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In vitro Study of Namnam (*Cynometra cauliflora*) Leaf Extract on Glucose Uptake by Rat Diaphragm

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Abstract: Diabetes Mellitus (DM) is a group of metabolic disorder characterized by hyperglycemia due to damage in insulin secretion, insulin action or both. One of the diabetes mellitus group is diabetes mellitus type 2. Diabetes accounts type 2 for most of the data of DM patients which reached 90-95% of the total diabetics population. One of the plants as a potential alternative antidiabetic drugs is (*Cynometra cauliflora*) Namnam plant. This research will test the ability of glucose absorption by muscles of the isolated diaphragm of Sprague Dawley rat by Namnam (*Cynometra cauliflora*) leaf methanol extract at various concentrations. The result shows that an increase in glucose uptake by diaphragm when concentration of Namnam (*Cynometra cauliflora*) leaf extract and the highest increases in the concentration of 450 mg dL^{-1} with the uptake of 42.54±3.23 mg dL^{-1}/30 min. Increased uptake of glucose also occurs when insulin 0.25 IU mL^{-1} is combined with methanol extract of namnam leaf (EMDN) at concentration of 450 mg dL^{-1} of 92.19±9.74 mg dL^{-1}/30 min. Therefore, the namnam leaf (*Cynometra cauliflora*) plant has potential as antidiabetes type 2 drug through mechanism of the increased glucose uptake into cells.

INTRODUCTION

Diabetes mellitus is a group of metabolic disorder characterized by hyperglycemia due to damage in insulin secretion, insulin action or both[1]. Diabetes mellitus type 2 (DMT2) responsible for most of DM patients data[2] which reached 90-95% of the total diabetics population.

The number of therapies have been done to improve the status of diabetes through different mechanisms including inhibition of the enzyme metabolism of carbohydrates, glucose transporter modification, ß-cell regeneration and increased activity of insulin release[3]. Those mechanisms are still relying on therapeutic use of hypoglycemic agents and insulin as the main pillar of diabetes treatment. However, it has side effect that

stand out and limited use[4]. Therefore, many patients with diabetes are likely to use alternative therapies or traditional herbal medicine. One of the plants as a potential alternative antidiabetic drug is Namnam (*Cynometra cauliflora*) plant derived from Family Fabaceae.

Previous research has shown that this plant is a potential for natural antioxidant and safe[5, 6], cytotoxic against cells HL-60[7], an inhibitor of lipase in the leaves[8], antidiarrheal[9]. Analysis results of bioactive compounds in this plant also showed that kaempferol 3-O-rhamnoside isolated from ethyl acetate fraction leaves of *C. cauliflora*[8], phenolic and flavonoid[5], prosianidin trimers, tetramers prosianidin, prosianidin hexamer, taksifolin pentosida, catechins, viteksin, isoviteksin, kaempferol heksosida, quercetin pentosida, quercetin hexosida, apigenin-6-C-glucoside-8-C-glucoside, caempferol-coumaril hexosida and isorhamnetin hexosida[10]. This indicates that *Cynometra cauliflora*, especially, the leaf is rich source of bioactive and favorable for fitomedika development and functional food products included as antidiabetic.

This study conducted to determine the anti-diabetic effects of the methanol extract of *Cynometra cauliflora* leaf through a mechanism of glucose uptake *in vitro* by cell diaphragm. This research is expected to provide information Namnam leaf extract (*Cynometra cauliflora*) capability to absorb glucose into cells as one of the mechanisms of anti-diabetic agent DMT2.

MATERIALS AND METHODS

Tools used in this study are glass, UV-Vis spectrophotometer, rotary evaporator, micro pipette, animal cage (and equipment), gas cylinders, water bath, rat surgical equipment and other supporting tools.

The test materials used in this study are Namnam (*Cynometra cauliflora*) leaf obtained from Cintaratu Village, Parigi sub-district, Pangandaran District, West Java-Indonesia and has been identified by Bogor-based Research Center for Biology, Indonesian Institute of Sciences as *Cynometra cauliflora* L. the standard rat feed, Glucose Kit (Human), Insulin (NovoRapid® FlexPen®) 100 IU mL^{-1}, as well as the pure chemical analysis. Animal experiments used white rats (*Rattus norvegicus*) strain Sprague Dawley 3.5-4 months old and weighing 200-250 g as many as 30 individuals.

