# JBCPP_MANUSCRIPT
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# Inhibitory activity of *Urena lobata* leaf extract on alpha-amylase and alpha-glucosidase: *in vitro* and *in silico* approach

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**Abstract**

**Background**: In food ingestion, alpha-glucosidase (α-glucosidase) and alpha-amylase (α-amylase) are enzyme that responsible for conversion carbohydrate into glucose. Inhibition both of the enzyme can prolong absorption of glucose in intestine and control blood glucose concentration, moreover, it is beneficial for type 2 diabetes treatment. Empirically, *Urena lobata (U. lobata)* is used to cure diabetes, however the inhibitory activity on α-glucosidase and α-amylase have not been evaluated. The objective of study to examine anti-diabetic potency of *U. lobata* leaf extract through inhibition of α-amylase and α-glucosidase.
**Methods**: *U. lobata* leaf extract was obtained through extraction process using ethanol, therefore active compounds in the extract was analyzed by Liquid Chromatography–Mass Spectra (LC-MS). The inhibitory activity of *U. lobata* on α-glucosidase and α-amylase were evaluated by *in-silico* using docking server, meanwhile, *in-vitro* study using paranitrophenyl-α-D-glucopyranoside (α-NPG) and strach as substrat. The data was stated as the mean ± SD and the IC-50 value was calculated by linear regression curve fit using SPSS.
**Results**: *U. lobata* leaf extract showed inhibitory activity on α-glucosidase and α-amylase with the IC-50 value was 43.73 µg/ml and 83.73 respectively, meanwhile, acarbose as standart have IC-50 value at 1.14 µg/ml and 0.08 g/mL. Molecular docking study indicated β-sitosterol and stigmasterol from *U. lobata* extract have a huge inhibitory activity both on α-amylase and α-glucosidase based on Inhibition constant (Ki) value.
**Conclusions**: Ethanolic extract of *U. lobata* have inhibition activity on α-glucosidase stronger than on α-amylase as anti-diabetic.

**Keywords:** *anti-diabetic, enzyme, molecular docking*
**DOI :**
**Received :**

## Introduction

Carbohydrate metabolism is regulated by enzyme that breaks polysacharide into monosacharide. In food ingestion, alpha-amylase (α-amylase) and alpha-glucosidase (α-glucosidase) are enzyme that responsible for conversion a strach complex become a simple strach[1]. Enzyme that metabolize carbohydrate are produced by salivary gland and pancreatic gland become maltosa, dextrin and maltotriosa. The product of carbohydrate metabolism is delivered to small intestinal mucosa, therefore, they are hydrolyzed by α-glucosidase become glucose and absorbed into blood circulation[2]. It contributes an increase blood glucose level post prandial and need to be controlled to avoid hyperglycemia. The inhibitory activity both of α-amylase and α-glucosidase will reduces carbohydrate metabolism and glucose absorption, moreover it prevent an increase of blood glucose level especially for diabetes mellitus[1,2]. Herbs is one of the choices to suppress activity both of α-amylase and α-glucosidase in therapy of diabetes mellitus. It have some benefit such as less adverse reaction, economize, and, mostly anti-diabetic herbs work through inhibitory activity of α-amylase and α-glucosidase[3].

Pulutan (*Urena lobata)* is herbs which used Nigerian people to cure many diseases including diabetes mellitus[4]. The herbs have a bitter taste therefore it can be used to cure diabetes based on their intuition. The herbs are used both of in single and combination with other herbs to overcome diabetes by traditional healers. The research indicated the administration of *U. lobata* leaf and roots extract has hypoglycemic activity on rat induced streptozotocin [4,5]. It involved to active compounds in herbs such as sterol group, alkaloid and flavonoid [6,7]. Anti-diabetic activity of *U.lobata* leaf extract have not been evaluated especially on the α-amylase and α-glucosidase inhibitory activity. Therefore, it open a chance to increase herbs become candidate of phytoteraphy. The objective of the study was to evaluate anti diabetic potency of *U. lobata* through inhibitory activity of α-glucosidase and α-amylase.

## Materials and Methods

### Chemical

α-amylase was obtained from porcine pancreatic and α-glucosidase meanwhile Paranitrophenyl-α-D-glucopyranoside (NPG), strach substrat, DNS pewarna, Gly-pro-p-nitroanilide (GPPN), Tris-HCl buffer. The chemicals were purchased from Sigma aldrich, meanwhile ethanol from E. Merck pro analysis grade.

### Sampel preparation

Simplisia of *U. lobata* leaves were obtained from Balai Materia Medika Batu, Malang, Indonesia with certificate number 074/027/101.8/2015. Therefore, 50 g of the herbs materials was extracted in 250 ml ethanol 80 % for 4 hours using water bath shacker and it was repeated 2 times using fresh solvent. The extract were evaporated to produce a paste form and dilute with solvent according to concentration which have been fixed.

### Identification of active substances

Ethanolic extract of *U. lobata* leaf was performed a qualitative analysis using Liquid Chromatography–Mass Spectra (LC-MS) Accela 1250 pump. Mobile phase contains 0.1 % formic acid in methanol and water combination. The identification included the 10 active compounds from phytosterol, flavonoid and alkaloid groups.

### Alpha (α)-amylase inhibitory assay

A pre-incubation volume of 150 µl α-amylase 5 unit/mL in 20 mM buffer phosphat pH 6.9 and 100 µl various concentration of test material or standard. This mixture was incubated at 37ºC for 30 minutes moreover followed by addition of 250 µl strach 1 % in aquadest as substrate. The mixture solution was incubated for 10 minutes at 37ºC therefore added 50 µl dinitrosalicylic acid (DNSA) 1 % and heated with a water bath. The solution was cooled to room temperature and the sample absorbance was measured with microplate reader at 540 nm. Acarbose was used as reference drugs of α-amylase inhibitor.

### Alpha(α)-glucosidase inhibitory assay

Various concentration of test material or standard were mixed with 320 µl buffer phospat 100 mM pH 6.8 and 50 µl paranitrophenyl-α-D-glucopyranoside (α-NPG) 10 mM as substrate This mixture was incubated at 30ºC for 5 minutes, followed by addition of 20 µl α-glucosidase enzyme in buffer phospat. The reaction of mixture was incubated for 5 minutes at 30ºC and the reaction was stopped by addition 3 mL sodium hydroxide 50 mM after the incubation. The sample absorbance was measured at 410 nm with microplate reader and acarbose was used as reference drugs of α-glucosidase inhibitor.

### Molecular docking study

Activity of active substances in *U.lobata* leaf extracts both on *a-amylase* and *a-glucosidase* were evaluated by *in silico* approach using a web-based software application (www.dockingserver.com) for ligand molecular docking and protein target. Inhibition constant, free energy of binding and surface interactions were observed by this method to examine theirs activity on *a-amylase* and *a-glucosidase*.

### Statistical Analysis

The IC-50 value was calculated by linear regression curve fit using SPPS version 16.0 and the data are stated as the mean ± SD.

## Results

### Identification of active substances in *U.lobata* leaf extracts

The Active compounds from ethanolic extract of *U.lobata* leaf can be seen in the figure 1 and table 1.

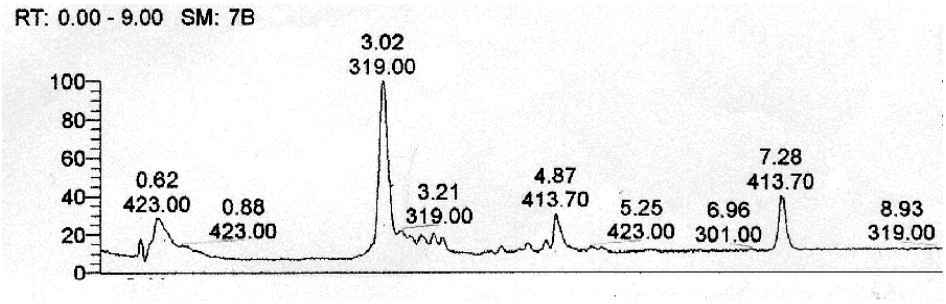


**Figure 1**. Chromatogram öf *U.lobata* leaf extract identified by LC-MS

**Table 1.** Active compounds in *U.lobata* leaf extracts

| No | Active compounds | Molecule weight | 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 qualitative analysis using LC-MS indicated that the most abundant of active substances from *U. lobata* leaf extract were gossypetin and stigmasterol. Active compounds such as β-sitosterol, chrysoeriol and mangiferin were found also in the extracts of *U.lobata*, however, the concentration was low.

### Molecular docking of *U.lobata* on α–amylase and α–glucosidase

Activity of *U.lobata* leaf extracts both of on *a-amylase* and *a-glucosidase* were evaluated by *in-silico* approach and the results can be seen at Table 2 and 3.

**Table 2.** Molecular docking of active substances in *U.lobata* leaf extracts with *a-amylase*

| No | Active compounds | Est. Free Energy of Binding (Kcal/mol) | Est. Inhibition Constant Ki (µM) | Interact. Surface |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| 1 | Stigmasterol | -9.63 | 0.0875 | 892.16 |
| 2 | β -Sitosterol | -8.66 | 0.4500 | 793.55 |
| 3 | Mangiferin | -7.84 | 1.80 | 702.30 |
| 4 | Gossypetin | -6.40 | 20.36 | 621.77 |
| 5 | Chrysoeriol | -6.50 | 17.13 | 653.91 |
| 6 | Acarbose | -8.78 | 0.3470 | 1087.32 |

**Table 3.** Molecular docking of active substances in *U.lobata* leaf extracts with *a-glucosidase*

| No | Active compounds | Est. Free Energy of Binding (Kcal/mol) | Est. Inhibition Constant Ki (μM) | Interact. Surface |
|---|---|---|---|---|
| 1 | Stigmasterol | -10.20 | 0.0331 | 891.01 |
| 2 | β -Sitosterol | -9.59 | 0.0931 | 894.65 |
| 3 | Mangiferin | -7.98 | 1.4100 | 764.01 |
| 4 | Gossypetin | -7.20 | 5.2400 | 687.04 |
| 5 | Chrysoeriol | -6.70 | 12.190 | 705.30 |
| 6 | Acarbose | -.3.98 | 0.0012 | 457.77 |

Molecular docking studies indicated that stigmasterol, β-sitosterol and mangiferin have a low value in both of the inhibition constant and the free energy of binding, however, the surface interaction was high actually. Meanwhile, gossypetin and chrysoeriol have a higher value on binding free energy and inhibition constant compare to other substances above. The differences in each parameter value causes the distinction of inhibitory activity both of on *a-glucosidase* and *a-amylase.* Based on inhibition constant, stigmasterol and β-sitosterol in *U.lobata* leaf extracts more potent to inhibit an activity both on *a-glucosidase* and *a-amylase,* even stronger than acarbose as a drug reference especially on *a-amylase*.

### α-glucosidase inhibitory activity and α–amylase of *U.lobata* leaf extract

Ethanolic extract of *U. lobata* leaf were tested both on *a-amylase* and *a-glucosidase* inhibitory assay by *in vitro* method and the result is shown in Table 4 and 5.

