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Different doses of purified extract of cacao bean *(Theobroma cacao)* to IgM antibody profile in Wistar rat stimulated by ovalbumin antigen

[1*]Emelda, A., [2]Wahid, S., [2]Massi, N. and [3]Wahyudin, E.

[1]*Faculty of Pharmacy, Universitas Muslim Indonesia, Jl.UripSumaharjo KM.05, Makassar 90231 Indonesia*
[2]*Faculty of Medicine, Universitas Hasanuddin, Jl.Perintis Kemerdekaan KM.10, Makassar 90245 Indonesia*
[3]*Faculty of Pharmacy, Universitas Hasanuddin Jl.Perintis Kemerdekaan KM.10, Makassar 90245 Indonesia*

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Abstract

Cacao bean is a rich source of polyphenol. It possesses antioxidant activity and can modulate an immune system. This study was conducted to test various doses of purified extract of cacao seeds, rich in polyphenols, to IgM antibody profile of wistar white rats on the fifth and seventh day after being stimulated by ovalbumin antigen. The production of IgM antibody on the fifth and seventh day were assayed by Elisa. The oral feeding of the purified extract at a dose of 30 mg/100 g body weight of rats did not elevate the number of IgM compared to the control group. The level of IgM production after administration of purified extract at dose of 60 mg/100 g body weight was increased significantly on the fifth day to the control group. The enhancing of the IgM level on the fifth day also was showed at the dose of 120 mg/100 g to the control group but its IgM level is less than the IgM level of the dose of 60 mg/100 g body weight. This research also presented that the amount of IgM on the seventh day decreased compared to the fifth day. The research showed that the effective treatment is at the dose of 60 mg/100 g body weight of rat.

Introduction

Immunity is a resistance to some kinds of illness, particularly infection. The aggregation of cells, molecules and tissues which has an important role to infection is called immune system. The reaction is coordinated by cells, molecules and other substances of microbe is called immune response. The immune system is needed to maintain its function to hazardous substances by nature. The scientists have been conducting some researches to find a substance produced by nature which is able to strengthen the immune system. One of the substance that is used as antioxidants and can improve the immune system is polyphenol (Arlorio *et al*., 2005).

The use of antioxidant and polyphenol is recommended for preventive purpose. Antioxidant is a microconstituent which is capable to inhibit the oxidation of lipid, by inhibiting the chain propagation in oxidation process and being involved in free radical scavenging. Foods, such as fruits and vegetables, have been reported having antioxidant component from a various variety, especially polyphenol (Katalinic *et al*., 2004; Chedea *et al*., 2010). Cocoa bean possesses a polyphenol content and its antioxidant ability is higher than tea, red wine and some other fruits (Lee *et al*., 2003; Crozier *et al*., 2011). Furthermore, unfermented dried cocoa beans provide higher level of polyphenol and antioxidant than partially fermented dried cocoa beans (Prayoga *et al*., 2013).

Cacao seed has a high content in flavonoid. The flavonoid components of the cacao is not easy to form. They depend on some factors such as geography, climates, and storage condition (Jayasekera *et al*., 2011). The purified extract of cacao seed from west Sulawesi Indonesia contains a total polyphenol of 391 mg/g calculated as tanic acid (Emelda *et al*., 2013). The 4% and 10% of natural diet of cacao showed the secretion of IL-2 in limponodus cell culture, but it showed the decrease of IL-4 production in lymph cell and lymponodus. At the same mouse, it showed an increase of interferon-γ. The result of the research stated that cacao diet lead to down-regulation of Th2 imune response (Ramiro *et al*., 2007).

The mechanism of polyphenol of purified extract of cacao bean *(Theobroma cacao)* in regulating immune system and its effective dose are still unclear. Therefore, this research was conducted to assess the effect of the polyphenol of purified extract of cacao

*Corresponding author.
Email: *a_emelda@yahoo.com*

beans administration on immune response of wistar rats based on the different doses of treatment.

Materials and Methods

Materials

Cacao seeds were collected from Polman regency, South Sulawesi, Indonesia. They were kept in unfermented condition. Assessment of IgM level used Elisa kit (Rat IgM E-25M). The animals were the wistar white rats from the University of Airlangga, Surabaya, Indonesia. This research was conducted in the University of Hasanuddin, Makassar, Indonesia (Ethical clearance: No.0134/H4.8.4.5.31/PP36-KOMETIK/2012).

Extraction and purification

Cacao beans were powdered and extracted by maceration method using n-hexane solvent. The maceration process was done by putting the the powder of cacao seed into maceration chamber then adding n-hexane solvent till the whole samples soaked. The chamber was tightly closed and let for one day while being stirred many times. The samples were filtered then remacerated. The maceration process was repeated until the solvent was colourless. Furthermore, the residue was re-extracted by maceration method with a mixed solvent of acetone and water (70:30 v/v) and then evaporated at temperature lower than 300C. The remaining water in purified extracts was removed by lyophilisation process using freeze drying until obtaining dried purified extracts that is rich in polyphenol content (Emelda *et al.*, 2013).

Animals

The wistar white rats were obtained from faculty of pharmacy, University of Airlangga, six weeks of age and in healthy condition. They were placed on free-pathogen condition and adapted into laboratory condition for two weeks. They were fed with usual nutrition and exposed to light cycle with twelve hours in dark and twelve hours in shiny condition.

