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Inhibitory activity of *Urena lobata* leaf extract on Dipeptidyl Peptidase-4 (DPP-4): is it different by in vitro and in vivo test ?

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ABSTRACT

Objective : To examine anti diabetic effect of *U. lobata* leaf extract through DPP-4 inhibitory activity both of in vitro and in vivo.

Methods: *U.lobata* leaf was extracted in ethanol solvent and hot water therefore evaporated until pasta form. In vitro test using *Gly-pro-p-nitroanilide* (GPPN) as substrat of DPP-4 and Vildagliptin as standard reference. p-nitroanilida, a product of the reactions between GPPN and DPP-4 was observed by microplatereader with λ=405 nm moreover the IC-50 value was determined by non linear regression curve fit. In vivo study utilize animal model of T2DM which divided into 2 control group and 6 test group (n=4) therefore DPP-4 level, GLP-1 level and AUC of blood glucose were examined after administration of *U. lobata* leaf extract. All data are expressed as the mean ± SD and analyzed with *one way anova* and then continued with LSD or Dunnet c ($p<0.05$).

Results : DPP-4 inhibitory activity of ethanolic extract from *U.lobata* was stronger than water extract by in *vitro* test however the opposite occurs in *vivo* study. The conformation change of active substances in ethanolic extract result a poor solubility and gastrointestinal absorption which contribute to decrease their biology activity.

Keywords: DPP-4, *in vitro*, *in vivo*, *Urena lobata.*

1. Introduction

Recently, treatment of type 2 Diabetes mellitus (T2DM) is proposed on incretin hormon. The major of incretin hormon is Glucagon like Peptide-1 (GLP-1) secreted by intestinal due to induction of oral nutrition[1]. GLP-1 play a role to maintain blood glucose level related to their biology activity such as to stimulate insulin secretion, increase β-cell proliferation, inhibit glucagon secretion, reduce the rate of gastric emptying and induce satiety[1,2]. In T2DM patient, GLP-1 bioavailability decrease moreover secretion of insulin reduce and the clinical effect is hyperglycemic chronic[3].

GLP-1 have a potency to cure T2DM however GLP-1 is metabolized by Dipeptidyl peptidase-4 (DPP-4) excessively become inactive forms [2,3]. Inhibition of DPP-4 is effective to treat T2DM therefore GLP-1 bioavailability can be retained moreover it was able to regulate blood glucose level[3]. Treatment of T2DM with *incretin like* drugs or DPP-4 inhibitor show less side effect eventhough the safety of this drugs have not been obtained the completely data[5]. However, incretin like drug have side effect also such as flu like symptoms, skin reaction, gastrointestinal problem and this effect are able increase in long term use of drugs[6,7]. This phenomenon attract people attention to find medicinal plant as alternative to treat T2DM by DPP-4 inhibition.

One of traditional plants which have anti-diabetic effect is *Urena lobata*. Root and leaf extract of *U. lobata* have been used empirically by Nigeria people to treat DM. Preclinical test of *U. lobata* root extract show anti hyperglycemic effect on streptozotocin-induced rat[8]. In Indonesia, *U. lobata* is known by Pulutan, the plant showed anti-bacterial effect based on preliminary study[25]. Some study have showed anti diabetic potency of *U. lobata* however the mechanism of herbs trough inhibition of DPP-4 activity not yet investigated[8-10]. Therefore the study aims to examine anti-diabetic effect of *U. lobata* leaf extract on DPP-4 inhibition.

2. Material and methods

2.1 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, the *U.lobata* leaf powder (50 g) was extracted according to decoction method in 250 ml hot water at 90ºC for 30 minutes. The other *U.lobata* leaf powder (50 g) was extracted by digestion method in 250 ml ethanol for five hours using shaker waterbath and repeated two times using fresh solvent. Both of extract were evaporated until resulting concentrated extract.

2.2 DPP-4 in vitro assays

The assay was performed in 96 micro well plates. A pre-incubation volume of 50 µl solution contains of 35 µl Tris-HCl buffer, 15 µl DPP-4 enzyme and various concentration of test material or

standard. This mixture was incubated at 37ºC for 10 minutes, followed by addition of 50 µl Gly-pro-p-nitroanilide as substrate. The reaction of mixture was incubated for 30 minutes at 37ºC and the absorbance was measured at 405 nm every 10 seconds. Vildagliptin was used as DPP-IV inhibitor.

2.3 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 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 eight 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 ethanolic extract (EEU) and water of *U.lobata* (WEU) and 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.

2.4 DPP-4 in vivo assay

DPP-4 serum level was analysed by rat DPP-4 ELISA kit (Elabscience E-EL-R0337). 100 µl samples were incubated for 90 minutes at 37 ºC, added 100 µl Biotinylated detection Ab and then incubated for 60 minutes at 37 ºC. After aspirating and washing then sample was added 100 µl HRP conjugate and incubated for 30 minutes at 37 ºC. Added 90 µl substrat reagent then was added 50 µl *stop solution*. Samples were read with microplate reader at $\lambda = 450$ nm.

2.5 GLP-1 in vivo 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.

2.6 Oral Glucosa Tolerance Test

For glucose tolerance test, the glucose were administered orally in a dose of 2 g/kg body weight after overnight fasting. The blood samples were collected from the tail vein at 0 (before glucose administered), 15, 30, 60, 90 and 120 min after glucose administered. They were measured immediately using a commercially available glucometer. The integrated area under the postprandial glucose curves (AUCs) was calculated by the trapezoidal method.

2.7 Statistical Analysis

The data were expressed as means ± S.E.M. Statistical analysis was performed by one-way ANOVA. The least significant difference (LSD) test or Dunnet C were used for mean comparisons and then $P < 0.05$ was considered to be statistically significant.

3. Results

3.1 DPP-4 inhibitory activity of U. lobata by in vitro test

The DPP-4 inhibitory activity of *U. lobata* leaf extract is shown in Table 1. Based on these results, ethanolic extract of *U.lobata* showed that the inhibitory activity on DPP-4 was stronger about 4 times folds compared to water extract ($p<0.05$).

Table 1. DPP-4 inhibitory activity of *U. lobata* leaf extracts

Group	Sample	n	Concentration (ppm)	% inhibition	IC-50 (ppm)
1	Water extract of *U.lobata*	3	625	0.00 ± 0.00	6489.88[a]
		3	1250	13.33 ± 0.00	
		3	2500	26.67 ± 0.00	
		3	5000	42.22 ± 3.85	
		3	10000	62.22 ± 3.85	
2	Ethanolic extract of *U.lobata*	3	625	36.17 ± 0.00	1654.64[b]
		3	1250	48.94 ± 0.00	
		3	2500	55.32 ± 0.00	
		3	5000	61.70 ± 0.00	
		3	10000	74.47 ± 0.00	
3	Vildagliptin	3	6.25	8.93 ± 0.00	57.44[c]
		3	12.50	16.07 ± 4.12	
		3	25.00	37.50 ± 0.00	
		3	50.00	46.63 ± 3.85	
		3	100.00	60.71 ± 0.00	

a,b,c = different letter showed the differences of the potency ($p<0.05$, LSD test)

It is regulated by the differences both of active compounds and their proportions in these extracts. Semi qualitative test of *U.lobata* leaf extract by LC-MS showed the compositions of Gossypetin in ethanolic extract is higher than in water extract whereas Stigmasterol is more dominant in water extract[11]. Active compounds such as Stigmasterol, β- sitosterol and Gossypetin are soluble in semi-polar solvents like alcohol eventhough Mangiferin and Hypolaetin is insoluble.The differences of solubility of active compounds in the solvents will affect to the percentages of active compounds in the extracts. Solubility of active compounds in the solvents will contribute on their compositions in the extracts. However, the DPP-4 inhibitory activity on both of water and ethanolic *U. lobata* extracts are still lower, approximetly 30-100 times folds, compared to Vildagliptin as reference drugs of DPP-4 inhibitor ($p<0.05$).

3.2 DPP-4 inhibitory activity of U. lobata by in vivo test

DPP-4 serum level of diabetic rat administrated *U.lobata* leaf extract can be shown at Figure 1. The diabetic group shows significant increase in DPP-4 level, which is about 4-fold from the normal group ($p<0.05$). Administration of water extract *U. lobata* 250 mg/kg, 500 mg/kg and 1000 mg/kg bw decrease DPP-4 level respectively about 60%, 70% and 70% compared to diabetic group ($p<0.05$) whereas ethanolic extract 60%, 50% and 40% ($p<0.05$). At the same dose, water extract from *U.lobata* is able to decrease more DPP-4 level compared to ethanolic extract ($p<0.05$).

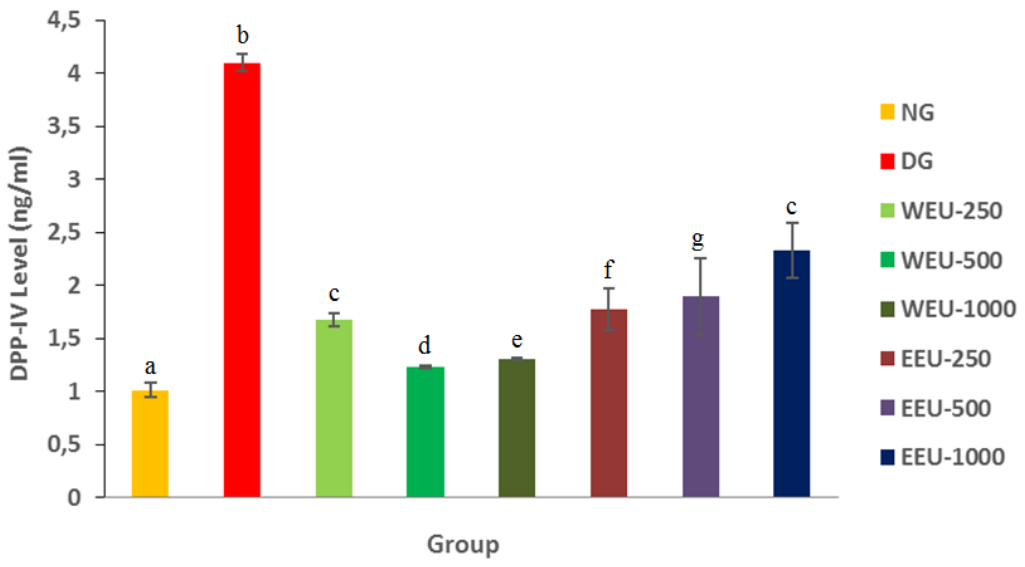

Figure 1. DPP-4 level administrated *U.lobata* leaf extract; a, b, c etc. showed the differences of potency ($p<0.05$, Dunnet C test)

Both of water and ethanolic extract from *U.lobata* significantly inhibit DPP-4 activity of diabetic rats. The effects are regulated by active compounds of *U.lobata* such as mangiferin, stigmasterol and β-sitosterol based on in-silico study [11]. The increase doses of water extract *U.lobata* more inhibit DPP-4 activity whereas ethanolic extract increase DPP-4 precisely. It is

caused by the conformation shift of active compounds particulary sterol group in ethanolic extract, furthermore they produced a complex compounds in post binding[18,19]. The change of active compounds structure can alter the solubility of active substances and its absorption therefore modulate inhibitory activity on DPP-4[18]. Ethanolic extract of *U.lobata* is more semi polar than water extract, it affect solubility of active compound and its absorption in gastrointestinal moreover their bioactivity decrease. Water extract of *U.lobata* more inhibit DPP-4 activity than ethanolic extract. Complex form of active substances in water extract of *U.lobata* fasciltate inhibitory activity on DPP-4. Some of active compound in water extract of *U.lobata* have synergistic interaction moreover reinforce their inhibitory activity on DPP-4[4]. Inhibition of DPP-4 activity will increase the bioavailability of incretin hormone which contribute in carbohydrate metabolism[3].

The differences of DPP-4 inhibitory activity between in vitro test and in vivo can be explained also trough pharmacokinetic aspect. The poor of active compound solubility in ethanolic extract maybe affect its absorption and distribution in blood therefore decrease their activities to inhibit DPP-4 activity.

The principal biological activity of DPP-4/CD26 is its enzymatic function. The enzymatic activity of DPP-4/CD26 is exhibited by the membrane-spanning form of the molecule, and the slightly smaller circulating soluble form[14]. The substrates of CD26/DPP-4 are not specific to a certain peptides. Proline or alanine containing peptides are the substrates of DPP-4 and include growth factors, chemokines, neuropeptides and vasoactive peptides[12]. DPP-4 prefers substrates with an amino-terminal proline or alanine at position 2 eventhough may also cleave substrates with non-preferred amino acids at position 2[12,15]. The structure of incretin hormone such as GLP-1 and GIP reveals a highly conserved to alanine at position 2, rendering these peptides ideal putative substrates for the aminopeptidase DPP-4[15].

DPP-4 inhibitor prevents the degradation of active GLP-1 however it does not increase the levels of circulating total GLP-1 and does not prevent the kidney from rapidly clearing GLP-1. Using DPP-4 inhibitors, primarily for the treatment of diabetes, related to the potential effects of these inhibition on immune function. CD26/DPP-4 plays an important role in tumor biology, and is useful as a marker for various cancers, with its levels either on the cell surface or in the serum increased in some neoplasms and decreased in others[17]. It is related to the inhibition of T cell proliferation in immune system moreover it lose their biological activity to protect against neoplasm[14,17].

3.3 Effect of U. lobata leaf extract on GLP-1 serum level

GLP-1 serum level of diabetic rat administrated *U.lobata* leaf extract can be shown Figure 2. There is a significant decrease of GLP-1 levels on diabetic group about 8-fold compared to normal group observed ($p<0.05$). Water extract of *U. Lobata* at doses of 250 mg/kg bw, 500 mg/kg bw and

1000 mg/kg bw prevent degradation of GLP-1 respectively about 3-fold, 5-fold and 7-fold compared to diabetic group ($p<0.05$) whereas ethanolic extract which show respectively about 5-fold, 2-fold and 2.5-fold ($p<0.05$). At the same dose, water extract from *U.lobata* is able to inhibit more the degradation of GLP-1 compared to ethanolic extract ($p<0.05$) except ethanolic extract of *U.lobata* at dose of 250 mg/kg bw.

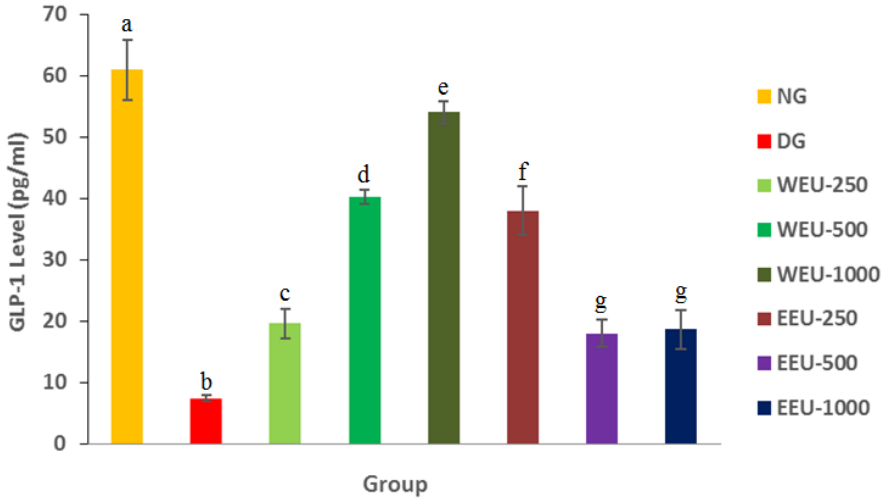

Figure 2. GLP-1 level administrated *U.lobata* leaf extract; a, b, c etc. showed the differences of potency ($p<0.05$, Dunnet C test)

Both administration of water and ethanolic extract from *U.lobata* significantly maintain GLP-1 bioavailability of diabetic rats. Mangiferin, stigmasterol and β-sitosterol in the extract are able to prevent degradation of GLP-1 by DPP-4. Based on our previous study, active compounds in *U.lobata* above act as DPP-4 inhibitor. The water extract of *U.lobata* maintain more GLP-1 bioavailability compared to ethanolic. It is caused by the difference of active compounds which inhibit GLP-1 degradation by DPP-4. It also relate to DPP-4 inhibitory activity of active compound both in water and ethanolic extract from *U.lobata*. The effect is regulated by DPP-4 inhibitory activity due to a synergistic interaction of active compounds and production of complex compound in water extract[4]. As the result, inhibition of DPP-4 will prevent GLP-1 metabolism from inactivation therefore their bioavailability is able to retain.

GLP-1 has a potency for T2DM therapy but it is metabolized excessively by DPP-4 become inactive form[3,7]. GLP-1 has a short half-life, approximately for 2-5 minutes, it is caused by 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. 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[20].

GLP-1 is a super family peptide of glucagon which have a similarity degree about 48 %. The similarity of amino acid sequence between GLP-1 and glucagon became one of this causa. Pro glucagon gen was located at chromosome 2q36-q37 and only found in some tissues whereas the messenger RNA (mRNA) of pro glucagon was met at α-cells pancreas, L-cells intestine and brain in hypothalamus part. Proglucagon production was started from transcription of preproglucagon gen and then was continued by translation process. The regulation of GLP-1 release from L-cells intestine are a complex mechanism that involve combinations of nutrition, hormone and neural stimuli[21]. GLP-1 receptor is classified in *G protein-coupled* receptor that is found at liver, muscle and pancreas cells. This receptor have a specific character by activation of adenilcyclase and result cAMP. After GLP-1 binding with the receptor, it will activate cAMP and Mitogen Activated Protein Kinase (MAPK).

3.4 U. Lobata effect on Glucose Tolerance Test of Diabetic Rats

Blood glucose level of rat administrated *U.lobata* after stimulating glucose can be shown at Figure 3 and 4. Based on these results, there is a significant increase at AUC of glucose on diabetic group up to 70% compared to normal group observed ($p<0.05$). The administration of water extract *U.lobata* at dose of 250 mg/kg bw, 500 mg/kg bw and 1000 mg/kg bw decrease AUC of glucose respectively 50% ,60% and 50% compare to diabetic group ($p<0.05$) whereas ethanolic extract respectively 50%, 40% and 20% ($p<0.05$). Water extract of *U.lobata* stronger control AUC of blood glucose compared to ethanolic extract ($p<0.05$) after glucose stimulation.

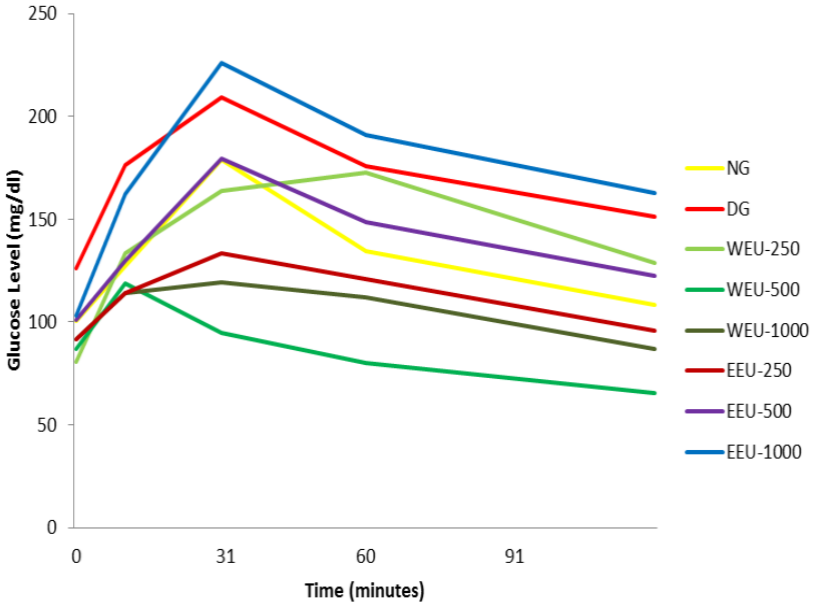

Figure 3. Blood glucose level administrated *U.lobata* extract after induction of glucose.

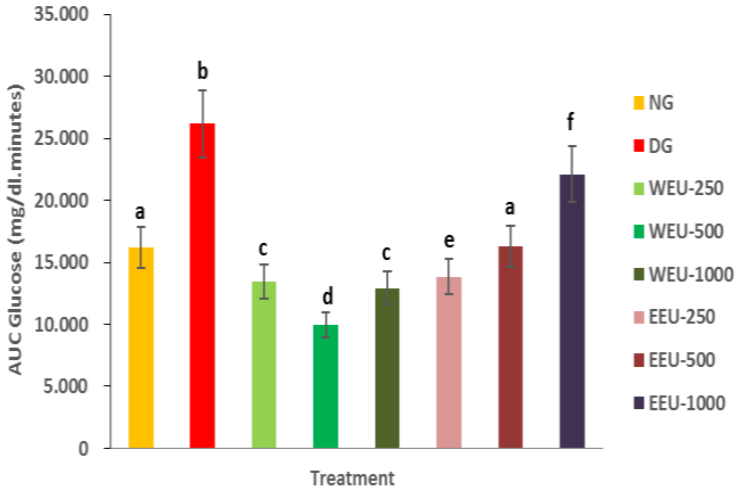

Figure 4. AUC of glucose administrated *U.lobata* extract; a, b, c etc. showed the differences of potency ($p<0.05$, LSD test)

Administration of ethanolic and water extract from *U.lobata* significantly decrease AUC of glucose of diabetic rats post glucose induction. It is controlled by *U.lobata* active compounds which has DPP-4 inhibitory activity therefore GLP-1 bioavailability can be retained to regulate blood glucose level increase after stimulating of oral nutrition[3,5]. 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 beneficial to prevent the increase of blood glucose level post prandial[5,6].

Water extract of *U.lobata* regulate more blood glucose level post glucose stimulation compared to ethanolic extract of *U.lobata*. It is predicted due to the poor of active compound solubility and absorption in ethanolic extract, therefore affect their activities to control blood glucose level. The reducing of active compounds solubility occur due to the formation of complex compounds and the change of active compound conformation furthermore decrease inhibitory activity on DPP-4 that contribute to maintain blood glucose level on diabetic groups[16,18].

4. Conclusions

DPP-4 inhibitory activity of ethanolic extract from *U.lobata* was stronger than water extract by in *vitro* test eventhough the opposite occurs in *vivo* study. Water extract of *U. lobata* stronger decrease DPP-4 level and AUC of blood glucose and retain GLP-1 bioavailability compared to ethanolic extract.

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Inhibitory activity of *Urena lobata* leaf extract on Dipeptidyl Peptidase-4 (DPP-4): is it different by *in vitro* test and *in vivo* ?

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Abstract

This study was aimed to examine anti diabetic effect of *Urena lobata* leaf extract through DPP-4 inhibitory activity both of *in vitro* and *in vivo*. *Urena lobata* leaf was extracted in ethanol solvent and hot water. *In vitro* test using *Gly-pro-p-nitroanilide* (GPPN) as substrat of DPP-4 and vildagliptin as standard reference. A product of the reactions between GPPN and DPP-4, *p*-nitroanilida was observed by microplatereader at λ=405 nm while ~~moreover~~ the IC_{50} value was determined by non linear regression curve ~~fit~~. *In vivo* study utilize animal model of T2DM with ~~which divided into~~ 2 control groups and 6 test groups (n=4), in which ~~therefore~~ DPP-4 level, GLP-1 level and AUC of blood glucose were examined after extract administration. All data were analyzed with one-way Anova and then continued with LSD or Dunnet c ($p<0.05$). The *in vitro* DPP-4 inhibitory activity of ethanolic extract from *U. lobata* was stronger than water extract ~~by in *vitro* test~~. However, vice versa results were observed *in vivo* study. ~~DPP-4 inhibitory activity of ethanolic extract from *U.lobata* was stronger than water extract by in *vitro* test eventhough the opposite occurs in *vivo* study~~. In addition, the *U. lobata* water extract ~~of~~ exhibit stronger decrease DPP-4 level and AUC of blood glucose, as well as retain the GLP-1 bioavailability compared to ethanolic extract.

Keywords: DPP-4, *in vitro*, *in vivo*, *Urena lobat*, *Gly-pro-p-nitroanilide (GPPN)* .

Introduction

Recently, treatment of type 2 Diabetes mellitus (T2DM) is proposed on incretin hormon. The major of incretin hormon is Glucagon like Peptide-1 (GLP-1) secreted by intestinal due to induction of oral nutrition (Drucker, 2007). GLP-1 plays a vital role to maintain blood glucose level related to their biology activity such as to stimulate insulin secretion, increase β-cell proliferation, inhibit glucagon secretion, reduce the rate of gastric emptying and induce satiety (Drucker, 2007; Chia and Egan, 2008). In T2DM patient, GLP-1 bioavailability decrease ~~moreover~~ the secretion of insulin reduce and the clinical effect is hyperglycemic chronic (Drucker, 2002).

GLP-1 has a potency to cure T2DM, however GLP-1 is metabolized by Dipeptidyl

peptidase-4 (DPP-4) excessively become inactive forms (Drucker, 2002; Chia and Egan, 2008). Inhibition of DPP-4 is effective to treat T2DM, therefore GLP-1 bioavailability can be retained in addition ~~moreover~~ ~~it was able~~ to regulate blood glucose level (Brunton et al., 2006). Treatment of T2DM with *incretin like* drugs or DPP-4 inhibitor show less side effect eventhough the safety of this drugs have not been obtained the complete~~ly~~ data (Holst and Orskov, 2004). ~~However~~ Nevertheless, incretin like drug have side effects, ~~also~~ such as flu like symptoms, skin reaction, gastrointestinal problem; ~~and~~ These ~~this~~ effects ~~are able~~ increases with the ~~in long term~~ use of drugs for long term (Bailey, 2008; Salehi et al., 2008). This phenomenon attracts people attention to find medicinal plant as alternative to treat T2DM by DPP-4 inhibition.

