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Elaeocarpus grandiflorus Leaves Extract Increased Immunoglobulin G Activity and Total Protein of SRBC-Induced Rat

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Abstract

The immune system plays a critical role in defending against viral infections such as Covid-19. *Elaeocarpus grandiflorus* contains various active compounds, including flavonoids, saponins, polyphenols, and tannins. Among these, the primary flavonoids identified in *E. grandiflorus* are kaempferol, quercetin, procyanidin, naringin, orientin, iso-orientin, vitexin, isovitexin, rutin, luteolin, and epicatechin. Kaempferol and quercetin have been particularly noted for their anti-inflammatory properties and potential to enhance the immune system. This study aimed to investigate the effects of *E. grandiflorus* leaf extract on immunoglobulin G (IgG) activity and total protein levels in rats induced by sheep red blood cells (SRBC). Twenty-five Wistar rats were randomly assigned to five groups: P1, P2, P3, Negative control, and Positive control. The extract suspension was administered from day 1 to day 7 at concentrations of 100 mg/kg BW (P1), 200 mg/kg BW (P2), and 400 mg/kg BW (P3). On day 7, rats were intraperitoneally injected with 1 mL of a 2% v/v solution of SRBC as an antigen. IgG activity and total protein levels were assessed on day 12. The average agglutination titters were as follows: 3.65 (Negative control), 3.89 (Positive control), 3.77 (P1), 3.65 (P2), and 4.61 (P3). Additionally, the average total protein values for the groups were 1.44 mg/dL (Positive control), 1.00 mg/dL (Negative control), 1.18 mg/dL (P1), 1.22 mg/dL (P2), and 1.46 mg/dL (P3). Significant differences were observed in IgG activity and total serum protein levels among the groups. These findings suggest a potential immunostimulant effect of *E. grandiflorus* leaf extract and warrant further exploration for its development.

Keywords *Elaeocarpus grandiflorus*, immunoglobulin G, immunostimulant

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1.0 INTRODUCTION

The immune system is very important for homeostasis. It prevents and fights disease from external and internal sources. Immunostimulants are substances that can increase the activity of the immune system. Immunostimulants are widely used to increase or improve the body's immune system and even in alternative ways. Many plants have shown effects as immunostimulants in health and disease [1].

Rejasa (*Elaeocarpus grandiflorus*) is a plant commonly used in traditional medicine. *E. grandiflorus* showed an effect as anti-inflammatory, antidiabetic, fever-reducing, and astringent [2]. The bioactive compounds in the *E. grandiflorus* also help heal the disease. Almost all parts of the *E. grandiflorus* plant are used as herbal medicines. Bioactive compounds can be found in almost all parts of the *E. grandiflorus* plant, namely in the leaves, fruit, and bark. The content of these compounds included flavonoids, saponins, polyphenols and tannins. The main flavonoids in young plants are Kaempferol and Quercetin [3,4].

Kaempferol is a polyphenolic compound that has a role in inhibiting inflammation [5,6,7]. Several studies have shown anti-inflammatory effects both in vitro and in vivo. Kaempferol significantly inhibited T-cell proliferation and NO release on cell

culture, indicating its role as an antioxidant. The antioxidant activity of Kaempferol is better than glycoside, indicating that Kaempferol may have better anti-inflammatory activity [8]. Kaempferol also affects the repair of nerve cell damage [9] and the prevention of cardiovascular disease [10].

Quercetin is a flavanol group with many benefits. Quercetin has been widely studied for its effect on the inflammatory process, especially in preventing COVID-19 infection [11, 12, 13]. Quercetin also has an effect on inhibiting the inflammatory process and enhancing the immune response [14, 15]. Based on the contents of Kaempferol and Quercetin, research to explore the effect of *E. grandiflorus* extract on the immune response needs to be carried out. This study is useful for providing a basic understanding of *E. grandiflorus* as an immunostimulant.

