Antidiabetic Activity of Okra Fruit (Abelmoschus esculentus (L) Moench) Extract and Fractions in Two Conditions of Diabetic Rats

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ABSTRACT

Okra (Abelmoschus esculentus (L) Moench) fruit is empirically used in type 2 diabetes mellitus treatment. This research aims to know the antihyperglycemic activity of okra fruit extract and fractions in streptozotocin-nicotinamide (STZ-NA) induced as well as in insulin resistance diabetic rats, the effect on pancreatic cells regeneration, and the effect on immunohistochemical expression of glucose transporter-4. This study used a group of 35 male Wistar rats for STZ-NA induced diabetic model and another group of 35 rats for insulin resistance diabetic model. Gliclazide (0.72mg/kg BW) and metformin (45mg/kg BW) were used as drug control in STZ-NA induced and insulin resistance diabetes, respectively. Okra fruit ethanol extract, n-hexane, ethyl acetate, and water fraction were orally administered with dose of 200; 107; 6 and 86mg/kg BW, respectively, for 28 days after diabetic condition was obtained. Blood glucose level was measured every week. Hematoxylin-eosin staining was used to evaluate the pancreatic cells regeneration, while immunohistochemistry was used to evaluate the expression of glucose transporter-4 in muscle membrane cells, at the end of the treatment. The results revealed that ethyl acetate fraction was the most effective in lowering blood glucose level in both condition of diabetes. Ethyl acetate fraction decreased the necrosis of pancreatic cells in STZ-NA induced diabetic rats and increased the expression of glucose transporter-4 in muscle cell of insulin resistance diabetic rats.

Keywords: Abelmoschus esculentus (L) Moench, STZ-NA, insulin resistance diabetes, pancreatic cell regeneration, glucose transporter-4.

INTRODUCTION

Type 2 diabetes mellitus (DM) is the most common form of DM characterized by hyperglycemia, insulin resistance, and relative insulin deficiency (Olokoba et al., 2012). This type of DM is resulted from the interaction between genetic, environmental and behavioral factors. Type 2 DM also associated with the expression of GLUT-4 protein in muscle cells, protein GLUT-4 indicated has crucial role in whole body glucose homeostasis (Bryant et al., 2002). Chronic uncontrolled hyperglycemic generates the developing of various complication like micro vascular (nephropathy, retinopathy, nephropathy) and macro vascular (peripheral vascular disease and coronary heart diseases). Type 2 DM can be well controlled by oral antidiabetic drugs; however, they can cause serious side effects for the patients. The length of DM therapy also often induces in compliance of drug administration. The medicinal herbs are usually used as alternative therapy of DM due to the minor or lack of side effects (Verma et al., 2018).

Okra (Abelmoschus esculentus (L) Moench) is a tropical vegetable that has been used extensively in traditional medicine for DM treatment. Previous researches reported the scientific evidences that okra fruit extract reduced the blood glucose level in normal, in alloxan-induced as well as in streptozotocin (STZ)-induced diabetic test animals (Tomoda et al., 1989; Ramachandran, et al., 2010; Sabitha et al., 2012). Water soluble fraction of this fruit significantly reduced the intestinal absorption of glucose in fasting rats (Khatun et al., 2011). Two flavonols in okra seeds, isoquercetin and quercetin-3-O-beta-glucopyranosyl-glucoside, are reported as alpha-glucosidase inhibitors (Thanakosai and Phuwapraisirisan, 2013). Isoquercitrin and
quercetin 3-O-gentiobioside contained in ethanol extract of okra fruit reduced blood glucose and serum insulin levels and improved glucose tolerance in obese mice (Fan et al., 2014).

In this study, fractionation of okra fruit extracts was conducted for further investigation of the bioactive compounds in different solvents (Azmir et al., 2013). Two conditions of diabetic rats i.e. STZ-NA induced, and insulin resistance diabetic rats were generated to investigate the effect of okra fruit extract and fractions. The effect on pancreatic cells regeneration was determined in STZ-NA induced model, while the effect on immunohistochemical expression of glucose transporter-4 was determined in insulin resistance model.

