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Extract of cell culture Rejasa (*Elaeocarpus grandiflorus*) Decrease Blood Glucose Through Insulin Receptor Pathway

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Abstract. Diabetes mellitus is a metabolic disease characterized by the high blood glucose levels. The high prevalence of Diabetes Mellitus needed an innovation in prevention, treatment and control of case. The aim of research was to explore the potency of Rejasa (*Elaeocarpus grandiflorus*) as an antidiabetic. The pretest and posttest control group design were conducted to 30 hyperglycemic Wistar rat. The rats were divided into 5 groups, that were negative control (K-), positive control (K+, given glibenclamide 0.072 mg/200 kgBW), and experimental groups that given *E. glandiflorus* cell extract 1 mg/kgBW (P1), 10 mg/kgBW (P2), and 100 mg/kgBW (P3). The extract was given orally for 10 days. Measurement of blood glucose levels was carried out on day 0 and day 10. The mechanism of antidiabetic effect of *E. glandiflorus* was explored by in silico. The mean of blood glucose levels on day 0 were 455.2 mg/dL (K), 422.8 mg/dL (K+), 469.8 mg/dL (P1), 355.5 mg/dL (P2) and 446 mg/dL (P3). The blood glucose levels on day 10 were 367.8 mg/dL (K-), 89.6 mg/dL (K+), 285.6 mg/dL (P1), 136.8 mg/dL (P2) and 104.8 (P3). Statistical analysis showed the difference between K- from P2($p=0.015$) and P3 ($p<0.001$). When compared with K+, only P3 showed no difference ($p=0.873$). Flavonoid of *E. glandiflorus* act on insulin receptor pathway and involved HK2, PTPN1, AKT1, PI3KR1, HRAS and GSK3B protein. The conclusion and the new finding of research that extract cell of *E. glandiflorus* have antidiabetic activity through insulin receptor pathway.

Key words: Blood glucose, Elaeocarpus grandiflorus, Insulin receptor pathway

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INTRODUCTION

Diabetes mellitus is a metabolic disease characterized by the high blood glucose levels. The high prevalence of Diabetes Mellitus needed an innovation in prevention, treatment and control of case. The use herbal medicine is an alternative for diabetes prevention and controlling blood glucose levels. Rejasa (*Elaeocarpus grandiflorus*) is one of the plants that has the potential to be developed as an antidiabetic. *E. grandiflorus* is widely used in the field of medicine because it has certain properties such as anti-inflammatory, anti-diabetic, fever-reducing, and astringent (Ganevi, 2020). In vitro study showed the potential mechanism of flavonoid as antioxidant and antidiabetic (Sarian, et.al; 2017). The content of bioactive compounds contained in the *E. grandiflorus* can help in healing diseases. Almost all parts of the *E. grandiflorus* plant are used as herbal medicine. Bioactive compounds can be found in almost all parts of the *E. grandiflorus*, namely in the leaves, fruit, and bark of the *E. grandiflorus* stem.

E. grandiflorus is a wild plant that can be found in nature. Unfortunately, *E. grandiflorus* is one of the rare and rarely found plants in Indonesia (Rahayu et al., 2018). *E. grandiflorus* grows slowly so it is difficult to get enough plant material. Plant tissue culture technology offers an alternative to obtain secondary metabolites by cell suspension culture. *E. grandiflorus* cell suspension culture can produce a variety of secondary metabolites. The content of these compounds included flavonoids, saponins, polyphenols and tannins. The main flavonoids found in cassava plants are kaempferol, quercetin, naringin, orientin, vitexin and iso vitexin (Sagala, 2018; Habibah et al., 2021)

Quercetin had neuroprotective effect against oxidative stress-mediated injury on some types of neuronal cells (Jazvinšćak et al., 2018; Jazvinšćak et al., 2021; Tseng et al., 2012; Costa et al., 2016). Besides quercetin, kaempferol also has a neuroprotective effect too (Silva Dos Santos, 2021). Kaempferol associated with the cytoprotective effect of β-pancreatic cells from the

hyperglycemic cell's environment, Kaempferol improved β-pancreatic cells that led increasing of insulin secretion (Zhang & Liu, 2011). Glibenclamide as a standard therapy of diabetes mellitus plays a role in the improvement of pancreatic beta cell function and regeneration. Based on quercetin and kaempferol containing, this research aimed to explore the effect *E. grandiflorus* on decreasing blood glucose of hyperglycemic-induced alloxan rats.

