JTCM_MANUSCRIPT AWAL

INCRETIN EFFECT OF *Urena lobata* LEAVES EXTRACT ON STRUCTURE AND FUNCTION OF RATS ISLET β-CELLS

Purnomo Y[1*], Soeatmadji DW[2], Sumitro SB[3], Widodo MA[4]

[1]Department of Pharmacology, Faculty of Medicine, Islamic University of Malang
[2] Department of Internal Medicine, School of Medicine, University of Brawijaya
[3] Department of Biology, Faculty of Science, University of Brawijaya
[4] Department of Pharmacology, School of Medicine, University of Brawijaya

*Corresponding author : Yudi Purnomo
Email : y_purnomo92@yahoo.com
Pharmacology Department, Faculty of Medicine, Islamic University of Malang
MT. Haryono 193 Malang 65144, East Java Indonesia

ABSTRACT

Recently, incretin hormone such as Glucagon Like Peptide-1 (GLP-1) has become therapeutic target of type 2 diabetes mellitus (T2DM). However, this hormone is known to have a short half-life time. Prolonging of GLP-1 bioavailability is useful to regulate blood glucose level. *Urena lobata* is a plant having anti-diabetes potency eventhough the incretin effects on islet β-cells has not been evaluated. This study aims to determine the incretin effects of *U.lobata* leaves extract on the structure and function of rats islet β-cells.

This study utilize male spraque dawley rats divided into 2 control group and 3 test group (n=5). Diabetic rats were induced with High Fructose Diet (HFD) and single dose intra-peritoneal streptozotocin 25 mg/kgbw. Aqueous leaves extract of *U.lobata* was prepared by decoction methods and administrated orally with doses of 250, 500 and 1000 mg/kgbw for 4 weeks then incretin effect was evaluated by measuring serum GLP-1, insulin and blood glucose levels. Histology of islet β-cells was evaluated using photomicroscope by analyzing size, shape and number. Data were analyzed using ANOVA test followed by LSD test and $p \leq 0.05$ is considered significant.

Oral administration of aqueous extract *U.lobata* leaves at doses of 250, 500 and 1000 mg/kg body weight were able to prolong GLP-1 bioavailability by 3-fold, 5-fold and 7-fold respectively when compared to the diabetic group whereas blood glucose level were decreased about 30%, 35% and 40% respectively ($p<0.05$). Extract at doses of 500 and 1000 mg/kgbw also increased insulin level by 4-fold and 8-fold respectively compared to the diabetic group and the islet β-cells were repaired. Active compound in *U.lobata* leaves extract are suggested to prevent degradation of GLP-1 by inhibition of DPP-4 activity. Aqueous extract of *U.lobata* also improved the structure and function of islet β-cells by increasing of GLP-1 bioavailability.

Key words: islet β-cells, GLP-1, incretin, insulin, *U.lobata*.

INTRODUCTION

Modulation of incretins in the treatment of type 2 diabetes mellitus (T2DM) have received attention in the recent search for potent anti-diabetes. Glucagon Like Peptide-1

(GLP-1) and Glucose Dependent Insulinotropic Polypeptide (GIP) are major incretin hormone secreted by intestinal due to induction of oral nutrition[1]. GLP-1 plays a role in maintaining blood glucose level because of their biological activity such as stimulating insulin secretion, increasing β-cell proliferation, inhibiting glucagon secretion, reducing the rate of gastric emptying and inducing satiety[2,3]. In patient with T2DM, chronic hyperglycemia is caused by a decreasing of GLP-1 bioavailability therefore the secretion of insulin reduced[1,2].

Incretin hormone especially GLP-1 has potency as anti-diabetes. However, GLP-1 is metabolized by Dipeptidyl peptidase-4 (DPP-4) excessively to become inactive forms[3]. GLP-1 have a short half life, approximately 2-5 minutes due to DPP-4 activity [1,3]. The inhibition of DPP-4 is effective to treat T2DM because GLP-1 bioavailability can be retained moreover it was able to regulate blood glucose level [3,4].

Therapy T2DM through inhibition of DPP-4 show less side effect[6] although the data of drugs safety in long term use is still limited [7]. Adverse reaction of Oral Anti Diabetic (OAD) such as body weight gain and hypoglycemia are seldom in using of incretin like drug [4]. The less side effect of drugs is affected by GLP-1 activity that could suppress appetite and it does not have insulin secretory effect [3,5]. However, incretin like drug have also side effect such as flu like symptoms, skin reaction, gastrointestinal problem and this effect are able increase in long term use of drugs. This phenomenone induce people to search a medicinal plant as alternative therapy for T2DM trough controlling of incretin bioavailability [7].

Herbs are becoming popular medications of choices in the managements of diseases due to their perceived less side effect and holistic care property. One of traditional plants which have anti-diabetes effect is Caesar weed (*Urena lobata*). The root and leaf extract of *U. lobata* has been used empirically by Nigeria people to treat diabetes mellitus [8,28]. Pre clinical study of *U. lobata* root extract demonstrate anti-hyperglycemic effect on streptozotocin-

induced rat [8,32]. Bioactivity of *U. lobata* is regulated by its active substances such as sterol, alkaloid and flavonoid [9,32]. In Indonesia, *U. lobata* is known by Pulutan and this plant showed the anti-bacterial effect based on preliminary study[33]. Some study have showed anti-diabetic effect of *U. lobata* extract [8,9] however the mechanism of *U.lobata* on incretin activity has not been investigated. Therefore this study aims to examine anti-diabetes effect of *U. lobata* leaf extract trough incretin activity focus on structure and function of rats islet β-cells.

MATERIAL AND METHODS

Preparation of *U.lobata* leaf extract

U.lobata leaf powder were obtained from Balai Materia Medika Batu Malang with certificate number 074/027/101.8/2015. In brief, 50 g *U.lobata* leaf powder was extracted according to decoction method in 250 ml hot water at 90ºC for 30 minutes therefore the extract were evaporated until resulting concentrated extract.

Animals and treatments

Male Sprague-Dawley (SD) rats (180-200 g) were obtained from Gajah Mada University Yogyakarta Indonesia. ~~They were conducted~~ The study was conducted according to the ethical guidelines which were approved by the Commision of Ethical Research Brawijaya University Malang Indonesia with certificate number 245-KEP-UB. SD rats were housed in individual cage and automatically controlled animal room at 25 ± 1º C on a 12:12-h light–dark cycle. They were fed by standard food, water *ad libitum* and fasted overnight before the experiments. Normal diet (ND) and a high-fructosa diet (HFD) food were freshly mixed in every two days. Diabetic rats were induced by HFD (65% fructose and 35% ND food) and single dose of streptozotocin 25 mg/kg BB intra peritoneal refer to Guo *et al* with minor modification. Rats were stated diabetic if the fasting blood glucose level more than 126 mg/dL[10]. The experiment were assigned into five groups for five rats each. For eight weeks, the normal group (NG) received ND whereas the diabetic (DG) and treatment groups

received HFD. The treatment groups were given aqueous extract of *U.lobata* (AEU) at a dose of 250 mg/kg, 500 mg/kg, and 1000 mg/kg bw for four weeks after the rats was classified as diabetic according to Shirwaikar *et al*. Body weight and food consumption were monitored weekly. Blood samples were obtained 15 minutes after orally glucose stimulation in dose of 2 g/kg body weight and taken from tail vein after overnight fasted. Blood sample were immediatley centrifuge 4500 rpm. The serum was separated and saved under -20 ºC.

GLP-1 assay

GLP-1 serum level was analyzed by rat GLP-1 ELISA kit (USCN CEA804). 50 µl samples were added 50 µl detection reagent A and then incubated for 60 minutes at 37 ºC. After aspirating and washing, samples were added 100 µl detection reagent B and incubated for 30 minutes at 37ºC. Added 90 µl substrat reagent then was added 50 µl *stop solution*. Samples were read with microplate reader at $\lambda = 450$ nm.

Insulin assay

Insulin serum level was analyzed by rat insulin ELISA kit (Elabscience E-EL-R0023). 50 µl samples were added 50 µl Biotinylated detection Ab and incubated for 45 minutes at 37 ºC. After aspirating and washing then samples were added 100 µl HRP conjugate and incubated for 30 minutes at 37ºC. Added 90 µl substrat reagent then incubated for 15 minutes at 37ºC. 50 µl *stop solution* was added then read with microplate reader at $\lambda = 450$ nm.

Blood Glucosa assay

The blood samples were collected from the tail vein after overnight fasted and at 15 minutes after oral glucose administration. They were measured immediately using a commercially available glucometer (AccuCheck).

Histopatology of islet β-cells

Pancreas tissue were taken by section methods and continued by Hematoxylin Eosin (H-E) staining. Mostly islet cells containing β-cells were observed including shape, size, number

each view under microscope with magnification 400 time.

Statistical Analysis

The data were expressed as means ± SD. Statistical analysis was performed by one-way ANOVA. The least significant difference (LSD) test and Dunnet C were used for mean comparisons and then $p \leq 0.05$ was considered to be statistically significant.

RESULTS

The effect of *U. lobata* leaf extract on body weight, food consumption, glucose and insulin level of diabetic rats

In the end of the treatment, there is not a significant decrease of body weight on test group compared to diabetic group ($p>0.05$) meanwhile food consumption is decreased ($p\leq 0.05$) (refer to table 1). The oral administration of *U. lobata* leaf extract decrease fasting blood glucose level compared to diabetic group ($p\leq 0.05$) whereas insulin level were increased ($p\leq 0.05$).

Table 1: Body weight, food consumption, blood glucose and insulin level of diabetic rats

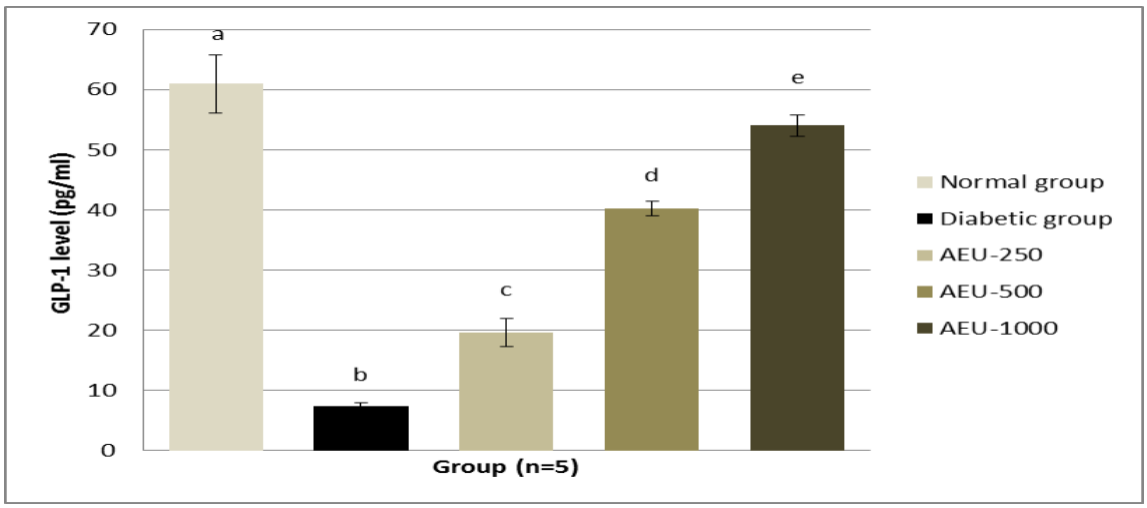
	Normal group	Diabetic group	AEU-250	AEU-500	AEU-1000
Body weight (g)	298.0 ± 13#	239.5 ± 19*	223.0 ± 11*	222.0 ± 16*	229.0 ± 12*
Food consumption (g)	25.0 ± 0	24.1 ± 3	15.4 ± 2#	14.8 ± 2#	20.2 ± 3#
Food consumption (%)	100.0 ± 0	96.0 ± 11	61.6 ± 7#	59.0 ± 6#	80.0 ± 8#
Fasting Blood Glucose (mg/dL)	101.0 ± 8#	129.0 ± 6*	96.0 ± 10#	87.0 ± 5#	92.0 ± 9#
Fasting Serum Insulin (pg/ml)	1242.9 ± 47#	226.9 ± 30*	350.8 ± 30#	536.2 ± 39#	699.2 ± 24#

Result are expressed as means ± SD, (n=5)
* different compared to normal group ($p\leq 0.05$, LSD test)
# different compared to diabetic group ($p\leq 0.05$, LSD test)

The effect of *U. lobata* leaf extract on GLP-1 serum level of diabetic rats

There is a significant decrease of GLP-1 levels on diabetic group about 8-fold compared to normal group observed ($p\leq 0.05$) refer to Fig.1. Aqueous extract of *U. lobata* at

doses 250 mg/kg bw, 500 mg/kg bw and 1000 mg/kg bw can prevent degradation of GLP-1 respectively about 3-fold, 5-fold and 7-fold compared to diabetic group ($p \leq 0.05$). An increase dose of *U.lobata* leaves extract prolong and enhance GLP-1 bioavailability.

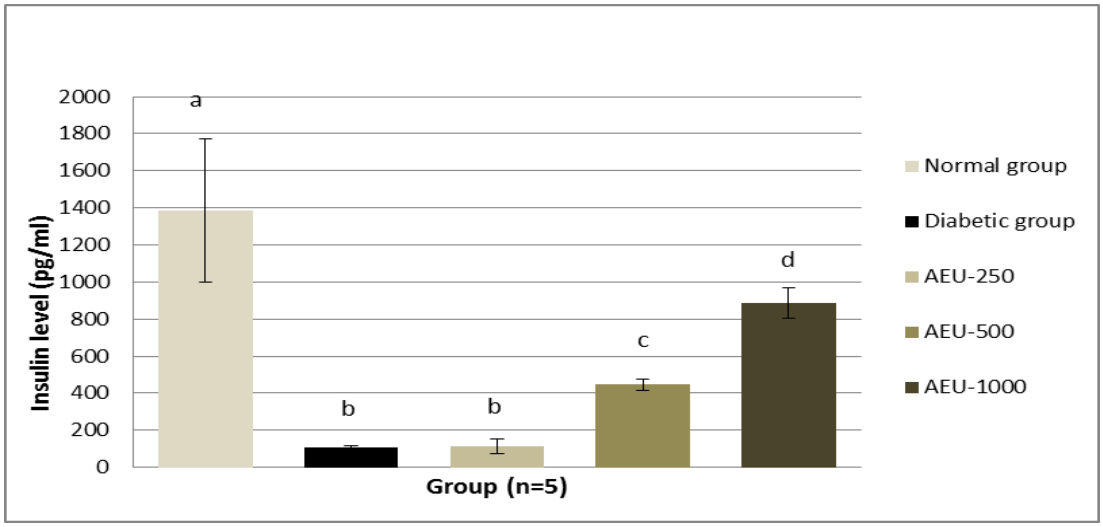


Description: a,b,c = different letter showed the differences of effect ($p \leq 0.05$, Dunnet C test)

Fig.1. GLP-1 level supplemented *U.lobata* extract

The effect of *U. lobata* leaf extract on insulin serum level of diabetic rats

There is a significant decrease of insulin levels on diabetic group approximately 14-fold compare to normal group observed ($p \leq 0.05$) refer to Fig.2. The administration of aqueous extract *U.lobata* 500 and 1000 mg/kg bw increase insulin level 4-fold, 8-fold respectively compared to diabetic group ($p \leq 0.05$) whereas the dose of 250 mg/kg bw cannot increase insulin level. The more increase dose of water extract *U. lobata*, the more insulin level was escalate.

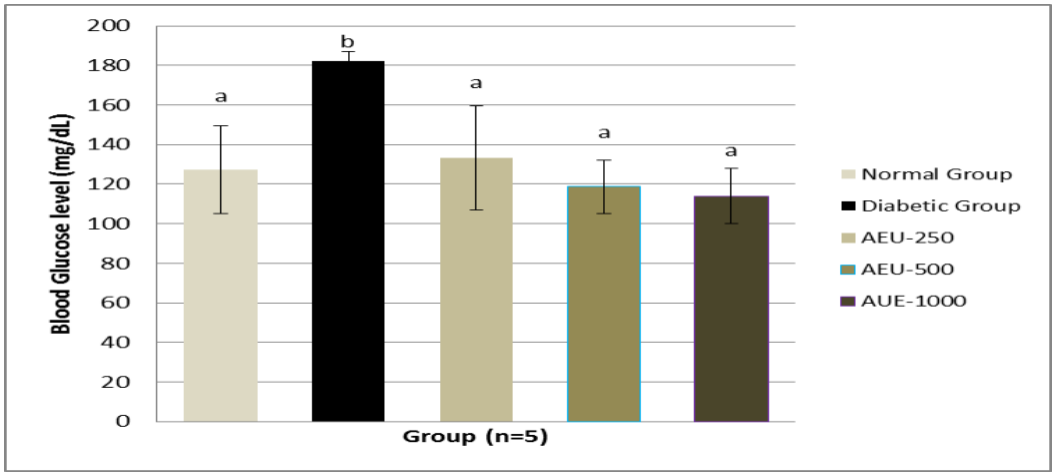


Description: a,b,c …= different letter showed the differences of potency ($p \leq 0.05$, LSD test)

Fig. 2 Insulin level supplemented *U.lobata* extract

The effect of *U. lobata* leaf extract on blood glucose level of diabetic rats

Based on these results at Fig.3, there is a significant increase at blood glucose level on diabetic group up to 70% compared to normal group observed ($p \leq 0.05$). The administration of aqueous extract *U.lobata* at dose of 250 mg/kg bw, 500 mg/kg bw and 1000 mg/kg bw can decrease glucose level respectively 30%, 35% and 40% compare to diabetic group ($p \leq 0.05$) after glucose stimulation. Blood glucose level is not different significantly on an increase of dose *U.lobata* ($p>0.05$).



Description: a,b,c …= different letter showed the differences of potency ($p \leq 0.05$, LSD test)

Fig. 3 Blood glucose level supplemented *U.lobata* extract

The effect of *U. lobata* leaf extract on islet β-cells of diabetic rats

The normal group (Fig.4.A) shows the shape of cells are round, nucleated and in a huge number, whereas the diabetic groups (Fig.4.B) cells shows longer, not nucleated and in a small number. Administration of aqueous extract *U. lobata* at dose of 500 and 1000 mg/kg bw could inhibit cells damage which shown as round cells, nucleated and in a huge number (Fig.4.C-D). Test groups have islet β-cells in slightly bigger size than normal group, except aqueous extract at dose of 1000 mg/kg bw. The bigger size of cells show a swelling cells and injury indications. The administration of aqueous extract *U.lobata* at dose of 1000 mg/kg bw are able to inhibit cells damage therefore the shape, size and number are similar to islet cells at normal group.

