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RESEARCH ARTICLE

Immunostimulatory Activity of Ethanol Extract from *Calotropis gigantea* L. Flower in Rats against *Salmonella typhimurium* Infection

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ABSTRACT:

Calotropis gigantea L. flower contains a high value of flavonoid and polyphenol that potentially as an immunostimulatory agent. This research has been conducted to observe and acquire the immunostimulator effect of ethanol extract from *Calotropis gigantea* L. flower (EECGF) in rats, which were induced with *Salmonella typhimurium* (*S. typhi*). Rats were grouped into six, namely normal, negative control, positive control, EECGF dose 40mg/200g, 80mg/200g, and 120mg/200g body weight. Rats were treated for 12 days according to their respective groups, on the sixth day, rats were induced by *S. typhi* except for the normal group. Three days after infection, the Widal test was evaluated, and on the 12th day, an immune system parameter was evaluated. The results obtained, EECGF can increase the value of leukocytes, lymphocytes, monocytes, and neutrophils, followed by an increase in CD_4 T cell values proportionally due to *S. typhi* infection. It was concluded that EECGF had immunostimulatory activity, EECGF dose 80mg/200g had the same character with positive control. However, the highest dose of extract 120mg/200g can cause splenomegaly.

KEYWORDS: *Calotropis gigantea* L., immunostimulator, CD_4 T cell, leukocytes, *Salmonella typhimurium*, chemometrics.

INTRODUCTION:

Humans have the risk of being infected with various types of microorganisms from food with a low level of hygiene. In contrast to diabetes, which is more on metabolic function problems[1]. Microorganism infections can be transmitted through food, for example, *Salmonella typhimurium* (*S. typhi*), which can cause typhoid fever[2,3,4]. Infectious agents such as *S. typhi* that enter the body can inhibit the function of phagolysosomes so that these bacteria are difficult to remove[5]. This bacterial infection can cause splenomegaly one week after entering the blood circulation. *S. typhi* will activate CD_4 T cells by specific immune system mechanisms through primary histocompatibility complex class 2 (MHC-II) and facilitate macrophages to carry out phagocytosis[6,7]. Therefore, a strong immune system is needed to prevent and accelerate the elimination of this infectious agent.

Immunostimulator is a material that can enhance and improve immune function. Immunostimulator agents from natural ingredients have been developed and applied to prevent or as adjunct therapies[3,8,9]. One of the potentials as an immunostimulator agent is the *Calotropis gigantea* plant[10,11]. There are flavonoids, phenolic, alkaloid, sterol, tannin, and anthraquinone compounds in flowers from *Calotropis gigantea*[12,13]. The ethanol extract of this flower is known to have a high flavonoid and phenolic content[14]. This is supported by the presence of free radical scavenger activities[15], hepatoprotective[16,17], and has acted in protection against mast cell degranulation[18]. Flavonoids can increase lymphocyte proliferation, which affects CD_4 T cells and Th_1 (T-helper) activation so that macrophages are activated and increase phagocyte activity to kill bacteria or pathogenic microorganisms[5,6,19].

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Theoretically, flavonoids and phenolics have proven to be useful as immunostimulator agents. However, as far as our scientific knowledge is concerned, there is no publication of interest from *Calotropis gigantea* as an immunostimulator agent, especially in rats infected with *S. typhi*. Therefore, ethanolic flower extract from *Calotropis gigantea* flower (EECGF) is important to investigate its immunostimulatory activity in rats infected with *S. typhi* bacteria. Immunostimulator parameters measured included CD_4 T cells, leukocytes, lymphocytes, monocyte, neutrophils, and macroscopic spleen. It is expected to be developed in nutraceutical and pharmaceutical products as an immunostimulatory agent.

MATERIAL AND METHODS:

Extract Preparation:

The EECGF preparation procedure follows the existing method with some modifications[12]. Fresh *Calotropis gigantea* flowers were collected from Panjang, Bengkulu Indonesia, and identified by certificate number 290/K-ID/ANDA/XI/2015 at the Herbarium Laboratory of Universitas Andalas, Padang Indonesia. The dried flower was macerated using 96% ethanol (Bratachem) solvent. Maceration results are filtered and evaporated with a rotary evaporator (Buchi, Germany) at a temperature of 70°C.