This research is important for developing new herbal medicines based on experiments. In addition to effectiveness data, this study provides an analysis of how the mechanism of bioactive substances from *E. grandiflorus* can provide an antidiabetic effect.

METHODS

This research is an experimental study using hyperglycemic rats as experimental animals. The independent variable in this study was the oral administration of *E. grandiflorus* extract, while the dependent variable was blood sugar levels. Hyperglycemic rats were given *E. grandiflorus* extract for 10 days to see the effect of extract on blood sugar levels. The mechanism of the bioactive compounds' activity of *E. grandiflorus* on blood glucose levels was explored using *in silico.*

Extraction of Plant Cell Culture procedure

E. grandiflorus cell cultures were obtained from callus grown in liquid Woody Plant Medium (WPM) with 2,4-D 2.5 ppm. Leaf stalk explants were taken from the 2-year-old *E. grandiflorus* plant which wa maintained at Semarang State University . Young stalks was sterilized by soaking in fungicides for 15 minutes and bactericides for 10 minutes. The stalk was rinsed with sterile aquadest. The young stalk was sterilized with 20 % bleach solution (containing sodium hypochlorite 2,4%) in laminar air flow followed by rinsing young stalks with sterile water. Callus induced on solid WPM medium with addition of 2.4-D 2.5 ppm.

The 5 months old callus used for cell culture induction. Cell suspension culture formation was carried out by transferring 1 g of callus into a 100 ml erlenmeyer containing 20 ml of WPM medium. The culture was shaken at a speed of 120 rpm. The culture was maintained for 30 days in dark condition. At harvest, cells were filtered and weighed. The cell was then dried in the oven for 48 hours at 60°C. The method of extraction of flavonoids used is the Hao et al. method (2009). Dry cells are mashed with mortar and pestle. The powder is extracted using methanol containing 1%(v/v) HCl, followed by the addition of 2 N HCl (of the same volume) and inchabited for an hour at 90°C. The extract is then dried and suspended in the aquadest.

Animal experiment

This research was a laboratory experimental research with Pretest and Posttest Control Group Design. The study involved 30 white rats (*Rattus norvegicus*) Wistar strain with an age of ± 3 months and body weight of 120-200 grams. Alloxan monohydrate were induced through intraperitoneal at a dose of 125 mg/kg BB dissolved NaCl 0.9%. Measurement of blood glucose levels starts from day 3 after being alloxan induced until get blood glucose levels was above 200 mg / dL. The use of experimental animals in this study has received approval from the health research ethics commission of Semarang State University with a letter number 327/KEPK/EC/2021

The thirty hyperglycemic rats were divided into 5 groups randomly, 6 rats per group, that were negative control (K-), positive control (K+) (glibenclamide 0.072 mg), and experiment groups which were given *E. glandiflorus* cell extract at a dose of 1 mg/kgBW (P1), 10 mg/kgBW (P2), and 100 mg/kgBW (P3). Rats were marked and housed in group cages. During experiment, the rats were given standard feed and drunk ad libitum.

Blood glucose measurement

The animals were checked blood glucose levels by being satisfied first for 8 hours by drinking water at day before treatment (day 0). The various doses of *E.grandiflorus* cell extract and glibenclamide were given with oral probe for 10 days. Measurement of blood glucose levels was carried out on day 10 as posttest. Blood collection was carried out after the rats were fasted for 10 hours and were only given water ad libitum.

Data analysis and in silico methods

The data of blood glucose level were tested of normality and homogeneity before compared between groups. The difference of blood glucose level among groups were exam by anova. Exploration using the in silico method was carried out through PubChem, SEA (The Similarity ensemble approach), String-db and KEGG Pathway (Kyoto Encyclopedia of Genes and Genomes).

repair of pancreatic beta cells after administration

Table 1. The blood glucose level after given *E. glandiflorus* extract on hyperglycemic rat

Groups	Blood Glucose Level (mg/mL)	
	Day 0	Day 10
Negative control (K-)	455.2 ± 56.7	367.8 ± 54.6
Positive control (K+)	422.8 ± 105.9	89.6 ± 8.0
P1 (1 mg/kgBW)	469.8 ± 63.4	285.6 ± 65.7
P2 (10 mg/kgBW)	355.6 ± 88	136.8 ± 4.3
P3 (100 mg/kgBW)	446.0 ± 56.4	104.8 ± 14.9

RESULTS AND DISCUSSION

Stalks of *E. grandiflorus* leaves grown on the Woody Plant Medium (WPM) medium with the addition of 2.4-D 2.5 ppm can produce callus. Callus is a friable which is a good material to be used as a cell suspension culture. By the optimum incubating the suspension culture resulted a brownish-yellow cell aggregate. The cell aggregate prepared to orally treatment by added aquadest.