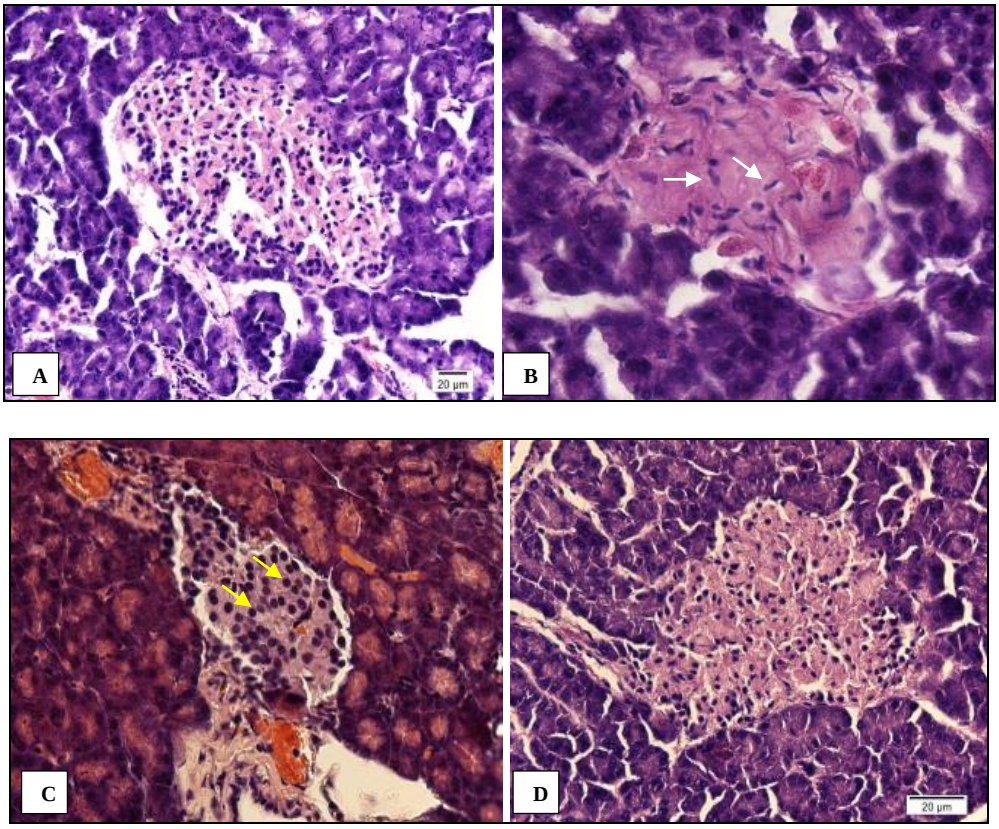


Fig. 4 Islet β-cells were stained by Hematoxylin Eosin and observed under photomicroscope with magnitude 400x. (A). Normal group (B). Diabetic group (C). AEU 500 mg/kb bw (D). AEU-1000 mg/kg bw. Magnification 400x. The white arrow show unnucleated cells and longer shape whereas yellow arrow show the swelling cells.

DISCUSSION

The effect of *U. lobata* leaf extract on GLP-1 serum level of diabetic rats

Oral administration of aqueous extract *U.lobata* significantly maintain GLP-1 bioavaibility of diabetic rats. Based on our previous study, active compounds in *U.lobata* such as mangiferin, stigmasterol and β-sitosterol are able to prevent degradation of GLP-1 by inhibition of DPP-4 [13]. DPP-4 inhibitor substances prevents the degradation of active GLP-1 eventhough it does not increase the levels of total circulating GLP-1 and does not prevent the kidney from rapidly clearing GLP-1[12].

GLP-1 is incretin hormone produced by L-cell intestine and the secretion depends on oral nutrition. GLP-1 has a potency for T2DM therapy but it is metabolized excessively by DPP-4 into inactive form [7]. GLP-1 has a short half-life, approximately for 2-5 minutes, it is caused of DPP-4 activity [3,6]. The active form of GLP-1 are GLP-1 (7-36) amides and GLP-1 (7-37) which are rapidly inactivated by DPP-4 through cleave N-terminal dipeptide His-Ala[12,19]. It produces inactive form of GLP-1, they are GLP-1 (9-36) amide and GLP-1 (9-37) isopeptides [6,7]. A number study showed that the importance of DPP-4 mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity [1,12].

GLP-1 is a super family peptide of glucagon which have a similarity degree approximatelly 48 % [14]. The similarity of amino acid sequence between GLP-1 and glucagon become one of this causa. Pro glucagon gen is located at chromosome 2q36-q37 and only found in some tissues whereas the messenger RNA (mRNA) of pro glucagon is met at α-cells pancreas, L cells intestine and brain[15]. Pro glucagon production is started from transcription of preproglucagon gen and then is continued by translation process [3,14]. The regulation of GLP-1 released from L cells intestine are a complex mechanism that involve combinations of nutrition, hormone and neural stimuli [14]. GLP-1 receptor is classified in *G protein-coupled* receptor that is found at liver, muscle and pancreas cells [2,3]. This receptor have a specific character by activation of adenilcyclase and result cAMP [15]. After GLP-1 binding with the receptor, it will activate cAMP and Mitogen Activated Protein Kinase (MAPK) [3,7].

The biological activities of GLP-1 are various and depend on the organ target. GLP-1 activity in pancreas has functions in stimulating the insulin secretion by cAMP activation, increasing β-cell masses by MAPK pathway and inhibiting the secretion of glucagon [3,5]. In brain, it will reduce the rate of gastric-emptying, induce satiety and neuroprotection whereas in liver, fatty acid metabolism will be decreased and glucose utilization increased [14,15]. All of them contribute to regulate blood glucose level in T2DM [1, 2.]

GLP-1 level is low in diabetic rat due to it is degradated by DPP-4. A number study showed that the importance of DPP-4 mediated inactivation of GLP-1 as a key determinant of GLP-1 bioactivity[2,3]. DPP-4 inhibition prevents the degradation of active GLP-1 therefore increase the bioavailability of GLP-1 which contribute in carbohydrate metabolism [1,3].

The effect of *U. lobata* leaf extract on Insulin serum level of diabetic rats

Aqueous extract of *U.lobata* significantly increase insulin synthesis of diabetic rats. It is controlled by active compounds in the extract through activity of GLP-1. The oral administration will maintains GLP-1 bioavaibility moreover the insulin biosynthesis can be increased. GLP-1 has a potency to retain the insulinotropic activity for treating T2DM [5,6]. In this study, the increase of insulin secrection is caused by the active compounds of *U.lobata* extract to maintain GLP-1 bioavaibility trough inhibitory of DPP-4 activity [13].

GLP-1 stimulates pro insulin biosynthesis and transcription of pro insulin gene. GLP-1 contributes to provide insulin deposition which loses from islet β-cells trough biosynthesis process[5]. GLP-1 is different with oral anti-diabetic sulphonylurea in stimulating of insulin formation because the sulphonylurea only stimulate insulin, not the biosynthesis of insulin [4,5]. GLP-1 is incretin hormone which is potential to increase islet β-cells proliferation and anti-apoptosis furthermore it is able to increase insulin secretion [2,6].

Hiperinsulinemia occurs in prediabetic condition or insulin resistance and then the secretion decline due to β-cell exhaustion or overwork [2]. The biological effect of insulin is

divided into two major groups, they are metabolic and mitogenic effect [11]. The metabolic effect are glucose transport, lipid metabolism, protein and glycogen synthesis whereas the mitogenic effect are the cell growth and mitogenesis [11].

This study showed also that the administrations of *U.lobata* extract give a good description of islet β-cell. It is shown by the shape, size and number of β-cell in better condition compared to diabetic groups. This conditions support the function of β-cell to produce insulin in order to maintain blood glucose level [14,15]. However, the diabetic group shows β-cells destruction which is signaled by a decrease number of islet β-cell and structure damage therefore it affect their performance to release insulin.

The effect of *U. lobata* leaf extract on blood glucose level of diabetic rats

Administration of aqueous extract *U.lobata* significantly decrease blood glucose level of diabetic rats. It is controlled by active compounds of *U.lobata* which has DPP-4 inhibitory activity like stigmasterol, mangiferin and β-sitosterol furthermore GLP-1 bioavaibility can be retained for insulin biosynthesis when the blood glucose level increase after stimulating of oral nutrition [13,18,32]. GLP-1 acts outside of metabolism purpose, that is inhibiting of gastric juices secretion, inhibiting of the GIT motility and inhibiting of the rate of gastric-emptying [2,3]. It is benefit to prevent the increase of blood glucose level at post prandial [5,6].

Insulin works to maintain blood glucose level after induction of glucose by metabolic pathway. This hormone transports glucose from blood to the tissue and then synthesis it into glycogen in muscle in order to reduce blood gucose level [11,14]. In diabetic groups, the insulin secretion is disrupted therefore they lose their's control to maintain blood glucose level [5,11]. This is showed by blood glucose level in diabetic group which is higher than normal and also treatment groups.

Histopatology of islet β-cell supplemented *U. lobata* extract

Oral administration of aqueous extract *U. lobata* are able to prevent islet β-cells death of

diabetic group. The effect of active compounds in *U.lobata* that have potency such as increasing β-cells proliferations and inhibiting β-cells apoptosis through GLP-1 activation [5,9,32]. Bioavailability of GLP-1 could be retained due to DPP-4 inhibitor substances in the extract such as stigmasterol, mangiferin and β-sitosterol [13,31]. It affects to the integrity of β-cells indirectly in test group, it is shown on the shape of cells, size and number which is close to normal groups. In some of test groups show swelling cells, it indicates cells damage at the first step eventhough the shape and number of cells are normal [20,29].

The active compounds of *U.lobata* leaves extract such as gossypetin, chrysoeriol and mangiferin could protect cell damage from free radical [22,24,27,29]. They work as antioxidant by donating electron to unstabil compounds in order to stabilize it [23]. Besides it, mangiferin and gossypetin act also as scavenger free radical moreover it could decrease oxidant level causing oxidative damage [16,22,27]. Hiperglycemiae in diabetes increases the production of free radical furthermore it occurs imbalance between oxidant and antioxidant [21,23]. This condition is caused by oxidative stress which lead to oxidative damage in tissue or organ and an increase of diabetic complication risk [16,23].

The effect of *U. lobata* leaf extract on body weight, food consumption, glucose level and insulin of diabetic rats

Aqueous extract of *U.lobata* reduces food consumption therefore it affects body weight gain of diabetic rats. It is related to active compound such as stigmasterol, mangiferin and β-sitosterol in *U.lobata* that maintain biavailability GLP-1 and their's interaction with GLP-1 receptor in brain could reduce the rate of gastric-emptying and also induce satiety [13,16,17,18]. The oral administration of *U.lobata* leaf extract decrease fasting blood glucose level and increase insulin level. GLP-1 activity in pancreas has functions in stimulating the secretion of insulin by cAMP activation, increasing β cell masses by MAPK pathway and inhibiting the secretion of glucagon [3,5]. In liver, it increase utilization of glucose and decrease fatty acid

metabolism. In T2DM, all of them contribute to maintain blood glucose level [1, 2.]

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest

AKNOWLEDGEMENTS

This study was funded by Doctorate Research Grant of Directorate General of Higher Education Indonesia (No. 053/B.07/U.III/LPPM/2014

REFERENCES

1. Drucker DJ. Dipeptidyl Peptidase-4 Inhibition and the Treatment of Type 2 Diabetes, Diabetes care 2007; 30(6):1335 - 1343. doi:10.2337/dc07-0228.
2. Chia CW, Egan JM. Incretin-based therapies in type 2 diabetes mellitus. J Clin Endocrinol Metab 2008; 93(10), 3703-3716. doi: 10.1210/jc.2007-2109.
3. Drucker DJ. Biological actions and therapeutic potential of the glucagon like peptides. Gastroenterology 2002; 122(2): 531-544. PMID:11832466.
4. Goodman LS, Gilman A, *et al*., Goodman & Gilman's the pharmacological basis of therapeutics. McGraw-Hill, New York, 2006.
5. Holst JJ, Orskov C. The incretin approach for diabetes treatment. Modulation of islet hormone release by GLP-1 agonism. Diabetes 2004; 53(3): S197-204
6. Salehi M, Aulinger AB, D'alessio AD. Targeting -cell mass in type 2 diabetes: Promise and limitation of new drugs based on incretins. Endocrine Reviews 2008; 29(3): 367-379. doi: http://dx.doi.org/10.1210/er.2007-0031
7. Bailey C. Incretin-based therapies. Endocrin abstract 2008; 15: S41.
8. Onoagbe IO, Negbenebor EO, Ogbeide VO, Dawha IH, Attah V, Lau HU, and Omonkhua AA. A study of the anti-diabetic effects of *Urena lobata* and *Sphenostylis stenocarpa* in streptozotocin-induced diabetic rats. Eur J Sci Res 2010; 43(1): 6-14.
9. Awika JM, Rooney LW. Sorghum Phytochemicals and their potential Impact on human Health. Phytochemistry 2004; 65(9): 1199-1221. doi:10.1016/j.phytochem.2004.04.001
10. Shirwaikar A, Rajendran K, Barik R. Effect of aqueous bark extract of Garuga pinnata Roxb. in streptozotocin-nicotinamide induced type-II diabetes mellitus. Journal of ethnopharmacology, 2006;107(2):285–290.
11. Rhodes CJ, White MF. Molecular insight into insulin action and secretion. Eur J Clin. Invest 2002; 32(S3): 3-13. PMID: 12028370
12. Rosenstock J, Zinman B. Dipeptidyl peptidase-4 inhibitors and the management of type 2 diabetes mellitus. Current opinion in endocrinology, diabetes and obesity 2007;14(2):98–107.
13. Purnomo Y, Soeatmadji DW, Sumitro SB, Widodo MA. Anti-diabetic Potential of *Urena lobata* Leaf Extract through Inhibition of Dipeptidyl Peptidase IV (DPP-4) Activity. *Asian Pacific Journal of Tropical Biomedicines* 2015;5(8)630-634.
14. Brubacker PL, Drucker DJ. Minireview : Glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut and central nervous system. Endocrinology 2004;145(6): 2653-2659.
15. Aronoff SL, Berkowitz K, Shreiner B *et al*., Glucose Metabolism and Regulation: Beyond Insulin and Glucagon. Diabetes spectrum 2004; 17(3): 183-190. doi:10.2337/diaspect.17.3.183
16. Sosa A, and Rosquete C. Flavonoid from *Urena sinuata* L. Avances en Química 2010; 5(2): 95 - 98.
17. Ros MM. *et al*., Phytosterol consumption and the anabolic steroid boldenone in humans: a hypothesis piloted. Food additives and contaminants 2007;24(7):679–684.

18. Rudkowska I. *et al.*, Cholesterol-lowering efficacy of plant sterols in low-fat yogurt consumed as a snack or with a meal. Journal of the American College of Nutrition. 2008;27(5):588–95
19. Mentlein R, and Gallwitz B. Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36) amide, peptide histidine methionine and is responsible for their degradation in human serum. Eur J Biochem 1993; 214(3): 829-35. DOI: 10.1111/j.1432-1033.1993.tb17986.x
20. Robbins Kumar. Pathologic Basic of diseases. 6th ed. WB Saunders Company, Philadelphia, 2002; pp.12-21.
21. Miura T. *et al.*, Antidiabetic activity of a xanthone compound, mangiferin. Phytomedicine: International Journal of phytotherapy and phytopharmacology. 2010;8(2):85–87.
22. Stoilova I. *et al.*, Antimicrobial and Antioxidant Activity of the Polyphenol Mangiferin. Herba Polonica 2005;51(1/2): 37–44. ISSN 0018-0599.
23. Halliwel B, Gutteridge JM. Free Radical in Biology and Medicine, Third edition, Oxford Science Publication, Oxford. 1999.
24. Matławska I, Sikorska M., Flavonoid compounds in the flowers of Abutilon indicum (L.) Sweet (Malvaceae). Acta poloniae pharmaceutica 2002;59(3):227–229.
25. Mazumder UK. et al. Antibacterial activity of Urena lobata root. Fitoterapia 2001;72(8): 927–929.
26. Panda S. et al., Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from butea monosperma. Fitoterapia 2009;80(2):123-126.
27. De las Heras B. et al., Anti-inflammatory and antioxidant activity of plants used in traditional medicine in Ecuador. Journal of ethnopharmacology 1998;61(2):161-166.
28. Omonkhua AA, Onoagbe IO. Preliminary proximate and phytochemical analyses of some medicinal plants used to treat diabetes mellitus in Nigeria. Inventi Impact Ethnopharmacol 2010; 1: 68-70
29. Sellamuthu PS, Arulselvan P, Kamalraj S, Fakurazi S, Kandasamy M. Protective nature of Mangifera on oxidative stress and antioxidant status in tissues of streptozotocin-induced diabetic rats. ISRN pharmacol 2013; doi: 10.1155/2013/750109.
30. Saeidnia S, Manayi A, Gohari AR, Abdollahi M. The story of beta sitosterol-a review. Eur J Med Plants 2014; 4(5): 590-609.
31. Chang CL, Lin Y, Bartolome AP, Chen YC, Chiu SC, Yang WC. Herbal therapies for type 2 diabetes mellitus: chemistry, biology, and potential application of selected plants and compounds. Evid Based Complement Alternat Med 2013; doi: 10.1155/2013/378657.
32. Islam MH, Rahman KMH, Rahman S, Rahmatullah M. Preliminary antihyperglycemic, antinociceptive activity, phytochemical analysis and toxicity studies on leaves of *Urena lobata* L. J Chem Pharm Res 2015;7(4): 559-63.
33. Nurfauziah C, Mulyani S., Anti-bacterial potency of *Urena lobata* leaf extract on *B. subtilis* and *E.Coli* also the profile of Thin Layer Chomatography. Final paper. Faculty of Pharmacy Gajah Mada University Yogyakarta 1999.
34. Guo H, Ling W, Wang Q, *et al*. Effect of anthocyanin-rich extract from black rice (Oryza sativa L. indica) on hyperlipidemia and insulin resistance in fructose-fed rats. Plant Foods Hum.Nutr. 2007;62: 1–6.