Condition and Design Animal:

The use of rats as test animals has received ethical code with certificate number 144/kepkrsmhfkunsri/2016. Rats were divided into six treatment groups, namely normal, infection, positive control, used a branded product containing Phyllanti extract at a dose of 0.54mL/200g, EECGF therapeutic dose of 40, 80, and 120mg/200g. Rats were adapted for seven days by providing standard drinking and eating and the treatment process for twelve days. Bacterial infection was carried out on the 6th day of all groups except normal rats by giving 0.5mL of *S. typhi* suspension of 10^5 CFU/mL intraperitoneal[2,3]. Widal test is done after three days of infection. The number of leukocytes, CD_4 T cells, lymphocytes, monocyte, neutrophils, and macroscopic spleen were examined on the 12th day.

Widal Assay:

As many as 1mL of blood was taken trough *retro-orbital plexus* and serum inserted into the tube. Blood samples were left to clot and centrifuged at 5000rpm for 10 minutes. Blood plasma is contained in the upper layer was taken using a micropipette and put into a plastic sample tube. A total of 20mL of serum is dripped onto each circle contained on the surface of the glass slide, added one drop reagent, and observed Widal agglutination occurs. Dilution is done until there is no agglutination when mixed Widal serum and reagents (Primaco)[20,21].

Determination of CD_4 T Cells:

A total of 25μL of blood was taken using a micropipette and put into a CD_4 Pima test cartridge (Abbott, US). The sample volume is observed until it reaches the maximum limit. Measurements were made by inserting a cartridge into the Alere PimaTM (Abbott, US). Data results were obtained in the form of CD_4 T cell counts in μL cells/blood units.

Determination of Total Leukocytes:

A 50μL blood sample is inserted into the sample withdrawal tube contained in the Sysmex KX-21N hematology analyzer (Sysmex, Kobe, Japan) and press the sample bar. The calculation of total leukocytes is processed when the withdrawal hose enters the sample automatically into the device. The results obtained in the form of total leukocytes 10^3 cells/mL.

Determination of Lymphocytes, Monocytes, and Neutrophils:

Smear preparations are made by dripping a sample of fresh blood on a glass object. After being allowed to dry, the preparation was fixed with methanol for 5 minutes. The preparations were stained using Giemsa coloring (1:9 dilution with phosphate buffer pH 6.8 - 7.2) for 30 minutes. Observation and calculation of lymphocytes, monocytes, and neutrophils are done under a microscope Olympus CX21 (Olympus, UK) with a magnification of 40 times.

Evaluation Macroscopic of Spleen:

The macroscopic examination of the spleen includes the size, weight, color, and consistency of the spleen. The identification of each parameter follows the existing protocol[22].

Data Analysis:

The data are presented in mean±SD, and statistical analysis using software assistance (SPSS Inc., Chicago, IL, USA). A value of $p<0.05$ was expressed where and indicated there were differences between treatment groups. All groups and responses were analyzed using a chemometric approach with principal component analysis (PCA) (Minitab, State College, PA, USA).

RESULT AND DISCUSSION:

Widal Test:

Increased body temperature is a clinical represent of typhoid fever that occurs when infected with *S. typhi* bacteria. Increased body temperature occurs to eliminate bacteria triggered by thermoregulators in the hypothalamus. An increase in body temperature indicates the activation of the immune system[23].

Widal test is done on the 3rd day after infection by identifying the antibodies that are formed. All infected

rats had *S. typhi* antibodies at 10mL observation with an antibody titer of 1:160. The agglutination that occurs indicates a positive Widal test and indicates a clinically significant level of antibody response in the serum. Widal tests generally show positive results one week or more after infection with bacteria through the digestive tract. However, in this study, it occurred on the 3rd day after infection because the induction of *S. typhi* was done intraperitoneally. This modified induction procedure causes within 24 hours the bacteria will enter the blood and form antibodies more quickly.