The rats were divided into four groups which each group consisted of 25 rats. Group I is a control group. The control groups was a group without purified extract administration. Group II, III, and IV were the treatment groups. The groups were fed orally with the purified extracts of cacao bean daily for 15 – 21 days at following doses respectively: 30 mg/100 g body weight, 60 mg/100 g body weight, and 120 mg/100 g body weight.

Stimulation of ovalbumin antigen

Stimulation of ovalbumin antigen, 10 mg/kg body weight of rat in adjuvant, was intraperitoneally conducted on fifteenth day to the all groups. The bloods of the rats were taken at fifth and seventh day after being stimulated with ovalbumin antigen. Before taking the blood, the rats were firstly given anesthetic by using ether. The blood was then accumulated and centrifuged to produce serum. The serum was then kept at -20^{0}C (Berezo *et al.*, 2009).

IgM antibody secretion test

The assessment of the IgM levels was performed by using Rat Elisa Kit with suitable method according to the KIT procedures. The absorbance was then read by the Elisa reader (microplate model 680, Bio Rad).

Results and Discussion

IgM antibody is a product of humoral immune system. The selection of B cell occurs in primary lymphoid organ that is bone marrow. The activation of B cell is initiated with recognition phase of specific antigen by surface receptors. IgM is the first antigen to be produced according to primary response rather than IgG. Most of B cells express the IgM surface as antigen receptors (Abbas *et al*., 2012).

The assay of secretion of IgM antibody in rats was carried out from polyphenol compound of cacao bean. It has been reported that the polyphenol total of purified extracts of cacao bean with high level is 39 mg/g calculated as tanic acid (Emelda *et al*., 2013) and the total flavonoid is 272 mg/g calculated as cathecin (Emelda and Wahyudin, 2014). Furthermore, the IgM level was determined by Elisa kit on the fifth and seventh day after the rats being stimulated by ovalbumin antigen. The data were analyzed statistically by one way analysis of variance (ANOVA) using SPSS 20.

Figure 1 dan Table 1 show the IgM immune response between groups of varying doses and the control group on the fifth and seventh day after being stimulated by antigen ovalbumin. It has been shown that administration of the purified extract at a dose of 30 mg/100 g body weight of rat did not enhance the production of IgM on the fifth and seventh day compared with the control group ($P>0,05$). In contrast, administration of the purified extract at a dose of 60 mg/100 g body weight elevated significantly ($p<0.01$) the number of IgM on fifth day compared to the control group. These results are supported by previous study (Berezzo *et al.*, 2009) in which the dose of 4% and 10% natural dietary cocoa (in vivo) showed production of IL-2 were higher

Table 1. Mean ratio and standard deviation of serum IgM levels in control group and treatment groups

Sample	Levels of IgM (µg/ml)	
	Fifth Day Mean ± SD	Seventh Day Mean ± SD
Control	322,59 ± 52,836	302,31 ± 113,11
Dose of 30 mg/100 gr Body Weight Rat	299,12 ± 13,042	306,23 ± 93,688
Dose of 60 mg/100 gr Body Weight Rat	668,02 ± 204,92	430,43 ± 200,72
Dose of 120 mg/100 gr Body Weight Rat	422,95±76,573	236,17 ± 25,260

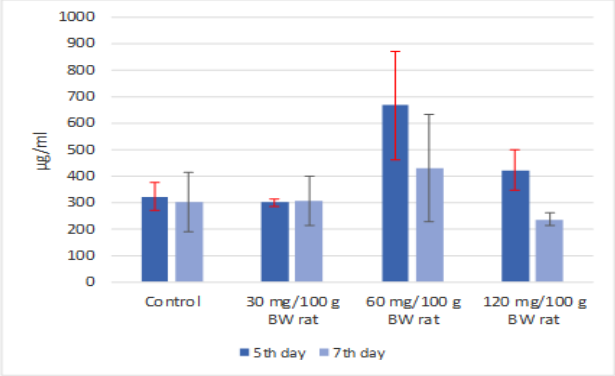

Figure 1.Production of IgM antibody

than control on the response of cells culture of lymph node cells that are activated by PMA/ionomycin *(in vitro)*. The IL-2 increases the proliferation and differentiation of others immune cells (NK cells, B cells, T cells) (Abbas *et al*., 2012). Furthermore, the level of IgM on seventh day did not significantly increase ($p>0.05$) compared to the control group. The decrease level of IgM on the seventh day might be caused by the antibody isotypes switching to another antibody (IgG) (Abbas *et al*., 2012). At a dose of 120 mg/100 g body weight of rat of purified extract, the level of IgM rised on the fifth day compared to the control group (P>0,05) but its number was lower than the dose of 60 mg/100 g. On day seventh, the amount of IgM level dropped and was lower than the control group (P>0,05).

Conclusion

The administration of different doses of purified extract of cacao beans has different effects on the production of IgM antibody in rat. The IgM level increases significantly at a dose of 60 mg/100 g body weight of rat on the fifth day. Therefore, the effective treatment is at the dose of 60 mg/100 g body weight of rat.

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