One of traditional plants which have anti-diabetic effect is *Urena lobata*. Root and leaf extract of *U. lobata* have been used empirically by Nigeria people to treat DM. Preclinical test of *U. lobata* root extract show anti-hyperglycemic effect on streptozotocin-induced rat (Onoagbe et al., 2010). In Indonesia, *U. lobata* is known by Pulutan, the plant showed anti-bacterial effect based on preliminary study (Nurfauziah and Mulyani, 1999). Some studies have showed anti-diabetic potency of *U. lobate*, however the mechanism of herbs ~~trough~~ on the inhibition of DPP-4 activity has not been investigated yet (Nurfauziah and Mulyani, 1999; Vats et al., 2002; Awika and Rooney, 2004; Onoagbe et al., 2010). Therefore, the study aims to examine the anti-diabetic effect of *U. lobata* leaf extract on DPP-4 inhibition.

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,~~ The *U.lobata* leaf powder (50 g) was extracted according to decoction method in which the leaf powder was soaked in 250 mL hot water (90ºC) for 30 minutes. The other sample of *U.lobata* leaf powder (50 g) was extracted based on the digestion method by soaking the sample in 250 mL ethanol for five hours using shaker waterbath. The extraction process was repeated two times using fresh solvent. Both of extract solution were removed its solvent using a rotary vapor to obtain crude exracts. ~~evaporated until resulting concentrated extract.~~

DPP-4 in vitro assay~~s~~

The assay was performed in 96 micro well plates. A pre-incubation volume of 50 µL solution contained of 35 µ L Tris-HCl buffer, 15 µ L DPP-4 enzyme and various concentration of test material or standard. This mixture was incubated at 37 ºC for 10 minutes, followed by addition of 50 µL Gly-pro-p-nitroanilide as substrate. The reaction of mixture was incubated for 30 minutes

at 37 ºC and the absorbance was measured at 405 nm at 10 seconds interval. Vildagliptin was used as the standard reference of DPP-IV inhibitor.

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 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~~ with 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 (Awika and Rooney, 2004). The experiments were assigned into eight 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 the ethanolic extract (EEU) and water extract of *U.lobata* (WEU) and at a dose of 250 mg/kg, 500 mg/kg, and 1000 mg/kg bw for four weeks after the rats ~~was~~ were classified as diabetic according to Shirwaikar *et al*. (2006). Body weight and food consumption were monitored weekly. Blood samples were obtained 15 minutes after given the glucose stimulation in dose of 2 g/kg body weight orally. The blood samples were also taken from tail vein after overnight fasted. Blood samples were immediatley centrifuged at 4500 rpm. The serum was separated and saved under -20 ºC.

DPP-4 in vivo assay

DPP-4 serum level was analysed by rat DPP-4 ELISA kit (Elabscience E-EL-R0337). 100 µL samples were incubated for 90 minutes at 37 ºC, added 100 µL Biotinylated detection Ab and then incubated for 60 minutes at 37 ºC. After aspirating and washing, then the sample was added 100 µL HRP conjugate and incubated for 30 minutes at 37 ºC. A~~dded~~ 90 µL substrat reagent was ~~was~~ added with 50 µL *stop solution*. The data of samples were measured with a microplate reader at λ = 450 nm.

GLP-1 in vivo assay

GLP-1 serum level was analyzed by rat GLP-1 ELISA kit (USCN CEA804). 50 µL samples were added with 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. A~~dded~~ 90 µL substrat reagent ~~then~~ was then added with 50 µL *stop solution*. The data of samples were obtained using a microplate reader at a wavelength of 450 nm.

Oral Glucosa Tolerance Test

For glucose tolerance test, the glucose were administered orally in a dose of 2 g/kg body weight after overnight fasting. The blood samples were collected from the tail vein at 0 (before glucose administered), 15, 30, 60, 90 and 120 min after glucose administered. They were measured immediately using a commercial~~ly available~~ glucometer. The integrated area under the postprandial glucose curves (AUCs) was calculated by the trapezoidal method.

Statistical Analysis

The data were expressed as means ± S.E.M. Statistical analysis was performed by one-way ANOVA. The least significant difference (LSD) test or Dunnet C were used for mean comparisons and then $P < 0.05$ was considered to be statistically significant.

Results

DPP-4 inhibitory activity of U. lobata by in vitro test

The DPP-4 inhibitory activity of *U. lobata* leaf extract is shown in Table 1. Based on the~~se~~ results, the *U. lobata* ethanolic extract showed that the inhibitory activity on DPP-4 was stronger (about 4 times folds) compared to that of water extract ($p<0.05$).

Table 1. DPP-4 inhibitory activity of *U. lobata* leaf extracts

Group	Sample	Concentration (ppm)	% inhibition	IC_{50} (ppm)
1	Water extract of *U.lobata*	625	0.00 ± 0.00	6489.88[a]
		1250	13.33 ± 0.00	
		2500	26.67 ± 0.00	
		5000	42.22 ± 3.85	
		10000	62.22 ± 3.85	
2	Ethanolic extract of *U.lobata*	625	36.17 ± 0.00	1654.64[b]
		1250	48.94 ± 0.00	
		2500	55.32 ± 0.00	
		5000	61.70 ± 0.00	
		10000	74.47 ± 0.00	

3	Vildagliptin	6.25	8.93 ± 0.00	57.44[c]
		12.50	16.07 ± 4.12	
		25.00	37.50 ± 0.00	
		50.00	46.63 ± 3.85	
		100.00	60.71 ± 0.00	

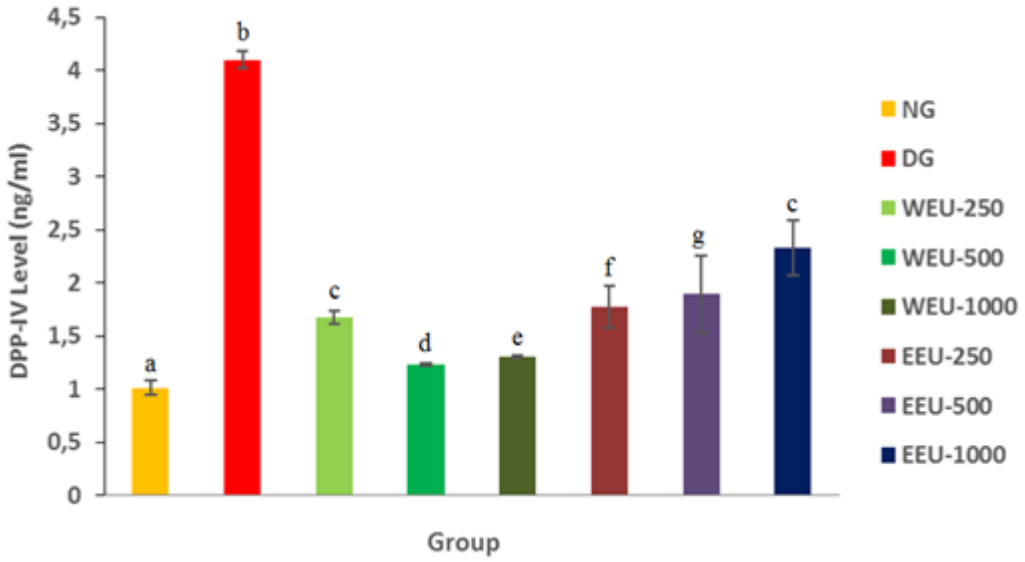
a,b,c = different letter showed the differences of the potency ($p<0.05$, LSD test)

The experiments were repeated in triplicate.

The results of % inhibition were expressed as means ± S.E.M

DPP-4 inhibitory activity of U. lobata by in vivo test

DPP-4 serum level of diabetic rat administrated *U.lobata* leaf extract are ~~can be~~ shown in ~~at~~ Figure 1. The diabetic group shows significant increase in DPP-4 level, which is about 4-fold from the normal group ($p<0.05$). Administration of *U. lobata* water extract at 250 mg/kg, 500 mg/kg and 1000 mg/kg bw decrease DPP-4 level about 60%, 70% and 70%, respectively compared to diabetic group ($p<0.05$) whereas that of ethanolic extract decrease DPP-4 level about 60%, 50% and 40% ($p<0.05$). The results demonstrated that the water extract ~~from *U.lobata*~~ is able to decrease more DPP-4 level compared to the ethanolic extract ($p<0.05$) of same dosage.



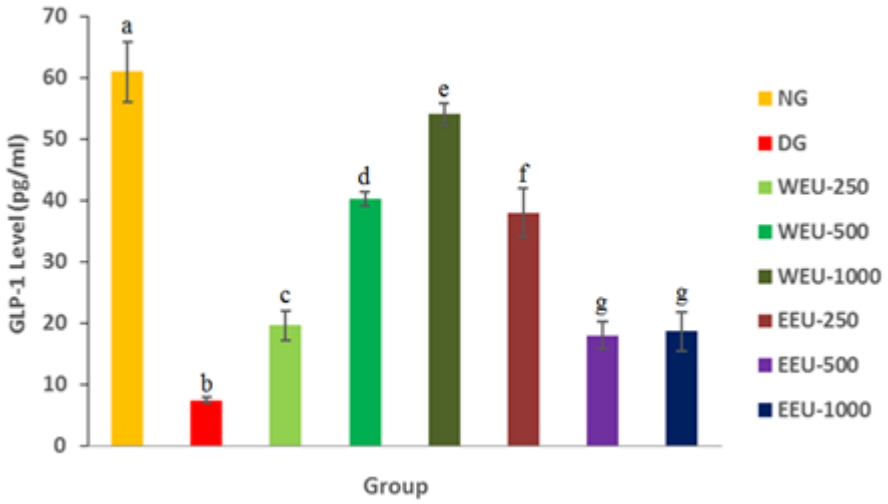
Note: a, b, c etc. showed the differences of potency ($p<0.05$, Dunnet C test)

Figure 1. DPP-4 level administrated *U.lobata* leaf extract

Effect of U. lobata leaf extract on GLP-1 serum level

GLP-1 serum level of diabetic rat administrated *U. lobata* leaf extract is displayed ~~can be shown~~ in Figure 2. There is a significant decrease of GLP-1 levels on diabetic group about 8-fold

compared to normal group observed ($p<0.05$). The water extract of *U. Lobata* at doses of 250 mg/kg bw, 500 mg/kg bw and 1000 mg/kg bw prevent degradation of GLP-1 about 3-fold, 5-fold and 7-fold, respectively compared to that of diabetic group ($p<0.05$) which ~~whereas~~ the ethanolic extract ~~which~~ show about 5-fold, 2-fold and 2.5-fold ($p<0.05$), respectively. For the same dosage, the water extract ~~from *U.lobata*~~ is able to inhibit more the degradation of GLP-1 compared to the ethanolic extract ($p<0.05$) except ~~ethanolic extract of *U.lobata*~~ at a dose of 250 mg/kg bw.



Note: a, b, c etc. showed the differences of potency ($p<0.05$, Dunnet C test)

Figure 2. GLP-1 level administrated *U.lobata* leaf extract

Both administration of the water and ethanolic extracts ~~from *U.lobata*~~ significantly maintain GLP-1 bioavailability of diabetic rats. Mangiferin, stigmasterol and β-sitosterol in the extract are able to prevent degradation of GLP-1 by DPP-4. Based on our previous study, active compounds in *U. lobata* extracts ~~above~~ act as DPP-4 inhibitor. The water extract ~~of *U. lobata*~~ maintain more GLP-1 bioavailability compared to the ethanolic extract. It is caused by the difference of active compounds which inhibit GLP-1 degradation by DPP-4. It is also relate to DPP-4 inhibitory activity of active compounds in both water and ethanolic extracts ~~from *U.lobata*~~. The effect is regulated by DPP-4 inhibitory activity due to a synergistic interaction of active compounds and production of complex compounds in water extract (Brunton et al., 2006). As the result, inhibition of DPP-4 ~~will~~ prevents the GLP-1 metabolism from inactivation and resuted retaintion of ~~therefore~~ their bioavailability ~~is able to retain~~.

The effect of U. Lobata on Glucose Tolerance Test of Diabetic Rats

Blood glucose level of rat administrated *U. lobata* after stimulating glucose ~~can be~~ are shown ~~at~~ in Figure 3 and 4. ~~Based on these~~ The results indicated that there is a significant increase

at AUC of glucose on diabetic group up to 70% compared to normal group observed ($p<0.05$). The administration of *U. lobata* water extract at dose of 250 mg/kg bw, 500 mg/kg bw and 1000 mg/kg bw decrease AUC of glucose 50% ,60% and 50%, respectively compared to that of diabetic group ($p<0.05$) whereas the ethanolic extract decrease AUC of glucose 50%, 40% and 20%, respectively ($p<0.05$). The *U.lobata* Water extract exhibits stronger control AUC of blood glucose compared to that of ethanolic extract ($p<0.05$) after glucose stimulation.

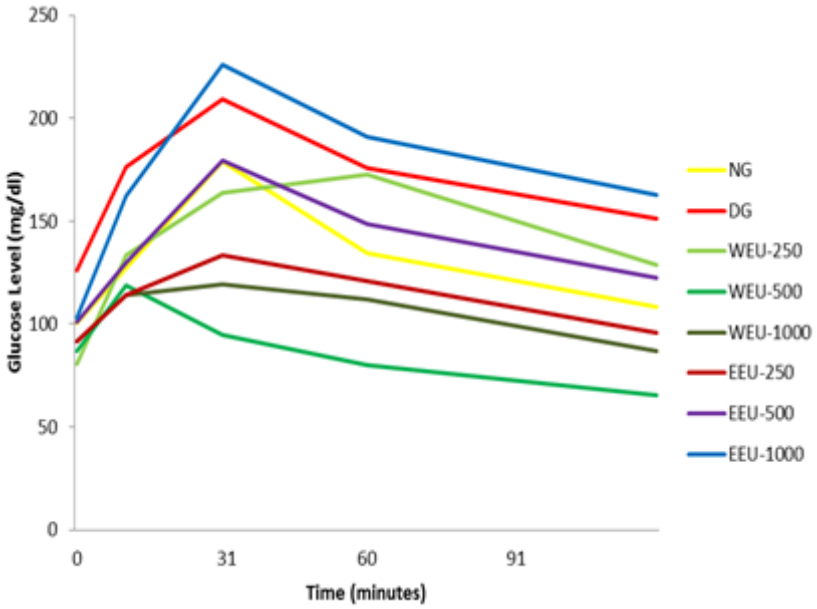
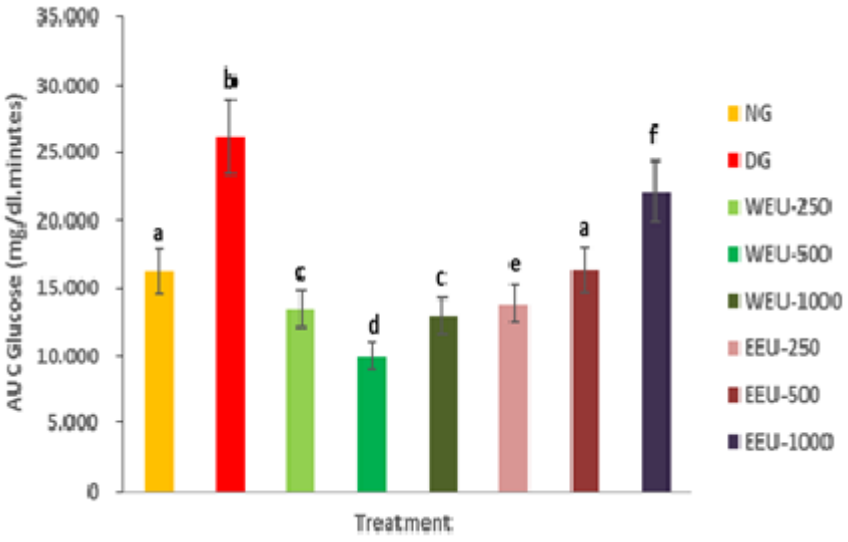


Figure 3. Blood glucose level administrated *U. lobata* extract after induction of glucose.



Note: a, b, c etc. showed the differences of potency ($p<0.05$, LSD test)

Figure 4. AUC of glucose administrated *U.lobata* extract;

Discussion

~~It is regulated by the differences both of active compounds and their proportions in these extracts.~~

The semi qualitative test of *U.lobata* leaf extract by LC-MS showed the compositions of gossypetin (**1**) in the ethanolic extract are ~~is~~ higher than that of in the water extract whereas stigmasterol is more dominant in the water extract (Purnomo et al., 2015). Active compounds such as stigmasterol (**2**), β-sitosterol (**3**) and gossypetin (**1**) are soluble in less ~~semi-~~polar solvents like alcohol eventhough mangiferin (**4**) and hypolaetin is insoluble. The differences ~~of~~ solubilities of active compounds in the solvents ~~will~~ affect ~~to~~ the ~~percentages~~ amount of active compounds extracted from ~~in~~ the extracts. ~~Solubility of active compounds in the solvents will contribute on their compositions in the extracts.~~ However, the DPP-4 inhibitory activity on both of the water and ethanolic ~~*U. lobata*~~ extracts are still lower, approximetly 30-100 times folds, compared to vildagliptin, the standard reference drugs of DPP-4 inhibitor ($p<0.05$).

Gossypetin (**1**)

Stigmasterol (**2**),

B-Sitosterol (**3**)

Mangiferin (**4**)

Figure 5. Structures of some chemical compositions found in *U.lobata* leaf extract

Both of the *U. lobata* water and ethanolic extracts ~~from *U. lobata*~~ significantly inhibit DPP-4 activity of diabetic rats. These effects are regulated by active compounds **2**, **3**, **4** in ~~of~~ *U.lobata*

~~such as mangiferin, stigmasterol and β sitosterol~~ based on *in-silico* study (Purnomo et al., 2015). The increase doses of *U.lobata* water extract ~~more~~ inhibit more DPP-4 activity whereas the ethanolic extract increase DPP-4 precisely. It is caused by the conformation shift of active compounds, particulary the sterol group in ethanolic extract which ~~, furthermore they~~ produced a complex compounds in post binding (Morris and Lim-Wilby, 2008; Setevens and Honerkamp-Smith, 2010). The change of active compounds structure can alter the solubility of active substances and its absorption and resultant a ~~therefore~~ modulate inhibitory activity on DPP-4 (Stevens and Honerkamp-Smith, 2010). Due to the ethanolic extract ~~of *U.lobata*~~ is ~~more semi~~ less polar than the water extract, it affect solubilities of active compounds and its absorption in gastrointestinal in addition to ~~moreover~~ the~~ir~~ bioactivity decrease. The water extract ~~of *U.lobata*~~ inhibit more DPP-4 activity than that of ethanolic extract. Complex form of active substances in the *U. lobata* water extract ~~of *U.lobata*~~ fasciltate the inhibitory activity on DPP-4. Some of active compounds in the water extract ~~of *U.lobata*~~ have synergistic interaction besides the ~~moreover~~ reinforcement of their inhibitory activity on DPP-4 (Brunton et al., 2006). Inhibition of DPP-4 activity ~~will~~ increases the bioavailability of incretin hormone which contribute in carbohydrate metabolism (Drucker, 2002).

The differences of DPP-4 inhibitory activity between *in vitro* and *in vivo* tests can be explained ~~also trough~~ pharmacokinetically ~~aspect~~. The poor solubility of active compounds ~~solubility~~ in the ethanolic extract may~~be~~ affect its absorption and distribution in blood, as a result to ~~therefore~~ decrease ~~their activities to~~ the inhibition of ~~inhibit~~ DPP-4 activity.

The principal biological activity of DPP-4/CD26 is its enzymatic function. The enzymatic activity of DPP-4/CD26 is exhibited by the membrane-spanning form of the molecule, and the slightly smaller circulating soluble form (Chen, 2006). The substrates of CD26/DPP-4 are not specific to ~~a~~ certain peptides. Proline or alanine containing peptides are the substrates of DPP-4 and include growth factors, chemokines, neuropeptides and vasoactive peptides (Mentlein and Gallwitz, 1993). DPP-4 prefers substrates with an amino-terminal proline or alanine at position 2 eventhough may also cleave substrates with non-preferred amino acids at position 2 (Mentlein and Gallwitz, 1993; Gopalan et al., 2010). The structure of incretin hormone such as GLP-1 and GIP reveals a highly conserved to alanine at position 2, rendering these peptides ideal putative substrates for the aminopeptidase DPP-4 (Gopalan et al., 2010).

DPP-4 inhibitor prevents the degradation of active GLP-1 however it does not increase the levels of circulating total GLP-1 and it does not prevent the kidney from rapidly clearing GLP-1. Using DPP-4 inhibitors, primarily for the treatment of diabetes, related to the potential effects of these inhibition on immune function. CD26/DPP-4 plays an important role in tumor biology; It is useful as a marker for various cancers, with its levels either on the cell surface or in the serum increased in some neoplasms and decreased in others (Havre et al., 2008). It is related to the

inhibition of T cell proliferation in immune system moreover it loses its ~~their~~ biological activity to protect against neoplasm (Chen, 2006; Havre et al., 2008).

GLP-1 has a potency for T2DM therapy but it is metabolized excessively by DPP-4 become inactive form (Drucker, 2002; Bailey, 2008). GLP-1 has a short half-life, approximately for 2-5 minutes and it is caused by DPP-4 activity (Drucker, 2002; Salehi et al., 2008). 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. It produces inactive form of GLP-1, they are GLP-1 (9-36) amide and GLP-1 (9-37) isopeptides (Bailey, 2008; Salehi et al., 2008). Previous studies showed that the importance of DPP-4 mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity (Stevens and Honerkamp-Smith, 2010).

GLP-1 is a super family peptide of glucagon which have a similarity degree (about 48 %). The similarity of amino acid sequence between GLP-1 and glucagon became one of this causa. Pro glucagon gen was located at chromosome 2q36-q37 and only found in some tissues whereas the messenger RNA (mRNA) of pro glucagon was met at α-cells pancreas, L-cells intestine and brain in hypothalamus part. Proglucagon production was started from transcription of preproglucagon gen and then was continued by translation process. The regulation of GLP-1 release from L-cells intestine are a complex mechanism that involve combinations of nutrition, hormone and neural stimuli (Morris and Lim-Wilby, 2008). GLP-1 receptor is classified in *G protein-coupled* receptor that is found at liver, muscle and pancreas cells. This receptor has a specific character by activation of adenilcyclase and result cAMP. After GLP-1 binding with the receptor, it will activate cAMP and Mitogen Activated Protein Kinase (MAPK).

Administration of *the U. lobata* ethanolic and water extracts have significantly decreased AUC of glucose of diabetic rats post glucose induction. It is controlled by the active compounds in *U. lobata* which has DPP-4 inhibitory activity that can retain the GLP-1 bioavailability ~~can be retained~~ to regulate blood glucose level increase after stimulating of oral nutrition (Drucker, 2002; Holst and Orskov, 2004). 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 (Drucker, 2002; Chia and Egan, 2008). Furthermore, it is beneficial to prevent the increase of blood glucose level post prandial (Holst and Orskov, 2004; Salehi et al., 2008)

The *U.lobata* water extract regulate more blood glucose level and post glucose stimulation compared to that of ethanolic extract ~~of *U.lobata*~~. It is predicted due to the poor solubility of active compounds and absorption in the ethanolic extract. As such, it also ~~therefore~~ affects the activity to control blood glucose level. The occurance of reducing solubility of active compounds is due to the formation of complex compounds and the conformation change of active compound, as well as the

~~furthermore~~ decrease inhibitory activity on DPP-4 that contribute to maintain blood glucose level on diabetic groups (Aronoff et al., 2004; Stevens and Honerkamp-Smith, 2010).

DPP-4 inhibitory activity of the *U. lobata* ethanolic extract ~~from *U.lobata*~~ was stronger than that of water extract ~~by~~ *in vitro* test but vise versa results was found ~~eventhough the opposite occurs~~ in *in-vivo* study. In addition, the *U. lobata* water extract ~~of *U. lobata*~~ demonstrated stronger decrease DPP-4 level and AUC of blood glucose and retain GLP-1 bioavailability compared to the ethanolic extract.