Evaluation CD_4 T Cells:

CD_4 T cells are a type of lymphocyte cells with CD_4 markers on their cell surfaces, which are the most critical part of the immune system. Optimal CD_4 T cell levels characterize an excellent immune system. The choice of CD_4 T cell immunity parameters is based on its primary function as a system that regulates the formation of a specific immune system against certain microbes.

Based on Fig.1, there was an increase in the average number of CD_4 T cells in the EECGF treatment group. CD_4 T cells increase with increasing dose of EECGF given. This pattern of increasing CD4 T cells is similar to the case of aqueous extract of *Vernonia amygdalina* in rats that are not antigen-induced[24]. EECGF doses of 120 mg/200 g showed the highest increase in the number of CD_4 T cells compared with doses of 40 and 80 mg/200 g. Based on statistical analysis, CD_4 and leukocyte data were normally distributed ($p>0.05$), but the variation of the data was not homogeneous ($p<0.05$). Therefore, the analysis used the Kruskal-Wallis test and obtained $p<0.05$, which means that there were significant differences between treatment groups. The increased dose of extract causes an increase in the effect of immunostimulators in the body. The EECGF group also had an average CD_4 T cell count higher than the normal and infection groups, and this showed the extract was able to boost the immune system.

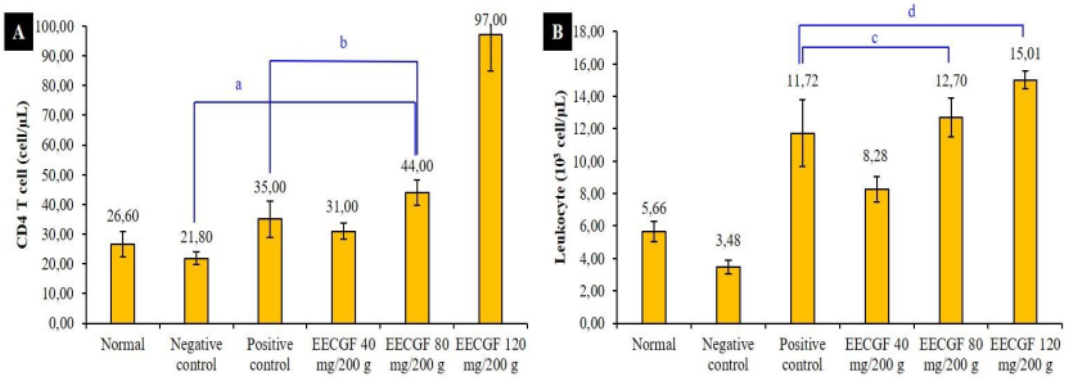

Fig.1: The Results of Measurements of CD_4 T cells (A) and Total Leukocytes (B) in Each Treatment Group, Mann-Whitney Test with significance value; (a) 0.008, (b) 0.043, (c) 0.347, and (d) 0.016

Increased CD_4 T cells can be influenced by active compounds of flavonoids and polyphenols in EECGF. Flavonoids and polyphenols act as immunostimulators by increasing IL-2 activity and lymphocyte proliferation[9,25]. Lymphocyte proliferation that is affected by CD_4 T cells will cause Th_1 cells to be activated. Activated Th_1 cells affect IFNγ, which can activate macrophages that function to phagocyte antigens. CD_4 T cells also play a role in the formation of antibodies as an identifier in the event of repeated infections[24,26]. Alkaloid compounds found in *Calotropis gigantea* flowers can also inhibit bacterial growth so that the activity of *S. typhi* can be suppressed.

Evaluation Leukocytes Total:

Antigens (*S. typhi*) that enter the blood circulation will stimulate leukocytes to eliminate them. Therefore, leukocytes become a crucial parameter in evaluating EECGF immunostimulatory activity[27,28,29]. Based on the results shown in Fig.-1b, there was an increase in total leukocytes in the EECGF group. Total leukocytes tend to increase with increasing doses. This indicates that the specific immune system activity is formed. The flavonoid content in EECGF acts as an immunostimulator, which can accelerate the formation of the immune system[30,31].