MATERIAL AND METHODS
Preparation, extraction and fractionation of plant materials

The okra fruits were obtained from Batu, Malang, East Java, were harvested 34-36 days after fruit appears (Bortey and Dzomoku, 2016). Determination by Department of Biology, Faculty of Mathematics and Natural Science UNS Surakarta, Central of Java, confirmed the desired species of Abelmoschus esculentus (certificate number of 58/UN27.9.6.4/LAB/2018).

The dried okra fruit powder was macerated in ethanol 96% for 5 days. The dried extract then was fractionated by the liquid-liquid partition method, to obtained n-hexane, ethyl acetate as well as water soluble fractions.

Experimental design

All the experimental protocols were approved by Health Research Ethic Committee Faculty of Medicine Muhammadiyah University Surakarta (Letter number of 1539/A.1/KEPK-FKUMS/XI/2018). STZ-NA (45 and 110 mg/kg BW) was induced intra peritonealy for 3 days to obtained diabetic condition. High fat diet i.e. 5 mL pork oil and fructose (180 mg/100g BW) were orally administered for 60 days, followed by intra peritoneal injection of STZ (30 mg/kg BW) on 30th and 44th, were conducted to obtained insulin resistance diabetic condition (Ai et al., 2005; Zhang et al., 2008). Normal diet (15g/rat) was administered daily.

Antidiabetic activity measurement

After each of diabetic condition was obtained, the rats were divided into 6 groups, namely diabetic control, drug control (gliclazide 0.72 mg/kg BW in STZ-NA induced diabetic rats and metformin 45 mg/kg BW in insulin resistance diabetic rats), and treatment groups (okra fruit ethanol extract, n-hexane, ethyl acetate, and water fraction were orally administered with dose of 200; 107; 6 and 86 mg/kg BW). The fraction dose was calculated based on the weight ratio of each fraction yield compared to the weight of the whole fraction, multiplied by the extract dose. The oral administration was conducted for 28 days, while the blood glucose level was determined every 7 days. On 28th day, the animals were sacrificed, the pancreas and skeletal muscle tissue were removed for morphology and immunohistochemistry study.

Morphology of pancreas tissue

The paraffin-embedded pancreas was sectioned at 4 µm, then were mounted on glass slides using a hot plate. The tissue sections then were deparaffinized by xylene and rehydrated by different graded ethanol dilution. Hematoxylin and eosin (HandE) stains was used for recognizing the morphologic profiles in pancreas tissue (Nurdiana et al., 2017).

Immunohistochemistry

The immunohistochemistry procedure was conducted in three stages, namely preparation of soleus muscle tissue sample slide; optimization of the dilution and operating time of anti-GLUT-4 antibody; and immunohistochemistry on sample, photomicroscope and semiquantitative of GLUT-4 protein expression (Feitosa et al., 2018).

Data analysis

All values are expressed as means ± standard errors (SEs). Data were analyzed using one-way analysis of variance with Post Hoc test. Differences with p values of less than 0.05 were considered statistically significant.

RESULT AND DISCUSSION
Antihyperglycemic activity and pancreatic cells regeneration in STZ-NA induced DM

Induction of STZ in test animals caused the degradation of the islet of β- pancreatic cells, due to the toxicity effect of the nitrous amide group of STZ (Szkudelski, 2012). Concomitant administration of nicotinamide reduced the STZ toxicity or protect Langerhans β-cells because nicotinamide acted as a Poly(ADP-ribose)-polymerase (PARP-1) inhibitor. Inhibition of PARP-1 cause the synthesis of cellular NAD⁺ was increase due to a decrease in the use of NAD⁺ as material to form ADP-ribose.
Increasing of NAD$^+$ followed by increasing of ATP and synthesis or secretion of insulin, so inhibited the apoptosis and necrosis of Langerhans β-cells (Alenzi, 2009).

