cell lineage. The FCM test on the DPSC of this study shows the results according to Figure 4 below.

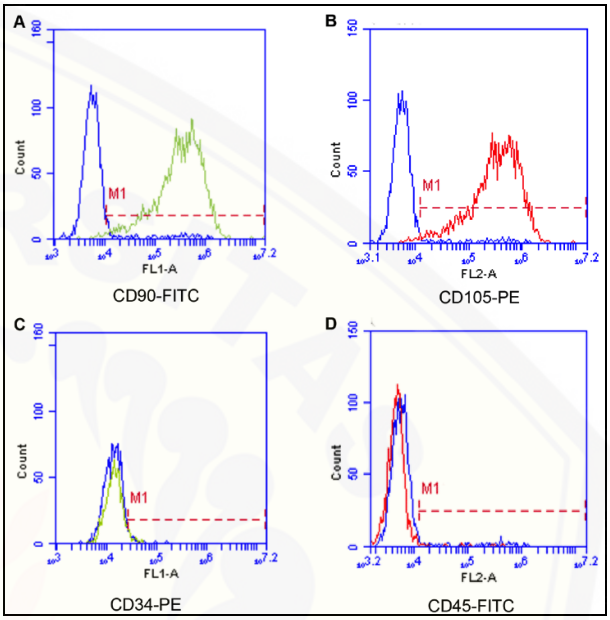


**Figure 4. DPSC FCM test histogram graph. (A, B) DPSCs express the cell surface protein markers CD90 and CD105 which are shown in the green and red diagrams on the right side of the histogram. (C, D) While DPSC did not express the CD34 and CD45 cell surface protein markers which were indicated by the absence of green and red diagrams on the right side of the histogram.**

## Robusta Coffee Bean Extract Toxicity Analysis on DPSC:

The results of the toxicity test of robusta coffee bean extract at various concentrations on DPSC showed results as shown in Figure 5. Robusta coffee bean extract at concentrations of 0.5% and 0.25% caused toxicity to DPSC at 24, 48, and 72 hours of observation. This was indicated by the percentage of DPSC living cells <80% after the concentration was given. Meanwhile, robusta coffee bean extract concentrations of 0.125% and 0.0625% were not toxic to DPSC.

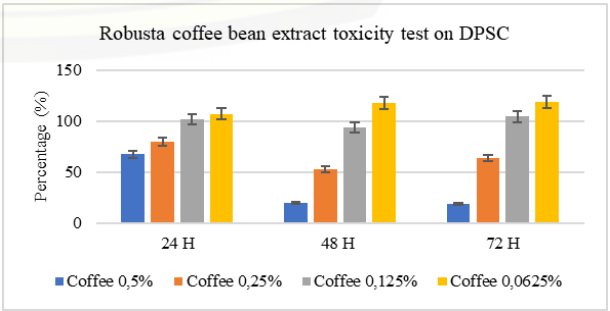


**Figure 5. Graph of Robusta coffee bean extract toxicity test against DPSC. Concentrations that do not cause toxicity are 0.125% and 0.0625%.**

## DISCUSSION:

The chlorogenic acid content of robusta coffee bean extract in this study was 30.49%. This fractionation result is the highest concentration of chlorogenic acid that has been successfully fractionated. The most chlorogenic acid is contained in robusta coffee beans compared to other herbal sources. This content is mostly contained in robusta coffee beans without the roasting process. [22,23] Farhaty's research (2016) states that the average chlorogenic acid content in robusta coffee beans is around 6.1-11.3%.[3]

Chlorogenic acid has biological properties such as anti-inflammatory, antioxidant, antimicrobial, antifungal, and antiviral activities.[5,6] In addition, several studies have revealed that chlorogenic acid is an effective anti-inflammatory, antipyretic, and analgesic agent in both in vitro and in vivo experiments. [7–10] The utilization of chlorogenic acid in stem cells as an agent to increase the anti-inflammatory potential of cells is currently the focus of researchers to develop the success rate of stem cell treatments. In addition, the application of chlorogenic acid to stem cells aims to increase cell proliferation and regeneration, so that cells are susceptible to premature aging. However, the use of chlorogenic acid in periodontal tissue engineering therapy using DPSC still requires further research.

Based on observations of morphological characteristics carried out in this study, it was confirmed that DPSCs are fusiform in shape with a tapered tip, a cell nucleus in the middle, and a large cytoplasm, which is often referred to as a fibroblast-like spindle formation. These results are following the previous theory, which confirmed that DPSCs, which are mesenchymal stem cells, have a morphology similar to fibroblast cells.[24–26] In this study, DPSCs can also grow and attach to plastic substrates and can colonize. These results are in line with the research of Martens et al. (2010) who investigated the characteristics and surface expression of DPSC in vitro. His research explains that in the first 24 hours of culture, cells are able to grow and migrate out of the cultured pulp tissue and will stick to the bottom of the plastic media container which will then form colonies.[27]

Observation of other characteristics by analyzing DPSC surface expression markers was performed using flow cytometry (FCM) test. The results of the FCM test showed the expression of CD90 and CD105, while CD34 and CD45 were not expressed. CD90 and CD105 specific antibodies are markers of mesenchymal stem cell lineage. While the specific antibodies CD34 and CD45 are markers of hematopoietic stem cell lineage. The results of this study are following pre-existing theories.[24,25,28] Martens et al. (2010) in their research in vitro also showed the same results.[27]

A cell has the criteria for mesenchymal stem cells if it has the following criteria: Able to stick to plastic medium containers when cultured,Expressing CD105, CD90, CD73 and CD44, and lacking expression of CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR, Can differentiate into osteoblasts, adipocytes, and chondroblasts in vitro[29]. Although this study did not test the ability of cells to differentiate into osteoblasts, adipocytes, or chondroblasts, the other two criteria for mesenchymal stem cells were met.

Toxicity test of robusta coffee bean extract against DPSC showed that 30.49% robusta coffee bean extract at a concentration of 0.125% and 0.0625% was not toxic to DPSC, with the percentage of DPSC living cells >80%. Meanwhile, robusta coffee bean extract concentrations of 0.5% and 0.25% caused toxicity to DPSC. The cytotoxicity level of each concentration of antibacterial agent was calculated as a percentage of cell viability including absorbance values obtained for each treatment. Based on ISO 10993-5, there are cytotoxicity criteria in the viability test, including: The percentage of cell viability > 80% is considered as non-cytotoxic;The percentage of cell viability 80%-60% is considered as weak cytotoxicity;Cell viability percentage 60%-40% is considered as moderate cytotoxicity;The percentage of cell viability < 40% is considered as strong cytotoxicity.[30]

## CONCLUSION:

Robusta coffee bean extract with a chlorogenic acid content of 30.49% at concentrations of 0.125% and 0.0625% did not cause toxicity to DPSC. This concentration is relatively safe to be used as a stem cell combination in periodontal tissue engineering to increase the anti-inflammatory and antioxidant power of stem cells.

## CONFLICT OF INTEREST:

The authors have no conflicts of interest regarding this investigation.

## ACKNOWLEDGMENTS:

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