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Research Article

Optimum Dose and Formulation of Centella asiatica L. Urban Extract Against IgG of Wistar Strain Male Mices which Induced by BCG Vaccine

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ABSTRACT

Objective: *Centella asiatica* L.Urban contains triterphenoid saphonin, flavonoid, and pectin that believed to have an immunomodulatory effect toward the Immunoglobulin-G in Wistar strain male mices which Induced by BCG vaccine. A study was conducted to find out the optimum dose and formulation of in enhancing on the level of IgG. It's therefore expected that *Cantella asiatica* L.Urban can be used as an immunomodulatory suplement in both animals and human.

Methods: *Centella asiatica* L.Urban were macerated with ethanol 50%. This was an experimental study with the post-test control group design. The samples in this study were 35 Wistar strain male mices which divided into 7 groups: negative control [Aquadest 0,5mL/20g BW]; control immunosupresant [Prednison® 0,06mg/20g BW]; control immunostimulant [*Levamisol*® 0,445mg/20g BW]; ethanolic extract of *Cantella asiatica* L.Urban with a dose of 50mg; 100mg; 150mg; 200mg/Kg B.W were administered orally fo 10 day, after the treatments implemented, BCG [Bacillus Calmette Guerin] vaccine was infected intraperitoneally on day 10^{th}, 14^{th}, 21^{st}, and 28^{th}. The most effective doses of ethanolic extract of *Cantella asiatica* L.Urban was further studied for its effect on hormonal assay using ELISA reader which compared with the effects of Prednison® and Levamisol®. Based on simplex lattice design to find the optimum proportion of coefficients a, b, and ab of the equation Y = a (A) + b (B) + ab (A) (B), where A is lactose and B is amylum.

Results : Oral administration of ethanolic extract of *Cantella asiatica* L.Urban at dose of 50mg and 100mg/Kg B.W increased the immunity which showed elevated levels of IgG in the blood serum in Wistar strain male mices that had been induced by BCG vaccine. The optimum formula capsul of Centella asiatica L.Urban extract that use combination of 80% Amylum and 20% Lactose which produce the optimum parameters of disintegration time (≤15minutes) and CV of weight uniformity (≤5%)with the test results had no significant difference to the prediction results of *Simplex Lattice Design* [p>0.05].

Keywords: Centella asiatica L.Urban extract, Immunomodulator, simplex lattice design

1. Introduction

Tuberculosis is an infectious disease caused by the bacterium *Mycobacterium tuberculosis*[13]. Most infections do not have symptoms, about 10% of latent infections progress to active disease which, if left untreated, kills about half of those infected. The classic symptoms of active tuberculosis are chronic cough with blood containing sputum, fever, night sweats, and weight loss[13]. In the world population is thought to be infected with tuberculosis, new infections occur in about 1% of the population each year. In 2014, there were 9,6 million cases of active tuberculosis which resulted in 1,5 million deaths. More than 95% of deaths occurred in developing countries such as Indonesia, China, and India. The number of new cases each year has decreased since 2000[14].

Centella asiatica L.Urban, contains not less than 6% of total triterpenoid derivatives expressed as asiaticoside [9,10]. *Centella asiatica* L.Urban more commonly called pegagan, has been famous for traditional medicine for years. Pegagan is also used for wound healing and improving memory span. It can also increase hyperplasia cell activity and the existence of collagen in wound tissue[1]. Jayathirta and Mishra, 2004[5] believed that *C. asiatica* extract from 100 to 500 mg/kg BW in mice could significantly increase the total of white blood cells and macrophage phagocyte ability against carbon molecules in those mice. Yun Astuti, 2004[12] said that immunostimulatory effect of Centella asiatica L. Urban induced IgG in sheep red blood cells. The study suggests that Centella asiatica L. Urban contains enzymes proxeronase, an alkaloid proxeronase that this enzyme in the intestinal wall will change the proxeronine into xeronine active substances to be absorbed into the bloodstream to cells and other tissues. These activities can enable active proteins so that antibody production is going well for the main constituent is a protein antibody[6].