Acknowledgement

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Ethical Clearance

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

Conflict of Interest

The authors have no conflict of interest to declare

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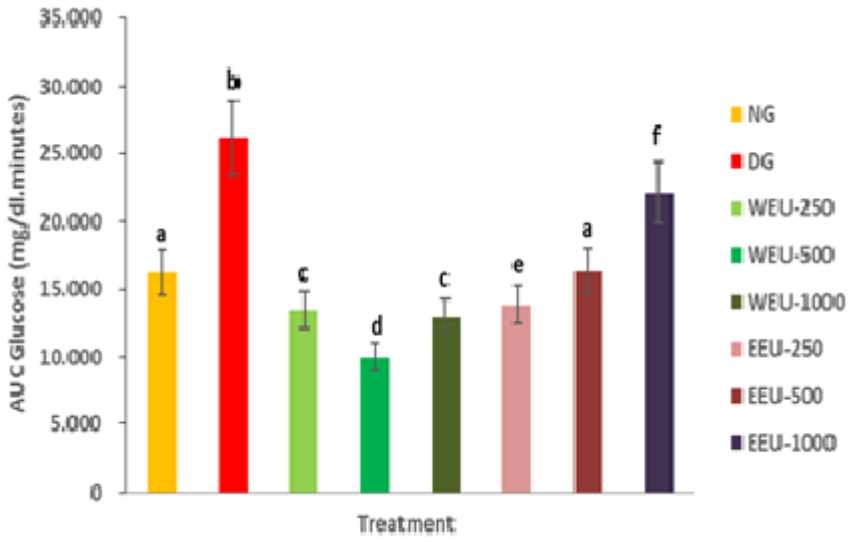
FIGURES LEGEND

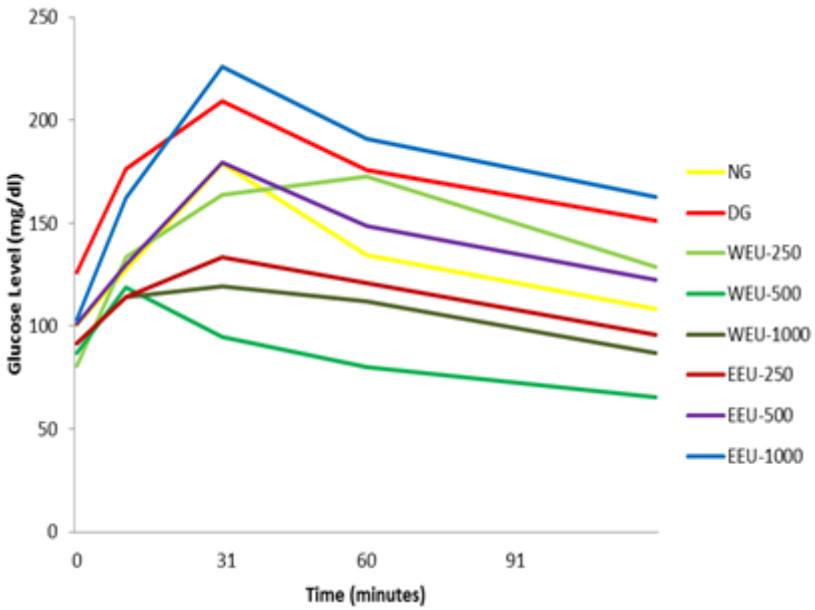
Figure 1. DPP-4 level administrated *U.lobata* leaf extract; a, b, c etc. showed the differences of potency ($p<0.05$, Dunnet C test)

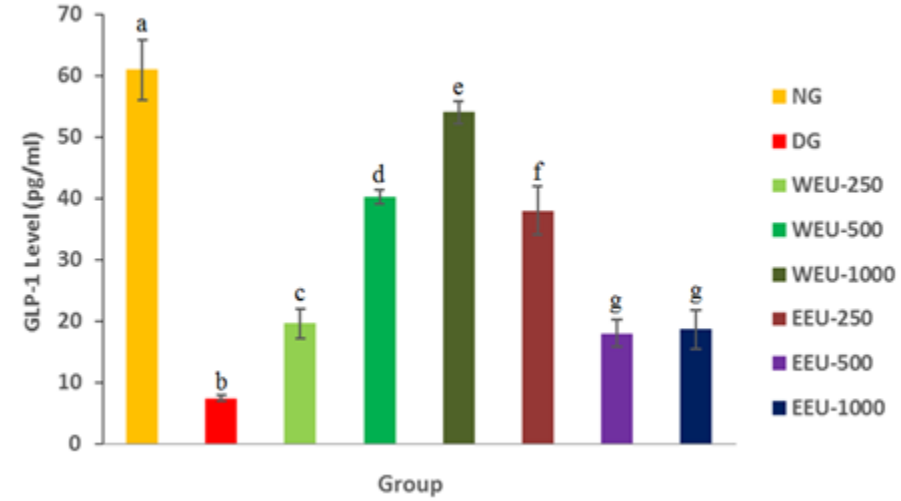
Figure 2. GLP-1 level administrated *U.lobata* leaf extract; a, b, c etc. showed the differences of potency ($p<0.05$, Dunnet C test)

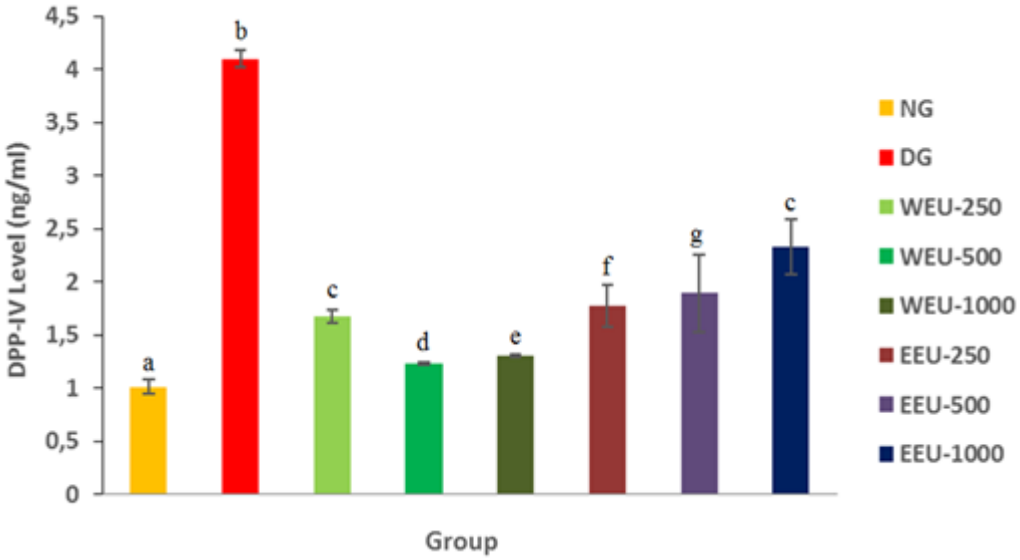
Figure 3. Blood glucose level administrated *U.lobata* extract after induction of glucose.

Figure 4. AUC of glucose administrated *U.lobata* extract; a, b, c etc. showed the differences of potency ($p<0.05$, LSD test)









FIGURES LEGEND

Figure 1. DPP-4 level administrated *U.lobata*leaf extract; a, b, c etc. showed the differences of potency ($p<0.05$, Dunnet C test)

Figure 2.GLP-1 level administrated *U.lobata*leaf extract;a, b, c etc. showed the differences of potency ($p<0.05$, Dunnet C test)

Figure 3. Blood glucose level administrated *U.lobata* extract after induction of glucose.

Figure 4.AUC of glucose administrated *U.lobata* extract;a, b, c etc. showed the differences of potency ($p<0.05$, LSD test)

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Inhibitory activity of *Urena lobata* leaf extract on dipeptidyl peptidase-4 (DPP-4): is it different *in vitro* and *in vivo*?

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Abstract

This study sought to assess the anti-diabetic effect of *Urena lobata* leaf extract through DPP-4 inhibitory activity both *in vitro* and *in vivo*. *Urena lobata* leaf was extracted in an ethanol solvent and hot water. *In vitro* testing using *gly-pro-p-nitroanilide* (GPPN) as a substrate of DPP-4 and vildagliptin as a standard reference was carried out. A product of the reactions between GPPN and DPP-4, *p*-nitroanilide was observed by microplate reader at λ=405 nm while the IC_{50} value was determined by non-linear regression curve. The *in vivo* investigation utilized an animal model of T2DM with two control groups and six test groups (n=4), where DPP-4 levels, GLP-1 levels and the AUC of blood glucose were determined after extract administration. All data were analyzed with one-way ANOVA and then continued with LSD or Dunnet c ($p<0.05$). The *in vitro* DPP-4 inhibitory activity of ethanolic extract from *U. lobata* was stronger than water extract. However, the results vice versa were observed during the *in vivo* study. In addition, the *U. lobata* water extract exhibited a stronger decrease in DPP-4 levels and the AUC of blood glucose as well as retaining GLP-1 bioavailability compared to ethanolic extract.

Keywords: DPP-4, *in vitro*, *in vivo*, *Urena lobata*, *gly-pro-p-nitroanilide (GPPN* .

Introduction

Recently, treatment of type II diabetes mellitus (T2DM) has been proposed with incretin hormone. The majority of incretin hormone is glucagon-like peptide-1 (GLP-1) secreted by intestinal tissues based on the induction of oral nutrition (Drucker, 2007). GLP-1 plays a vital role in maintaining blood glucose levels related to their biology activity, such as to stimulating insulin secretion, increasing β-cell proliferation, inhibiting glucagon secretion, reducing the rate of gastric emptying, and promoting satiety (Drucker, 2007; Chia and Egan, 2008). In T2DM patients, GLP-1 bioavailability decreases the secretion of insulin and the clinical effect is chronic hyperglycemia (Drucker, 2002).

GLP-1 is sufficiently potent to cure T2DM, however, GLP-1 is metabolized by dipeptidyl peptidase-4 (DPP-4) excessively and becomes inactive (Drucker, 2002; Chia and Egan, 2008). Inhibition of DPP-4 is effective in treating T2DM, therefore GLP-1 bioavailability can be retained in addition to regulating blood glucose levels (Brunton et al., 2006). Treatment of T2DM with *incretin-like* drugs or DPP-4 inhibitors shows lesser side effects even though the safety of this drug has not been evaluated with respect to a complete data set (Holst and Orskov, 2004). Nevertheless, incretin-like drugs have side effects, such as flu-like symptoms, skin reactions, and gastrointestinal problems. These effects are exacerbated by the use of the drugs over the long-term (Bailey, 2008; Salehi et al., 2008). As such, these phenomena have attracted attention to finding medicinal plants as alternatives to treat T2DM by DPP-4 inhibition.

One of the traditional plants known to have anti-diabetic effects is *Urena lobata*. Root and leaf extract of *U. lobata* have been used empirically by Nigerian people to treat DM. Preclinical testing of *U. lobata* root extract indicated there were anti-hyperglycemic effects on streptozotocin-induced rats (Onoagbe et al., 2010). In Indonesia, *U. lobata* is known by Pulutan, and the plant exhibited anti-bacterial effects based on a preliminary study (Nurfauziah and Mulyani, 1999). Further, a number of studies have shown the anti-diabetic potency of *U. lobata*, however the mechanism of the inhibition of DPP-4 activity has not been investigated as of yet (Nurfauziah and Mulyani, 1999; Vats et al., 2002; Awika and Rooney, 2004; Onoagbe et al., 2010). Therefore, the current study sought to examine the anti-diabetic effects of *U. lobata* leaf extract on DPP-4 inhibition.

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. The *U.lobata* leaf powder (50 g) was extracted according to the decoction method in which the leaf powder was soaked in 250 mL hot water (90ºC) for 30 minutes. The other sample of *U.lobata* leaf powder (50 g) was extracted based on the digestion method by soaking the sample in 250 mL ethanol for five hours using a shaker water bath. The extraction process was repeated twice with fresh solvent. Both of the extract solutions had the solvent removed using a rotary evaporator to obtain crude extracts.

DPP-4 in vitro assay

The assay was performed in 96-well micro plates. A pre-incubation volume of 50 µL solution contained 35 µL Tris-HCl buffer, 15 µL DPP-4 enzyme, and various concentrations of test material or standards. This mixture was incubated at 37 ºC for 10 minutes followed by addition of 50 µL gly-pro-p-nitroanilide as the substrate. The reaction mixture was incubated for 30 minutes at 37 ºC and the absorbance was measured at 405 nm at 10-second intervals. Vildagliptin was used as the standard reference of the DPP-4 inhibitor.

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 approved by the Commision of Ethical Research, Brawijaya University, Malang, Indonesia, with certificate number 245-KEP-UB. SD rats were housed in individual cages and automatically controlled animal rooms at 25 ± 1º C on a 12:12-h light–dark cycle. They were fed with standard food and water *ad libitum* and fasted overnight before the experiments. Normal diet (ND) and a high-fructose diet (HFD) foods 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 intraperitoneally [refer to Guo *et al.* ( ) with minor modification]. Rats were stated to be diabetic if the fasting blood glucose level was more than 126 mg/dL (Awika and Rooney, 2004). The experiments were assigned to eight groups of 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 the ethanolic extract (EEU) and water extract of *U.lobata* (WEU) at dosages 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.* (2006). Body weight and food consumption were monitored weekly. Blood samples were obtained 15 minutes after being given the glucose stimulation in dosages of 2 g/kg body weight orally. The blood

samples were also taken from the tail vein after overnight fasting. Blood samples were immediately centrifuged at 4500 rpm. The serum was separated and saved under temperature -20 ºC.

DPP-4 in vivo assay

DPP-4 serum levels were analyzed by rat DPP-4 ELISA kit (Elabscience E-EL-R0337). 100 µL samples were incubated for 90 minutes at 37 ºC, and 100 µL of biotinylated detection Ab ( ) was added and the mixture was then incubated for 60 minutes at 37 ºC. After aspirating and washing, 100 µL HRP conjugate was added and the mixture was incubated for 30 minutes at 37 ºC. A 90 µL substrate reagent ( ) was then added to 50 µL *stop solution*. The data of samples were measured with a microplate reader at λ = 450 nm.

GLP-1 in vivo assay

GLP-1 serum levels were determined by rat GLP-1 ELISA kit (USCN CEA804). 50 µL samples were added with 50 µL of detection reagent A and then incubated for 60 minutes at 37 ºC. After aspirating and washing, 100 µL of detection reagent B was added to the samples that were next incubated for 30 minutes at 37 ºC. A 90 µL substrate reagent was then added to 50 µL *stop solution*. The data of samples were obtained using a microplate reader at λ = 450 nm.

Oral glucose tolerance test

For the glucose tolerance test, glucose was administered orally at a dose of 2 g/kg body weight after overnight fasting. The blood samples were collected from the tail vein at 0 (before glucose administered), 15, 30, 60, 90, and 120 min after glucose administration and they were measured immediately using a commercial glucometer ( ). The integrated area under the postprandial glucose curves (AUCs) was calculated by the trapezoidal method.

Statistical analysis

The data were expressed as means ± S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA). The least significant difference (LSD) test or Dunnet C were employed for mean comparisons and $P < 0.05$ was considered to be statistically significant.

Results

DPP-4 inhibitory activity of U. lobata via in vitro testing

The DPP-4 inhibitory activity of *U.lobata* leaf extract is presented in Table 1. Based on the results, the *U.lobata* ethanolic extract had greater inhibitory activity on DPP-4 (roughly four-fold) compared to that of water extract ($P < 0.05$).

Table 1. DPP-4 inhibitory activity of *U.lobata* leaf extract

Group	Sample	Concentration (ppm)	% inhibition	IC_{50} (ppm)
1	Water extract of *U.lobata*	625	0.00 ± 0.00	6489.88[a]
		1250	13.33 ± 0.00	
		2500	26.67 ± 0.00	
		5000	42.22 ± 3.85	
		10000	62.22 ± 3.85	
2	Ethanolic extract of *U.lobata*	625	36.17 ± 0.00	1654.64[b]
		1250	48.94 ± 0.00	
		2500	55.32 ± 0.00	
		5000	61.70 ± 0.00	
		10000	74.47 ± 0.00	
3	Vildagliptin	6.25	8.93 ± 0.00	57.44[c]
		12.50	16.07 ± 4.12	
		25.00	37.50 ± 0.00	
		50.00	46.63 ± 3.85	
		100.00	60.71 ± 0.00	

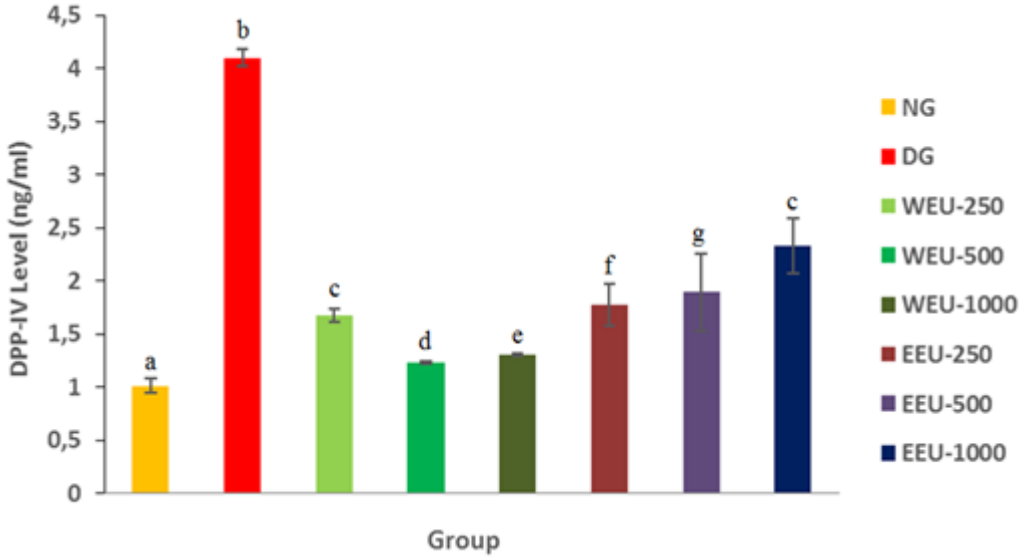
a,b,c = different letter showed the differences in potency ($P < 0.05$, LSD test)

The experiments were repeated in triplicate.

The results of % inhibition were expressed as means ± S.E.M.

DPP-4 inhibitory activity of U.lobata via in vivo testing

DPP-4 serum levels of diabetic rats with administered *U.lobata* leaf extract are shown in Figure 1. The diabetic group exhibited a significant increase in DPP-4 levels, which is approximately four-fold higher than the normal group ($P < 0.05$). Administration of *U.lobata* water extract at 250 mg/kg, 500 mg/kg, and 1000 mg/kg BW decreased DPP-4 by roughly 60%, 70%, and 70%, respectively, compared to the diabetic group ($P < 0.05$), whereas that of the ethanolic extract diminished DPP-4 levels by 60%, 50%, and 40%, respectively ($P < 0.05$). The results demonstrated that the water extract was able to decrease DPP-4 levels more so compared to the ethanolic extract ($P < 0.05$) at the same dosages.

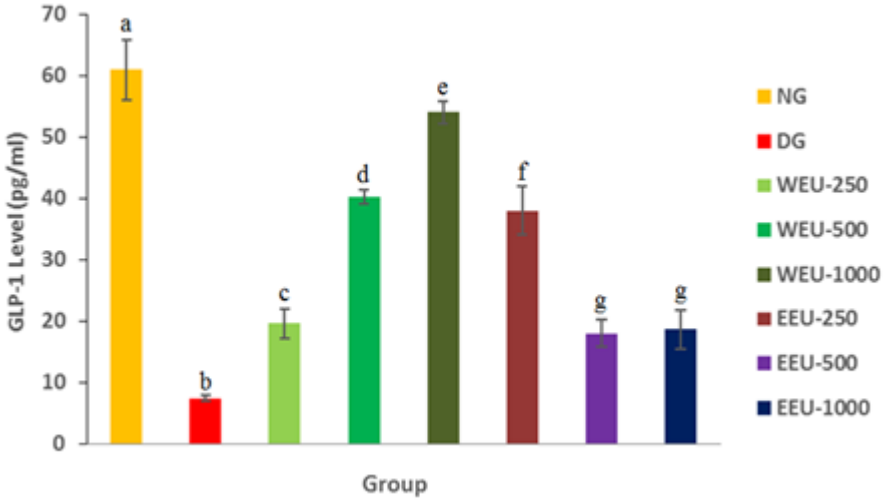


Note: a, b, c, etc. indicates the differences in potency ($P < 0.05$, Dunnet C test)

Figure 1. DPP-4 levels with administered *U.lobata* leaf extract.

Effect of U.lobata leaf extract on GLP-1 serum levels

GLP-1 serum levels of diabetic rats that had been administered *U.lobata* leaf extract are portrayed in Figure 2. There is a significant reduction in GLP-1 within the DG by nearly eight-fold compared to the NG ($P < 0.05$). The water extract of *U.lobata* at dosages of 250 mg/kg, 500 mg/kg, and 1000 mg/kg bw prevented degradation of GLP-1 by roughly three-fold, five-fold, and seven-fold, respectively, compared to that of the DG ($P < 0.05$), of which the ethanolic extract showed approximately five-fold, two-fold, and 2.5-fold ($P < 0.05$), respectively. For the same dosage, the water extract was able to inhibit more degradation of GLP-1 compared to the ethanolic extract ($P < 0.05$) excepting at a dose of 250 mg/kg BW.



Note: a, b, c, etc. showed the differences of potency ($P < 0.05$, Dunnet C test)

Figure 2. GLP-1 levels with administered *U.lobata* leaf extract.

Both administration of water and ethanolic extracts significantly maintained GLP-1 bioavailability in diabetic rats. Mangiferin, stigmasterol, and β-sitosterol in the extract were able to prevent degradation of GLP-1 by DPP-4. Based on our previous work, active compounds in *U.lobata* extract act as DPP-4 inhibitors. With this, the water extract ensured more GLP-1 bioavailability compared to the ethanolic extract, and this is because of the difference in active compounds that inhibit GLP-1 degradation by DPP-4. It is also related to the DPP-4 inhibitory activity of active compounds in both water and ethanolic extracts. The effect is regulated by DPP-4 inhibitory activity owing to a synergistic interaction of active compounds and production of complex compounds in the water extract (Brunton et al., 2006). As the result, inhibition of DPP-4 prevents GLP-1 metabolism from inactivation and resulted in the retention of their bioavailability.

The effect of U. lobata on glucose tolerance testing in diabetic rats

Blood glucose levels of rat administered *U.lobata* extract after stimulating glucose are depicted in Figures 3 and 4. The results indicated that there is a significant increase of the AUC of glucose in the DG by up to 70% compared to the NG ($P < 0.05$). The administration of *U.lobata* water extract at dosages of 250 mg/kg BW, 500 mg/kg, and 1000 mg/kg BW decreased the AUC of glucose by 50%, 60%, and 50%, respectively, compared to that of the DG ($P < 0.05$) whereas the ethanolic extract diminished the AUC of glucose by 50%, 40%, and 20%, respectively ($P < 0.05$). The *U.lobata* water extract exhibited stronger control over the AUC of blood glucose compared to that of ethanolic extract ($P < 0.05$) after glucose stimulation.

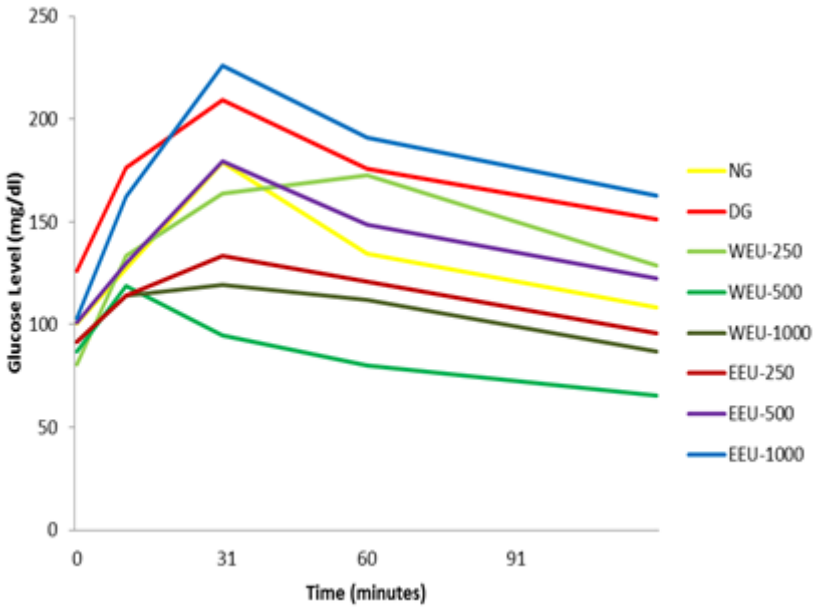
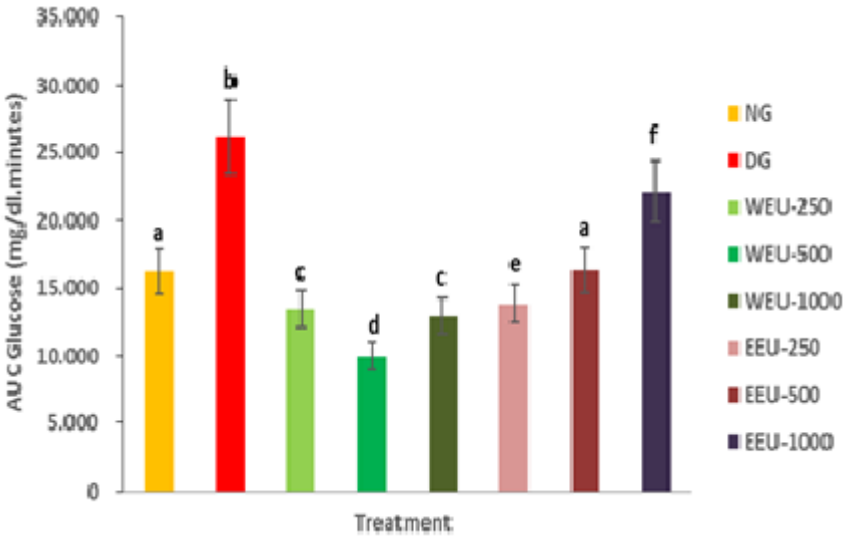


Figure 3. Blood glucose level administered *U. lobata* extract after induction of glucose.