STZ induction often caused significant and acute weight loss resulting from the acute onset and progression of diabetes, and also due to the repeated fasting of the animals as well as the acute toxicity of STZ (Nørgaard, 2018). Increasing of muscle wasting and loss of tissue protection also contributed to the body weight reduction. The weight loss in diabetic control group after STZ-NA induction (Figure 1). It was observed that rats treated with gliclazide show gradual and significant increase in body weight, which indicates the prevention of muscle tissue damage due to hyperglycemic condition (Cheng, 2013). Oral administration of okra fruit ethanol extract and fractions also increased the body weight, however only okra fruit ethanol extract showed comparable effect to gliclazide.

The antihyperglycemic effect of okra fruit ethanol extract and fractions, compared to the normal, diabetic and gliclazide treated group (Table I). After 28 days of oral administration, the extract and fractions showed antihyperglycemic effects, however only ethyl acetate fraction showed the comparable effect to gliclazide. This was an interesting fact because the dose of ethyl acetate fraction was the lowest among other fractions.

Ethyl acetate fraction contained semi polar compounds mainly the flavonoids (quercetin glycosides) and triterpenoids. Flavonoids posed antihyperglycemic activity because of antioxidant and aldose reductase inhibition activity.
Flavonoids, such as quercetin, acted as radical scavenger and inhibited the formation of free radicals that can damage pancreatic beta cells by transferring the hydrogen atoms (H) from their phenolic groups and binding with free radical substituents (R•) to form flavonoid radicals (Banjarnahor and Artanti, 2014).

Triterpenoids play a role in lowering of blood glucose level by inhibition of aldose reductase enzyme, an enzyme that catalyzes the conversion of glucose into sorbitol through the reduction of NADPH to NADP⁺ in the polyol pathway. Normally, glucose is metabolized through the glycolysis pathway, then enters the Krebs cycle to produce ATP. When hyperglycemia occurs, the aldose reductase enzyme becomes active and glucose metabolism occurs in the polyol pathway (Nazaruk and Borzym-Kluczyk, 2015). This causes an increase of sorbitol levels in cells and a decrease of ATP production in the mitochondria. Furthermore, it also results in increased apoptosis and necrosis in pancreatic β-cells. Therefore, flavonoids and triterpenoids play a role in regeneration of pancreatic β-cells and trigger the release of insulin.

Table 2. Profiles of regeneration of pancreatic cells after administration of okra fruit extract and fraction

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of cells (mean ± SD)</th>
<th>Picnosis</th>
<th>Caryorexis</th>
<th>Caryolysis</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic</td>
<td></td>
<td>21.7±2.5a</td>
<td>40.3±2.5b</td>
<td>6.3±0.6b</td>
<td>31.7±4.9</td>
</tr>
<tr>
<td>Glicazide</td>
<td></td>
<td>13.7±1.5a</td>
<td>27.0±2.6a</td>
<td>3.3±0.6a</td>
<td>56.0±2.6</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td></td>
<td>19.0±1.0ab</td>
<td>35.0±3.6ab</td>
<td>4.3±0.6ab</td>
<td>41.7±2.5</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td></td>
<td>18.3±2.1ab</td>
<td>32.0±3.5ab</td>
<td>3.7±0.6ab</td>
<td>46.0±2.0</td>
</tr>
</tbody>
</table>

*Significantly different to diabetic group (p<0.05); b significantly different to glicazide group (p<0.05)
Histological changes were observed in the pancreatic cells of diabetic group, which were indicated by the invisible form of acini and intact islet cells, resulting in clumping or thickening of the cell nucleus (picnosis), cell nucleus rupture (cariorexis) and cell nucleus lysis (cariolysis). Numbers of damaged and normal cells of each group (Table II). Glicazide decreased the number of damaged cells, correlated to its ability in controlling of glycemic condition. The okra fruit ethanol extract and ethyl acetate fraction also decreased the number of damaged cells, although lowered than glicazide therapy.

Antihyperglycemic activity and pancreatic cells regeneration in insulin resistance DM

High fat diet are considered one of the most important environmental factors in the pathophysiology of obesity. Induction of high fat diet for 60 days increased the body weight average of 138 g/rat, compare to average increasing of 27 g/rat in normal diet group. Weight increasing after 4 weeks administration of metformin (Figure 3). Metformin effectively inhibits dipeptidyl peptidase IV (DPPIV) activity in type 2 diabetic patients, slowing the gastric emptying due to the anorectic action, and has a direct action on neurons in the central nervous system involved in appetite regulation (Matsui et al., 2010). Oral administration of okra fruit ethanol extract and fractions showed higher effect in lowering the body weight in obesity rats. It was reported that okra polysaccharide can lower body weight (Durazzo, 2018).