The aims of this study were what the ethanolic extract of Centella asiatica L. Urban has an influence on titter immunoglobulin [IgG] and to get the optimum formula of capsul of Centella asiatica L. Urban. The results of this study are expected to be one of the scientific based for the use of Centella asiatica L. Urban in improving of health (immunity), and it may be developed into a fitofarmaka dosage form.

2. Materials and Methods

2.1. Collection and Preparation of Centella Asiatica L.Urban

The Centella asiatica L. Urban were collected from Kaliurang Km.19, Sleman, Yogyakarta, Indonesia on March 2008, identified and authenticated by experts in plant morphology and taxonomy at the Laboratory of Faculty of Biology, Gadjah Mada University, Yogyakarta, Indonesia. The Centella asiatica L. Urban herb ovened at 50±2°C then powdered using *National*® blander. Centella asiatica L. Urban powder masetaed with ethanol 50% for 3 days and remaserated for five days then filtered. The filtrate was evaporated to near dryness on a water bath, weighted and kept at -4°C in refrigerator [*Toshiba*®] until further use.

2.2. Phytochemical Screening

The presence of various constituents in the Centella asiatica L. Urban extract (triterpenoid) were determined by TLC. Centella asiatica L. Urban extract of 25,0 mg was dissolved in 50,0 mL of ethanol 50% [Bratachem], vortexed for one minutes and then filtered. The filtrate then spotted onto silicagel 60 $F_{254.}$ Mobile phase was BAW (Butanol : Asam Asetat : Air) 4 : 1 : 5, and after elutioned, the TLC was sprayed with Vanilin H_2SO_4 and *Lieberman burchard* reagent, then dried in an oven with temperature of 110±2°C for five minutes. It was then monitored under UV light (UV_{254} ang UV_{366}) then calculated the Rf value of terpenoid group[7].

2.3. Group Treatment of Wistar Strain Male Mices

Wistar strain male mices [laboratory of pharmacology, Faculty of Pharmacy, UGM, Yogyakarta, Indonesia] of 35 were separated into 7 groups comprising of 5 mices each. The mices were treated with extract or reference drug for 10 days before induced with BCG vaccine [Biofarma Laboratories, Indonesia][12].

Group I: Aquadest served as control neutral [p.o daily 0,5mL/20 g B.W]

Group II: Immunosupresant [Prednisone® p.o. daily 0,06mg/20 g B.W]

Group III: Immunostimulant [Levamisole® p.o. daily 0,455mg/20 g B.W]

Group IV: Centella asiatica L. Urban extract p.o. daily doses of 50mg/Kg B.W

Group V: Centella asiatica L. Urban extract p.o. daily doses of 100mg/Kg B.W

Group VI: Centella asiatica L. Urban extract p.o. daily doses of 150mg/Kg B.W

Group VII: Centella asiatica L. Urban extract p.o. daily doses of 200mg/Kg B.W

2.4. Vaccinated of Wistar Male Mices All Group Treatment

At these studied to analysis antibody [IgG] with ELISA reader. Before 10 days of treatments to mices of all respective groups, the blood from vena orbitalis were taked in to effendrof 1,5 mL, then sentrifuges for 10 minute at 14.000 rpm. The blood serum from each groups were kept at -80°C in refrigerator [Toshiba®] until analysis. At days 10^{th} all mices of respective groups were vaccinated intraperitoneally with BCG vaccine at doses of 6,5 µL/20g B.W and replayed at days 21^{st} and 31^{st}. The blood from vena orbitalis were taked in to effendrof 1,5 mL after vaccinated at days 14^{th}, 28^{th}, and 32^{th} then sentrifuges for 10 minute at 14.000 rpm. The blood serum from each groups were kept at -80°C in refrigerator until analysis with ELISA reader.

2.5. ELISA Reader: Antibody Assay

Microtiter plate coated with BCG vaccine at levels of approximately 2,0 ug/mL in carbonate buffer of 100,0

mL each of the wells and incubated overnight at temperature of 40±2°C. Washed the wells with PBS + 0.05% Tween 80 three times and added a solution of 1% BSA in 100,0 mL of PBS each of the wells and incubated 1 hour at temperature of 370°C, then washing it again with PBST each of the wells three times[8].

