APJTB_MANUSCRIPT AWAL

Anti-diabetic Potential of *Urena lobata* Leaf Extract through Inhibition of Dipeptidyl Peptidase IV (DPP-IV) Activity.

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ABSTRACT

Objective: Glucagon Like Peptide-1 (GLP-1) is one of incretin hormones which has been proposed as a new treatment for Type 2 Diabetes Mellitus (T2DM). However, this hormone is metabolized excessively by Dipeptidyl Peptidase IV (DPP-IV) into inactive form. The inhibition of DPP-IV can enhance GLP-1 bioavailability and regulate blood glucose level, therefore it would be beneficial in the treatment of T2DM. *Urena lobata* is the plant that can be found in Indonesia and has been used to cure many diseases. The objective of this study was to evaluate the anti-diabetic potential of leaf extract from *U. lobata* on DPP-IV inhibitory activity.

Methods: *U. lobata* leaf was extracted in hot water and ethanol. The activity of DPP-IV inhibitor was tested by in-vitro study using *Gly-pro-p-nitroanilide* (GPPN) as substrat of DPP-IV and Vildagliptin, as standard reference. p-nitroanilida, a product of the reactions between GPPN and DPP-IV, was observed by microplatereader with λ=405 nm. All data were expressed as the mean ± SD and the IC-50 value was determined by non linear regression curve fit. Active substances in leaf extract of *U. lobata* was analyzed by Liquid Chromatography–Mass Spectra (LC-MS). DPP-IV inhibitory activity of active compounds was evaluated by in-silico using docking server.

Results: The ethanolic extract of *U. lobata* showed that DPP-IV inhibitor activity were stronger than water extract with the IC-50 value was 1654.64 and 6489.88 μg/ml, respectively. Vildagliptin, based on standard reference for DPP-IV inhibitor activity, have IC-50 value at 57.44 μg/ml. Based on in-silico analysis, Mangiferin, Stigmasterol and β-sitosterol in *U. lobata* extract have a strong inhibitory activity on DPP-IV.

Conclusions: *U. lobata* showed that DPP-IV inhibitory activity is related to its active compounds such as Mangiferin, Stigmasterol and β-sitosterol.

KEYWORDS*:* anti-diabetic, Dipeptidyl peptidase-IV, in-silico, in-vitro, *U.lobata*

1. Introduction

Recently, the treatment of type 2 Diabetes mellitus (T2DM) is focused on incretin hormone. Glucagon Like Peptide-1 (GLP-1) and Glucose Dependent Insulinotropic Polypeptide (GIP) are a major incretin hormone which is secreted by intestinal cells. GLP-1 plays a role in regulation of blood glucose level due to their biological actions, such as

stimulating the secretion of insulin, increasing β-cell masses, inhibiting the secretion of glucagon, reducing the rate of gastric-emptying and inducing satiety [1,2]. However, GLP-1 is rapidly metabolized by the enzyme called Dipeptidyl peptidase-IV (DPP-IV) into inactive forms. Therefore, the GLP-1 has a short half-life, approximately for 1-2 minutes. Inhibition of DPP-IV maintain the level of endogenous active GLP-1 and prolongs its half life [1,3].

DPP-IV inhibitor has the potential to be a novel, efficient and considerable as approaching method to treat T2DM [3]. The usage of DPP-IV inhibitor drugs have less side effects like hypoglycemia, increasing body weight and GIT disorders [4]. The studies of oral glucose tolerance test on animals showed that genetic deletion of DPP-IV have been improved in glucose tolerance and increased the insulin secretion [5]. In the other hand, the complete data of long terms use of synthetic drugs of DPP-IV inhibitor have not been obtained yet, especially on its safety [6]. It induces the research of DPP-IV inhibitor compounds from herbs that have less side effects, cheaper and easier to get.

Urena lobata is the plant that can be found in Indonesia and has been used to cure many diseases. Based on experiences, Nigerian people used *U. lobata* to treat DM because of their biology activities [7]. The study showed that administration of *U. lobata* roots extract had anti-hyperglycemic on rat which has been induced before by streptozotocin [8]. It related to active substances in *U.lobata* such as sterol groups, alkaloid and flavonoid [9,10]. Anti-diabetic potential of *U. lobata* has not been evaluated yet, especially on the DPP-IV inhibitor activity, therefore it is an opportunity to expand herbs that can become candidate of phytopharmaca. The aim of this study was to know the anti-diabetic potencial of *U. lobata* leaf extract on DPP-IV inhibitor activity.

2. Material and methods

2.1 Chemical Sample

DPP-IV was obtained from porcine kidney, Gly-pro-p-nitroanilide (GPPN), Tris-HCl buffer. All chemicals were purchased from Sigma aldrich.

2.2 Sample prepration

U. lobata leaf powder was obtained from Materia Medika Batu Malang with certificate number 074/027/101.8/2015. Then, 50 g of the powdered plant materials were extracted in 250 ml hot water 90ºC for 30 minutes or called a decoctation methods. In the same weight, *U.lobata* powder was extracted in 250 ml ethanol for 4 hours by waterbath shacker and repeated 2 times with fresh ethanol. Both of the extracts were then evaporated.

2.3 Identification of active compounds

Both of water and ethanol extract were analyzed on a semi qualitative scale by Liquid Chromatography–Mass Spectra (LC-MS) Accela 1250 pump. Liquid phase contains 0.1 % formic acid in methanol and water. The identification included the 10 active substances from alkaloid, fitosterol and flavonoid groups.

2.4 Dipeptidyl peptidase IV 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-IV 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 [11].

2.4 Molecular docking studies

DPP-IV inhibitor activity of active compounds in *U.lobata* leaf extracts was evaluated by in silico study using a web-based software application (www.dockingserver.com) for protein and ligand molecular docking. Free energy binding, inhibition constanta and surface interactions were analyzed by this methods to measure the inhibitor activity of active compounds on DPP-IV.

2.6 Statistical Analysis

All data are expressed as the mean ± SD. The IC-50 was determined by non-linear regression curve fit. The statistical data were done by SPSS One Way ANOVA test and then followed by Least Significant Difference (LSD) with significant-value ($p < 0.05$).

3. Results

3.1 DPP-IV inhibitory activity of U. lobata

Both of water and ethanol *U. lobata* leaf extracts were tested on Dipeptidyl peptidase-IV (DPP-IV) inhibitory assay by in vitro method. The DPP-IV inhibitor activity is shown in Table 1.

Table 1. DPP-IV inhibitory activity of *U. lobata* leaf extracts and Vildagliptin

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)

Based on these results, ethanolic extract of *U.lobata* showed that the activity on DPP-IV inhibition was stronger, about 4 times folds, compared to water extract ($p<0.05$). However, the DPP-IV 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-IV inhibitor ($p<0.05$).

3.2 Identification of active compounds in U.lobata leaf extracts

The Active compounds, both in water and ethanol leaf extract of *U.lobata,* can be seen in the Table 2.

Table 2. Active compounds in *U.lobata* leaf extracts

No	Active compounds	Molecule weight	Water extract	Ethanolic extract
1	Stigmasterol	413	(+++)	(+++)
2	B-Sitosterol	415	(++)	(+)
3	Mangiferin	423	(+)	(+)
4	Quercetine	303	(-)	(-)
5	Kaempferol	286	(-)	(-)
6	Hypolaetin	302	(-)	(-)
7	Gossypetin	318	(+)	(++)
8	Luteolin	286	(-)	(-)
9	Apigenin	270	(-)	(-)
10	Chrysoeriol	300	(+)	(+)

The semi-qualitative analysis by LC-MS showed that the most abundant of active compounds both in water and ethanolic extract of *U. lobata* were stigmasterol, gossypetin and β-sitosterol. Active compounds such as mangiferin and chrysoeriol were also identified both in water and ethanolic extracts of *U.lobata* and showed that the concentration value was low.

3.3 Molecular docking of U.lobata leaf extracts on DPP-IV

Inhibitor activity of *U.lobata* leaf extracts on DPP-IV was evaluated by in-silico study. Active compounds that were identified in *U.lobata* as ligand then docked with DPP-IV as protein target and the results can be seen at Table 3.

Table 3. Molecular docking of active compounds in *U.lobata* leaf extracts

No	Active compounds	Est. Free Energy of Binding (Kcal/mol)	Est. Inhibition Constant Ki (µM)	Interact. Surface
1	Stigmasterol	-7.42	3.62	962.48
2	B-Sitosterol	-6.59	14.67	886.91
3	Mangiferin	-7.66	2.43	742.75
4	Gossypetin	-5.2	153.42	552.29
5	Chrysoeriol	-4.66	386.05	539.84

Docking studies showed that mangiferin, stigmasterol and β-sitosterol have a low value in both of the binding free energy and the inhibition constant but the surface interaction was high. However, gossypetin and chrysoeriol have a higher value on binding free energy and inhibition constant than other substances above. The differences in each parameter value causes the distinction in inhibitory activity on DPP-IV.

4. Discussion

4.1 Identification of Active compounds in U.lobata leaf extracts

Five active compounds were identified in *U.lobata* leaf extract and had been found on both of in water extract and ethanol. It is only different in the quantity or composition of active compounds in both extract. They are stigmasterol gossypetin, β-sitosterol, mangiferin and chrysoeriol. All of them are classified in secondary metabolite groups and have biological activity that can be used to cure the diseases. Stigmasterol is one of a group of plant sterols or phytosterols that are chemically similar to animal cholesterol. Phytosterols are insoluble in water but soluble in most organic solvents and contain one alcohol functional group. Stigmasterol is an unsaturated plant sterol occurring in the plant fats or oils of soybean, calabar bean, rape seed, and in various medicinal herbs. Studies about laboratory animals treated by stigmasterol found that both cholesterol and sitosterol absorption decreased 23% and 30%, respectively, over a 6-week period. It also possesses a potential antioxidant, hypoglycemic and thyroid inhibiting properties [12,13].

Gossypetin is flavonol or flavone, a type of flavonoid. It has been isolated originally from the flowers and the calyx of Hisbiscus species. Gossypetin shows a high antioxidant, anti-microbial, anti-mutagenic and anti-atherosclerotic [14]. This compound is very soluble in chloroform and benzene, and also moderately soluble in ethanol and ether, but insoluble in water.