Figure 3. Decreasing of body weight after oral administration of okra fruit ethanol extract and fractions

Table III. Antihyperglycemic activity of okra fruit extract and fraction in insulin resistance diabetic rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose level (mg/dL)</th>
<th>After insulin resistance induction</th>
<th>Day 7th</th>
<th>Day 14th</th>
<th>Day 21th</th>
<th>Day 28th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td>68.6±5.9</td>
<td>68.6bc</td>
<td>68.6bc</td>
<td>68.8bc</td>
</tr>
<tr>
<td>Diabetic (CMC Na)</td>
<td></td>
<td></td>
<td>248.8±29.0</td>
<td>249.0ac</td>
<td>249.8bc</td>
<td>249.6ac</td>
</tr>
<tr>
<td>Metformin 45 mg/kg bw</td>
<td></td>
<td></td>
<td>251.6±30.1</td>
<td>119.4ab</td>
<td>105.4ab</td>
<td>98.6ab</td>
</tr>
<tr>
<td>Ethanol extract 200 mg/kg bw</td>
<td></td>
<td></td>
<td>241.4±27.5</td>
<td>184.2abc</td>
<td>165.6abc</td>
<td>160.6abc</td>
</tr>
<tr>
<td>n-Hexane fraction 107 mg/kg bw</td>
<td></td>
<td></td>
<td>223.0±6.7</td>
<td>208.6abc</td>
<td>176.2abc</td>
<td>147.4abc</td>
</tr>
<tr>
<td>Ethyl acetate fraction 6 mg/kg bw</td>
<td></td>
<td></td>
<td>228.4±7.0</td>
<td>129.0abc</td>
<td>123.0abc</td>
<td>119.2abc</td>
</tr>
<tr>
<td>Water fraction 86 mg/kg bw</td>
<td></td>
<td></td>
<td>260.4±38.9</td>
<td>200.8abc</td>
<td>192.6abc</td>
<td>173.2abc</td>
</tr>
</tbody>
</table>

* significantly different to normal group (p<0.05);  b significantly different to diabetic group (p<0.05);  c significantly different to glicazide group (p<0.05)
Blood glucose levels after 60 days of high fat diet, fructose and low-dose STZ induction, were increased significantly compared to the normal group. The high calorie intake derived from fructose caused excessive energy stored as triglycerides in adipose tissue and was used as an energy reserve. The formation of abdominal adipose tissue was important in the development of dyslipidemia, hyperglycemia and hypertension. Excessive triglycerides formation reduced the insulin receptor sensitivity, lowered the glucose uptake by insulin-sensitive tissue. This triggered the lipolysis process resulted more free fatty acids and glycerol were produced. Free fatty acids and glycerol entered the tissues adipose as triglycerides. This cycle continued so more triglycerides were formed. It was concluded that the decrease of insulin receptor sensitivity was related to the state of hypertriglyceridemia, which furthermore triggered hyperglycemia (Mamikutty et al., 2014).

Antihyperglycemic effect after 28 days’ administration of okra fruit extract and fraction, compared to normal, diabetic, and metformin control (Table III). The extract and fractions showed antihyperglycemic effects, however the effects were lower than that’s of metformin. The ethyl acetate fraction showed the highest antihyperglycemic effect among the other fractions. The difference of blood glucose level lowering effect between fractions was caused by the difference in the contained compounds in each fraction which had different mechanisms in reducing blood glucose levels. The water fraction of okra seeds contain two types of flavonol glycosides i.e. isoquercetin and quercetin-3-O-beta-glucopyranosyl-glucoside which acted as α-glucosidase inhibitor, so inhibited the absorption of maltase and sucrose in the intestine (Dubey and Mishra, 2017). However, this mechanism only occurred in the post prandial period and did not affect insulin levels or insulin receptor sensitivity. The n-hexane fraction contained nonpolar compound such phytosterol and fatty acids, which delayed the rate of gastric emptying, decreasing the average glucose absorption from gastrointestinal tract (Bawa and Badrie, 2016). Ethyl acetate fractions contained alkaloids, terpenoids and many flavonoids. The suggested mechanism of action of these compounds are: the alkaloid increased the GLUT-4 translocation; terpenoids increased the GLUT-4 translocation through suppressing pro-inflammatory formation (TNFα) and increased the number of anti-inflammatory mediator (adiponectin). Flavonoids as antioxidants reduced the expression and secretion of cytokines that

