Potential of Chlorogenic Acid from *Coffea canephora* to Improving Innate Immunity System Components among BALB / c Mice

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Abstract

The aim of this study was to verify the phagocytosis activity which displayed in CD11b and B220 markers and also the markers of erythroid 2 nuclear factor related factor 2 (Nrf2) as a key regulatory transcription factor on various antioxidant gene expressions and Superoxide dismutase (SOD) as an antioxidant marker that related with protein. Nrf2 plays an important role to inhibit the ROS accumulation and eliminate free radicals. The active compound of chlorogenic acid in robusta coffee type (Coffea canephora) in Indonesian Coffee and Cocoa Research Center was used in three groups of mice in this study and it was gave different dose in each groups. The assessment samples were taken from the peritoneal fluid of mice than it was analyzed by using Flow Cytometry method to find phagocytosis function and antioxidant activity. The results of ANOVA statistical test was $p < 0.05$ in all parameters, this mean that there was indicated that the active compound of chlorogenic acid in coffee was involved in natural immune system mechanism and it was seen in increasing of phagocytic activity and antioxidant levels.

Keywords: *Coffea Canephora, chlorogenic acid, phagocytosis, antioxidant*

Introduction

The natural immune system is a non-specific defence mechanism and quickly responds to antigen exposure in the body. The natural immune response is main weapon in host use to prevent or reduce pathogen replication in early stages of infection [(1).] The natural immune system as first body defence against infection (non-self) or tissue injury (damaged-self) with involves many cell and molecular components. Antibodies and complement was plays in Identification molecule solvent and cellular components plays in phagocytic cells (macrophages), antigen presenting cells (dendrite cells) and killer cells (NK cells). Another addition molecular is T and B lymphocyte cells who involve in natural immunity [(2).]

Phagocytosis process is one of an important aspect in natural immune response because it plays a role to inhibit an adaptive immune response [(3),] a highly-conserved mechanism occurs in first increasing before the development of other multicellular [(4),] the efficiency processes is eliminates pathogens attack and help in homeostasis repair process[(5).]

Macrophages and neutrophils are one of the key regulators in inflammatory process because it can produce reactive oxygen species (ROS) as a defence system to clean the microorganism components. ROS has two different ways. First, in high level as a as effector molecules against intracellular pathogens and second, in low level as a signalling messengers to inflammatory expression [(6).] The role of B lymphocytes as phagocytic cells is to produces ROS, and it happen

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because of the results of mitochondrial respiration due to high production of immunoglobulin or activation of NADPH oxidase complex (NOS)[7].

The popularity of coffee drinks related with health effects and it was done in many studies on coffee and this provided some evidence such as coffee was reduced risk of cancer, improved neurological conditions, body metabolism and liver[8]. The phenolic compounds in coffee produced secondary metabolism which it have sentinel activity against free radicals and a role as antioxidants in body. The main phenolic compounds are phenolic acids, flavonoids and tannins. The phenolic acid family are include hydroxynamide and chlorogenic acids[9]. Based on this case, the researchers will assessed the phagocytosis activity by the description of CD11b and B220 also markers of nuclear factor erythroid 2 related factor 2 (Nrf2) as key transcription factor on various antioxidant gene expressions and Superoxide dismutase (SOD) as an antioxidant marker that was closely related with Nrf2 protein and it plays an important role to inhibit the accumulation of ROS and eliminating free radicals.

Selection of coffee beans

Four types of coffee beans were taken from several cities in East Java, Indonesia. Two types of arabica (Coffea arabica) and robusta (Coffea canephora) coffee beans were taken from one of the coffee bean suppliers on the Ijen Mount, and one of types of robusta coffee beans was taken from coffee plantations in Jember and one type of robusta coffee bean was from coffee planter in f Sumber Asin Malang area.

Measurement of chlorogenic acid levels

The HPLC-LC-MS / MS test used to measured the levels of chlorogenic acid and it was applied on four different types of coffee beans, and the highest concentration of chlorogenic acid used in research process. Analyzes process used Thermo Surveyor HPLC LC-MS / MS system. A 2ul sample injected into LC at 16 °C, the column controlled at 30 °C, and the autosampler compartment was set in 16 °C. Columns used are Hypersil Gold specifications (50mm x 2.1mm x 1.9µm). UHPLC brand of ACCELLA type 1250 made by Thermo Scientific which consists of a vacuum degasser, quartener pump, thermostatic autosampler controlled by Personal computer through the x-calibur 2.1 program. Solvent A = 0.1% formic acid in Water and B = 0.1% formic acid in Acetonitrile. A mobile phase gradient with a speed of 300 µl / minute at a setting of 0.0-0.6.00 minutes 5% B, 0.6-3.0 minutes 75% B, 3.0-3.5 minutes 75% B, 4.0-5.5 minutes 5% B. The quantification compared with chlorogenic acid standard at 325nm of wavelength with ranged of 5-750 ng.