β-sitosterol is one of several phytosterols or plant sterols with chemical structure similar to that of cholesterol. Sterols are isoprenoid-derived molecules that have essential functions typically in eukaryotes, and especially in higher plants. β-sitosterol are white, waxy powder

with the specific characteristic of odor. They are hydrophobic and soluble in ethanol and chloroform but insoluble in water [15]. It can be found in avocados, cucurbita pepo, corn oil and soy beans also it showed anti cholesterol, anti-inflamatory and immunomodulator effects [16].

Mangiferin is a xanthonoid, and a glucoside of norathyriol. It was found in Mangoes, *Iris unguicularis* and *Anemarrhena asphedelous*. Mangiferin is soluble in hot dilutes ethanol and methanol but insoluble in water. Laboratory study has identified a variety of pharmacology effect that associated with mangiferin including anti-microbial, antioxidant activity, and anti-diabetic effect in rodent [17,18].

Chrysoeriol is a flavon, one of major flavonoid classes. They provide many health-promoting benefits such as anti inflammation and anti histamine. It is soluble in alkalies solution and sufficiently soluble in water [19].

The presence of active compounds in extract were influenced by polarity and extract solvent. Type of extract solvent certain the composition of active compound in extract due to the difference of their solubility in solvent. Secondly, polarity of active compound also contribute to their solubility in solvent. Alkaloid, terpenoid and steroid soluble in non polar solvent like acetone, diethyl ether and hexane. Meanwhile, flavonoid, phenol and glycoside solve in polar solvent such as water and methanol [20,21]. It is appropriate with the determinate solubility theory “like dissolve like” that polar substances will dissolve in polar solvent and vice versa [20, 22].

Generally, plants contain two major substances that they are a nutrition compound and non nutrition. Primary metabolite or nutrition compounds such as carbohydrate, protein, fatty acids and phytosterol can be found in a huge proportion but they do not have pharmacology effect. On the other hand, non nutrition compounds or secondary metabolite like alkaloid, terpenoid, flavonoid and steroid are met in a small concentration but it have pharmacology effect in certain dose [20]. Secondary metabolites are derived from metabolism of primary metabolite in plant but sometimes they have a toxic effect especially if it is used in large high dose. Most of flavonoid and terpenoid in herbs have a potency as antioxidant, antiseptic and anti-inflammatory whereas steroid as anti-inflammatory and sex hormone. But, the pharmacology effect of alkaloid is difficult to be predicted in medicinal plants due to they have so many biological activity activities[23].

Anti-diabetic effect of herbs are indicated by their potency to decrease blood glucose level. The hypoglycemia effect is controlled by active compounds likes terpenoid, steroid, alkaloid and flavonoid but their mechanisms of work are different. Some of herbs works as anti-diabetes by mechanisms such as insulin sensitizers, insulin secretory, DPP-IV inhibitor and α-glucosidase inhibitor [24]. Anti-diabetic herbs have many active compounds so that it have a possibility to work by multiple action and result an interactions either synergistic or antagonistic effect. Sometimes the interaction have both of negative and positive pharmacology effect [4].

4.2 Molecular docking of U.lobata leaf extracts on DPP-IV

Molecular docking is now widely used to discover new ligands for target of known structure. Potential compound can screened by free energy binding. The score of free energy

binding represent of binding affinity of ligand to the target protein, the lower free energy binding, the higher binding affinity [25]. In addition inhibition constant (Ki) can be predicted using bioinformatics approach. The lowest Ki is the most potential compound. Other parameter is surface interaction, it represent the molecular recognition between ligand and target protein. The higher value of surface interaction, the higher the interaction possibilities of compounds interacting with the target protein [26]. Based on those categories, mangiferin have the lowest value of inhibition constants and followed by stigmasterol activity on DPP-IV. It is related to binding free energy and surface interaction of these compounds. In this study, stigmasterol have the highest value of surface interaction that followed by β–sitosterol and mangiferin respectively. A great result of surface interaction showed a stronger bond between ligand and protein target so that the biology activity is higher. Based on the in silico analysis, mangiferin have the lowest value in binding free energy while stigmasterol and β–sitosterol were in the second and third position. The lowest value of binding free energy produces a strong binding molecule and then causes the potential biology activity. Free energy binding and surface interaction between ligand and protein target affects the inhibitory activity of *U.lobata* leaf extract on DPP-IV.

Molecular docking studies are widely used to predict the potential candidates of drugs in the pharmaceutical industry. Binding orientation of these small molecules or active compounds to their protein targets reveals their affinity and activity as possible candidates of drugs.

4.3 DPP-IV inhibitory activity of U. lobata

DPP-IV inhibitory activity of ethanolic extract of *U.lobata* are stronger than that of water extract. 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 Stigmasterol, β- sitosterol, Gossypetin and Chrysoeriol which higher than that of Mangiferin, Quercetine and Hypolaetin. Active compounds such as Stigmasterol, β-sitosterol and Gossypetin are soluble in semi-polar solvents like alcohol but 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.

Both of ethanolic and water extracts of *U.lobata* contain the same active compounds but different in amounts. Composition of Stigmasterol, β-sitosterol and Gossypetin are lower in water extract meanwhile the proportion of Chrysoeriol, Mangiferin, Quercetine and Hypolaetin are similar both of in water and ethanolic extract. Non-polar compounds such as Stigmasterol, β-sitosterol and Gossypetin could be extracted in water solvent eventhough in small amount. When the water is boiled, their polarity will decrease so that it could be extracted from semi-polar until non-polar compounds [27].

Molecular docking study of *U. lobata* leaf extract showed inhibitory activity on DPP-IV. Three active compounds such as Mangiferin, Stigmasterol and β-sitosterol have a low binding free energy. It means that the ability of binding between ligand and molecule target is easy so that causing a strong DPP-IV inhibitory activity. It is also supported by a low value from Inhibitions constant of Mangiferin, Stigmasterol and β-sitosterol which showed a high DPP-

IV inhibitory activity. The lower value of inhibitions constant means that just by low doses of them is able to inhibit the DPP-IV activity. Surface interaction between DPP-IV and three compounds above showed a high score, as following: Stigmasterol (962.48), β-sitosterol (886.91) and Mangiferin (742.75). The higher value of surface interaction has the potential to binding between ligand and molecule target so that can be predicted a stronger biological activity.

DPP-IV or CD26 is a membrane-associated peptidase of 766 amino acids that is widely distributed in numerous tissues. DPP-IV is hydrolase enzyme and also exists as a soluble circulating form in plasma and significant DPP-IV-like activity is detectable in plasma from humans and rodents. DPP-IV (CD26) exerts its biological effects via two distinct mechanisms of action. First, as a membrane-spanning protein, it binds adenosine deaminase and when activated, conveys intracellular signals independent of its enzymatic function via dimerization and activation of intracellular signaling pathways. The signaling properties of membrane-associated CD26 have been most extensively characterized in T cells [27]. The second principal biological activity of CD26 (DPP-IV) is its enzymatic function. The enzymatic activity of CD26 is exhibited by the membrane-spanning form of the molecule, and by the slightly smaller circulating soluble form [27,28].

The substrates of CD26/DPPIV are not specific to a particular peptides. The substrates of CD26/DPPIV are proline or alanine containing peptides and include growth factors, chemokines, neuropeptides and vasoactive peptides. DPP-4 prefers substrates with an amino-terminal proline or alanine at position 2, but may also cleave substrates with non-preferred amino acids at position 2. The structure of incretin hormone such as GLP-1 and GIP reveals a highly conserved alanine at position 2, rendering these peptides ideal putative substrates for the aminopeptidase DPP-IV [29].

A number of study showed that the importance of DPP-IV mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity [30]. DPP-IV inhibition prevents the degradation of active GLP-1 but does not increase the levels of circulating total GLP-1 and does not prevent the kidney from rapidly clearing GLP-1. DPP-IV inhibition also acutely decreases L cell secretion of GLP-1, likely via negative feedback on the L cell. The biological activities of GLP-1 are stimulating the secretion of insulin, increasing β-cell masses, inhibiting the secretion of glucagon, reducing the rate of gastric-emptying and inducing satiety that contribute to maintain blood glucose level in T2DM [1,2].

Using of DPP-4 inhibitors, primarily for the treatment of diabetes, relates to the potential effects of these inhibition on immune function. DPP-IV/CD26 is expressed on T cells, plays a functional role in T cell activation, and activates CD26 sets in motion a well-defined signaling cascade in the T cell. CD26 associates with CD45, and modulation of CD26 activity is frequently associated with enhanced T cell proliferation in immune system [29]. CD26/DPPIV 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 [31].

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Anti-diabetic potency of *Urena lobata* leaf extract on the inhibition of Dipeptidyl Peptidase IV (DPP-IV) activity

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Abstract

Objective : Glucagon Like Peptide-1 (GLP-1) is one of incretin hormones which has been proposed as a new treatment for Type 2 Diabetes Mellitus (T2DM). However, this hormone is metabolized excessively by Dipeptidyl Peptidase IV (DPP-IV) into inactive form. The inhibition of DPP-IV can enhance GLP-1 bioavailability and regulate blood glucose level, therefore it would be beneficial in the treatment of T2DM. *Urena lobata* is the plant~~s growing~~ that can be found in Indonesia and has been used to cure many diseases. The objective of this study was to evaluate the anti-diabetic potential ~~anti diabetic potency~~ of leaf extract from *U. lobata* ~~leaf extract~~ on DPP-IV inhibitory activity.

Method: *U. lobata* leaf was extracted in hot water and ethanol ~~solvent~~. The activity of DPP-IV inhibitor was tested by in-vitro study ~~use~~ using *Gly-pro-p-nitroanilide* (GPPN) as substrat of DPP-IV and Vildagliptin, as standard reference ~~standard~~. p-nitroanilida, ~~as~~ a product of the reactions between GPPN and DPP-IV, was observed ~~with~~ by microplatereader with λ=405 nm. All data ~~are~~ were expressed as the mean ± SD and the IC-50 value was determined by non linear regression curve fit. Active substances in leaf extract of *U. lobata* ~~leaf extract~~ was analyzed by Liquid Chromatography –Mass Spectra (LC-MS). DPP-IV inhibitory activity of active compounds was evaluated by in-silico ~~study~~ ~~used~~ using docking server ~~software~~.

Result: The ethanolic extract of *U. lobata* showed that DPP-IV inhibitor activity were stronger than water extract with ~~an~~ the ~~Inhibitory Concentration 50~~ (IC-50) value ~~of~~ was 1654.64 and 6489.88 µg/ml, respectively. Vildagliptin, ~~used as~~ based on standard reference ~~standart~~ ~~of~~ for DPP-IV inhibitor activity, ~~have~~ had IC-50 value at 57.44 µg/ml. Mangiferin, Stigmasterol and β-sitosterol in *U. lobata* extract have a strong inhibitory activity on DPP-IV, based on in-silico test.