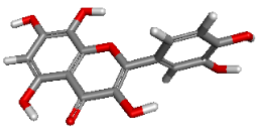


Note: a, b, c, etc. indicates the differences in potency ($P < 0.05$, LSD test)

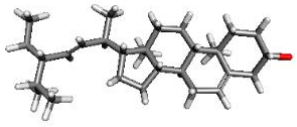
Figure 4. AUC of glucose with administered *U.lobata* extract.

Discussion

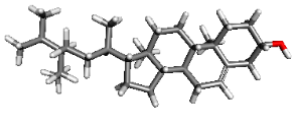
The semi-qualitative testing of *U.lobata* leaf extract by LC-MS showed the compositions of gossypetin (**1**) in the ethanolic extract are higher than that of in the water extract whereas stigmasterol is more predominant in the water extract (Purnomo et al., 2015). Active compounds, such as stigmasterol (**2**), β-sitosterol (**3**), and gossypetin (**1**) are soluble in less polar solvents, like alcohol, even though mangiferin (**4**) and hypoxemia are insoluble. The difference in solubilities of the active compounds in the solvents affects the number of active compounds extracted from the extracts. However, the DPP-4 inhibitory activity of both the water and ethanolic extracts are still lower, approximately 30-100-fold, compared to vildagliptin, the standard reference DPP-4 inhibitor ($P < 0.05$).

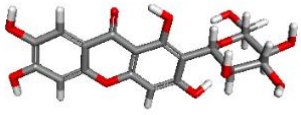


Gossypetin (**1**)



Stigmasterol (**2**)





B-Sitosterol (**3**) Mangiferin (**4**)

Figure 5. Chemical structures of certain molecules found in *U.lobata* leaf extract.

Both of the *U.lobata* water and ethanolic extracts significantly inhibit the DPP-4 activity of diabetic rats. These effects are regulated by active compounds **2**, **3**, and **4** from *U.lobata* based on *in-silico* analysis (Purnomo et al., 2015). The increase dosages of *U.lobata* water extract inhibit more DPP-4 activity whereas the ethanolic extract raises DPP-4 activity precisely. This is based on the conformational shift of active compounds, particularly the sterol group in the ethanolic extract which produces a complex of compounds post-binding (Morris and Lim-Wilby, 2008; Setevens and Honerkamp-Smith, 2010). The change in active compound structure can alter the solubility of active substances and their absorption and resultant modulation of inhibitory activity on DPP-4 (Stevens and Honerkamp-Smith, 2010). Owing to the ethanolic extract being less polar than the water extract, it affects solubilities of active compounds and their absorption within the gastrointestinal system in addition to bioactivity decreasing. The water extract inhibited more DPP-4 activity than that of the ethanolic extract. Complex formed of active substances from the *U. lobata* water extract facilitate the inhibitory activity of DPP-4. A number of active compounds within the water extract have synergistic interactions besides the reinforcement of their inhibitory activity on DPP-4 (Brunton et al., 2006). Inhibition of DPP-4 activity increases the bioavailability of the incretin hormone, thereby contributing to carbohydrate metabolism (Drucker, 2002).

The differences in DPP-4 inhibitory activity between *in vitro* and *in vivo* testing can be explained pharmacokinetically. The poor solubility of active compounds within the ethanolic extract may affect its absorption and distribution in the blood as a result of decreasing the inhibition of DPP-4 activity.

The principal biological activity of DPP-4/CD26 is its enzymatic function. The enzymatic activity of DPP-4/CD26 is exhibited by the membrane-spanning form of the molecule, and the slightly smaller circulating soluble form (Chen, 2006). The substrates of CD26/DPP-4 are not specific to certain peptides. Proline- or alanine-containing peptides are the substrates of DPP-4 and include growth factors, chemokines, neuropeptides, and vasoactive peptides (Mentlein and Gallwitz, 1993). DPP-4 prefers substrates with an amino-terminal proline or alanine at position 2 even though they may also cleave substrates with non-preferred amino acids at position 2 (Mentlein and Gallwitz, 1993; Gopalan et al., 2010). The structure of

incretin hormones, such as GLP-1 and GIP, reveals a highly conserved alanine in position 2, rendering these peptides ideal putative substrates for the aminopeptidase, DPP-4 (Gopalan et al., 2010).

The DPP-4 inhibitor prevents the degradation of active GLP-1; however, it does not elevate the levels of circulating total GLP-1 and, further, does not prevent the kidney from rapidly clearing GLP-1. Using DPP-4 inhibitors, primarily for the treatment of diabetes, is related to the potential effects of them on immune function. CD26/DPP-4 plays an important role in tumor biology; It is valuable as a marker for various cancers with its levels either on the cell surface or in the serum increased in certain neoplasms and reduced in others (Havre et al., 2008). It is related to the inhibition of T cell proliferation in the immune system and, moreover, it loses its biological activity in protecting against neoplasms (Chen, 2006; Havre et al., 2008).

GLP-1 has a potency for T2DM therapy but it is metabolized excessively by DPP-4 and becomes the inactive form (Drucker, 2002; Bailey, 2008). GLP-1 has a short half-life, approximately for two to five minutes, and this is based on DPP-4 activity (Drucker, 2002; Salehi et al., 2008). The active forms of GLP-1 are GLP-1 (7-36) amides and GLP-1 (7-37), which are rapidly inactivated by DPP-4 through cleavage of the N-terminal dipeptide, His-Ala. This results in the production of the inactive forms of GLP-1, namely GLP-1 (9-36) amide and GLP-1 (9-37) isopeptides (Bailey, 2008; Salehi et al., 2008). Previous studies have shown that the importance of DPP-4-mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity (Stevens and Honerkamp-Smith, 2010).

GLP-1 is a super family peptide of glucagon which has a similarity degree of approximately 48%. The similarity of the amino acid sequences between GLP-1 and glucagon is one of the reasons. Pro-glucagon gen was located at chromosome 2q36-q37 and only found in certain tissues whereas the messenger RNA (mRNA) of pro-glucagon was located in the α-cells of the pancreas as well as the L-cells of the intestine and brain's hypothalamus. Pro-glucagon production commences from transcription of preproglucagon gen and is then continued by the translation process. The regulation of GLP-1 release from L-cells of the intestine is a complex mechanism that involves combinations of nutrition, hormones, and neural stimuli (Morris and Lim-Wilby, 2008). The GLP-1 receptor is classified as a *G protein-coupled* receptor that is found in liver, muscle and pancreas cells. This receptor has a specific character by activation of adenyl cyclase and resultant cAMP. After GLP-1 binding with the receptor, it will activate cAMP and mitogen-activated protein kinase (MAPK).

Administration of *the U. lobata* ethanolic and water extracts significantly decreased the AUC of glucose in diabetic rats post-glucose induction. It is controlled by the active compounds in *U. lobata* which inhibit DPP-4 activity, leading to retention of the GLP-1 bioavailability to regulate blood glucose level increases after stimulation through oral nutrition (Drucker, 2002; Holst and Orskov, 2004). GLP-1 acts beyond metabolic purposes, specifically inhibiting gastric juice secretion, GIT motility, and the rate of gastric-emptying functioning (Drucker, 2002; Chia and Egan, 2008). Furthermore, it is beneficial to prevent increasing blood glucose levels post-prandial (Holst and Orskov, 2004; Salehi et al., 2008)

The *U.lobata* water extract regulated more blood glucose levels and post-glucose stimulation compared to the ethanolic extract. This was predicted based on the poor solubility of the active compounds and absorption of the ethanolic extract. As such, it also affects the activities constituting controlling blood glucose levels. The occurrence of reducing the solubility of active compounds is based on the formation of complex compounds and the conformational changes in active compounds as well as the decrease in inhibitory activity of DPP-4 that contributes to maintaining blood glucose levels in the DG (Aronoff et al., 2004; Stevens and Honerkamp-Smith, 2010).

DPP-4 inhibitory activity by the *U. lobata* ethanolic extract was stronger than that of the water extract during *in-vitro* testing but the opposite was found with the *in-vivo* study. In addition, the *U.lobata* water extract demonstrated a more pronounced decrease of DPP-4 levels and the AUC of blood glucose as well as retention of GLP-1 bioavailability compared to the ethanolic extract.

Acknowledgement

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Ethical Clearance

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

Conflict of Interest

The authors have no conflict of interest to declare

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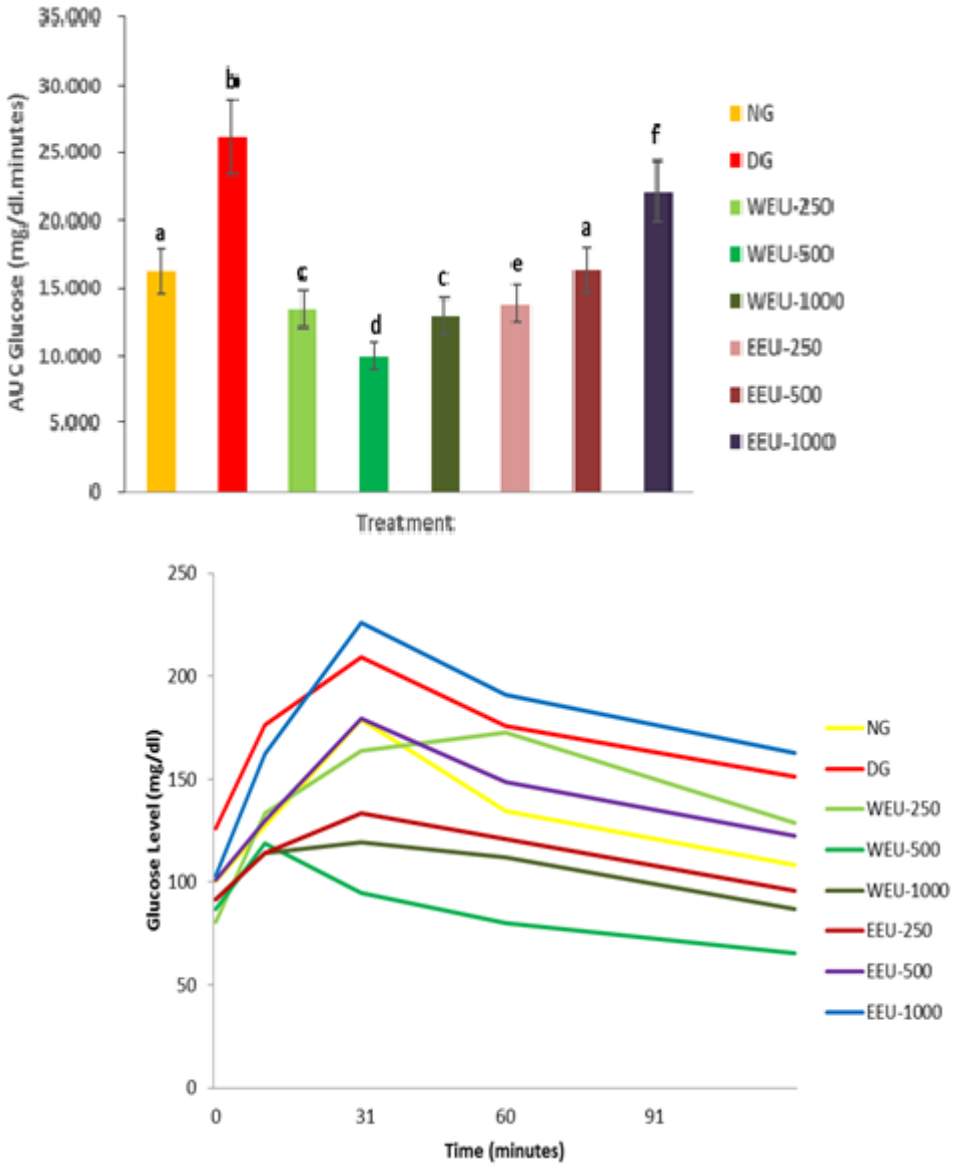
FIGURES LEGEND

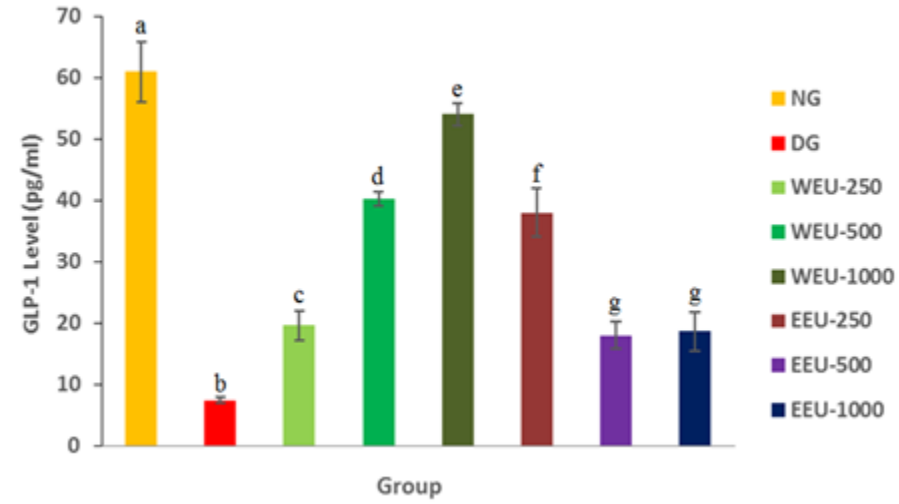
Figure 1. DPP-4 level administrated *U.lobata* leaf extract; a, b, c etc. showed the differences of potency ($p<0.05$, Dunnet C test)

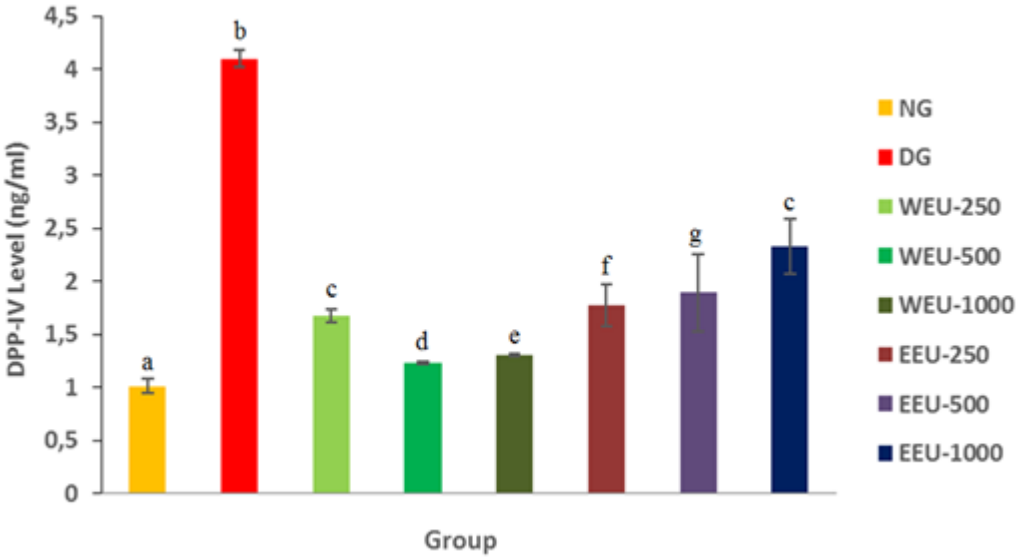
Figure 2. GLP-1 level administrated *U.lobata* leaf extract; a, b, c etc. showed the differences of potency ($p<0.05$, Dunnet C test)

Figure 3. Blood glucose level administrated *U.lobata* extract after induction of glucose.

Figure 4. AUC of glucose administrated *U.lobata* extract; a, b, c etc. showed the differences in potency ($p<0.05$, LSD test)







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Inhibitory activity of *Urena lobata* leaf extract on dipeptidyl peptidase-4 (DPP-4): is it different *in vitro* and *in vivo*?

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Abstract

This study was aimed to compare the anti diabetic effect of *Urena lobata* leaf extract through DPP-4 inhibitory activity by *in vitro* and *in vivo*. *Urena lobata* leaf was extracted in ethanol and hot water to evaluate its activity on DPP-4 both of *in vitro* and *in vivo*. *In vitro* test using *Gly-pro-p-nitroanilide* (GPPN) as substrate of DPP-4 and the reactions product of them was observed by microplate reader at λ=405 nm furthermore the IC_{50} value was determined. *In vivo* study utilize an animal model of diabetes with 2 control groups and 6 test groups (n=4), in which DPP-4 level, GLP-1 level and AUC of blood glucose were examined after extract administration. The *in vitro* DPP-4 inhibitory activity of ethanolic extract from *U. lobata* is stronger than water extract with the IC_{50} value of 1654,64 and 6489,88 µg/ml respectively. However, the water extract of *U. lobata* exhibits stronger decrease DPP-4 level (60-70%) compared to ethanolic extract (40-60%) *in vivo* study as well as the AUC of blood glucose were reduced by 50-60 % and 20-50 % respectively. Meanwhile, GLP-1 level could be retained more by the water extract of *U. lobata* administration (3-7 fold) compared to ethanolic extract (2-5 fold) due to the reducing of DPP-4 activity.

Keywords: DPP-4, *in vitro*, *in vivo*, *Urena lobata*, *Gly-pro-p-nitroanilide (GPPN)*.

Introduction

Recently, treatment of type II diabetes mellitus (T2DM) has been proposed with incretin hormone. The majority of incretin hormone is glucagon-like peptide-1 (GLP-1) secreted by intestinal tissues based on the induction of oral nutrition (Drucker, 2007). GLP-1 plays a vital role in maintaining blood glucose levels related to their biology activity, such as to stimulating insulin secretion, increasing β-cell proliferation, inhibiting glucagon secretion, reducing the rate of gastric emptying, and promoting satiety (Drucker, 2007; Chia and Egan, 2008). In T2DM patients, GLP-1 bioavailability decreases the secretion of insulin and the clinical effect is chronic hyperglycemia (Drucker, 2002).

GLP-1 is sufficiently potent to cure T2DM, however, GLP-1 is metabolized by dipeptidyl peptidase-4 (DPP-4) excessively and becomes inactive (Drucker, 2002; Chia and Egan, 2008). Inhibition of DPP-4 is effective in treating T2DM, therefore GLP-1 bioavailability can be retained in addition to regulating blood glucose levels (Brunton *et al.*, 2006). Treatment of T2DM with *incretin-like* drugs or DPP-4 inhibitors shows lesser side effects even though the safety of this drug has not been evaluated with respect to a complete data set (Holst and Orskov, 2004). Nevertheless, incretin-like drugs have side effects, such as flu-like symptoms, skin reactions, and gastrointestinal problems. These effects are exacerbated by the use of the drugs over the long-term (Nauck *et al.*, 2009; Salehi *et al.*, 2008). As such, these phenomena have attracted attention to finding medicinal plants as alternatives to treat T2DM by DPP-4 inhibition.

One of the traditional plants known to have anti-diabetic effects is *Urena lobata*. Root and leaf extract of *U. lobata* have been used empirically by Nigerian people to treat DM. Preclinical testing of *U. lobata* root extract indicated there were anti-hyperglycemic effects on streptozotocin-induced rats (Onoagbe *et al.*, 2010). In Indonesia, *U. lobata* is known by Pulutan, and the plant exhibited anti-bacterial effects based on a preliminary study (Nurfauziah and Mulyani, 1999). Further, a number of studies have shown the anti-diabetic potency of *U. lobata*, however the mechanism of the inhibition of DPP-4 activity has not been investigated as of yet (Nurfauziah and Mulyani, 1999; Vats *et al.*, 2002; Awika and Rooney, 2004; Onoagbe *et al.*, 2010). Therefore, the current study sought to examine the anti-diabetic effects of *U. lobata* leaf extract on DPP-4 inhibition.

Material and methods

Preparation of U. lobata leaf extract

Urena lobata leaf powder was obtained from Balai Materia Medika Batu Malang with certificate number 074/027/101.8/2015. The *U. lobata* leaf powder (50 g) was extracted according to the decoction method in which the leaf powder was soaked in 250 mL hot water (90ºC) for 30 minutes. The other sample

of *U. lobata* leaf powder (50 g) was extracted based on the digestion method by soaking the sample in 250 mL ethanol for five hours using a shaker water bath. The extraction process was repeated twice with fresh solvent. Both of the extract solutions had the solvent removed using a rotary evaporator to obtain crude extracts.

DPP-4 in vitro assay

The assay was performed in 96-well micro plates. A pre-incubation volume of 50 µL solution contained 35 µL Tris-HCl buffer, 15 µL DPP-4 enzyme, and various concentrations of test material or standards. This mixture was incubated at 37 ºC for 10 minutes followed by addition of 50 µL gly-pro-p-nitroanilide as the substrate. The reaction mixture was incubated for 30 minutes at 37 ºC and the absorbance was measured at 405 nm at 10-second intervals. Vildagliptin was used as the standard reference of the DPP-4 inhibitor.

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 approved by the Commision of Ethical Research, Brawijaya University, Malang, Indonesia, with certificate number 245-KEP-UB. SD rats were housed in individual cages and automatically controlled animal rooms at 25 ± 1º C on a 12:12-h light–dark cycle. They were fed with standard food and water *ad libitum* and fasted overnight before the experiments. Normal diet (ND) and a high-fructose diet (HFD) foods 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 intraperitoneally. Rats were stated to be diabetic if the fasting blood glucose level was more than 126 mg/dL (Awika and Rooney, 2004). The experiments were assigned to eight groups of 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 the ethanolic extract (EEU) and water extract of *U. lobata* (WEU) at dosages 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*. (2006). Body weight and food consumption were monitored weekly. Blood samples were obtained 15 minutes after being given the glucose stimulation in dosages of 2 g/kg body weight orally. The blood samples were also taken from the tail vein after overnight fasting. Blood samples were immediately centrifuged at 4500 rpm. The serum was separated and saved under temperature -20 ºC.

DPP-4 in vivo assay

DPP-4 serum levels were analyzed by rat DPP-4 ELISA kit (Elabscience E-EL-R0337). 100 µL samples were incubated for 90 minutes at 37 ºC, and 100 µL of biotinylated detection Ab ( ) was added and the mixture was then incubated for 60 minutes at 37 ºC. After aspirating and washing, 100 µL HRP

conjugate was added and the mixture was incubated for 30 minutes at 37 ºC. A 90 µL substrate reagent ( ) was then added to 50 µL *stop solution*. The data of samples were measured with a microplate reader at λ = 450 nm.

GLP-1 in vivo assay

GLP-1 serum levels were determined by rat GLP-1 ELISA kit (USCN CEA804). 50 µL samples were added with 50 µL of detection reagent A and then incubated for 60 minutes at 37 ºC. After aspirating and washing, 100 µL of detection reagent B was added to the samples that were next incubated for 30 minutes at 37 ºC. A 90 µL substrate reagent was then added to 50 µL *stop solution*. The data of samples were obtained using a microplate reader at λ = 450 nm.

Oral glucose tolerance test

For the glucose tolerance test, glucose was administered orally at a dose of 2 g/kg body weight after overnight fasting. The blood samples were collected from the tail vein at 0 (before glucose administered), 15, 30, 60, 90, and 120 min after glucose administration and they were measured immediately using a commercial glucometer ( ). The integrated area under the postprandial glucose curves (AUCs) was calculated by the trapezoidal method.

Statistical analysis

The data were expressed as means ± S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA). The least significant difference (LSD) test or Dunnet C were employed for mean comparisons and $P < 0.05$ was considered to be statistically significant.

Results

The DPP-4 inhibitory activity of U. lobata via in vitro testing

The DPP-4 inhibitory activity of *U. lobata* leaf extract is presented in Table 1. Based on the results, the *U. lobata* ethanolic extract had greater inhibitory activity on DPP-4 (roughly four-fold) compared to that of water extract ($P < 0.05$).

Table 1. The DPP-4 inhibitory activity of *U. lobata* leaf extract

Group	Sample	Concentration (ppm)	% inhibition	IC_{50} (ppm)
1	Water extract of *U.lobata*	625	0.00 ± 0.00	6489.88
		1250	13.33 ± 0.00	
		2500	26.67 ± 0.00	
		5000	42.22 ± 3.85	
		10000	62.22 ± 3.85	

2	Ethanolic extract of *U.lobata*	625	36.17 ± 0.00	1654.64
		1250	48.94 ± 0.00	
		2500	55.32 ± 0.00	
		5000	61.70 ± 0.00	
		10000	74.47 ± 0.00	
3	Vildagliptin	6.25	8.93 ± 0.00	57.44
		12.50	16.07 ± 4.12	
		25.00	37.50 ± 0.00	
		50.00	46.63 ± 3.85	
		100.00	60.71 ± 0.00	

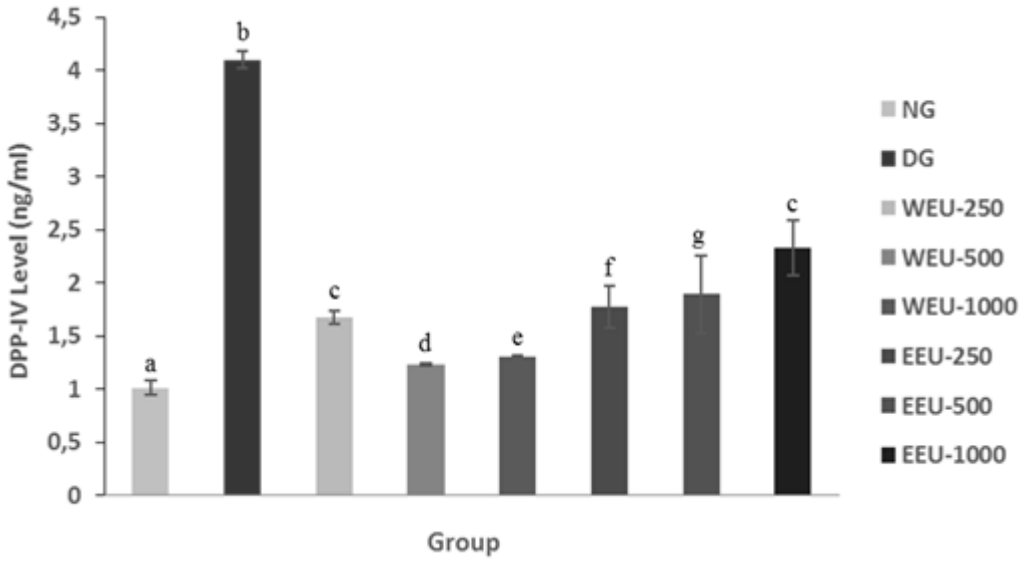
a,b,c = different letter showed the differences in potency ($P < 0.05$, LSD test)

The experiments were repeated in triplicate.