The EECGF group, with a dose of 120 mg/200 g, had the largest total leukocytes with a high CD_4 T cell count. Products with Phyllanti extract, as a positive control, has high leukocytes, but lower CD_4 T cell counts. In this condition, it is suspected that the non-specific immune system is more dominant, while in EECGF, the formation of specific immune systems occurs more quickly to eliminate antigens. Total leukocyte data alone cannot provide specific information about immune

status, so it is necessary to calculate the number of leukocyte cell differentiation, namely lymphocytes, monocytes, and neutrophils[32].

Lymphocytes, Monocytes, and Neutrophils:

Lymphocytes are cells that play a significant role in the specific immune system. Each T cell can only interact specifically with antigens that are present on the surface of the antigen-presenting cell (APC) that binds to the major histocompatibility complex (MHC). Observation lymphocytes have large round nuclei that occupy most cells. Sizes vary from 7 to 15 microns. Monocytes can phagocyte and develop into macrophages when they come out of blood vessels and enter tissues. Macrophages also function in processing antigens that are induced at an early stage in the initiation of an immune response[33,34]. Chemotactic factors pull monocytes into damaged tissue or microbial invasion. Monocytes are classified as mononuclear system cells that play a role in phagocytosis of antigens, destroying foreign particles, and dead tissue and then processed to evoke immune responses. Monocytes have round or long cell nuclei, such as kidneys or horseshoe-like, and have deep indentations. The percentage of normal monocytes is 2-10% of the total components in white blood cells. Neutrophils were chosen as one of the parameters of immunity because of their ability to recognize pathogens directly. Neutrophils are capable of destroying microbes through independent oxygen pathways (lysozyme, lactoferrin, ROI, proteolytic enzymes, cathepsin G, and cationic protein) and oxygen.

Lymphocyte, monocyte, and neutrophil cell profiles are presented in Fig.2. Lymphocyte cells will increase at increasing EECGF doses. A comparison of the number of monocytes and neutrophils is presented in Fig.2b and 2c. When compared with the normal group, the administration of positive control and EECGF provides an increase in all responses determined by either lymphocytes, monocytes, or neutrophils.