**Table 4.** *a-amylase inhibitory* activity of *U. lobata* leaf extracts and acarbose

| Group | Sample | n | Concentration (μg/ml) | % inhibition | IC-50 (μg/mL) |
|---|---|---|---|---|---|
| 1 | Ethanolic extract of *U.lobata* | 3 | 6.25 | 20.62 ± 3.79 | 83.73 |
| | | 3 | 12.50 | 22.36 ± 1.24 | |
| | | 3 | 25.00 | 27.33 ± 1.24 | |
| | | 3 | 50.00 | 40.99 ± 4.35 | |
| | | 3 | 100.00 | 54.65 ± 8.14 | |
| 2 | Acarbose | 3 | 0.05 | 47.77 ± 0.99 | 0.08 |
| | | 3 | 0.10 | 53.40 ± 3.05 | |
| | | 3 | 0.15 | 58.74 ± 0.44 | |
| | | 3 | 0.20 | 61.04 ± 2.48 | |
| | | 3 | 0.25 | 61.47 ± 4.12 | |

**Table 5.** *a-glucosidase inhibitory* activity of *U. lobata* leaf extracts and acarbose

| Group | Sample | n | Concentration (μg/ml) | % inhibition | IC-50 (μg/mL) |
|---|---|---|---|---|---|
| 1 | Ethanolic extract of *U.lobata* | 3 | 6.25 | 20.00 ± 0.00 | 43.73 |
| | | 3 | 12.5 | 30.00 ± 0.00 | |
| | | 3 | 25.0 | 45.00 ± 0.00 | |
| | | 3 | 50.0 | 60.00 ± 3.85 | |
| | | 3 | 100.0 | 80.00 ± 3.85 | |
| 2 | Acarbose | 3 | 0.25 | 16.37 ± 11.52 | 1.14 |
| | | 3 | 0.50 | 29.09 ± 0.11 | |
| | | 3 | 0.75 | 31.98 ± 6.85 | |
| | | 3 | 1.00 | 44.74 ± 1.69 | |
| | | 3 | 1.25 | 52.02 ± 12.44 | |

Based on these results, ethanolic extract of *U.lobata* showed that the inhibition activity on *a-glucosidase* was stronger about 2 times folds, compared to on α-amylase. However, the inhibitory activity of *U. lobata* extracts both on *a-glucosidase* and α-amylase are still lower, compared to acarbose as reference drugs.

## Discussion

## Identification of active compounds in *U. lobata* leaf extracts

Ethanolic extracts of *U. lobata* leaves has been reported to contain non-nutritional substances having pharmacologoical bioactive effects [8,21]. These include flavone or flavonol compounds, such as Gossypetin, which was shown to be an effective antioxidant and possess anti-microbial, anti-atherosclerotic and anti-mutagenic properties[12]. A flavon, Chrysoeriol, was also reported in these extracts, and was shown to be an anti-histamine, and therefore, anti-inflammatory [17]. Gossypetin is known to be very soluble in non-polar eluents (such as chloroform and benzene), and moderately soluble in semi-polar eluents (in this case, ether and ethanol), however insoluble in water. Aside from compounds in the flavonoid class, other classes of compounds were also reported. Stigmasterol, a group of unsaturated plant sterols commonly found in many medicinal herbs in plant fats or oils [9,10], was also reported to be found in the extract. Meanwhile Stigmasterol was found to be insoluble in water (similar to other sterols), it was soluble in most organic solvents containing at least one alcohol functional group. Stigmasterol was found to be able to prevent hyperglycemia and could inhibit thyroid levels, as well as being an antioxidant [10,11]. β-sitosterol, also a hydrophobic phytosterol [13], was found to be anti-cholesterol and could act as an immunomodulator [14]. Mangiferin, a glucoside of norathyriol and a semipolar xanthonoid reported to be soluble in hot ethanol and methanol, was found to be anti-microbial, anti-glycemic and an antioxidant [15, 16].

The composition of extracted active compounds was found to be influenced by the polarity of the solvents used in the extraction, which would dictate obtained active compounds based on its polarity and solubility in that solvent. Non-polar solvents, such as acetone, diethy ether and hexane, would draw also non-polar compounds, such as alkaloids, terpenoids and steroids, while polar solvents, such as water and methanol, would draw flavonoids, phenols and glycosides [18, 19]. The solubility of polar substances in polar solvents, and vice versa, concurs with the basic determinate solubility theory ("like dissolves like") [20].

The determination of herbal medicine, or herbal extract, to act as an anti-diabetic was due to its potency in reducing blood glucose levels, and often resulted in the presence of active substances in the terpenoid, steroid, alkaloid and flavonoid classes. However, these compounds often have different and varied mechanisms of action. Some may act by increasing insulin secretion or insulin sensitivity, and other may act by inhibiting α-glucosidase and DPP-4 [3,5]. Furthermore, as an extract could contain many active substances with similar bioactivities (but different drug targets) may interact synergistically or even antagonistically, and may result in a negative or positive pharmacological activity [23].

## Molecular docking of *U.lobata* on α–glucosidase and α–amylase

In the pharmaceutical field, molecular docking is often used to screen and predict the potential candidates the drug target of ligands with a known structure, based on its free energy binding, inhibition constant, and surface interaction. Free energy binding would inversely correspond to the binding affinity of a ligand to a target molecule (a lower free energy binding value would indicate higher binding affinity) [24]. The inhibition constant (Ki) is used in *in silico* studies to predict the inhibitory activity of a ligand to a drug target. Similarly, this score also operates inversely, in which a lower Ki score would indicate a higher inhibition activity of a protein aterget. Lastly, surface interaction represents the surface area and molecular recognition between a ligand and a binding pocket in a protein target. A higher surface interaction would indicate a higher number of interactions between a ligand and a molecule target [25]. Based on the molecular docking in this study, stigmasterol and β-sitosterol was found to have a lowest inhibition constant when tested against α-glucosidase and α-amylase. Free energy binding score showed that stigmasterol, followed by β-sitosterol and mangiferin, had the lowest free energy binding, respectively, and a high surface interaction in the same order. Both scores indicate a stronger binding with the drug target and may indicate a strong biological activity [24, 25] and therefore indicating the inhibitory activity of *U. lobata* leaf extracts on α-glucosidase and α-amylase.

Based on those categories, stigmasterol have the lowest score of inhibition constant and followed by β-sitosterol either on *a-glucosidase* and *a-amylase*. It is related to free energy of binding and surface interaction of these substances. In this research, stigmasterol have the highest score of surface interaction that followed by β–sitosterol and mangiferin respectively. A high score of surface interaction indicated a stronger bond between ligand and molecule target, moreover, it results a great biology activity. *In silico* analysis showed that stigmasterol have the lowest score in the free energy of binding, meanwhile β–sitosterol and mangiferin were in the second and third position. The lowest score of binding free energy results a strong binding molecule, furthermore, it causes an increase of theirs biology activity [24,25]. Free energy of binding and surface interaction between molecule target and ligand influences the inhibitory activity of *U.lobata* leaf extract both on *a-glucosidase* and *a-amylase*.

Molecular docking research is widely used to predict the potential candidates of drugs in the pharmaceutical fields. Binding orientation of these active substances to their molecule targets reveals their activity and affinity as possible candidates of drugs [25].

## α-glucosidase and α–amylase inhibitory activity of *U.lobata* leaf extract by *in vitro*

Inhibitory activity of *U.lobata* leaf extract on *a-glucosidase* was stronger than on *a-amylase*. It is controlled by the differences of active compounds which interacts with both *a-glucosidase* and *a-amylase. U lobata* contains active substances such as phenol, tanin, stigmasterol, beta sitosterol, mangiferin, quercetin and also some flavon compounds such as gossypetin dan chrysoeriol, with total a phenol content 25 %. Active compounds found to inhibit *a-glucosidase* activity are those from the flavonoid, alkaloid and tanin group [26]. However, this depend on other factors such as are isoflavone level which is contained in herbs, molecule size and structur variation of tanin. Meanwhile, molecule configuration on binding site of active situs from *a –glucosidase* contribute also to its activity. Generally, a more complex and large tannin structure would be more effective in inhibiting α-*glucosidase* [27]. Isoflavone is active compounds having an inhibition activity of *a –glucosidase* stronger than flavone compounds [26].

Flavonoid inhibits *a–glucosidase* by hydroxylation binding and substitution on the β-flavonoid ring. Inhibition activity of flavonoid is correlated with the number of hydroxyls on β-flavonoid ring. The more hydrogen binding between hydroxyl and polyphenol ligand with catalytic residues from the binding site of enzyme glucosidase, the more strongly the inhibition activity against *a*-glucosidase. Inhibition of *a–glucosidase* retain carbohydrat hydrolysis into maltose, therefore, it decreases glucose level post prandially[1]. *U. lobata* contains stigmasterol and β-sterol from phytosterol group and also mangiferin from xanthone glucosidase group, and, based on *in silico* study previously, *U. lobata* is able to suppress *a–glucosidase* activity.

Other active substances inhibiting *α–glucosidase* is sterol and xanthonoid [27,28]. *α–glucosidase* is a glucoamylase enzyme that hidrolyzes polysaccharides, disaccharides, oligosaccharides on the brush border of the microvili in epitel intestine into glucose monomers. Activity of α–glucosidase is influenced by temperature and acidity level. Optimum temperature and acidity level for enzyme are 37°C and 6,8 respectively. This condition is suitable with reactions occuring in the human body[29]. Active substances found to be able to supress *α–amylase* are flavonoid, fenol, alkaloid, miscellaneous,terpenoid, xanthone, glucosidase and sterol [27,28].

## Conclusions

*U. lobata* have anti-diabetic potency more on *α-glucosidase* than on *α-amylase* through its inhibitory activity.

## Conflict of interest statement

We declare that we have no conflict of interest

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# JBCPP_MANUSCRIPT DIREVISI KE-1

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**Abstract**

**Background**: In food ingestion, alpha-glucosidase (α-glucosidase) and alpha-amylase (α-amylase) are enzymes that are responsible to convert a carbohydrate into glucose. Inhibition of both enzyme activities can prolong absorption of glucose in intestine and reduce post-prandial increase of blood glucose concentration, thus, it is beneficial for type-2 diabetes treatment. Traditionally, *Urena lobata (U. lobata)* has been used to manage diabetes, but the scientific proof of this claim remains scarce. Therefore, the objective of this study to examine the anti-diabetic potential of *U. lobata* leaf extract through inhibition of α-amylase and α-glucosidase.
**Methods**: *U. lobata* leaf extract was obtained through extraction process using ethanol and the chemical compounds in the extract were analyzed by Liquid Chromatography–Mass Spectra (LC-MS). The inhibitory activity of *U. lobata* on α-glucosidase and α-amylase were evaluated by *in-silico* using docking server, whereas *in-vitro* enzymatic assays using *para*-nitrophenyl-α-D-glucopyranoside (α-NPG) and starch as substrates. The data was presented as mean ± SD and the $IC_{50}$ value was calculated using SPSS.
**Results**: *U. lobata* leaf extract showed inhibitory activity on α-glucosidase and α-amylase with the $IC_{50}$ value was 43.73 µg/mL and 83.73 µg/mL respectively, meanwhile, acarbose as standart have $IC_{50}$ value at 1.14 µg/mL and 0.08 µg/mL. Molecular docking study indicated β-sitosterol and stigmasterol from *U. lobata* extract have a huge inhibitory activity both on α-amylase and α-glucosidase based on Inhibition constant (Ki) value.
**Conclusions**: Ethanolic extract of *U. lobata* showed inhibition activity on α-glucosidase stronger than on α-amylase as anti-diabetic.

**Keywords:** *anti-diabetic, enzyme, metabolism, molecular docking, polysaccharides*
**DOI :**
**Received :**

## Introduction

Carbohydrate metabolism is regulated by enzyme that breaks polysaccharides into monosaccharides. In food ingestion, alpha-amylase (α-amylase) and alpha-glucosidase (α-glucosidase) are enzymes that are responsible for the conversion of a starch complex into a simple starch[1]. alpha-amylase are produced by the salivary glands and pancreatic glands which metabolizes starch into maltosa, dextrin and maltotriosa. These products are then delivered in to the small intestinal mucosa, where they are then hydrolyzed by α-glucosidase and results in glucose and which are absorbed into the blood[2]. It contributes to an increase in blood glucose level post-prandial, which needs to be controlled to avoid hyperglycemia. The inhibitory activity both of α-amylase and α-glucosidase will reduce carbohydrate metabolism and glucose absorption, and thus preventing an increase of blood glucose level, beneficial especially in patients with diabetes mellitus[1,2]. Herbal remedies is one of the choices to suppress activity of both α-amylase and α-glucosidase in the therapy of diabetes mellitus. Some benefit of herbal remedies includes less adverse reaction and is relatively cheaper. Most anti-diabetic herbal remedies work through inhibitory activity of α-amylase and α-glucosidase[3].