Flow Cytometry analysis

Experimental animals

The research subjects were male BALB / c mice aged 10-12 weeks with an average weight 20-25 grams, white skin color, active conditions and normal behavior, no visible anatomic.. 24 mice were selected by using random method than they were divided into 4 groups and 6 mice in each group. Namely each group were: group 1 (healthy mice without coffee intervention), group 2 with coffee intervention per sonde of 0,5g / kgBW / day, group 3 with coffee intervention per sonde of 1.5g / kgBB / day and group 4 with coffee intervention per sonde of 2.5g / kgBB / day. The coffee intervention with different doses were carried out for 14 days, then the peritoneal fluid was taken in all mice and than it analyzed by using the Flow Cytometry method of phagocytosis function and antioxidant levels.

Macrophages Isolation process

The mice were dislocated on the neck, put on a sterile surgical board. The all part of mice body soaked with 70% EtOH and gave sterile injection of 10 ml PBS in an intraperitoneal. The ventral part (stomach) was tapped, then slowly taken peritoneal fluid by using a syringe and than the peritoneal fluid was put in a sterile 15ml propylene tube, than it centrifuged at 2500 rpm for 5 minutes at 10^{o}C. The pellets were resuspended on 3 ml RPMI completed and homogenized, so in the final got macrophages.

E. coli bacterial coloring and phagocytosis

The 10^8 cells / ml E. coli culture on sterile PZ media was transferred to a sterile 15ml propylene tube as much as 10ml than it was centrifugated 8000 rpm for 5 second at 4^{o}C, than the supernatant was discarded, the pellets were resuspended with 0.5 ml sterile PBS and homogenized. The suspension heated on 80^{o}C for 5 minutes. At normal temperature, the suspension add

5ul coloring solvent of carboxyfluorescein succinimydil ester (CFSE). Incubation process wad did for 20 minutes at 4ºC in the dark room. The stained bacterial suspension added to the macrophage cells that had been prepared before, than put in a 30mm petri dish, and cultured macrophage cells at 37ºC CO_2 5%.

Flow Cytometry interpretation

Macrophage cell culture carried out for 4 hours, than the suspension transferred to 15 ml propylene tube (avoided from direct exposure to light). After that, it was centrifuged in 2500 rpm for 5 second at 10ºC. The supernatant removed, the pellets resuspended with 50ul working solution of specific antibodies (PE conjugated anti-CD11b, anti-B220, anti-superoxide dismustase (SOD) 1 antibody EP1727Y, and anti-Nrf2 antibody ab89443). Incubation process at 4ºC for 20 minutes in dark room, then added 0.5 ml of PBS and homogenized. Then the suspension transferred to the FCM cuvette (polystyrene tube 12x75 mm) and read on a Flow Cytometry tool.

Data Analysis

Data analyzed by using SPSS with version 17 and the sample analyzed by using ANOVA and the significance data were continued test with Tukey's test.

Results and Discussion

Chlorogenic acid HPLC analysis

Table 1. The comparison of chlorogenic acid levels

Number	Kind of coffee beans	Sample	Weigh Spl (g)	Area	CONS. TKR (µg/ml)	F.P.	Weigh (µg/ml)	CONS. TKR (µg/ml)	Level (%)
1.	Robusta of Jember	Spl_1_1	0.5109	21699	5.43	500	2,715.34	5,315.34	0.53
2.	Robusta of Sumber Asin	Spl_2_1	0.4244	26120	6.54	500	3,269.74	7,704.39	0.77
3.	Arabica BWI	Spl_3_1	0.3685	15087	3.77	500	1,886.92	5,120.54	0.51
4.	Robusta BWI	Spl_4_1	0.4059	10521	2.63	500	1,314.59	3,238.72	0,32
5.	Spray	Spl_5_1	0.4385	11062	2.76	500	1,382.50	3,152.79	0,32

The results of HPLC analysis showed that robusta coffee beans in area of Sumber Asin Malang was the highest levels of chlorogenic acid