Table.2. GLP-1

Treatments	n	Means ± SD (pg/ml)
Normal group	5	60.95 ± 4.8[a]
Diabetic group	5	7.47 ± 0.42[b]
AEU-250 mg/kg bw	5	19.62 ± 2.40[c]
AEU-500 mg/kg bw	5	40.27 ± 1.19[d]
AEU-1000 mg/kg bw	5	54.01 ± 1.76[e]

Table 3. Insulin

Treatment	n	Means ± SD (pg/ml)
Normal group	5	1387.63 ± 315.48[a]
Diabetic group	5	106.00 ± 6.14[b]
AEU-250 mg/kg bw	5	113.85 ± 32.16[b]
AEU-500 mg/kg bw	5	446.96 ± 24.24[c]
AEU-1000 mg/kg bw	5	887.42 ± 67.19[d]

Table. Blood Glucose

Treatments	n	Means ± SD (mg/dl)
Normal group	5	127.25 ± 22.1[a]
Diabetic group	5	182.25 ± 4.57[b]
AEU-250 mg/kg bw	5	133.33 ± 26.31[a]
AEU-500 mg/kg bw	5	118.67 ± 13.61[a]
AEU-1000 mg/kg bw	5	114.00 ± 14.00[a]

JTCM_RESPON/KOMENTAR PENULIS

RESPONSE TO REVIEWERS

Q.1 Reviewer #1: Comments:
The study is an interesting one and publishable in a reputable journal of such, but I think the following comments will enrich the outreach of the work and convey the intended information to readers, also making it reproducible.

Introduction:

1. The first sentence could be recast as "Modulation of incretins in the treatment of type 2 diabetes mellitus have received increasing attention attention in the recent search for potent anti-diabetes".
2. Hormone(s) (not hormon(s)).
3. Hyperglycemia (Not hyperglycemic) in the first two paragraphs.
4. Incretin hormone especially GLP-1 has effect to cure T2DM patient. ? This is not a clear sentence.
5. I think the authors should reconstruct the information in the second and third paragraphs to flow.
6. Therapy (not theraphy)
7. I think the sentence "Herbs is one of medication choices because they have less side effect and holistic care property", can be replaced with "Herbs are becoming popular medications of choice in the managements of diseases due to their perceived less side effects …"
8. I think the sentence "One of traditional plants which have anti-diabetic effect is Caesar weed (Urenalobata)" Should be expunged, or authors could move to the body of the same paragraph with relevant ref if they wish to retain.
9. The authors may find it required to redraft the last sentences, focusing on U. lobota and its reported efficacies in the literature.

Answer: Thank you for the suggestion. I have revised the introduction section according to reviewer comments.

Q.2 Material and Methods:

1. Authors should carefully edit this section for minor typographical mistakes.
2. Animals and Treatments (The study was conducted). I think not they were conducted
3. $P \leq 0.05$ or $P < 0.05$ was considered to be statistical significant (I think you used 95% CI)

Answer: Thank you for the suggestion. I have revised the material and method section according to reviewer comments.

Q.3 Grouping and Treatments:

1. You did not state anywhere in the methodology (abstract and body), if you firstly induced diabetes in the U lobota extract treated animals.

Answer: To make animal model diabetes, We gave fructose diet for 3 weeks, on third weeks, we gave streptozotocin 20 mg/kg bw intraperitoneal. 3 days after injection, we checked fasting blood glucose level. We stated diabetes when fasting blood glucose level was more than 126 mg/dL therefore we gave fructose diet until the end of study.

Q.4 It is stated in your abstract that the animals were divided into 2 control groups and 3 test goups. Diabetic rats were induced with High Fructose Diet (HFD) and single dose intra-peritoneal streptozotocin. Aqueous leaves extract of U. lobata was administrated orally with doses of 250, 500 and 1000mg/kgbw for 4 weeks (?).

Answer: Control groups, there was normal groups (given normal diet/chow papas for 8 weeks) and diabetic group (given fructose for 8 weeks and streptozotocin 20 mg/kg bw i.p). Test group treated with Aqueous leaves extract of U. Lobata with doses of 250 250, 500 and 1000 mg/kgbw for 4 weeks after they were stated diabetes

Q.5 In the methods section, it is written "For eight weeks, the normal group (NG) received ND whereas the diabetic (DG) and treatment groups received HFD. The treatment groups were given aqueous extract of U. lobata (AEU) at a dose of250 mg/kg, 500 mg/kg, and 1000 mg/kgbw for four weeks (?)."

Answer: Normal group (NG) was given normal diet for 8 weeks**.** Diabetic group (DG) and test group were given high fructose diet for 8 weeks therefore at third weeks induced streptozotocin 20 mg/kg bw single dose i.p until stated diabetes. Test or treatment group treated with Aqueous leaves extract of U. Lobata with doses of 250 250, 500 and 1000 mg/kgbw for 4 weeks after they are stated diabetes.

Q.6 Results, discussion, conclusion and infrences:
I think statements below written before the results will be a repetition of the legends and can be expunged "Body weight, food consumption, blood glucose and insulin level of diabetic rat supplementedwith U.lobataleaf extract can be shown Table 1." "GLP-1 serum level of diabetic rat supplemented withU.lobata leaf extract can be shown Figure 1." "Insulin serum level of diabeticrat supplemented withU.lobata leaf extractcan be shown at Figure 2." "Blood glucose level of rat supplemented with U.lobata after stimulating glucose oral can be shown at Figure 3." "Islet <beta>-cellswere observed under microscope at 400x magnification as shown at Figure 4."

Answer: Thank you for the suggestion. I have revised the The results, discussion, conclusion sections.

Q.7 If these are your treatments, without pre-induction of diabetes in the EAU treated groups, do you not think that the EAU affecting both Serum Insulin level, Blood glucose level, GLP-1, body weight and food consumption when compared with the normal control animals, in the same way like the diabetic groups.
Answer: We induced diabetes (by HFD and streptozotocin) and they were administrated by EAU 250, 500 and 1000 mg/kg bw after stated diabetes for treatment group. In the same way for diabetic group but without EAU administration

Q.8. What do the authors think may be responsible for the low GLP-1 level in the diabetic rats, knowing that the Secretagogues of GLP-1 is nutrition, and there is high food consumption in the rats.
Answer: The low GLP-1 level in diabetic group due to the increase of DPP-4 activity moreover destroy and decrease GLP-1. The other bioactivity of GLP-1 reduce the rate of gastric-emptying, induce satiety therefore the low GLP-1 level increase food consumption.

Q.9 Please, redefine the # in table 1, as it also indicate significant difference in your treatment groups to both the control and the diabetic in the Food consumption results.
Answer: We used asteric superscript (*) for indicating significant different
Q.10. There is need to define the superscripts (a, b, c and d) as presented in tables 2,3
Answer: We have revised the superscript (a,b,c and d) on the figure caption
Q.11 I don't think it is also necessary to present a single result as table and figure (as of GPI-1, insulin and blood glucose levels).
Answer: Thank you for the suggestion. We used figure for data representation (GLP-1, insulin, Blood glucose level)

Q.12 Representative photomicrographs:
Q.12.1 Why is the EAU-250 mg/kg bw not represented
Answer: Islet B cells at EAU-250 mg/kg bw was not different significant compare to diabetic group therefore I do not show it in this paper.

Q.12.2 The authors used the verb "PREVENT" in defining the activities of EAU in the discussion and conclusion.
Answer: I have changed with" inhibit"

Q.12.3 If it is clear that EAU was administered before inducing diabetes, then they are 100% correct. But if diabetes induction precedes EAU treatments, I suggest they use "AMELIORATE".
These observations are intended to better the presentation of the work.
Answer: administration of EAU after diabetes induced or rats are stated diabetes

****

JTCM_MANUSCRIPT
DIREVISI

INCRETIN EFFECT OF *Urena lobata* LEAVES EXTRACT ON STRUCTURE AND FUNCTION OF RATS ISLET β-CELLS

Purnomo Y[1*], Soeatmadji DW[2], Sumitro SB[3], Widodo MA[4]

[1]Department of Pharmacology, Faculty of Medicine, Islamic University of Malang
[2] Department of Internal Medicine, School of Medicine, University of Brawijaya
[3] Department of Biology, Faculty of Science, University of Brawijaya
[4] Department of Pharmacology, School of Medicine, University of Brawijaya

*Corresponding author : Yudi Purnomo
Email : y_purnomo92@yahoo.com
Pharmacology Department, Faculty of Medicine, Islamic University of Malang
MT. Haryono 193 Malang 65144, East Java Indonesia

ABSTRACT

Recently, incretin hormone such as Glucagon-Like Peptide-1 (GLP-1) has become the therapeutic target of type 2 diabetes mellitus (T2DM). However, this hormone is known to have a short half-life time. The prolonging of GLP-1 bioavailability is useful to regulate blood glucose level. *Urena lobata* is a plant having anti-diabetes potency even though the incretin effects on islet β-cells has not been evaluated. This study aims to determine the incretin effects of *U.lobata* leaves extract on the structure and function of rats islet β-cells.

This study utilizes male Sprague-Dawley rats divided into 2 control group and 3 test group (n=5). Diabetic rats were induced with High Fructose Diet (HFD) and single dose intraperitoneal streptozotocin 25 mg/kg bw. Aqueous leaves extract of *U.lobata* was prepared by decoction methods and administrated orally with doses of 250, 500 and 1000 mg/kg bw for 4 weeks then incretin effect was evaluated by measuring serum GLP-1, insulin and blood glucose levels. Histology of islet β-cells was evaluated using photomicroscopy by analyzing size, shape, and number. Data were analyzed using ANOVA test followed by LSD test and $p \leq 0.05$ is considered significant.

Oral administration of aqueous extract *U.lobata* leaves at doses of 250, 500 and 1000 mg/kg body weight were able to prolong GLP-1 bioavailability by 3-fold, 5-fold and 7-fold respectively when compared to the diabetic group whereas blood glucose level were decreased about 30%, 35% and 40% respectively ($p<0.05$). Extract at doses of 500 and 1000 mg/kg bw also increased insulin level by 4-fold and 8-fold respectively compared to the diabetic group and the islet β-cells were repaired. The active compound in *U.lobata* leaves extract are suggested to prevent degradation of GLP-1 by inhibition of DPP-4 activity. Aqueous extract of *U.lobata* also improved the structure and function of islet β-cells by increasing of GLP-1 bioavailability.

Keywords: islet β-cells, GLP-1, incretin, insulin, *U.lobata*.

INTRODUCTION

Modulation of incretins in the treatment of type 2 diabetes mellitus (T2DM) has received attention in the recent search for potent anti-diabetes. Glucagon-Like Peptide-1 (GLP-1) and

Glucose-Dependent Insulinotropic Polypeptide (GIP) are major incretin hormone secreted by intestinal due to induction of oral nutrition[1]. GLP-1 plays a role in maintaining blood glucose level because of their biological activity such as stimulating insulin secretion, increasing β-cell proliferation, inhibiting glucagon secretion, reducing the rate of gastric emptying and inducing satiety[2,3]. In a patient with T2DM, chronic hyperglycemia is caused by a decreasing of GLP-1 bioavailability, therefore the secretion of insulin reduced[1,2].

Incretin hormone especially GLP-1 has potency as anti-diabetes. However, GLP-1 is metabolized by Dipeptidyl peptidase-4 (DPP-4) excessively to become inactive forms[3]. GLP-1 have a short half-life, approximately 2-5 minutes due to DPP-4 activity [1,3]. The inhibition of DPP-4 is effective to treat T2DM because GLP-1 bioavailability can be retained moreover it was able to regulate blood glucose level [3,4].

Therapy T2DM through inhibition of DPP-4 show less side effect[6] although the data of drugs safety in long term use is still limited [7]. Adverse reaction of Oral Anti-Diabetic (OAD) such as body weight gain and hypoglycemia are seldom in using of incretin-like drug [4]. The less side effect of drugs is affected by GLP-1 activity that could suppress appetite and it does not have insulin secretory effect [3,5]. However, incretin-like drug has also side effects such as flu-like symptoms, skin reaction, gastrointestinal problem, and this effect is able increase in long-term use of drugs. This phenomenon induce people to search a medicinal plant as an alternative therapy for T2DM trough controlling of incretin bioavailability [7].

Herbs are becoming popular medications of choices in the managements of diseases due to their perceived less side effect and holistic care property. One of the traditional plants which have anti-diabetes effect is Caesar weed (*Urena lobata*). The root and leaf extract of *U. lobata* have been used empirically by Nigeria people to treat diabetes mellitus [8,28]. Preclinical study of *U. lobata* root extract demonstrates the anti-hyperglycemic effect on streptozotocin-induced rat [8,32]. Bioactivity of *U. lobata* is regulated by its active substances such as a sterol,

alkaloid and flavonoid [9,32]. In Indonesia, *U. lobata* is known by Pulutan and this plant showed the anti-bacterial effect based on preliminary study[33]. Some study showed the anti-diabetic effect of *U. lobata* extract [8,9] however the mechanism of *U.lobata* on incretin activity has not been investigated. Therefore this study aims to examine the anti-diabetes effect of *U. lobata* leaf extract trough incretin activity focus on structure and function of rats islet β-cells.

MATERIAL AND METHODS

Preparation of *U.lobata* leaf extract

U.lobata leaf powder was obtained from Balai Materia Medika Batu Malang with certificate number 074/027/101.8/2015. In brief, 50 g *U.lobata* leaf powder was extracted according to decoction method in 250 ml hot water at 90ºC for 30 minutes therefore the extract was evaporated until resulting concentrated extract.

Animals and treatments

Male Sprague-Dawley (SD) rats (180-200 g) were obtained from Gajah Mada University Yogyakarta Indonesia. The study was conducted according to the ethical guidelines which were approved by the Commission of Ethical Research Brawijaya University Malang Indonesia with certificate number 245-KEP-UB. SD rats were housed in an individual cage and automatically controlled animal room at 25 ± 1º C on a 12:12-h light–dark cycle. They were fed by standard food, water *ad libitum* and fasted overnight before the experiments. Normal diet (ND) and a high-fructose diet (HFD) food were freshly mixed in every two days. Diabetic rats were induced by HFD (65% fructose and 35% ND food) and a single dose of streptozotocin 25 mg/kg BB intraperitoneal refer to Guo *et al* with minor modification. Rats were stated diabetic if the fasting blood glucose level more than 126 mg/dL[10]. The experiment was assigned into five groups for five rats each. For eight weeks, the normal group (NG) received ND whereas the diabetic (DG) and treatment groups received HFD. The treatment groups were given an aqueous extract of *U.lobata* (AEU) at a dose of 250 mg/kg,

500 mg/kg, and 1000 mg/kg bw for four weeks after the rats were classified as diabetic according to Shirwaikar *et al*. Body weight and food consumption were monitored weekly. Blood samples were obtained 15 minutes after orally glucose stimulation in a dose of 2 g/kg body weight and taken from tail vein after overnight fasted. A blood sample was immediately centrifuged 4500 rpm. The serum was separated and saved under -20 ºC.

GLP-1 assay

GLP-1 serum level was analyzed by rat GLP-1 ELISA kit (USCN CEA804). 50 µl samples were added 50 µl detection reagent A and then incubated for 60 minutes at 37 ºC. After aspirating and washing, samples were added 100 µl detection reagent B and incubated for 30 minutes at 37ºC. Added 90 µl substrate reagents then was added 50 µl *stop solution*. Samples were read with a microplate reader at λ = 450 nm.

Insulin assay

Insulin serum level was analyzed by rat insulin ELISA kit (Elabscience E-EL-R0023). 50 µl samples were added 50 µl Biotinylated detection Ab and incubated for 45 minutes at 37 ºC. After aspirating and washing then samples were added 100 µl HRP conjugate and incubated for 30 minutes at 37ºC. Added 90 µl substrate reagents then incubated for 15 minutes at 37ºC. 50 µl *stop solution* was added then read with a microplate reader at λ = 450 nm.

Blood Glucose assay

The blood samples were collected from the tail vein after overnight fasted and at 15 minutes after oral glucose administration. They were measured immediately using a commercially available glucometer (AccuCheck).

Histopathology of islet β-cells

Pancreas tissue was taken by section methods and continued by Hematoxylin-Eosin (H-E) staining. Mostly islet cells containing β-cells were observed including shape, size, number each view under the microscope with magnification 400 times.

Statistical Analysis

The data were expressed as means ± SD. Statistical analysis was performed by one-way ANOVA. The least significant difference (LSD) test and Dunnet C were used for mean comparisons and then $p \leq 0.05$ was considered to be statistically significant.

RESULTS

The effect of *U. lobata* leaf extract on body weight, food consumption, glucose and insulin level of diabetic rats

In the end of the treatment, there is not a significant decrease of body weight on test group compared to diabetic group ($p>0.05$) meanwhile food consumption is decreased ($p\leq 0.05$) (table 1). The oral administration of *U. lobata* leaf extract decrease fasting blood glucose level compared to diabetic group ($p\leq 0.05$) whereas insulin level was increased ($p\leq 0.05$).

Table 1: Body weight, food consumption, blood glucose and insulin level of diabetic rats

	Normal group	Diabetic group	AEU-250	AEU-500	AEU-1000
Body weight (g)	298.0 ± 13**	239.5 ± 19*	223.0 ± 11*	222.0 ± 16*	229.0 ± 12*
Food consumption (g)	25.0 ± 0	24.1 ± 3	15.4 ± 2**	14.8 ± 2**	20.2 ± 3**
Food consumption (%)	100.0 ± 0	96.0 ± 11	61.6 ± 7**	59.0 ± 6**	80.0 ± 8**
Fasting Blood Glucose (mg/dL)	101.0 ± 8**	129.0 ± 6*	96.0 ± 10**	87.0 ± 5**	92.0 ± 9**
Fasting Serum Insulin (pg/ml)	1242.9 ± 47**	226.9 ± 30*	350.8 ± 30**	536.2 ± 39**	699.2 ± 24**

Result are expressed as means ± SD, (n=5)
*significant different compared to normal group ($p\leq 0.05$, LSD test)
**significant different compared to diabetic group ($p\leq 0.05$, LSD test)

The effect of *U. lobata* leaf extract on GLP-1 serum level of diabetic rats

There is a significant decrease of GLP-1 levels on the diabetic group about 8-fold compared to normal group observed ($p\leq 0.05$) Fig.1. Aqueous extract of *U. lobata* at doses 250 mg/kg bw, 500 mg/kg bw and 1000 mg/kg bw can prevent degradation of GLP-1 respectively about 3-fold, 5-fold and 7-fold compared to diabetic group ($p\leq 0.05$). An increased dose of *U.lobata* leaves extract prolong and enhance GLP-1 bioavailability.