Conclusion: *U. lobata* showed that DPP-IV inhibitory activity is related to ~~it's~~ its active compounds such as Mangiferin, Stigmasterol and β-sitosterol.

Key words: *U.lobata, anti-diabetic, Dipeptidyl peptidase-IV.*

INTRODUCTION

Recently, the treatment of type 2 Diabetes mellitus (T2DM) is focused on incretin hormone. Glucagon Like Peptide-1 (GLP-1) and Glucose Dependent Insulinotropic Polypeptide (GIP) are a major incretin hormone which is secreted by intestinal cells. GLP-1 plays a role ~~to regulate~~ in regulation of blood glucose level due to their ~~biology~~ biological actions, such as ~~to stimulate~~ stimulating ~~insulin secretion~~ the secretion of insulin, ~~increase~~ increasing β-cell masses, ~~inhibit~~ inhibiting ~~glucagon secretion~~ the secretion of glucagon, ~~reduce~~ reducing the rate of gastric-emptying and ~~induce~~ inducing satiety [1,2]. However, GLP-1 is rapidly metabolized by the enzyme called Dipeptidyl peptidase-IV (DPP-IV) into inactive forms. ~~It results a short half-life of GLP-1 of only approximately 1-2 minutes~~. Therefore, the GLP-1 has a short half-life, approximately for 1-2 minutes. Inhibition of DPP-IV maintain the level of endogenous active GLP-1 and prolongs its half life [1,3].

DPP-IV inhibitor has the potential to be a novel, efficient and ~~tolerable~~ considerable as ~~approach~~ approaching method to treat T2DM [3]. ~~Use~~ The usage of DPP-IV inhibitor drugs have less ~~adverse reaction~~ side effects like hypoglycemia, ~~increase of~~ increasing body weight and GIT disorders [4]. The studies on animals showed that genetic deletion of DPP-IV have been improved in glucose tolerance and increased the insulin secretion ~~in~~ as the results ~~respon~~ to oral of glucose [5]. In the other hand, the complete data of long terms use of synthetic drugs of DPP-IV inhibitor ~~has~~ have not been obtained ~~a complete data~~ yet, especially on ~~the~~ its safety [6]. It induces the ~~search~~ research of DPP-IV inhibitor compounds from herbs that have ~~having~~ less side effects, ~~cheaps~~ cheaper and easier to get.

Urena lobata is the plant ~~growing~~ that can be found in Indonesia and has been used to cure many diseases. Based on experiences, Nigerian people used *U. lobata* to treat DM ~~empirically~~ ~~due to~~ because of their biology ~~activity~~ activities [7]. The study showed that administration of *U. lobata* roots extract ~~has~~ had anti-hyperglycemic on rat which has been induced before by streptozotocin [8]. It related to active substances in *U.lobata* such as sterol

groups, alkaloid and flavonoid [9,10]. Anti-diabetic ~~potency~~ potential of *U. lobata* has not been evaluated yet, especially on the DPP-IV inhibitor activity, therefore it is ~~open the~~ an opportunity to expand herbs that can become candidate of phytopharmaca. The aim of ~~the~~ this study was to know the anti-diabetic ~~potency~~ potencial of *U. lobata* leaf extract on DPP-IV inhibitor activity.

MATERIAL AND METHODS

Chemical used: DPP-IV was obtained from porcine kidney, Gly-pro-p-nitroanilide (GPPN), Tris-HCl buffer. All chemicals were purchased from Sigma aldrich.

Sample prepration: *U. lobata* leaf powder was obtained from Materia Medika Batu Malang with certificate number 074/027/101.8/2015. Then, 50 g of the powdered plant materials were extracted in 250 ml hot water 90ºC for 30 minutes or called a decoctation method~~s~~. In the same weight, ~~of~~ *U.lobata* powder was extracted in 250 ml ethanol for 4 hours by ~~shacker waterbath~~ waterbath shacker and repeated 2 times with ~~fresh~~ new /fresh ~~solven~~ ethanol. Both of the extracts were then evaporated. ~~until produce pasta form.~~

Identification of active compounds : Both of water and ethanol extract were analyzed on a semi qualitative scale by Liquid Chromatography–Mass Spectra (LC-MS) Accela 1250 pump. Liquid phase contains 0.1 % formic acid in methanol and water. The identification ~~covers~~ included ~~exhibited~~ the 10 active substances from alkaloid, fitosterol and flavonoid groups.

Dipeptidyl peptidase IV assays: The assay was performed as per Bharti *et al*, 2012. In briefly, ~~the assay~~ it was performed in 96 micro well plates. A pre-incubation volume of 50 µl solution ~~contained~~ contains of 35 µl Tris-HCl buffer, ~~and~~ 15 µl DPP-IV 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 based on reference [11].

***Molecular docking studies* :** DPP-IV inhibitor activity of active compounds in *U.lobata* leaf extracts was evaluated by in-silico study using a web-based software application (www.dockingserver.com) for protein and ligand molecular docking. Free energy binding, inhibition constanta and surface interactions were analyzed by this methods to measure the inhibitor activity of active compounds on DPP-IV.

Statistical Analysis: All datas are expressed as the mean ± SD. The IC-50 was determined by non linear regression curve fit. The statistical data were ~~evaluated~~ done by ~~using~~ SPSS One Way ANOVA test and then followed by Least Significant Difference (LSD) with significant value ($p<0.05$).

RESULTS

DPP-IV inhibitory activity of *U. lobata*

Both of water and ethanol *U. lobata* leaf extracts were tested on Dipeptidyl peptidase-IV (DPP-IV) inhibitory assay by in vitro methods. The DPP-IV inhibitor activity is ~~showed~~ shown in ~~table~~ Table-1 and ~~figure~~ Figure-1. ~~bellow~~

Table-1 : DPP-IV inhibitory activity of *U. lobata* leaf extracts and Vildagliptin

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 ~~different~~ differences of the potency ($p<0.05$, LSD test)

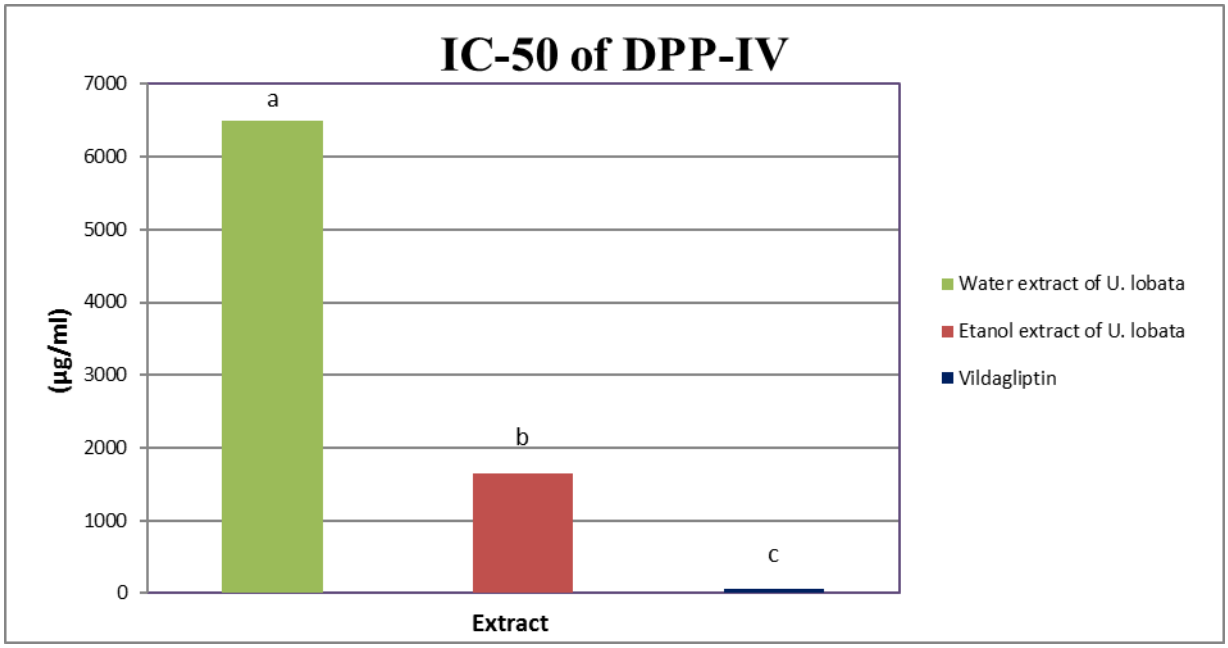

Figure-1 : IC-50 of DPP-IV on *U.lobata* leaf extract with ~~standart~~ Vildagliptin as standard

Based on ~~these~~ these ~~result~~ results, ethanolic extract of *U.lobata* showed that the activity on DPP-IV inhibition was stronger, about 4 times folds, compared to water extract ($p<0.05$). However, ~~the inhibitory activity on DPP-IV~~ the DPP-IV inhibitory activity on both of water and ethanolic *U. lobata* ~~*extract*~~ extracts are still lower, approximetly 30-100 times folds, compared to Vildagliptin as reference drugs of DPP-IV inhibitor ($p<0.05$).

Identification of Active compounds in *U.lobata* leaf extracts

The Active compounds, both ~~of~~ in water and ethanol leaf extract of *U.lobata*, can be ~~shown~~ seen in the ~~table~~ Table-2 and ~~figure~~ Figure-2.

Tabel-2 : Active compounds in *U.lobata* leaf extracts

No	Active compounds	Molecule weight	Water extract	Ethanolic extract
1	Stigmasterol	413	(+++)	(+++)
2	B-Sitosterol	415	(++)	(+)
3	Mangiferin	423	(+)	(+)
4	Quercetine	303	(-)	(-)
5	Kaempferol	286	(-)	(-)
6	Hypolaetin	302	(-)	(-)
7	Gossypetin	318	(+)	(++)
8	Luteolin	286	(-)	(-)
9	Apigenin	270	(-)	(-)
10	Chrysoeriol	300	(+)	(+)

~~Based on~~ The semi qualitative analysis by LC-MS showed that the ~~dominant~~ most abundant of active compounds both ~~of~~ in water and ethanolic extract of *U. lobata* ~~are~~ were

stigmasterol, gossypetin and β-sitosterol. Active ~~substances~~ compounds such as mangiferin and chrysoeriol were also identified both ~~of~~ in water and ethanolic extracts of *U.lobata* ~~but~~ and showed that the concentration value was low.

