 ISBN 978-602-60569-3-1

EFFECTS OF ROBUSTA COFFEE BEAN EXTRACT (*Coffea robusta*) ON THE VIABILITY OF NEUTROPHILS EXPOSED BY *Porphyromonas gingivalis*

Tantin Ermawati, Microbiology Laboratory of Biomedical Sciences Department, Dental Faculty of Jember University, Jl. Kalimantan No. 37, 68121, tantin.ermawati@gmail.com

INTRODUCTION

The new paradigm evolving today in the treatment of periodontal infection is the use a host modulator therapy i.e. therapy that aims to improve the host immune response to bacterial infection. Good immune response is necessary in increasing the phagocytic function that in this case is played by neutrophil cells, monocytes and macrophages, which served as the first line of defense against bacterial infections. One of the bacteria causing periodontal infection is *P. gingivalis*. It has the biggest role in causing periodontal disease, where in the proteolytic enzyme such as *gingipain* contributes to the bacterial pathogenicity.[1, 2] During the initial invasion *P. gingivalis* will colonize the gingival sulcus and invade into the gingival tissues and will induce an inflammatory response and continue to alveolar bone resorption.[3]

The immune response that acts first against invading bacteria is neutrophils. The presence of bacterial adhesion to neutrophils allows neutrophils secrete microbicidal substance by destroying the bacterial membrane and fagocyting bacteria. A neutrophil can phagocyte bacteria before becoming inactive or cell lysis. Phagocytosis that occurs can affect the lysis of neutrophils. This can emerge very fatal impacts because if the neutrophils undergo lysis then chemical components and enzymes may break into the tissue leading to degradation of various organic molecules of its adjacent tissue.[4] Nowdays Indonesia has developed a lot of research on medicinal plants, such as Robusta coffee beans. Robusta coffee beans naturally have substances such as caffeine, phenolic compounds, trigonellin and chlorogenic acid which have antibacterial activity. Besides as antibacterial chlorogenic it also has antioxidant properties.[5] Based on the above background, the researchers want to develop Robusta coffee beans as host modulator therapy to maintain the viability of neutrophils by observing neutrophil cell viability against *P. gingivalis* applied by Robusta coffee bean extract which will be useful for preventing periodontal tissue infections of the oral cavity.

MATERIALS AND METHODS

The study was carried out experimentally *in vitro* using post test only control group design. The independent variable is the coffee bean extract concentration of 25%, 50% and 100%. The dependent variable is the viability of neutrophils.

Robusta Coffee Bean Extract

The coffee bean extract is obtained by blending dried Robusta coffee beans into tiny pieces and subsequently crushed into powder and macerated in 97% ethanol solution for 24 hours using a shaker bath. After the samples were filtered using a vacuum pump and concentrated using a rotary evaporator, and concentrated extract 100% was obtained. Then Robusta coffee bean extract was divided into several concentrations of 100%, 50% and 25% with serial dilution method.

Culture of *P.gingivalis*

P.gingivalis bacteria were obtained from the Laboratory of Microbiology Faculty of Dentistry University of Jember. Culture was performed on BHI-B media enriched with 10 µl of vitamin K, 50 µl of hemin and 50 µl of yeast extract. One ose of pure cultures of bacteria was inserted into the test tube closed using cotton and put in a desicator to obtain facultative anaerobic atmosphere. Furthermore, the suspension was incubated in an incubator for 3x24 hours at a temperature of 370^{C} and *P.gingivalis* suspension was shaker using *Thermolyne* and its turbidity levels were measured using a spectrophotometer with a standard solution Mc.farland 0.5. Scale absorbance of the *P.gingivalis* suspension using a wavelength of 560 nm.

Neutrophil Isolation

The technique conducted for neutrophil isolation was performed by density gradient technique using Histopaque 1199 and ficoll.[5] Blood was collected from a peripheral vein of respondents who were willing to participate in research. The collected blood was inserted into three heparin tubes and all stored in a falcon tube. Furthermore 3 cc of Histopaque 1199 was layered on the falcon tube, and 3 cc Ficoll layer was subsequently superimposed on Histopaque 1119. Subsequently, 6 cc of blood was carefully superimposed from falcon tube onto Histopaque 1199 and Ficoll layers, then centrifuged at a speed of 1900 rpm for 30 minutes at

a temperature 25˚C. The results will form six layers i.e. plasma layer, blood mononuclear cells, Ficoll, granulocytes (neutrophils), Histopaque 1119, and erythrocytes. Taking the first 3 layers followed by taking granulocytes (neutrophils) layer and adding 1000 µl of HBSS in the layer of granulocytes (neutrophils). Centrifugation speed of 1700 rpm was conducted for 10 minutes at a temperature of 37˚ C. The supernatant (top layer) was taken and then adding 2000 µl of HBSS in the layer of granulocytes (neutrophils). Monocytes were observed under inverted microscope with a magnification of 400x.