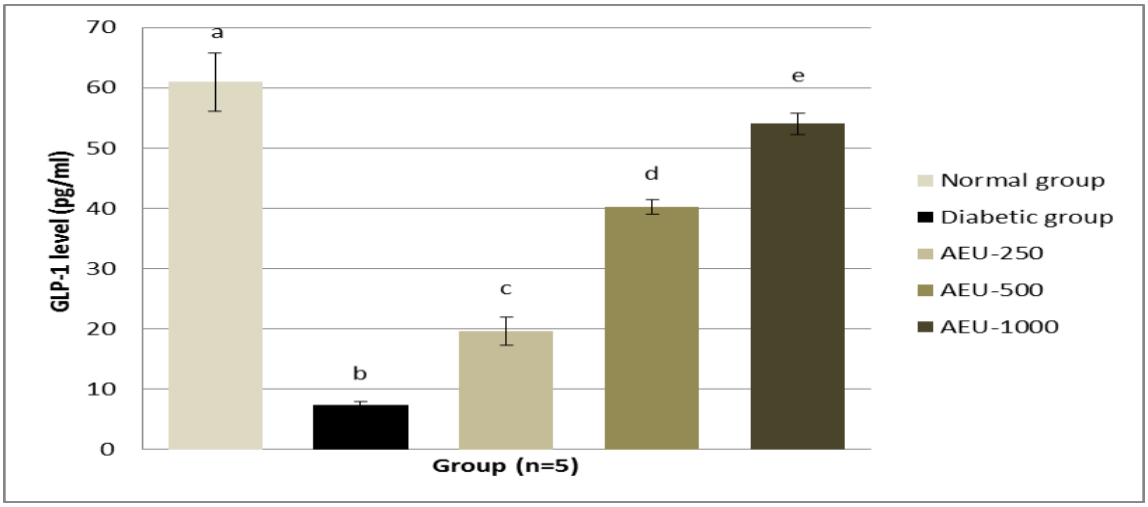


Fig.1. GLP-1 level supplemented *U.lobata* extract. Means with different letters are significantly different (p≤ 0.05, Dunnet C test)

The effect of *U. lobata* leaf extract on insulin serum level of diabetic rats

There is a significant decrease of insulin levels on diabetic group approximately 14-fold compared to normal group observed (p≤ 0.05) refer to Fig.2. The administration of aqueous extract *U.lobata* 500 and 1000 mg/kg bw increase insulin level 4-fold, 8-fold respectively compared to diabetic group (p≤ 0.05) whereas the dose of 250 mg/kg bw cannot increase insulin level. The more increase dose of water extract *U. lobata*, the more insulin level escalated.

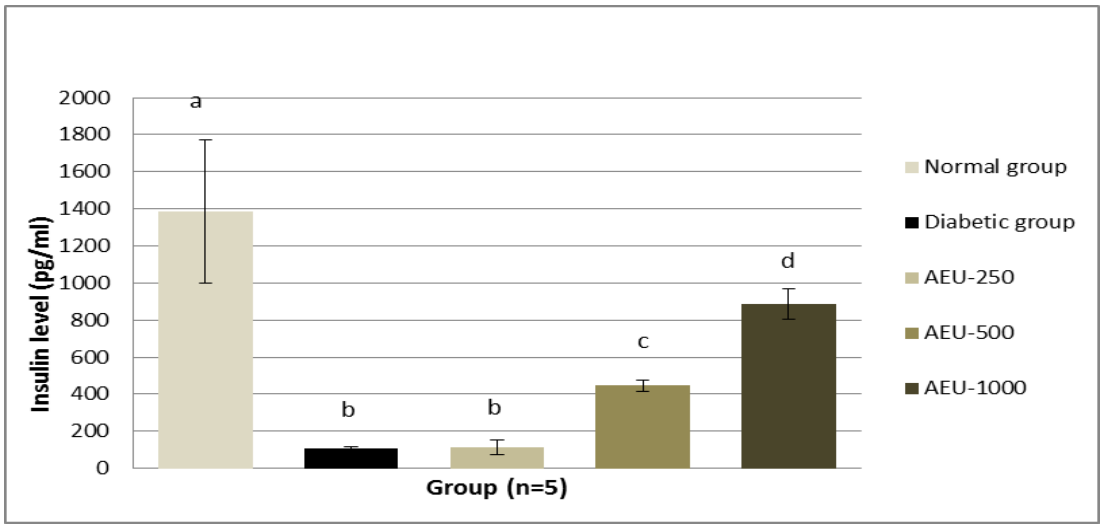


Fig. 2 Insulin level supplemented *U.lobata* extract. Means with different letters are significantly different (p≤ 0.05, LSD test)

The effect of *U. lobata* leaf extract on blood glucose level of diabetic rats

Based on these results at Fig.3, there is a significant increase at blood glucose level on a diabetic group up to 70% compared to normal group observed (p≤ 0.05). The administration of aqueous extract *U.lobata* at dose of 250 mg/kg bw, 500 mg/kg bw and 1000 mg/kg bw can decrease glucose level respectively 30%, 35% and 40% compare to the diabetic group (p≤ 0.05) after glucose stimulation. Blood glucose level is not different significantly on an increase of dose *U.lobata* (p>0.05).

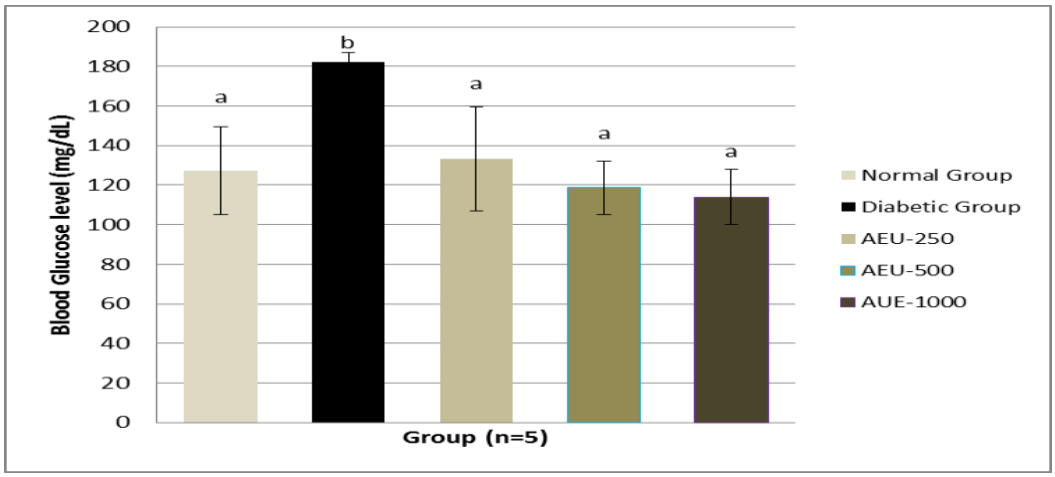


Fig. 3 Blood glucose level supplemented *U.lobata* extract. Means with different letters are significantly different (p≤ 0.05, LSD test)

The effect of *U. lobata* leaf extract on islet β-cells of diabetic rats

The normal group (Fig.4.A) shows the shape of cells are round, nucleated and in a huge number, whereas the diabetic groups (Fig.4.B) cells show longer, not nucleated and in a small number. Administration of aqueous extract *U. lobata* at dose of 500 and 1000 mg/kg bw could inhibit cells damage which shown as round cells, nucleated and in a huge number (Fig.4.C-D). Test groups have islet β-cells in slightly bigger size than normal group, except aqueous extract at dose of 1000 mg/kg bw. The bigger size of cells show a swelling cells and injury indications. The administration of aqueous extract *U.lobata* at dose of 1000 mg/kg bw

are able to inhibit cells damage therefore the shape, size, and number are similar to islet cells at the normal group.

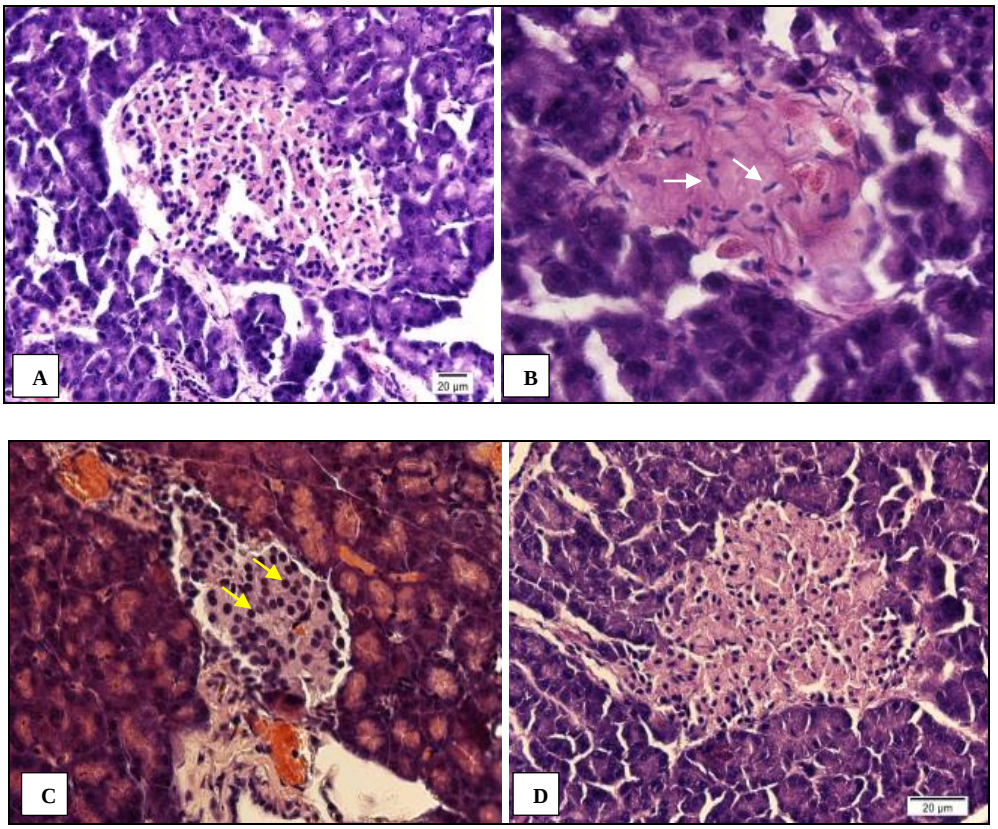


Fig. 4 Islet β-cells were stained by Hematoxylin-Eosin and observed under photomicroscope with magnitude 400x. (A). Normal group (B). Diabetic group (C). AEU 500 mg/kb bw (D). AEU-1000 mg/kg bw. Magnification 400x. The white arrow show unnucleated cells and longer shape whereas yellow arrow show the swelling cells.

DISCUSSION

The effect of *U. lobata* leaf extract on GLP-1 serum level of diabetic rats

Oral administration of aqueous extract *U.lobata* significantly maintains GLP-1 bioavailability of diabetic rats. Based on our previous study, active compounds in *U.lobata* such as mangiferin, stigmasterol and β-sitosterol are able to prevent degradation of GLP-1 by inhibition of DPP-4 [13]. DPP-4 inhibitor substances prevent the degradation of active GLP-1 even though it does not increase the levels of total circulating GLP-1 and does not prevent the kidney from rapidly clearing GLP-1[12].

GLP-1 is incretin hormone produced by L-cell intestine and the secretion depends on oral nutrition. GLP-1 has a potency for T2DM therapy but it is metabolized excessively by DPP-4 into inactive form [7]. GLP-1 has a short half-life, approximately for 2-5 minutes, it is caused of DPP-4 activity [3,6]. The active form of GLP-1 is GLP-1 (7-36) amides and GLP-1 (7-37) which are rapidly inactivated by DPP-4 through cleave N-terminal dipeptide His-Ala[12,19]. It produces an inactive form of GLP-1, they are GLP-1 (9-36) amide and GLP-1 (9-37) isopeptides [6,7]. A number study showed that the importance of DPP-4 mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity [1,12].

GLP-1 is a superfamily peptide of glucagon which has a similarity degree approximately 48 % [14]. The similarity of amino acid sequence between GLP-1 and glucagon become one of this cause. Proglucagon gene is located at chromosome 2q36-q37 and only found in some tissues whereas the messenger RNA (mRNA) of proglucagon is met at α-cells pancreas, L cells intestine, and brain[15]. Proglucagon production is started from transcription of preproglucagon gene and then is continued by translation process [3,14]. The regulation of GLP-1 released from L cells intestine is a complex mechanism that involves combinations of nutrition, hormone and neural stimuli [14]. The GLP-1 receptor is classified in *G protein-coupled* receptor that is found in liver, muscle and pancreas cells [2,3]. This receptor has a specific character by activation of adenyl cyclase and results cAMP [15]. After GLP-1 binding with the receptor, it will activate cAMP and Mitogen-Activated Protein Kinase (MAPK) [3,7].

The biological activities of GLP-1 are various and depend on the organ target. GLP-1 activity in the pancreas has functions in stimulating the insulin secretion by cAMP activation, increasing β-cell masses by MAPK pathway and inhibiting the secretion of glucagon [3,5]. In the brain, it will reduce the rate of gastric emptying, induce satiety and neuroprotection whereas in liver, fatty acid metabolism will be decreased and glucose utilization increased [14,15]. All of them contribute to regulate blood glucose level in T2DM [1, 2.]

The effect of *U. lobata* leaf extract on Insulin serum level of diabetic rats

Aqueous extract of *U.lobata* significantly increases insulin synthesis of diabetic rats. It is controlled by active compounds in the extract through the activity of GLP-1. The oral administration will maintain GLP-1 bioavailability moreover the insulin biosynthesis can be increased. GLP-1 has a potency to retain the insulinotropic activity for treating T2DM [5,6]. In this study, the increase of insulin secretion is caused by the active compounds of *U.lobata* extract to maintain GLP-1 bioavailability trough inhibition of DPP-4 activity [13].

GLP-1 stimulates proinsulin biosynthesis and transcription of proinsulin gene. GLP-1 contributes to provide insulin deposition which loses from islet β-cells trough biosynthesis process[5]. GLP-1 is different with oral anti-diabetic sulphonylurea in stimulating of insulin formation because the sulphonylurea only stimulates insulin, not the biosynthesis of insulin [4,5]. GLP-1 is incretin hormone which is potential to increase islet β-cells proliferation, and anti-apoptosis furthermore it is able to increase insulin secretion [2,6].

Hyperinsulinemia occurs in prediabetic condition or insulin resistance and then the secretion decline due to β-cell exhaustion or overwork [2]. The biological effect of insulin is divided into two major groups, they are metabolic and mitogenic effect [11]. The metabolic effect is glucose transport, lipid metabolism, protein, and glycogen synthesis whereas the mitogenic effect is the cell growth and mitogenesis [11].

This study showed also that the administrations of *U.lobata* extract give a good description of islet β-cell. It is shown by the shape, size and number of β-cell in better condition compared to diabetic groups. These conditions support the function of β-cell to produce insulin in order to maintain blood glucose level [14,15]. However, the diabetic group shows β-cells destruction which is signaled by a decreasing number of islet β-cell and structure damage therefore it affect their performance to release insulin.

The effect of *U. lobata* leaf extract on blood glucose level of diabetic rats

Administration of aqueous extract *U.lobata* significantly decreases blood glucose level of diabetic rats. It is controlled by active compounds of *U.lobata* which has DPP-4 inhibitory activity like stigmasterol, mangiferin and β-sitosterol furthermore GLP-1 bioavailability can be retained for insulin biosynthesis when the blood glucose level increase after stimulating of oral nutrition [13,18,32]. GLP-1 acts outside of metabolism purpose, that is inhibiting of gastric juices secretion, inhibiting of the GIT motility and inhibiting of the rate of gastric emptying [2,3]. It is a benefit to prevent the increase of blood glucose level at postprandial [5,6].

Insulin works to maintain blood glucose level after induction of glucose by a metabolic pathway. This hormone transports glucose from blood to the tissue and then synthesise it into glycogen in muscle in order to reduce blood glucose level [11,14]. In diabetic groups, the insulin secretion is disrupted therefore they lose their's control to maintain blood glucose level [5,11]. This is showed by blood glucose level in the diabetic group which is higher than normal and also treatment groups.

Histopathology of islet β-cell supplemented *U. lobata* extract

Oral administration of aqueous extract *U. lobata* are able to prevent islet β-cells death of diabetic group. The effect of active compounds in *U.lobata* that has potency such as increasing β-cells proliferations and inhibiting β-cells apoptosis through GLP-1 activation [5,9,32]. Bioavailability of GLP-1 could be retained due to DPP-4 inhibitor substances in the extract such as stigmasterol, mangiferin and β-sitosterol [13,31]. It affects the integrity of β-cells indirectly in the test group, it is shown in the shape of cells, size and number which is close to normal groups. Some tests show swelling cells, it indicates cells damage at the first step even though the shape and number of cells are normal [20,29].

The active compounds of *U.lobata* leaves extract such as gossypetin, chrysoeriol and mangiferin could protect cell damage from free radical [22,24,27,29]. They work as an antioxidant

by donating an electron to unstable compounds in order to stabilize it [23]. Besides it, mangiferin and gossypetin act also as scavenger free radical moreover it could decrease oxidant level causing oxidative damage [16,22,27]. Hyperglycemia in diabetes increases the production of free radical furthermore it occurs imbalance between oxidant and antioxidant [21,23]. This condition is caused by oxidative stress which leads to oxidative damage in tissue or organ and an increase of diabetic complication risk [16,23].

The effect of *U. lobata* leaf extract on body weight, food consumption, glucose level and insulin of diabetic rats

Aqueous extract of *U.lobata* reduces food consumption therefore it affects body weight gain of diabetic rats. It is related to active compound such as stigmasterol, mangiferin, and β-sitosterol in *U.lobata* that maintains bioavailability GLP-1 and their's interaction with GLP-1 receptor in the brain could reduce the rate of gastric emptying and also induce satiety [13,16,17,18]. The oral administration of *U.lobata* leaf extract decreases fasting blood glucose level and increase insulin level. GLP-1 activity in the pancreas has functions in stimulating the secretion of insulin by cAMP activation, increasing β cell masses by MAPK pathway and inhibiting the secretion of glucagon [3,5]. In liver, it increases utilization of glucose and decrease fatty acid metabolism. In T2DM, all of them contribute to maintain blood glucose level [1, 2.]

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest

ACKNOWLEDGEMENTS

This study was funded by Doctorate Research Grant of Directorate General of Higher Education Indonesia (No. 053/B.07/U.III/LPPM/2014.