Pulutan (*Urena lobata)* is a herbal remedy used by Nigerian people to manage many diseases, including diabetes mellitus[4]. This plant has a bitter taste, which is the basis of the intuition that it may be able to treat diabetes. Traditional healers used *U. lobata* in both in single and in combination with other herbs to manage diabetes. Research indicates that the administration of *U. lobata* leaf and roots extracts has hypoglycemic activity on rats induced with streptozotocin[4,5]. This is due to several active compounds in the herbs, such as sterol groups, alkaloids and flavonoids[6,7]. Anti-diabetic activity of *U. lobata* leaf extracts has not been evaluated, especially regarding its α-amylase and α-glucosidase inhibitory activity. The objective of the study was to evaluate anti diabetic potency of *U. lobata* through inhibitory activity of α-glucosidase and α-amylase.

## Materials and Methods

### Chemical

α-amylase was obtained from porcine pancreatic and α-glucosidase, meanwhile *para*-nitrophenyl-α-D-glucopyranoside (α-NPG), strach substrat, dinitrosalicylic acid (DNSA), Gly-pro-p-nitroanilide (GPPN), Tris-HCl buffer were purchased from Sigma aldrich, meanwhile ethanol was from Merck (pro analysis grade).

### Sampel preparation

Leaves of *U. lobata* leaves were obtained from Balai Materia Medika Batu, Malang, Indonesia with a certificate number of 074/027/101.8/2015. About 50 g of the sample materials were extracted in 250 ml ethanol 80% for 4 hours using water bath shaker. The extraction was repeated for another two times using fresh solvent. The extracts was evaporated using a rotary evaporator to produce a paste form (weight) and dilutde with solvent according to the designated concentration.

### Identification of active substances
Ethanolic extract of *U. lobata* leaf was subjected to a qualitative analysis using Liquid Chromatography–Mass Spectra (LC-MS) Accela 1250 pump. Mobile phase contains 0.1 % formic acid in methanol and water combination. The identification included the 10 active substances, considered based on previous study compounds including phytosterol, flavonoid and alkaloid groups.

### α-amylase inhibitory assay
About 100 µl of sample or standard solution was added to 150 µl of 5 unit/ml α-amylase and 20 mM of phosphat buffer pH 6.9. This mixture was incubated at 37°C for 30 minutes followed by addition of 250 µl of 1% starch in distilled water. The mixture solution was incubated again for 10 minutes at 37°C. Then, 50 µl of 1% DNSA was added and the mixture was and heated on water bath. The solution was cooled to room temperature and the sample absorbance was measured with microplate reader at 540 nm. Acarbose was used as standard.

### α-glucosidase inhibitory assay
Various concentration of test material or standard were mixed with 320 µl 100 mM phospate buffer pH 6.8 and 50 µl of 10 mM α-NPG. This mixture was incubated at 30°C for 5 minutes, followed by addition of 20 µl of α-glucosidase in phospate buffer. The mixture was incubated for another 5 minutes at 30°C and the reaction was stopped by adding 3 ml of 50 mM sodium hydroxide 50 mM. The sample absorbance was measured at 410 nm with a microplate reader. Acarbose was used as a standard.

### Molecular docking study
Activity of identified substances in *U. lobata* leaf extracts both on *α-amylase* (P0DTE8) and *α-glucosidase* (O43451) were evaluated by *in silico* approach using a web-based software application (www.dockingserver.com). The structure of identified compounds were obtained from PubChem database and protein target (enzymes) from Uniprot database. Molecular docking was performed by uploading both of chemical compound and protein target on software. The prediction value of parameters include inhibitory constant (Ki), free energy of binding and surface interactions.

### Statistical Analysis
The $IC_{50}$ value was calculated by linear regression curve fit using SPPS version 16.0 and the data are presented as the mean ± SD.

## Results
### Identification of active substances in *U. lobata* leaf extracts
The Active compounds from ethanolic extract of *U.lobata* leaf can be seen in the Figure 1 and Table 1.

**Figure 1**. LC-MS Chromatogram öf *U. lobata* leaf extract

**Table 1.** Identified compounds in *U. lobata* leaf extract by LC-MS

The qualitative analysis using LC-MS indicated that the most abundant secondary metabolites in *U. lobata* leaf extract was gossypetin and stigmasterol. Other compounds such as β-sitosterol, chrysoeriol and mangiferin were also identified in the extracts of *U. lobata*, however, the concentration was low.

### Molecular docking of *U. lobata* on α–amylase and α–glucosidase
The *in silico* inhibitory activity of the identified compounds in *U. lobata* leaf extract on α-amylase and α-glucosidase results can be seen at Table 2 and 3.

**Table 2.** Molecular docking of identified compounds *U. lobata* leaf extracts with α-amylase

**Table 3.** Molecular docking of identified substances in *U. lobata* leaf extracts with α-glucosidase

Molecular docking studies indicated that stigmasterol, β-sitosterol and mangiferin have low inhibition constant and free energy of binding, however, the surface interaction showed high value. Meanwhile, gossypetin and chrysoeriol have higher values on binding free energy and inhibition constant as compared to stigmasterol, β-sitosterol and mangiferin. The differences in each parameter value signified distinct inhibitory activity both of on α-glucosidase and α-amylase activities. Based on inhibition constant, stigmasterol and β-sitosterol in showed higher inhibition activity towards both α-glucosidase and α-amylase. These activities are more prominent than acarbose, especially on α-amylase.

### α-glucosidase inhibitory activity and α–amylase of *U. lobata* leaf extract
The inhibitory activity of ethanolic extract of *U. lobata* leaf on α-amylase and α-glucosidase were evaluated *in vitro* using enzymatic assay and the result are shown in Table 4 and 5.

**Table 4.** α-amylase inhibitory activity of *U. lobata* leaf extract and acarbose

**Table 5.** α-glucosidase inhibitory activity of *U. lobata* leaf extract and acarbose
Based on these results, the inhibition activity of the ethanolic extract of *U. lobata* on α-glucosidase was 2 times stronger than α-amylase. However, the inhibitory activities are lower than acarbose.

# Discussion

## Identification of active compounds in *U. lobata* leaf extracts

Ethanolic extracts of *U. lobata* leaves has been reported to contain non-nutritional substances having pharmacological effects [8,21]. These include flavone or flavonol compounds, such as gossypetin, which was shown to be an effective antioxidant and possess anti-microbial, anti-atherosclerotic and anti-mutagenic properties[12]. A flavon, chrysoeriol, was also reported in these extracts, and was shown to be an anti-histamine and anti-inflammatory activities[17]. Gossypetin is known to be very soluble in non-polar eluents (such as chloroform and benzene), moderately soluble in semi-polar eluents (in this case, ether and ethanol), and insoluble in water. Aside from compounds in the flavonoid class, other classes of compounds were also reported. Stigmasterol, a plant sterol commonly found in many medicinal herbs in plant fats or oils [9,10], was also reported to be found in the extract. This compound was insoluble in water (similar to other sterols), and soluble in most organic solvents containing at least one alcohol functional group. Stigmasterol showed to be able to prevent hyperglycemia and could inhibit thyroid levels, as well as being an antioxidant [10,11]. Other hydrophobic phytosterol, β-sitosterol[13], showed anti-cholesterol activity and could act as an immunomodulator [14]. Mangiferin, a glucoside of norathyriol and a semipolar xanthonoid reported to be soluble in hot ethanol and methanol, was found to be anti-microbial, anti-glycemic and an antioxidant [15, 16].

The identified compounds in the extract of *U. lobata* leaf were found to be influenced by the polarity of the solvents used in the extraction. Non-polar solvents, such as acetone, diethy ether and hexane, would extract non-polar compounds, such as alkaloids, terpenoids and steroids, while polar solvents, such as water and methanol, would extract flavonoids, phenols and glycosides [18, 19]. The solubility of polar substances in polar solvents, and vice versa, concurs with the basic determinate solubility theory ("like dissolves like") [20].

Herbal medicine, or herbal extract, is considered as an anti-diabetic due to its potency in reducing blood glucose levels, which often resulted from the phytochemical compounds, such as terpenoid, steroid, alkaloid and flavonoid classes through different mechanisms of action. Some may act by increasing insulin secretion or insulin sensitivity, and other may act by inhibiting α-glucosidase and DPP-4[3,5]. Furthermore, since an extract could contain many compounds, the activity of the extract may result in synergistic or even antagonistic interactions of some compounds existing in the extract[23].

## Molecular docking of *U.lobata* on α–glucosidase and α–amylase

In pharmaceutical field, molecular docking is often used to screen and predict the potential candidates the drug target of ligands with a known structure, based on its free energy binding, inhibition constant, and surface interaction. Free energy binding would inversely correspond to the binding affinity of a ligand to a target molecule (a lower free energy binding value would indicate higher binding affinity) [24]. The inhibition constant (Ki) is used in *in silico* studies to predict the inhibitory activity of a ligand to a drug target. Similarly, this score also operates inversely, in which a lower Ki score would indicate a higher inhibition activity of a protein target. Lastly, surface interaction represents the surface area and molecular recognition between a ligand and a binding pocket in a protein target. A higher surface interaction would indicate a higher number of interactions between a ligand and a molecule target [25].

Based on the molecular docking in this study, stigmasterol and β-sitosterol were found to have the lowest inhibition constant when tested against α-glucosidase and α-amylase. Free energy binding score showed that stigmasterol, followed by β-sitosterol and mangiferin, had the lowest free energy binding, respectively, and a high surface interaction in the same order. Both scores indicate a stronger binding with the drug target and may indicate a strong biological activity [24, 25] and therefore indicating the inhibitory activity of *U. lobata* leaf extracts on α-glucosidase and α-amylase.

Based on those categories, stigmasterol have the lowest score of inhibition constant and followed by β-sitosterol either on α-glucosidase and α-amylase. It is related to free energy of binding and surface interaction of these substances. In this research, stigmasterol have the highest score of surface interaction that followed by β–sitosterol and mangiferin respectively. A high score of surface interaction indicated a stronger bond between ligand and molecule target, moreover, it results a great biology activity. *In silico* analysis showed that stigmasterol have the lowest score in the free energy of binding, meanwhile β–sitosterol and mangiferin were in the second and third position. The lowest score of binding free energy results a strong binding molecule, furthermore, it causes an increase of their biology activity [24,25]. Free energy of binding and surface interaction between molecule target and ligand influences the inhibitory activity of *U. lobata* leaf extract both on *α-glucosidase* and *α-amylase*.

Molecular docking research is widely used to predict the potential candidates of drugs in the pharmaceutical fields. Binding orientation of these active substances to their molecule targets reveals their activity and affinity as possible candidates of drugs [25].

## α-glucosidase and α–amylase inhibitory activity of *U. lobata* leaf extract by *in vitro*

Our results showed that inhibitory activity of *U.lobata* leaf extract on α-glucosidase was stronger than on α-amylase. It may be due to the different chemical composition of the extracts which interacts with α-glucosidase and α-amylase. *U. lobata* contains compounds such as phenol, tanin, stigmasterol, beta sitosterol, mangiferin, quercetin and also some flavon compounds such as gossypetin dan chrysoeriol, with total a phenol content of 25 %. Active compounds found to inhibit α-glucosidase activity are those from the flavonoid, alkaloid and tanin group [26]. However, this depend on other factors such as are isoflavone level which is contained in herbs, molecule size and structur variation of tanin. Meanwhile, molecular configuration on binding site of α–glucosidase active sites also contributes to its activity. Generally, a more complex and large tannin structure would be more effective in inhibiting α-glucosidase [27]. Isoflavone is active compounds having an inhibition activity of α–glucosidase stronger than flavone compounds [26].

