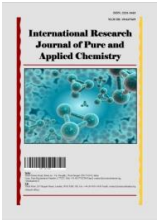

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A Study on the Inhibitory Potential of Dpp-Iv Enzyme by Lobeline through *In silico* and *In vivo* Approaches

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Authors' contributions

This work was carried out in collaboration among all authors. Author BK under supervision of authors GS and RL. Authors WK and GS conceived and designed the in silico study. Author RL conceived and designed the In vivo study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the inhibitory activity of Lobeline natural alkaloid against dipeptidyl peptidase IV (DPP IV) enzyme by *in silico* and in vivo experiments.
Study Design: Evaluation of Antidiabetic Activity of Lobeline alkaloid.
Place and Duration of Study: Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Aleppo University, Aleppo, Syria, between March 2020 and December 2020.
Methodology: *in silico* study was carried out using iGEM docking software to predict the binding affinity of lobeline with DPP IV enzyme in comparison with the reference synthetic compound Sitagliptin. Then in vivo experiment was performed on HFD/alloxan induced diabetic mice to evaluate the anti hyperglycemic effect of lobeline. After treatment duration of 21 days, FBG and the inhibitory effect on DPP IV enzyme activity were measured.
Results: Lobeline bound efficiently to the active site of DPP IV enzyme and consumed less binding energy than Sitagliptin. This finding was confirmed by the in vivo study.

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Administration of lobe line at a dose of 25 mg/kg in HFD/alloxan induced diabetic mice produced a significant reduction in blood glucose level and in DPP IV activity compared to the diabetic control group (*P* value< .01).
Conclusion: Lobe line could be a good candidate to be developed as a natural compound for treating diabetes mellitus.

Keywords: Type2 diabetes; alkaloids; lobeline; DPP IV enzyme inhibitor; molecular docking; In vivo.

1. INTRODUCTION

Diabetes Mellitus (DM) is a metabolic disease with a highly increasing prevalence which the International Diabetes Federation (IDF) expects it to reach 700 million in 2045 [1].

DM occurs due to either insulin deficiency and/or insulin resistance [2] and it can be classified into four categories according to their pathophysiology [3].

Type 1 with an absolute deficiency of insulin, type 2 resulting from insulin resistance and various degrees of β-cell dysfunction, gestational diabetes diagnosed during pregnancy and diabetes due to other specific causes, e.g. diseases of the exocrine pancreas and drug- or chemical-induced diabetes.

In 2016, DM caused death to 1.6 million according to World Health Organization report [4].

However, type 2 diabetes accounts for greater than 90% of cases [5].

If DM is not treated properly, patients may develop life- threatening complications like: retinopathy, nephropathy, neuropathy and cardiovascular diseases [6].

Searching for new antidiabetics has always been a big concern for researchers, that is because most of drugs available to date have failed to achieve a long term glycemic control due to lack of efficacy, emerging of side effects or the high cost [7].

Over centuries, Natural compounds have been a highly attractive source to investigate new effective, safe and inexpensive drug [8]., These natural compounds might be a promising drug to treat diabetes by targeting specific enzymes involved in the mechanism of stimulating insulin secretion. Dipeptidyl peptidase IV (DPP IV) enzyme was chosen as a target due to its vital role in incretin axis [9].

Incretins are peptides secreted from the small intestine in presence of nutrients and they are responsible for 50- 70% of insulin postprandial secretion [10]. Two main incretins are glucagon like peptide- 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). These incretins stimulate the secretion of insulin by binding to their receptors on β pancreatic cells [11]. But they have very short half-lives due to inactivation by dipeptidyl peptidase IV enzyme (DPP IV) which cleaves two peptides at N- terminal [12].Therefore, inhibition of DPP IV enzyme delays the degradation of the GLP- 1 in plasma and prolongs its effect positively on insulin and negatively on glucagon secretion [13](Fig.1).

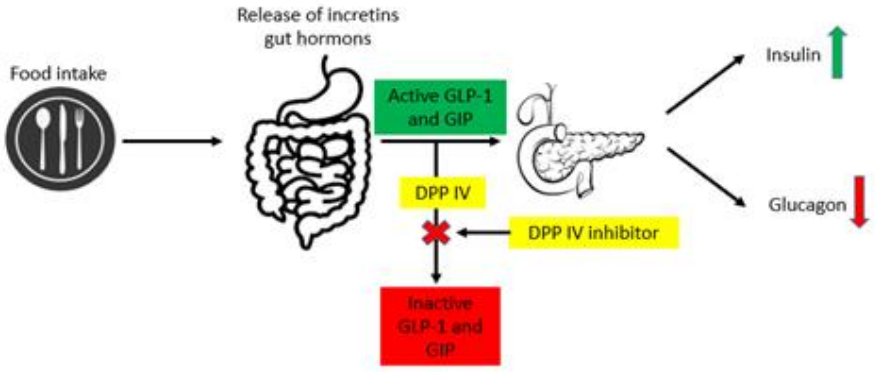

Fig. 1. Incretin signaling pathway and the role of DPPIV

DPP IV is a homodimer enzyme comprised of 766 amino acids (Fig. 2), three residues Ser 630, Asp708 and His740 are forming the active site and located in the C- terminus extracellular part [14].