2.0 EXPERIMENTAL

2.1 Animal and ethical approval

This research was conducted with a post-test-only control group design. A total of 25 Wistar rats were randomly divided into 5 groups. The first group was a negative control, and the second was a positive control. The experiment groups were marked as P1, P2, and P3. The rats were maintained in group cages and were given standard food and water ad-lib. The animal experiment has been approved by the Health Research Ethics Committee Universitas Negeri Semarang with the number 303/KEPK/EC/2022.

2.2 Extraction of *E. grandiflorus*

E. grandiflorus leaf ethanol extract was made using the maceration method. A total of 1 kg of fresh *E. grandiflorus* leaves were washed and then air dried. The air-dried leaves were dried in the oven at 600 C and crushed using a blender. The powder is put into a maceration tube containing 70% alcohol. Screening was carried out over 5-6 days. The concentrated extract was dried by rotary evaporation.

2.3 Experiment procedure

The experiment groups were given *E. grandiflorus* extract. The P1 groups were given 100 mg/kg BW, P2 was given 200 mg/kg BW, and P3 was given 400 mg/kg BW. The extract was given for 5 days by gavage sonde. On day 6, all rats were injected with Sheep Red Blood Cell (SRBC) 2% v/v suspension as much as 1 mL intraperitoneally. Blood was collected from the intra-o orbital vein on day 10. Blood was centrifugated 500 g for 15 minutes to get the serum.

2.4 Agglutination test of immunoglobulin G activity

The 96 wells were marked according to the sample order. The serum was diluted by double dilution with PBS NaCl pH 7.4 in a ratio of 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, and 1/ 512. First, 75 µL of PBS NaCl pH 7.4 and 25 µL of serum were pipetted and mixed to achieve the lowest dilution (1/4). Then, 50 µL of PBS NaCl pH 7.4 was pipetted from the ¼ dilution to make 1/8, and so on until the 1/512 dilution. 50 µL from a 1/4 dilution was pipetted to a 1/8 dilution, then homogenized. 50 µL from 1/8 dilution was pipetted to 1/16 dilution, then homogenized. The same procedure was carried out for each dilution until the highest dilution was 1/512; hence, each dilution's volume was 50 µL. 50 µL SRBC 2% v/v was pipetted into each dilution so that the volume became 100 µL, then homogenized. Then, it was incubated at 37ºC for 60 minutes and allowed to stand for 1x24 hours at room temperature, and the agglutination in the base of wells was observed.

2.4 Total protein serum test

The total protein serum analysis was done using a photometric test based on the biuret method. Protein was determined at 550 nm wavelength. The collected serum was measured at the Regional Health Laboratory of Semarang, Central Java, Indonesia.

2.5 Data analysis

For each repetition, the highest dilution showing positive agglutination was noted. This value was converted by the 2Log(titter)+1 formula and then analyzed by ANOVA. Data on total serum protein values obtained were analyzed using one-way ANOVA with a significance level of 5% and continued with the LSD Post Hoc test.

3.0 RESULTS AND DISCUSSION

The agglutination test was used to examine the activity of immunoglobulin activity when the organism was induced antigen. SRBCs were an antigen used in experiments that can activate the immune system. Antigen-antibody binding between sheep

red blood and IgG formed agglutination. The agglutination test results and value titter conversion are presented in Tables 1 and 2.

Table 1 The serum agglutination test after induction of *E. grandiflorus* extract and SRBC.

dilution	Negative control					Positive control					P1					P2					P3				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1/512																									
1/256																									
1/128																							+	+	+
1/64										+													+	+	+
1/32	+		+	+		+	+			+			+	+	+	+	+					+	+	+	+
1/16	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1/8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
¼	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2 The conversion of titer by 2Log(titer)+1 formula.

Group	IgG activity					Mean±std.dev
	1	2	3	4	5	
Negative control	4.01	3.41	4.01	4.01	2.81	3.65±.537
Positive control	4.01	4.01	3.41	3.41	4.61	3.89±.343
P1	3.41	3.41	4.01	4.01	4.01	3.77±.653
P2	4.01	4.01	3.41	3.41	3.41	3.65±.377
P3	3.41	4.01	5.21	5.21	5.21	4.61±.219

The significant difference between the serum titer of the experimental group at a dose of 400 mg/kgBW and that of other groups indicated that the extract of *E. grandiflorus* showed an increasing immunoglobulin G activity at 400 mg/kgBW. The dose of less than 400 mg/kgBW showed a similar effect to the negative and positive control. The total protein is presented in Table 3.