Flavonoid inhibits α–glucosidase by hydroxylation binding and substitution on the β-flavonoid ring. Inhibition activity of flavonoid is correlated with the number of hydroxyls on β-flavonoid ring. The more hydrogen binding between hydroxyl and polyphenol ligand with catalytic residues from the binding site of enzyme glucosidase, the more strongly the inhibition activity against α-glucosidase. Inhibition of α–glucosidase retains carbohydrate hydrolysis into maltose, therefore, it decreases glucose level post prandially[1]. *U. lobata* contains stigmasterol and β-

sterol from phytosterol group and also mangiferin from xanthone glucosidase group. Based on *in silico* study previously, *U. lobata* is able to suppress α–glucosidase activity controlled by active compound mentioned above.

Previous study showed anti-diabetic of *U. lobata* leaf extract through inhibitory activity of dipeptdyl peptidase-4 (DPP-4) using *in vitro* and *in vivo* approach [5,6]. Other study indicated both of aqueous and methanolic extract of *U.lobata* leaf have anti-hyperglycemic or hypoglycemic effect on rats and alkaloids, flavonoids, saponins, tannins present in the extracts may be responsible for the effects [4,9]. Study in rabbits showed *U.lobata* aqueous extract of roots significantly reduced fasting glucose and body weight [8].

Other active substances inhibiting α–glucosidase are sterol and xanthonoid[27,28]. Enzyme α–glucosidase is a glucoamylase enzyme that hidrolyzes polysaccharides, disaccharides, oligosaccharides on the brush border of the microvili in epitel intestine into glucose monomers. Activity of α–glucosidase is influenced by temperature and acidity level. Optimum temperature and acidity level for enzyme are 37°C and 6.8 respectively. This condition is suitable with reactions occuring in the human body[29]. Active substances found to be able to supress α–amylase are flavonoid, fenol, alkaloid, miscellaneous,terpenoid, xanthone, glucosidase and sterol [27,28,30].

## Conclusions

Ethanolic extract of *U. lobata* leaf have inhibition activity stronger on α-glucosidase than on α-amylase as anti-diabetic.

## Conflict of interest statement

We declare that we have no conflict of interest

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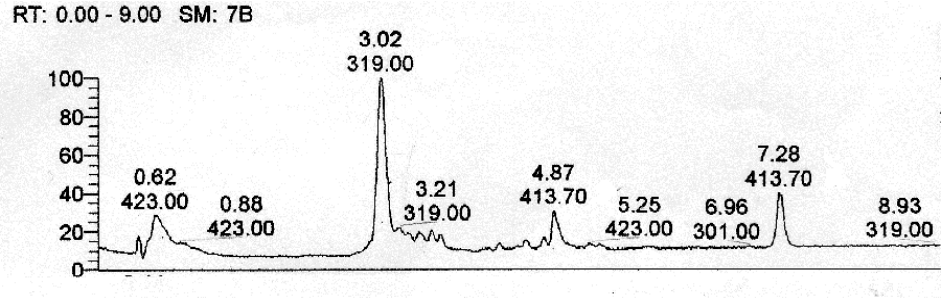

**Figure 1**. Chromatogram öf *U.lobata* leaf extract identified by LC-MS

**Table 1.** Active compounds in *U.lobata* leaf extracts

| No | Active compounds | Molecule weight | 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 | (+) |

Note (-) : negative, (+) : weak, (++) : moderate, (+++) : strong

**Table 2.** Molecular docking of active substances in *U.lobata* leaf extracts with *a-amylase*

| No | Active compounds | Estimation Free Energy of Binding (Kcal/mol) | Estimation of Inhibition Constant Ki (µM) | Interaction Surface |
|---|---|---|---|---|
| 1 | Stigmasterol | -9.63 | 0.0875 | 892.16 |
| 2 | β -Sitosterol | -8.66 | 0.4500 | 793.55 |
| 3 | Mangiferin | -7.84 | 1.80 | 702.30 |
| 4 | Gossypetin | -6.40 | 20.36 | 621.77 |
| 5 | Chrysoeriol | -6.50 | 17.13 | 653.91 |
| 6 | Acarbose | -8.78 | 0.3470 | 1087.32 |

**Table 3.** Molecular docking of active substances in *U.lobata* leaf extracts with *a-glucosidase*

| No | Active compounds | Estimation Free Energy of Binding (Kcal/mol) | Estimation of Inhibition Constant Ki (µM) | Interaction Surface |
|---|---|---|---|---|
| 1 | Stigmasterol | -10.20 | 0.0331 | 891.01 |
| 2 | β -Sitosterol | -9.59 | 0.0931 | 894.65 |
| 3 | Mangiferin | -7.98 | 1.4100 | 764.01 |
| 4 | Gossypetin | -7.20 | 5.2400 | 687.04 |
| 5 | Chrysoeriol | -6.70 | 12.190 | 705.30 |
| 6 | Acarbose | -.3.98 | 0.0012 | 457.77 |

**Table 4.** *a-amylase inhibitory* activity of *U. lobata* leaf extracts and acarbose

| Group | Sample | n | Concentration (µg/ml) | % inhibition | $IC_{50}$ (µg/mL) |
|---|---|---|---|---|---|
| 1 | Ethanolic extract of *U.lobata* | 3 | 6.25 | 20.62 ± 3.79 | 83.73 |
| | | 3 | 12.50 | 22.36 ± 1.24 | |
| | | 3 | 25.00 | 27.33 ± 1.24 | |
| | | 3 | 50.00 | 40.99 ± 4.35 | |
| | | 3 | 100.00 | 54.65 ± 8.14 | |
| 2 | Acarbose | 3 | 0.05 | 47.77 ± 0.99 | 0.08 |
| | | 3 | 0.10 | 53.40 ± 3.05 | |
| | | 3 | 0.15 | 58.74 ± 0.44 | |
| | | 3 | 0.20 | 61.04 ± 2.48 | |
| | | 3 | 0.25 | 61.47 ± 4.12 | |

**Table 5.** *a-glucosidase inhibitory* activity of *U. lobata* leaf extracts and acarbose

| Group | Sample | n | Concentration (µg/ml) | % inhibition | $IC_{50}$ (µg/mL) |
|---|---|---|---|---|---|

| | | | | | |
|---|---|---|---|---|---|
| 1 | Ethanolic extract of *U.lobata* | 3 | 6.25 | 20.00 ± 0.00 | 43.73 |
| | | 3 | 12.5 | 30.00 ± 0.00 | |
| | | 3 | 25.0 | 45.00 ± 0.00 | |
| | | 3 | 50.0 | 60.00 ± 3.85 | |
| | | 3 | 100.0 | 80.00 ± 3.85 | |
| 2 | Acarbose | 3 | 0.25 | 16.37 ± 11.52 | 1.14 |
| | | 3 | 0.50 | 29.09 ± 0.11 | |
| | | 3 | 0.75 | 31.98 ± 6.85 | |
| | | 3 | 1.00 | 44.74 ± 1.69 | |
| | | 3 | 1.25 | 52.02 ± 12.44 | |

# JBCPP_MANUSCRIPT
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# Inhibitory activity of *Urena lobata* leaf extract on alpha-amylase and alpha-glucosidase: *in vitro* and *in silico* approach

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**Abstract**

**Background**: In food ingestion, alpha-glucosidase (α-glucosidase) and alpha-amylase (α-amylase) are enzymes that are responsible to convert a carbohydrate into glucose. Inhibition of both enzyme activities can prolong absorption of glucose in intestine and reduce post-prandial increase of blood glucose concentration, thus, it is beneficial for type-2 diabetes treatment. Traditionally, *Urena lobata (U. lobata)* has been used to manage diabetes, but the scientific proof of this claim remains scarce. Therefore, the objective of this study to examine the anti-diabetic potential of *U. lobata* leaf extract through inhibition of α-amylase and α-glucosidase.
**Methods**: *U. lobata* leaf extract was obtained through extraction process using ethanol and the chemical compounds in the extract were analyzed by Liquid Chromatography–Mass Spectra (LC-MS). The inhibitory activity of *U. lobata* on α-glucosidase and α-amylase was evaluated by *in-silico* using docking server, whereas *in-vitro* enzymatic assays were using *para*-nitrophenyl-α-D-glucopyranoside (α-NPG) and starch as substrates. The data were presented as mean ± SD and the $IC_{50}$ value was calculated using SPSS.
**Results**: *U. lobata* leaf extract showed inhibitory activity on α-glucosidase and α-amylase with the $IC_{50}$ value was 43.73 µg/mL and 83.73 µg/mL, respectively, meanwhile, acarbose as standard has $IC_{50}$ value at 1.14 µg/mL and 0.08 µg/mL. Molecular docking study indicated β-sitosterol and stigmasterol from *U. lobata* extract have a huge inhibitory activity both on α-amylase and α-glucosidase based on inhibition constant (Ki) value.
**Conclusions**: Ethanolic extract of *U. lobata* showed inhibition activity on α-glucosidase stronger than on α-amylase as anti-diabetic.

**Keywords:** *anti-diabetic, enzyme, metabolism, molecular docking, polysaccharides*
**DOI :**
**Received :**

## Introduction

Carbohydrate metabolism is regulated by enzymes that break polysaccharides into monosaccharides. In food ingestion, alpha-amylase (α-amylase) and alpha-glucosidase (α-glucosidase) are enzymes that are responsible for the conversion of a starch complex into a simple starch[1]. Alpha-amylase are produced by the salivary glands and pancreatic glands, which metabolizes starch into maltosa, dextrin and maltotriosa. These products are then delivered in to the small intestinal mucosa, where they are then hydrolyzed by α-glucosidase and results in glucose and which are absorbed into the blood[2]. It contributes to an increase in blood glucose level post-prandial, which needs to be controlled to avoid hyperglycemia. The inhibitory activity both of α-amylase and α-glucosidase will reduce carbohydrate metabolism and glucose absorption, and, thus, preventing an increase of blood glucose level, beneficial especially in patients with diabetes mellitus[1,2]. Herbal remedies are one of the choices to suppress activity of both α-amylase and α-glucosidase in the therapy of diabetes mellitus. Some benefit of herbal remedies includes less adverse reaction and is relatively cheaper. Most anti-diabetic herbal remedies work through inhibitory activity of α-amylase and α-glucosidase[3].

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## Materials and Methods

### Chemical

α-amylase was obtained from porcine pancreatic and α-glucosidase, meanwhile *para*-nitrophenyl-α-D-glucopyranoside (α-NPG), starch substrate, dinitrosalicylic acid (DNSA), Gly-pro-p-nitroanilide (GPPN), Tris-HCl buffer were purchased from Sigma aldrich, meanwhile ethanol was from Merck (pro analysis grade).

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Leaves of *U. lobata* leaves were obtained from Balai Materia Medika Batu, Malang, Indonesia with a certificate number of 074/027/101.8/2015. About 50 g of the sample materials were extracted in 250 ml ethanol 80% for 4 hours using water bath shaker. The extraction was repeated for another two times using fresh solvent. The extracts were evaporated using a rotary evaporator to produce a paste form (weight) and diluted with solvent according to the designated concentration.

### Identification of active substances
Ethanolic extract of *U. lobata* leaf was subjected to a qualitative analysis using Liquid Chromatography–Mass Spectra (LC-MS) Accela 1250 pump. Mobile phase contains 0.1 % formic acid in methanol and water combination. The identification included the 10 active substances, considered based on previous study compounds including phytosterol, flavonoid and alkaloid groups.