REFERENCES

1. Drucker DJ. Dipeptidyl Peptidase-4 Inhibition and the Treatment of Type 2 Diabetes, Diabetes care 2007; 30(6):1335 - 1343. doi:10.2337/dc07-0228.
2. Chia CW, Egan JM. Incretin-based therapies in type 2 diabetes mellitus. J Clin Endocrinol Metab 2008; 93(10), 3703-3716. doi: 10.1210/jc.2007-2109.
3. Drucker DJ. Biological actions and therapeutic potential of the glucagon like peptides. Gastroenterology 2002; 122(2): 531-544. PMID:11832466.
4. Goodman LS, Gilman A, *et al*., Goodman & Gilman's the pharmacological basis of therapeutics. McGraw-Hill, New York, 2006.
5. Holst JJ, Orskov C. The incretin approach for diabetes treatment. Modulation of islet hormone release by GLP-1 agonism. Diabetes 2004; 53(3): S197-204
6. Salehi M, Aulinger AB, D'alessio AD. Targeting -cell mass in type 2 diabetes: Promise and limitation of new drugs based on incretins. Endocrine Reviews 2008; 29(3): 367-379. doi: http://dx.doi.org/10.1210/er.2007-0031
7. Bailey C. Incretin-based therapies. Endocrin abstract 2008; 15: S41.
8. Onoagbe IO, Negbenebor EO, Ogbeide VO, Dawha IH, Attah V, Lau HU, and Omonkhua AA. A study of the anti-diabetic effects of *Urena lobata* and *Sphenostylis stenocarpa* in streptozotocin-induced diabetic rats. Eur J Sci Res 2010; 43(1): 6-14.
9. Awika JM, Rooney LW. Sorghum Phytochemicals and their potential Impact on human Health. Phytochemistry 2004; 65(9): 1199-1221. doi:10.1016/j.phytochem.2004.04.001
10. Shirwaikar A, Rajendran K, Barik R. Effect of aqueous bark extract of Garuga pinnata Roxb. in streptozotocin-nicotinamide induced type-II diabetes mellitus. Journal of ethnopharmacology, 2006;107(2):285–290.
11. Rhodes CJ, White MF. Molecular insight into insulin action and secretion. Eur J Clin. Invest 2002; 32(S3): 3-13. PMID: 12028370
12. Rosenstock J, Zinman B. Dipeptidyl peptidase-4 inhibitors and the management of type 2 diabetes mellitus. Current opinion in endocrinology, diabetes and obesity 2007;14(2):98–107.
13. Purnomo Y, Soeatmadji DW, Sumitro SB, Widodo MA. Anti-diabetic Potential of *Urena lobata* Leaf Extract through Inhibition of Dipeptidyl Peptidase IV (DPP-4) Activity. *Asian Pacific Journal of Tropical Biomedicines* 2015;5(8)630-634.
14. Brubacker PL, Drucker DJ. Minireview : Glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut and central nervous system. Endocrinology 2004;145(6): 2653-2659.
15. Aronoff SL, Berkowitz K, Shreiner B *et al*., Glucose Metabolism and Regulation: Beyond Insulin and Glucagon. Diabetes spectrum 2004; 17(3): 183-190. doi:10.2337/diaspect.17.3.183
16. Sosa A, and Rosquete C. Flavonoid from *Urena sinuata* L. Avances en Química 2010; 5(2): 95 - 98.
17. Ros MM. *et al*., Phytosterol consumption and the anabolic steroid boldenone in humans: a hypothesis piloted. Food additives and contaminants 2007;24(7):679–684.

18. Rudkowska I. *et al.*, Cholesterol-lowering efficacy of plant sterols in low-fat yogurt consumed as a snack or with a meal. Journal of the American College of Nutrition. 2008;27(5):588–95
19. Mentlein R, and Gallwitz B. Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36) amide, peptide histidine methionine and is responsible for their degradation in human serum. Eur J Biochem 1993; 214(3): 829-35. DOI: 10.1111/j.1432-1033.1993.tb17986.x
20. Robbins Kumar. Pathologic Basic of diseases. 6th ed. WB Saunders Company, Philadelphia, 2002; pp.12-21.
21. Miura T. *et al.*, Antidiabetic activity of a xanthone compound, mangiferin. Phytomedicine: International Journal of phytotherapy and phytopharmacology. 2010;8(2):85–87.
22. Stoilova I. *et al.*, Antimicrobial and Antioxidant Activity of the Polyphenol Mangiferin. Herba Polonica 2005;51(1/2): 37–44. ISSN 0018-0599.
23. Halliwel B, Gutteridge JM. Free Radical in Biology and Medicine, Third edition, Oxford Science Publication, Oxford. 1999.
24. Matławska I, Sikorska M., Flavonoid compounds in the flowers of Abutilon indicum (L.) Sweet (Malvaceae). Acta poloniae pharmaceutica 2002;59(3):227–229.
25. Mazumder UK. et al. Antibacterial activity of Urena lobata root. Fitoterapia 2001;72(8): 927–929.
26. Panda S. et al., Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from butea monosperma. Fitoterapia 2009;80(2):123-126.
27. De las Heras B. et al., Anti-inflammatory and antioxidant activity of plants used in traditional medicine in Ecuador. Journal of ethnopharmacology 1998;61(2):161-166.
28. Omonkhua AA, Onoagbe IO. Preliminary proximate and phytochemical analyses of some medicinal plants used to treat diabetes mellitus in Nigeria. Inventi Impact Ethnopharmacol 2010; 1: 68-70
29. Sellamuthu PS, Arulselvan P, Kamalraj S, Fakurazi S, Kandasamy M. Protective nature of Mangifera on oxidative stress and antioxidant status in tissues of streptozotocin-induced diabetic rats. ISRN pharmacol 2013; doi: 10.1155/2013/750109.
30. Saeidnia S, Manayi A, Gohari AR, Abdollahi M. The story of beta sitosterol-a review. Eur J Med Plants 2014; 4(5): 590-609.
31. Chang CL, Lin Y, Bartolome AP, Chen YC, Chiu SC, Yang WC. Herbal therapies for type 2 diabetes mellitus: chemistry, biology, and potential application of selected plants and compounds. Evid Based Complement Alternat Med 2013; doi: 10.1155/2013/378657.
32. Islam MH, Rahman KMH, Rahman S, Rahmatullah M. Preliminary antihyperglycemic, antinociceptive activity, phytochemical analysis and toxicity studies on leaves of *Urena lobata* L. J Chem Pharm Res 2015;7(4): 559-63.
33. Nurfauziah C, Mulyani S., Anti-bacterial potency of *Urena lobata* leaf extract on *B. subtilis* and *E.Coli* also the profile of Thin Layer Chomatography. Final paper. Faculty of Pharmacy Gajah Mada University Yogyakarta 1999.
34. Guo H, Ling W, Wang Q, *et al*. Effect of anthocyanin-rich extract from black rice (Oryza sativa L. indica) on hyperlipidemia and insulin resistance in fructose-fed rats. Plant Foods Hum.Nutr. 2007;62: 1–6.

JTCM_GALLEY PROOF

Journal of Traditional and Complementary Medicine xxx (2016) 1–6

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Original Article

Incretin effect of *Urena lobata* leaves extract on structure and function of rats islet β-cells

Y. Purnomo [a, *], D.W. Soeatmadji [b], S.B. Sumitro [c], M.A. Widodo [d]

[a] Department of Pharmacology, Faculty of Medicine, Islamic University of Malang, Indonesia
[b] Department of Internal Medicine, School of Medicine, University of Brawijaya, Indonesia
[c] Department of Biology, Faculty of Science, University of Brawijaya, Indonesia
[d] Department of Pharmacology, School of Medicine, University of Brawijaya, Indonesia

ARTICLE INFO

Article history:
Received 16 May 2016
Received in revised form
22 August 2016
Accepted 25 October 2016
Available online xxx

Keywords:
Islet β-cells
GLP-1
Incretin
Insulin
U. lobata

ABSTRACT

This study aims to determine the incretin effects of *Urena lobata* leaves extract on the structure and function of rats islet β-cells. This study utilizes male Sprague-Dawley rats divided into 2 control group and 3 test group (n = 5). Diabetic rats were induced with High Fructose Diet (HFD) and single dose intraperitoneal streptozotocin 25 mg/kg bw. Aqueous leaves extract of *U. lobata* was prepared by decoction methods and administrated orally with doses of 250, 500, and 1000 mg/kg bw for 4 weeks then incretin effect was evaluated by measuring serum GLP-1, insulin, and blood glucose levels. Histology of islet β-cells was evaluated using photomicroscopy by analyzing size, shape, and number. Data were analyzed using ANOVA test followed by LSD test and $p \leq 0.05$ is considered significant. Oral administration of aqueous extract *U. lobata* leaves at doses of 250, 500, and 1000 mg/kg body weight were able to prolong GLP-1 bioavailability by 3-fold, 5-fold, and 7-fold respectively when compared to the diabetic group whereas blood glucose level were decreased about 30%, 35%, and 40% respectively ($p < 0.05$). Extract at doses of 500 and 1000 mg/kg bw also increased insulin level by 4-fold and 8-fold respectively compared to the diabetic group and the islet β-cells were repaired. The active compound in *U. lobata* leaves extract are suggested to prevent degradation of GLP-1 by inhibition of DPP-4 activity. Aqueous extract of *U. lobata* also improved the structure and function of islet β-cells by increasing of GLP-1 bioavailability.

1. Introduction

Modulation of incretins in the treatment of type 2 diabetes mellitus (T2DM) has received attention in the recent search for potent anti-diabetes. Glucagon-Like Peptide-1 (GLP-1) and Glucose-Dependent Insulinotropic Polypeptide (GIP) are major incretin hormone secreted by intestinal due to induction of oral nutrition.[1] GLP-1 plays a role in maintaining blood glucose level because of their biological activity such as stimulating insulin secretion, increasing β-cell proliferation, inhibiting glucagon secretion, reducing the rate of gastric emptying and inducing satiety.[2,3] In a patient with T2DM, chronic hyperglycemia is caused by a decreasing of GLP-1 bioavailability, therefore the secretion of insulin reduced.[1,2]

Incretin hormone especially GLP-1 has potency as anti-diabetes. However, GLP-1 is metabolized by Dipeptidyl peptidase-4 (DPP-4) excessively to become inactive forms.[3] GLP-1 have a short half-life, approximately 2–5 min due to DPP-4 activity.[1,3] The inhibition of DPP-4 is effective to treat T2DM because GLP-1 bioavailability can be retained moreover it was able to regulate blood glucose level.[3,4]

Therapy T2DM through inhibition of DPP-4 show less side effect[6] although the data of drugs safety in long-term use is still limited.[7] Adverse reaction of Oral Anti-Diabetic (OAD) such as body weight gain and hypoglycemia are seldom in using of incretin-like drug.[4] The less side effect of drugs is affected by GLP-1 activity that could suppress appetite and it does not have insulin secretory effect.[3,5] However, incretin-like drug has also side effects such as flu-like symptoms, skin reaction, gastrointestinal problem, and this effect is able increase in long-term use of drugs. This phenomenon induces people to search a medicinal plant as an alternative therapy for T2DM trough controlling of incretin bioavailability.[7]

* Corresponding author. Pharmacology Department, Faculty of Medicine, Islamic University of Malang, MT. Haryono 193, Malang 65144, Indonesia.
E-mail address: y_purnomo92@yahoo.com (Y. Purnomo).

Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

http://dx.doi.org/10.1016/j.jtcme.2016.10.001

Herbs are becoming popular medications of choices in the managements of diseases due to their perceived less side effect and holistic care property. One of the traditional plants which have anti-diabetes effect is Caesar weed (*Urena lobata*). The root and leaf extract of *U. lobata* have been used empirically by Nigeria people to treat diabetes mellitus.[8,28] Preclinical study of *U. lobata* root extract demonstrates the anti-hyperglycemic effect on streptozotocin-induced rat.[8,32] Bioactivity of *U. lobata* is regulated by its active substances such as a sterol, alkaloid, and flavonoid.[9,32] In Indonesia, *U. lobata* is known by Pulutan and this plant showed the anti-bacterial effect based on preliminary study.[25,33] Some study showed the anti-diabetic effect of *U. lobata* extract[8,9] however the mechanism of *U. lobata* on incretin activity has not been investigated. Therefore, this study aims to examine the anti-diabetes effect of *U. lobata* leaf extract trough incretin activity focus on structure and function of rats islet β-cells.

2. Material and methods

2.1. Preparation of U. lobata leaf extract

U. lobata leaf powder was obtained from Balai Materia Medika Batu Malang with certificate number 074/027/101.8/2015. In brief, 50 g *U. lobata* leaf powder was extracted according to decoction method in 250 ml hot water at 90 °C for 30 min therefore the extract was evaporated until resulting concentrated extract.

2.2. Animals and treatments

Male Sprague-Dawley (SD) rats (180–200 g) were obtained from Gajah Mada University Yogyakarta Indonesia. The study was conducted according to the ethical guidelines which were approved by the Commission of Ethical Research Brawijaya University Malang Indonesia with certificate number 245-KEP-UB. SD rats were housed in an individual cage and automatically controlled animal room at 25 ± 1 °C on a 12:12-h light–dark cycle. They were fed by standard food, water *ad libitum* and fasted overnight before the experiments. Normal diet (ND) and a high fructose diet (HFD) food were freshly mixed in every two days. Diabetic rats were induced by HFD (65% fructose and 35% ND food) and a single dose of streptozotocin 25 mg/kg BB intraperitoneal refer to Guo *et al* with minor modification. Rats were stated diabetic if the fasting blood glucose level more than 126 mg/dL.[10] The experiment was assigned into five groups for five rats each. For eight weeks, the normal group (NG) received ND whereas the diabetic (DG) and treatment groups received HFD. The treatment groups were given an aqueous extract of *U. lobata* (AEU) at a dose of 250 mg/kg, 500 mg/kg, and 1000 mg/kg bw for four weeks after the rats were classified as diabetic according to Shirwaikar *et al*. Body weight and food consumption were monitored weekly. Blood samples were obtained 15 min after orally glucose stimulation in a dose of 2 g/kg body weight and taken from tail vein after overnight fasted. A blood sample was immediately centrifuged 4500 rpm. The serum was separated and saved under −20 °C.

2.3. GLP-1 assay

GLP-1 serum level was analyzed by rat GLP-1 ELISA kit (USCN CEA804). 50 μl samples were added 50 μl detection reagent A and then incubated for 60 min at 37 °C. After aspirating and washing, samples were added 100 μl detection reagent B and incubated for 30 min at 37 °C. Added 90 μl substrate reagents then was added 50 μl *stop solution*. Samples were read with a microplate reader at λ = 450 nm.

2.4. Insulin assay

Insulin serum level was analyzed by rat insulin ELISA kit (Elabscience E-EL-R0023). 50 μl samples were added 50 μl Biotinylated detection Ab and incubated for 45 min at 37 °C. After aspirating and washing then samples were added 100 μl HRP conjugate and incubated for 30 min at 37 °C. Added 90 μl substrate reagents then incubated for 15 min at 37 °C. 50 μl *stop solution* was added then read with a microplate reader at λ = 450 nm.

2.5. Blood glucose assay

The blood samples were collected from the tail vein after overnight fasted and at 15 min after oral glucose administration. They were measured immediately using a commercially available glucometer (AccuCheck).

2.6. Histopathology of islet β-cells

Pancreas tissue was taken by section methods and continued by Hematoxylin–Eosin (H–E) staining. Mostly islet cells containing β-cells were observed including shape, size, number each view under the microscope with magnification 400 times.

2.7. Statistical analysis

The data were expressed as means ± SD. Statistical analysis was performed by one-way ANOVA. The least significant difference (LSD) test and Dunnet C were used for mean comparisons and then $p \leq 0.05$ was considered to be statistically significant.

3. Results

3.1. The effect of U. lobata leaf extract on body weight, food consumption, glucose, and insulin level of diabetic rats

In the end of the treatment, there is not a significant decrease of body weight on test group compared to diabetic group ($p > 0.05$) meanwhile food consumption is decreased ($p \leq 0.05$) (Table 1). The oral administration of *U. lobata* leaf extract decrease fasting blood glucose level compared to diabetic group ($p \leq 0.05$) whereas insulin level was increased ($p \leq 0.05$).

3.2. The effect of U. lobata leaf extract on GLP-1 serum level of diabetic rats

There is a significant decrease of GLP-1 levels on the diabetic group about 8-fold compared to normal group observed ($p \leq 0.05$) Fig. 1. Aqueous extract of *U. lobata* at doses 250 mg/kg bw, 500 mg/kg bw, and 1000 mg/kg bw can prevent degradation of GLP-1 respectively about 3-fold, 5-fold, and 7-fold compared to diabetic group ($p \leq 0.05$). An increased dose of *U. lobata* leaves extract prolong and enhance GLP-1 bioavailability.

3.3. The effect of U. lobata leaf extract on insulin serum level of diabetic rats

There is a significant decrease of insulin levels on diabetic group approximately 14-fold compared to normal group observed ($p \leq 0.05$) refer to Fig. 2. The administration of aqueous extract *U. lobata* 500, and 1000 mg/kg bw increase insulin level 4-fold, 8-fold respectively compared to diabetic group ($p \leq 0.05$) whereas the dose of 250 mg/kg bw cannot increase insulin level. The more increase dose of water extract *U. lobata*, the more insulin level escalated.

Please cite this article in press as: Purnomo Y, et al., Incretin effect of *Urena lobata* leaves extract on structure and function of rats islet β-cells, Journal of Traditional and Complementary Medicine (2016), http://dx.doi.org/10.1016/j.jtcme.2016.10.001

Table 1
Body weight, food consumption, blood glucose, and insulin level of diabetic rats.

	Normal group	Diabetic group	AEU-250	AEU-500	AEU-1000
Body weight (g)	298.0 ± 13[b]	239.5 ± 19[a]	223.0 ± 11[a]	222.0 ± 16[a]	229.0 ± 12[a]
Food consumption (g)	25.0 ± 0	24.1 ± 3	15.4 ± 2[b]	14.8 ± 2[b]	20.2 ± 3[b]
Food consumption (%)	100.0 ± 0	96.0 ± 11	61.6 ± 7[b]	59.0 ± 6[b]	80.0 ± 8[b]
Fasting blood glucose (mg/dL)	101.0 ± 8[b]	129.0 ± 6[a]	96.0 ± 10[b]	87.0 ± 5[b]	92.0 ± 9[b]
Fasting serum insulin (pg/ml)	1242.9 ± 47[b]	226.9 ± 30[a]	350.8 ± 30[b]	536.2 ± 39[b]	699.2 ± 24[b]

Result is expressed as means ± SD, (n = 5).
[a] Significant different compared to normal group ($p \leq 0.05$, LSD test).
[b] Significant different compared to diabetic group ($p \leq 0.05$, LSD test).

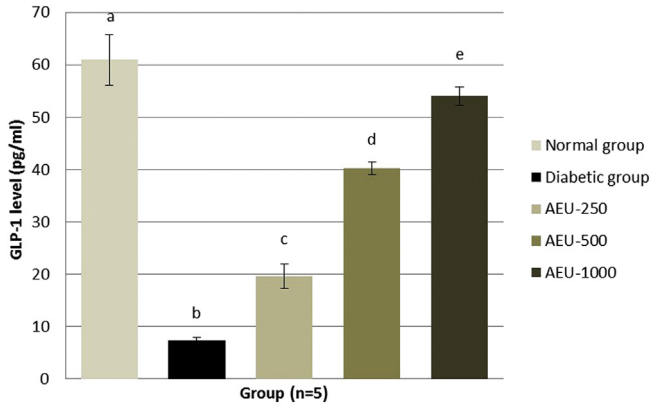


Fig. 1. GLP-1 level supplemented *U. lobata* extract. Means with different letters are significantly different ($p \leq 0.05$, Dunnet C test).