The results of % inhibition were expressed as means ± S.E.M.

The DPP-4 inhibitory activity of U. lobata via in vivo testing

DPP-4 serum levels of diabetic rats with administered *U. lobata* leaf extract are shown in Figure 1. The diabetic group exhibited a significant increase in DPP-4 levels, which is approximately four-fold higher than the normal group ($P < 0.05$). Administration of *U. lobata* water extract at 250 mg/kg, 500 mg/kg, and 1000 mg/kg BW decreased DPP-4 by roughly 60%, 70%, and 70%, respectively, compared to the diabetic group ($P < 0.05$), whereas that of the ethanolic extract diminished DPP-4 levels by 60%, 50%, and 40%, respectively ($P < 0.05$). The results demonstrated that the water extract was able to decrease DPP-4 levels more so compared to the ethanolic extract ($P < 0.05$) at the same dosages.

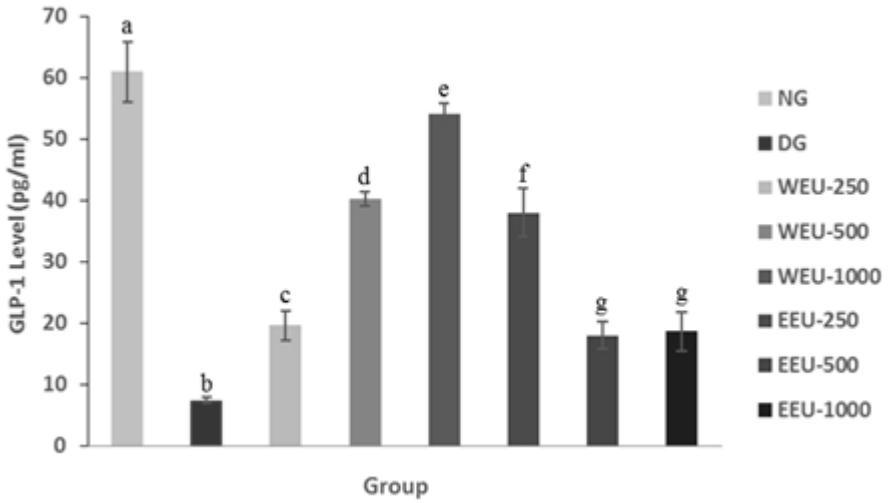


Note: a, b, c, etc. indicates the differences in potency ($P < 0.05$, Dunnet C test)

Figure 1. DPP-4 levels with administered *U. lobata* leaf extract.

Effect of U. lobata leaf extract on GLP-1 serum levels

GLP-1 serum levels of diabetic rats that had been administered *U. lobata* leaf extract are portrayed in Figure 2. There is a significant reduction in GLP-1 within the DG by nearly eight-fold compared to the NG ($P < 0.05$). The water extract of *U. lobata* at dosages of 250 mg/kg, 500 mg/kg, and 1000 mg/kg bw prevented degradation of GLP-1 by roughly three-fold, five-fold, and seven-fold, respectively, compared to that of the DG ($P < 0.05$), of which the ethanolic extract showed approximately five-fold, two-fold, and 2.5-fold ($P < 0.05$), respectively. For the same dosage, the water extract was able to inhibit more degradation of GLP-1 compared to the ethanolic extract ($P < 0.05$) excepting at a dose of 250 mg/kg BW.



Note: a, b, c, etc. showed the differences of potency ($P < 0.05$, Dunnet C test)

Figure 2. GLP-1 levels with administered *U. lobata* leaf extract.

Both administration of water and ethanolic extracts significantly maintained GLP-1 bioavailability in diabetic rats. Mangiferin, stigmasterol, and β-sitosterol in the extract were able to prevent degradation of GLP-1 by DPP-4. Based on our previous work, active compounds in *U. lobata* extract act as DPP-4 inhibitors. With this, the water extract ensured more GLP-1 bioavailability compared to the ethanolic extract, and this is because of the difference in active compounds that inhibit GLP-1 degradation by DPP-4. It is also related to the DPP-4 inhibitory activity of active compounds in both water and ethanolic extracts. The effect is regulated by DPP-4 inhibitory activity owing to a synergistic interaction of active compounds and production of complex compounds in the water extract (Brunton *et al.*, 2006). As the result, inhibition of DPP-4 prevents GLP-1 metabolism from inactivation and resulted in the retention of their bioavailability.

The effect of U. lobata on glucose tolerance testing in diabetic rats

Blood glucose levels of rat administered *U. lobata* extract after stimulating glucose are depicted in Figures 3 and 4. The results indicated that there is a significant increase of the AUC of glucose in the DG by

up to 70% compared to the NG ($P < 0.05$). The administration of *U. lobata* water extract at dosages of 250 mg/kg BW, 500 mg/kg, and 1000 mg/kg BW decreased the AUC of glucose by 50%, 60%, and 50%, respectively, compared to that of the DG ($P < 0.05$) whereas the ethanolic extract diminished the AUC of glucose by 50%, 40%, and 20%, respectively ($P < 0.05$). The *U. lobata* water extract exhibited stronger control over the AUC of blood glucose compared to that of ethanolic extract ($P < 0.05$) after glucose stimulation.

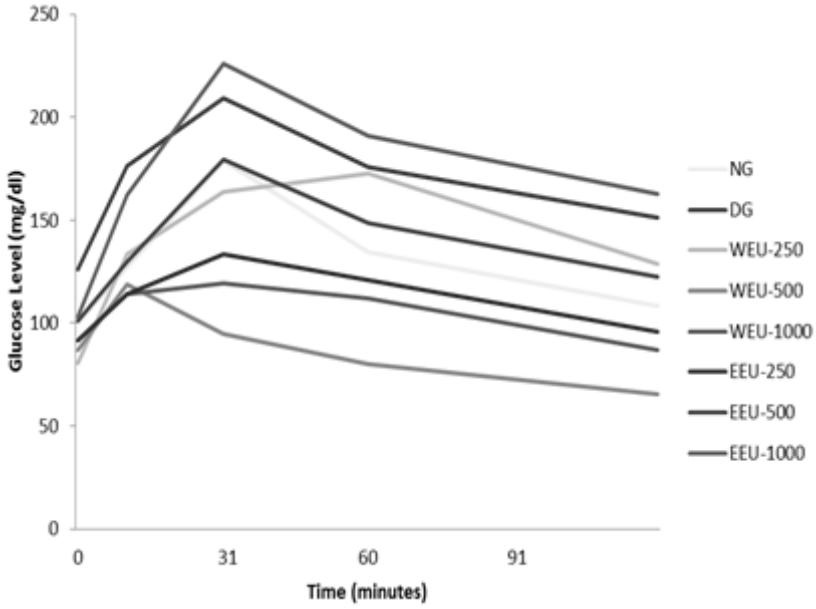
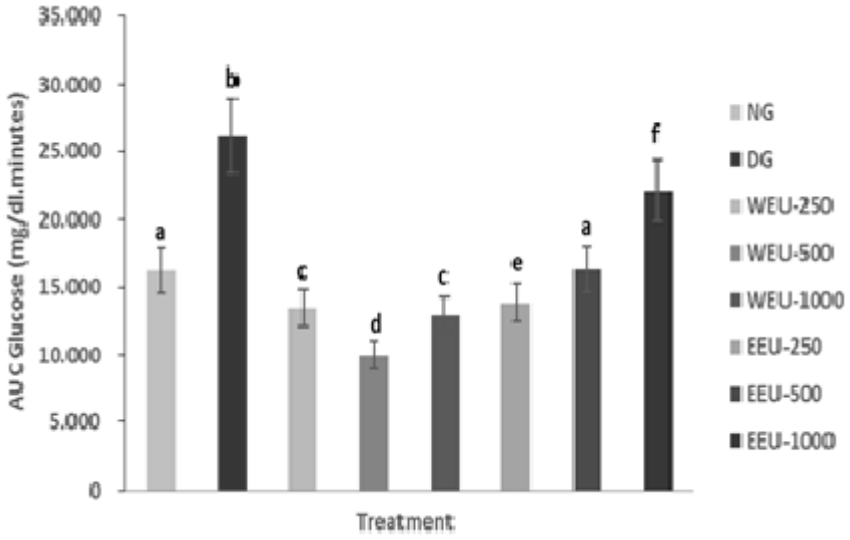


Figure 3. Blood glucose level administered *U. lobata* extract after induction of glucose.

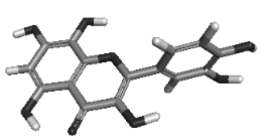


Note: a, b, c, etc. indicates the differences in potency ($P < 0.05$, LSD test)

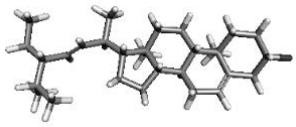
Figure 4. AUC of glucose with administered *U. lobata* extract.

Discussion

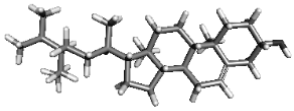
The semi-qualitative testing of *U. lobata* leaf extract by LC-MS showed the compositions of gossypetin (**1**) in the ethanolic extract are higher than that of in the water extract whereas stigmasterol is more predominant in the water extract (Purnomo *et al.*, 2015). Active compounds, such as stigmasterol (**2**), β-sitosterol (**3**), and gossypetin (**1**) are soluble in less polar solvents, like alcohol, even though mangiferin (**4**) and hypoxemia are insoluble. The difference in solubilities of the active compounds in the solvents affects the number of active compounds extracted from the extracts. However, the DPP-4 inhibitory activity of both the water and ethanolic extracts are still lower, approximately 30-100-fold, compared to vildagliptin, the standard reference DPP-4 inhibitor ($P < 0.05$).



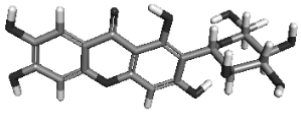
Gossypetin (**1**)



Stigmasterol (**2**)



B-Sitosterol (**3**)



Mangiferin (**4**)

Figure 5. Chemical structures of certain molecules found in *U. lobata* leaf extract.

Both of the *U. lobata* water and ethanolic extracts significantly inhibit the DPP-4 activity of diabetic rats. These effects are regulated by active compounds **2**, **3**, and **4** from *U. lobata* based on *in-silico* analysis (Purnomo *et al.*, 2015). The increase dosages of *U. lobata* water extract inhibit more DPP-4 activity whereas the ethanolic extract raises DPP-4 activity precisely. This is based on the conformational shift of active compounds, particularly the sterol group in the ethanolic extract which produces a complex of compounds post-binding (Morris and Lim-Wilby, 2008; Setevens and Honerkamp-Smith, 2010). The change in active compound structure can alter the solubility of active substances and their absorption and resultant modulation of inhibitory activity on DPP-4 (Stevens and Honerkamp-Smith, 2010). Owing to the ethanolic extract being less polar than the water extract, it affects solubilities of active compounds and their absorption within the gastrointestinal system in addition to bioactivity decreasing. The water extract inhibited more DPP-4 activity than that of the ethanolic extract. Complex formed of active substances from the *U. lobata*

water extract facilitate the inhibitory activity of DPP-4. A number of active compounds within the water extract have synergistic interactions besides the reinforcement of their inhibitory activity on DPP-4 (Brunton *et al.*, 2006). Inhibition of DPP-4 activity increases the bioavailability of the incretin hormone, thereby contributing to carbohydrate metabolism (Drucker, 2002).

The differences in DPP-4 inhibitory activity between *in vitro* and *in vivo* testing can be explained pharmacokinetically. The poor solubility of active compounds within the ethanolic extract may affect its absorption and distribution in the blood as a result of decreasing the inhibition of DPP-4 activity.

The principal biological activity of DPP-4/CD26 is its enzymatic function. The enzymatic activity of DPP-4/CD26 is exhibited by the membrane-spanning form of the molecule, and the slightly smaller circulating soluble form (Chen, 2006). The substrates of CD26/DPP-4 are not specific to certain peptides. Proline- or alanine-containing peptides are the substrates of DPP-4 and include growth factors, chemokines, neuropeptides, and vasoactive peptides (Mentlein and Gallwitz, 1993). DPP-4 prefers substrates with an amino-terminal proline or alanine at position 2 even though they may also cleave substrates with non-preferred amino acids at position 2 (Mentlein and Gallwitz, 1993; Gopalan *et al.*, 2010). The structure of incretin hormones, such as GLP-1 and GIP, reveals a highly conserved alanine in position 2, rendering these peptides ideal putative substrates for the aminopeptidase, DPP-4 (Gopalan *et al.*, 2010).

The DPP-4 inhibitor prevents the degradation of active GLP-1; however, it does not elevate the levels of circulating total GLP-1 and, further, does not prevent the kidney from rapidly clearing GLP-1. Using DPP-4 inhibitors, primarily for the treatment of diabetes, is related to the potential effects of them on immune function. CD26/DPP-4 plays an important role in tumor biology; It is valuable as a marker for various cancers with its levels either on the cell surface or in the serum increased in certain neoplasms and reduced in others (Havre *et al.*, 2008). It is related to the inhibition of T cell proliferation in the immune system and, moreover, it loses its biological activity in protecting against neoplasms (Chen, 2006; Havre *et al.*, 2008).

GLP-1 has a potency for T2DM therapy but it is metabolized excessively by DPP-4 and becomes the inactive form (Drucker, 2002; Nauck *et al.*, 2009). GLP-1 has a short half-life, approximately for two to five minutes, and this is based on DPP-4 activity (Drucker, 2002; Salehi *et al.*, 2008). The active forms of GLP-1 are GLP-1 (7-36) amides and GLP-1 (7-37), which are rapidly inactivated by DPP-4 through cleavage of the N-terminal dipeptide, His-Ala. This results in the production of the inactive forms of GLP-1, namely GLP-1 (9-36) amide and GLP-1 (9-37) isopeptides (Nauck *et al.*, 2009; Salehi *et al.*, 2008). Previous studies have shown that the importance of DPP-4-mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity (Stevens and Honerkamp-Smith, 2010).

GLP-1 is a super family peptide of glucagon which has a similarity degree of approximately 48%. The similarity of the amino acid sequences between GLP-1 and glucagon is one of the reasons. Pro-glucagon

gen was located at chromosome 2q36-q37 and only found in certain tissues whereas the messenger RNA (mRNA) of pro-glucagon was located in the α-cells of the pancreas as well as the L-cells of the intestine and brain's hypothalamus. Pro-glucagon production commences from transcription of preproglucagon gen and is then continued by the translation process. The regulation of GLP-1 release from L-cells of the intestine is a complex mechanism that involves combinations of nutrition, hormones, and neural stimuli (Morris and Lim-Wilby, 2008). The GLP-1 receptor is classified as a *G protein-coupled* receptor that is found in liver, muscle and pancreas cells. This receptor has a specific character by activation of adenyl cyclase and resultant cAMP. After GLP-1 binding with the receptor, it will activate cAMP and mitogen-activated protein kinase (MAPK).

Administration of *the U. lobata* ethanolic and water extracts significantly decreased the AUC of glucose in diabetic rats post-glucose induction. It is controlled by the active compounds in *U. lobata* which inhibit DPP-4 activity, leading to retention of the GLP-1 bioavailability to regulate blood glucose level increases after stimulation through oral nutrition (Drucker, 2002; Holst and Orskov, 2004). GLP-1 acts beyond metabolic purposes, specifically inhibiting gastric juice secretion, GIT motility, and the rate of gastric-emptying functioning (Drucker, 2002; Chia and Egan, 2008). Furthermore, it is beneficial to prevent increasing blood glucose levels post-prandial (Holst and Orskov, 2004; Salehi *et al.,* 2008)

The *U. lobata* water extract regulated more blood glucose levels and post-glucose stimulation compared to the ethanolic extract. This was predicted based on the poor solubility of the active compounds and absorption of the ethanolic extract. As such, it also affects the activities constituting controlling blood glucose levels. The occurrence of reducing the solubility of active compounds is based on the formation of complex compounds and the conformational changes in active compounds as well as the decrease in inhibitory activity of DPP-4 that contributes to maintaining blood glucose levels in the DG (Aronoff *et al.,* 2004; Stevens and Honerkamp-Smith, 2010).

DPP-4 inhibitory activity by the *U. lobata* ethanolic extract was stronger than that of the water extract during *in-vitro* testing but the opposite was found with the *in-vivo* study. In addition, the *U. lobata* water extract demonstrated a more pronounced decrease of DPP-4 levels and the AUC of blood glucose as well as retention of GLP-1 bioavailability compared to the ethanolic extract.

Acknowledgment

The authors gratefully acknowledge the support of Balai Materia Medica, Batu, East Java, Indonesia for providing the *U. lobata* sample. The authors also would like to thank Gajah Mada University, Yogyakarta, Indonesia for the supply of animals which needed for this research.

Ethical Clearance

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

Conflict of Interest

The authors have no conflict of interest to declare

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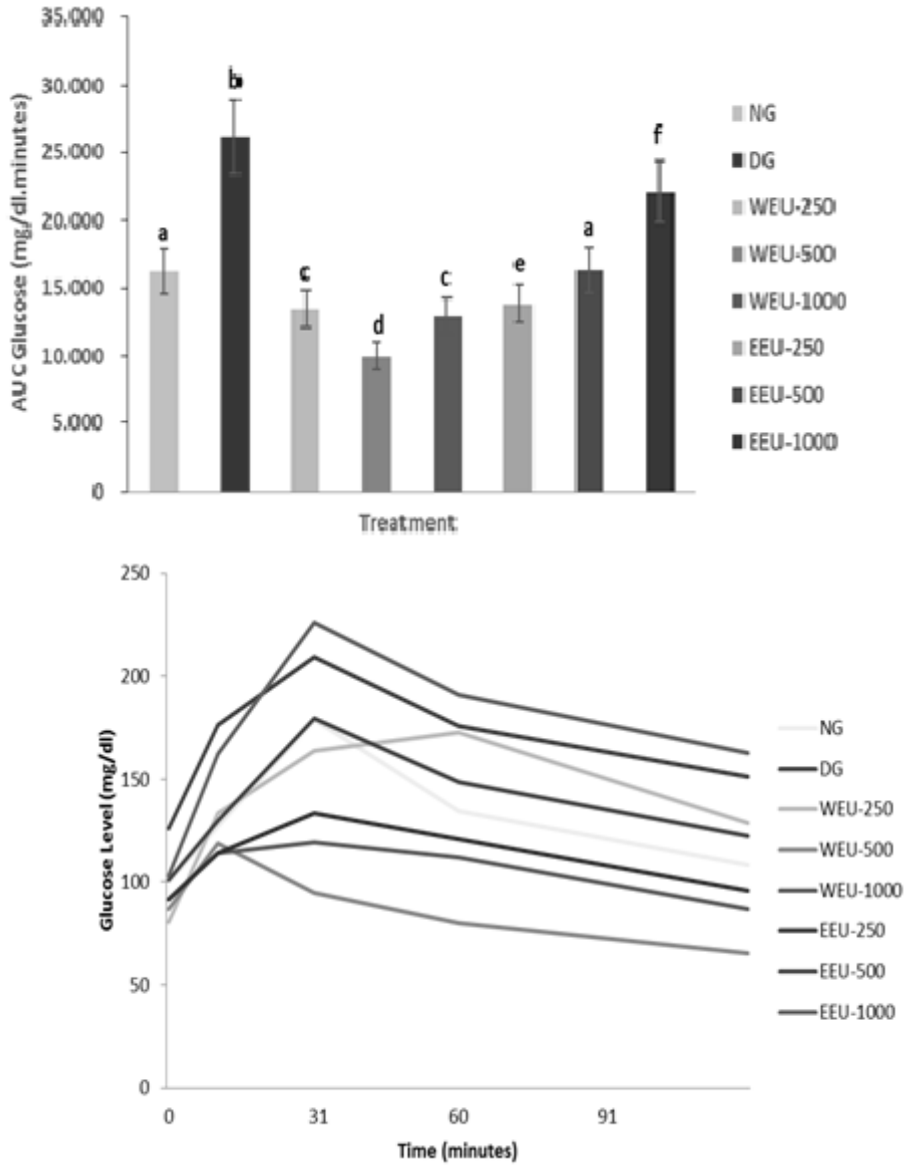
FIGURES LEGEND

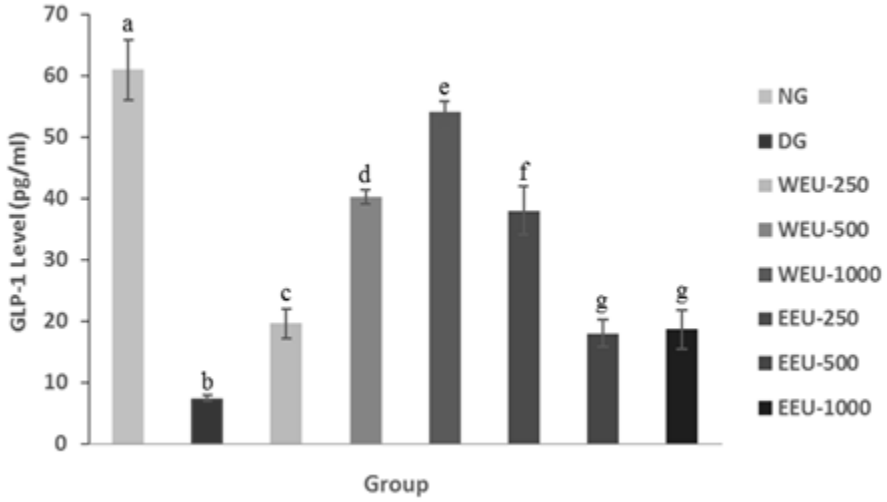
Figure 1. DPP-4 level administrated *U. lobata* leaf extract; a, b, c etc. showed the differences of potency ($p<0.05$, Dunnet C test)

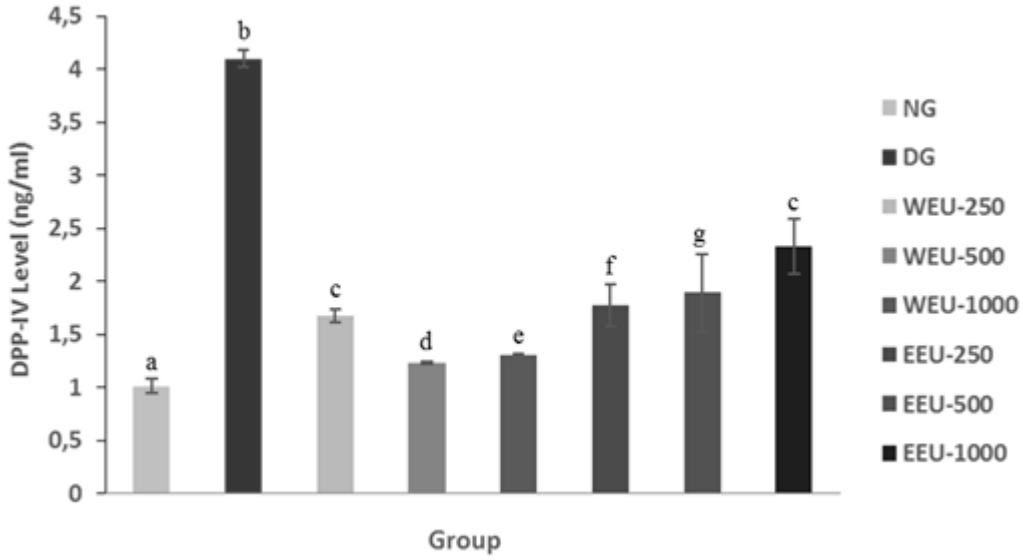
Figure 2. GLP-1 level administrated *U. lobata* leaf extract; a, b, c etc. showed the differences of potency ($p<0.05$, Dunnet C test)

Figure 3. Blood glucose level administrated *U. lobata* extract after induction of glucose.

Figure 4. AUC of glucose administrated *U. lobata* extract; a, b, c etc. showed the differences in potency ($p<0.05$, LSD test)







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IJPRI_PERBAIKAN BAHASA INGGRIS PADA MANUSCRIPT

CJ468

Inhibitory activity of *Urena lobata* leaf extract on dipeptidyl peptidase-4 (DPP-4): is it different *in vitro* and *in vivo* ?