Sample preparation and extraction: Namnam (*Cynometra cauliflora* L.) leaf washed with flowing water, sorted and then dried by the Sun for 30 h until the moisture content 9-10%. Dried Namnam leaf samples (*Cynometra cauliflora*) sorted and then crushed with blender, in order to obtain a fine powder.

Namnam leaf powders (*Cynometra cauliflora*) as many as 100 g soaked in 500 mL of methanol p.a and do maceration for 24 h. Results of maceration filtered with Whatman filter paper No. 1, so that, the filtrate obtained first. Then the residue of re-macerated Namnam leaf (remaserasi) with methanol p.a 250 mL for 9 h, in order to obtain a second filtrate. Furthermore, the first filtrate and second filtrate are mixed and concentrated using a vacuum rotary evaporator at temperature of 45-50°C, so that, all that remains is Namnam leaf extract form crude extract. This extract is made to 150, 300 and 450 mg mL^{-1} to test the glucose uptake by rat diaphragm.

Glucose uptake by isolated rat diaphragm[11]**:** Rat diaphragm preparation method is used to examine peripheral glucose utilization. Tyrode solution made by dissolving 8 g NaCl, 0.2 g KCl; 0.2 g $CaCl_2$; 0.1 $MgCl_2$ in 250 mL of distilled as solution A and 0.05 g NaH_2PO_4; 1 g $NaHCO_3$ in 250 mL of distilled water as a solution B and 1 g of glucose in 500 mL of distilled water as solution C. After that mix 250 mL of solution A and solution B 250 and 500 mL solution C. Rat weighing 150-200 g maintained with standard ration, water *ad libitum*. Then fasted overnight before being taken diaphragm. Rat turned off by dislocation cervical and diaphragm is taken quickly to prevent traumatics. After that, the diaphragm is washed using tyrode solution without glucose. Samples in the form of Namnam leaf extract analysis. Measurement of glucose uptake by cells of the diaphragm on the samples was done by grouping (Table 1 and 2).

After the reaction solution was prepared, then rat diaphragm is inserted into a test tube and incubated. Incubation performed for 30 min at 37°C with O_2 95% with rocking incubator 140 rpm. After incubation, the diaphragm is rinsed with distilled water, then dried and weighed exterior with analytical balance. Tyrode solution which has been added to the sample glucose or insulin levels measured before and after incubation with the diaphragm. The determination of glucose is conducted by glucose oxidase method (GOD-PAP). Glucose level that has been obtained then divided by weight of the diaphragm of each tube.

Date analysis: Measurement data were analyzed using SPSS Software-20.0. The data obtained is treated as data related as evidenced by one-way ANOVA test. For the test group had significant differences continued with Tukey's test. Limit of significance used was $p<0.05$.

Table 1: Grouping and treatment of glucose uptake by diaphragm muscle at several concentrations of methanol extract of Namnam leaves

Groups	Treatment
1	Tyrode solution 2 mL (contain glucose) and 2 mL aquades as control
2	Tyroide solution 2 mL (contain glucose) dan 2 mL Insulin (400 µL Insulin 100 IU in 100 mL) as control positive
3	Tyrode solution 2 mL (contain glucose) and 2 mL EMDN 150 mg mL^{-1}
4	Tyrode solution 2 mL (contain glucose) and 2 mL EMDN 300 mg mL^{-1}
5	Tyrode solution 2 mL (contain glucose) and 2 mL EMDN 450 mg mL^{-1}

EMDN = Namnam leaves methanol extract

Table 2: Grouping and treatment of glucose uptake by diaphragm muscle at some EMDN concentrations and insulin