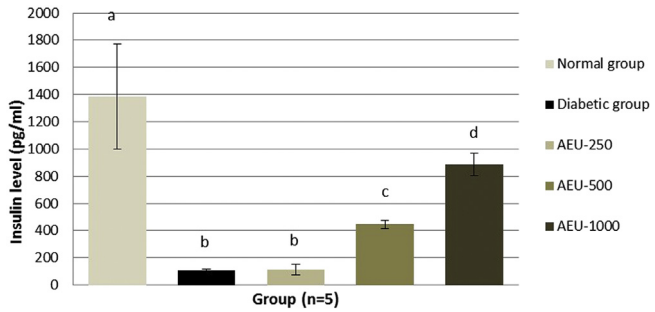


Fig. 2. Insulin level supplemented *U. lobata* extract. Means with different letters are significantly different ($p \leq 0.05$, LSD test).

3.4. The effect of U. lobata leaf extract on blood glucose level of diabetic rats

Based on these results at Fig. 3, there is a significant increase at blood glucose level on a diabetic group up to 70% compared to normal group observed ($p \leq 0.05$). The administration of aqueous extract *U. lobata* at dose of 250 mg/kg bw, 500 mg/kg bw, and 1000 mg/kg bw can decrease glucose level respectively 30%, 35%, and 40% compare to the diabetic group ($p \leq 0.05$) after glucose stimulation. Blood glucose level is not different significantly on an increase of dose *U. lobata* ($p > 0.05$).

3.5. The effect of U. lobata leaf extract on islet β-cells of diabetic rats

The normal group (Fig. 4A) shows the shape of cells are round, nucleated, and in a huge number, whereas the diabetic groups (Fig. 4B) cells show longer, not nucleated, and in a small number. Administration of aqueous extract *U. lobata* at dose of 500 and 1000 mg/kg bw could inhibit cells damage which shown as round cells, nucleated, and in a huge number (Fig. 4C-D). Test groups have islet β-cells in slightly bigger size than normal group, except aqueous extract at dose of 1000 mg/kg bw. The bigger size of cells shows a swelling cells and injury indications. The administration of aqueous extract *U. lobata* at dose of 1000 mg/kg bw are able to inhibit cells damage therefore the shape, size, and number are similar to islet cells at the normal group.

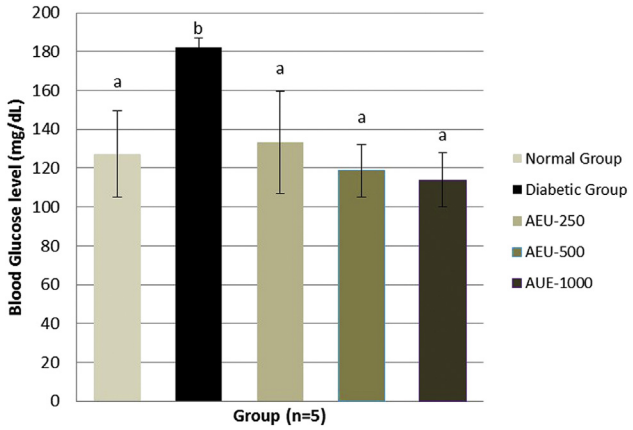


Fig. 3. Blood glucose level supplemented *U. lobata* extract. Means with different letters are significantly different ($p \leq 0.05$, LSD test).

4. Discussion

4.1. The effect of U. lobata leaf extract on GLP-1 serum level of diabetic rats

Oral administration of aqueous extract *U. lobata* significantly maintains GLP-1 bioavailability of diabetic rats. Based on our previous study, active compounds in *U. lobata* such as mangiferin, stigmasterol, and β-sitosterol are able to prevent degradation of GLP-1 by inhibition of DPP-4.[13] DPP-4 inhibitor substances prevent the degradation of active GLP-1 even though it does not increase the levels of total circulating GLP-1 and does not prevent the kidney from rapidly clearing GLP-1.[12]

GLP-1 is incretin hormone produced by L cell intestine and the secretion depends on oral nutrition. GLP-1 has a potency for T2DM therapy but it is metabolized excessively by DPP-4 into inactive form.[7] GLP-1 has a short half-life, approximately for 2–5 min, it is caused of DPP-4 activity.[3,6] The active form of GLP-1 is GLP-1 (7–36) amides and GLP-1 (7–37) which are rapidly inactivated by DPP-4 through cleave N-terminal dipeptide His-Ala.[12,19] It produces an inactive form of GLP-1, they are GLP-1 (9–36) amide and GLP-1 (9–37) isopeptides.[6,7] A number study showed that the importance of DPP-4 mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity.[1,12]

Please cite this article in press as: Purnomo Y, et al., Incretin effect of *Urena lobata* leaves extract on structure and function of rats islet β-cells, Journal of Traditional and Complementary Medicine (2016), http://dx.doi.org/10.1016/j.jtcme.2016.10.001

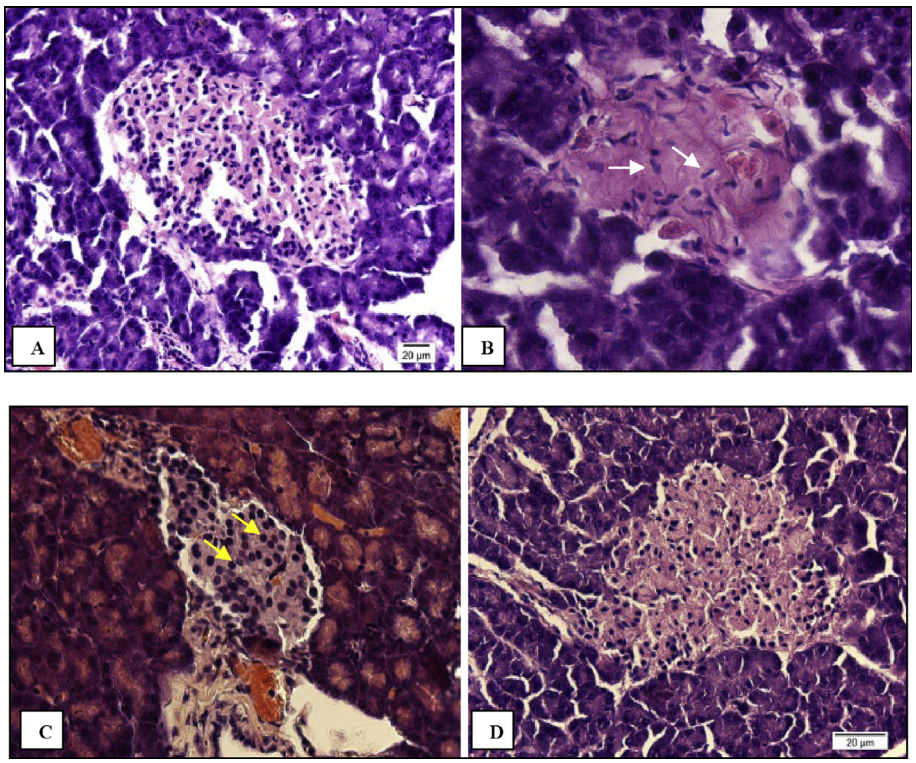


Fig. 4. Islet β-cells were stained by Hematoxylin–Eosin and observed under photomicroscope with magnitude 400×. (A). Normal group, (B). Diabetic group, (C). AEU 500 mg/kb bw, (D). AEU-1000 mg/kg bw. Magnification 400×. The white arrow shows unnucleated cells and longer shape whereas yellow arrow shows the swelling cells.

GLP-1 is a superfamily peptide of glucagon which has a similarity degree approximately 48%.[14] The similarity of amino acid sequence between GLP-1 and glucagon become one of this cause. Proglucagon gene is located at chromosome 2q36-q37 and only found in some tissues whereas the messenger RNA (mRNA) of proglucagon is met at α-cells pancreas, L cells intestine, and brain.[15] Proglucagon production is started from transcription of preproglucagon gene and then is continued by translation process.[3,14] The regulation of GLP-1 released from L cells intestine is a complex mechanism that involves combinations of nutrition, hormone, and neural stimuli.[14] The GLP-1 receptor is classified in *G protein-coupled* receptor that is found in liver, muscle, and pancreas cells.[2,3] This receptor has a specific character by activation of adenyl cyclase and results cAMP.[15] After GLP-1 binding with the receptor, it will activate cAMP and Mitogen-Activated Protein Kinase (MAPK).[3,7]

The biological activities of GLP-1 are various and depend on the organ target. GLP-1 activity in the pancreas has functions in stimulating the insulin secretion by cAMP activation, increasing β-cell masses by MAPK pathway and inhibiting the secretion of glucagon.[3,5] In the brain, it will reduce the rate of gastric emptying, induce satiety, and neuroprotection whereas in liver, fatty acid metabolism will be decreased and glucose utilization increased.[14,15] All of them contribute to regulate blood glucose level in T2DM.[1,2]

4.2. The effect of U. lobata leaf extract on insulin serum level of diabetic rats

Aqueous extract of *U. lobata* significantly increases insulin synthesis of diabetic rats. It is controlled by active compounds in the extract through the activity of GLP-1. The oral administration will maintain GLP-1 bioavailability moreover the insulin biosynthesis can be increased. GLP-1 has a potency to retain the insulinotropic activity for treating T2DM.[5,6] In this study, the increase of insulin secretion is caused by the active compounds of *U. lobata* extract to maintain GLP-1 bioavailability trough inhibition of DPP-4 activity.[13]

GLP-1 stimulates proinsulin biosynthesis and transcription of proinsulin gene. GLP-1 contributes to provide insulin deposition which loses from islet β-cells trough biosynthesis process.[5] GLP-1 is different with oral anti-diabetic sulphonylurea in stimulating of insulin formation because the sulphonylurea only stimulates insulin, not the biosynthesis of insulin.[4,5] GLP-1 is incretin hormone which is potential to increase islet β-cells proliferation, and anti-apoptosis furthermore it is able to increase insulin secretion.[2,6]

Hyperinsulinemia occurs in prediabetic condition or insulin resistance and then the secretion decline due to β-cell exhaustion or overwork.[2] The biological effect of insulin is divided into two major groups, they are metabolic and mitogenic effect.[11] The metabolic effect is glucose transport, lipid metabolism, protein, and glycogen synthesis whereas the mitogenic effect is the cell growth and mitogenesis.[11]

This study showed also that the administrations of *U. lobata* extract give a good description of islet β-cell. It is shown by the shape, size, and number of β-cell in better condition compared to diabetic groups. These conditions support the function of β-cell to produce insulin in order to maintain blood glucose level.[14,15] However, the diabetic group shows β-cells destruction which is signaled by a decreasing number of islet β-cell and structure damage therefore it affect their performance to release insulin.

4.3. The effect of U. lobata leaf extract on blood glucose level of diabetic rats

Administration of aqueous extract *U. lobata* significantly decreases blood glucose level of diabetic rats. It is controlled by active compounds of *U. lobata* which has DPP-4 inhibitory activity like stigmasterol, mangiferin, and β-sitosterol furthermore GLP-1

Please cite this article in press as: Purnomo Y, et al., Incretin effect of *Urena lobata* leaves extract on structure and function of rats islet β-cells, Journal of Traditional and Complementary Medicine (2016), http://dx.doi.org/10.1016/j.jtcme.2016.10.001

bioavailability can be retained for insulin biosynthesis when the blood glucose level increase after stimulating of oral nutrition.[13,18,30,32] GLP-1 acts outside of metabolism purpose, that is inhibiting of gastric juices secretion, inhibiting of the GIT motility and inhibiting of the rate of gastric emptying.[2,3] It is a benefit to prevent the increase of blood glucose level at postprandial.[5,6]

Insulin works to maintain blood glucose level after induction of glucose by a metabolic pathway. This hormone transports glucose from blood to the tissue and then synthesize it into glycogen in muscle in order to reduce blood glucose level.[11,14] In diabetic groups, the insulin secretion is disrupted therefore they lose their's control to maintain blood glucose level.[5,11] This is showed by blood glucose level in the diabetic group which is higher than normal and also treatment groups.

4.4. Histopathology of islet β-cell supplemented U. lobata extract

Oral administration of aqueous extract *U. lobata* is able to prevent islet β-cells death of diabetic group. The effect of active compounds in *U. lobata* that has potency such as increasing β-cells proliferations and inhibiting β-cells apoptosis through GLP-1 activation.[5,9,32] Bioavailability of GLP-1 could be retained due to DPP-4 inhibitor substances in the extract such as stigmasterol, mangiferin, and β-sitosterol.[13,26,31] It affects the integrity of β-cells indirectly in the test group, it is shown in the shape of cells, size, and number which is close to normal groups. Some tests show swelling cells, it indicates cells damage at the first step even though the shape and number of cells are normal.[20,29]

The active compounds of *U. lobata* leaves extract such as gossypetin, chrysoeriol, and mangiferin could protect cell damage from free radical.[22,24,27,29] They work as an antioxidant by donating an electron to unstable compounds in order to stabilize it.[23] Besides it, mangiferin and gossypetin act also as scavenger free radical moreover it could decrease oxidant level causing oxidative damage.[16,22,27] Hyperglycemia in diabetes increases the production of free radical furthermore it occurs imbalance between oxidant and antioxidant.[21,23] This condition is caused by oxidative stress which leads to oxidative damage in tissue or organ and an increase of diabetic complication risk.[16,23]

4.5. The effect of U. lobata leaf extract on body weight, food consumption, glucose level and insulin of diabetic rats

Aqueous extract of *U. lobata* reduces food consumption therefore it affects body weight gain of diabetic rats. It is related to active compound such as stigmasterol, mangiferin, and β-sitosterol in *U. lobata* that maintains bioavailability GLP-1 and their's interaction with GLP-1 receptor in the brain could reduce the rate of gastric emptying and also induce satiety.[13,16–18] The oral administration of *U. lobata* leaf extract decreases fasting blood glucose level and increase insulin level. GLP-1 activity in the pancreas has functions in stimulating the secretion of insulin by cAMP activation, increasing β cell masses by MAPK pathway and inhibiting the secretion of glucagon.[3,5] In liver, it increases utilization of glucose and decrease fatty acid metabolism. In T2DM, all of them contribute to maintain blood glucose level.[1,2]

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study was funded by Doctorate Research Grant of Directorate General of Higher Education Indonesia (No. 053/B.07/U.III/LPPM/2014).

References

1. Drucker DJ. Dipeptidyl peptidase-4 inhibition and the treatment of type 2 diabetes. *Diabetes Care*. 2007;30(6):1335–1343. http://dx.doi.org/10.2337/dc07-0228.
2. Chia CW, Egan JM. Incretin-based therapies in type 2 diabetes mellitus. *J Clin Endocrinol Metab*. 2008;93(10):3703–3716. http://dx.doi.org/10.1210/jc.2007-2109.
3. Drucker DJ. Biological actions and therapeutic potential of the glucagon like peptides. *Gastroenterology*. 2002;122(2):531–544. PMID: 11832466.
4. Brunton L, Chabner B, Knollman B. *Goodman & Gilman's the Pharmacological Basis of Therapeutics*. New York: McGraw-Hill; 2006.
5. Holst JJ, Orskov C. The incretin approach for diabetes treatment. Modulation of islet hormone release by GLP-1 agonism. *Diabetes*. 2004;53(3):S197–S204.
6. Salehi M, Aulinger AB, D'alessio AD. Targeting -cell mass in type 2 diabetes: promise and limitation of new drugs based on incretins. *Endocr Rev*. 2008;29(3):367–379. http://dx.doi.org/10.1210/er.2007-0031.
7. Bailey C. Incretin-based therapies. *Endocrin Abstr*. 2008;15:S41.
8. Onoagbe IO, Negbenebor EO, Ogbeide VO, et al. A study of the anti-diabetic effects of *Urena lobata* and *Sphenostylis stenocarpa* in streptozotocin-induced diabetic rats. *Eur J Sci Res*. 2010;43(1):6–14.
9. Awika JM, Rooney LW. Sorghum phytochemicals and their potential Impact on human health. *Phytochemistry*. 2004;65(9):1199–1221. http://dx.doi.org/10.1016/j.phytochem.2004.04.001.
10. Shirwaikar A, Rajendran K, Barik R. Effect of aqueous bark extract of *Garuga pinnata* Roxb. in streptozotocin–nicotinamide induced type-II diabetes mellitus. *J Ethnopharmacol*. 2006;107(2):285–290.
11. Rhodes CJ, White MF. Molecular insight into insulin action and secretion. *Eur J Clin Invest*. 2002;32(S3):3–13. PMID: 12028370.
12. Rosenstock J, Zinman B. Dipeptidyl peptidase-4 inhibitors and the management of type 2 diabetes mellitus. *Curr Opin Endocrinol Diabetes Obes*. 2007;14(2):98–107.
13. Purnomo Y, Soeatmadji DW, Sumitro SB, Widodo MA. Anti-diabetic potential of *Urena lobata* leaf extract through inhibition of dipeptidyl peptidase IV (DPP-4) Activity. *Asian Pac J Trop Biomed*. 2015;5(8):630–634.
14. Brubacker PL, Drucker DJ. Minireview: glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut and central nervous system. *Endocrinology*. 2004;145(6):2653–2659.
15. Aronoff SL, Berkowitz K, Shreiner B, et al. Glucose metabolism and regulation: beyond insulin and glucagon. *Diabetes Spectr*. 2004;17(3):183–190. http://dx.doi.org/10.2337/diaspect.17.3.183.
16. Sosa A, Rosquete C. Flavonoid from *Urena sinuata* L. *Av Quím*. 2010;5(2):95–98.
17. Ros MM, Sterk S, Verhagens H, Stalenhoef AF, De-Jong N. Phytosterol consumption and the anabolic steroid boldenone in humans: a hypothesis piloted. *Food Addit Contam*. 2007;24(7):679–684.
18. Rudkowska I, AbuMweis SS, Nicolle C, Jones PJ. Cholesterol-lowering efficacy of plant sterols in low-fat yogurt consumed as a snack or with a meal. *J Am Coll Nutr*. 2008;27(5):588–595.
19. Mentlein R, Gallwitz B. Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36) amide, peptide histidine methionine and is responsible for their degradation in human serum. *Eur J Biochem*. 1993;214(3):829–835. http://dx.doi.org/10.1111/j.1432-1033.1993.tb17986.x.
20. Kumar Robbins. *Pathologic Basic of Diseases*. 6th ed. Philadelphia: WB Saunders Company; 2002:pp.12–21.
21. Miura T, Ichiki H, Iwamoto N, et al. Antidiabetic activity of a xanthone compound, mangiferin. *Phytomedicine*. 2010;8(2):85–87.
22. Stoilova I, Gargova S, Stoyanova A, Ho L. Antimicrobial and antioxidant activity of the polyphenol mangiferin. *Herba Pol*. 2005;51(1/2):37–44. ISSN 0018–0599.
23. Halliwel B, Gutteridge JM. *Free Radical in Biology and Medicine*. 3rd ed. Oxford: Oxford Science Publication; 1999.
24. Matławska I, Sikorska M. Flavonoid compounds in the flowers of *Abutilon indicum* (L.) sweet (Malvaceae). *Acta Pol Pharm*. 2002;59(3):227–229.
25. Mazumder UK, Gupta M, Malikandan L, Bhattacharya S. Antibacterial activity of *Urena lobata* root. *Fitoterapia*. 2001;72(8):927–929.
26. Panda S, Jafri M, Kar A, Maheta BK. Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from *Butea monosperma*. *Fitoterapia*. 2009;80(2):123–126.
27. De las Heras B, Slowing K, Benedi J, et al. Anti-inflammatory and antioxidant activity of plants used in traditional medicine in Ecuador. *J Ethnopharmacol*. 1998;61(2):161–166.
28. Omonkhua AA, Onoagbe IO. Preliminary proximate and phytochemical analyses of some medicinal plants used to treat diabetes mellitus in Nigeria. *Inven Impact Ethnopharmacol*. 2010;1:68–70.