Abstract

This study sought to assess the anti-diabetic effect of *Urena lobata* leaf extract through DPP-4 inhibitory activity both *in vitro* and *in vivo*. *Urena lobata* leaf was extracted in ethanol solvent and hot water. *In vitro* testing using *gly-pro-p-nitroanilide* (GPPN) as a substrate of DPP-4 and vildagliptin as a standard reference was carried out. A product of the reactions between GPPN and DPP-4, *p*-nitroanilida was observed by microplate reader at λ=405 nm while the IC_{50} value was determined by non-linear regression curve. The *in vivo* investigation utilized an animal model of T2DM with two control groups and six test groups (n=4), where DPP-4 levels, GLP-1 levels and the AUC of blood glucose were determined after extract administration. All data were analyzed with one-way ANOVA and then continued with LSD or Dunnet c ($p<0.05$). The *in vitro* DPP-4 inhibitory activity of ethanolic extract from *U. lobata* was stronger than water extract. However, the results vice versa were observed during the *in vivo* study. In addition, the *U. lobata* water extract exhibited a stronger decrease in DPP-4 levels and the AUC of blood glucose as well as retaining GLP-1 bioavailability compared to ethanolic extract.

Keywords: DPP-4, *in vitro*, *in vivo*, *Urena lobata, gly-pro-p-nitroanilide (GPPN .*

Introduction

Recently, treatment of type II diabetes mellitus (T2DM) has been proposed with incretin hormone. The majority of incretin hormone is glucagon-like peptide-1 (GLP-1) secreted by intestinal tissues based on the induction of oral nutrition (Drucker, 2007). GLP-1 plays a vital role in maintaining blood glucose levels related to their biology activity, such as to stimulating insulin secretion, increasing β-cell proliferation, inhibiting glucagon secretion, reducing the rate of gastric emptying, and promoting satiety (Drucker, 2007; Chia and

Egan, 2008). In T2DM patients, GLP-1 bioavailability decreases the secretion of insulin and the clinical effect is chronic hyperglycemia (Drucker, 2002).

GLP-1 is sufficiently potent to cure T2DM, however GLP-1 is metabolized by dipeptidyl peptidase-4 (DPP-4) excessively and becomes inactive (Drucker, 2002; Chia and Egan, 2008). Inhibition of DPP-4 is effective in treating T2DM, therefore GLP-1 bioavailability can be retained in addition to regulating blood glucose levels (Brunton et al., 2006). Treatment of T2DM with *incretin-like* drugs or DPP-4 inhibitors show lesser side effects even though the safety of this drug has not been evaluated with respect to a complete data set (Holst and Orskov, 2004). Nevertheless, incretin-like drugs have side effects, such as flu-like symptoms, skin reactions, and gastrointestinal problems. These effects are exacerbated with the use of the drugs over the long-term (Bailey, 2008; Salehi et al., 2008). As such, these phenomena have attracted attention to finding medicinal plants as alternatives to treat T2DM by DPP-4 inhibition.

One of the traditional plants known to have anti-diabetic effects is *Urena lobata*. Root and leaf extract of *U. lobata* have been used empirically by Nigerian people to treat DM. Preclinical testing of *U. lobata* root extract indicated there were anti-hyperglycemic effects on streptozotocin-induced rats (Onoagbe et al., 2010). In Indonesia, *U. lobata* is known by Pulutan, and the plant exhibited anti-bacterial effects based on preliminary study (Nurfauziah and Mulyani, 1999). Further, a number of studies have showed anti-diabetic potency of *U. lobata*, however the mechanism of the inhibition of DPP-4 activity has not been investigated as of yet (Nurfauziah and Mulyani, 1999; Vats et al., 2002; Awika and Rooney, 2004; Onoagbe et al., 2010). Therefore, the current study sought to examine the anti-diabetic effects of *U. lobata* leaf extract on DPP-4 inhibition.

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. The *U.lobata* leaf powder (50 g) was extracted according to the decoction method in which the leaf powder was soaked in 250 mL hot water (90ºC) for 30 minutes. The other sample of *U.lobata* leaf powder (50 g) was extracted based on the digestion method by soaking the sample in 250 mL ethanol for five hours using a shaker water bath. The extraction process was repeated twice with fresh solvent. Both of the extract solutions had the solvent removed using a rotary evaporator to obtain crude extracts.

DPP-4 in vitro assay

The assay was performed in 96-well micro plates. A pre-incubation volume of 50 µL solution contained 35 µL Tris-HCl buffer, 15 µL DPP-4 enzyme, and various concentrations of test material or standards. This mixture was incubated at 37 ºC for 10 minutes followed by addition of 50 µL gly-pro-p-nitroanilide as the

substrate. The reaction mixture was incubated for 30 minutes at 37 ºC and the absorbance was measured at 405 nm at 10-second intervals. Vildagliptin was used as the standard reference of the DPP-4 inhibitor.

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 approved by the Commision of Ethical Research, Brawijaya University, Malang, Indonesia, with certificate number 245-KEP-UB. SD rats were housed in individual cages and automatically controlled animal rooms at 25 ± 1º C on a 12:12-h light–dark cycle. They were fed with standard food and water *ad libitum* and fasted overnight before the experiments. Normal diet (ND) and a high-fructosa diet (HFD) foods 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 intra peritoneally [refer to Guo *et al.* ( ) with minor modification]. Rats were stated to be diabetic if the fasting blood glucose level was more than 126 mg/dL (Awika and Rooney, 2004). The experiments were assigned into eight 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 the ethanolic extract (EEU) and water extract of *U.lobata* (WEU) at dosages 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*. (2006). Body weight and food consumption were monitored weekly. Blood samples were obtained 15 minutes after being given the glucose stimulation in dosages of 2 g/kg body weight orally. The blood samples were also taken from the tail vein after overnight fasting. Blood samples were immediately centrifuged at 4500 rpm. The serum was separated and saved under -20 ºC.

DPP-4 in vivo assay

DPP-4 serum levels were analyzed by rat DPP-4 ELISA kit (Elabscience E-EL-R0337). 100 µL samples were incubated for 90 minutes at 37 ºC, and 100 µL of biotinylated detection Ab ( ) was added and the mixture was then incubated for 60 minutes at 37 ºC. After aspirating and washing, 100 µL HRP conjugate was added and the mixture was incubated for 30 minutes at 37 ºC. A 90 µL substrat reagent ( ) was then added with 50 µL *stop solution*. The data of samples were measured with a microplate reader at λ = 450 nm.

GLP-1 in vivo assay

GLP-1 serum levels were determined by rat GLP-1 ELISA kit (USCN CEA804). 50 µL samples were added with 50 µL of detection reagent A and then incubated for 60 minutes at 37 ºC. After aspirating and washing,

100 µL of detection reagent B was added to the samples that were next incubated for 30 minutes at 37 ºC. A 90 µL substrat reagent was then added with 50 µL *stop solution*. The data of samples were obtained using a microplate reader at λ = 450 nm.

Oral glucosa tolerance test

For the glucose tolerance test, glucose was administered orally at a dose of 2 g/kg body weight after overnight fasting. The blood samples were collected from the tail vein at 0 (before glucose administered), 15, 30, 60, 90, and 120 min after glucose administration and they were measured immediately using a commercial glucometer ( ). The integrated area under the postprandial glucose curves (AUCs) was calculated by the trapezoidal method.

Statistical analysis

The data were expressed as means ± S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA). The least significant difference (LSD) test or Dunnet C were employed for mean comparisons and $P < 0.05$ was considered to be statistically significant.

Results

DPP-4 inhibitory activity of U. lobata via in vitro testing

The DPP-4 inhibitory activity of *U.lobata* leaf extract is presented in Table 1. Based on the results, the *U.lobata* ethanolic extract had greater inhibitory activity on DPP-4 (roughly four-fold) compared to that of water extract ($P < 0.05$).

Table 1. DPP-4 inhibitory activity of *U.lobata* leaf extract

Group	Sample	Concentration (ppm)	% inhibition	IC_{50} (ppm)
1	Water extract of *U.lobata*	625	0.00 ± 0.00	6489.88[a]
		1250	13.33 ± 0.00	
		2500	26.67 ± 0.00	
		5000	42.22 ± 3.85	
		10000	62.22 ± 3.85	
2	Ethanolic extract of *U.lobata*	625	36.17 ± 0.00	1654.64[b]
		1250	48.94 ± 0.00	
		2500	55.32 ± 0.00	
		5000	61.70 ± 0.00	
		10000	74.47 ± 0.00	

3	Vildagliptin	6.25	8.93 ± 0.00	57.44[c]
		12.50	16.07 ± 4.12	
		25.00	37.50 ± 0.00	
		50.00	46.63 ± 3.85	
		100.00	60.71 ± 0.00	

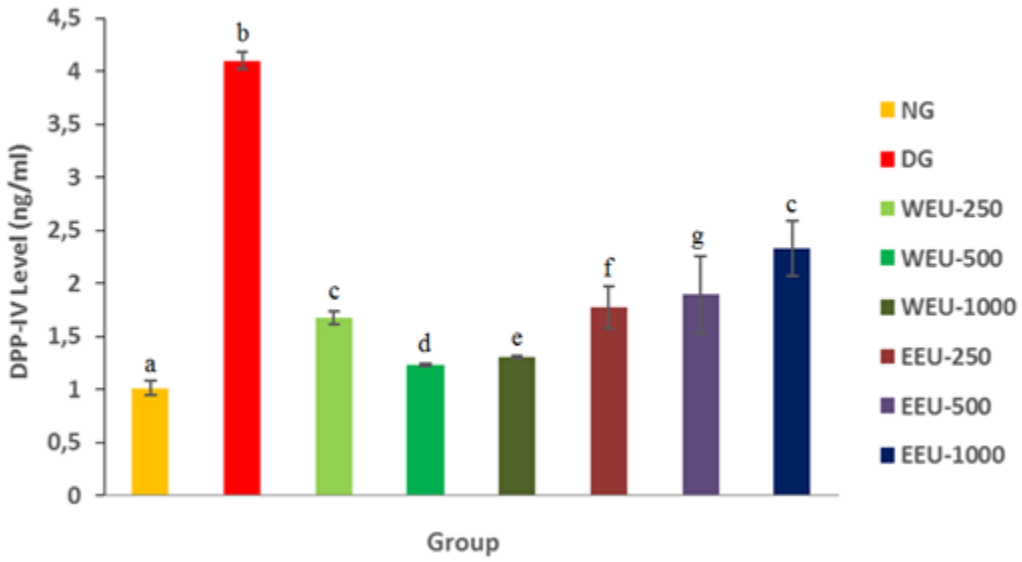
a,b,c = different letter showed the differences in potency (P < 0.05, LSD test)

The experiments were repeated in triplicate.

The results of % inhibition were expressed as means ± S.E.M.

DPP-4 inhibitory activity of U.lobata via in vivo testing

DPP-4 serum levels of diabetic rats with administered *U.lobata* leaf extract are shown in Figure 1. The diabetic group exhibited a significant increase in DPP-4 levels, which is approximately four-fold higher than the normal group (P < 0.05). Administration of *U.lobata* water extract at 250 mg/kg, 500 mg/kg, and 1000 mg/kg bw decreased DPP-4 by roughly 60%, 70%, and 70%, respectively, compared to the diabetic group (P < 0.05), whereas that of the ethanolic extract diminished DPP-4 levels by 60%, 50%, and 40%, respectively (P < 0.05). The results demonstrated that the water extract was able to decrease DPP-4 levels more so compared to the ethanolic extract (P < 0.05) at the same dosages.



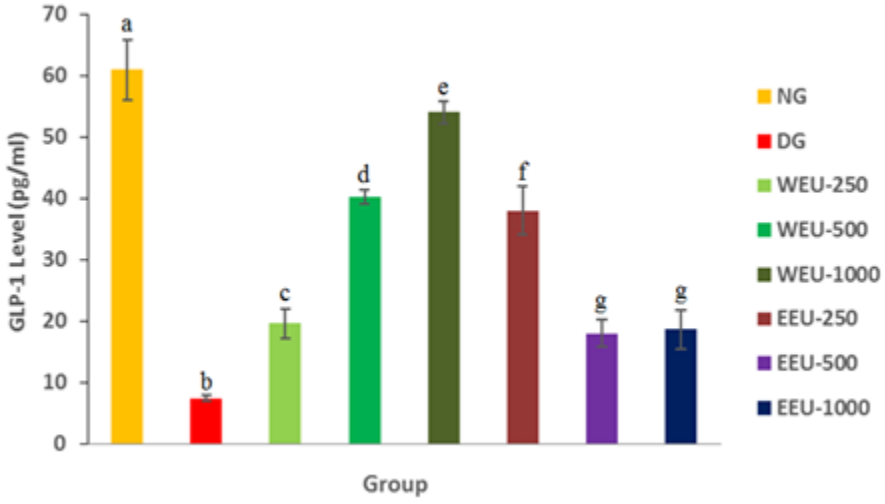
Note: a, b, c, etc. indicates the differences in potency (P < 0.05, Dunnet C test)

Figure 1. DPP-4 levels with administered *U.lobata* leaf extract.

Effect of U.lobata leaf extract on GLP-1 serum levels

GLP-1 serum levels of diabetic rats that had been administered *U.lobata* leaf extract are portrayed in Figure 2. There is a significant reduction in GLP-1 within the DG by nearly eight-fold compared to the NG (P < 0.05). The water extract of *U.lobata* at dosages of 250 mg/kg, 500 mg/kg,

and 1000 mg/kg bw prevented degradation of GLP-1 by roughly three-fold, five-fold, and seven-fold, respectively, compared to that of the DG ($P < 0.05$), of which the ethanolic extract showed approximately five-fold, two-fold, and 2.5-fold ($P < 0.05$), respectively. For the same dosage, the water extract was able to inhibit more degradation of GLP-1 compared to the ethanolic extract ($P < 0.05$) excepting at a dose of 250 mg/kg bw.



Note: a, b, c, etc. showed the differences of potency ($P < 0.05$, Dunnet C test)

Figure 2. GLP-1 levels with administered *U.lobata* leaf extract.

Both administration of water and ethanolic extracts significantly maintained GLP-1 bioavailability in diabetic rats. Mangiferin, stigmasterol, and β-sitosterol in the extract were able to prevent degradation of GLP-1 by DPP-4. Based on our previous work, active compounds in *U.lobata* extract act as DPP-4 inhibitors. With this, the water extract ensured more GLP-1 bioavailability compared to the ethanolic extract, and this is because of the difference in active compounds that inhibit GLP-1 degradation by DPP-4. It is also related to DPP-4 inhibitory activity of active compounds in both water and ethanolic extracts. The effect is regulated by DPP-4 inhibitory activity owing to a synergistic interaction of active compounds and production of complex compounds in the water extract (Brunton et al., 2006). As the result, inhibition of DPP-4 prevents GLP-1 metabolism from inactivation and resulted in the retention of their bioavailability.

The effect of U. lobata on glucose tolerance testing in diabetic rats

Blood glucose levels of rat administered *U.lobata* extract after stimulating glucose are depicted in Figures 3 and 4. The results indicated that there is a significant increase of the AUC of glucose in the DG by up to 70% compared to the NG ($P < 0.05$). The administration of *U.lobata* water extract at dosages of 250 mg/kg bw, 500 mg/kg, and 1000 mg/kg bw decreased the AUC of glucose by 50%, 60%, and 50%, respectively, compared to that of the DG ($P < 0.05$) whereas the ethanolic extract

diminished the AUC of glucose by 50%, 40%, and 20%, respectively ($P < 0.05$). The *U.lobata* water extract exhibited stronger control over the AUC of blood glucose compared to that of ethanolic extract ($P < 0.05$) after glucose stimulation.

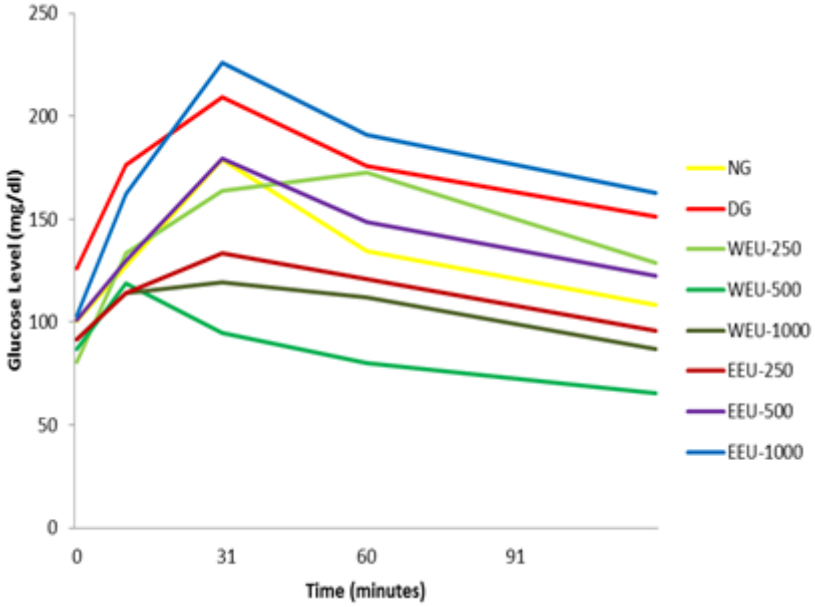
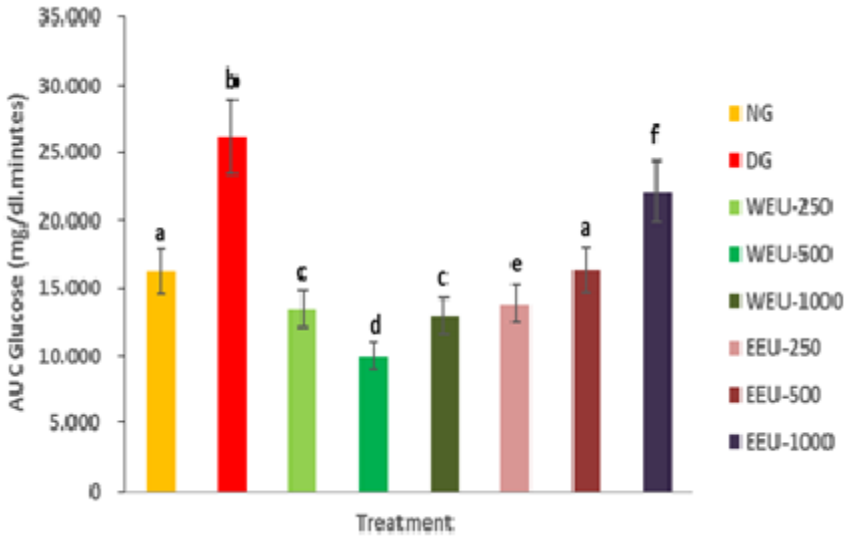


Figure 3. Blood glucose level administered *U. lobata* extract after induction of glucose.



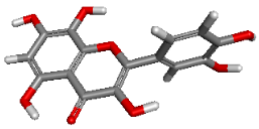
Note: a, b, c, etc. indicates the differences in potency ($P < 0.05$, LSD test)

Figure 4. AUC of glucose with administered *U.lobata* extract.

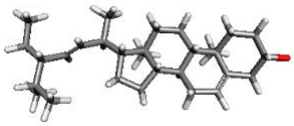
Discussion

The semi-qualitative testing of *U.lobata* leaf extract by LC-MS showed the compositions of gossypetin (**1**) in the ethanolic extract are higher than that of in the water extract whereas stigmasterol is more predominant

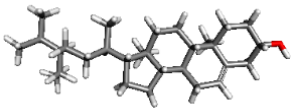
in the water extract (Purnomo et al., 2015). Active compounds, such as stigmasterol (**2**), β-sitosterol (**3**), and gossypetin (**1**) are soluble in less polar solvents, like alcohol, even though mangiferin (**4**) and hypolaetin are insoluble. The difference in solubilities of the active compounds in the solvents affect the amount of active compounds extracted from the extracts. However, the DPP-4 inhibitory activity of both the water and ethanolic extracts are still lower, approximately 30-100-fold, compared to vildagliptin, the standard reference DPP-4 inhibitor ($P < 0.05$).



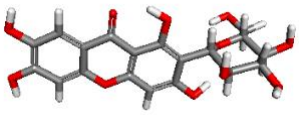
Gossypetin (**1**)



Stigmasterol (**2**)



B-Sitosterol (**3**)



Mangiferin (**4**)

Figure 5. Chemical structures of certain molecules found in *U.lobata* leaf extract.

Both of the *U.lobata* water and ethanolic extracts significantly inhibit the DPP-4 activity of diabetic rats. These effects are regulated by active compounds **2**, **3**, and **4** from *U.lobata* based on *in-silico* analysis (Purnomo et al., 2015). The increase dosages of *U.lobata* water extract inhibit more DPP-4 activity whereas the ethanolic extract raise DPP-4 activity precisely. This is based on the conformational shift of active compounds, particularly the sterol group in ethanolic extract which produces a complex of compounds post-binding (Morris and Lim-Wilby, 2008; Setevens and Honerkamp-Smith, 2010). The change in active compound structure can alter the solubility of active substances and their absorption and resultant modulation of inhibitory activity on DPP-4 (Stevens and Honerkamp-Smith, 2010). Owing to the ethanolic extract being less polar than the water extract, it affects solubilities of active compounds and their absorption within the

gastrointestinal system in addition to bioactivity decreasing. The water extract inhibited more DPP-4 activity than that of the ethanolic extract. Complex formed of active substances from the *U. lobata* water extract facilitate the inhibitory activity on DPP-4. A number of active compounds within the water extract have synergistic interactions besides the reinforcement of their inhibitory activity on DPP-4 (Brunton et al., 2006). Inhibition of DPP-4 activity increases the bioavailability of the incretin hormone, thereby contributing to carbohydrate metabolism (Drucker, 2002).

The differences in DPP-4 inhibitory activity between *in vitro* and *in vivo* testing can be explained pharmacokinetically. The poor solubility of active compounds within the ethanolic extract may affect its absorption and distribution in the blood as a result of decreasing the inhibition of DPP-4 activity.

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The DPP-4 inhibitor prevents the degradation of active GLP-1; however, it does not elevate the levels of circulating total GLP-1 and, further, does not prevent the kidney from rapidly clearing GLP-1. Using DPP-4 inhibitors, primarily for the treatment of diabetes, is related to the potential effects of them on immune function. CD26/DPP-4 plays an important role in tumor biology; It is valuable as a marker for various cancers with its levels either on the cell surface or in the serum increased in certain neoplasms and reduced in others (Havre et al., 2008). It is related to the inhibition of T cell proliferation in the immune system and, moreover, it loses its biological activity in protecting against neoplasms (Chen, 2006; Havre et al., 2008).

GLP-1 has a potency for T2DM therapy but it is metabolized excessively by DPP-4 and becomes the inactive form (Drucker, 2002; Bailey, 2008). GLP-1 has a short half-life, approximately for two to five minutes, and this is based on DPP-4 activity (Drucker, 2002; Salehi et al., 2008). The active forms of GLP-1 are GLP-1 (7-36) amides and GLP-1 (7-37), which are rapidly inactivated by DPP-4 through cleavage of the N-terminal dipeptide, His-Ala. This results in the production of the inactive forms of GLP-1, namely GLP-1 (9-36) amide and GLP-1 (9-37) isopeptides (Bailey, 2008; Salehi et al., 2008). Previous studies have shown that the importance of DPP-4-mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity (Stevens and Honerkamp-Smith, 2010).

GLP-1 is a super family peptide of glucagon which have a similarity degree of approximately 48%. The similarity of the amino acid sequences between GLP-1 and glucagon are one of the reasons. Pro-glucagon gen was located at chromosome 2q36-q37 and only found in certain tissues whereas the messenger RNA (mRNA) of pro-glucagon was located in the α-cells of the pancreas as well as the L-cells of the intestine and brain's hypothalamus. Pro-glucagon production commences from transcription of prepro-glucagon gen and is then continued by the translation process. The regulation of GLP-1 release from L-cells of the intestine is a complex mechanism that involves combinations of nutrition, hormones, and neural stimuli (Morris and Lim-Wilby, 2008). The GLP-1 receptor is classified as a *G protein-coupled* receptor that is found in liver, muscle and pancreas cells. This receptor has a specific character by activation of adenylcyclase and resultant cAMP. After GLP-1 binding with the receptor, it will activate cAMP and mitogen-activated protein kinase (MAPK).

Administration of *the U. lobata* ethanolic and water extracts significantly decreased the AUC of glucose in diabetic rats post-glucose induction. It is controlled by the active compounds in *U. lobata* which inhibit DPP-4 activity, leading to retention of the GLP-1 bioavailability to regulate blood glucose level increases after stimulation through oral nutrition (Drucker, 2002; Holst and Orskov, 2004). GLP-1 acts beyond metabolic purposes, specifically inhibiting gastric juice secretion, GIT motility, and the rate of gastric-emptying functioning (Drucker, 2002; Chia and Egan, 2008). Furthermore, it is beneficial to prevent for increasing blood glucose levels post-prandial (Holst and Orskov, 2004; Salehi et al., 2008)

The *U.lobata* water extract regulated more blood glucose levels and post-glucose stimulation compared to the ethanolic extract. This was predicted based on the poor solubility of the active compounds and absorption of the ethanolic extract. As such, it also affects the activities constituting controlling blood glucose levels. The occurrence of reducing the solubility of active compounds is based on the formation of complex compounds and the conformational changes in active compounds as well as the decrease in inhibitory activity of DPP-4 that contributes to maintaining blood glucose levels in the DG (Aronoff et al., 2004; Stevens and Honerkamp-Smith, 2010).

