Elaeocarpus grandiflorus induced immunoglobulin G activity on B Cell Receptor (BCR) Pathway

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Abstract

Immune system is important factor to prevent virus attacks such as Covid 19. *Elaeocarpus grandiflorus* contains active compounds including flavonoids, saponins, polyphenols and tannins. The main flavonoids found in *E. grandiflorus* are kaempferol, quercetin, procyanidin, naringin, orientin, iso orientin, vitexin, isovitexin, rutin, luteolin and epicatechin. Kaempferol and quercetin show their role in anti-inflammatory and immune system enhancement. The purpose of this study was to examine the effect of *E. grandiflorus* leaf extract on the immunoglobulin G activity of rat induced by sheep red blood cells. A total of 25 Wistar rats were randomly divided into 5 groups. A suspension of ethanol extract of *E. glandiflorus* leaves was given on day 1-7 with a concentration of 0.04% w/v (P1), 0.08% w/v (P2), 0.16% w/v (P3), Na.CMC 1% w/v (Neg control) and mix herbal immunostimulant (positive control). On day 7 the rat was intraperitoneally injected 1 mL 2% v/v sheep red blood cells as antigen. The blood was taken from the orbital vein on day 12. The IgG activity was examined by agglutination test. The average of agglutination titers was 3.77 (neg control), 3.89 (pos control), 3.77 (P1), 3.65 (P2) and 4.61 (P3). By the statistics analyzed only P3 group that showed the difference. Increased IgG activity due to *E. grandiflorus* extract can occur through the B Cell Receptor (BCR) pathway. This pathway involved 5 proteins: CD22, HRAS, PIK3R1 and GSK3B and AKT1. It can be concluded that the extract of *E. grandiflorus* has the potential to be developed as an immunostimulant.

Key word: *E. grandiflorus, BCR Pathway, immunostimulant, kaempferol, quercetin*

INTRODUCTION

Immune system is very important in homeostasis. The immune system prevents and fights disease from the external and internal. included covid 19. The immune system can be boosted in many ways. 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 alternative ways. Many plants showed the effect as immunostimulants in health and disease (Sethl & Singh, 2015)

Rejasa (*Elaeocarpus grandiflorus*) is a plant commonly used a traditional medicine. *E. grandiflorus* showed an effect as anti-inflammatory, antidiabetic, fever-reducing, and astringent [1](Ganevi, et.al, 2020). The bioactive compounds contained in the *E. grandiflorus* also help in healing 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 of *E. grandiflorus*. The content of these compounds included flavonoids, saponins, polyphenols and tannins. The main flavonoids found in young plants are Kaempferol and Quercetin [2,3] (Habibah, et.al, 2021; Sagala,2018).

Kaempferol is a polyphenolic compound role in inhibiting inflammation (Chen & Chen, 2013; Ren, et.al, 2019; Alam, et.al, 2020). 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, indicated the role as antioxidant. The antioxidant activity of Kaempferol better than glycoside indicated that Kaempferol may have better anti-inflammatory activity (Wang, et.al, 2018). Kaempferol also affected on the repair of nerve cell damage (Silva dos Santos, 2021), and prevention of cardiovascular disease (Dabeek&Marra,2019).

Quercetin is a flavonol group with many benefits. Quercetin has been widely studied for its effect on the inflammatory process, especially in the prevention of COVID-19 infection (Colunga-Biancatelli, et.al, 2020; Saeedi-Boroujeni & Mahmoudian-Sani, 2021; Di Pierro, et.al, 2021). Quercetin also shown its effect on the inhibition of the inflammatory process and the enhancement of the immune response (Tang, et.al, 2015;Li, et.al, 2016). Based on the contains of Kaempferol and Quercetin, research to explore the effect of *E. grandiflorus* extract on the immune response needs to be carried out. This study useful to give a basic understanding of *E. grandiflorus* as immunostimulant

METHODS

Animal and ethical approval

This research conducted by the post test only control group design. A total 25 Wistars rat were randomly divided into 5 groups. The first group was negative control and the second group was positive control. The experiment groups were marked as P1, P2 and P3. The rats were maintenance in groups cage and were given standard food and water ad lib. The experiment involved animal have been approved by Komisi Etik Penelitian Kesehatan (KEPK) Universitas Negeri Semarang with the number 303/KEPK/EC/2022.

Experiment Procedure

The experiment groups were given *E. grandiflorus* extract. The P1 groups was given 100 mg/kg BW, P2 was gives 200 mg/kgBW, and P3 was given 400 mg/kgBW. The extract was given along 5 days by gavage sonde. In day 6, all rat were injected Sheep Red Blood Cell (SRBC) 2% v/v suspension as much as 1 mL intraperitoneally. Blood was collected from intra orbital vein on day 10. Blood were centrifugated 3000 rpm for 15 minutes to get the serum.