Please cite this article in press as: Purnomo Y, et al., Incretin effect of *Urena lobata* leaves extract on structure and function of rats islet β-cells, Journal of Traditional and Complementary Medicine (2016), http://dx.doi.org/10.1016/j.jtcme.2016.10.001

29. Sellamuthu PS, Arulselvan P, Kamalraj S, Fakurazi S, Kandasamy M. Protective nature of *Mangifera* on oxidative stress and antioxidant status in tissues of streptozotocin-induced diabetic rats. *ISRN Pharmacol*. 2013:1–10. http://dx.doi.org/10.1155/2013/750109.
30. Saeidnia S, Manayi A, Gohari AR, Abdollahi M. The story of beta sitosterol-a review. *Eur J Med Plants*. 2014;4(5):590–609.
31. Chang CL, Lin Y, Bartolome AP, Chen YC, Chiu SC, Yang WC. Herbal therapies for type 2 diabetes mellitus: chemistry, biology, and potential application of selected plants and compounds. *Evid Based Complement Altern Med*. 2013: 1–33. http://dx.doi.org/10.1155/2013/378657.
32. Islam MH, Rahman KMH, Rahman S, Rahmatullah M. Preliminary anti-hyperglycemic, antinociceptive activity, phytochemical analysis and toxicity studies on leaves of *Urena lobata* L. *J Chem Pharm Res*. 2015;7(4):559–563.
33. Nurfauziah C, Mulyani S. *Anti-bacterial Potency of Urena lobata Leaf Extract on B. subtilis and E.Coli Also the Profile of Thin Layer Chomatography*. Final paper. Faculty of Pharmacy Gajah Mada University Yogyakarta. 1999.

Please cite this article in press as: Purnomo Y, et al., Incretin effect of *Urena lobata* leaves extract on structure and function of rats islet β-cells, Journal of Traditional and Complementary Medicine (2016), http://dx.doi.org/10.1016/j.jtcme.2016.10.001

JTCM_MANUSCRIPT
DIPUBLIKASKAN

Journal of Traditional and Complementary Medicine 7 (2017) 301–306

Contents lists available at ScienceDirect

Journal of Traditional and Complementary Medicine

journal homepage: http://www.elsevier.com/locate/jtcme

Original Article

Incretin effect of *Urena lobata* leaves extract on structure and function of rats islet β-cells

Y. Purnomo [a, *], D.W. Soeatmadji [b], S.B. Sumitro [c], M.A. Widodo [d]

[a] Department of Pharmacology, Faculty of Medicine, Islamic University of Malang, Indonesia
[b] Department of Internal Medicine, School of Medicine, University of Brawijaya, Indonesia
[c] Department of Biology, Faculty of Science, University of Brawijaya, Indonesia
[d] Department of Pharmacology, School of Medicine, University of Brawijaya, Indonesia

ARTICLE INFO

Article history:
Received 16 May 2016
Received in revised form
22 August 2016
Accepted 25 October 2016
Available online 24 November 2016

Keywords:
Islet β-cells
GLP-1
Incretin
Insulin
U. lobata

ABSTRACT

This study aims to determine the incretin effects of *Urena lobata* leaves extract on the structure and function of rats islet β-cells. This study utilizes male Sprague-Dawley rats divided into 2 control group and 3 test group ($n = 5$). Diabetic rats were induced with High Fructose Diet (HFD) and single dose intraperitoneal streptozotocin 25 mg/kg bw. Aqueous leaves extract of *U. lobata* was prepared by decoction methods and administrated orally with doses of 250, 500, and 1000 mg/kg bw for 4 weeks then incretin effect was evaluated by measuring serum GLP-1, insulin, and blood glucose levels. Histology of islet β-cells was evaluated using photomicroscopy by analyzing size, shape, and number. Data were analyzed using ANOVA test followed by LSD test and $p \leq 0.05$ is considered significant. Oral administration of aqueous extract *U. lobata* leaves at doses of 250, 500, and 1000 mg/kg body weight were able to prolong GLP-1 bioavailability by 3-fold, 5-fold, and 7-fold respectively when compared to the diabetic group whereas blood glucose level were decreased about 30%, 35%, and 40% respectively ($p < 0.05$). Extract at doses of 500 and 1000 mg/kg bw also increased insulin level by 4-fold and 8-fold respectively compared to the diabetic group and the islet β-cells were repaired. The active compound in *U. lobata* leaves extract are suggested to prevent degradation of GLP-1 by inhibition of DPP-4 activity. Aqueous extract of *U. lobata* also improved the structure and function of islet β-cells by increasing of GLP-1 bioavailability.

1. Introduction

Modulation of incretins in the treatment of type 2 diabetes mellitus (T2DM) has received attention in the recent search for potent anti-diabetes. Glucagon-Like Peptide-1 (GLP-1) and Glucose-Dependent Insulinotropic Polypeptide (GIP) are major incretin hormone secreted by intestinal due to induction of oral nutrition.[1] GLP-1 plays a role in maintaining blood glucose level because of their biological activity such as stimulating insulin secretion, increasing β-cell proliferation, inhibiting glucagon secretion, reducing the rate of gastric emptying and inducing satiety.[2,3] In a patient with T2DM, chronic hyperglycemia is caused by a decreasing of GLP-1 bioavailability, therefore the secretion of insulin reduced.[1,2]

Incretin hormone especially GLP-1 has potency as anti-diabetes. However, GLP-1 is metabolized by Dipeptidyl peptidase-4 (DPP-4) excessively to become inactive forms.[3] GLP-1 have a short half-life, approximately 2–5 min due to DPP-4 activity.[1,3] The inhibition of DPP-4 is effective to treat T2DM because GLP-1 bioavailability can be retained moreover it was able to regulate blood glucose level.[3,4]

Therapy T2DM through inhibition of DPP-4 show less side effect[6] although the data of drugs safety in long-term use is still limited.[7] Adverse reaction of Oral Anti-Diabetic (OAD) such as body weight gain and hypoglycemia are seldom in using of incretin-like drug.[4] The less side effect of drugs is affected by GLP-1 activity that could suppress appetite and it does not have insulin secretory effect.[3,5] However, incretin-like drug has also side effects such as flu-like symptoms, skin reaction, gastrointestinal problem, and this effect is able increase in long-term use of drugs. This phenomenon induces people to search a medicinal plant as an alternative therapy for T2DM trough controlling of incretin bioavailability.[7]

* Corresponding author. Pharmacology Department, Faculty of Medicine, Islamic University of Malang, MT. Haryono 193, Malang 65144, Indonesia.
E-mail address: y_purnomo92@yahoo.com (Y. Purnomo).
Peer review under responsibility of The Center for Food and Biomolecules, National Taiwan University.

http://dx.doi.org/10.1016/j.jtcme.2016.10.001

Herbs are becoming popular medications of choices in the managements of diseases due to their perceived less side effect and holistic care property. One of the traditional plants which have anti-diabetes effect is Caesar weed (*Urena lobata*). The root and leaf extract of *U. lobata* have been used empirically by Nigeria people to treat diabetes mellitus.[8,28] Preclinical study of *U. lobata* root extract demonstrates the anti-hyperglycemic effect on streptozotocin-induced rat.[8,32] Bioactivity of *U. lobata* is regulated by its active substances such as a sterol, alkaloid, and flavonoid.[9,32] In Indonesia, *U. lobata* is known by Pulutan and this plant showed the anti-bacterial effect based on preliminary study.[25,33] Some study showed the anti-diabetic effect of *U. lobata* extract[8,9] however the mechanism of *U. lobata* on incretin activity has not been investigated. Therefore, this study aims to examine the anti-diabetes effect of *U. lobata* leaf extract trough incretin activity focus on structure and function of rats islet β-cells.

2. Material and methods

2.1. Preparation of U. lobata leaf extract

U. lobata leaf powder was obtained from Balai Materia Medika Batu Malang with certificate number 074/027/101.8/2015. In brief, 50 g *U. lobata* leaf powder was extracted according to decoction method in 250 ml hot water at 90 °C for 30 min therefore the extract was evaporated until resulting concentrated extract.

2.2. Animals and treatments

Male Sprague-Dawley (SD) rats (180–200 g) were obtained from Gajah Mada University Yogyakarta Indonesia. The study was conducted according to the ethical guidelines which were approved by the Commission of Ethical Research Brawijaya University Malang Indonesia with certificate number 245-KEP-UB. SD rats were housed in an individual cage and automatically controlled animal room at 25 ± 1 °C on a 12:12-h light–dark cycle. They were fed by standard food, water *ad libitum* and fasted overnight before the experiments. Normal diet (ND) and a high fructose diet (HFD) food were freshly mixed in every two days. Diabetic rats were induced by HFD (65% fructose and 35% ND food) and a single dose of streptozotocin 25 mg/kg BB intraperitoneal refer to Guo *et al* with minor modification. Rats were stated diabetic if the fasting blood glucose level more than 126 mg/dL.[10] The experiment was assigned into five groups for five rats each. For eight weeks, the normal group (NG) received ND whereas the diabetic (DG) and treatment groups received HFD. The treatment groups were given an aqueous extract of *U. lobata* (AEU) at a dose of 250 mg/kg, 500 mg/kg, and 1000 mg/kg bw for four weeks after the rats were classified as diabetic according to Shirwaikar *et al*. Body weight and food consumption were monitored weekly. Blood samples were obtained 15 min after orally glucose stimulation in a dose of 2 g/kg body weight and taken from tail vein after overnight fasted. A blood sample was immediately centrifuged 4500 rpm. The serum was separated and saved under −20 °C.

2.3. GLP-1 assay

GLP-1 serum level was analyzed by rat GLP-1 ELISA kit (USCN CEA804). 50 μl samples were added 50 μl detection reagent A and then incubated for 60 min at 37 °C. After aspirating and washing, samples were added 100 μl detection reagent B and incubated for 30 min at 37 °C. Added 90 μl substrate reagents then was added 50 μl *stop solution*. Samples were read with a microplate reader at λ = 450 nm.

2.4. Insulin assay

Insulin serum level was analyzed by rat insulin ELISA kit (Elabscience E-EL-R0023). 50 μl samples were added 50 μl Biotinylated detection Ab and incubated for 45 min at 37 °C. After aspirating and washing then samples were added 100 μl HRP conjugate and incubated for 30 min at 37 °C. Added 90 μl substrate reagents then incubated for 15 min at 37 °C. 50 μl *stop solution* was added then read with a microplate reader at λ = 450 nm.

2.5. Blood glucose assay

The blood samples were collected from the tail vein after overnight fasted and at 15 min after oral glucose administration. They were measured immediately using a commercially available glucometer (AccuCheck).

2.6. Histopathology of islet β-cells

Pancreas tissue was taken by section methods and continued by Hematoxylin–Eosin (H–E) staining. Mostly islet cells containing β-cells were observed including shape, size, number each view under the microscope with magnification 400 times.

2.7. Statistical analysis

The data were expressed as means ± SD. Statistical analysis was performed by one-way ANOVA. The least significant difference (LSD) test and Dunnet C were used for mean comparisons and then $p \leq 0.05$ was considered to be statistically significant.

3. Results

3.1. The effect of U. lobata leaf extract on body weight, food consumption, glucose, and insulin level of diabetic rats

In the end of the treatment, there is not a significant decrease of body weight on test group compared to diabetic group ($p > 0.05$) meanwhile food consumption is decreased ($p \leq 0.05$) (Table 1). The oral administration of *U. lobata* leaf extract decrease fasting blood glucose level compared to diabetic group ($p \leq 0.05$) whereas insulin level was increased ($p \leq 0.05$).

3.2. The effect of U. lobata leaf extract on GLP-1 serum level of diabetic rats

There is a significant decrease of GLP-1 levels on the diabetic group about 8-fold compared to normal group observed ($p \leq 0.05$) Fig. 1. Aqueous extract of *U. lobata* at doses 250 mg/kg bw, 500 mg/kg bw, and 1000 mg/kg bw can prevent degradation of GLP-1 respectively about 3-fold, 5-fold, and 7-fold compared to diabetic group ($p \leq 0.05$). An increased dose of *U. lobata* leaves extract prolong and enhance GLP-1 bioavailability.

3.3. The effect of U. lobata leaf extract on insulin serum level of diabetic rats

There is a significant decrease of insulin levels on diabetic group approximately 14-fold compared to normal group observed ($p \leq 0.05$) refer to Fig. 2. The administration of aqueous extract *U. lobata* 500, and 1000 mg/kg bw increase insulin level 4-fold, 8-fold respectively compared to diabetic group ($p \leq 0.05$) whereas the dose of 250 mg/kg bw cannot increase insulin level. The more increase dose of water extract *U. lobata*, the more insulin level escalated.

Table 1
Body weight, food consumption, blood glucose, and insulin level of diabetic rats.

	Normal group	Diabetic group	AEU-250	AEU-500	AEU-1000
Body weight (g)	298.0 ± 13[b]	239.5 ± 19[a]	223.0 ± 11[a]	222.0 ± 16[a]	229.0 ± 12[a]
Food consumption (g)	25.0 ± 0	24.1 ± 3	15.4 ± 2[b]	14.8 ± 2[b]	20.2 ± 3[b]
Food consumption (%)	100.0 ± 0	96.0 ± 11	61.6 ± 7[b]	59.0 ± 6[b]	80.0 ± 8[b]
Fasting blood glucose (mg/dL)	101.0 ± 8[b]	129.0 ± 6[a]	96.0 ± 10[b]	87.0 ± 5[b]	92.0 ± 9[b]
Fasting serum insulin (pg/ml)	1242.9 ± 47[b]	226.9 ± 30[a]	350.8 ± 30[b]	536.2 ± 39[b]	699.2 ± 24[b]

Result is expressed as means ± SD, (n = 5).
[a] Significant different compared to normal group ($p \leq 0.05$, LSD test).
[b] Significant different compared to diabetic group ($p \leq 0.05$, LSD test).

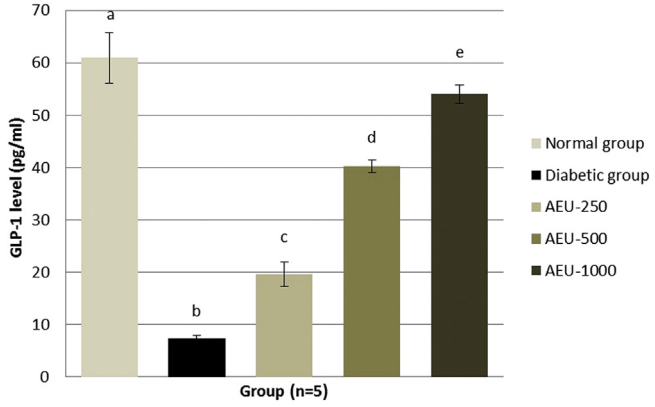


Fig. 1. GLP-1 level supplemented *U. lobata* extract. Means with different letters are significantly different ($p \leq 0.05$, Dunnet C test).

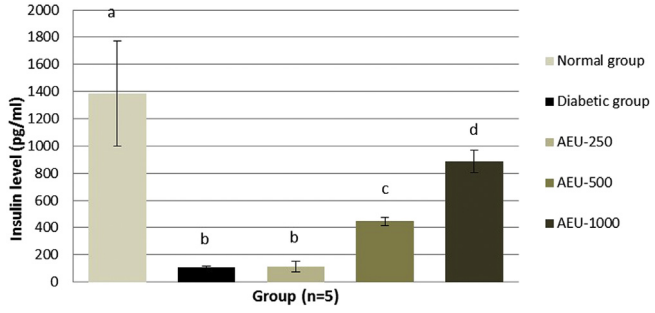


Fig. 2. Insulin level supplemented *U. lobata* extract. Means with different letters are significantly different ($p \leq 0.05$, LSD test).

3.4. The effect of U. lobata leaf extract on blood glucose level of diabetic rats

Based on these results at Fig. 3, there is a significant increase at blood glucose level on a diabetic group up to 70% compared to normal group observed ($p \leq 0.05$). The administration of aqueous extract *U. lobata* at dose of 250 mg/kg bw, 500 mg/kg bw, and 1000 mg/kg bw can decrease glucose level respectively 30%, 35%, and 40% compare to the diabetic group ($p \leq 0.05$) after glucose stimulation. Blood glucose level is not different significantly on an increase of dose *U. lobata* ($p > 0.05$).

3.5. The effect of U. lobata leaf extract on islet β-cells of diabetic rats

The normal group (Fig. 4A) shows the shape of cells are round, nucleated, and in a huge number, whereas the diabetic groups (Fig. 4B) cells show longer, not nucleated, and in a small number. Administration of aqueous extract *U. lobata* at dose of 500 and 1000 mg/kg bw could inhibit cells damage which shown as round cells, nucleated, and in a huge number (Fig. 4C-D). Test groups have islet β-cells in slightly bigger size than normal group, except aqueous extract at dose of 1000 mg/kg bw. The bigger size of cells shows a swelling cells and injury indications. The administration of aqueous extract *U. lobata* at dose of 1000 mg/kg bw are able to inhibit cells damage therefore the shape, size, and number are similar to islet cells at the normal group.