DPP-4 inhibitory activity by the *U. lobata* ethanolic extract was stronger than that of the water extract during *in-vitro* testing but the opposite was found with the *in-vivo* study. In addition, the *U.lobata* water extract demonstrated a more pronounced decrease of DPP-4 levels and the AUC of blood glucose as well as retention of GLP-1 bioavailability compared to the ethanolic extract.

GENERAL EDITORIAL COMMENTS:

- ***This was a very high-quality document featuring an extremely intriguing bioethics research topic – well done!!!***

- *This writing, overall, is superb, but there are a number of issues that, if corrected, will improve it such that it will become even better. They are as follows:*

  - *It is ALWAYS best to use different words/terms even when describing the same sorts of things (ie. "increase" v.s. "elevate" v.s. "raise", etc.) where possible. This makes the reader more interested in what has been written. Feel free to use a Thesaurus when writing – that is a best practice, and a good one is available using Google (www.google.com → just type in the word + "definition" or "synonym").*

  - *It is more appropriate to use the word, "due", when referring to timing or duration – better to say "because of" or "as a result of".*

  - *Better to be consistent with the use of 2nd-to-last item commas in lists (ex. "… biology, physics, and chemistry.") – make sure ALL lists are in the same style throughout document.*

  - *Review the rules for hyphenation below and see the changes made in this document that were related:*

    - *http://www.grammarbook.com/punctuation/hyphens.asp*

  - *There were quite a few comma problems – see links below for assistance:*

    - *http://grammar.ccc.commnet.edu/grammar/commas.htm*

    - *https://owl.english.purdue.edu/owl/resource/607/02/*

- ***Please ensure that all Comments made during this round of revisions are reviewed.***

Again, great work! Good luck!

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Research Article

Inhibitory activity of *Urena lobata* leaf extract on dipeptidyl peptidase-4 (DPP-4): is it different *in vitro* and *in vivo*?

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ABSTRACT

This study was aimed to compare the anti diabetic effect of *Urena lobata* leaf extract through DPP-4 inhibitory activity by *in vitro* and *in vivo*. *Urena lobata* leaf was extracted in ethanol and hot water to evaluate its activity on DPP-4 both of *in vitro* and *in vivo*. *In vitro* test using *Gly-pro-p-nitroanilide* (GPPN) as substrate of DPP-4 and the reactions product of them was observed by microplate reader at λ=405 nm furthermore the IC_{50} value was determined. *In vivo* study utilize an animal model of diabetes with 2 control groups and 6 test groups (n=4), in which DPP-4 level, GLP-1 level and AUC of blood glucose were examined after extract administration. The *in vitro* DPP-4 inhibitory activity of ethanolic extract of *U. lobata* is higher than water extract with the IC_{50} value of 1654,64 and 6489,88 µg/ml respectively. However, the water extract of *U. lobata* exhibits stronger decrease DPP-4 level (60-70%) compared to ethanolic extract (40-60%) *in vivo* study as well as the AUC of blood glucose were reduced by 50-60% and 20-50%, respectively. Meanwhile, GLP-1 level could be retained more by the water extract of *U. lobata* administration (3-7 fold) compared to ethanolic extract (2-5 fold) due to the reducing of DPP-4 activity.

Keywords: DPP-4, *in vitro*, *in vivo*, *Urena lobata*, Gly-pro-p-nitroanilide (GPPN)

INTRODUCTION

Recently, treatment of type 2 diabetes mellitus (T2DM) has been proposed with incretin hormone. The majority of incretin hormone is glucagon-like peptide-1 (GLP-1) secreted by intestinal tissues based on the induction of oral nutrition (Drucker, 2007). GLP-1 plays a vital role in maintaining blood glucose levels related to their biology activity, such as to stimulating insulin secretion, increasing β-cell proliferation, inhibiting glucagon secretion, reducing the rate of gastric emptying, and promoting satiety (Drucker, 2007; Chia and Egan, 2008). In T2DM patients, GLP-1 bioavailability decreases the secretion of insulin and the clinical effect is chronic hyperglycemia (Drucker, 2002).

GLP-1 is sufficiently potent to cure T2DM, however, GLP-1 is metabolized by dipeptidyl peptidase-4 (DPP-4) excessively and becomes inactive (Drucker, 2002; Chia and Egan, 2008). Inhibition of DPP-4 is effective in treating T2DM, therefore GLP-1 bioavailability can be

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retained in addition to regulating blood glucose levels (Brunton *et al.*, 2006). Treatment of T2DM with *incretin-like* drugs or DPP-4 inhibitors shows lesser side effects even though the safety of this drug has not been evaluated with respect to a complete data set (Holst and Orskov, 2004). Nevertheless, incretin-like drugs have side effects, such as flu-like symptoms, skin reactions, and gastrointestinal problems. These effects are exacerbated by the use of the drugs over the long-term (Nauck *et al.*, 2009; Salehi *et al.,* 2008). As such, these phenomena have attracted attention to finding medicinal plants as alternatives to treat T2DM by DPP-4 inhibition.

One of the traditional plants known to have anti-diabetic effects is *Urena lobata*. Root and leaf extract of *U. lobata* have been used empirically by Nigerian people to treat DM. Preclinical testing of *U. lobata* root extract indicated there were anti-hyperglycemic effects on streptozotocin-induced rats (Onoagbe *et al.,* 2010). In Indonesia, *U. lobata* is known by Pulutan, and the plant exhibited anti-bacterial effects based on a preliminary study (Nurfauziah and Mulyani, 1999). Further, a number of studies have shown the anti-diabetic potency of *U. lobata*, however the mechanism of the inhibition of DPP-4 activity has not been investigated yet (Nurfauziah and Mulyani, 1999; Vats *et al.,* 2002; Awika and Rooney, 2004; Onoagbe *et al.,* 2010). Therefore, the current study sought to examine the anti-diabetic effects of *U. lobata* leaf extract on DPP-4 inhibition.

MATERIAL AND METHODS

Preparation of *U. lobata* leaf extract

Urena lobata leaf powder was obtained from Balai Materia Medika Batu Malang with certificate number 074/027/101.8/2015. The *U. lobata* leaf powder (50 g) was extracted according to the decoction method in which the leaf powder was soaked in 250 mL hot water (90ºC) for 30 minutes. The other sample of *U. lobata* leaf powder (50 g) was extracted based on the digestion method by soaking the sample in 250 mL ethanol for five hours using a shaker water bath. The extraction process was repeated twice with fresh solvent. Both of the extract solutions had the solvent removed using a rotary evaporator to obtain crude extracts.

DPP-4 *in vitro* assay

The assay was performed in 96-well micro plates. A pre-incubation volume of 50 µL solution contained 35 µL Tris-HCl buffer, 15 µL DPP-4 enzyme, and various concentrations of test material or standards. This mixture was incubated at 37ºC for 10 minutes followed by addition of 50 µL gly-pro-p-nitroanilide as the substrate. The reaction mixture was incubated for 30 minutes at 37ºC and the absorbance was measured at wavelength 405 nm at 10-second intervals. Vildagliptin was used as the standard reference of the DPP-4 inhibitor. The IC_{50} value was determined by non-linear regression.

Animals and treatments

Male Sprague-Dawley (SD) rats with body weight (180-200 g) were obtained from Gajah Mada University Yogyakarta, Indonesia. The study was conducted according to the ethical guidelines approved by the Commision of Ethical Research, Brawijaya University, Malang, Indonesia, with certificate number 245-KEP-UB. SD rats were housed in individual cages and automatically controlled animal rooms at 25±1ºC on a 12:12-h light–dark cycle. They were fed with standard food and water *ad libitum* and fasted overnight before the experiments. Normal diet (ND) and a high-fructose diet (HFD) foods 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 intraperitoneally. Rats were stated to be diabetic if the fasting blood glucose level was more than 126 mg/dL (Shirwaikar *et al.,* 2016). The experiments were assigned to eight groups of 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 the ethanolic extract (EEU) and water extract of *U. lobata* (WEU) at dosages 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*. (2006). Body weight and food consumption were monitored weekly. Blood samples were obtained 15 minutes after being given the glucose stimulation in dosages of 2 g/kg body weight orally. The blood samples were also taken from the tail vein after overnight fasting. Blood samples were immediately centrifuged at 4500 rpm. The serum was separated and saved under temperature -20ºC.

DPP-4 *in vivo* assay

DPP-4 serum levels were analyzed by rat DPP-4 ELISA kit (Elabscience E-EL-R0337). 100 µL samples were incubated for 90 minutes at 37ºC, and 100 µL of biotinylated detection Ab was added and the mixture was

then incubated for 60 minutes at 37ºC. After aspirating and washing, 100 µL HRP conjugate was added and the mixture was incubated for 30 minutes at 37ºC. A 90 µL substrate reagent was then added to 50 µL *stop solution*. The data of samples were measured with a microplate reader at λ = 450 nm.

GLP-1 *in vivo* assay

GLP-1 serum levels were determined by rat GLP-1 ELISA kit (USCN CEA804). 50 µL samples were added with 50 µL of detection reagent A and then incubated for 60 minutes at 37 ºC. After aspirating and washing, 100 µL of detection reagent B was added to the samples that were next incubated for 30 minutes at 37ºC. A 90 µL substrate reagent was then added to 50 µL *stop solution*. The data of samples were obtained using a microplate reader at λ = 450 nm.

Oral glucose tolerance test

For the glucose tolerance test, glucose was administered orally at a dose of 2 g/kg body weight after overnight fasting. The blood samples were collected from the tail vein at 0 (before glucose administered), 15, 30, 60, 90, and 120 min after glucose administration and they were measured immediately using a commercial glucometer. The integrated area under the postprandial glucose curves (AUCs) was calculated by the trapezoidal method.

Statistical analysis

The data were expressed as means±S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA). The least significant difference (LSD) test or Dunnet C were employed for mean comparisons and $P < 0.05$ was considered to be statistically significant.

RESULTS

The DPP-4 inhibitory activity of *U. lobata* via *in vitro* testing

The DPP-4 inhibitory activity of *U. lobata* leaf extract is presented in Table 1. Based on the results, the *U. lobata* ethanolic extract had greater inhibitory activity on DPP-4 (roughly four-fold) compared to that of water extract.

The DPP-4 inhibitory activity of *U. lobata* via *in vivo* testing

DPP-4 serum levels of diabetic rats with administered *U. lobata* leaf extract are shown in Figure 1. The diabetic group exhibited a significant increase in DPP-4 levels, which is approximately four-fold higher than the normal group ($P < 0.05$). Administration of *U. lobata* water extract at 250 mg/kg, 500 mg/kg, and 1000 mg/kg BW decreased DPP-4 by roughly 60%, 70%, and 70%, respectively, compared to the diabetic group ($P < 0.05$), whereas that of the ethanolic extract diminished DPP-4 levels by 60%, 50%, and 40%, respectively ($P < 0.05$). The results demonstrated that the water extract was able to decrease

Table 1: The DPP-4 inhibitory activity of *U. lobata* leaf extract

Group	Sample	Concentration (ppm)	Percent inhibition	IC_{50} (ppm)
1	Water extract of *U. lobata*	625	0.00±0.00	6489.88
		1250	13.33±0.00	
		2500	26.67±0.00	
		5000	42.22±3.85	
		10000	62.22±3.85	
2	Ethanolic extract of *U. lobata*	625	36.17±0.00	1654.64
		1250	48.94±0.00	
		2500	55.32±0.00	
		5000	61.70±0.00	
		10000	74.47±0.00	
3	Vildagliptin	6.25	8.93±0.00	57.44
		12.50	16.07±4.12	
		25.00	37.50±0.00	
		50.00	46.63±3.85	
		100.00	60.71±0.00	

Letter showed the differences in potency ($P < 0.05$)
The experiments were repeated in triplicate.
The results of % inhibition were expressed as means±S.E.M.

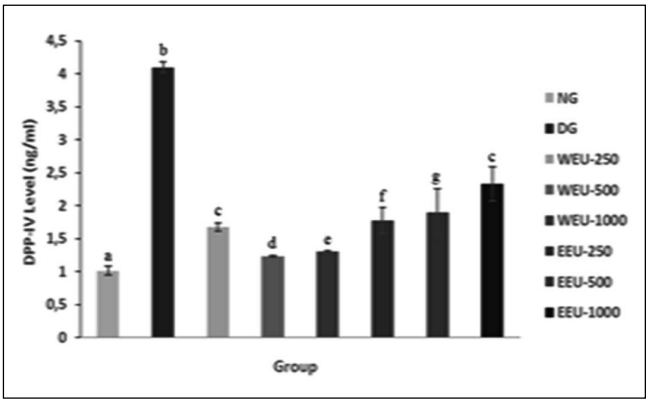

Figure 1: DPP-4 levels with administered *U. lobata* leaf extract.
Note: a, b, c, etc. indicates the differences in potency ($P < 0.05$, Dunnet C test)

DPP-4 levels more so compared to the ethanolic extract ($P < 0.05$) at the same dosages.

Effect of *U. lobata* leaf extract on GLP-1 serum levels

GLP-1 serum levels of diabetic rats that had been administered *U. lobata* leaf extract are portrayed in Figure 2. There is a significant reduction in GLP-1 within the DG by nearly eight-fold compared to the NG ($P < 0.05$). The water extract of *U. lobata* at dosages of 250 mg/kg, 500 mg/kg, and 1000 mg/kg bw prevented degradation of GLP-1 by roughly three-fold, five-fold, and seven-fold, respectively, compared to that of the DG ($P < 0.05$), of which the ethanolic extract showed approximately 5-fold, 2-fold, and 2.5-fold ($P < 0.05$), respectively. For the same dosage, the water extract was able to inhibit more degradation of GLP-1 compared to the ethanolic extract ($P < 0.05$) excepting at a dose of 250 mg/kg BW.

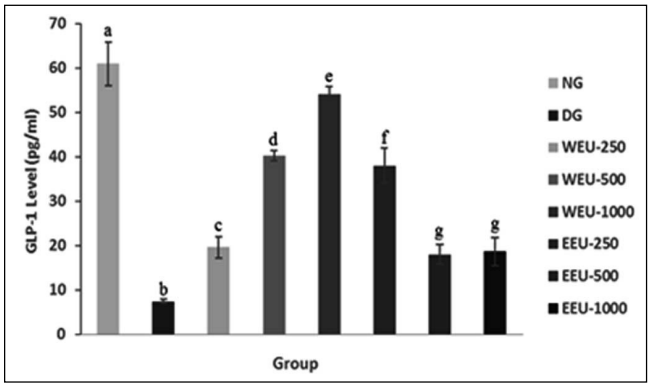

Figure 2: GLP-1 levels with administered *U. lobata* leaf extract
Note: a, b, c, etc. showed the differences of potency ($P < 0.05$, Dunnet C test)

Both administration of water and ethanolic extracts significantly maintained GLP-1 bioavailability in diabetic rats. Mangiferin, stigmasterol, and β-sitosterol in the extract were able to prevent degradation of GLP-1 by DPP-4 inhibition. Based on our previous work, active compounds in *U. lobata* extract act as DPP-4 inhibitors. With this, the water extract ensured more GLP-1 bioavailability compared to the ethanolic extract, and this is because of the difference in active compounds that inhibit GLP-1 degradation by DPP-4. It is also related to the DPP-4 inhibitory activity of active compounds in both water and ethanolic extracts. The effect is regulated by DPP-4 inhibitory activity owing to a synergistic interaction of active compounds and production of complex compounds in the water extract (Brunton *et al.,* 2006). As the result, inhibition of DPP-4 prevents GLP-1 metabolism from inactivation and resulted in the maintenance of their bioavailability.

The effect of *U. lobata* on glucose tolerance testing in diabetic rats

Blood glucose levels of rat administered *U. lobata* extract after stimulating glucose are depicted in Figures 3 and 4. The results indicated that there is a significant increase of the AUC of glucose in the DG by up to 70% compared to the NG ($P < 0.05$). The administration of *U. lobata* water extract at dosages of 250 mg/kg BW, 500 mg/kg, and 1000 mg/kg BW decreased the AUC of glucose by 50%, 60%, and 50%, respectively, compared to that of the DG ($P < 0.05$) whereas the ethanolic extract diminished the AUC of glucose by 50%, 40%, and 20%, respectively ($P < 0.05$).

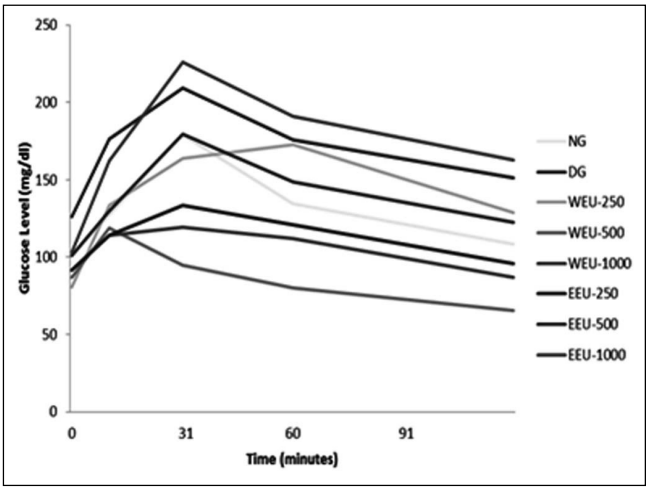

Figure 3: Blood glucose level administered *U. lobata* extract after induction of glucose

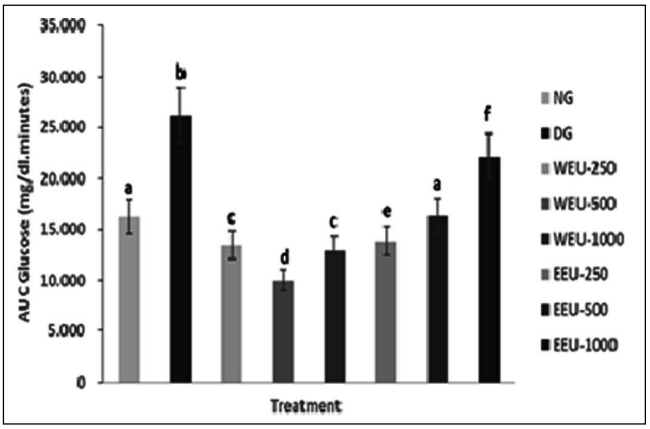

Figure 4: AUC of glucose with administered *U. lobata* extract
Note: a, b, c, etc. indicates the differences in potency ($P < 0.05$, LSD test)

The *U. lobata* water extract exhibited stronger control over the AUC of blood glucose compared to that of ethanolic extract ($P < 0.05$) after glucose stimulation.

DISCUSSION

The semi-qualitative testing of *U. lobata* leaf extract by LC-MS showed the compositions of gossypetin (**1**) in the ethanolic extract are higher than that of in the water extract whereas stigmasterol is more predominant in the water extract (Purnomo *et al.,* 2015). Active compounds, such as stigmasterol (**2**), β-sitosterol (**3**), and gossypetin (**1**) are soluble in less polar solvents, like alcohol, even though mangiferin (**4**) are insoluble. The difference in solubilities of the active compounds in the solvents affects the number of active compounds extracted from the extracts. However, the DPP-4 inhibitory activity of both the water and ethanolic extracts are still lower, approximately 30-100-fold, compared to vildagliptin, the standard reference DPP-4 inhibitor.

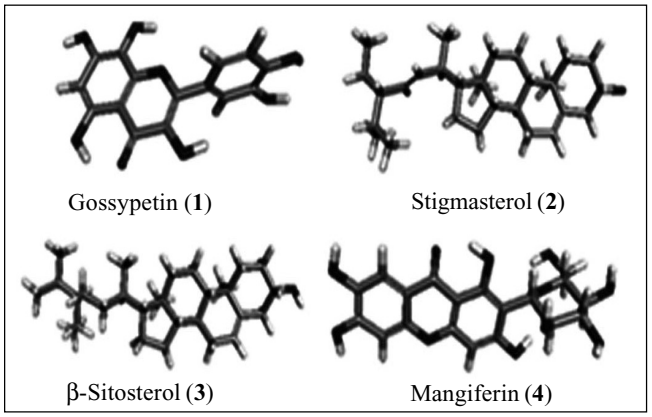

Figure 5: Chemical structures of certain molecules found in *U. lobata* leaf extract

Both of the *U. lobata* water and ethanolic extracts significantly inhibit the DPP-4 activity of diabetic rats. These effects are regulated by active compounds **2**, **3**, and **4** from *U. lobata* based on *in-silico* analysis (Purnomo *et al.,* 2015). The increase dosages of *U. lobata* water extract inhibit more DPP-4 activity whereas the ethanolic extract raises DPP-4 activity precisely. This is based on the conformational shift of active compounds, particularly the sterol group in the ethanolic extract which produces a complex of compounds post-binding (Morris and Lim-Wilby, 2008; Setevens and Honerkamp-Smith, 2010). The change in active compound structure can alter the solubility of active substances and their absorption and resultant modulation of inhibitory activity on DPP-4 (Stevens and Honerkamp-Smith, 2010). Owing to the ethanolic extract being less polar than the water extract, it affects solubilities of active compounds and their absorption within the gastrointestinal system in addition to bioactivity decreasing. The water extract inhibited more DPP-4 activity than that of the ethanolic extract. Complex formed of active substances from the *U. lobata* water extract facilitate the inhibitory activity of DPP-4. A number of active compounds within the water extract have synergistic interactions besides the reinforcement of their inhibitory activity on DPP-4 (Brunton *et al.,* 2006). Inhibition of DPP-4 activity increases the bioavailability of the incretin hormone, thereby contributing to carbohydrate metabolism (Drucker, 2002).

The differences in DPP-4 inhibitory activity between *in vitro* and *in vivo* testing can be explained pharmacokinetically. The poor solubility of active compounds within the ethanolic extract may affect its absorption and distribution in the blood as a result of decreasing the inhibition of DPP-4 activity.

The principal biological activity of DPP-4/CD26 is its enzymatic function. The enzymatic activity of DPP-4/CD26 is exhibited by the membrane-spanning form of the molecule, and the slightly smaller circulating soluble form (Chen, 2006). The substrates of CD26/DPP-4 are not specific to certain peptides. Proline- or alanine-containing peptides are the substrates of DPP-4 and include growth factors, chemokines, neuropeptides, and vasoactive peptides (Mentlein and Gallwitz, 1993). DPP-4 prefers substrates with an amino-terminal proline or alanine at position 2 even though they may also cleave substrates with non-preferred amino acids at position 2 (Mentlein and Gallwitz, 1993; Gopalan *et al.,* 2010). The structure of incretin hormones, such as GLP-1 and GIP, reveals a highly conserved alanine in position 2, rendering these peptides ideal putative substrates for the aminopeptidase, DPP-4 (Gopalan *et al.,* 2010).

The DPP-4 inhibitor prevents the degradation of active GLP-1; however, it does not elevate the levels of circulating total GLP-1 and, further, does not prevent the kidney from rapidly clearing GLP-1. Using DPP-4 inhibitors, primarily for the treatment of diabetes, is related to the potential effects of them on immune function. CD26/DPP-4 plays an important role in tumor biology; It is valuable as a marker for various cancers with its levels either on the cell surface or in the serum increased in certain neoplasms and reduced in others (Havre *et al.,*

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GLP-1 is a super family peptide of glucagon which has a similarity degree of approximately 48%. The similarity of the amino acid sequences between GLP-1 and glucagon is one of the reasons. Pro-glucagon gen was located at chromosome 2q36-q37 and only found in certain tissues whereas the messenger RNA (mRNA) of pro-glucagon was located in the α-cells of the pancreas as well as the L-cells of the intestine and brain's hypothalamus. Pro-glucagon production commences from transcription of preproglucagon gen and is then continued by the translation process. The regulation of GLP-1 release from L-cells of the intestine is a complex mechanism that involves combinations of nutrition, hormones, and neural stimuli (Morris and Lim-Wilby, 2008). The GLP-1 receptor is classified as a *G protein-coupled* receptor that is found in liver, muscle and pancreas cells. This receptor has a specific character by activation of adenyl cyclase and resultant cAMP. After GLP-1 binding with the receptor, it will activate cAMP and mitogen-activated protein kinase (MAPK).

Administration of *the U. lobata* ethanolic and water extracts significantly decreased the AUC of glucose in diabetic rats post-glucose induction. It is controlled by the active compounds in *U. lobata* which inhibit DPP-4 activity, leading to retention of the GLP-1 bioavailability to regulate blood glucose level increases after stimulation through oral nutrition (Drucker, 2002; Holst and Orskov, 2004). GLP-1 acts beyond metabolic purposes, specifically inhibiting gastric juice secretion, GIT motility, and the rate of gastric-emptying functioning (Drucker, 2002; Chia and Egan, 2008). Furthermore, it is beneficial to prevent increasing blood glucose levels post-prandial (Holst and Orskov, 2004; Salehi *et al.,* 2008)

The *U. lobata* water extract regulated more blood glucose levels and post-glucose stimulation compared to the ethanolic extract. This was predicted based on the poor solubility of the active compounds and absorption of the ethanolic extract. As such, it also affects the activities constituting controlling blood glucose levels. The occurrence of reducing the solubility of active compounds is based on the formation of complex compounds and the conformational changes in active compounds as well as the decrease in inhibitory activity of DPP-4 that contributes to maintaining blood glucose levels in the DG (Aronoff *et al.,* 2004; Stevens and Honerkamp-Smith, 2010).

DPP-4 inhibitory activity by the *U. lobata* ethanolic extract was stronger than that of the water extract during *in-vitro* testing but the opposite was found with the *in-vivo* study. In addition, the *U. lobata* water extract demonstrated a more pronounced decrease of DPP-4 levels and the AUC of blood glucose as well as retention of GLP-1 bioavailability compared to the ethanolic extract.