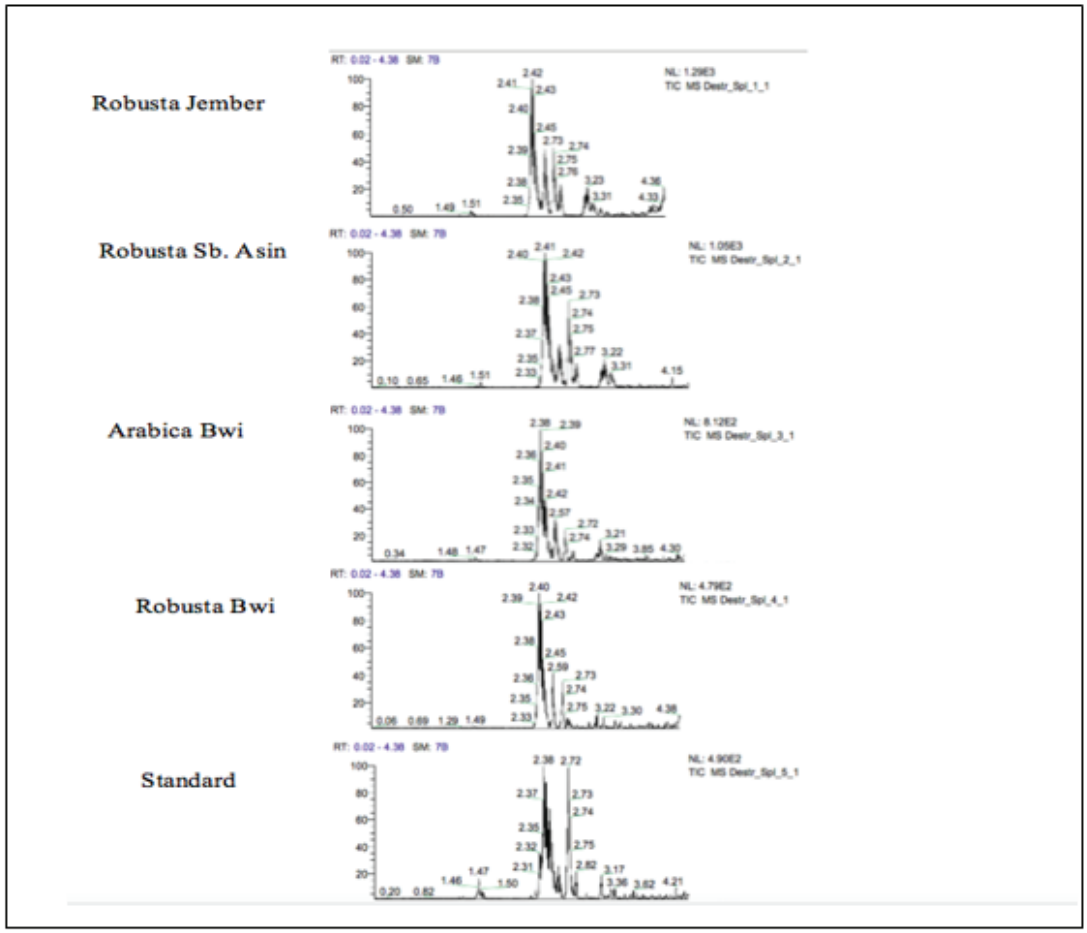

Figure 1. Graph of Chlorogenic Acid Fractionation

Results of Phagocytosis and Antioxidant Activities

Table 2. Results of the Quantification of Total (%) CD11b, B220, SOD, NRF2 by using ANOVA Test

Variable	n	N Mean ± SD	T1 Mean ± SD	T2 Mean ± SD	T3 Mean ± SD	p
CD11b(%)	6	13.48±1.90 ^{b}	16.40±3.99 ^{b}	2.00±0.35 ^{a}	3.35±1.05 ^{a}	0.00
B220 (%)	6	14.38± 1.91^{a}	23.65± 4.28^{b}	16.41±2.45 ^{a}	34.22±6.64^{c}	0,00
SOD (%)	6	14.07± 2.97^{ab}	20.28± 1.75^{c}	10.61±2.96 ^{a}	17.53±3.9 ^{bc}	0.00
NRF2 (%)	6	12.91±2.59^{a}	12.71±2.67 ^{ab}	8,9±3.18 ^{ab}	14.63±2.44 ^{b}	0,012

Note: * significant at α = 0.05, the ^{ab}suprscript showed that there was no differences in each groups (ANOVA). n = number of samples, SOD = Superoxide dismutase, NRF2 = nuclear factor erythroid 2 – related factor 2, N = mice without treatment, T1 = mice with treatment 1 (0.5 mg coffee powder), T2 = mice with treatment 2 (1.5 mg coffee powder), T3 = mice with treatment 3 (2.5 mg of coffee powder).