Blood serum of mices added in to the each wells of 100,0 mL which had been diluted 100 times using PBS/H_2O in each of the wells and incubated for 1 hour at a temperature of 370°C, then washed again with PBST. Conjugate alkaline phospatase was added of 100,0 mL then diluted 3.000 times with PBS and incubated for 1 hour at temperature of 370°C. Washed again with PBST each wells three times. OPD (Phenil Nitro Phosphate) was added once, then blended with OPD buffer (Diethanolamin) and also added 10,0 mL of H_2O_2 and waited for 10 minutes in the dark room. The reaction was stopped using H_2SO_4 and observed the color formed. After that, the analysed by ELISA Reader at a wavelength of 405 nm as the final stage of the experiment[8].

2.6. Formulation of Capsules with Experimental Design

Based simplex lattice design to find the coefficients a, b, and ab of the equation Y = a (A) + b (B) + ab (A) (B) it is necessary to study three formulas. In this study, the total weight of capsules are made of 500 mg, the optimum doses of *Centella asiatica* L. Urban extract was used each capsule. The formulation based on simplex lattice design[2], presented in table 1.

Table 1. Formula of Centella Asiatica L. Urban Based on Simplex Lattice Design

Components of Capsule Formula	*Simplex Lattice Design*		
	A	B	C
***C.asiatica* L.Urban extract**	Optimum dose	Optimum dose	Optimum dose
Lakctose	100%	0%	50%
Amylum	0%	100%	50%
Gelatine	qs	qs	qs
Mg Stearate	qs	qs	qs
Talk	qs	qs	qs

A Simplex Lattice Design (SLD) was adopted to optimize the formulation variables. In order to optimize the preparation of formulations, the amount of Lactose [Bratachem] (*A*), and amount of Amylum [Bratachem] (*C*), were chosen as independent variables. The average of weight uniformity of capsules (X_1), and disintegration time (X_2) were taken as response variables. Manual equation results to describe the relationships among the factors on dependent variables are expressed by mathematical equations[2].

$$R_{total} = R_1 + R_2 + R_3 + \ldots + R_n \quad \ldots\ldots\ldots\ldots\ldots(1)$$

Where $R_{1, 2, 3, n}$ is the response of the physical properties of the granules, each response given value. Total value was 1. If the units of each response is not the same, it is necessary to standardized response assessment using the formula :

$$N = \frac{X - Xmin}{Xmax - Xmin} \quad \ldots\ldots\ldots\ldots\ldots(2)$$

Where :

X = responses obtained from experiments
Xmin = minimum desired response
Xmax = maximum desired response

R is calculated by multiplying N by a predetermined value. R_{total} calculation becomes : R_{total} = (Value x N weight uniformity) + (Value x N disintegration time). Optimum formula determined by looking at the highest response rates[2].

DATA ANALYSIS

Student's T-Test at p-value ≥ 0,05 significance level was used to evaluate data homogenity. While, the group differences were analyzed by using p-value ≥ 0,05 0,05 significance level in analysis of variants. All of data were analyzed by statistic analysis using Microsoft Excel®.

3. Results and Discussion

3.1. Phytochemical Screening

Chromatogram with vanilin sulfuric acid reagent formed five spots of color is pink, green, purple, green blue and light green. Rf value of 0,75 with color purple is a positive color for terpenoids that allegedly spotting the compound of class of terpenoids. In the chromatogram with reagent Liebermann-Burchard formed eight spots of color compounds. Rf value of 0,75 on leave green color indicates a positive reaction to terpenoids. There suitability of the results of the chromatogram with coloring vanilin sulfuric acid and Lieberman-Burchard that area at Rf value of 0,75 suspected to be spotting compound from the class terpenoids[7]. Based on reference that Centella asiatica L.Urban, triterpenoid saponins present in it possesses immunomodualtory activity [5,10]. Preclinical studies performed in mice have reported that the alcoholic extract of the Centella asiatica L.Urban when injected intravenously known to possess stimulatory effect on reticuloendothelial system followed by 24 hour latency period[3].

3.2. ELISA Reader

The aim of BCG vaccination are to stimulate proliferate of lymphocytes and diffuses into plasma cells capable of producing antibodies. Vaccination is done three times to the more selective B-cell antigen recognition and memory cells formed[6]. The blood serum of 35 mices in this study were tested for IgG on ELISA test. The result of IgG level can be seen in figure 1.