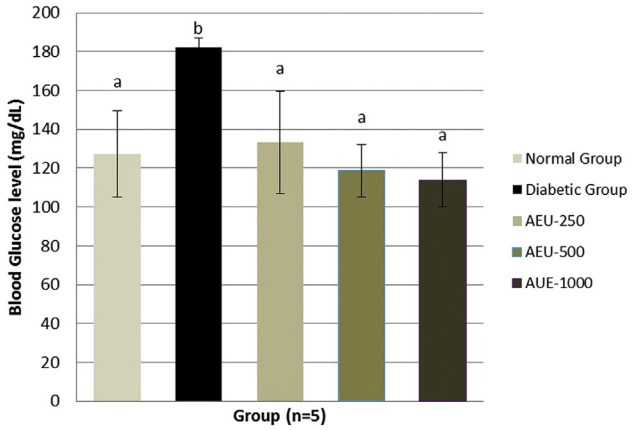


Fig. 3. Blood glucose level supplemented *U. lobata* extract. Means with different letters are significantly different ($p \leq 0.05$, LSD test).

4. Discussion

4.1. The effect of U. lobata leaf extract on GLP-1 serum level of diabetic rats

Oral administration of aqueous extract *U. lobata* significantly maintains GLP-1 bioavailability of diabetic rats. Based on our previous study, active compounds in *U. lobata* such as mangiferin, stigmasterol, and β-sitosterol are able to prevent degradation of GLP-1 by inhibition of DPP-4.[13] DPP-4 inhibitor substances prevent the degradation of active GLP-1 even though it does not increase the levels of total circulating GLP-1 and does not prevent the kidney from rapidly clearing GLP-1.[12]

GLP-1 is incretin hormone produced by L cell intestine and the secretion depends on oral nutrition. GLP-1 has a potency for T2DM therapy but it is metabolized excessively by DPP-4 into inactive form.[7] GLP-1 has a short half-life, approximately for 2–5 min, it is caused of DPP-4 activity.[3,6] The active form of GLP-1 is GLP-1 (7–36) amides and GLP-1 (7–37) which are rapidly inactivated by DPP-4 through cleave N-terminal dipeptide His-Ala.[12,19] It produces an inactive form of GLP-1, they are GLP-1 (9–36) amide and GLP-1 (9–37) isopeptides.[6,7] A number study showed that the importance of DPP-4 mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity.[1,12]

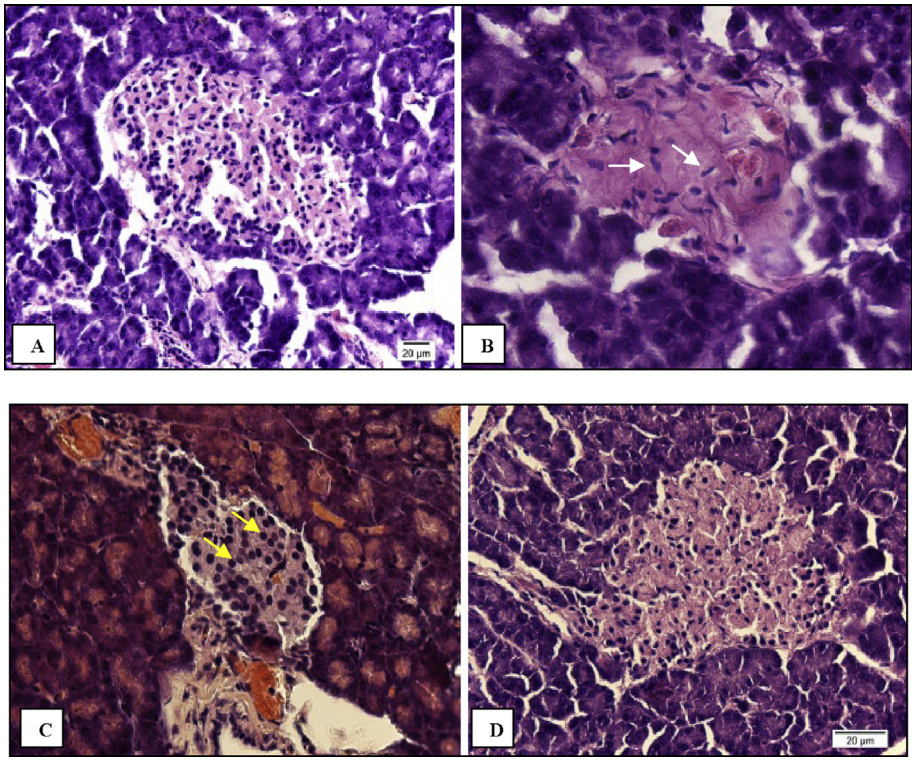


Fig. 4. Islet β-cells were stained by Hematoxylin–Eosin and observed under photomicroscope with magnitude 400×. (A). Normal group, (B). Diabetic group, (C). AEU 500 mg/kb bw, (D). AEU-1000 mg/kg bw. Magnification 400×. The white arrow shows unnucleated cells and longer shape whereas yellow arrow shows the swelling cells.

GLP-1 is a superfamily peptide of glucagon which has a similarity degree approximately 48%.[14] The similarity of amino acid sequence between GLP-1 and glucagon become one of this cause. Proglucagon gene is located at chromosome 2q36-q37 and only found in some tissues whereas the messenger RNA (mRNA) of proglucagon is met at α-cells pancreas, L cells intestine, and brain.[15] Proglucagon production is started from transcription of pre-proglucagon gene and then is continued by translation process.[3,14] The regulation of GLP-1 released from L cells intestine is a complex mechanism that involves combinations of nutrition, hormone, and neural stimuli.[14] The GLP-1 receptor is classified in *G protein-coupled* receptor that is found in liver, muscle, and pancreas cells.[2,3] This receptor has a specific character by activation of adenyl cyclase and results cAMP.[15] After GLP-1 binding with the receptor, it will activate cAMP and Mitogen-Activated Protein Kinase (MAPK).[3,7]

The biological activities of GLP-1 are various and depend on the organ target. GLP-1 activity in the pancreas has functions in stimulating the insulin secretion by cAMP activation, increasing β-cell masses by MAPK pathway and inhibiting the secretion of glucagon.[3,5] In the brain, it will reduce the rate of gastric emptying, induce satiety, and neuroprotection whereas in liver, fatty acid metabolism will be decreased and glucose utilization increased.[14,15] All of them contribute to regulate blood glucose level in T2DM.[1,2]

4.2. The effect of U. lobata leaf extract on insulin serum level of diabetic rats

Aqueous extract of *U. lobata* significantly increases insulin synthesis of diabetic rats. It is controlled by active compounds in the extract through the activity of GLP-1. The oral administration will maintain GLP-1 bioavailability moreover the insulin biosynthesis can be increased. GLP-1 has a potency to retain the insulinotropic activity for treating T2DM.[5,6] In this study, the increase of insulin secretion is caused by the active compounds of *U. lobata* extract to maintain GLP-1 bioavailability trough inhibition of DPP-4 activity.[13]

GLP-1 stimulates proinsulin biosynthesis and transcription of proinsulin gene. GLP-1 contributes to provide insulin deposition which loses from islet β-cells trough biosynthesis process.[5] GLP-1 is different with oral anti-diabetic sulphonylurea in stimulating of insulin formation because the sulphonylurea only stimulates insulin, not the biosynthesis of insulin.[4,5] GLP-1 is incretin hormone which is potential to increase islet β-cells proliferation, and anti-apoptosis furthermore it is able to increase insulin secretion.[2,6]

Hyperinsulinemia occurs in prediabetic condition or insulin resistance and then the secretion decline due to β-cell exhaustion or overwork.[2] The biological effect of insulin is divided into two major groups, they are metabolic and mitogenic effect.[11] The metabolic effect is glucose transport, lipid metabolism, protein, and glycogen synthesis whereas the mitogenic effect is the cell growth and mitogenesis.[11]

This study showed also that the administrations of *U. lobata* extract give a good description of islet β-cell. It is shown by the shape, size, and number of β-cell in better condition compared to diabetic groups. These conditions support the function of β-cell to produce insulin in order to maintain blood glucose level.[14,15] However, the diabetic group shows β-cells destruction which is signaled by a decreasing number of islet β-cell and structure damage therefore it affect their performance to release insulin.

4.3. The effect of U. lobata leaf extract on blood glucose level of diabetic rats

Administration of aqueous extract *U. lobata* significantly decreases blood glucose level of diabetic rats. It is controlled by active compounds of *U. lobata* which has DPP-4 inhibitory activity like stigmasterol, mangiferin, and β-sitosterol furthermore GLP-1

bioavailability can be retained for insulin biosynthesis when the blood glucose level increase after stimulating of oral nutrition.[13,18,30,32] GLP-1 acts outside of metabolism purpose, that is inhibiting of gastric juices secretion, inhibiting of the GIT motility and inhibiting of the rate of gastric emptying.[2,3] It is a benefit to prevent the increase of blood glucose level at postprandial.[5,6]

Insulin works to maintain blood glucose level after induction of glucose by a metabolic pathway. This hormone transports glucose from blood to the tissue and then synthesize it into glycogen in muscle in order to reduce blood glucose level.[11,14] In diabetic groups, the insulin secretion is disrupted therefore they lose their's control to maintain blood glucose level.[5,11] This is showed by blood glucose level in the diabetic group which is higher than normal and also treatment groups.

4.4. Histopathology of islet β-cell supplemented U. lobata extract

Oral administration of aqueous extract *U. lobata* is able to prevent islet β-cells death of diabetic group. The effect of active compounds in *U. lobata* that has potency such as increasing β-cells proliferations and inhibiting β-cells apoptosis through GLP-1 activation.[5,9,32] Bioavailability of GLP-1 could be retained due to DPP-4 inhibitor substances in the extract such as stigmasterol, mangiferin, and β-sitosterol.[13,26,31] It affects the integrity of β-cells indirectly in the test group, it is shown in the shape of cells, size, and number which is close to normal groups. Some tests show swelling cells, it indicates cells damage at the first step even though the shape and number of cells are normal.[20,29]

The active compounds of *U. lobata* leaves extract such as gossypetin, chrysoeriol, and mangiferin could protect cell damage from free radical.[22,24,27,29] They work as an antioxidant by donating an electron to unstable compounds in order to stabilize it.[23] Besides it, mangiferin and gossypetin act also as scavenger free radical moreover it could decrease oxidant level causing oxidative damage.[16,22,27] Hyperglycemia in diabetes increases the production of free radical furthermore it occurs imbalance between oxidant and antioxidant.[21,23] This condition is caused by oxidative stress which leads to oxidative damage in tissue or organ and an increase of diabetic complication risk.[16,23]

4.5. The effect of U. lobata leaf extract on body weight, food consumption, glucose level and insulin of diabetic rats

Aqueous extract of *U. lobata* reduces food consumption therefore it affects body weight gain of diabetic rats. It is related to active compound such as stigmasterol, mangiferin, and β-sitosterol in *U. lobata* that maintains bioavailability GLP-1 and their's interaction with GLP-1 receptor in the brain could reduce the rate of gastric emptying and also induce satiety.[13,16–18] The oral administration of *U. lobata* leaf extract decreases fasting blood glucose level and increase insulin level. GLP-1 activity in the pancreas has functions in stimulating the secretion of insulin by cAMP activation, increasing β cell masses by MAPK pathway and inhibiting the secretion of glucagon.[3,5] In liver, it increases utilization of glucose and decrease fatty acid metabolism. In T2DM, all of them contribute to maintain blood glucose level.[1,2]

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study was funded by Doctorate Research Grant of Directorate General of Higher Education Indonesia (No. 053/B.07/U.III/LPPM/2014).

References

1. Drucker DJ. Dipeptidyl peptidase-4 inhibition and the treatment of type 2 diabetes. *Diabetes Care*. 2007;30(6):1335–1343. http://dx.doi.org/10.2337/dc07-0228.
2. Chia CW, Egan JM. Incretin-based therapies in type 2 diabetes mellitus. *J Clin Endocrinol Metab*. 2008;93(10):3703–3716. http://dx.doi.org/10.1210/jc.2007-2109.
3. Drucker DJ. Biological actions and therapeutic potential of the glucagon like peptides. *Gastroenterology*. 2002;122(2):531–544. PMID: 11832466.
4. Brunton L, Chabner B, Knollman B. *Goodman & Gilman's the Pharmacological Basis of Therapeutics*. New York: McGraw-Hill; 2006.
5. Holst JJ, Orskov C. The incretin approach for diabetes treatment. Modulation of islet hormone release by GLP-1 agonism. *Diabetes*. 2004;53(3):S197–S204.
6. Salehi M, Aulinger AB, D'alessio AD. Targeting -cell mass in type 2 diabetes: promise and limitation of new drugs based on incretins. *Endocr Rev*. 2008;29(3):367–379. http://dx.doi.org/10.1210/er.2007-0031.
7. Bailey C. Incretin-based therapies. *Endocrin Abstr*. 2008;15:S41.
8. Onoagbe IO, Negbenebor EO, Ogbeide VO, et al. A study of the anti-diabetic effects of *Urena lobata* and *Sphenostylis stenocarpa* in streptozotocin-induced diabetic rats. *Eur J Sci Res*. 2010;43(1):6–14.
9. Awika JM, Rooney LW. Sorghum phytochemicals and their potential Impact on human health. *Phytochemistry*. 2004;65(9):1199–1221. http://dx.doi.org/10.1016/j.phytochem.2004.04.001.
10. Shirwaikar A, Rajendran K, Barik R. Effect of aqueous bark extract of *Garuga pinnata* Roxb. in streptozotocin–nicotinamide induced type-II diabetes mellitus. *J Ethnopharmacol*. 2006;107(2):285–290.
11. Rhodes CJ, White MF. Molecular insight into insulin action and secretion. *Eur J Clin Invest*. 2002;32(S3):3–13. PMID: 12028370.
12. Rosenstock J, Zinman B. Dipeptidyl peptidase-4 inhibitors and the management of type 2 diabetes mellitus. *Curr Opin Endocrinol Diabetes Obes*. 2007;14(2):98–107.
13. Purnomo Y, Soeatmadji DW, Sumitro SB, Widodo MA. Anti-diabetic potential of *Urena lobata* leaf extract through inhibition of dipeptidyl peptidase IV (DPP-4) Activity. *Asian Pac J Trop Biomed*. 2015;5(8):630–634.
14. Brubacker PL, Drucker DJ. Minireview: glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut and central nervous system. *Endocrinology*. 2004;145(6):2653–2659.
15. Aronoff SL, Berkowitz K, Shreiner B, et al. Glucose metabolism and regulation: beyond insulin and glucagon. *Diabetes Spectr*. 2004;17(3):183–190. http://dx.doi.org/10.2337/diaspect.17.3.183.
16. Sosa A, Rosquete C. Flavonoid from *Urena sinuata* L. *Av Quím*. 2010;5(2):95–98.
17. Ros MM, Sterk S, Verhagens H, Stalenhoef AF, De-Jong N. Phytosterol consumption and the anabolic steroid boldenone in humans: a hypothesis piloted. *Food Addit Contam*. 2007;24(7):679–684.
18. Rudkowska I, AbuMweis SS, Nicolle C, Jones PJ. Cholesterol-lowering efficacy of plant sterols in low-fat yogurt consumed as a snack or with a meal. *J Am Coll Nutr*. 2008;27(5):588–595.
19. Mentlein R, Gallwitz B. Dipeptidyl-peptidase IV hydrolyses gastric inhibitory polypeptide, glucagon-like peptide-1(7-36) amide, peptide histidine methionine and is responsible for their degradation in human serum. *Eur J Biochem*. 1993;214(3):829–835. http://dx.doi.org/10.1111/j.1432-1033.1993.tb17986.x.
20. Kumar Robbins. *Pathologic Basic of Diseases*. 6th ed. Philadelphia: WB Saunders Company; 2002:pp.12–21.
21. Miura T, Ichiki H, Iwamoto N, et al. Antidiabetic activity of a xanthone compound, mangiferin. *Phytomedicine*. 2010;8(2):85–87.
22. Stoilova I, Gargova S, Stoyanova A, Ho L. Antimicrobial and antioxidant activity of the polyphenol mangiferin. *Herba Pol*. 2005;51(1/2):37–44. ISSN 0018–0599.
23. Halliwel B, Gutteridge JM. *Free Radical in Biology and Medicine*. 3rd ed. Oxford: Oxford Science Publication; 1999.
24. Matławska I, Sikorska M. Flavonoid compounds in the flowers of *Abutilon indicum* (L.) sweet (Malvaceae). *Acta Pol Pharm*. 2002;59(3):227–229.
25. Mazumder UK, Gupta M, Malikandan L, Bhattacharya S. Antibacterial activity of *Urena lobata* root. *Fitoterapia*. 2001;72(8):927–929.
26. Panda S, Jafri M, Kar A, Maheta BK. Thyroid inhibitory, antiperoxidative and hypoglycemic effects of stigmasterol isolated from *Butea monosperma*. *Fitoterapia*. 2009;80(2):123–126.
27. De las Heras B, Slowing K, Benedi J, et al. Anti-inflammatory and antioxidant activity of plants used in traditional medicine in Ecuador. *J Ethnopharmacol*. 1998;61(2):161–166.
28. Omonkhua AA, Onoagbe IO. Preliminary proximate and phytochemical analyses of some medicinal plants used to treat diabetes mellitus in Nigeria. *Inven Impact Ethnopharmacol*. 2010;1:68–70.

29. Sellamuthu PS, Arulselvan P, Kamalraj S, Fakurazi S, Kandasamy M. Protective nature of *Mangifera* on oxidative stress and antioxidant status in tissues of streptozotocin-induced diabetic rats. *ISRN Pharmacol*. 2013:1–10. http://dx.doi.org/10.1155/2013/750109.
30. Saeidnia S, Manayi A, Gohari AR, Abdollahi M. The story of beta sitosterol-a review. *Eur J Med Plants*. 2014;4(5):590–609.
31. Chang CL, Lin Y, Bartolome AP, Chen YC, Chiu SC, Yang WC. Herbal therapies for type 2 diabetes mellitus: chemistry, biology, and potential application of selected plants and compounds. *Evid Based Complement Altern Med*. 2013: 1–33. http://dx.doi.org/10.1155/2013/378657.
32. Islam MH, Rahman KMH, Rahman S, Rahmatullah M. Preliminary anti-hyperglycemic, antinociceptive activity, phytochemical analysis and toxicity studies on leaves of *Urena lobata* L. *J Chem Pharm Res*. 2015;7(4):559–563.
33. Nurfauziah C, Mulyani S. *Anti-bacterial Potency of Urena lobata Leaf Extract on B. subtilis and E.Coli Also the Profile of Thin Layer Chomatography*. Final paper. Faculty of Pharmacy Gajah Mada University Yogyakarta. 1999.