The results of this study showed that there were significant differences in the amount (%) of CD11b, B220, SOD, NRF2 with ANOVA test. In T1 group (mice with treatment 1 (0.5 mg of coffee powder), the percentage of CD11b was higher than normal group. T2 and T3 groups were significantly different from the normal group. The highest percentage of B220 was in T3 group of mice with treatment 3 (2.5 mg of coffee powder). The highest percentage of SOD was in T1 group and the highest percentage of NRF2 was in T3 group and Tukey's test results found that there were significant different of each groups with normal group.

Nuclear factor-Errythroid-related factor 2 (Nrf2) is a transcription of the basic leucine zipper redox-sensitive factor, namely pleiotropic proteins of regulate basal and induce basal expression of antioxidants and various other genes related to cell protection through binding of sequence enhancers (enhancers) and known as elements response of antioxidants. In normal condition, the Nrf2 level will be at low level but in stressful conditions such as oxidative stimuli, the Nrf2 will increases the transcription activation in targets that undergo protective conversion against various stress-inducing the environments [10].

There was no differences of the Nrf2 level in each group, it mean that there was no environmental stress, but the active compound of Chlorogenic acid gave a different responses in each intervention group and described a response toward antioxidant function of Chlorogenic acid in coffee.

In this study, different doses were gave in each group of mice. There were differences of SOD enzyme in normal group and intervention group, there was higher antioxidant activity in low dose group compared with other groups. SOD as an endogenous antioxidant enzyme is a protein that acts as a first line of defense against ROS which clean superoxide radicals. The formation of ROS can find in a removing the potential of oxidants to become relatively stable compounds [11].

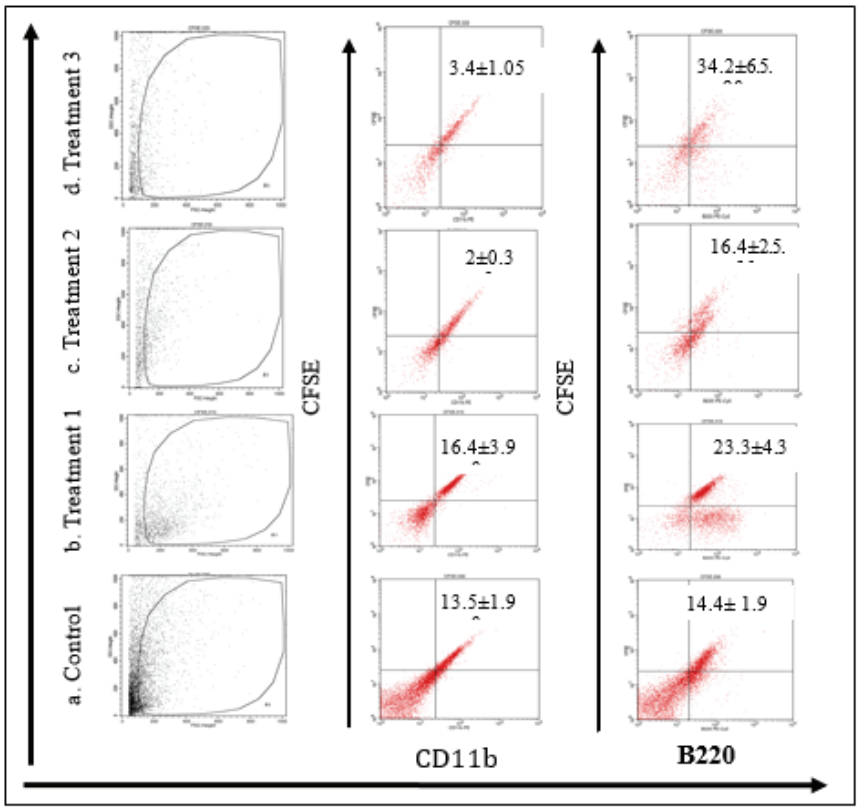

Figure 3. Phagocytosis by using Flow Cytometry method a). Untreated mice expressed that CD11b and B220 b molecules). Mice with treatment 1 (0.5 mg of coffee powder) express CD11b and B220 c molecules). Mice with treatment 2 (1.5 mg of coffee powder) expressed CD11b and B220 d molecules). Mice with treatment 2 (2.5 mg of coffee powder) expressed CD11b and B220 molecules

CD11b is an integrin component that mediates monocytes, macrophages and granular cells to attach by using iC3b Opsonization which leads to phagocytosis, neutrophil aggregation and Chemotaxis. The expression of markers is related with T cell activation. Chlorogenic acid compounds as part of polyphenols which are present in large concentrations in coffee beans and it was proved in various clinical studies to reduce the risk of various diseases [12]. n this study, the increasing of CD11b expressed that medium doses and high doses can be seen as a response to active compounds, especially Chlorogenic acid in coffee.