Molecular docking of *U.lobata* leaf extracts on DPP-IV

Inhibitor activity of *U.lobata* leaf extracts on DPP-IV was evaluated by in-silico study. Active compounds that were identified in *U.lobata* as ligand then docked with DPP-IV as protein target and the results can be ~~showed~~ seen at ~~table~~ Table-3.

Table-3 : Molecular docking of active compounds in *U.lobata* leaf extracts

No	Active compounds	Est. Free Energy of Binding (kcal/mol)	Est. Inhibition Constant Ki (µM)	Interact. Surface
1	**Stigmasterol**	-7.42	3.62	962.48
2	**B-Sitosterol**	-6.59	14.67	886.91
3	**Mangiferin**	-7.66	2.43	742.75
4	**Gossypetin**	-5.2	153.42	552.29
5	**Chrysoeriol**	-4.66	386.05	539.84

Docking studies showed that mangiferin, stigmasterol and β-sitosterol have a low value in both of the ~~binding free~~ binding free energy and the inhibition constant~~s~~ but the ~~interaction surface~~ surface interaction was high. However, gossypetin and chrysoeriol have a ~~free energy of binding~~ higher value on binding free energy and ~~an~~ inhibition constant~~s~~ ~~higher~~ than ~~three~~ other substances above. The ~~different~~ differences in each parameter value ~~result~~ causes ~~a differences~~ the distinction in inhibitory activity on DPP-IV.

DISCUSSION

Identification of Active compounds in *U.lobata* leaf extracts

Five active compounds were identified in *U.lobata* leaf extract and ~~the substances were~~ had been found on ~~same~~ both of ~~in~~ water extract and ethanol. It is only differs in the quantity or composition of active compounds in both extract. They are stigmasterol gossypetin, β-sitosterol, mangiferin and chrysoeriol. All of them are classified in secondary metabolite groups and have biology activity that can be used to cure the diseases. Stigmasterol is one of a group of plant sterols or phytosterols that are chemically similar to

animal cholesterol. Phytosterols are insoluble in water but soluble in most organic solvents and contain one alcohol functional group. Stigmasterol is an unsaturated plant sterol occurring in the plant fats or oils of soybean, calabar bean, rape seed, and in ~~a number of~~ various medicinal herbs. Studies ~~with~~ about laboratory animals ~~fed~~ treated by stigmasterol found that both cholesterol and sitosterol absorption decreased 23% and 30%, respectively, over a 6-week period. It also possesses ~~potent~~ a potencial antioxidant, hypoglycemic and thyroid inhibiting properties [13,14].

Gossypetin is flavonol or flavone, a type of flavonoid. It has been isolated originally from the flowers and the calyx of Hisbiscus species. Gossypetin shows a ~~strong~~ high antioxidant, anti-microbial, anti-mutagenic and anti-atherosclerotic (15). This compound~~s~~ is very soluble in chloroform and benzene, and also moderately soluble in ethanol and ether, but ~~it is~~ insoluble in water.

β-sitosterol is one of several phytosterols or plant sterols with chemical structure similar to that of cholesterol. Sterols are isoprenoid-derived molecules that have essential functions typically in eukaryotes ~~in general~~, and especially in higher plants ~~in particular~~. β-sitosterol are white, waxy powder with the specific characteristic of odor. They are hydrophobic and soluble in ethanol and chloroform but insoluble in water [16]. It can be found in avocados, cucurbita pepo, corn oil and soy beans also it ~~exhibits~~ showed anti cholesterol, anti-inflamatory and immunomodulator effects (17).

Mangiferin is a xanthonoid~~, this molecule is~~ and a glucoside of norathyriol. It ~~is~~ was found in Mangoes, *Iris unguicularis* and *Anemarrhena asphedelous*. Mangiferin is soluble in hot dilutes ethanol and methanol but insoluble in water. Laboratory study has identified a variety of pharmacology effect that associated with mangiferin including anti-microbial, antioxidant activity, and anti-diabetic effect in rodent [18,19].

Chrysoeriol is a flavon, one of major flavonoid classes. They provide many health-promoting benefits such as anti inflammation and anti histamine. It is soluble in alkalies solution and ~~sparingly~~ sufficiently soluble in water [20].

The presence of active compounds in extract were influenced by polarity and extract solven. Type of extract solven certain the composition of active compound in extract due to the difference of their solubility in solven. Second, polarity of active compound also contribute to their solubility in solven. Alkaloid, terpenoid and steroid soluble in non polar solven like aceton, diethyl eter and hexane. Meanwhile, flavonoid, fenol and glycoside solve in polar solven such as water and methanol [25,28]. It is appropriate with the determinate

solubility theory “like dissolve like” that polar substances will dissolve in polar solven and vice versa [25, 29].

Generally, plants contain two major substances they are a nutrition compound and non nutrition. Primary metabolite or nutrition compounds such as carbohydrate, protein, fatty acids and phytosterol can be found in a huge proportion but they do not have pharmacology effect. On the other hand, non nutrition compounds or secondary metabolite like alkaloid, terpenoid, flavonoid and steroid are met in a small concentration but it have pharmacology effect in certain dose [25,26]. Secondary metabolites are derived from metabolism of primary metabolite in plant but sometimes they have a toxic effect especially if it is used in large dose. Most of flavonoid and terpenoid in herbs have a potency as antioxidant, antiseptic and anti-inflammatory whereas steroid as anti-inflammatory and sex hormone. But, the pharmacology effect of alkaloid is difficult to be predicted in medicinal plants due to they have so many biology activity [27].

Anti-diabetic effect of herbs are signaled by their potency to decrease blood glucose level. The hypoglycemiae effect is controlled by active compounds likes terpenoid, steroid, alkaloid and flavonoid but their mechanisms of work are different. Some of herbs works as anti-diabetes by mechanisms such as insulin sensitizers, insulin secretory, DPP-IV inhibitor and α-glucosidase inhibitor [30]. Anti-diabetic herbs have many active compounds so that it have a possibility to work by multiple action and result an interactions both synergistic or antagonistic effect. Sometimes the interaction have both of negative and positive pharmacology effect [4].

Molecular docking of *U.lobata* leaf extracts on DPP-IV

Mangiferin have the lowest value of inhibition constants ~~that~~ and followed by stigmasterol activity on DPP-IV. ~~It’s~~ It is related to ~~binding free~~ binding free energy and ~~interaction surface~~ surface interaction of these ~~substances~~ compounds. The higher value of ~~interaction surface~~ surface interaction, ~~indicate~~ the higher the interaction possibilities of compounds interacting with the target protein ~~possibility of ligand to interact with protein target is bigger~~. In this study, stigmasterol have the highest value of ~~interaction surface~~ surface interaction that followed by β–sitosterol and mangiferin respectively. A great ~~chance~~ result of surface interaction ~~result~~ showed a stronger bond between ligand and protein target so that the biology activity is ~~bigger~~ higher. In the other hand, the lower value of ~~binding free~~ binding free energy showed ~~a chance~~ an easier probability of ligand to binding with protein target ~~is easer~~. Based on analysis, mangiferin have the lowest value in ~~free energy of binding~~ binding free energy while stigmasterol and β–sitosterol were in the second and third position.

The lowest value of ~~binding free energy~~ binding free energy produces a strong binding molecule ~~binding~~ and then ~~result~~ causes the potential biology activity. Free energy ~~of~~ binding and ~~interaction surface~~ surface interaction between ligand and protein target ~~influence~~ affects the ~~an~~ inhibitory activity of *U.lobata* leaf extract on DPP-IV.

~~In the field of~~ ~~bioinformatics,~~ Molecular docking studies are widely used to predict the ~~suitable~~ potential candidates of drugs ~~candidates~~ in the ~~pharmaceutical drug designing~~ pharmaceutical industry. Binding orientation of these small molecules or active ~~ingredients~~ compounds to their protein targets reveals their affinity and activity as possible candidates of drugs ~~candidates~~.

DPP-IV inhibitory activity of *U. lobata*

DPP-IV inhibitory activity of ethanolic extract of *U.lobata* are stronger than that of water extract. It is regulated by the differences both of active compounds and their proportions in ~~this~~ these extracts. Semi qualitative test of *U.lobata* leaf extract by LC-MS ~~exhibits~~ showed the compositions of Stigmasterol, β- sitosterol, Gossypetin and Chrysoeriol which higher than that of Mangiferin, Quercetine and Hypolaetin. Active ~~substances~~ compounds such as Stigmasterol, β- sitosterol and Gossypetin are soluble in semi-polar solvents like alcohol but Mangiferin and Hypolaetin is insoluble. The differences of solubility of active compounds in the solvents will ~~influence~~ affect to the percentages of active compounds in the extracts. Solubility of active compounds in the solvents will contribute on their~~'s~~ ~~extract composition~~ compositions in the extracts.

Both of ethanolic and water extracts of *U.lobata* ~~and water~~ contain the same ~~of~~ active compounds but different in amounts. Composition of Stigmasterol, β- sitosterol and Gossypetin are ~~smaller~~ lower in water extract ~~though~~ meanwhile the proportion of Chrysoeriol, Mangiferin, Quercetine and Hypolaetin ~~in the extract~~ are ~~same~~ similar both of ~~in~~ water and ethanol~~ic~~ extract. Non-polar compounds such as Stigmasterol, β- sitosterol and Gossypetin could be extracted in water solvent eventhough in small ~~numbers~~ amount. When the water ~~was~~ is boiled, their polarity will decrease so that it could be extracted from semi-polar until non-polar ~~substances~~ compounds (12).

Molecular docking study of *U. lobata* leaf extract showed inhibitory activity on DPP-IV. Three active ~~substances~~ compounds such as Mangiferin, Stigmasterol and β-sitosterol have a low ~~free energy of binding~~ binding free energy. It means that the ability of binding between ligand and molecule target ~~more easily~~ is easy so that ~~result~~ causing a strong DPP-IV inhibitory activity. ~~It's~~ It is also supported ~~also~~ by a low value from Inhibitions constant ~~value~~ of Mangiferin, Stigmasterol and β-sitosterol which showed a ~~strong~~ high DPP-IV inhibitory

activity. The lower value of inhibitions constant means that just by ~~with~~ low doses of them ~~has been~~ is able to inhibit the DPP-IV activity. ~~Interaction surface~~ surface interaction between DPP-IV and three compounds above showed a high score, ~~in the~~ as following: ~~order~~ Stigmasterol (962.48), β-sitosterol (886.91) and Mangiferin (742.75). The higher value of ~~Interaction surface~~ surface interaction has the potential to binding between ligand and molecule target so that can be predicted a stronger biological activity.