Figure 4. Immunohistochemical staining of normal control(a), metformin control(b), diabetic control(c), and ethyl acetate fraction (d). The arrows indicated GLUT-4 protein.
resulted the increasing of GLUT-4 translocation (Aba and Asuzu, 2018).

Immunohistochemistry observation on GLUT-4 in rat soleus muscle cells (Figure 4). Expression of GLUT-4 protein in membrane of soleus muscle cells could be observed by immunohistochemical exami-nation using anti-
GLUT-4 polyclonal antibodies. We used the indirect method based on the formation of antigen-
antibody complex. The antigen was the GLUT-4 protein in membrane of soleus muscle cells, the primary antibody was the polyclonal anti-GLUT-4 antibody, while the secondary antibody was biotinylated (labeled) antibody, so the antigen-
antibody complex could be observed as brown spots on the cytoplasm.

The GLUT-4 expression in diabetic control was the lowest, proved that the long-term administration of high fat diet and fructose caused the desensitization and down regulation of insulin receptors, then interfered the GLUT4 translocation to the plasma membrane of skeletal muscle cells, resulting of hyperglycemia. Ethyl acetate fraction increased the GLUT-4 protein translocation of soleus muscle cells in insulin resistance diabetic condition. The suggested mechanism of ethyl acetate fraction in increasing the GLUT-4 expression was including the role of the flavonoids (quercetin glycosides) through the inhibition of inflammatory mediators such as reactive oxygen species (ROS) and nitric oxide (NO); regulating the activity of inflammatory enzymes including cyclooxygenases (COXs) and inducible nitric oxide synthase (iNOS); reducing the production and expression of several cytokines and modulating transcription factors such as x-light chain enhancer of activated B-cells (NF-κB) and activation of protein-1 (AP-1). The inflammatory response increased the concentration of inflammatory cytokines, especially Interleukin 6 (IL-6) (Leyva-lópez, Gutierrez-grijalva, Ambriz-perez, and Heredia, 2016). Furthermore, IL-6 triggered the expression of suppressor of cytokine signaling-
(SOCS3) which can result in decreased expression of GLUT-4 and IRS-1 in insulin-sensitive target tissues and trigger insulin resistance (Chen et al., 2015).

Immunohistochemistry result also revealed that long term induction of high fat diet caused lipid accumulation in soleus muscle intra cells (figure 4c). Oral administration of metformin decreased the lipid accumulation (figure 4b). It was reported that metformin stimulated fatty acid oxidation, and decreased triglyceride synthesis (Wang, 2014).

Ethyl acetate fraction of okra fruit ethanol extract reduced the accumulation of lipid, although the effect was lower than metformin. Ethyl acetate fraction contains flavonoid and quercetin glycosides. Quercetin has been reported to have an anti-obese effect, and inhibit the adipogenesis of muscle cells by suppressing the transcription of adipogenic markers (Funakoshi et al., 2018).

CONCLUSION
The results revealed that okra fruit ethanol extract, n-hexane, ethyl acetate and water fraction showed antihyperglycemic effect on both of STZ-NA induced and insulin resistance diabetic rats, however only ethyl acetate fraction showed comparable effect to drug control. Ethyl acetate fraction regenerated the pancreatic cell in STZ-NA induced diabetic rats. Ethyl acetate fraction increased the translocation of GLUT-4 proteins comparable to metformin on the insulin resistance diabetic rats.

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