Groups	Treatment
1	Tyrode solution 2 mL (contain glucose) and 2 mL aquades as control
2	Tyrode solution 2 mL (contain glucose) and 2 mL Insulin 1 IU mL^{-1} as control positive
3	Tyrode solution 2 mL (contain glucose) and 2 mL Insulin 0,5 IU mL^{-1} as control positive
4	Tyrode solution 2 mL (contain glucose) and 2 mL Insulin 0,25 IU mL^{-1} as control positive
5	Tyrode solution 2 mL (contain glucose), 2 mL EMDN 150 mg mL^{-1} and Insulin 1 IU mL^{-1}
6	Tyrode solution 2 mL (contain glucose), 2 mL EMDN 150 mg mL^{-1} and Insulin 0.5 IU mL^{-1}
7	Tyrode solution 2 mL (contain glucose), 2 mL EMDN 150 mg mL^{-1} and Insulin 0.25 IU mL^{-1}
8	Tyrode solution 2 mL (contain glucose), 2 mL EMDN 300 mg mL^{-1} and Insulin 1 IU mL^{-1}
9	Tyrode solution 2 mL (contain glucose), 2 mL EMDN 300 mg mL^{-1} and Insulin 0.5 IU mL^{-1}
10	Tyrode solution 2 mL (contain glucose), 2 mL EMDN 300 mg mL^{-1} and Insulin 0.25 IU mL^{-1}
11	Tyrode solution 2 mL (contain glucose), 2 mL EMDN 450 mg mL^{-1} and Insulin 1 IU mL^{-1}
12	Tyrode solution 2 mL (contain glucose), 2 mL EMDN 450 mg mL^{-1} and Insulin 0.5 IU mL^{-1}
13	Tyrode solution 2 mL (contain glucose), 2 mL EMDN 450 mg mL^{-1} and Insulin 0.25 IU mL^{-1}

RESULTS AND DISCUSSION

Antidiabetic agent can affect multiple pathways of glucose metabolism such as insulin secretion, glucose uptake by targeted organ and the absorption of nutrients. Therefore, the calculation of the content of glucose in rat's hemidiafragma is a method for determining the efficiency of *in vitro* plants absorbs glucose in peripheral[12]. The use of the diaphragm muscle for glucose uptake *in vitro* test for the network is known to have insulin receptors[13]. Skeletal muscle accounts for approximately 40% of the body mass at every stage of life and the major networks involved in the induction stimulation of insulin on glucose uptake[14].

The analysis showed that Namnam leaf extract has ability to stimulate diaphragm muscles to absorb glucose. This capability is higher with the higher concentration of Namnam leaves methanol extract (EMDN). This was shown by the amount of glucose absorbed 42.54±3.23 mg dL^{-1}/30 min at a concentration of 450 mg mL^{-1} compared with control. EMDN ability is even higher than when just using insulin alone is 29.83±6.48 mg dL^{-1}/30 minutes (Table 3). That is where EMDN at concentration of 450 mg dL^{-1} is able of stimulating insulin to improve its ability to encourage the absorption of glucose into cell diaphragm. Glucose absorption capability is even higher compared to metformin 1 mg mL^{-1} of 16.17±0.20 mg g^{-1}/30 min, H. Isora 4.85±0.25 mg g^{-1}/30 min[15]. However, this difference may be caused by concentration of natural materials (active ingredients) contained in the sample.

On the other hand, the higher EMDN concentration also showed a trend increase in the rat diaphragm ability to absorb glucose. This phenomenon is possible because of the increased concentration of EMDN will increase the content of bioactive components that work to stimulate glucose uptake. Nevertheless in EMDN concentration of 150 and 350 mg mL^{-1} are still under control (insulin and water only).

Somani *et al*.[16] research also showed that methanol extract of leaves Couroupita guianensis significantly inhibit the enzyme activity of the metabolism of carbohydrates and increase the use of glucose by peripheral tissues. The same thing when Ghosh *et al*.[17] found that the extract of plant (*Ficus hispida* skin) provides glucose uptake in the hemi-diaphragm higher than when only given insulin only. The high absorption than insulin plant extracts have also been raised by her Suthar *et al*.[15] who conducted research on the hot water extract of the fruit Helicteres isora. The extract is able to increase glucose uptake by isolated rat hemidiafragma, but less effective than insulin.