Neutrophil Viability Test

In the viability test, briefly, the suspension of neutrophils were divided into four test groups i.e. normal group, and the group treated with Robusta coffee bean extract incubation in concentration of 25%, 50%, 100%, and as a control was neutrophils incubated with RPMI. Incubation was performed for 18 hours in a roller tool. Furthermore, neutrophils were exposed using suspension of *P. gingivalis* $3x10^8$ CFU / ml and incubated for 1 hour, subsequently viability test was conducted using trypane blue dye staining. The observation of viability of neutrophils was performed using a hemocytometer under inverted microscope with magnification of 400x. The viable neutrophils did not absorb trypane blue thus it looked translucent or transparent, while the dead neutrophils absorbed blue trypane blue.

RESULTS

Neutrophil Isolation

The research was conducted in the Bio Science Laboratory of Dental Hospital, Faculty of Dentistry, University of Jember. This research used a sample of neutrophil isolates that were isolated from the blood of healthy people. Isolated neutrophils can be seen in Figure 1.

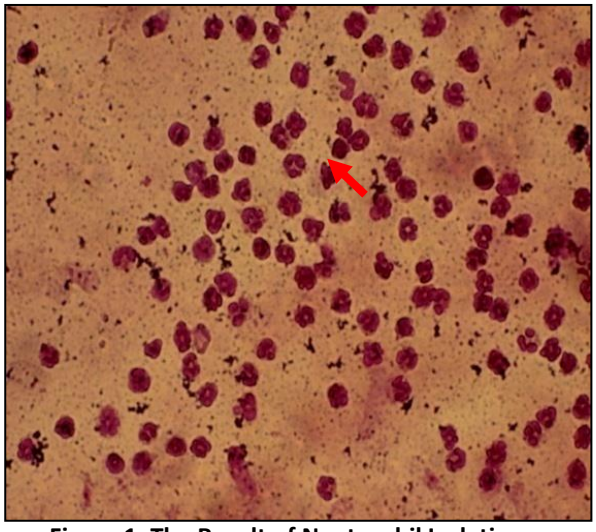

Figure 1. The Result of Neutrophil Isolation. Showing neutrophils colored purplish pink (Staining Giemsa, magnification 400x)

The Results of Neutrophil Viability Test

Viability test data of neutrophils exposed to *P gingivalis* and incubated with Robusta coffee bean extract can be seen in Table.1

Table. 1 Mean of Neutrophil Viability between Control group and Treatment Group

	Neutrophil Viability			
	Group 1(N)	Group 2 (25%)	Group 3 (50%)	Group 4 (100%)
X±SD	60,31± 3,19	26,89± 2,45	37,49 ± 2,17	29,67 ± 0,66

Notes:

Group 1: Neutrophils + Complete Media

Group 2: Neutrophils incubated with coffee bean extract 25% +exposed by *P.gingivalis*

Group 3: Neutrophils incubated with coffee bean extract 50%+exposed by *P.gingivalis*

Group 4: Neutrophils incubated with coffee bean extract 100%+exposed by *P.gingivalis*

Based on the Table 1. it can be observed that among the groups treated with Robusta coffee bean extract hat has the greater ability to live (viable) is the group 3 (Neutrophils incubated with coffee bean extract 50%+exposed by *P.gingivalis*), while in the concentration of 100% of extracts the number of viable cells decreased. The calculation of each sample was conducted using a hemocytometer viewed using inverted microscope as depicted in Figure 2

