Alkaloid Fraction of *Litsea glutinosa* Leaves Provides an Important Precursor for Inhibition of Dipeptidyl Peptidase 4 Activity

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**Abstract.** Dipeptidyl Peptidase-4 (DPP-4) is a serine protease that plays an important role in metabolic and immunological functions. DPP-4 inhibitor is an alternative oral drug for the therapy of patients with Type 2 Diabetes Mellitus who do not respond to the standard therapy (metformin). However, long term uses of this drug remain unknown. Therefore, this study aimed to identify a phytochemical that could inhibit the DPP-4 and to extract it. This biocomputational study used a molecular docking method. Three-dimensional structure of DPP-4 and sitagliptin was downloaded from Protein Data Bank with access code PDB 3F8S and ZINC database with access code ZINC22007143. Indonesian phytochemicals were selected as research samples, which had 3D structure and met the criteria of Lipinski. Leaves of *L. glutinosa* were extracted using the soxhletation method with ethanol solvent of which alkaloid fraction was generated with phosphoric acid, n-hexane and chloroform solvents. Boldine concentration in extract and alkaloid fraction of *L. glutinosa* leaves was quantified using the High Performance Liquid Chromatography device. Actinodaphnine interacted with the catalytic triad of DPP4 (Ser 630 and His 740) and had three additional residues to bind to DPP-4 as same as sitagliptin (Glu 205, Glu 206 and Tyr 662). Naturally, actinodaphnine was synthesized from boldine. Total boldine concentration in *L. glutinosa* extract and alkaloid fraction were 1.11 and 2.14 mM respectively. Boldine as the precursor of actinodaphnine was successfully isolated from *L. glutinosa* leaves and will become a promising phytochemical for drug development of DPP-4 inhibitor.

1. **Introduction**

Type 2 Diabetes Mellitus (T2DM) is commonly found in patients with diabetes worldwide and most of them use oral anti diabetic (OAD) drugs. The first line therapy of T2DM is metformin but only 35% T2DM patients reach normoglycemic levels [20]. Long term use of metformin has reported association with B12 vitamin deficiency, renal and liver disfunctions, and increased lactic acidosis in patients with
congestive heart failure [3]. Therefore, metformin should be combined with other OAD drugs such as thiazolidinedione, alpha-glucosidase inhibitor, sulfonylureas (SU) or DPP-4 inhibitor [11].

Sitagliptin is the first generation of DPP-4 inhibitor and used as the second line OAD drug for patients with T2DM who are not responsive to metformin. This drug also becomes the third line OAD when dual therapies such as metformin and SU (Glyburide, glipizide and glimepiride) do not improve glycemic control. Sitagliptin has showed excellent selectivity and competitive inhibition against DPP-4 [11,25]. The advantages of sitagliptin for T2DM therapy can reduce appetite, satiety, weight gain and hypoglycemia. Furthermore, sitagliptin combined with metformin has a beneficial effect to optimize the pancreatic beta function [13,19]. However, Sakura et al. [24] and Naidoo et al. [18], reported that 50 – 100 mg/day sitagliptin administrated to 86 patients T2DM for 6 months have some side effects such as upper respiratory tract infections (6.3%), nasopharyngitis (5.2%) and headaches (5.1%). Therefore, development of alternative DPP-4 inhibitor from herbal compounds may minimize those side effects.

Around 30,000 plant species exist in Indonesia and 9,600 of them have pharmacological activities [31]. In silico study of herbal plants which inhibit DPP-4 enzyme is not available yet, whereas many in vivo studies have indicated that extracts of herbal plants are able to decrease of blood glucose level in mice and rats with T2DM. For example, administration of 50, 100, 200 and 400 mg/Kg BW methanol extract of L. glutinosa bark reduced blood gluce level in hyperglycemic mice [5]. Other studies by Manjunath et al. [16], administration of 200 and 400 mg/Kg BW Aloe vera leaf extract for 5 weeks produced hypoglicemic effect comparable with metformin 50 mg/Kg BW in alloxan induced diabetic rats.

However, it remains unknown the active compound that has an ability to reduce blood glucose level. Therefore, the aim of this study was to identify a phytochemical that could inhibit the DPP-4 enzyme and to extract it.

2. Methods

2.1. Preparation of DPP-4 protein and standard ligand
Molecular structure of DPP-4 protein was downloaded from a Protein Data Bank (www.rcsb.org/pdb/) with access code PDB ID: 3F8S whereas molecular structure of the DPP-4 inhibitor (sitagliptin) was obtained from the Zinc Database (www.zinc.docking.org/) with access code ZINC22007143. Before validation of sitagliptin – DPP-4 binding complex, the DPP-4 protein structure was modified by removing water molecules and adding hydrogen atoms in order to make polar charge in the catalytic side of DPP-4 protein. Modification of DPP-4 used the AutoDock Tools 1.5.6 program (www.mgltools.scripps.edu/downloads). Sitagliptin was molecularly docked with the modified DPP-4 protein for five times to get Root Mean Square Deviation (RMSD) < 2 [6].