In other observations made are also studies the ability of insulin and EMDN (Namnam Leaf Methanol Extract) combined (Table 3). In the current study observed the effect of rising concentration of insulin to change glucose uptake by rat diaphragm as observation model of the effect of rising insulin when diabetic type 2 due to insulin resistance (insulin insensitivity).

The test results of insulin showed that insulin is able to absorb glucose into the cells in diaphragm. Glucose uptake capability when no insulin is caused by the ability of insulin binds insulin receptors in rat diaphragm[18]. However, the higher concentration of insulin (0.25-1 IU mL^{-1}) decreased uptake of glucose significantly ($p<0.05$). The same trend is also happening typing insulin combined with EMDN (Table 4). This

Table 3: The ability of glucose uptake by cells *in vitro* diaphragm

Groups	Incubation medium	Glucose uptake (mg dL^{-1}/30 min)
1	Tyrode solution (contain glucose) and aquades as control.	8.98±1.03[a]
2	Tyrode solution (contain glucose) and Insulin as control positive	29.83±6.48[b]
3	Tyrode solution (contain glucose) and EMDN 150 mg mL^{-1}	-37.80±9.32[c]
4	Tyrode solution (contain glucose) and EMDN 300 mg mL^{-1}	4.88±4.03[d]
5	Tyrode solution (contain glucose) and EMDN 450 mg mL^{-1}	42.54±3.23[e]

EMDN = Namnam leaf methanol extract . Results are expressed as mean±SD (n = 4); [a, b, c, d] with different superscripts in the same column has pointed out, showed a difference significantly ($p<0.05$)

Table 4: Ability absorption of glucose by diaphragm muscles at some concentrations of EMDN and insulin

Groups	Incubation medium	Glukosa uptake (mg dL^{-1}/30 min)
1	Tyrode and aquades	5.79±3.29
2	Tyrode and Insulin 1 IU mL^{-1}	36.67±2.47[a]
3	Tyrode and Insulin 0.5 IU mL^{-1}	45.36±13.95[b]
4	Tyrode and Insulin 0.25 IU mL^{-1}	65.52±14.36[c]
5	Tyrode, EMDN 150 mg mL^{-1} and Insulin 1 IU mL^{-1}	-92.28±7.13[d]
6	Tyrode, EMDN 150 mg mL^{-1} and Insulin 0,5 IU mL^{-1}	1.96±17.10[e]
7	Tyrode, EMDN 150 mg mL^{-1} and Insulin 0,25 IU mL^{-1}	20.16±6.46[f]
8	Tyrode, EMDN 300 mg mL^{-1} and Insulin 1 IU mL^{-1}	-86.21±6.47[g]
9	Tyrode, EMDN 300 mg mL^{-1} and Insulin 0,5 IU mL^{-1}	13.08±3.99[h]
10	Tyrode, EMDN 300 mg mL^{-1} and Insulin 0,25 IU mL^{-1}	33.99±2.77[i]
11	Tyrode, EMDN 450 mg mL^{-1} and Insulin 1 IU mL^{-1}	-14.78±18.95[j]
12	Tyrode, EMDN 450 mg mL^{-1} and Insulin 0,5 IU mL^{-1}	22.62±8.35[k]
13	Tyrode, EMDN 450 mg mL^{-1} and Insulin 0,25 IU mL^{-1}	92.19±9.74[l]

EMDN = Namnam leaf methanol extract. Results are expressed as mean±SD from three observations. Figures followed by different letters in the same column indicate significantly different ($p<0.05$)

means that decrease in glucose uptake in insulin concentration is higher cannot be enhanced by the presence of EMDN. Such condition also occurred in some studies of combination of insulin with some *Houttuynia cordata* extract decreased the fraction of ethyl acetate, chloroform and hexane fraction[12]. A decrease in the ability of glucose uptake by diaphragm also occurs to *Hybanthus enneaspermus* and *Pedalium murex* combination with insulin[19]. This phenomenon indicates that some components of active ingredient contained in the active ingredient is still no antagonistic to insulin which reduce the absorption of glucose by the ability of diaphragm muscle cells as an *in vitro* model. This is possible because the samples used in these studies are still in the form of crude extract. It has been proved by Kumar *et al*.[12] which showed an increase in glucose uptake by diaphragm capabilities in combination with insulin standard compound quercetin (29.65±34.10±0.54 becomes 1.03 mg g^{-1}/30 min). Similarly, Jadhav and Puchchakayala[20] research which found an increased uptake of glucose as standard flavonoid compounds (Boswellic acid, ellagic acid, quercetin, rutin) in combination with insulin.