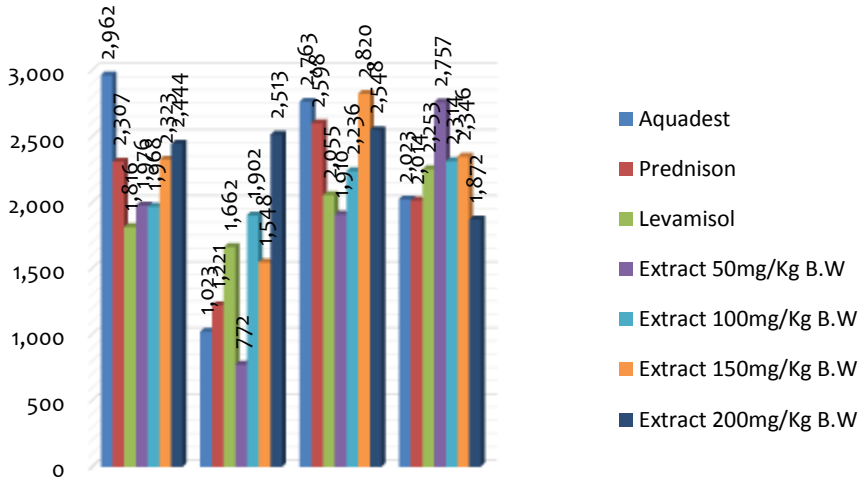

Figure 1. IgG Level from Each of Mices Group Treatments During Tree Times BCG Vaccine in Blood Serum

Table 2. The absorbance value of blood serum of mices each treatments groups at 405 nm that analysed using ELISA reader

Group Treatment	Serum I	Serum II	Serum III	Average±S.E
Aquadest	1, 14	2, 71	1,73	1,86±0,65
Prednisone®	1,22	2,60	2,01	1,94±0,57
Levamisol®	1,66	2,06	2,25	1,99±0,25
C. asiatica 50 mg	1,39	1, 74	2,63	1,92±0,52
C. asiatica 100 mg	1,81	1,76	2,41	1,99±0,29
C. asiatica 150 mg	1,58	2,60	2,25	2,14±0,42
C. asiatica 200 mg	1,93	2,51	1, 76	2,06±0,32

****S.E p ≤ 0,05**

IgG is a type of antibody that had two antigen binding sites. Representing approximately 75% of serum antibodies in humans, IgG is the most common type of antibody found in the circulation, created and released by plasma B cells. Antibodies are major components of humoral immunity. IgG is the main type of antibody found in blood and extracellular fluid allowing it to control infection of body tissues. By binding many kinds of pathogens such as viruses, bacteria, and fungi, IgG protects the body from infection[6].

The level of IgG each group treatments fluctuated depending on the dose of Centella asiatica L.Urban extract that given. Based on figure-1 and table-2 may confirm that Prednisone was IgG suppression effect that can be trusted just for vaccination I, while for vaccination II and III can be compared with Levamisole. All groups treatment of Centella asiatica L.Urban extract in vaccination I can stimulate level of IgG with optimal doses of 50 mg/Kg B.W and 100 mg/Kg B.W. Centella asiatica L.Urban extract in vaccination II and III can stimulate level of IgG proved as greater than Levamisole. After vaksinaksi III the level of IgG in groups of Centella asiatica L.Urban extract at doses of 200 mg/Kg B.W has down. According to index imunomodulatory, can be classified into tree level of IgG in blood serum there were: low [level of IgG serum 1,1-1,2], medium [level of IgG serum 1,2-1,4], and high [level of IgG serum >1,4]. Based on ELISA reader analysed of IgG serum level in Centella asiatica L.Urban extract all variant doses classified immunostimulant[5].

3.3. Optimum Formula of Centella Asiatica L.Urban Extract

Based on ELISA reader analysed, in formulation of capsule of Centella asiatica L.Urban extract, used the optimum doses of 50-100mg/Kg B.W as active ingredient of imunostimulant suplement. Doses for human was 388 mg-776 mg each capsule. Analysis of simplex lattice design used linier model for respone variable of average of weight uniformity of capsules (X_1), and disintegration time (X_2) that shown in Table 4.