2.2. Molecular docking of phytochemicals with DPP-4 protein
Indonesian herbal plants which were registered in a Herbal Data Base (HerbalDB), Faculty of Pharmacy, University of Indonesia (www.herbaldb.farmasi.ui.ac.id) and had molecular structure were used in this study. Phytochemicals of Indonesian plants were selected using the Lipinski’s Rule of Five Criteria and 3D structure of selected phytochemicals was searched in the PubChem NCBI.

To analyse binding affinity and binding sites between phytochemicals and DPP-4 protein, AutoDockVina version1.1.2, which was freely downloaded from www.pyrx.sourceforge.net was used. Phytochemicals-DPP-4 protein binding complexes were then visualized using PyMol version 1.7 (www.pymol.org) and Chimera version 1.9 (www.cgl.ucsf.edu/chimera).

2.3. Alkaloid extraction of L. glutinosa leaves that have DPP-4 inhibitor property
Fresh L. glutinosa leaves were obtained from the Center for Research and Development of Medicinal Plants and Traditional Medicines, Tawangmangu, Karanganyar, Indonesia. Dried L. glutinosa leaves were then extracted using a soxhletation method with 96% (v/v) ethanol (Merck, Darmstadt,
Germany) solvent, which was heated under reflux temperature [17,30]. Five gram extracted L. glutinosa leaves were dissolved in 30 mL 0.1 M phosphoric acid using a sonicator for 30 min and the acid solution was then filtered using Whatman filter paper (90 mm). Furthermore, filtrate was re-dissolved in 50 ml n-hexane (Merck, Darmstadt, Germany) and separated using a funnel (Pyrex). This process was done three times and collected acid solution was adjusted to pH ∼9 using 25% (v/v) ammonia hydroxide solution (Merck, Darmstadt, Germany). The basic solution was then partitioned for three times with 50 ml chloroform (Merck, Darmstadt, Germany) [21].

2.4. Determination of boldine concentration using HPLC

To determine boldine concentration in ethanol extract and alkaloid extract of L. glutinosa leaves, HPLC Waters e2695 was used. Standard boldine (Cat B3916, Sigma-Aldrich®, USA) and samples were dissolved in methanol (Merck, Darmstadt, Germany) and 10 µL of 100 – 1000 ppm diluted standard boldine and 1000 ppm samples was injected into a reverse phase column 5C18-MS-II, 4.6ID x 150 mm with a mobile phase that contains acetonitril/water/triethylamine (1:1:0.2 v/v/v), pH 4 adjusted with 10% solution of phosphoric acid with 0.6 mL min⁻¹ flow rate [29]. Peak area and retention time were used to determine boldine concentration in ethanol extract and alkaloid extract of L. glutinosa compared to standard boldine. All analyses were performed in triplicate and data were presented as mean ± standard deviation.

3. Results and Discussion

After running molecular docking between sitagliptin and DPP-4 protein, sitagliptin bound to the enzyme with -8.3 kcal / mol binding score and RMSD = 0. In addition, sitagliptin interacted with DPP-4 at Glu 205, Glu 206 and Tyr 662 residues. Interactions between H and O atoms occurred at Glu 205 and Tyr 662 residues while interaction between O and O atoms occurred at Glu 206 residue (Van der waals interaction). However, sitagliptin does not bind to the catalytic triad of DPP-4 (Ser 630, Asn 710 and His 740) but it is able to block substrate entry into the catalytic triad. Some evidence shows that sitagliptin competitively inhibits DPP-4 protein and has a reversible effect [4,7].

Table 1. Molecular Docking Scores Analysis of Sitagliptin and Phytochemicals of Indonesian Herbal Plants.

| No  | Docking Score (Kcal/ mol) | Binding Site | MW (<500) (Da) | H Bond Donor (<5) | H Bond Acceptor (<10) | Lipinski’s Rule of Five | Compound’s Lipophilicity (XLogP3- AA<5) |
|-----|--------------------------|--------------|----------------|-----------------|---------------------|------------------------|-------------------------------|
| 1.  | -8.3                     | Glu 205, Glu 206 and Tyr 662 | 400.35        | 3               | 4                   | 3.79                   |                               |
| 2.  | -8.5                     | Glu 205, Glu 206, Tyr 662, Ser 630, Asn 710 and His 740 | 311.33188 | 2               | 5                   | 2.4                    |                               |
| 3.  | -9.17                    | Glu 205, Glu 206, Ser 209 and Arg 358 | 327.37434 | 2               | 5                   | 2.6                    |                               |
| 4.  | -9.1                     | Arg 125, Tyr 662 and Asn 710 | 480.591       | 4               | 8                   | 2.6                    |                               |

From Table 1, some phytochemicals had binding score and binding sites, comparable to sitagliptin. Only actinodaphnine had lower binding score and occupied the catalytic triad and entry sites of DPP-4 than sitagliptin did. As seen in Figure 1, sitagliptin and actinodaphnine share molecular conformation that binds to DPP-4 protein. Meanwhile, coreximine had as same binding site as sitagliptin and -9.17 binding score. A lower binding score was observed in neoandrographolide, compared to sitagliptin but it had binding sites at Tyr 662 and Asp 710 residues against DPP-4 protein. Although three
phytochemicals have different properties, they meet criteria of the Lipinski's rule. Overall, actinodaphnine becomes a potential DPP-4 inhibitor better than sitagliptin.