Nevertheless, the results of this study indicate that when EMDN concentration enhanced, the ability of glucose uptake by rat diaphragm muscle cells also increased significantly ($p<0.05$) (Table 4). Even this increasing trend EMDN highest at concentration of 450 mg mL^{-1} 92.19± 9.74 mg g^{-1}/30 min. According to Patel *et al*.[19] differences in the uptake of glucose when combined with prior combination with insulin showed the interaction between fractions with insulin. The same thing has been done by Dawoud and Shayoub[21] which revealed an increase in glucose uptake by rat hemidiaphragm combination of two compounds *Eucalyptus camaldulensi* (ethanol extract and water in combination with insulin). This suggests a synergistic effect between the two combinations as hypoglycemic agents that can be used as bioactive compounds to treat diabetes. These results indicate that the presence of EMDN at certain concentration can increase the ability of diaphragm muscle cells to glucose uptake.

The glucose uptake by various studies hemidiaphragm can occur through the existence of different factions. This suggests that these fractions have a mechanism similar to extra hepatic glucose uptake by peripheral tissues. This allegation is almost similar to that reported by Chattopadhyay *et al*.[22] which showed that saponins from *M. cymbalaria* alleged to have insulin-like activity that increases glucose utilization and extra pancreatic effects.

Flavonoids are thought to have a role in increasing the glucose uptake by diaphragm muscles and Sumarlin *et al*.[9] have shown that methanol extract of leaves of this plant contains flavonoids. This was confirmed by Jadhav and Puchchakayala[20] who found an increased glucose uptake in the sample flavonoids such as quercetin. Kumar *et al*.[12] estimated the mechanisms of flavonoids as antidiabetic agents are the inhibition of glucose transport activity, glucose uptake by isolated rat hemi-diaphragm. Another possible mechanism is the ability of flavonoid apigenin-6-C-

(2"-O-a-L-ramnopiranosil)-ß-l-fukopiranosida isolated from the leaves *Averrhoa carambola* L. (Oxalidaceae) as triggers insulin secretion (insulin-secretagogue) and insulin-mimetic agent[23]. Even Zygmunt *et al*.[24] has made clear that Naringenin (flavonoids) improves glucose uptake of skeletal muscle cells through a mechanism associated with AMPK-dependent. This indicates that EMDN can be used as an antihyperglycemic agent through the increased glucose uptake by muscle.

The results of this analysis also indicate absorption value is very low and even negative (Table 4). According to Seethanathan *et al*.[25] a decrease in glucose uptake in rat diaphragm allegedly due to a decrease in glucose metabolism in the diaphragm or the presence of glucose absorption inhibitor, or both. However, the mechanisms are certainly in relation to premises EMDN needs to be further research. Each treatment also showed a significant difference ($p<0.05$). This illustrates that the combination of insulin and EMDN impact on the ability of glucose uptake by isolated rat diaphragm.

CONCLUSION

The analysis showed that the increased uptake of glucose by diaphragm when the concentration of Namnam leaf extract (*Cynometra cauliflora*) and the highest increases in the concentration of 450 mg mL^{-1} with the uptake of 42.64±3.23 mg dL^{-1}/30 min. Increased uptake of glucose also occurs when insulin 0.25 IU mL^{-1} is combined with methanol extract of Namnam leaf (EMDN) at concentration of 450 mg mL^{-1} of 92.19±9.74 mg dL^{-1}/30 min. This suggests that Namnam plant leaf (*Cynometra cauliflora*) has potential as antidiabetic drug DMT2 through mechanism of increased glucose uptake into cells.

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