Agglutination test of Immunoglobulin G activity

a. The wells were marked according to the sample order.
b. 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.
c. First, pipette as much as 75 µL of PBS NaCl pH 7.4 and 25 µL of serum, mix to result the lowest dilution (1/4). Then pipette as much as 50 µL of PBS NaCl pH 7.4 from the ¼ dilution to make 1/8, and so on until the 1/512 dilution.
d. Pipette 50 µL from a 1/4 dilution to a 1/8 dilution, then homogenize. Pipette 50 µL from 1/8 dilution to 1/16 dilution, then homogenize.
e. The same procedure was carried out for each dilution until the highest dilution was 1/512, so that the volume of each dilution was 50 µL.

f. Pipette 50 µL SRBC 2% v/v into each dilution so that the volume becomes 100 µL, then homogenize.
g. Then it was incubated at 37ºC for 60 minutes and allowed to stand for 1x24 hours at room temperature, then observed the agglutination in the base of well.

Data Analysis

The highest dilution that showed positive agglutination was noted for each repetition. This value was converted by 2Log(titer)+1 formula and, then was analyzed by anova.

RESULT AND DISCUSSION

The agglutination test used to examine the activity of immunoglobulin activity when the organism was induced antigen. Sheep red blood was an antigen used in experiment have an ability to activate immune system. Antigen-antibody binding between sheep red blood and IgG formed agglutination. The result of agglutination test, titer agglutination and value titer conversion were presented in Table 1, 2 and 3.

Table 1. The positive agglutination test of serum after induction of *Elaeocarpus grandiflorus* extract and sheep red blood cell

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	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
1/4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Table 2. The dilution value that given positive agglutination

	Negative control	Positive control	P1	P2	P3
1	1/32	1/32	1/16	1/32	1/16
2	1/32	1/32	1/16	1/32	1/32
3	1/32	1/16	1/32	1/16	1/128
4	1/32	1/16	1/32	1/16	1/128
5	1/8	1/64	1/32	1/16	1/128

Table 3. The conversion of titer by 2Log(titer)+1 formula

	Negative control	Positive control	P1	P2	P3
1	4.01	4.01	3.41	4.01	3.41
2	4.01	4.01	3.41	4.01	4.01
3	4.01	3.41	4.01	3.41	5.21

4	4.01	3.41	4.01	3.41	5.21
5	2.81	4.61	4.01	3.41	5.21
average	3.77	3.89	3.77	3.65	4.61

The significant difference between serum titer of experimental group at dose 400 mg/kgBW indicated that the extract of *E. grandiflorus* showed increaseing of immunoglobulin G activity in dose 400 mg/kgBW. The dose less than 400 mg/kgBW showed the simillar effect with the negative and even positive control.

Antibody was 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 cell leads the formation of a specific antibody. The first specific antibody not release but is stored in the plasma membrane. This specific antibody role as receptor for sheep red blood cell antigen.The second meeting with the sheep red blood cell will activated membrane receptor signaling to produced specific antibody. This specific antibody will released to the blood or tissue. After BCR ligation by antigen, the protein tyrosine kinases (PTKs) : the SRC-family kinase (LYN and SYK) and the TEC-family kinase (BTK) were activated. Phosphatidylinositol 3-kinase (PI3K) and phospholipase C-gamma 2 (PLC-gamma 2) are important downstream effectors of BCR signalling

BCR involved many protein that might be influenced by external stimulus. *In silico* exploration found that *E. grandiflorus* associated with many protein involved in the process of antibody activity. Cluster of differentiation-22 (CD22), Phosphatidylinositol 3-kinase regulatory subunit alpha (PIK3R1), HRAS, Glycogen synthase kinase-3β (GSK3B), and AKT were associated with active compound of *E. grandiflorus* to increase B cell response against sheep red blood cell.

B cell secreted antibody by specific antigen induced. The first stimulus by a specific antigen leads the formation of a specific antibody which is stored in the plasma membrane, where they serve as receptors for antigen .these receptors is stably associated with a complex of transmembrane proteins that activate intracellular signaling pathways when antigen binds to the receptor. When a naïve or memory B cell is activated by antigen (with the aid of a helper T cell), it proliferates and differentiates into an antibody-secreting effector cell. Such cells make and secrete large amounts of soluble (rather than membrane-bound) antibody, which has the same unique antigen-binding site as the cell-surface antibody that served earlier as the antigen receptor.