Figure 1. Visualisation of actinodaphnine and sitagliptin with DPP-4 protein. Left figure showed atom interaction between actinodaphnine and DPP-4 using Pymol 1.7 software. green = C atom, red = O atom, white = H atom, blue = N atom. Right figure showed visualisation by software Chimera 1.9. red = sitagliptin, green = actinodaphnine, blue rectangular = interaction location, yellow circle = similarity of interaction location with sitagliptin, black circle = catalytic triad.

There are some Indonesian herbal plants that have actinodaphnine compound such as _L. glutinosa_, _Cassytha filiformis_, _Cylcodaphne sebifera_ and _Litsea sebifera_. _L. glutinosa_ leaves were extracted using 96% ethanol solvent with soxhletation method and then fractionated using phosphoric acid, n-hexane, chloroform and ammonia hydroxide solutions. Because pure actinodaphnine is not available, we used boldine as a standard compound. From molecular pathways, boldine is the molecular precursor for actinodaphnine synthesis [1].

Figure 2. HPLC chromatograms of standard boldine and _L. glutinosa_ extract and alkaloid fraction.

Total boldine concentration in extract and alkaloid fraction of _L. glutinosa_ leaves were 1.11 and 2.14 mM respectively. So far, boldine concentration in _L. glutinosa_ plant has not been determined yet using HPLC. However, a recent study conducted by Sukma [26] reported that ethanol extract and
alkaloid fraction of *L. glutinosa* leaves contained 13.47 and 53.4 nM boldine respectively. She used Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS) rather than HPLC devices.

Boldine concentration in extract and alkaloid fraction of *L. glutinosa* leaves using HPLC device were higher than boldine concentration using LC-MS/MS device. There were different results because mobile phase separation of HPLC in our study used isocratic, while in Sukma’s study used gradient separation in LC-MS/MS. Isochratic separation work in equilibrium conditions in the column and the actual velocity of compounds moving through the column are constant. However separation power is low, so that causes such as the longer of component to retaind in the column, the lower and the wider of peak area [22], the peak area of HPLC chromatogram show coincide with each other and the retention time stops at almost the same time [8]. Otherwise, gradient separation in elution of mobile phase in her study has the optimum result.

The other causes of different results in our studies were different of flow rate and injection volume parameters. Flow rate of this study was regulated at 0.6 mL min\(^{-1}\) and injection volume was 10 µL, while flow rate of Sukma’s study was regulated at 0.3 mL min\(^{-1}\) and injection volume was 5 µL. Therefore, there were effected to results [15]. If the large injection volume, which the column can be overload, so it causes such as lead to peak broadening and peak tailing (Figure 2, (B) and (C)). The best of injection volume is accuracy inject to achieve the absolute value. In order to achieve good reproducibility for quantification run to run, injection precision is importance which inject the same amount of sample over many runs. Whereas, the difference of flow rate will be effect to choice of a column. For simple analytical sample use short column to reduce analysis time. For higher resolution use longer column. The longer column is the higher of flow rate pump to match the flow requirement of a preparative column. Unnecessary longer column may lead to a loss of efficiency and more tails in chromatographic result [2].

Otherwise, solvent type of mobile phase using HPLC and LC-MS/MS devices had same solvents. There were acetonitrile, phosphoric acid and methanol solvent types. Phosphate buffer is soluble in methanol, that will be a low pH in mobile phase which reduced the peak tailing and increased retention time. The similarity of solvent types will effect to defend of resolution, selectivity and efficiency which have good resolution between peaks, minimum tailing and retention time repeatability [22,27].

LC-MS/MS methode has fast response, high specificity properties, short runtimes, more sensitive and specific compared to HPLC [9,12]. LC-MS/MS is analysis device with high performance which can use for quantitative and structural analysis, so that have specific result for determine a metabolite. LCMS/MS is not only the retention time observed but also the separation of ions in the compound and accuracy of mass spectrometric detection [14,23,28].

4. Conclusion
Actinodaphnine has molecular properties, binding affinity and binding sites to DPP-4 better than sitagliptin. Boldine as the precursor of actinodaphnine was successfully isolated from *L. glutinosa* leaves. Boldine concentration in alkaloid fraction of *L. glutinosa* leaves is higher than boldine concentration in ethanol extract of *L. glutinosa* leaves. Further studies are required to figure out the effects of ethanol extract and alkaloid fraction of *L. glutinosa* leaves on blood glucose level in rats model T2DM.

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