DPP-IV or CD26 is a membrane-associated peptidase of 766 amino acids that is widely distributed in numerous tissues. DPP-IV is hydrolase enzyme and also exists as a soluble circulating form in plasma and significant DPP-IV-like activity is detectable in plasma from humans and rodents []. DPP-IV (CD26) exerts its biological effects via two distinct mechanisms of action. First, as a membrane-spanning protein, it binds adenosine deaminase and when activated, conveys intracellular signals independent of its enzymatic function via dimerization and activation of intracellular signaling pathways. The signaling properties of membrane-associated CD26 have been most extensively characterized in T cells [22].

The second principal biological activity of CD26 (DPP-IV) is its enzymatic function. The enzymatic activity of CD26 is exhibited both by the membrane-spanning form of the molecule, and by the slightly smaller circulating soluble form [21,22].

The substrates of CD26/DPPIV are proline or alanine containing peptides and include growth factors, chemokines, neuropeptides and vasoactive peptides. DPP-4 prefers substrates with an amino-terminal proline or alanine at position 2, but may also cleave substrates with non-preferred amino acids at position 2. The structure of incretin hormone such as GLP-1 and GIP reveals a highly conserved alanine at position 2, rendering these peptides ideal putative substrates for the aminopeptidase DPP-IV [24].

A number study showed the importance of DPP-IV –mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity [21]. DPP-IV inhibition prevents the degradation of active GLP-1 but does not increase the levels of circulating total GLP-1 and does not prevent the kidney from rapidly clearing GLP-1. DPP-IV inhibition also acutely decreases L cell secretion of GLP-1, likely via negative feedback on the L cell. The biology actifity of GLP-1 are stimulating the secretion of insulin, increasing β-cell masses, inhibiting the secretion of glucagon, reducing the rate of gastric-emptying and inducing satiety that contribute to maintain blood glucose level in T2DM [1,2].

Use of DPP-4 inhibitors, primarily for the treatment of diabetes, relates to the potential effects of these inhibition on immune function. DPP-IV/CD26 is expressed on T cells, plays a functional role in T cell activation, and activation of CD26 sets in motion a

well-defined signaling cascade in the T cell. CD26 associates with CD45, and modulation of CD26 activity is frequently associated with enhanced T cell proliferation in immune system [22]. CD26/DPPIV 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 [23]

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Anti-diabetic potential of *Urena lobata* leaf extract through inhibition of dipeptidyl peptidase IV activity

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ABSTRACT

Objective: To evaluate the anti-diabetic potential of leaf extract from *Urena lobata* (*U. lobata*) through dipeptidyl peptidase IV (DPP-IV) inhibitory activity.
Methods: *U. lobata* leaf was extracted in hot water and ethanol. The activity of DPP-IV inhibitor was tested by *in vitro* study using gly-pro-p-nitroanilide as substrat of DPP-IV and vildagliptin, as standard reference. A product of the reactions between gly-pro-p-nitroanilide and DPP-IV, was observed by microplate readers with λ = 405 nm. All data were expressed as mean ± SD and the IC_{50} value was determined by non linear regression curve fit. Active substances in leaf extract of *U. lobata* was analyzed by liquid chromatography-mass spectrometry. DPP-IV inhibitory activity of active compounds was evaluated *in silico* using docking server.
Results: The ethanolic extract of *U. lobata* showed stronger DPP-IV inhibitor activity than water extract with the IC_{50} values of 1 654.64 and 6 489.88 μg/mL, respectively. Vildagliptin, based on standard reference for DPP-IV inhibitor activity, has IC_{50} value of 57.44 μg/mL. Based on *in silico* analysis, mangiferin, stigmasterol and β-sitosterol in *U. lobata* extract have a strong inhibitory activity on DPP-IV.
Conclusions: The results showed that DPP-IV inhibitory activity of *U. lobata* is related to its active compounds such as mangiferin, stigmasterol and β-sitosterol.

1. Introduction

Recently, the treatment of type 2 diabetes mellitus is focused on incretin hormone. Glucagon like peptide-1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP) are the major incretin hormones which are secreted by intestinal cells. GLP-1 plays a role in regulation of blood glucose level due to their biological actions, such as stimulating the secretion of insulin, increasing β-cell mass, inhibiting the secretion of glucagon, reducing the rate of gastric emptying and inducing satiety[1,2]. However, GLP-1 is rapidly metabolized by the enzyme called dipeptidyl peptidase IV (DPP-IV) into inactive forms. Therefore, the GLP-1 has a short half life, approximately for 1-2 min. Inhibition of DPP-IV maintains the level of endogenous active GLP-1 and prolongs its half life[1,3].

DPP-IV inhibitor has the potential to be a novel, efficient and considerable agent to treat type 2 diabetes mellitus[3]. The usage of DPP-IV inhibitor has less side effects like hypoglycemia, increasing body weight and GIT disorders[4]. The studies on oral glucose tolerance test for animals showed that genetic deletion of DPP-IV has been improved in glucose tolerance and increased the insulin secretion[5]. In the other hand, the complete data of long term use of synthetic drugs of DPP-IV inhibitor have not been obtained yet, especially on its safety[6]. It induces the research of DPP-IV inhibitor compounds from herbs that are of less side effects, cheaper and easier to get.

Urena lobata (*U. lobata*) is the plant that can be found in Indonesia and has been used to cure many diseases. Based on experiences, Nigerian people used *U. lobata* to treat diabetes mellitus because of their biology activities[7]. The study showed that administration of *U. lobata* roots extract had anti-hyperglycemic effect on rat induced by streptozotocin before[8]. It related to active substances in *U. lobata* such as sterol groups, alkaloid and flavonoid[9,10]. Anti-diabetic potential of *U. lobata* has not been evaluated yet, especially the inhibition of DPP-IV activity. Therefore it is an opportunity to expand herbs that can become candidate of phytopharmaca. The aim of this study was to know the anti-diabetic potencial of *U. lobata* leaf extract through inhibition of DPP-IV activity.

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2. Materials and methods

2.1. Chemicals used

DPP-IV was obtained from porcine kidney. Gly-pro-p-nitroanilide and Tris-HCl buffer were used. All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Sample preparation

U. lobata leaf powder was obtained from Materia Medika Batu Malang with certificate number 074/027/101.8/2015. Then, 50 g of the powdered plant materials were extracted in 250 mL hot water at 90 ℃ for 30 min. Similarly, 50 g *U. lobata* powder was extracted in 250 mL ethanol for 4 h by waterbath shaker and repeated 2 times with fresh ethanol. Both of the extracts were then evaporated.

2.3. Identification of active compounds

Both water and ethanol extract were analyzed on a semi qualitative scale by liquid chromatography-mass spectrometry (LC-MS) Accela 1250 pump for identification of active compounds. Liquid phase contained 0.1% formic acid in methanol and water.

2.4. DPP-IV assays

The assay was performed in 96 micro well plates. A pre-incubation solution (50 μL) contained 35 μL Tris-HCl buffer, 15 μL DPP-IV enzyme and various concentration (625, 1 250, 2 500, 5 000 and 10 000 μg/mL) of the extracts or standard (6.25, 12.5, 25, 50 and 100 μg/mL). This mixture was incubated at 37 ℃ for 10 min, followed by addition of 50 μL gly-pro-p-nitroanilide as substrate. The reaction mixture was incubated for 30 min at 37 ℃ and the absorbance was measured by microplate readers at λ = 405 nm every 10 seconds. Vildagliptin was used as the standard DPP-IV inhibitor[11].

2.4. Molecular docking studies

DPP-IV inhibitory activity of active compounds in *U. lobata* leaf extracts was evaluated by *in silico* study using a web-based software application (www.dockingserver.com) for protein and ligand molecular docking. Free energy binding, inhibition constant and surface interactions were analyzed by this method to measure the DPP-IV inhibitory activity of active compounds.

2.6. Statistical analysis

All data are expressed as mean ± SD. The IC_{50} was determined by non-linear regression curve fit. The statistical data were analyzed by SPSS One-way ANOVA test followed by least significant difference test with significant value at $P < 0.05$.

3. Results

3.1. DPP-IV inhibitory activity of U. lobata

Both water and ethanol *U. lobata* leaf extracts were tested on DPP-IV inhibitory assay by *in vitro* method. The DPP-IV inhibitory activity is shown in Table 1.

Table 1
DPP-IV inhibitory activity of *U. lobata* leaf extracts and vildagliptin.

Sample (n = 3)	Concentration (μg/mL)	% Inhibition	IC_{50} (μg/mL)
Water extract of *U. lobata*	625.00	00.00 ± 0.00	6 489.88[a]
	1 250.00	13.33 ± 0.00	
	2 500.00	26.67 ± 0.00	
	5 000.00	42.22 ± 3.85	
	10 000.00	62.22 ± 3.85	
Ethanolic extract of *U. lobata*	625.00	36.17 ± 0.00	1 654.64[b]
	1 250.00	48.94 ± 0.00	
	2 500.00	55.32 ± 0.00	
	5 000.00	61.70 ± 0.00	
	10 000.00	74.47 ± 0.00	
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 letters showed the differences of the potency ($P < 0.05$, Least Significant Difference test).

The results obtained in the DPP-IV inhibitory assay showed that ethanolic extract of *U. lobata* showed stronger activity in DPP-IV inhibition, about 4 times folds, compared to water extract ($P < 0.05$). However, the DPP-IV inhibitory activity of both water and ethanolic *U. lobata* extracts are still lower, approximetly 30-100 times folds, compared to vildagliptin as reference drugs of DPP-IV inhibitor ($P < 0.05$).

3.2. Identification of active compounds in U. lobata leaf extracts

Ten active substances from alkaloid, fitosterol and flavonoid groups were indentified in extracts of *U. lobata*. The active compounds, both in water and ethanol leaf extract of *U. lobata*, can be seen in the Table 2. The semi-qualitative analysis by LC-MS showed that the most abundant active compounds both in water and ethanolic extract of *U. lobata* were stigmasterol, gossypetin and β-sitosterol. Active compounds such as mangiferin and chrysoeriol were also identified

in both water and ethanolic extracts of *U. lobata* with less content.

Table 2

Active compounds in *U. lobata* leaf extracts.