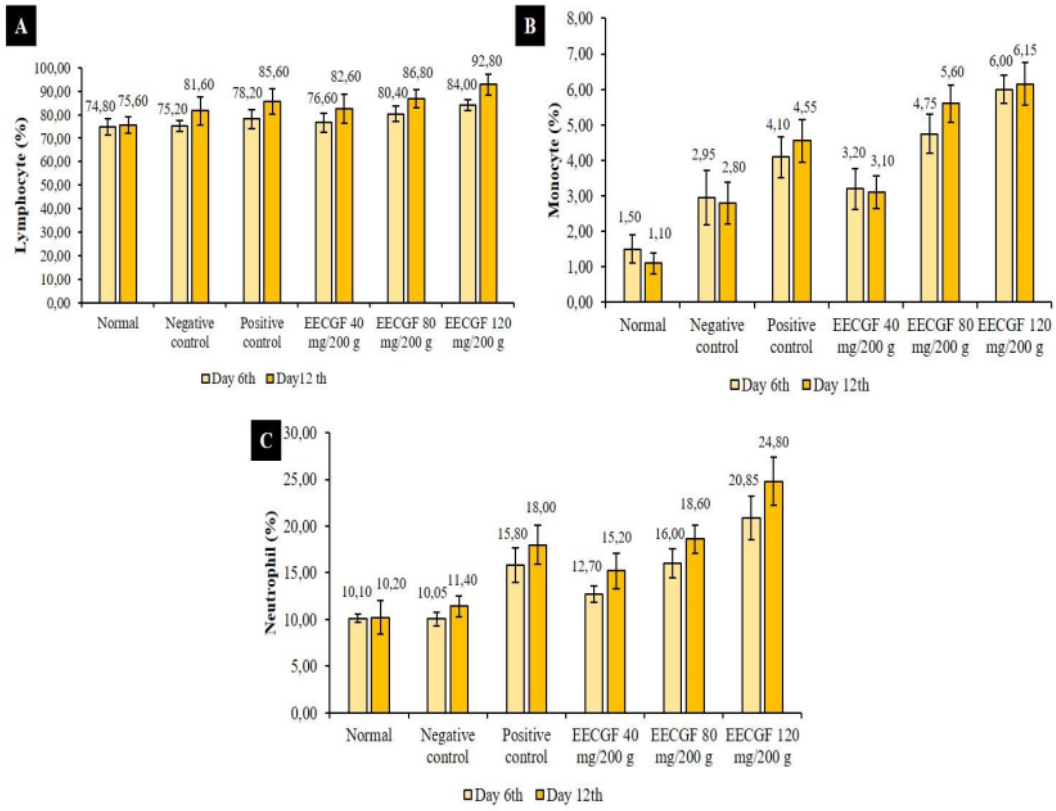

Fig.2: Measurements of Lymphocytes (A), Monocytes (B), and Neutrophils (C)

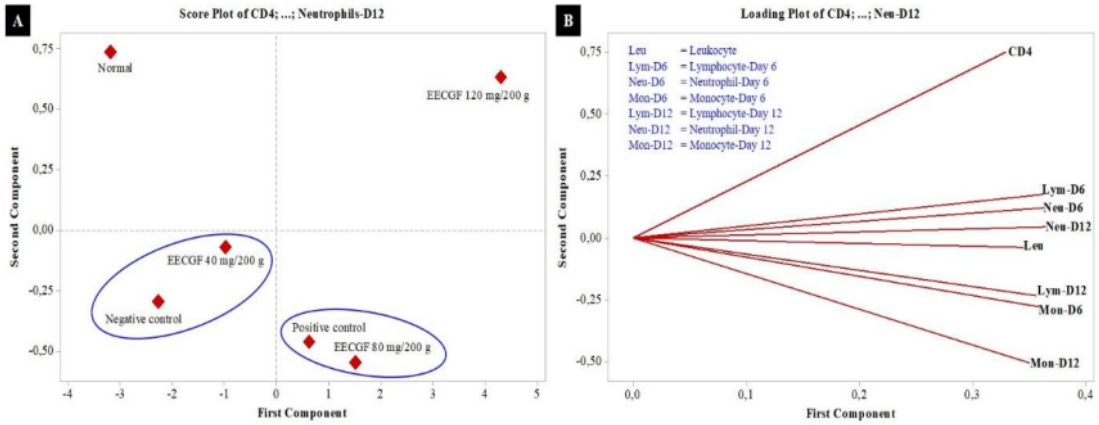

Fig.3: The Results of Principal Component Analysis, (A) Score Plot, and (B) Loading Plot

The chemometric approach with the principal component analysis (PCA) technique obtained results such as Fig.3. This analysis allows us to be able to enter the overall response of all treatment groups and analyze simultaneously. The score plot (Fig.3a) depicts groups with high similarity to be close together[35]. The positive control with EECGF 80mg/200g had similar properties based on the variables used. Variables or values observed from CD_4 T cells, leukocytes, lymphocytes, monocytes, and neutrophils in the two groups are similar. These results indicate that the administration of extract samples at a dose of 80mg/200g body weight can have pharmacological effects similar to the positive controls used. The infection group and EECGF dose 40 mg/200 are located at adjacent points. EECGF dose of 40mg/200g has not had pharmacological effects because it has properties similar to the infection group.

The loading plot (Fig.3b) is used to reinforce the correlation between the variables being evaluated. The formation of a narrow-angle shows a positive correlation. Some variables that are positively correlated are lymphocytes (day 6) with neutrophils (day 6), neutrophils (day 12) with leukocytes, and lymphocytes with monocytes (day 6). The chemometrics analysis is constructive in grouping and can easily explain the relationship between responses[36,37,38].

Evaluation Macroscopic of Spleen:

The spleen is a lymphoid organ that plays a role in the formation of the immune system, which produces lymphocytes B, T, and macrophages. T cell proliferation and the presence of intracellular microorganisms such as *S. typhi* can cause splenomegaly. Evaluation of spleen organs of mice was carried out on a macroscopic change consisting of changes in shape, color, consistency, and weight of the organ.