Alloxan inducing resulted the hyperglycemic rats that reach after 8 days intraperitoneal treatment. The pretest and posttest experiment showed decreasing of blood glucose level after ten days treatment by *E. glandiflorus* cell extract (Table 1). The mean of blood glucose level on day 10 were compared by anova test to analyze the difference between groups. Statistical analysis showed the difference between negative control group (K-) compared with P2($p=0.015$) and P3 ($p<0.001$). When compared with K+, only P3 showed no difference ($p=0.873$). These results suggested that extract cell of *E. glandiflorus* have antidiabetic activity begin at the dose 10 mg/kgBW and optimum at the dose 100 mg/kgBW.

E. grandiflorus cell suspension culture has the potential to have antidiabetic activity. Their contain of flavonoids have been shown the beneficial effects in against diabetes mellitus. Quercetin and Kaempferol were main flavonoid effected to blood glucose level (Al-Ishaq et al., 2019; Vinayagam & Xu, 2015). The decreasing of blood glucose levels might through the ability of flavonoid to reduce glucose uptake and by increasing glucose tolerance. The flavonoids containing *E. grandiflorus* can function as antioxidants that repair damage to pancreatic beta cells due to the administration of alloxan (Coskun et al., 2005). The decrease in blood glucose in the experimental group may have occurred due to the of *E. grandiflorus* extract. This condition might be due to the pharmacodynamic properties of glibenclamide which can stimulate pancreatic beta cells to secrete insulin. Glibenclamide plays a role in the improvement of pancreatic beta cell function and regeneration due to alloxan induction. The damage of pancreatic beta cells due to alloxan induction can be temporary if immediately stimulated for regeneration.

The antidiabetic mechanism of *E. grandiflorus* was carried out by *in silico* secondary data tracking showed its effect on the insulin receptor pathway. Insulin affects in multiple physiological process to maintain blood glucose level, included increasing of glucose uptake, glycogen synthesis, lipogenesis, protein synthesis, gene expression, DNA synthesis, amino acid up takes and Na+/K+ pump. The insulin also affects in decreasing of lipolysis, gluconeogenesis, apoptosis and autophagy (Meyts, 2016). Insulin receptor pathway starts from the bond between insulin and insulin receptors to form insulin receptor signaling (IRS). IRS have three major biochemical steps:1) tyrosine phosphorylation of the receptor, 2) activation of the phosphoinositide-3-kinase (PI3K), and 3) activation of multiple serine/threonine kinases (AKT) (Haeusler et al., 2018).

Insulin receptor signaling involved many proteins, enzymes, hormones and compounds. Each component of the IRS can be a target of internal and external bioactives and affected to insulin homeostasis and glucose regulatory. Quercetin, orientin and kaempferol from *E grandiflorus* acts on several protein in the insulin receptor pathway, namely Hexokinase 2 (HK2), Tyrosine-protein phosphatase non-receptor type 1 (PTPN1), serine/threonine kinase 1 (AKT1), Phosphoinositide-3-kinase regulatory subunit alpha (PI3KR1), HRAS (Harvey Rat Sarcoma) and Glycogen synthase kinase-3 beta (GSK3B).

Quercetin is involved in several biological

Table 2. The protein target of *E. grandiflorus* bioactive compound on insulin receptor pathway

Bioactive compound	Protein target
Quercetin	PI3KR GSK3B AKT1
Kaempferol	PTPN1
Orientin	HRAS HK2

actions in correlation with diabetes mellitus mechanism included glucose homeostasis; insulin sensitizing and secreting; glucose utilization in peripheral tissues; the inhibition of intestinal glucose absorption (Eid et al., 2017). The first step quercetin's role reduces GLUT2 expression which decreased glucose absorption in small intestine (Borghi et al., 2017). Then, quercetin decreased blood glucose by inhibit insulin dependent activation of Phosphoinositide-3-kinase regulatory subunit alpha (PI3KR1). Together with AKT, PI3K forms a key component of many signaling pathways that involve the binding of membrane-bound ligands such as receptor tyrosine kinases. This pathway resulted increasing of lipogenesis and glycogenesis, so that blood glucose levels decrease. Quercetin binds to PI3KR1 and activated (phosphorylated) protein-Tyr kinases, through its Src homology 2 (SH2) domain. SH2 domain associated to membrane plasma was mediated by p110 catalytic unit, resulted increase in glucose uptake and glycogen synthesis.