Active compounds	Molecule weight	Water extract	Ethanolic extract
Stigmasterol	413	+++	+++
β-Sitosterol	415	++	+
Mangiferin	423	+	+
Quercetine	303	-	-
Kaempferol	286	-	-
Hypolaetin	302	-	-
Gossypetin	318	+	++
Luteolin	286	-	-
Apigenin	270	-	-
Chrysoeriol	300	+	+

3.3. Molecular docking of active compounds in U. lobata leaf extracts

Inhibitory activity of *U. lobata* leaf extracts on DPP-IV was evaluated by *in silico* study. Active compounds identified in *U. lobata* as ligand were docked with DPP-IV as protein target and the results can be seen at Table 3.

Table 3

Molecular docking of active compounds in *U. lobata* leaf extracts.

Active compounds	Est. free energy of binding (Kcal/mol)	Est. inhibition constant (μmol/L)	Interact. surface
Stigmasterol	-7.42	3.62	962.48
β-Sitosterol	-6.59	14.67	886.91
Mangiferin	-7.66	2.43	742.75
Gossypetin	-5.20	153.42	552.29
Chrysoeriol	-4.66	386.05	539.84

Docking studies showed that mangiferin, stigmasterol and β-sitosterol have a low value in both the binding free energy and the inhibition constant but the surface interaction was high. However, gossypetin and chrysoeriol have a higher value in binding free energy and inhibition constant than other substances above. The differences in each parameter value caused the distinction in inhibitory activity on DPP-IV.

4. Discussion

4.1. Identification of active compounds in U. lobata leaf extracts

Five active compounds were identified in *U. lobata* leaf extract and had been found in both water and ethanol extract. It is only different in the quantity or amount of active compounds in both extracts. The active compounds are stigmasterol, gossypetin, β-sitosterol, mangiferin and chrysoeriol. All of them are classified into secondary metabolite groups and have biological activity that can be used to cure diseases. Stigmasterol is one of a group of plant sterols or phytosterols that are chemically similar to animal cholesterol. Phytosterols are insoluble in water but soluble in most organic solvents and contain one alcohol functional group. Stigmasterol is an unsaturated plant sterol in the plant fats or oils of soybean, calabar bean, rape seed, and in various medicinal herbs. Studies about laboratory animals treated by stigmasterol found that both cholesterol and sitosterol absorption decreased 23% and 30%, respectively, over a 6-week period. It also possesses potential antioxidant, hypoglycemic and thyroid inhibiting properties[12,13].

Gossypetin is flavonol or flavone, a type of flavonoid. It has been isolated originally from the flowers and the calyx of *Hibiscus* species. Gossypetin shows potential antioxidant, anti-microbial, anti-mutagenic and anti-atherosclerotic activities[14]. This compound is very soluble in chloroform and benzene, and also moderately soluble in ethanol and ether, but insoluble in water.

β-sitosterol is one of several phytosterols or plant sterols with chemical structure similar to that of cholesterol. Sterols are isoprenoid-derived molecules that have essential functions typically in eukaryotes, and especially in higher plants. β-sitosterol are white, waxy powder with characteristic odor. They are hydrophobic and soluble in ethanol and chloroform but insoluble in water[15]. It can be found in avocados, cucurbita pepo, corn oil and soy beans; it also showed anti-cholesterol, anti-inflamatory and immunomodulator effects[16].

Mangiferin is a xanthonoid, and a glucoside of norathyriol. It was found in mangoes, *Iris unguicularis* and *Anemarrhena asphedelous*. Mangiferin is soluble in hot diluted ethanol and methanol but insoluble in water. Laboratory study has identified a variety of pharmacology effect that associated with mangiferin including anti-microbial, antioxidant activity, and anti-diabetic effect in rodent[17,18].

Chrysoeriol is a flavon, one of major flavonoid classes. They exhibit many activities such as anti-inflammation and anti-histamine activities. It is soluble in alkalies solution and sufficiently soluble in water[19].

The presence of active compounds in extract was influenced by polarity and extract solvent. Type of extract solvent impacts the amount of active compounds in extract due to the difference of their solubility in solvent. Secondly, polarity of active compound also contribute to their solubility in solvent. Alkaloid, terpenoid and steroid are soluble in non polar solvent like acetone, diethyl ether and hexane. Meanwhile, flavonoid, phenol and glycoside dissolve in polar solvent such as water and methanol[20,21]. It is appropriate with the determinate solubility theory "like dissolve like" that polar substances will dissolve in polar solvent and vice versa[20,22].

Generally, plants contain two major substances; they are nutrition and non nutrition compounds. Primary metabolite or nutrition compounds such as carbohydrate, protein, fatty acids and phytosterol can be found in a huge proportion but they do not have pharmacology effect. On the other hand, non nutrition compounds or secondary metabolite like alkaloid, terpenoid, flavonoid and steroid are found in a small concentration but it have pharmacology effect at certain dose[20]. Secondary metabolites are derived from metabolism of primary metabolite in plant but sometimes they have a toxic effect especially if it is used in high dose. Most of flavonoid and terpenoid in herbs have a potency as antioxidant, antiseptic and anti-inflammatory whereas steroid as anti-inflammatory and sex hormone. But, the pharmacology effect of alkaloid is difficult to be predicted in medicinal plants because they have so many biological activities[23].

Anti-diabetic effect of herbs are indicated by their potency to decrease blood glucose level. The hypoglycemia effect is controlled by active compounds likes terpenoid, steroid, alkaloid and flavonoid but their mechanisms of work are different. Some herbs work as anti-diabetes by mechanisms such as insulin sensitizers, insulin secretory, DPP-IV inhibition and α-glucosidase inhibition[24]. Anti-diabetic herbs have many active compounds so that they have a possibility to work by multiple action and result in interactions either synergistic or antagonistic. Sometimes the interactions have both negative and positive pharmacology effect[4].

4.2. Molecular docking of U. lobata leaf extracts

Molecular docking is now widely used to discover new ligands for target of known structure. Potential compound can be screened by free energy binding. The score of free energy binding represents binding affinity of ligand to the target protein; the lower free energy binding, the higher binding affinity[25]. In addition, inhibition constant can be predicted using bioinformatics approach. The lowest inhibition constant indicates the most potential compound. Other parameter is surface interaction. It represents the molecular recognition between ligand and target protein. The higher value of surface interaction, the higher interaction possibilities of compounds interacting with the target protein[26]. Based on the findings in the present study, mangiferin have the lowest value of inhibition constants followed by stigmasterol. It is related to binding free energy and surface interaction of these compounds. In this study, stigmasterol has the highest value of surface interaction followed by β-sitosterol and mangiferin. A great result of surface interaction showed a stronger binding between ligand and protein target, so that the biology activity is higher. Based on the *in silico* analysis, mangiferin has the lowest value in binding free energy while stigmasterol and β-sitosterol were in the second and third position. The lowest value of binding free energy produces a strong binding molecule and then causes the potential biology activity. Free energy binding and surface interaction between ligand and protein target affects the inhibitory activity of *U. lobata* leaf extract on DPP-IV.

Molecular docking studies are widely used to predict the potential candidates of drugs in the pharmaceutical industry. Binding orientation of these small molecules or active compounds to their protein targets reveals their affinity and activity as possible candidates of drugs.

4.3. DPP-IV inhibitory activity of U. lobata

DPP-IV inhibitory activity of ethanolic extract of *U. lobata* are stronger than that of water extract. It is regulated by the differences of both active compounds and their proportions in these extracts. Semi qualitative test of *U. lobata* leaf extract by LC-MS showed the contents of stigmasterol, β-sitosterol, gossypetin and chrysoeriol which are higher than that of mangiferin, quercetine and hypolaetin. Active compounds such as stigmasterol, β-sitosterol and gossypetin are soluble in semi-polar solvents like alcohol but mangiferin and hypolaetin are insoluble. The differences of solubility of active compounds in the solvents will affect the percentages of active compounds in the extracts.

Both ethanolic and water extracts of *U. lobata* contain the same active compounds but different in amounts. Contents of stigmasterol, β-sitosterol and gossypetin are lower in water extract meanwhile the proportion of chrysoeriol, mangiferin, quercetine and hypolaetin are similar in both water and ethanolic extract. Non-polar compounds such as stigmasterol, β-sitosterol and gossypetin could be extracted in water solvent even though in small amount. When the water is boiled, their polarity will decrease so that it could be extracted from semi-polar until non-polar compounds[27].

Molecular docking study of *U. lobata* leaf extract showed inhibitory activity on DPP-IV. Three active compounds such as mangiferin, stigmasterol and β-sitosterol showed a low value in binding free energy. It means that the binding between ligand and molecule target is easy so that cause a strong DPP-IV inhibitory activity. It is also supported by a low value from inhibitions constant of mangiferin, stigmasterol and β-sitosterol which showed a high DPP-IV inhibitory activity. The lower value of inhibitions constant means that these compounds with low doses are able to inhibit the DPP-IV activity. Surface interaction between DPP-IV and three compounds above showed a high score (stigmasterol: 962.48, β-sitosterol: 886.91, and mangiferin: 742.75). The compound with higher value of surface interaction has the potential to binding ligand and molecule target, predicting a stronger biological activity.

DPP-IV or CD26 is a membrane-associated peptidase of 766 amino acids that is widely distributed in numerous tissues. DPP-IV is hydrolase enzyme and also exists with a soluble circulating form in plasma, and significant DPP-IV-like activity is detectable in plasma from humans and rodents. DPP-IV (CD26) exerts its biological effects via two distinct mechanisms of action. First, as a membrane-spanning protein, it binds adenosine deaminase and when activated, conveys intracellular signals independent of its enzymatic function via dimerization and activation of intracellular signaling pathways. The signaling properties of membrane-associated CD26 have been most extensively characterized in T cells[27]. The second principal biological activity of CD26 (DPP-IV) is its enzymatic function. The enzymatic activity of CD26 is exhibited by the membrane-spanning form of the molecule, and by the slightly smaller circulating soluble form[27,28].

The substrates of CD26/DPP-IV are not specific to a particular peptides. The substrates of CD26/DPP-IV are proline or alanine containing peptides and include growth factors, chemokines, neuropeptides and vasoactive peptides. DPP-IV prefers substrates with an amino-terminal proline or alanine at position 2, but may also cleave substrates with non-preferred amino acids at position 2. The structure of incretin hormone such as GLP-1 and GIP reveals a highly conserved alanine at position 2, rendering these peptides ideal putative substrates for the aminopeptidase DPP-IV[29].