### α-amylase inhibitory assay
About 100 µl of sample or standard solution was added to 150 µl of 5 unit/ml α-amylase and 20 mM of phosphate buffer pH 6.9. This mixture was incubated at 37ºC for 30 minutes followed by addition of 250 µl of 1% starch in distilled water. The mixture solution was incubated again for 10 minutes at 37ºC. Then, 50 µl of 1% DNSA was added and the mixture was and heated on a water bath. The solution was cooled to room temperature and the sample absorbance was measured with microplate reader at 540 nm. Acarbose was used as standard.

### α-glucosidase inhibitory assay
Various concentrations of test material or standard were mixed with 320 µl 100 mM phosphate buffer pH 6.8 and 50 µl of 10 mM α-NPG. This mixture was incubated at 30ºC for 5 minutes, followed by addition of 20 µl of α-glucosidase in phosphate buffer. The mixture was incubated for another 5 minutes at 30ºC and the reaction was stopped by adding 3 ml of 50 mM sodium hydroxide. The sample absorbance was measured at 410 nm with a microplate reader. Acarbose was used as a standard.

### Molecular docking study
Activity of identified substances in *U. lobata* leaf extracts both on *α-amylase* (P0DTE8) and *α-glucosidase* (O43451) was evaluated by *in silico* approach using a web-based software application (www.dockingserver.com). The structures of identified compounds were obtained from PubChem database and protein target (enzymes) from Uniprot database. Molecular docking was performed by uploading both chemical compound and protein target on software. The prediction value of parameters include inhibitory constant (Ki), free energy of binding and surface interactions.

### Statistical Analysis
The $IC_{50}$ value was calculated by linear regression curve fit using SPPS version 16.0 and the data are presented as the mean ± SD.

## Results
### Identification of active substances in *U. lobata* leaf extracts
The active compounds from ethanolic extract of *U.lobata* leaf can be seen in the Figure 1 and Table 1.

**Figure 1**. LC-MS Chromatogram öf *U. lobata* leaf extract

**Table 1.** Identified compounds in *U. lobata* leaf extract by LC-MS

The qualitative analysis using LC-MS indicated that the most abundant secondary metabolites in *U. lobata* leaf extract was gossypetin and stigmasterol. Other compounds such as β-sitosterol, chrysoeriol and mangiferin were also identified in the extracts of *U. lobata*; however, the concentration was low.

### Molecular docking of *U. lobata* on α–amylase and α–glucosidase
The *in silico* inhibitory activity of the identified compounds in *U. lobata* leaf extract on α-amylase and α-glucosidase results can be seen at Table 2 and 3.

**Table 2.** Molecular docking of identified compounds *U. lobata* leaf extracts with α-amylase

**Table 3.** Molecular docking of identified substances in *U. lobata* leaf extracts with α-glucosidase

Molecular docking studies indicated that stigmasterol, β-sitosterol and mangiferin have low inhibition constant and free energy of binding; however, the surface interaction showed high value. Meanwhile, gossypetin and chrysoeriol have higher values on binding free energy and inhibition constant as compared to stigmasterol, β-sitosterol and mangiferin. The differences in each parameter value signified distinct inhibitory activity both of on α-glucosidase and α-amylase activities. Based on inhibition constant, stigmasterol and β-sitosterol showed higher inhibition activity toward both α-glucosidase and α-amylase. These activities are more prominent than acarbose, especially on α-amylase.

### α-glucosidase inhibitory activity and α–amylase of *U. lobata* leaf extract

The inhibitory activity of ethanolic extract of *U. lobata* leaf on α-amylase and α-glucosidase were evaluated *in vitro* using enzymatic assay and the results are shown in Table 4 and 5.

**Table 4.** α-amylase inhibitory activity of *U. lobata* leaf extract and acarbose

**Table 5.** α-glucosidase inhibitory activity of *U. lobata* leaf extract and acarbose

Based on these results, the inhibition activity of the ethanolic extract of *U. lobata* on α-glucosidase was two times stronger than α-amylase. However, the inhibitory activities are lower than acarbose.

## Discussion

### Identification of active compounds in *U. lobata* leaf extracts

Ethanolic extracts of *U. lobata* leaves have been reported to contain non-nutritional substances having pharmacological effects [8,21]. These include flavone or flavonol compounds, such as gossypetin, which was shown to be an effective antioxidant and possess anti-microbial, anti-atherosclerotic and anti-mutagenic properties[12]. A flavon, chrysoeriol, was also reported in these extracts, and was shown to be have anti-histamine and anti-inflammatory activities[17]. Gossypetin is known to be very soluble in non-polar eluents (such as chloroform and benzene), moderately soluble in semi-polar eluents (in this case, ether and ethanol), and insoluble in water. Aside from compounds in the flavonoid class, other classes of compounds were also reported. Stigmasterol, a plant sterol commonly found in many medicinal herbs in plant fats or oils [9,10], was also reported to be found in the extract. This compound was insoluble in water (similar to other sterols), and soluble in most organic solvents containing at least one alcohol functional group. Stigmasterol showed to be able to prevent hyperglycemia and could inhibit thyroid levels, as well as being an antioxidant [10,11]. Other hydrophobic phytosterol, β-sitosterol[13], showed anti-cholesterol activity and could act as an immunomodulator [14]. Mangiferin, a glucoside of norathyriol and a semipolar xanthonoid reported to be soluble in hot ethanol and methanol, was found to be anti-microbial, anti-glycemic and an antioxidant [15, 16].

The identified compounds in the extract of *U. lobata* leaf were found to be influenced by the polarity of the solvents used in the extraction. Non-polar solvents, such as acetone, diethy ether and hexane, would extract non-polar compounds, such as alkaloids, terpenoids and steroids, while polar solvents, such as water and methanol, would extract flavonoids, phenols and glycosides [18, 19]. The solubility of polar substances in polar solvents, and vice versa, concurs with the basic determinate solubility theory ("like dissolves like") [20].

Herbal medicine, or herbal extract, is considered as an anti-diabetic due to its potency in reducing blood glucose levels, which is often resulted from the phytochemical compounds, such as terpenoid, steroid, alkaloid and flavonoid classes through different mechanisms of action. Some may act by increasing insulin secretion or insulin sensitivity, and others may act by inhibiting α-glucosidase and DPP-4[3,5]. Furthermore, since an extract could contain many compounds, the activity of the extract may result in synergistic or even antagonistic interactions of some compounds existing in the extract[23].

### Molecular docking of *U.lobata* on α–glucosidase and α–amylase

In the pharmaceutical field, molecular docking is often used to screen and predict the potential candidates the drug target of ligands with a known structure, based on its free energy binding, inhibition constant, and surface interaction. Free energy binding would inversely correspond to the binding affinity of a ligand to a target molecule (a lower free energy binding value would indicate higher binding affinity) [24]. The inhibition constant (Ki) is used in *in silico* studies to predict the inhibitory activity of a ligand to a drug target. Similarly, this score also operates inversely, in which a lower Ki score would indicate a higher inhibition activity of a protein target. Lastly, surface interaction represents the surface area and molecular recognition between a ligand and a binding pocket in a protein target. A higher surface interaction would indicate a higher number of interactions between a ligand and a molecule target [25].

Based on the molecular docking in this study, stigmasterol and β-sitosterol were found to have the lowest inhibition constant when tested against α-glucosidase and α-amylase. Free energy binding score showed that stigmasterol, followed by β-sitosterol and mangiferin, had the lowest free energy binding, respectively, and a high surface interaction in the same order. Both scores indicate a stronger binding with the drug target and may indicate a strong biological activity [24, 25] and, therefore, indicating the inhibitory activity of *U. lobata* leaf extracts on α-glucosidase and α-amylase.

Based on those categories, stigmasterol has the lowest score of inhibition constant and followed by β-sitosterol either on α-glucosidase and α-amylase. It is related to free energy of binding and surface interaction of these substances. In this research, stigmasterol has the highest score of surface interaction followed by β–sitosterol and mangiferin, respectively. A high score of surface interaction indicated a stronger bond between ligand and molecule target, moreover, it results a great biology activity. *In silico* analysis showed that stigmasterol has the lowest score in the free energy of binding, meanwhile β–sitosterol and mangiferin were in the second and third position. The lowest score of binding free energy results a strong binding molecule, furthermore, it causes an increase of their biology activity [24,25]. Free energy of binding and surface interaction between molecule target and ligand influences the inhibitory activity of *U. lobata* leaf extract, both on *α-glucosidase* and *α-amylase*.

Molecular docking research is widely used to predict the potential candidates of drugs in the pharmaceutical fields. Binding orientation of these active substances to their molecule targets reveals their activity and affinity as possible candidates of drugs [25].

### α-glucosidase and α–amylase inhibitory activity of *U. lobata* leaf extract by *in vitro*

Our results showed that inhibitory activity of *U.lobata* leaf extract on α-glucosidase was stronger than on α-amylase. It may be due to the different chemical composition of the extracts, which interacts with α-glucosidase and α-amylase. *U. lobata* contains compounds such as phenol, tanin, stigmasterol, beta sitosterol, mangiferin, quercetin and also some flavon compounds such as gossypetin dan chrysoeriol, with total phenol content of 25%.

Active compounds found to inhibit α-glucosidase activity are those from the flavonoid, alkaloid and tanin group [26]. However, this depends on other factors, such as isoflavone level which is contained in herbs, molecule size and structural variation of tanin. Meanwhile, molecular configuration on binding site of α–glucosidase active sites also contributes to its activity. Generally, a more complex and large tannin structure would be more effective in inhibiting α-glucosidase [27]. Isoflavones are active compounds having an inhibition activity of α–glucosidase stronger than flavone compounds [26].

Flavonoid inhibits α–glucosidase by hydroxylation binding and substitution on the β-flavonoid ring. Inhibition activity of flavonoid is correlated with the number of hydroxyls on β-flavonoid ring. The more hydrogen binding between hydroxyl and polyphenol ligand with catalytic residues from the binding site of enzyme glucosidase, the more strongly the inhibition activity against α-glucosidase. Inhibition of α–glucosidase retains carbohydrate hydrolysis into maltose; therefore, it decreases glucose level post prandially[1]. *U. lobata* contains stigmasterol and β-sterol from phytosterol group and also mangiferin from xanthone glucosidase group. Based on previous *in silico* study , *U. lobata* is able to suppress α–glucosidase activity controlled by active compounds mentioned above.

Previous study showed anti-diabetic of *U. lobata* leaf extract through inhibitory activity of dipeptdyl peptidase-4 (DPP-4) using *in vitro* and *in vivo* approach [5,6]. Other study indicated both aqueous and methanolic extract of *U.lobata* leaf have anti-hyperglycemic or hypoglycemic effect on rats and alkaloids, flavonoids, saponins, tannins present in the extracts may be responsible for the effects [4,9]. Study in rabbits showed *U.lobata* aqueous extract of roots significantly reduced fasting glucose and body weight [8].

Other active substances inhibiting α–glucosidase are sterol and xanthonoid[27,28]. Enzyme α–glucosidase is a glucoamylase enzyme that hidrolyzes polysaccharides, disaccharides, oligosaccharides on the brush border of the microvili in epitel intestine into glucose monomers. Activity of α–glucosidase is influenced by temperature and acidity level. Optimum temperature and acidity level for enzyme are 37°C and 6.8, respectively. This condition is suitable with reactions occurring in the human body[29]. Active substances found to be able to suppress α–amylase are flavonoid, fenol, alkaloid, miscellaneous,terpenoid, xanthone, glucosidase and sterol [27,28,30].

## Conclusions

Ethanolic extract of *U. lobata* leaf has inhibition activity stronger on α-glucosidase than on α-amylase as anti-diabetic.