Table 3. Caracteristic of Optimized Formulation

Response variable	Formula		
	A	B	C
Average of weight uniformity (%)	390±1,8%	433±2%	377±2,6%
Disintegration time (minutes)	20,74 ±0,23	24,55±0,53	18,85±0,70

****S.E p ≤ 0,05**

Table 4. The analysis based on Simplex Lattice Design [response variable]

Response variable	Matematical equation	Matematical modelling
Average of weight uniformity	Y = 0,93 (A) + 4 (B) + 4,54 (A)(B)	linier
Disintegration time	Y = 16,76 (A) + 23,89 (B) + 2,38(A)	linier

The composition of amylum in the mix formula made the size of granule become bigger and rised the amount of it, so made the CV [coefisient variation] value of weight uniformity becomes higher than only 100% of lactose in formula which was dominated by small-sized granules. Increasing the proportion of amylum made disintegration time become longer, because amylum condensed period would block the penetration of water into the capsule.

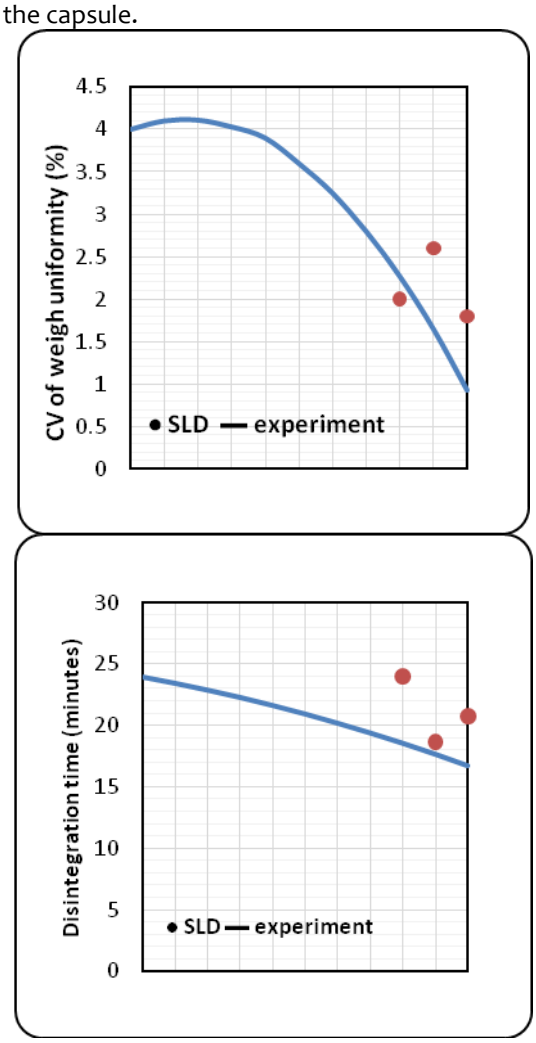

Figure 2. Diagram plot of CV of weight uniformity and disintegration time of optimum formula granul of Centella asiatica L. Urban extract based on prediction of Simplex Lattice Design and Experiments

The experiment test results of physical properties of formula optimum capsule of Centella asiatica L. Urban compared with a prediction of SLD approach results using statistical analysis students t-test. The combination of 80 % amylum and 20 % of lactose was the optimum proportion, base on the analysis of Simplex Lattice Design with statistic analised using student's t-test [p>0.05]. Students t-test was conducted to determine significant differences between the experiment formula with the predictions of the SLD approach.

4. Conclusion

Oral administration of ethanolic extract of *Cantella asiatica* L.Urban at dose of 50mg and 100mg/Kg B.W increased the immunity which showed elevated levels of IgG in the blood serum in Wistar strain male mices that had been induced by BCG vaccine. The optimum formula capsul of Centella asiatica L.Urban extract that use combination of 80% Amylum and 20% Lactose which produce the optimum parameters of disintegration time (≤15minutes) and CV of weight uniformity (≤5%) with the test results had no significant difference to the prediction results of *Simplex Lattice Design* [p> 0.05].

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