A number of study showed that the importance of DPP-IV mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity[30]. DPP-IV inhibition prevents the degradation of active GLP-1 but does not increase the levels of circulating total GLP-1 and does not prevent the kidney from rapidly clearing GLP-1. DPP-IV inhibition also acutely decreases L cell secretion of GLP-1, likely via negative feedback on the L cell. The biological activities of GLP-1 are stimulating the secretion of insulin, increasing β-cell masses, inhibiting the secretion of glucagon, reducing the rate of gastric-emptying and inducing satiety that contribute to maintain blood glucose level in type 2 diabetes mellitus[1,2].

Using of DPP-IV inhibitors, primarily for the treatment of diabetes, relates to the potential effects of these inhibition on immune function. DPP-IV/CD26 is expressed on T cells, plays a functional role in T cell activation, and activates CD26 sets in motion a well-defined signaling cascade in the T cell. CD26 associates with CD45,

and modulation of CD26 activity is frequently associated with enhanced T cell proliferation in immune system[29]. CD26/DPP-IV 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[31].

Conflict of interest statement

We declare that we have no conflict of interest.

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Anti-diabetic potential of *Urena lobata* leaf extract through inhibition of dipeptidyl peptidase IV activity

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ABSTRACT

Objective: To evaluate the anti-diabetic potential of leaf extract from *Urena lobata* (*U. lobata*) through dipeptidyl peptidase IV (DPP-IV) inhibitory activity.
Methods: *U. lobata* leaf was extracted in hot water and ethanol. The activity of DPP-IV inhibitor was tested by *in vitro* study using gly-pro-p-nitroanilide as substrat of DPP-IV and vildagliptin, as standard reference. A product of the reactions between gly-pro-p-nitroanilide and DPP-IV, was observed by microplate readers with λ = 405 nm. All data were expressed as mean ± SD and the IC_{50} value was determined by non linear regression curve fit. Active substances in leaf extract of *U. lobata* was analyzed by liquid chromatography-mass spectrometry. DPP-IV inhibitory activity of active compounds was evaluated *in silico* using docking server.
Results: The ethanolic extract of *U. lobata* showed stronger DPP-IV inhibitor activity than water extract with the IC_{50} values of 1 654.64 and 6 489.88 μg/mL, respectively. Vildagliptin, based on standard reference for DPP-IV inhibitor activity, has IC_{50} value of 57.44 μg/mL. Based on *in silico* analysis, mangiferin, stigmasterol and β-sitosterol in *U. lobata* extract have a strong inhibitory activity on DPP-IV.
Conclusions: The results showed that DPP-IV inhibitory activity of *U. lobata* is related to its active compounds such as mangiferin, stigmasterol and β-sitosterol.

1. Introduction

Recently, the treatment of type 2 diabetes mellitus is focused on incretin hormone. Glucagon like peptide-1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP) are the major incretin hormones which are secreted by intestinal cells. GLP-1 plays a role in regulation of blood glucose level due to their biological actions, such as stimulating the secretion of insulin, increasing β-cell mass, inhibiting the secretion of glucagon, reducing the rate of gastric emptying and inducing satiety [1,2]. However, GLP-1 is rapidly metabolized by the enzyme called dipeptidyl peptidase IV (DPP-IV) into inactive forms. Therefore, the GLP-1 has a short half life, approximately for 1–2 min. Inhibition of DPP-IV maintains the level of endogenous active GLP-1 and prolongs its half life [1,3].

DPP-IV inhibitor has the potential to be a novel, efficient and considerable agent to treat type 2 diabetes mellitus [3]. The usage of DPP-IV inhibitor has less side effects like hypoglycemia, increasing body weight and GIT disorders [4]. The studies of oral glucose tolerance test on animals showed that genetic deletion of DPP-IV have improved glucose tolerance and increased the insulin secretion [5]. In the other hand, the complete data of long term use of synthetic drugs of DPP-IV inhibitor have not been obtained yet, especially on its safety [6]. It induces the research of DPP-IV inhibitor compounds from herbs that are of less side effects, cheaper and easier to get.

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Urena lobata (*U. lobata*) is the plant that can be found in Indonesia and has been used to cure many diseases. Based on experiences, Nigerian people used *U. lobata* to treat diabetes mellitus because of their biology activities [7]. The study showed that administration of *U. lobata* roots extract had anti-hyperglycemic effect on rat induced by streptozotocin before [8]. It related to active substances in *U. lobata* such as sterol groups, alkaloid and flavonoid [9,10]. Anti-diabetic potential of *U. lobata* has not been evaluated yet, especially the inhibition of DPP-IV activity. Therefore it is an opportunity to expand herbs that can become candidate of phytopharmaca. The aim of this study was to know the anti-diabetic potential of *U. lobata* leaf extract through inhibition of DPP-IV activity.

2. Materials and methods

2.1. Chemicals used

DPP-IV was obtained from porcine kidney. Gly-pro-p-nitroanilide and Tris–HCl buffer were used. All chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Sample preparation

U. lobata leaf powder was obtained from Materia Medika Batu Malang with certificate number 074/027/101.8/2015. Then, 50 g of the powdered plant materials were extracted in 250 mL hot water at 90 °C for 30 min. Similarly, 50 g *U. lobata* powder was extracted in 250 mL ethanol for 4 h by waterbath shaker and repeated 2 times with fresh ethanol. Both of the extracts were then evaporated.

2.3. Identification of active compounds

Both water and ethanol extract were analyzed on a semi qualitative scale by liquid chromatography–mass spectrometry (LC–MS) Accela 1250 pump for identification of active compounds. Mobile phase contained 0.1% formic acid in a mixture of methanol and water.

2.4. DPP-IV assays

The assay was performed in 96 micro well plates. A pre-incubation solution (50 μL) contained 35 μL Tris–HCl buffer, 15 μL DPP-IV enzyme and various concentration (625, 1 250, 2 500, 5 000 and 10 000 μg/mL) of the extracts or standard (6.25, 12.5, 25, 50 and 100 μg/mL). This mixture was incubated at 37 °C for 10 min, followed by addition of 50 μL gly-pro-p-nitroanilide as substrate. The reaction mixture was incubated for 30 min at 37 °C and the absorbance was measured by microplate readers at λ = 405 nm every 10 s. Vildagliptin was used as the standard DPP-IV inhibitor [11]. % Inhibition was calculated by the following formula:

$$\%\ \text{Inhibition} = \frac{\text{DPP}-\text{IV activity (with extract)}}{\text{DPP}-\text{IV activity (without extract)}} \times 100$$

2.5. Molecular docking studies

DPP-IV inhibitory activity of active compounds in *U. lobata* leaf extracts was evaluated by *in silico* study using a web-based software application (www.dockingserver.com) for protein and ligand molecular docking. Free energy binding, inhibition constant and surface interactions were analyzed by this method to measure the DPP-IV inhibitory activity of active compounds.

2.6. Statistical analysis

All data are expressed as mean ± SD. The IC_{50} was determined by non-linear regression curve fit. The statistical data were analyzed by SPSS One-way ANOVA test followed by least significant difference test with significant value at $P < 0.05$.

3. Results

3.1. DPP-IV inhibitory activity of U. lobata

Both water and ethanol *U. lobata* leaf extracts were tested on DPP-IV inhibitory assay by *in vitro* method. The DPP-IV inhibitory activity is shown in Table 1.

The results obtained in the DPP-IV inhibitory assay showed that ethanolic extract of *U. lobata* showed stronger activity in DPP-IV inhibition, about 4 times folds, compared to water extract ($P < 0.05$). However, the DPP-IV inhibitory activity of both water and ethanolic *U. lobata* extracts are still lower, approximately 30–100 times folds, compared to vildagliptin as reference drugs of DPP-IV inhibitor ($P < 0.05$).

3.2. Identification of active compounds in U. lobata leaf extracts

Ten active substances from alkaloid, fitosterol and flavonoid groups were identified in extracts of *U. lobata*. The active compounds, both in water and ethanol leaf extract of *U. lobata*, can be seen in Table 2. The semi-qualitative analysis by LC–MS showed that the most abundant active compounds both in water and ethanolic extract of *U. lobata* were stigmasterol, gossypetin and β-sitosterol. Active compounds such as mangiferin and chrysoeriol were also identified in both water and ethanolic extracts of *U. lobata* with less content.

Table 1
DPP-IV inhibitory activity of *U. lobata* leaf extracts and vildagliptin.

Sample (n = 3)	Concentration (μg/mL)	% Inhibition	IC_{50} (μg/mL)
Water extract of *U. lobata*	625.00	00.00 ± 0.00	6 489.88[a]
	1 250.00	13.33 ± 0.00	
	2 500.00	26.67 ± 0.00	
	5 000.00	42.22 ± 3.85	
	10 000.00	62.22 ± 3.85	
Ethanolic extract of *U. lobata*	625.00	36.17 ± 0.00	1 654.64[b]
	1 250.00	48.94 ± 0.00	
	2 500.00	55.32 ± 0.00	
	5 000.00	61.70 ± 0.00	
	10 000.00	74.47 ± 0.00	
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 letters showed the differences of the potency ($P < 0.05$, Least Significant Difference test).

Table 2
Active compounds in *U. lobata* leaf extracts.

Active compounds	Molecule weight (Dalton)	Water extract	Ethanolic extract
Stigmasterol	413	+++	++
β-Sitosterol	415	+	+
Mangiferin	423	+	+
Quercetine	303	–	–
Kaempferol	286	–	–
Hypolaetin	302	–	–
Gossypetin	318	+	++
Luteolin	286	–	–
Apigenin	270	–	–
Chrysoeriol	300	+	+

+: Weak; ++: Moderate; +++: Strong; –: Negative.

Table 3
Molecular docking of active compounds in *U. lobata* leaf extracts.

Active compounds	Estimation of free energy of binding (Kcal/mol)	Estimation of inhibition constant (μmol/L)	Interaction surface
Stigmasterol	–7.42	3.62	962.48
β-Sitosterol	–6.59	14.67	886.91
Mangiferin	–7.66	2.43	742.75
Gossypetin	–5.20	153.42	552.29
Chrysoeriol	–4.66	386.05	539.84

3.3. Molecular docking of active compounds in U. lobata leaf extracts

Inhibitory activity of *U. lobata* leaf extracts on DPP-IV was evaluated by *in silico* study. Active compounds identified in *U. lobata* as ligand were docked with DPP-IV as protein target and the results can be seen at Table 3.