Sitagliptin (Januvia®) is the first DPP IV inhibitor got approval by the FDA in 2006, chemical structure is shown in (Fig.3). It has effectively reduced blood glucose and HbA1c. Studies on Sitagliptin also showed a positive effect on pancreatic *β*-cells regeneration and differentiation [15].

Treatment with DPP IV inhibitors may associate with adverse effects such as: nausea, headache, nasopharyngitis, pancreatitis, angioedema and others [16].

Therefore, this study focused on alkaloids as a widespread group in plants with several known therapeutic effects such as antidiabetic [17-21], antihypertensive [22], anti-cancer [23] and analgesic [24].

Pelletier S. defined alkaloids as organic heterocyclic compounds containing nitrogen and produced by plants, animals, insects, microbes and also humans as secondary metabolites [25].

We searched for DPP IV inhibitors depending on molecular docking, a computer-aided modern technique in drug discovery which allows to predict the binding affinity between various compounds and the target enzyme then ranks them according to the energy required for binding [26]. Computational docking analyses have been commonly used for designing inhibitors [27], screening of potential inhibitors [28] and explaining the differences in activity of drugs with different structures [29].

Comparing about 500 alkaloids to the currently available DPP IV inhibitors [30], Lobeline was one of the best alkaloids which showed more affinity to bind with the DPP IV.

Lobeline (Fig. 4) is an alkaloidal constituent of Lobelia inflata LINN, which has a long history of therapeutic usage ranging from emetic and respiratory stimulant [31] to tobacco smoking cessation agent [32] and treatment of drug abuse [33].

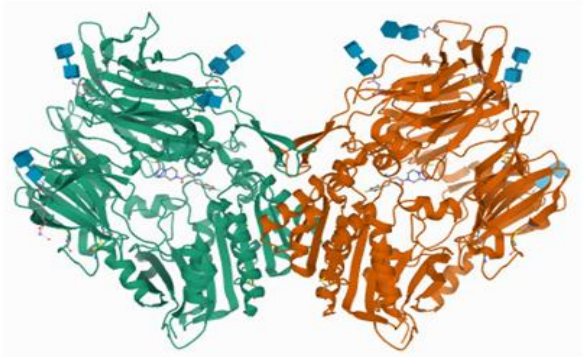

Fig. 2. DPP IV enzyme (PDB ID: 1X70)

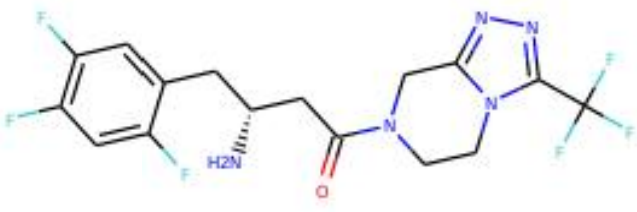

Fig. 3. Sitagliptin chemical structure and IUPAC name (Zinc ID: 1489478)

3-amino-1-[3-(trifluoromethyl)-6,8-dihydro-5H-[1,2,4] triazolo [4,3-a] pyrazin-7-yl] -4- (2,4,5-trifluorophenyl) butan-1-one

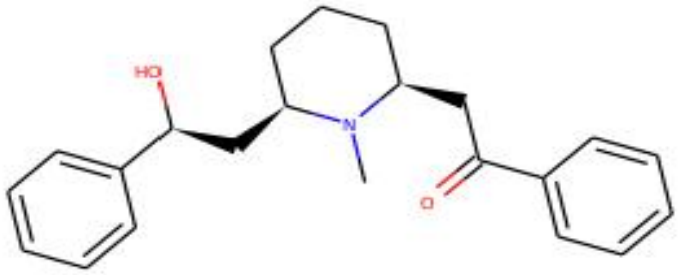

Fig. 4. Lobeline chemical structure and IUPAC name (Zinc ID: 1624)
2, 6-[2-hydroxy-2-phenylethyl]-1-methylpiperidin-2-yl]-1-phenylethanone

Since diabetes mellitus is still a major cause of morbidity and mortality despite the various available antidiabetic drugs. Natural compounds are considered as a compromising source for DM treatment. This study aimed to confirm the virtual inhibitory effect of candidate alkaloids on DPP IV enzyme involved in the mechanism of diabetes. Therefore, Lobeline was subjected for the in vivo study on diabetic mice.