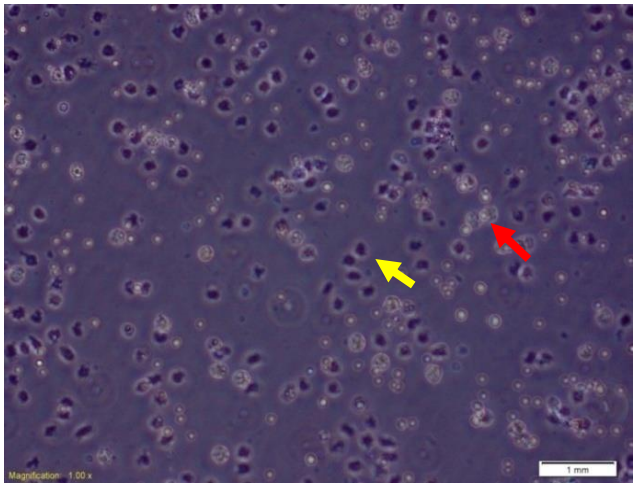

Figure 2. The viable neutrophil cells produced bright colors (red arrow), b. the dead neutrophil cells absorbed color to dark (yellow arrows).

Table 2. The Results of Kruskal Wallis Neutrophil Viability Test

Chi-Square	df	Asymp. Sig.
9,842	3	0,020*

Note :
df : Degrees of freedom
* Significant

Table 3. The Result of Mann Whitney Test on Neutrophil Cells

Group	1	2	3	4
1		0,00*	0,00*	0,00*
2			0,00*	1,50
3				0,00*
4				

Notes:
Group 1: Neutrophils + Complete Media
Group 2: Neutrophils incubated with coffee extract 25% +exposed by *P.gingivalis*
Group 3: Neutrophils incubated with coffee extract 50%+exposed by *P.gingivalis*
Group 4: Neutrophils incubated with coffee extract 100%+exposed by *P.gingivalis*
* : Signifikan

The results of Kruskal Wallis test (Table. 2) shows that the value of p <0.05, this means that there are significant differences in the number of neutrophil viability of the four treatment groups. Subsequently, *Mann Whitney* test was performed to observe the differences in each treatment group. Based on the Table 3 it can be observed the differences of the data among the groups (p <0.05), except in the group (2-4) that have no significant difference.

DISCUSSION

In the study conducted on the viability of neutrophils that had been exposed by *P. gingivalis* and incubated with coffee bean extract showed that the highest percentage of viable cells among the treatment groups was found in the group 3 (incubation with Robusta coffee bean extract 50%). While the lowest percentage was found in the group 2 (Robusta coffee bean extract 25%). In the control group, the number of viable neutrophil cells was the highest compared to the other treatment groups because there was no exposure of *P. gingivalis* in those groups, the neutrophil cells were only given M199 growth media, thus there were more viable neutrophil cells (alive). The results showed that Robusta coffee bean extract has the ability to improve the viability of neutrophils.

Cell viability is the ability of a cell to survive, grow and develop. Dead cells will appear blue, because the cells undergo lysis in the plasma protein that binds to trypan blue thus the cell becomes dark blue. This does not occur in living cells because of no damage on the cell membrane, so they still look round, lighter and transculent.[6] Cell viability is affected by the presence of free radicals that can cause cell damage. Free radicals attack the nearest stable molecule and takes electrons. The substance in which the electrons were taken would be unstable. The increasing number of free radicals leads to a situation where the level of toxic reactive oxygen intermediates (ROI) exceeds endogenous antioxidant defense called oxidative stress. Oxidative stress leads to lipid peroxidation, protein and cellular nucleic acids resulting in cell damage. Cell membrane is rich of poly unsaturated fatty acid (PUFA) source which is easily damaged by oxidants such as lipid peroxidation. The damage of the cell membrane results in disrupted cell biochemical activities, thus the cells are not able to sustain life. [7]

Excessive free radical formation in the body bean can be eliminated by the presence of antioxidants. Many natural antioxidants contained in natural plant bean derived from phenolic compounds such as flavonoid.[8] Robusta coffee bean contains of phenolic bean acids, flavonoids, and tannins. A research conducted by Nayeem *et al* showed that the content of phenolic acids, flavonoids and tannins in Robusta coffee beans has a greater percentage than any other coffee types like arabika.[9] This is supported by a research conducted by Yasin *et al* showing that coffee has the ability as an antioxidant and antiradical in vitro.[5] Robusta coffee bean extract has antioxidant properties that can reduce the excessive number of free radicals thus it can maintain the cell intact.