## Conflict of interest statement

We declare that we have no conflict of interest

# JBCPP_HASIL KOREKSI BAHASA INGGRIS MANUSCRIPT

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# Inhibitory activity of *Urena lobata* leaf extract on alpha-amylase and alpha-glucosidase: *in vitro* and *in silico* approach

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**Abstract**

**Background**: In food ingestion, alpha-glucosidase (α-glucosidase) and alpha-amylase (α-amylase) are enzymes that are responsible to convert a carbohydrate into glucose. Inhibition of both enzyme activities can prolong absorption of glucose in intestine and reduce post-prandial increase of blood glucose concentration, thus, it is beneficial for type-2 diabetes treatment. Traditionally, *Urena lobata (U. lobata)* has been used to manage diabetes, but the scientific proof of this claim remains scarce. Therefore, the objective of this study to examine the anti-diabetic potential of *U. lobata* leaf extract through inhibition of α-amylase and α-glucosidase.
**Methods**: *U. lobata* leaf extract was obtained through extraction process using ethanol and the chemical compounds in the extract were analyzed by Liquid Chromatography–Mass Spectra (LC-MS). The inhibitory activity of *U. lobata* on α-glucosidase and α-amylase was evaluated by *in-silico* using docking server, whereas *in-vitro* enzymatic assays were using *para*-nitrophenyl-α-D-glucopyranoside (α-NPG) and starch as substrates. The data were presented as mean ± SD and the $IC_{50}$ value was calculated using SPSS.
**Results**: *U. lobata* leaf extract showed inhibitory activity on α-glucosidase and α-amylase with the $IC_{50}$ value was 43.73 µg/mL and 83.73 µg/mL, respectively, meanwhile, acarbose as standard has $IC_{50}$ value at 1.14 µg/mL and 0.08 µg/mL. Molecular docking study indicated β-sitosterol and stigmasterol from *U. lobata* extract have a huge inhibitory activity both on α-amylase and α-glucosidase based on inhibition constant (Ki) value.
**Conclusions**: Ethanolic extract of *U. lobata* showed inhibition activity on α-glucosidase stronger than on α-amylase as anti-diabetic.

**Keywords:** *anti-diabetic, enzyme, metabolism, molecular docking, polysaccharides*
**DOI :**
**Received :**

## Introduction

Carbohydrate metabolism is regulated by enzymes that break polysaccharides into monosaccharides. In food ingestion, alpha-amylase (α-amylase) and alpha-glucosidase (α-glucosidase) are enzymes that are responsible for the conversion of a starch complex into a simple starch[1]. Alpha-amylase are produced by the salivary glands and pancreatic glands, which metabolizes starch into maltosa, dextrin and maltotriosa. These products are then delivered in to the small intestinal mucosa, where they are then hydrolyzed by α-glucosidase and results in glucose and which are absorbed into the blood[2]. It contributes to an increase in blood glucose level post-prandial, which needs to be controlled to avoid hyperglycemia. The inhibitory activity both of α-amylase and α-glucosidase will reduce carbohydrate metabolism and glucose absorption, and, thus, preventing an increase of blood glucose level, beneficial especially in patients with diabetes mellitus[1,2]. Herbal remedies are one of the choices to suppress activity of both α-amylase and α-glucosidase in the therapy of diabetes mellitus. Some benefit of herbal remedies includes less adverse reaction and is relatively cheaper. Most anti-diabetic herbal remedies work through inhibitory activity of α-amylase and α-glucosidase[3].

Pulutan (*Urena lobata)* is a herbal remedy used by Nigerian people to manage many diseases, including diabetes mellitus[4]. This plant has a bitter taste, which is the basis of the intuition that it may be able to treat diabetes. Traditional healers used *U. lobata* in both in single and in combination with other herbs to manage diabetes. Research indicates that the administration of *U. lobata* leaf and roots extracts has hypoglycemic activity on rats induced with streptozotocin[4,5]. This is due to several active compounds in the herbs, such as sterol groups, alkaloids and flavonoids[6,7]. Anti-diabetic activity of *U. lobata* leaf extracts has not been evaluated, especially regarding its α-amylase and α-glucosidase inhibitory activity. The objective of the study was to evaluate anti diabetic potency of *U. lobata* through inhibitory activity of α-glucosidase and α-amylase.

## Materials and Methods

### Chemical

α-amylase was obtained from porcine pancreatic and α-glucosidase, meanwhile *para*-nitrophenyl-α-D-glucopyranoside (α-NPG), starch substrate, dinitrosalicylic acid (DNSA), Gly-pro-p-nitroanilide (GPPN), Tris-HCl buffer were purchased from Sigma aldrich, meanwhile ethanol was from Merck (pro analysis grade).

### Sample preparation

Leaves of *U. lobata* leaves were obtained from Balai Materia Medika Batu, Malang, Indonesia with a certificate number of 074/027/101.8/2015. About 50 g of the sample materials were extracted in 250 ml ethanol 80% for 4 hours using water bath shaker. The extraction was repeated for another two times using fresh solvent. The extracts were evaporated using a rotary evaporator to produce a paste form (weight) and diluted with solvent according to the designated concentration.

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Ethanolic extract of *U. lobata* leaf was subjected to a qualitative analysis using Liquid Chromatography–Mass Spectra (LC-MS) Accela 1250 pump. Mobile phase contains 0.1 % formic acid in methanol and water combination. The identification included the 10 active substances, considered based on previous study compounds including phytosterol, flavonoid and alkaloid groups.

### α-amylase inhibitory assay
About 100 µl of sample or standard solution was added to 150 µl of 5 unit/ml α-amylase and 20 mM of phosphate buffer pH 6.9. This mixture was incubated at 37ºC for 30 minutes followed by addition of 250 µl of 1% starch in distilled water. The mixture solution was incubated again for 10 minutes at 37ºC. Then, 50 µl of 1% DNSA was added and the mixture was and heated on a water bath. The solution was cooled to room temperature and the sample absorbance was measured with microplate reader at 540 nm. Acarbose was used as standard.

### α-glucosidase inhibitory assay
Various concentrations of test material or standard were mixed with 320 µl 100 mM phosphate buffer pH 6.8 and 50 µl of 10 mM α-NPG. This mixture was incubated at 30ºC for 5 minutes, followed by addition of 20 µl of α-glucosidase in phosphate buffer. The mixture was incubated for another 5 minutes at 30ºC and the reaction was stopped by adding 3 ml of 50 mM sodium hydroxide. The sample absorbance was measured at 410 nm with a microplate reader. Acarbose was used as a standard.

### Molecular docking study
Activity of identified substances in *U. lobata* leaf extracts both on *α-amylase* (P0DTE8) and *α-glucosidase* (O43451) was evaluated by *in silico* approach using a web-based software application (www.dockingserver.com). The structures of identified compounds were obtained from PubChem database and protein target (enzymes) from Uniprot database. Molecular docking was performed by uploading both chemical compound and protein target on software. The prediction value of parameters include inhibitory constant (Ki), free energy of binding and surface interactions.

### Statistical Analysis
The $IC_{50}$ value was calculated by linear regression curve fit using SPPS version 16.0 and the data are presented as the mean ± SD.

## Results
### Identification of active substances in *U. lobata* leaf extracts
The active compounds from ethanolic extract of *U.lobata* leaf can be seen in the Figure 1 and Table 1.

**Figure 1**. LC-MS Chromatogram öf *U. lobata* leaf extract

**Table 1.** Identified compounds in *U. lobata* leaf extract by LC-MS

The qualitative analysis using LC-MS indicated that the most abundant secondary metabolites in *U. lobata* leaf extract was gossypetin and stigmasterol. Other compounds such as β-sitosterol, chrysoeriol and mangiferin were also identified in the extracts of *U. lobata*; however, the concentration was low.

### Molecular docking of *U. lobata* on α–amylase and α–glucosidase
The *in silico* inhibitory activity of the identified compounds in *U. lobata* leaf extract on α-amylase and α-glucosidase results can be seen at Table 2 and 3.

**Table 2.** Molecular docking of identified compounds *U. lobata* leaf extracts with α-amylase

**Table 3.** Molecular docking of identified substances in *U. lobata* leaf extracts with α-glucosidase

Molecular docking studies indicated that stigmasterol, β-sitosterol and mangiferin have low inhibition constant and free energy of binding; however, the surface interaction showed high value. Meanwhile, gossypetin and chrysoeriol have higher values on binding free energy and inhibition constant as compared to stigmasterol, β-sitosterol and mangiferin. The differences in each parameter value signified distinct inhibitory activity both of on α-glucosidase and α-amylase activities. Based on inhibition constant, stigmasterol and β-sitosterol showed higher inhibition activity toward both α-glucosidase and α-amylase. These activities are more prominent than acarbose, especially on α-amylase.

### α-glucosidase inhibitory activity and α–amylase of *U. lobata* leaf extract

The inhibitory activity of ethanolic extract of *U. lobata* leaf on α-amylase and α-glucosidase were evaluated *in vitro* using enzymatic assay and the results are shown in Table 4 and 5.

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Based on these results, the inhibition activity of the ethanolic extract of *U. lobata* on α-glucosidase was two times stronger than α-amylase. However, the inhibitory activities are lower than acarbose.

## Discussion

### Identification of active compounds in *U. lobata* leaf extracts

Ethanolic extracts of *U. lobata* leaves have been reported to contain non-nutritional substances having pharmacological effects [8,21]. These include flavone or flavonol compounds, such as gossypetin, which was shown to be an effective antioxidant and possess anti-microbial, anti-atherosclerotic and anti-mutagenic properties[12]. A flavon, chrysoeriol, was also reported in these extracts, and was shown to be have anti-histamine and anti-inflammatory activities[17]. Gossypetin is known to be very soluble in non-polar eluents (such as chloroform and benzene), moderately soluble in semi-polar eluents (in this case, ether and ethanol), and insoluble in water. Aside from compounds in the flavonoid class, other classes of compounds were also reported. Stigmasterol, a plant sterol commonly found in many medicinal herbs in plant fats or oils [9,10], was also reported to be found in the extract. This compound was insoluble in water (similar to other sterols), and soluble in most organic solvents containing at least one alcohol functional group. Stigmasterol showed to be able to prevent hyperglycemia and could inhibit thyroid levels, as well as being an antioxidant [10,11]. Other hydrophobic phytosterol, β-sitosterol[13], showed anti-cholesterol activity and could act as an immunomodulator [14]. Mangiferin, a glucoside of norathyriol and a semipolar xanthonoid reported to be soluble in hot ethanol and methanol, was found to be anti-microbial, anti-glycemic and an antioxidant [15, 16].

The identified compounds in the extract of *U. lobata* leaf were found to be influenced by the polarity of the solvents used in the extraction. Non-polar solvents, such as acetone, diethy ether and hexane, would extract non-polar compounds, such as alkaloids, terpenoids and steroids, while polar solvents, such as water and methanol, would extract flavonoids, phenols and glycosides [18, 19]. The solubility of polar substances in polar solvents, and vice versa, concurs with the basic determinate solubility theory ("like dissolves like") [20].

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Based on those categories, stigmasterol has the lowest score of inhibition constant and followed by β-sitosterol either on α-glucosidase and α-amylase. It is related to free energy of binding and surface interaction of these substances. In this research, stigmasterol has the highest score of surface interaction followed by β–sitosterol and mangiferin, respectively. A high score of surface interaction indicated a stronger bond between ligand and molecule target, moreover, it results a great biology activity. *In silico* analysis showed that stigmasterol has the lowest score in the free energy of binding, meanwhile β–sitosterol and mangiferin were in the second and third position. The lowest score of binding free energy results a strong binding molecule, furthermore, it causes an increase of their biology activity [24,25]. Free energy of binding and surface interaction between molecule target and ligand influences the inhibitory activity of *U. lobata* leaf extract, both on *α-glucosidase* and *α-amylase*.

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Active compounds found to inhibit α-glucosidase activity are those from the flavonoid, alkaloid and tanin group [26]. However, this depends on other factors, such as isoflavone level which is contained in herbs, molecule size and structural variation of tanin. Meanwhile, molecular configuration on binding site of α–glucosidase active sites also contributes to its activity. Generally, a more complex and large tannin structure would be more effective in inhibiting α-glucosidase [27]. Isoflavones are active compounds having an inhibition activity of α–glucosidase stronger than flavone compounds [26].