Macroscopic observation of the spleen (Fig.4f) showed that the EECGF 120 mg/200 g group was abnormal, the spleen enlarged, and the color darkened. The spleen profile in the EECGF 120 mg 200 g group (Fig.4f) was also similar to the group of infectious mice (Fig.4c). The evaluation results in Fig.4, EECGF doses of 40 and 80 mg/200 g show normal spleen.

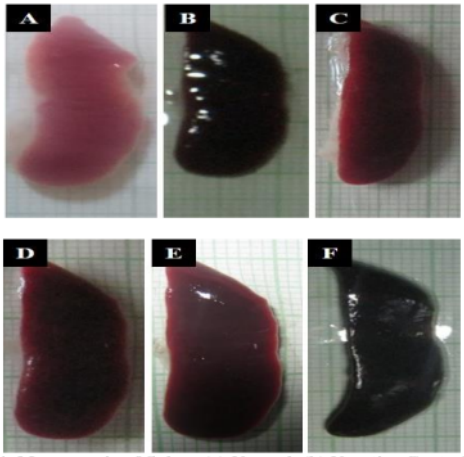

Fig.4: Macroscopic of Spleen (a) Normal, (b) Negative Control, (c) Positive Control, (d) EECGF 40 mg/200 g, (e) EECGF 80 mg/200 g, and (f) EECGF 120 mg/200 g

Fig.4e and f show macroscopic depictions of EECGF 80 and 120 mg/200 g splenic organs. Spleen organs at EECGF 80 mg/200 g showed normal conditions, no lumps, brownish-red color, smooth surface, supple consistency, and a normal size (Table 1). Whereas EECGF 120 mg/200 g did not show any solid mass that was formed, but there was an enlargement of the spleen organ as indicated by the addition of the size and weight of the spleen increased two times the normal state.

Table 1: Evaluate the Pathology of Spleen Organ Anatomy (*n*=5)

Anatomical Pathology	Treatment					
	Normal	**Infection**	**Positive control**	**EECGF 40 mg/200 g**	**EECGF 80 mg/200 g**	**EECGF 120 mg/200 g**
Color	Pink	Black	Brownish-red	Brownish-red	Brownish-red	Black
Shape	Normal	Normal	Normal	Normal	Normal	Normal
Weight (g)	0.50±0.06	0.80±0.10	0.51±0.08	0.51±0.07	0.53±0.08	0.86±0.05
Consistency	Chewy	Hard	Chewy little hard	Chewy little hard	Chewy little hard	Hard

There was an enlarged spleen organ in the EECGF group with a dose of 120mg/200 g. The content of polyphenols and flavonoids has a positive correlation with the ability to immunomodulate through stimulation of splenocyte proliferation in the spleen organs[39]. The administration of the highest dose of EECGF is thought to increase splenocyte proliferation faster than the doses of 40 and 80mg/200g, which can cause splenomegaly.

Free radicals due to bacterial infections and other metabolisms in the body can cause immune cells to be disrupted. The immunomodulatory effects of both flavonoids and phenolics are related to antioxidant activity[9,30,39]. Sources of flavonoids and phenolics as antioxidant agents from nature are very abundant, for example, white tea and green tea from *Camellia sinensis*[40,41,42], herbs *Phyllanti niruri*[9], and *Calotropis gigantea* flowers[14]. High antioxidant agents can increase redox reactions to stabilize free radicals so that they can control immune function[43,44].

CONCLUTION:

Ethanol extract of *Calotropis gigantea* L. flower has an immunostimulatory activity. Evaluation of immune system parameters, which include CD_4 T cell counts, total leukocytes, lymphocytes, monocytes, and neutrophils, leads to EECGF at a dose of 80mg/200g having similar activity with positive control. As for the EECGF dose of 120mg/200g, body weight can cause splenomegaly and cause more severe lymph damage. These results are scientific information on the pharmacology of natural materials, phytotherapy, and the basis for further product development.

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CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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