Table 3 Total serum protein values.

	N	Total protein serum value (mg/dL)					Mean±std. dev
		1	2	3	4	5	
Negative control	5	0.2	0.4	0.5	1.5	2.4	1.00±.930
Positive control	5	1.3	1.1	1.7	1.9	1.2	1.44±.344
P1	5	0.9	2.0	0.3	1.6	1.1	1.18±.654
P2	5	1.8	1.2	0.8	1.3	1.0	1.22±.377
P3	5	1.4	1.8	1.5	1.4	1.2	1.46±.219

Descriptive data of Table 3 showed that the highest mean value in treatment group three with a dose of 400 mg/kg bw was 1.46 mg/dL. The statistical analysis results of total serum protein values obtained by the negative control group against the experimental group had no significant difference. Various factors, including the recovery process, can influence this. The same thing happened in the positive control group against the experimental group. They both tend to have the same effect. Flavonoid compounds in ethanol extract of *E. grandiflorus* leaves can increase the production and activation of interleukin 2 (IL2). IL-2 secretion will increase the expansion and differentiation of CD4+ and CD8+ effector T cells to help activate B lymphocyte cells to produce antibodies and activate macrophages and other immune cells to eliminate infection by secreting cytokines and increasing cytotoxicity [16].

Antibodies were produced by B cell as the response to antigen stimulus. B cell produce and secrete millions of different antibody molecules, each of which recognizes a different (foreign) antigen. Sheep red blood cell-induced immune system by activated B Cell Receptor (BCR) pathway. The first stimulus of sheep red blood cells leads to the formation of a specific antibody. The first specific antibody is not released but stored in the plasma membrane. This specific antibody role is a receptor for sheep red blood cell antigen. The second meeting with the sheep red blood cell will activate membrane receptor signalling to produce specific antibodies. This specific antibody will be released into the blood or tissue. The binding between antigen and BCR activated many proteins, including protein tyrosine kinases (PTKs domain), the SRC-family kinase (LYN and SYK) and Btk (one of the Tec-family kinases.)

The differentiation of B lymphocytes into a clone of plasma cells releasing specific antibodies into the blood is a form of initiation of the humoral immune response. The specific antibodies in the form of immunoglobulins were secreted to extracellular. Antibodies may be found on the surface of B cells and serve as antigen receptors [17]. The increase in total blood protein, as in the average results of the 400 mg/kg BW dose treatment compared to the negative control group, can be caused by several factors, including the role of flavonoids in increasing the regulation of the immune system. An increase in the globulin fraction accompanied by an increase in immunoglobulins can occur in conditions of increased protein in the blood. Immunoglobulin is one type of globulin protein that acts as an antibody in the body [18]. Therefore, an infectious process can increase the body's total blood protein due to increased immunoglobulins.

BCR involves many proteins that might be influenced by external stimuli. In the *In silico* exploration, it was found that *E. grandiflorus* is associated with many proteins involved in the process of antibody activity. Three bioactive compounds of *E. grandiflorus*, Quercetin, Naringin, and Orientin, stimulate the BCR pathway. Quercetin suppresses the accumulation and activation of immune cells, including anti-inflammatory cells [15]. This stimulation occurs through the activation of 6 target proteins in the BCR pathway by bioactive *E. grandiflorus*, namely: Cluster of differentiation-22 (CD22), Phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1), HRAS, Glycogen synthase kinase-3β (GSK3B), and AKT. The KEGG analysis found that the BCR pathway was activated by molecular binding between Naringin-CD22, Orientin-HRAS, Quercetin-PI3K, Quercetin-AKT and Quercetin-GSK3B. The binding between the bioactive and the target protein causes an enhanced response associated with B cell response against sheep red blood cells SRBC.