Other content of Robusta coffee bean acting as an antibacterial is caffeine. Caffeine is a white crystals-shaped alkaloid compound, the caffeine content in Robusta coffee beans is 1.6 to 2.4 % . Besides being antibacterial, caffeine also has an ability to prevent free radicals and destroys the molecules that can damage DNA cells. Caffeine can also prevent lipid peroxidation in order to maintain cell viability allowing it to be able to survive against bacterial attack. [10,11]

The research on the neutrophil viability indicates that in the treatment group 4 (incubation extract 100%) shows decreased viability compared to group 2 and group 3. It is assumed that a concentration of 100% of coffee bean extract is toxic concentration, thus many cells are dead. Besides having the ability to improve cell viability of neutrophils, coffee bean in numerous concentration (100%) is found toxic and causes the death of neutrophil cells. Therefore, it is required to use appropriate concentrations of coffee bean extract to maintain neutrophil cell viability.

The purpose of using Robusta coffee bean as medicine in health sector is to develop the coffee extract a host modulator therapy. In general, the therapy conducted to eliminate a disease is on bacterial target or direct bacterial products, but it is sometimes failed. Hence the necessity for additional therapy i.e. host modulator therapy that function to improve the body's immune response, one of them is played by PMNs cells as a first line of defense against bacteria.[12]

Based on the above research it can be concluded Robusta coffee bean extract has capabilities on the viability of neutrophils. Neutrophil viability test is intended to correct the host cell to a lesion from the outside. Thus Robusta coffee bean extract can later be used as a basis for making topical ingredient in the oral cavity which has a role as an immunomodulator host therapy.

REFERENCE

1. Carranza F, Newman M, Takei H, dan Klokkevold P. 2006. *Clinical periodontology*. 10th edition. Philadelphia: WB Saunders
2. Pyrc K, Strzyz P, Milewska A, Golda A, Schildgen O, Potempa J. Porphyromonas gingivalis enzymes enhance infection with human metapneumovirus in vitro. *J Gen Virol* 2011; 92(10): 2324-32.
3. Kimura S, Nemoto YO, Shimoyana Y, Isikhawa T, Sasaki M. Pathogenic Factors of P.gingivalis and the host defense mechanism, pathogenesis and treatment of periodontitis. *InTech* 2012; ISBN : 978-953-307-924-0.
4. Nussbaum, G., Shapira, L. How Has Neutrophil Research Improved Our Understanding of Periodontal Pathogenesis. *J. Clin Periodontol* 2011; 38(11): 49-59.
5. Yashin A, Yashin Y, Wang JY, Nemzer B. Antioxidant and Antiradical activity of coffee. *Antioxidant* 2013: ISSN 2076-3921
6. Djajanegara I dan Wahyudi P. Pemakaian sel HeLa dalam Uji sitotoksisitas fraksi kloroform dan etanol ekstrak daun *annona squamosa*. *Jurnal ilmu kefarmasian Indonesia* 2009. Jakarta : P3 Teknologi Bioindustri BPPT.
7. Arief S. Radikal Bebas. Ilmu Kesehatan Anak FK Unair, Surabaya. 2007. Available at:http://www.sribd.com/doc/112189876/radikal-bebas. Diakses: 9 Maret 2014
8. Astuti S, Isoflavon kedelai dan potensinya sebagai penangkap radikal bebas. *Jurnal Teknologi Industri dan Hasil Pertanian* 2008; 13(2): 126-136
9. Nayeem N, Denny G, Mehta SK. Comparative phytochemical analysis, antimicrobial and antioxidant activity of the methanolic extracts of the leaves of coffea arabica and coffea robusta. *Scholars research Library* 2011. 3(1): 292-297. ISSN : 0975-5071
10. Nadanasabapthi S, Rufia J, Manju V. In vitro free radical scavenging activity and bioavaibility of dietary compounds caffeine,caffeic acid and their combination. *International food research journal* 2013; 20(6): 3159-65
11. Widyotomo S dan Sri M. Kafein*: Senyawa Penting Pada Biji Kopi*. warta Pusat Penelitian Kopi dan Kakao Indonesia 2007; 23 (1): 44 - 50
12. Reddy S, Prasad MGS, Kaul S, Asutkar H, Bhownik N, Amuda. Host modulation in periodontics. Reveiw article 2011. Available at : www.ejournaldentistry.com . Diakses 29 agustus 2013.