ACKNOWLEDGMENT

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ETHICAL CLEARANCE

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

CONFLICT OF INTEREST

The authors have no conflict of interest to declare

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Research Article

Inhibitory activity of *Urena lobata* leaf extract on dipeptidyl peptidase-4 (DPP-4): is it different *in vitro* and *in vivo*?

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ABSTRACT

This study was aimed to compare the anti diabetic effect of *Urena lobata* leaf extract through DPP-4 inhibitory activity by *in vitro* and *in vivo*. *Urena lobata* leaf was extracted in ethanol and hot water to evaluate its activity on DPP-4 both of *in vitro* and *in vivo*. *In vitro* test using *Gly-pro-p-nitroanilide* (GPPN) as substrate of DPP-4 and the reactions product of them was observed by microplate reader at λ=405 nm furthermore the IC_{50} value was determined. *In vivo* study utilize an animal model of diabetes with 2 control groups and 6 test groups (n=4), in which DPP-4 level, GLP-1 level and AUC of blood glucose were examined after extract administration. The *in vitro* DPP-4 inhibitory activity of ethanolic extract of *U. lobata* is higher than water extract with the IC_{50} value of 1654,64 and 6489,88 µg/ml respectively. However, the water extract of *U. lobata* exhibits stronger decrease DPP-4 level (60-70%) compared to ethanolic extract (40-60%) *in vivo* study as well as the AUC of blood glucose were reduced by 50-60% and 20-50%, respectively. Meanwhile, GLP-1 level could be retained more by the water extract of *U. lobata* administration (3-7 fold) compared to ethanolic extract (2-5 fold) due to the reducing of DPP-4 activity.

Keywords: DPP-4, *in vitro*, *in vivo*, *Urena lobata*, Gly-pro-p-nitroanilide (GPPN)

INTRODUCTION

Recently, treatment of type 2 diabetes mellitus (T2DM) has been proposed with incretin hormone. The majority of incretin hormone is glucagon-like peptide-1 (GLP-1) secreted by intestinal tissues based on the induction of oral nutrition (Drucker, 2007). GLP-1 plays a vital role in maintaining blood glucose levels related to their biology activity, such as to stimulating insulin secretion, increasing β-cell proliferation, inhibiting glucagon secretion, reducing the rate of gastric emptying, and promoting satiety (Drucker, 2007; Chia and Egan, 2008). In T2DM patients, GLP-1 bioavailability decreases the secretion of insulin and the clinical effect is chronic hyperglycemia (Drucker, 2002).

GLP-1 is sufficiently potent to cure T2DM, however, GLP-1 is metabolized by dipeptidyl peptidase-4 (DPP-4) excessively and becomes inactive (Drucker, 2002; Chia and Egan, 2008). Inhibition of DPP-4 is effective in treating T2DM, therefore GLP-1 bioavailability can be

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retained in addition to regulating blood glucose levels (Brunton *et al.*, 2006). Treatment of T2DM with *incretin-like* drugs or DPP-4 inhibitors shows lesser side effects even though the safety of this drug has not been evaluated with respect to a complete data set (Holst and Orskov, 2004). Nevertheless, incretin-like drugs have side effects, such as flu-like symptoms, skin reactions, and gastrointestinal problems. These effects are exacerbated by the use of the drugs over the long-term (Nauck *et al.*, 2009; Salehi *et al.,* 2008). As such, these phenomena have attracted attention to finding medicinal plants as alternatives to treat T2DM by DPP-4 inhibition.

One of the traditional plants known to have anti-diabetic effects is *Urena lobata*. Root and leaf extract of *U. lobata* have been used empirically by Nigerian people to treat DM. Preclinical testing of *U. lobata* root extract indicated there were anti-hyperglycemic effects on streptozotocin-induced rats (Onoagbe *et al.,* 2010). In Indonesia, *U. lobata* is known by Pulutan, and the plant exhibited anti-bacterial effects based on a preliminary study (Nurfauziah and Mulyani, 1999). Further, a number of studies have shown the anti-diabetic potency of *U. lobata*, however the mechanism of the inhibition of DPP-4 activity has not been investigated yet (Nurfauziah and Mulyani, 1999; Vats *et al.,* 2002; Awika and Rooney, 2004; Onoagbe *et al.,* 2010). Therefore, the current study sought to examine the anti-diabetic effects of *U. lobata* leaf extract on DPP-4 inhibition.

MATERIAL AND METHODS

Preparation of *U. lobata* leaf extract

Urena lobata leaf powder was obtained from Balai Materia Medika Batu Malang with certificate number 074/027/101.8/2015. The *U. lobata* leaf powder (50 g) was extracted according to the decoction method in which the leaf powder was soaked in 250 mL hot water (90ºC) for 30 minutes. The other sample of *U. lobata* leaf powder (50 g) was extracted based on the digestion method by soaking the sample in 250 mL ethanol for five hours using a shaker water bath. The extraction process was repeated twice with fresh solvent. Both of the extract solutions had the solvent removed using a rotary evaporator to obtain crude extracts.

DPP-4 *in vitro* assay

The assay was performed in 96-well micro plates. A pre-incubation volume of 50 µL solution contained 35 µL Tris-HCl buffer, 15 µL DPP-4 enzyme, and various concentrations of test material or standards. This mixture was incubated at 37ºC for 10 minutes followed by addition of 50 µL gly-pro-p-nitroanilide as the substrate. The reaction mixture was incubated for 30 minutes at 37ºC and the absorbance was measured at wavelength 405 nm at 10-second intervals. Vildagliptin was used as the standard reference of the DPP-4 inhibitor. The IC_{50} value was determined by non-linear regression.

Animals and treatments

Male Sprague-Dawley (SD) rats with body weight (180-200 g) were obtained from Gajah Mada University Yogyakarta, Indonesia. The study was conducted according to the ethical guidelines approved by the Commision of Ethical Research, Brawijaya University, Malang, Indonesia, with certificate number 245-KEP-UB. SD rats were housed in individual cages and automatically controlled animal rooms at 25±1ºC on a 12:12-h light–dark cycle. They were fed with standard food and water *ad libitum* and fasted overnight before the experiments. Normal diet (ND) and a high-fructose diet (HFD) foods 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 intraperitoneally. Rats were stated to be diabetic if the fasting blood glucose level was more than 126 mg/dL (Shirwaikar *et al.,* 2016). The experiments were assigned to eight groups of 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 the ethanolic extract (EEU) and water extract of *U. lobata* (WEU) at dosages 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*. (2006). Body weight and food consumption were monitored weekly. Blood samples were obtained 15 minutes after being given the glucose stimulation in dosages of 2 g/kg body weight orally. The blood samples were also taken from the tail vein after overnight fasting. Blood samples were immediately centrifuged at 4500 rpm. The serum was separated and saved under temperature -20ºC.

DPP-4 *in vivo* assay

DPP-4 serum levels were analyzed by rat DPP-4 ELISA kit (Elabscience E-EL-R0337). 100 µL samples were incubated for 90 minutes at 37ºC, and 100 µL of biotinylated detection Ab was added and the mixture was

then incubated for 60 minutes at 37ºC. After aspirating and washing, 100 µL HRP conjugate was added and the mixture was incubated for 30 minutes at 37ºC. A 90 µL substrate reagent was then added to 50 µL *stop solution*. The data of samples were measured with a microplate reader at λ = 450 nm.

GLP-1 *in vivo* assay

GLP-1 serum levels were determined by rat GLP-1 ELISA kit (USCN CEA804). 50 µL samples were added with 50 µL of detection reagent A and then incubated for 60 minutes at 37 ºC. After aspirating and washing, 100 µL of detection reagent B was added to the samples that were next incubated for 30 minutes at 37ºC. A 90 µL substrate reagent was then added to 50 µL *stop solution*. The data of samples were obtained using a microplate reader at λ = 450 nm.

Oral glucose tolerance test

For the glucose tolerance test, glucose was administered orally at a dose of 2 g/kg body weight after overnight fasting. The blood samples were collected from the tail vein at 0 (before glucose administered), 15, 30, 60, 90, and 120 min after glucose administration and they were measured immediately using a commercial glucometer. The integrated area under the postprandial glucose curves (AUCs) was calculated by the trapezoidal method.

Statistical analysis

The data were expressed as means±S.E.M. Statistical analysis was performed by one-way analysis of variance (ANOVA). The least significant difference (LSD) test or Dunnet C were employed for mean comparisons and $P < 0.05$ was considered to be statistically significant.

RESULTS

The DPP-4 inhibitory activity of *U. lobata* via *in vitro* testing

The DPP-4 inhibitory activity of *U. lobata* leaf extract is presented in Table 1. Based on the results, the *U. lobata* ethanolic extract had greater inhibitory activity on DPP-4 (roughly four-fold) compared to that of water extract.

The DPP-4 inhibitory activity of *U. lobata* via *in vivo* testing

DPP-4 serum levels of diabetic rats with administered *U. lobata* leaf extract are shown in Figure 1. The diabetic group exhibited a significant increase in DPP-4 levels, which is approximately four-fold higher than the normal group ($P < 0.05$). Administration of *U. lobata* water extract at 250 mg/kg, 500 mg/kg, and 1000 mg/kg BW decreased DPP-4 by roughly 60%, 70%, and 70%, respectively, compared to the diabetic group ($P < 0.05$), whereas that of the ethanolic extract diminished DPP-4 levels by 60%, 50%, and 40%, respectively ($P < 0.05$). The results demonstrated that the water extract was able to decrease

Table 1: The DPP-4 inhibitory activity of *U. lobata* leaf extract

Group	Sample	Concentration (ppm)	Percent inhibition	IC_{50} (ppm)
1	Water extract of *U. lobata*	625	0.00±0.00	6489.88
		1250	13.33±0.00	
		2500	26.67±0.00	
		5000	42.22±3.85	
		10000	62.22±3.85	
2	Ethanolic extract of *U. lobata*	625	36.17±0.00	1654.64
		1250	48.94±0.00	
		2500	55.32±0.00	
		5000	61.70±0.00	
		10000	74.47±0.00	
3	Vildagliptin	6.25	8.93±0.00	57.44
		12.50	16.07±4.12	
		25.00	37.50±0.00	
		50.00	46.63±3.85	
		100.00	60.71±0.00	

Letter showed the differences in potency ($P < 0.05$)
The experiments were repeated in triplicate.
The results of % inhibition were expressed as means±S.E.M.

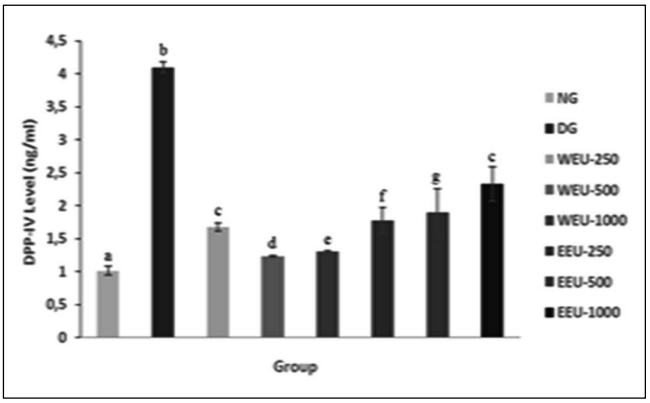

Figure 1: DPP-4 levels with administered *U. lobata* leaf extract.
Note: a, b, c, etc. indicates the differences in potency ($P < 0.05$, Dunnet C test)

DPP-4 levels more so compared to the ethanolic extract ($P < 0.05$) at the same dosages.

Effect of *U. lobata* leaf extract on GLP-1 serum levels

GLP-1 serum levels of diabetic rats that had been administered *U. lobata* leaf extract are portrayed in Figure 2. There is a significant reduction in GLP-1 within the DG by nearly eight-fold compared to the NG ($P < 0.05$). The water extract of *U. lobata* at dosages of 250 mg/kg, 500 mg/kg, and 1000 mg/kg bw prevented degradation of GLP-1 by roughly three-fold, five-fold, and seven-fold, respectively, compared to that of the DG ($P < 0.05$), of which the ethanolic extract showed approximately 5-fold, 2-fold, and 2.5-fold ($P < 0.05$), respectively. For the same dosage, the water extract was able to inhibit more degradation of GLP-1 compared to the ethanolic extract ($P < 0.05$) excepting at a dose of 250 mg/kg BW.

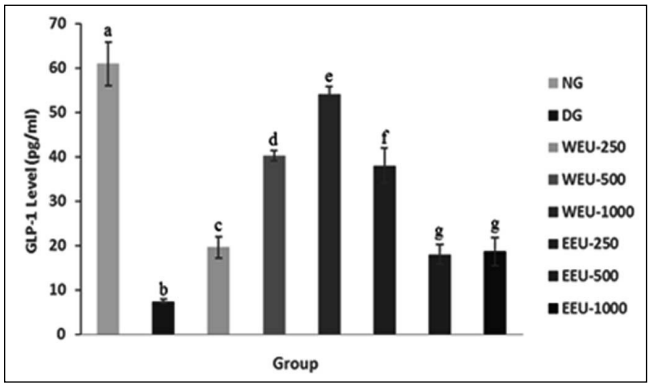

Figure 2: GLP-1 levels with administered *U. lobata* leaf extract
Note: a, b, c, etc. showed the differences of potency ($P < 0.05$, Dunnet C test)

Both administration of water and ethanolic extracts significantly maintained GLP-1 bioavailability in diabetic rats. Mangiferin, stigmasterol, and β-sitosterol in the extract were able to prevent degradation of GLP-1 by DPP-4 inhibition. Based on our previous work, active compounds in *U. lobata* extract act as DPP-4 inhibitors. With this, the water extract ensured more GLP-1 bioavailability compared to the ethanolic extract, and this is because of the difference in active compounds that inhibit GLP-1 degradation by DPP-4. It is also related to the DPP-4 inhibitory activity of active compounds in both water and ethanolic extracts. The effect is regulated by DPP-4 inhibitory activity owing to a synergistic interaction of active compounds and production of complex compounds in the water extract (Brunton *et al.,* 2006). As the result, inhibition of DPP-4 prevents GLP-1 metabolism from inactivation and resulted in the maintenance of their bioavailability.

The effect of *U. lobata* on glucose tolerance testing in diabetic rats

Blood glucose levels of rat administered *U. lobata* extract after stimulating glucose are depicted in Figures 3 and 4. The results indicated that there is a significant increase of the AUC of glucose in the DG by up to 70% compared to the NG ($P < 0.05$). The administration of *U. lobata* water extract at dosages of 250 mg/kg BW, 500 mg/kg, and 1000 mg/kg BW decreased the AUC of glucose by 50%, 60%, and 50%, respectively, compared to that of the DG ($P < 0.05$) whereas the ethanolic extract diminished the AUC of glucose by 50%, 40%, and 20%, respectively ($P < 0.05$).

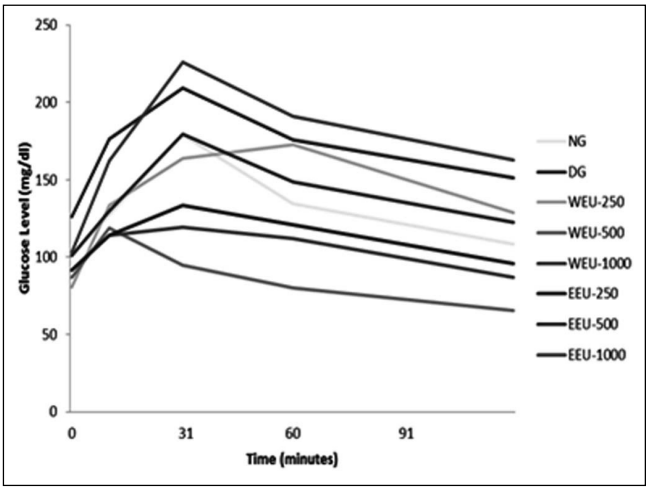

Figure 3: Blood glucose level administered *U. lobata* extract after induction of glucose

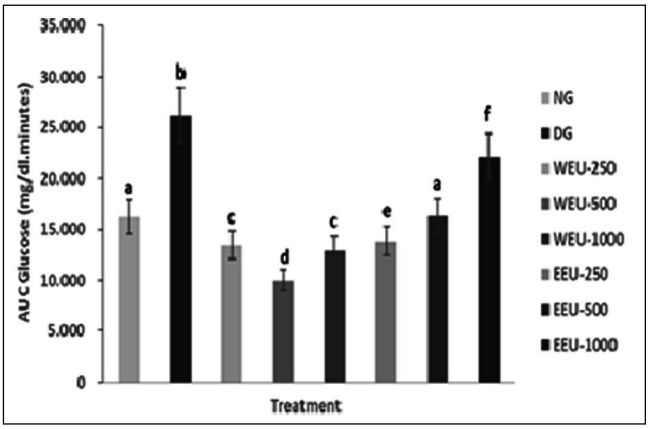

Figure 4: AUC of glucose with administered *U. lobata* extract
Note: a, b, c, etc. indicates the differences in potency ($P < 0.05$, LSD test)

The *U. lobata* water extract exhibited stronger control over the AUC of blood glucose compared to that of ethanolic extract ($P < 0.05$) after glucose stimulation.

DISCUSSION

The semi-qualitative testing of *U. lobata* leaf extract by LC-MS showed the compositions of gossypetin (**1**) in the ethanolic extract are higher than that of in the water extract whereas stigmasterol is more predominant in the water extract (Purnomo *et al.,* 2015). Active compounds, such as stigmasterol (**2**), β-sitosterol (**3**), and gossypetin (**1**) are soluble in less polar solvents, like alcohol, even though mangiferin (**4**) are insoluble. The difference in solubilities of the active compounds in the solvents affects the number of active compounds extracted from the extracts. However, the DPP-4 inhibitory activity of both the water and ethanolic extracts are still lower, approximately 30-100-fold, compared to vildagliptin, the standard reference DPP-4 inhibitor.

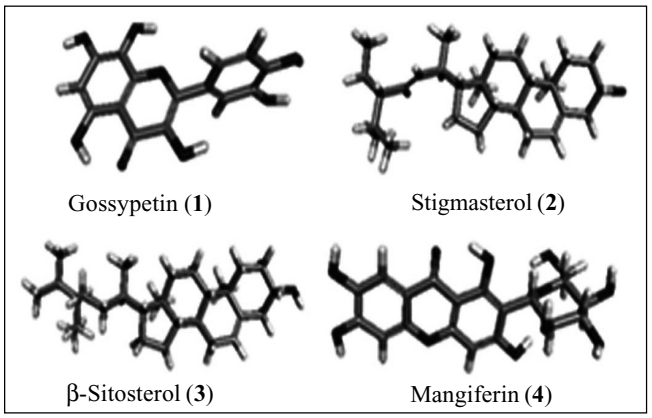

Figure 5: Chemical structures of certain molecules found in *U. lobata* leaf extract

Both of the *U. lobata* water and ethanolic extracts significantly inhibit the DPP-4 activity of diabetic rats. These effects are regulated by active compounds **2**, **3**, and **4** from *U. lobata* based on *in-silico* analysis (Purnomo *et al.,* 2015). The increase dosages of *U. lobata* water extract inhibit more DPP-4 activity whereas the ethanolic extract raises DPP-4 activity precisely. This is based on the conformational shift of active compounds, particularly the sterol group in the ethanolic extract which produces a complex of compounds post-binding (Morris and Lim-Wilby, 2008; Setevens and Honerkamp-Smith, 2010). The change in active compound structure can alter the solubility of active substances and their absorption and resultant modulation of inhibitory activity on DPP-4 (Stevens and Honerkamp-Smith, 2010). Owing to the ethanolic extract being less polar than the water extract, it affects solubilities of active compounds and their absorption within the gastrointestinal system in addition to bioactivity decreasing. The water extract inhibited more DPP-4 activity than that of the ethanolic extract. Complex formed of active substances from the *U. lobata* water extract facilitate the inhibitory activity of DPP-4. A number of active compounds within the water extract have synergistic interactions besides the reinforcement of their inhibitory activity on DPP-4 (Brunton *et al.,* 2006). Inhibition of DPP-4 activity increases the bioavailability of the incretin hormone, thereby contributing to carbohydrate metabolism (Drucker, 2002).

The differences in DPP-4 inhibitory activity between *in vitro* and *in vivo* testing can be explained pharmacokinetically. The poor solubility of active compounds within the ethanolic extract may affect its absorption and distribution in the blood as a result of decreasing the inhibition of DPP-4 activity.

The principal biological activity of DPP-4/CD26 is its enzymatic function. The enzymatic activity of DPP-4/CD26 is exhibited by the membrane-spanning form of the molecule, and the slightly smaller circulating soluble form (Chen, 2006). The substrates of CD26/DPP-4 are not specific to certain peptides. Proline- or alanine-containing peptides are the substrates of DPP-4 and include growth factors, chemokines, neuropeptides, and vasoactive peptides (Mentlein and Gallwitz, 1993). DPP-4 prefers substrates with an amino-terminal proline or alanine at position 2 even though they may also cleave substrates with non-preferred amino acids at position 2 (Mentlein and Gallwitz, 1993; Gopalan *et al.,* 2010). The structure of incretin hormones, such as GLP-1 and GIP, reveals a highly conserved alanine in position 2, rendering these peptides ideal putative substrates for the aminopeptidase, DPP-4 (Gopalan *et al.,* 2010).

The DPP-4 inhibitor prevents the degradation of active GLP-1; however, it does not elevate the levels of circulating total GLP-1 and, further, does not prevent the kidney from rapidly clearing GLP-1. Using DPP-4 inhibitors, primarily for the treatment of diabetes, is related to the potential effects of them on immune function. CD26/DPP-4 plays an important role in tumor biology; It is valuable as a marker for various cancers with its levels either on the cell surface or in the serum increased in certain neoplasms and reduced in others (Havre *et al.,*

2008). It is related to the inhibition of T cell proliferation in the immune system and, moreover, it loses its biological activity in protecting against neoplasms (Chen, 2006; Havre *et al.,* 2008).

GLP-1 has a potency for T2DM therapy but it is metabolized excessively by DPP-4 and becomes the inactive form (Drucker, 2002; Nauck *et al.*, 2009). GLP-1 has a short half-life, approximately for two to five minutes, and this is based on DPP-4 activity (Drucker, 2002; Salehi *et al.,* 2008). The active forms of GLP-1 are GLP-1 (7-36) amides and GLP-1 (7-37), which are rapidly inactivated by DPP-4 through cleavage of the N-terminal dipeptide, His-Ala. This results in the production of the inactive forms of GLP-1, namely GLP-1 (9-36) amide and GLP-1 (9-37) isopeptides (Nauck *et al.*, 2009; Salehi *et al.,* 2008). Previous studies have shown that the importance of DPP-4-mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity (Stevens and Honerkamp-Smith, 2010).

GLP-1 is a super family peptide of glucagon which has a similarity degree of approximately 48%. The similarity of the amino acid sequences between GLP-1 and glucagon is one of the reasons. Pro-glucagon gen was located at chromosome 2q36-q37 and only found in certain tissues whereas the messenger RNA (mRNA) of pro-glucagon was located in the α-cells of the pancreas as well as the L-cells of the intestine and brain's hypothalamus. Pro-glucagon production commences from transcription of preproglucagon gen and is then continued by the translation process. The regulation of GLP-1 release from L-cells of the intestine is a complex mechanism that involves combinations of nutrition, hormones, and neural stimuli (Morris and Lim-Wilby, 2008). The GLP-1 receptor is classified as a *G protein-coupled* receptor that is found in liver, muscle and pancreas cells. This receptor has a specific character by activation of adenyl cyclase and resultant cAMP. After GLP-1 binding with the receptor, it will activate cAMP and mitogen-activated protein kinase (MAPK).

Administration of *the U. lobata* ethanolic and water extracts significantly decreased the AUC of glucose in diabetic rats post-glucose induction. It is controlled by the active compounds in *U. lobata* which inhibit DPP-4 activity, leading to retention of the GLP-1 bioavailability to regulate blood glucose level increases after stimulation through oral nutrition (Drucker, 2002; Holst and Orskov, 2004). GLP-1 acts beyond metabolic purposes, specifically inhibiting gastric juice secretion, GIT motility, and the rate of gastric-emptying functioning (Drucker, 2002; Chia and Egan, 2008). Furthermore, it is beneficial to prevent increasing blood glucose levels post-prandial (Holst and Orskov, 2004; Salehi *et al.,* 2008)

The *U. lobata* water extract regulated more blood glucose levels and post-glucose stimulation compared to the ethanolic extract. This was predicted based on the poor solubility of the active compounds and absorption of the ethanolic extract. As such, it also affects the activities constituting controlling blood glucose levels. The occurrence of reducing the solubility of active compounds is based on the formation of complex compounds and the conformational changes in active compounds as well as the decrease in inhibitory activity of DPP-4 that contributes to maintaining blood glucose levels in the DG (Aronoff *et al.,* 2004; Stevens and Honerkamp-Smith, 2010).

DPP-4 inhibitory activity by the *U. lobata* ethanolic extract was stronger than that of the water extract during *in-vitro* testing but the opposite was found with the *in-vivo* study. In addition, the *U. lobata* water extract demonstrated a more pronounced decrease of DPP-4 levels and the AUC of blood glucose as well as retention of GLP-1 bioavailability compared to the ethanolic extract.

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ETHICAL CLEARANCE

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

CONFLICT OF INTEREST

The authors have no conflict of interest to declare

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