Docking studies showed that mangiferin, stigmasterol and β-sitosterol have a low value in both the binding free energy and the inhibition constant but the surface interaction was high. However, gossypetin and chrysoeriol have a higher value in binding free energy and inhibition constant than other substances above. The differences in each parameter value caused the distinction in inhibitory activity on DPP-IV.

4. Discussion

4.1. Identification of active compounds in U. lobata leaf extracts

Five active compounds were identified in *U. lobata* leaf extract and had been found in both water and ethanol extract. It is only different in the quantity or amount of active compounds in both extracts. The active compounds are stigmasterol, gossypetin, β-sitosterol, mangiferin and chrysoeriol. All of them are classified into secondary metabolite groups and have biological activity that can be used to cure diseases. Stigmasterol is one of a group of plant sterols or phytosterols that are chemically similar to animal cholesterol. Phytosterols are insoluble in water but soluble in most organic solvents and contain one alcohol functional group. Stigmasterol is an unsaturated plant sterol in the plant fats or oils of soybean, calabar bean, rape seed, and in various medicinal herbs. Studies about laboratory animals treated by stigmasterol found that both cholesterol and sitosterol absorption decreased 23% and 30%, respectively, over a 6-week period. It also possesses potential antioxidant, hypoglycemic and thyroid inhibiting properties [12,13].

Gossypetin is flavonol or flavone, a type of flavonoid. It has been isolated originally from the flowers and the calyx of *Hibiscus* species. Gossypetin shows potential antioxidant, antimicrobial, anti-mutagenic and anti-atherosclerotic activities [14]. This compound is very soluble in chloroform and benzene, and also moderately soluble in ethanol and ether, but insoluble in water.

β-sitosterol is one of several phytosterols or plant sterols with chemical structure similar to that of cholesterol. Sterols are isoprenoid-derived molecules that have essential functions typically in eukaryotes, and especially in higher plants. β-sitosterol are white, waxy powder with characteristic odor. They are hydrophobic and soluble in ethanol and chloroform but insoluble in water [15]. It can be found in avocados, cucurbita pepo, corn oil and soy beans; it also showed anti-cholesterol, anti-inflamatory and immunomodulator effects [16].

Mangiferin is a xanthonoid, and a glucoside of norathyriol. It was found in mangoes, *Iris unguicularis* and *Anemarrhena asphedelous*. Mangiferin is soluble in hot diluted ethanol and methanol but insoluble in water. Laboratory study has identified a variety of pharmacology effect that associated with mangiferin including anti-microbial, antioxidant activity, and anti-diabetic effect in rodent [17,18].

Chrysoeriol is a flavon, one of major flavonoid classes. They exhibit many activities such as anti-inflammation and anti-histamine activities. It is soluble in alkalies solution and sufficiently soluble in water [19].

The presence of active compounds in extract was influenced by polarity and extract solvent. Type of extract solvent impacts the amount of active compounds in extract due to the difference of their solubility in solvent. Secondly, polarity of active compound also contributes to their solubility in solvent. Alkaloid, terpenoid and steroid are soluble in non polar solvent like acetone, diethyl ether and hexane. Meanwhile, flavonoid, phenol and glycoside dissolve in polar solvent such as water and methanol [20,21]. It is appropriate with the determinate solubility theory “like dissolve like” that polar substances will dissolve in polar solvent and vice versa [20,22].

Generally, plants contain two major substances; they are nutrition and non nutrition compounds. Primary metabolite or nutrition compounds such as carbohydrate, protein, fatty acids and phytosterol can be found in a huge proportion but they do not have pharmacology effect. On the other hand, non nutrition compounds or secondary metabolite like alkaloid, terpenoid, flavonoid and steroid are found in a small concentration but it have pharmacology effect at certain dose [20]. Secondary metabolites are derived from metabolism of primary metabolite in plant but sometimes they have a toxic effect especially if it is used in high dose. Most of flavonoid and terpenoid in herbs have a potency as antioxidant, antiseptic and anti-inflammatory whereas steroid as anti-inflammatory and sex hormone. But, the pharmacology effect of alkaloid is difficult to be predicted in medicinal plants because they have so many biological activities [23].

Anti-diabetic effect of herbs is indicated by their potency to decrease blood glucose level. The hypoglycemia effect is controlled by active compounds like terpenoid, steroid, alkaloid and flavonoid but their mechanisms of work are different. Some herbs work as anti-diabetes by mechanisms such as insulin

sensitizers, insulin secretory, DPP-IV inhibitor and α-glucosidase inhibitor [24]. Anti-diabetic herbs have many active compounds so that they have a possibility to work by multiple action and result in interactions either synergistic or antagonistic. Sometimes the interactions have both negative and positive pharmacology effect [4].

4.2. Molecular docking of U. lobata leaf extracts

Molecular docking is now widely used to discover new ligands for target of known structure. Potential compound can be screened by free energy binding. The score of free energy binding represents binding affinity of ligand to the target protein; the lower free energy binding, the higher binding affinity [25]. In addition, inhibition constant can be predicted using bioinformatics approach. The lowest inhibition constant indicates the most potential compound. Other parameter is surface interaction. It represents the molecular recognition between ligand and target protein. The higher value of surface interaction, the higher interaction possibilities of compounds interacting with the target protein [26]. Based on the findings in the present study, mangiferin have the lowest value of inhibition constants followed by stigmasterol. It is related to binding free energy and surface interaction of these compounds. In this study, stigmasterol has the highest value of surface interaction followed by β-sitosterol and mangiferin. A great result of surface interaction showed a stronger binding between ligand and protein target, so that the biology activity is higher. Based on the *in silico* analysis, mangiferin has the lowest value in binding free energy while stigmasterol and β-sitosterol were in the second and third position. The lowest value of binding free energy produces a strong binding molecule and then causes the potential biology activity. Free energy binding and surface interaction between ligand and protein target affects the inhibitory activity of *U. lobata* leaf extract on DPP-IV.

Molecular docking studies are widely used to predict the potential candidates of drugs in the pharmaceutical industry. Binding orientation of these small molecules or active compounds to their protein targets reveals their affinity and activity as possible candidates of drugs.

4.3. DPP-IV inhibitory activity of U. lobata

DPP-IV inhibitory activity of ethanolic extract of *U. lobata* is stronger than that of water extract. It is regulated by the differences of both active compounds and their proportions in these extracts. Semi qualitative test of *U. lobata* leaf extract by LC–MS showed the contents of stigmasterol, β-sitosterol, gossypetin and chrysoeriol which are higher than that of mangiferin, quercetine and hypolaetin. Active compounds such as stigmasterol, β-sitosterol and gossypetin are soluble in semi-polar solvents like alcohol but mangiferin and hypolaetin are insoluble. The differences of solubility of active compounds in the solvents will affect the percentages of active compounds in the extracts.

Both ethanolic and water extracts of *U. lobata* contain the same active compounds but different in amounts. Content of gossypetin is lower in water extract meanwhile content of stigmasterol is higher in water extract than that in ethanolic extract, but the proportions of chrysoeriol, mangiferin and β-sitosterol are similar in both water and ethanolic extract. Non-polar compounds such as stigmasterol, β-sitosterol and gossypetin could be extracted in water solvent even though in small amount. When the water is boiled, their polarity will decrease so that it could be extracted from semi-polar until non-polar compounds [27].

Molecular docking study of *U. lobata* leaf extract showed inhibitory activity on DPP-IV. Three active compounds such as mangiferin, stigmasterol and β-sitosterol showed a low value in binding free energy. It means that the binding between ligand and molecule target is easy so that cause a strong DPP-IV inhibitory activity. It is also supported by a low value from inhibitions constant of mangiferin, stigmasterol and β-sitosterol which showed a high DPP-IV inhibitory activity. The lower value of inhibitions constant means that these compounds with low doses are able to inhibit the DPP-IV activity. Surface interaction between DPP-IV and three compounds above showed a high score (stigmasterol: 962.48, β-sitosterol: 886.91, and mangiferin: 742.75). The compound with higher value of surface interaction has the potential to binding ligand and molecule target, predicting a stronger biological activity.

DPP-IV or CD26 is a membrane-associated peptidase of 766 amino acids that is widely distributed in numerous tissues. DPP-IV is hydrolase enzyme and also exists with a soluble circulating form in plasma, and significant DPP-IV-like activity is detectable in plasma from humans and rodents. DPP-IV (CD26) exerts its biological effects via two distinct mechanisms of action. First, as a membrane-spanning protein, it binds adenosine deaminase and when activated, conveys intracellular signals independent of its enzymatic function via dimerization and activation of intracellular signaling pathways. The signaling properties of membrane-associated CD26 have been most extensively characterized in T cells [27]. The second principal biological activity of CD26 (DPP-IV) is its enzymatic function. The enzymatic activity of CD26 is exhibited by the membrane-spanning form of the molecule, and by the slightly smaller circulating soluble form [27,28].

The substrates of CD26/DPP-IV are not specific to a particular peptides. The substrates of CD26/DPP-IV are proline or alanine containing peptides and include growth factors, chemokines, neuropeptides and vasoactive peptides. DPP-IV prefers substrates with an amino-terminal proline or alanine at position 2, but may also cleave substrates with non-preferred amino acids at position 2. The structure of incretin hormone such as GLP-1 and GIP reveals a highly conserved alanine at position 2, rendering these peptides ideal putative substrates for the aminopeptidase DPP-IV [29].

A number of study showed that the importance of DPP-IV mediated inactivation of GLP-1 as a key determinant of GLP-1 and GIP bioactivity [30]. DPP-IV inhibition prevents the degradation of active GLP-1 but does not increase the levels of circulating total GLP-1 and does not prevent the kidney from rapidly clearing GLP-1. DPP-IV inhibition also acutely decreases L cell secretion of GLP-1, likely via negative feedback on the L cell. The biological activities of GLP-1 are stimulating the secretion of insulin, increasing β-cell masses, inhibiting the secretion of glucagon, reducing the rate of gastric-emptying and inducing satiety that contribute to maintain blood glucose level in type 2 diabetes mellitus [1,2].

Using of DPP-IV inhibitors, primarily for the treatment of diabetes, relates to the potential effects of these inhibition on immune function. DPP-IV/CD26 is expressed on T cells, plays a functional role in T cell activation, and activates CD26 as signaling cascade in the T cell. CD26 associates with CD45, and

modulation of CD26 activity is frequently associated with enhanced T cell proliferation in immune system [29]. CD26/DPP-IV 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 [31].

Conflict of interest statement

We declare that we have no conflict of interest.

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