B220 is a CD45 isoform and it expressed in all mice B lymphocyte cells and as a subset of B lymphocyte cells in human, as a protein tyrosine kinase with various isoforms that regulate activation through a range of T and B lymphocyte cell surface receptors, as well as at cytokine receptors. CD45 known as B220, Ly-5 and T200, this mean that it is a member of the tyrosine phosphatase (PTP) protein family with a molecular weight of 220kDa. The PTP family has a function as a signaling molecule in regulatory process on cell differentiation, cell division and development. CD45R is the most common form of protein and it obtained by excess glycosylation when it expressed on B lymphocytes. NK cells (BioRad). In general, this study found that in intervention group (medium and high doses) were significantly different with normal group. Active compounds, especially Chlorogenic acid in coffee, are able to induce the activity of B lymphocytes as part of the natural immune system related with the ability of cells to internalize the phagocytosis process [13].

Conclusion

This study proved that the active compound of Chlorogenic Acid in Robusta coffee beans (*Coffea canephora*) which taken from Sumber Asin Malang area was the higher levels and it gave an effect in every component of natural immune system including phagocytosis mechanisms. The increasing levels of antioxidant was related to the neutralization mechanism of reactive oxygen species (ROS).

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Conflict of Interest: There was no conflict of interests regarding the publication of this study

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References

1. Biondo C, Entini G, Beninati C, Teti G. .The dual role of innate immunity during influenza. Biomedical Journal 2019; 42(1): 8-18
2. Takashi H & Pierre CD. How the innate immune system senses trouble and causes trouble. Clinical Journal of the American Society of Nephrology 2015;10 (8): 1459-1469.
3. Richey T, Foster JS, Williams AD, Williams AB, Stroh A, Macy S., … Wall JS. Macrophage-mediated phagocytosis and dissolution of amyloid-like fibrils in mice, monitored by optical imaging. The American Journal of Pathology 2019;189(5):989-998
4. Stuart L M & Ezekowitz RA. Phagocytosis and comparative innate immunity: learning on the fly. Nature Reviews Immunology 2008; 8(2):131–141.
5. Uribe-Querol E & Rosales C. Control of phagocytosis by Microbial Pathogens. Front. Immunol 2017; 8:1368.
6. Rabadi SM, Sanchez BC, Varanat M, Ma Z, Catlett SV, Melendez JA., … Bakshi CS. Antioxidant Defenses ofFrancisella tularensisModulate Macrophage Function and Production of Proinflammatory Cytokines. Journal of Biological Chemistry 2015; 291(10):5009–5021.
7. Gilljam KM, Holm K, Zahoor M, Centonze FG, Farhan H & Blomhoff HK. Differential Effects of Reactive Oxygen Species on IgG versus IgM Levels in TLR-Stimulated B Cells. The Journal of Immunology 2020; ji1901131. doi:10.4049/jimmunol.
8. Poole R, Kennedy OJ, Roderick P, Fallowfield JA, Hayes PC & Parkes J. Coffee consumption and health: umbrella review of meta-analyses of multiple health outcomes. BMJ 2017; j5024. doi:10.1136/bmj.j5024
9. Król K, Gantner M, Tatarak A & Hallmann E. The content of polyphenols in coffee beans as roasting,

origin and storage effect. European Food Research and Technology 2020; **246**: 33–39.

10. Thimmulappa RK, Lee H, Rangasamy T, Reddy SP, Yamamoto M,Kensler TW and Biswal S. Nrf2 is a critical regulator of the innate immune response and survival during experimental sepsis. J. Clin. Invest 2006; 116:984–995 .
11. Aritanoga M, Effendi C, Herawati L Gayo-Arabica Cofee Decreases MDA and Increases SOD after Single Bout Submaximal Physical Exercise in Sedentary Men. Jurnal Sumberdaya 2019; 5(2): 58-63
12. Tajik N, Tajik M, Mack I & Enck P. The potential effects of chlorogenic acid, the main phenolic components in coffee, on health: a comprehensive review of the literature. European Journal of Nutrition 2017;56(7):2215–2244.
13. Milner ECB, Anolik J, Cappione A, and Sanz I. Human innate B cells: a link between host defense and autoimmunity?. Springer Semin Immunopathol 2005; 26(4): 433–452