CD22 is a protein ligand associated glucosa . In conclusion we have found that the ligand-binding domain of CD22 is important for regulation of B-cell Ca signaling. This control is mediated by ligand- dependent regulation of the association with the BCR. Although it has been well established that CD22 functions mainly as a negative regulator, further complexity arises by the ability of CD22 to associate with other signaling molecules otherwise involved in positive BCR signaling. Among others, PLCγ2, Syk, and PI3K have been reported to be recruited to CD22

Naringin from *E. grandiflorus* recruited to CD22 and positively increased B Cell activation, resulted increasing of Ig production. CD22 is a protein ligand associated glucosa. The ligand-binding domain of CD22 is important for regulation of B-cell Ca signaling. CD22

associated with other signaling molecules otherwise involved in positive BCR signaling: PLCγ2, Syk, and PI3K (Deenonpoe, et.al, 2019)

PIK3R1 is a member of the PI3K/AKT pathway, which is a key regulator of various cellular processes such as proliferation and apoptosis and is regulated by several sphingolipids (Oskouian & Saba, 2010). Phosphatidylinositol 3-kinase regulatory subunit alpha is an enzyme that in humans is encoded by the PIK3R1 gene. Phosphatidylinositol 3-kinase plays an important role in the metabolic actions of insulin, and a mutation in this gene has been associated with insulin resistance. The pathway can be activated by a range of signals, including hormones, growth factors and components of the extracellular matrix (ECM). Quercetin role as extracellular ligand bind to a receptor tyrosine kinase (RTK) in the plasma membrane, causing receptor dimerization and cross-phosphorylation of tyrosine residues in the intracellular domains.

Another target of *E. grandiflorus* is HRas. Orientin bound HRas and resulted Ig G production. HRas is a small G protein in the Ras subfamily. HRas will activate a Raf kinase like c-Raf, the next step in the MAPK/ERK pathway. Once activated, receptors stimulate signal transduction events in the cytoplasm, a process by which proteins and second messengers relay signals from outside the cell to the cell nucleus and instructs the cell to grow or divide. The HRas protein is a GTPase and is an early player in many signal transduction pathways and is usually associated with cell membranes due to the presence of an isoprenyl group on its C-terminus. HRAS acts as a molecular on/off switch, once it is turned on it recruits and activates proteins necessary for the propagation of the receptor's signal, such as c-Raf and PI 3-kinase. HRAS binds to GTP in the active state and possesses an intrinsic enzymatic activity that cleaves the terminal phosphate of this nucleotide converting it to GDP. Upon conversion of GTP to GDP, HRAS is turned off.

Glycogen synthase kinase-3 (GSK3) enhanced immune responses to counteract real, or stress-induced perceived, threats to survival. Thus, survival may have benefited from GSK3 promoting both innate and adaptive immune responses, increasing the production of multiple inflammatory cytokines and promoting the production of Th1 and Th17 inflammatory T cells, which should increase resistance to injury and infection (Beurel et al., 2015). Glycogen synthase kinase-3 (GSK3) have two isomer: GSK3α and GSK3β. Glycogen synthase kinase-3β (GSK-3β) has been shown to play an important role in tumor rogression particularly through the modulation of oncogenes, cell cycle regulators and mediators of epithelial–mesenchymal transition (Saraswat, et.al, 2018]) Recent studies have also demonstrated that aberrant overexpression of GSK-3β promotes tumor growth and chemotherapy resistance in various solid tumors including pancreatic, colorectal and prostate cancer through diferentialefects on pro-survival NF-κB and c-Myc pathways as well as on TNF-related apoptosis-inducing ligand (TRAIL) and p53-mediated apoptotic mechanisms (Ougolkov AV, et.al, 2005). GSK-3β represents therefore an important therapeutic target in human malignancies. Quercetin of *E. grandiflorus* bind to GSK3B and promoted Ig G production and increased their activity when induced SRBC.

AKT comprises three closely related isoforms AKT1, AKT2 and AKT3. AKT1 is related serine/threonine-protein kinases called the AKT kinase regulated many processes including metabolism, proliferation, cell survival, growth and angiogenesis. AKT isoforms are activated by growth factors and other extracellular stimuli as well as by oncogenic mutations

in key upstream regulatory proteins including Ras, PI3-kinase subunits and PTEN. There are also an ever increasing number of Akt substrates being identified that play a role in the regulation of the diverse array of biological effects of activated Akt; this includes the regulation of cell proliferation, survival and metabolism.

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 types of solid tumors (Lida, et.al, 2020). AKT also regulates glycogen synthase kinase-3, a kinase whose substrates include the nuclear factor of activated T cells (NF-AT)cl and beta-catenin transcriptional activators. PI3K-derived lipids also regulate the activity and localization of other targets of BCR signaling. Thus, a key event in BCR signaling is the recruitment of PI3K to the plasma membrane where its substrates are located. This is mediated by binding of the Src homology (SH) 2 domains in PI3K to phosphotyrosine-containing sequences on membrane-associated docking proteins

Conclusion

E. grandiflorus potentially to develop as an immunostimulant. The extract influence Ig G production through the B Cell Receptor (BCR) pathway. Quercetin, naringin and orientin bind to CD22, HRAS, PIK3R1, GSK3B and AKT1 resulted increasing of IgG activity.

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