Flavonoid inhibits α–glucosidase by hydroxylation binding and substitution on the β-flavonoid ring. Inhibition activity of flavonoid is correlated with the number of hydroxyls on β-flavonoid ring. The more hydrogen binding between hydroxyl and polyphenol ligand with catalytic residues from the binding site of enzyme glucosidase, the more strongly the inhibition activity against α-glucosidase. Inhibition of α–glucosidase retains carbohydrate hydrolysis into maltose; therefore, it decreases glucose level post prandially[1]. *U. lobata* contains stigmasterol and β-sterol from phytosterol group and also mangiferin from xanthone glucosidase group. Based on previous *in silico* study , *U. lobata* is able to suppress α–glucosidase activity controlled by active compounds mentioned above.

Previous study showed anti-diabetic of *U. lobata* leaf extract through inhibitory activity of dipeptdyl peptidase-4 (DPP-4) using *in vitro* and *in vivo* approach [5,6]. Other study indicated both aqueous and methanolic extract of *U.lobata* leaf have anti-hyperglycemic or hypoglycemic effect on rats and alkaloids, flavonoids, saponins, tannins present in the extracts may be responsible for the effects [4,9]. Study in rabbits showed *U.lobata* aqueous extract of roots significantly reduced fasting glucose and body weight [8].

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## Conclusions

Ethanolic extract of *U. lobata* leaf has inhibition activity stronger on α-glucosidase than on α-amylase as anti-diabetic.

## Conflict of interest statement

We declare that we have no conflict of interest

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Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.elsevier.com/locate/apjtb

Original article doi:



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

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ARTICLE INFO

*Article history:*
Received 29 Apr 2015
Received in revised form 11 May 2015
Accepted 28 May 2015
Available online 30 May 2015

*Keywords:*
Anti-diabetic
Dipeptidyl peptidase IV
*In silico*
*In vitro*
*Urena lobata*

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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Foundation Project: Supported by a grant of doctoral dissertation research from Education Ministry of Indonesia.

## 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.

## Acknowledgments

This work was supported by a grant of doctoral dissertation research from Education Ministry of Indonesia.

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# JBCPP_MANUSCRIPT DIPUBLIKASIKAN

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# Inhibitory activity of *Urena lobata* leaf extract on alpha-amylase and alpha-glucosidase: *in vitro* and *in silico* approach

https://doi.org/10.1515/jbcpp-2020-0430
Received November 28, 2020; accepted March 29, 2021

**Abstract**

**Objectives:** In food ingestion, alpha-glucosidase (α-glucosidase) and alpha-amylase (α-amylase) are enzymes that are responsible to convert a carbohydrate into glucose. Inhibition of both enzyme activities can prolong absorption of glucose in intestine and reduce post-prandial increase of blood glucose concentration, thus, it is beneficial for type-2 diabetes treatment. Traditionally, *Urena lobata* (*U. lobata*) has been used to manage diabetes, but the scientific proof of this claim remains scarce. Therefore, the objective of this study to examine the anti-diabetic potential of *U. lobata* leaf extract through inhibition of α-amylase and α-glucosidase.

**Methods:** *U. lobata* leaf extract was obtained through extraction process using ethanol and the chemical compounds in the extract were analyzed by liquid chromatography–mass spectra (LC–MS). The inhibitory activity of *U. lobata* on α-glucosidase and α-amylase was evaluated by *in silico* using docking server, whereas *in vitro* enzymatic assays were using *para*-nitrophenyl-α-D-glucopyranoside (α-NPG) and starch as substrates. The data were presented as mean ± SD and the $IC_{50}$ value was calculated using SPSS.

**Results:** *U. lobata* leaf extract showed inhibitory activity on α-glucosidase and α-amylase with the $IC_{50}$ value was 43.73 and 83.73 μg/mL, respectively, meanwhile, acarbose as standard has $IC_{50}$ value at 1.14 and 0.08 μg/mL. Molecular docking study indicated β-sitosterol and stigmasterol from *U. lobata* extract have a huge inhibitory activity both on α-amylase and α-glucosidase based on inhibition constant (Ki) value.

**Conclusions:** Ethanolic extract of *U. lobata* showed inhibition activity on α-glucosidase stronger than on α-amylase as antidiabetic.

**Keywords:** anti-diabetic; enzyme; metabolism; molecular docking; polysaccharides.

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## Introduction

Carbohydrate metabolism is regulated by enzymes that break polysaccharides into monosaccharides. In food ingestion, alpha-amylase (α-amylase) and alpha-glucosidase (α-glucosidase) are enzymes that are responsible for the conversion of a starch complex into a simple starch [1]. α-Amylase is produced by the salivary glands and pancreatic glands, which metabolizes starch into maltosa, dextrin, and maltotriosa. These products are then delivered in to the small intestinal mucosa, where they are then hydrolyzed by α-glucosidase and results in glucose which are absorbed into the blood [2]. It contributes to an increase in blood glucose level post-prandial, which needs to be controlled to avoid hyperglycemia. The inhibitory activity both of α-amylase and α-glucosidase will reduce carbohydrate metabolism and glucose absorption, and thus, preventing an increase of blood glucose level, beneficial especially in patients with diabetes mellitus [1, 2]. Herbal remedies are one of the choices to suppress activity of both α-amylase and α-glucosidase in the therapy of diabetes mellitus. Some benefit of herbal remedies includes less adverse reaction and is relatively cheaper. Most antidiabetic herbal remedies work through inhibitory activity of α-amylase and α-glucosidase [3].

Pulutan (*Urena lobata* [*U. lobata*]) is an herbal remedy used by Nigerian people to manage many diseases, including diabetes mellitus [4]. This plant has a bitter taste, which is the basis of the intuition that it may be able to treat diabetes. Traditional healers used *U. lobata* in both in single and in combination with other herbs to manage diabetes. Research indicates that the administration of *U. lobata* leaf and roots extracts has hypoglycemic activity on rats induced with streptozotocin [4, 5]. This is due to several active compounds in the herbs, such as sterol groups, alkaloids, and flavonoids [6, 7]. Antidiabetic

activity of *U. lobata* leaf extracts has not been evaluated, especially regarding its α-amylase and α-glucosidase inhibitory activity. The objective of the study was to evaluate antidiabetic potency of *U. lobata* through inhibitory activity of α-glucosidase and α-amylase.

# Materials and methods

### Chemical

α-amylase was obtained from porcine pancreatic and α-glucosidase, meanwhile *para*-nitrophenyl-α-D-glucopyranoside (α-NPG), starch substrate, dinitrosalicylic acid (DNSA), Gly-pro-*p*-nitroanilide (GPPN), and Tris-HCl buffer were purchased from Sigma Aldrich, meanwhile ethanol was from Merck (pro analysis grade).

### Sample preparation

Leaves of *U. lobata* were obtained from Balai Materia Medika Batu, Malang, Indonesia, with a certificate number of 074/027/101.8/2015. About 50 g of the sample materials were extracted in 250 mL ethanol 80% for 4 h using water bath shaker. The extraction was repeated for another two times using fresh solvent. The extracts were evaporated using a rotary evaporator to produce a paste form (weight) and diluted with solvent according to the designated concentration.

### Identification of active substances

Ethanolic extract of *U. lobata* leaf was subjected to a qualitative analysis using liquid chromatography–mass spectra (LC–MS) Accela 1250 pump. Mobile phase contains 0.1% formic acid in methanol and water combination. The identification included the 10 active substances, considered based on previous study compounds including phytosterol, flavonoid, and alkaloid groups.

### α-Amylase inhibitory assay

About 100 µL of sample or standard solution was added to 150 µL of 5 unit/mL α-amylase and 20 mM of phosphate buffer pH 6.9. This mixture was incubated at 37 °C for 30 min followed by addition of 250 µL of 1% starch in distilled water. The mixture was incubated again for 10 min at 37 °C. Then, 50 µL of 1% DNSA was added and the mixture was heated on a water bath. The solution was cooled down to room temperature and the sample absorbance was measured with microplate reader at 540 nm. Acarbose was used as standard.

### α-Glucosidase inhibitory assay

Various concentrations of test material or standard were mixed with 320 µL 100 mM phosphate buffer pH 6.8 and 50 µL of 10 mM α-NPG. This mixture was incubated at 30 °C for 5 min, followed by addition of 20 µL of α-glucosidase in phosphate buffer. The mixture was incubated for another 5 min at 30 °C and the reaction was stopped by adding 3 mL of 50 mM sodium hydroxide. The sample absorbance was measured at 410 nm with a microplate reader. Acarbose was used as a standard.

### Molecular docking study

Activity of identified substances in *U. lobata* leaf extracts both on *α-amylase* (P0DTE8) and *α-glucosidase* (O43451) was evaluated by *in silico* approach using a web-based software application (www.dockingserver.com). The structures of identified compounds were obtained from PubChem database and protein target (enzymes) from Uniprot database. Molecular docking was performed by uploading both chemical compound and protein target on software. The prediction value of parameters include inhibitory constant (Ki), free energy of binding and surface interactions.

### Statistical analysis

The $IC_{50}$ value was calculated by linear regression curve fit using SPPS version 16.0 and the data are presented as the mean ± SD.

# Results

## Identification of active substances in *U. lobata* leaf extracts

The active compounds from ethanolic extract of *U. lobata* leaf can be seen in Figure 1 and Table 1.

The qualitative analysis using LC–MS indicated that the most abundant secondary metabolites in *U. lobata* leaf extract was gossypetin and stigmasterol. Other compounds such as β-sitosterol, chrysoeriol, and mangiferin were also identified in the extracts of *U. lobata*; however, the concentration was low.

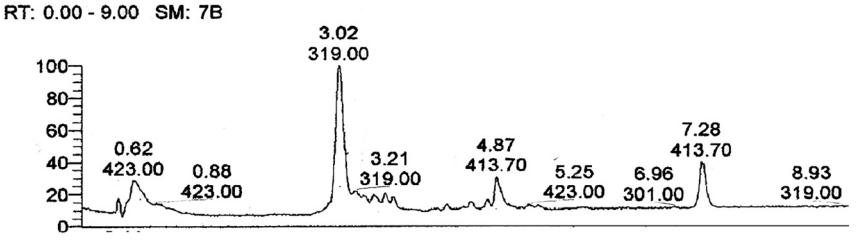


**Figure 1:** Chromatogram of *U. lobata* leaf extract identified by LC–MS [1–29].

**Table 1:** Active compounds in *U. lobata* leaf extracts.

| No | Active compounds | Molecule weight | Ethanolic extract |
|---|---|---|---|
| 1 | Stigmasterol | 413 | (++) |
| 2 | β-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 | (+) |

(–): negative, (+): weak, (++): moderate, (+++): strong.

## Molecular docking of *U. lobata* on α-amylase and α-glucosidase

The *in silico* inhibitory activity of the identified compounds in *U. lobata* leaf extract on α-amylase and α-glucosidase results can be seen at Tables 2 and 3.

Molecular docking studies indicated that stigmasterol, β-sitosterol, and mangiferin have low inhibition constant and free energy of binding; however, the surface interaction showed high value. Meanwhile, gossypetin and chrysoeriol have higher values on binding free energy and inhibition constant as compared to stigmasterol, β-sitosterol, and mangiferin. The differences in each parameter value signified distinct inhibitory activity both of on α-glucosidase and α-amylase activities. Based on inhibition constant, stigmasterol and β-sitosterol showed higher inhibition activity toward both α-glucosidase and α-amylase. These activities are more prominent than acarbose, especially on α-amylase.