Glycogen synthase kinase-3 (GSK3) is a protein that mediates the addition of phosphate molecules onto serine and threonine amino acid residue. The member of GSK family was GSK3α and GSK3β. The inhibition of GSK3 may have a particularly significant effect on disease-associated self-activating mechanisms (Beurel, et al, 2015). Glycogen synthase kinase-3 beta (GSK3B) is the one of GSK family. GSK3B acts as a negative regulator of glucose homeostasis. Inhibition of GSK3B action is one point that can be used as a target for diabetes therapy. Quercetin targeting on GSK3B protein induced glycogenesis.

Protein tyrosine phosphatases (PTPs) is a large family of enzymes in the insulin signaling. PTP role as a negative regulator of insulin signaling by dephosphorylating the phosphothyosine residues of insulin receptor kinase. One of PTPs is Tyrosine-protein phosphatase non-receptor type 1 (PTPN1), also known as PTP1B. Tyrosine-protein phosphatase non-receptor type 1 was encoded by PTPN1 gene, located in chromosome 20. PTPN1 plays a role in down-regulating insulin and leptin signaling for diabetes and obesity therapeutic (Tonks, 2013). The binding between Kaempferol and PTPN1 induced the inhibition of glycogenesis through AKT/PI3K signaling.

RAS proteins determined the on-off cycle between active guanosine triphosphate (GTP)-bound and inactive guanosine diphosphate (GDP)-bound states (Simanshu, 2017). HRAS is human RAS protein encoded by HRAS gene that located on the short (p) arm of chromosome 11. HRAS regulating the division or proliferation of cell. The growth hormone and other factors stimulated the cell and initiated to cell proliferation. HRAS induced the cell proliferation through the activation of Raf, MEK 1/2 (mitogen-activated protein kinase) and ERK ½ (extracellular signal-regulated kinases 1/2) proteins, which known as MAPK/ERK Pathway. Orientin *of E.grandiflorus* activates the HRAS protein thereby increasing cell proliferation. The beta pancreas cells were damaged by alloxan can be repaired by orientin so that insulin production increased.

Hexokinase (HK) 2 is the crucial enzymes for the initiation and end of glycolysis. Together with pyruvate kinase (PK), convert glucose to glucose-6-phosphatase and phosphoenolpyruvate to pyruvate. The first rate-limiting step of glycolysis is the conversion of glucose to glucose-6-phosphatase and phosphoenolpyruvate to pyruvate mediated by HK, whereas the PK based reaction is associated to glycolysis to lactic acid and amino acid metabolism. The binding of orientin to HK2 lead depletion of its. The depletion of HK2 inhibit glycolysis process and induced oxidative phosphorylation, resulted the decreasing of blood glucose (DeWall, et al., 2018).

E.grandiflorus extract which has many bioactive compounds can be developed as an antidiabetic. Oral administration *E.grandiflorus*

extract reduced blood glucose level of hyperglycemic rat. The new finding of our study is the pathway of *E.grandiflorus* bioactive compound as antidiabetic.The target proteins of quercetin, kaempferol and orientin have a very important role in insulin receptor signaling. Quercetin, kaempferol and orientin play a role in down-regulation of blood glucose levels. They act by reducing glucose absorption in the small intestine, increasing of cell glucose uptake, increasing of lipogenesis and glycogenesis. This research is limited to testing the effectiveness in experimental animals and tracing mechanisms by in silico. For the discovery and development of new drugs, a lot of data is still needed which includes pharmacokinetics, which describe the journey of extracts from entering the body to being excreted. Another data is also needed to ensure the safety of extract use in humans, which includes the data of toxicity extract to cells, tissues and organs, both acutely and chronically.

CONCLUSION

The high prevalence of diabetes mellitus led an innovative thinking to discover a new agent of antidiabetic. *E. grandiflorus* potentially to develop be herbal medicine. From this experimental study can be concluded that the *E. glandiflorus* cell culture extract had an effect on reducing blood glucose levels of hyperglycemic rats with the optimal at a dose of 100 mg/kg BW. The bioactive of *E.grandiflorus* that act as antidiabetic was quercetin, kaempferol and orientin. Quercetin, orientin and kaempferol of *E. grandiflorus* play important role in insulin receptor pathway to decrease blood glucose level. Further research is needed to provide a scientific basic before clinical trials in humans or diabetic patients, included acute and chronic toxicity, pharmacokinetic and pharmacodynamic.

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