B cell secreted antibodies by specific antigens were induced. The first stimulus by a specific antigen leads to the formation of a specific antibody. The specific antibodies were stored in the plasma membrane and acted as antigen receptors. When an antigen binds to the receptor, the intracellular signalling pathway is activated. The activated naïve or memory B cells will begin to process of proliferation and differentiation into antibody-secreting effector cells. Antibodies secreted by antibody-secreting effector cells are soluble antibodies that occupy the same unique antigen-binding site as the cell-surface antibody,

Naringin is a flavonoid that is found in many vegetables and fruits. Naringin plays a role in the immune response as an anti-inflammatory antioxidant. Naringin inhibits the inflammatory reaction by suppressing the release of inflammatory cytokines such as IL-1β, IL-6, and IL-18 [19]. The other study showed that Naringin reduced the production of the prostaglandin E2 (PGE2), nitric oxide (NO), interleukin-6 (IL-6), and tumour necrosis factor-α (TNF-α) [20]. Naringin from *E. grandiflorus* was recruited for CD22, which positively increased B Cell activation and Ig production. CD22 is a protein ligand-associated glucose. The ligand-binding domain of CD22 is important for regulating B-cell Ca signalling. CD22 is associated with other signalling molecules that are otherwise involved in positive BCR signalling: PLCγ2, Syk, and PI3K [21].

PI3K/Akt signalling play an important role in protein synthesis, cell survival, cell migration and proliferation, glucose metabolism, neuroscience and others. AKT comprises three closely related isoforms: AKT1, AKT2 and AKT3. In the BCR pathway, Akt stimulated protein synthesis through mTOR and p70SGK. Expression of AKT in macrophage associated with NO synthesis and cytokines. The interferon-γ production was promoted by AKT1 through negative regulation of GSK3β [22]. Activation of PI3K by extracellular stimuli results in activation of AKT in virtually all cells and tissue. Overexpression of phosphorylated AKT (pAKT) is a key defect in many solid tumours [23]. In this research, SRBC induction triggers AKT hyperactivation due to oxidative stress in the inflammatory process. Quercetin, a ubiquitous bioactive flavonoid, significantly decreased the phosphorylation of AKT and its role in down-regulation [24].

Another target of E. grandiflorus is HRas. Orientin-bound HRas, which resulted in Ig G production. HRas is an isoform of the RAS protein and a binary switch protein that can be activated or inactivated [25]. An extracellular agent or growth factor activates Ras by loading it with GTP, allowing it to bind its downstream effectors. RAS stimulation induced effector activation, including PI3K, RalGDS, BRAF and MEK1 or MEK2 [25]. As a member of the small GTPases, RAS regulates cell survival, growth, differentiation and polarization. Cell polarization associated with the complex process and multiple components will likely be recognized to play an even more vital role within the immune system [26].

Glycogen synthase kinase-3 (GSK3) is a protein role in protein synthesis. GSK3 is present in the cytosol, mitochondria, nucleus, and other subcellular compartments [27]. The presence of GSK3 inhibits protein synthesis in the BCR pathway. Stress or antigen stimulates the GSK3 expression and the immune response by producing inflammatory mediators and cytokines by T lymphocyte cells [27]. Intraperitoneal injection of SRBC-activated GSK3 to suppress protein synthesis, including globulin. Some agents and molecules influence the immune response by inactivating GSK3. Quercetin inactivated glycogen synthase kinase (GSK)-3β, which is phosphatidylinositol 3-kinase/Akt dependent [28],[29]. Quercetin of *E. grandiflorus* binds to GSK3B to promote Ig G production and increase their activity when induced SRBC.

4.0 CONCLUSION

E. grandiflorus has many bioactive compounds, including flavonoids, saponins, polyphenols and tannins. The main flavonoids are kaempferol, quercetin, procyanidin, naringin, orientin, iso orientin, vitexin, isovitexin, rutin, luteolin and epicatechin, Quercetin, naringin and orientin have the potential to be developed as an immunostimulant. These bioactive compounds affect IgG production through the B Cell Receptor (BCR) pathway and enhance immune regulation of total blood protein levels. Quercetin, naringin and orientin bind to CD22, HRAS, PIK3R1, GSK3B and AKT1, increasing IgG activity.

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