**Table 2:** Molecular docking of active substances in *U. lobata* leaf extracts with *α-amylase*.

| No | Active compounds | Estimation free energy of binding, Kcal/mol | Estimation of inhibition constant Ki, μM | Interaction surface |
|---|---|---|---|---|
| 1 | Stigmasterol | –9.63 | 0.0875 | 892.16 |
| 2 | β-Sitosterol | –8.66 | 0.4500 | 793.55 |
| 3 | Mangiferin | –7.84 | 1.80 | 702.30 |
| 4 | Gossypetin | –6.40 | 20.36 | 621.77 |
| 5 | Chrysoeriol | –6.50 | 17.13 | 653.91 |
| 6 | Acarbose | –8.78 | 0.3470 | 1,087.32 |

**Table 3:** Molecular docking of active substances in *U. lobata* leaf extracts with *α-glucosidase*.

| No | Active compounds | Estimation free energy of binding, Kcal/mol | Estimation of inhibition constant Ki, μM | Interaction surface |
|---|---|---|---|---|
| 1 | Stigmasterol | –10.20 | 0.0331 | 891.01 |
| 2 | β-Sitosterol | –9.59 | 0.0931 | 894.65 |
| 3 | Mangiferin | –7.98 | 1.4100 | 764.01 |
| 4 | Gossypetin | –7.20 | 5.2400 | 687.04 |
| 5 | Chrysoeriol | –6.70 | 12.190 | 705.30 |
| 6 | Acarbose | –0.3.98 | 0.0012 | 457.77 |

## α-Glucosidase inhibitory activity and α-amylase of *U. lobata* leaf extract

The inhibitory activity of ethanolic extract of *U. lobata* leaf on α-amylase and α-glucosidase were evaluated *in vitro* using enzymatic assay and the results are shown in Tables 4 and 5.

Based on these results, the inhibition activity of the ethanolic extract of *U. lobata* on α-glucosidase was two times stronger than α-amylase. However, the inhibitory activities are lower than acarbose.

# Discussion

## Identification of active compounds in *U. lobata* leaf extracts

Ethanolic extracts of *U. lobata* leaves have been reported to contain non nutritional substances having pharmacological effects [8, 9]. These include flavone or flavonol compounds, such as gossypetin, which was shown to be an effective antioxidant and possess antimicrobial, anti

**Table 4:** *α-Amylase inhibitory* activity of *U. lobata* leaf extracts and acarbose.

| Group | Sample | n | Concentration, μg/mL | % inhibition | $IC_{50}$, μg/mL |
|---|---|---|---|---|---|
| 1 | Ethanolic | 3 | 6.25 | 20.62 ± 3.79 | 83.73 |
| | extract of | 3 | 12.50 | 22.36 ± 1.24 | |
| | *U. lobata* | 3 | 25.00 | 27.33 ± 1.24 | |
| | | 3 | 50.00 | 40.99 ± 4.35 | |
| | | 3 | 100.00 | 54.65 ± 8.14 | |
| 2 | Acarbose | 3 | 0.05 | 47.77 ± 0.99 | 0.08 |
| | | 3 | 0.10 | 53.40 ± 3.05 | |
| | | 3 | 0.15 | 58.74 ± 0.44 | |
| | | 3 | 0.20 | 61.04 ± 2.48 | |
| | | 3 | 0.25 | 61.47 ± 4.12 | |

**Table 5:** *α-Glucosidase inhibitory* activity of *U. lobata* leaf extracts and acarbose.

| Group | Sample | n | Concentration, µg/mL | % inhibition | $IC_{50}$, µg/mL |
|---|---|---|---|---|---|
| 1 | Ethanolic | 3 | 6.25 | 20.00 ± 0.00 | 43.73 |
| | extract of | 3 | 12.5 | 30.00 ± 0.00 | |
| | *U. lobata* | 3 | 25.0 | 45.00 ± 0.00 | |
| | | 3 | 50.0 | 60.00 ± 3.85 | |
| | | 3 | 100.0 | 80.00 ± 3.85 | |
| 2 | Acarbose | 3 | 0.25 | 16.37 ± 11.52 | 1.14 |
| | | 3 | 0.50 | 29.09 ± 0.11 | |
| | | 3 | 0.75 | 31.98 ± 6.85 | |
| | | 3 | 1.00 | 44.74 ± 1.69 | |
| | | 3 | 1.25 | 52.02 ± 12.44 | |

atherosclerotic, and antimutagenic properties [10]. A flavon, chrysoeriol, was also reported in these extracts, and was shown to be having antihistamine and anti inflammatory activities [11]. Gossypetin is known to be very soluble in nonpolar eluents (such as chloroform and benzene), moderately soluble in semipolar eluents (in this case, ether and ethanol), and insoluble in water. Aside from compounds in the flavonoid class, other classes of compounds were also reported. Stigmasterol, a plant sterol commonly found in many medicinal herbs in plant fats or oils [12, 13], was also reported to be found in the extract. This compound was insoluble in water (similar to other sterols), and soluble in most organic solvents containing at least one alcohol functional group. Stigmasterol showed to be able to prevent hyperglycemia and could inhibit thyroid levels, as well as being an antioxidant [13, 14]. Other hydrophobic phytosterol, β-sitosterol [15], showed anticholesterol activity and could act as an immunomodulator [16]. Mangiferin, a glucoside of norathyriol and a semipolar xanthonoid reported to be soluble in hot ethanol, and methanol was found to be antimicrobial, antiglycemic, and an antioxidant [17, 18].

The identified compounds in the extract of *U. lobata* leaf were found to be influenced by the polarity of the solvents used in the extraction. Nonpolar solvents, such as acetone, diethyl ether, and hexane, would extract nonpolar compounds, such as alkaloids, terpenoids, and steroids, while polar solvents, such as water and methanol, would extract flavonoids, phenols, and glycosides [19, 20]. The solubility of polar substances in polar solvents, and *vice versa*, concurs with the basic determinate solubility theory ("like dissolves like") [20].

Herbal medicine, or herbal extract, is considered as an antidiabetic due to its potency in reducing blood glucose levels, which is often resulted from the phytochemical compounds, such as terpenoid, steroid, alkaloid, and flavonoid classes through different mechanisms of action [21]. Some may act by increasing insulin secretion or insulin sensitivity, and others may act by inhibiting α-glucosidase and DPP-4 [3, 5]. Furthermore, since an extract could contain many compounds, the activity of the extract may result in synergistic or even antagonistic interactions of some compounds existing in the extract [22].

## Molecular docking of *U. lobata* on α-glucosidase and α-amylase

In the pharmaceutical field, molecular docking is often used to screen and predict the potential candidates the drug target of ligands with a known structure, based on its free energy binding, inhibition constant, and surface interaction. Free energy binding would inversely correspond to the binding affinity of a ligand to a target molecule (a lower free energy binding value would indicate higher binding affinity) [23]. The inhibition constant (Ki) is used in *in silico* studies to predict the inhibitory activity of a ligand to a drug target. Similarly, this score also operates inversely, in which a lower Ki score would indicate a higher inhibition activity of a protein target. Lastly, surface interaction represents the surface area and molecular recognition between a ligand and a binding pocket in a protein target. A higher surface interaction would indicate a higher number of interactions between a ligand and a molecule target [24].

Based on the molecular docking in this study, stigmasterol and β-sitosterol were found to have the lowest inhibition constant when tested against α-glucosidase and α-amylase. Free energy binding score showed that stigmasterol, followed by β-sitosterol and mangiferin, had the lowest free energy binding, respectively, and a high surface interaction in the same order. Both scores indicate a stronger binding with the drug target and may indicate a strong biological activity [23, 24] and, therefore, indicating the inhibitory activity of *U. lobata* leaf extracts on α-glucosidase and α-amylase.

Based on those categories, stigmasterol has the lowest score of inhibition constant and followed by β-sitosterol either on α-glucosidase and α-amylase. It is related to free energy of binding and surface interaction of these substances. In this research, stigmasterol has the highest score of surface interaction followed by β-sitosterol and mangiferin, respectively. A high score of surface interaction indicated a stronger bond between ligand and molecule target, moreover, it results a great biology activity. *In silico* analysis showed that stigmasterol has the lowest score in

the free energy of binding, meanwhile β-sitosterol and mangiferin were in the second and third position. The lowest score of binding free energy results a strong binding molecule, furthermore, it causes an increase of their biology activity [23, 24]. Free energy of binding and surface interaction between molecule target and ligand influences the inhibitory activity of *U. lobata* leaf extract, both on *α-glucosidase* and *α-amylase*.

Molecular docking research is widely used to predict the potential candidates of drugs in the pharmaceutical fields. Binding orientation of these active substances to their molecule targets reveals their activity and affinity as possible candidates of drugs [24].

### α-Glucosidase and α-amylase inhibitory activity of *U. lobata* leaf extract by *in vitro*

Our results showed that inhibitory activity of *U. lobata* leaf extract on α-glucosidase was stronger than on α-amylase. It may be due to the different chemical composition of the extracts, which interacts with α-glucosidase and α-amylase. *U. lobata* contains compounds such as phenol, tanin, stigmasterol, beta sitosterol, mangiferin, quercetin, and also some flavon compounds such as gossypetin dan chrysoeriol, with total phenol content of 25%. Active compounds found to inhibit α-glucosidase activity are those from the flavonoid, alkaloid and tanin group [25]. However, this depends on other factors, such as isoflavone level which is contained in herbs, molecule size and structural variation of tanin. Meanwhile, molecular configuration on binding site of α-glucosidase active sites also contributes to its activity. Generally, a more complex and large tannin structure would be more effective in inhibiting α-glucosidase [26]. Isoflavones are active compounds having an inhibition activity of α-glucosidase stronger than flavone compounds [25].

Flavonoid inhibits α-glucosidase by hydroxylation binding and substitution on the β-flavonoid ring. Inhibition activity of flavonoid is correlated with the number of hydroxyls on β-flavonoid ring. The more hydrogen binding between hydroxyl and polyphenol ligand with catalytic residues from the binding site of enzyme glucosidase, the more strongly the inhibition activity against α-glucosidase. Inhibition of α-glucosidase retains carbohydrate hydrolysis into maltose; therefore, it decreases glucose level postprandially [1]. *U. lobata* contains stigmasterol and β-sterol from phytosterol group and also mangiferin from xanthone glucosidase group. Based on previous *in silico* study, *U. lobata* is able to suppress α-glucosidase activity controlled by active compounds mentioned above.

Previous study showed antidiabetic of *U. lobata* leaf extract through inhibitory activity of dipeptidyl peptidase-4 (DPP-4) using *in vitro* and *in vivo* approach [5, 6]. Other study indicated both aqueous and methanolic extract of *U. lobata* leaf have antihyperglycemic or hypoglycemic effect on rats and alkaloids, flavonoids, saponins, and tannins present in the extracts may be responsible for the effects [4, 12]. Study in rabbits showed *U. lobata* aqueous extract of roots significantly reduced fasting glucose and body weight [8].

Other active substances inhibiting α-glucosidase are sterol and xanthonoid [26, 27]. Enzyme α-glucosidase is a glucoamylase enzyme that hydrolyzes polysaccharides, disaccharides, and oligosaccharides on the brush border of the microvilli in epitel intestine into glucose monomers. Activity of α-glucosidase is influenced by temperature and acidity level. Optimum temperature and acidity level for enzyme are 37 °C and 6.8, respectively. This condition is suitable with reactions occurring in the human body [28]. Active substances found to be able to suppress α-amylase are flavonoid, fenol, alkaloid, miscellaneous, terpenoid, xanthone, glucosidase, and sterol [26, 27, 29].

## Conclusions

Ethanolic extract of *U. lobata* leaf has inhibition activity stronger on α-glucosidase than on α-amylase as antidiabetic.

**Research funding:** Self financing.
**Author contributions:** All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
**Competing interest:** We declare that we have no conflict of interest.
**Informed consent:** Not applicable.
**Ethical approval:** Not applicable.

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