2. MATERIALS AND METHODS

All the experimental works were carried out using analytical grade chemicals reagents and solvents, they were procured from commercial sources.

Alloxan monohydrate (ALX) purchased fromTitan Biotech LTD, India.

DPP-IV assay substrate Glycine- proline- para nitroanilide obtained from Cayman Chemical, USA.

DPP-IV inhibitor Sitagliptin was supplied by Ibn Al Haytham for pharmaceutical company, Syria.

Test alkaloid Lobeline hydrochloride purvhaced from Nanjing Sunsure Chemical Technology, China.

A glucometer (On Call® plus, ACON laboratories, Inc., USA) with a maximum measuring capacity of 600 mg/dl.

2.1 *In silico* Docking Study

2.1.1 Protein preparation

The targeted protein DPP IV crystal structure was retrieved from the Protein Data Bank, a free resource for 3D structures of proteins and other large molecules [34]. The human DPP IV enzyme (PDB ID: 1X70) was deposited by Kim D. et al. with a resolution factor 2.10 A°, in complex with the ligand Sitagliptin which was first developed by Merck & Co [35] (Fig. 5).

2.1.2 Ligands preparation

Lobeline (ID: 1624) and Sitagliptin (ID: 1489478) were downloaded in SDF format from Zinc free available database of compounds with formats ready for virtual screening [36].

The SDF files were then converted into MOL2 files using openbabel, a computer software to convert chemical file formats [37].

Lipinski and veber rules are commonly used to determine the drug-like properties of the compounds depend on physiochemical properties.

Lipinski rule of 5:

This rule was formulated to predict drug-likeness and oral bioavailability. It consists of four important properties, each related to the number 5 [38]:

1. The molecular weight of less than 500 mg/mol
2. Has a high lipophilicity (log p less than 5)
3. Hydrogen bond donors less than 5
4. Hydrogen bond acceptor is less than 10

2- Veber rule includes two following criteria [39]:

a) rotatable bond count>=10.
b) polar surface area (PSA) equal to or less than 140 A.

Lobeline satisfies Lipinski and Veber rules as illustrated in Table 1. Although natural

compounds could be excepted of these rules likely because of their high complexity and special conformational features [40].

2.1.3 Protein-ligand docking

To perform the docking study, we used iGEMDOCK v2.0 software, a graphical-automatic drug discovery system developed by the University of National Chiao Tung, Taiwan [41] and freely available at http://gemdock.life.nctu.edu.tw/dock/igemdock.php. This software was used as molecular docking tool in various previous researches [42-46]. iGEMDOCK affords an interactive interface to prepare the library of screening compounds and allow to define the binding site of the target protein from the selected bounded ligand, Sitagliptin, in the PDB file.

Lobeline and Sitagliptin were subjected to "accurate docking", by setting: population size of 800 with 80 generations and 10 solutions, to predict the bounded poses of protein- ligand complex.

Then the predicted ligand binding geometries generated were analyzed by post- analysis tool and binding scores were calculated based on the pharmacological interactions and energy-based scoring function of electrostatic (E), hydrogen-bonding (H), and Van der Waal's (V) interactions.

Energy = V bond+ H bond+ E bond

2.2 *In vivo* Study

2.2.1 Animals

Thirty male of albino mice (28 ± 2 g; 8-10 weeks old) were obtained from the Animal House Centre of faculty of pharmacy, Aleppo University. The animals were housed in standard polypropylene cages (6 mice/cage) and maintained under controlled room temperature and humidity with a 12:12 hour light and dark cycle. The mice had free access to water and food, either normal or high fat diet (HFD).

2.2.2 Diabetes induction

Alloxan (ALX) is one of the most commonly used substances to induce diabetes. It causes damage to beta cells proportional to the dose administered [47]. To mimic type 2 diabetes, chemical diabetogenic agent is combined with a high fat diet to develop insulin resistance with partial destroying of pancreatic cells [48].

Male mice had been allocated into high fat diet HFD 60% for three weeks, then after overnight fasting mice were injected intraperitoneally with a solution of alloxan in physiological saline (175 mg/kg). The animals were allowed to drink 5% glucose solution overnight to avoid induced hypoglycemia shock for 24 hours. After 72 hours of ALX administration, fasting blood glucose was measured and mice with FBG levels above 200 mg/dl were considered to be diabetic and included in the study.

2.2.3 Experimental design

After diabetes induction, mice were randomly divided into five different groups of six animals in each as detailed below:

Group (NC) - Normal Control
Group (DC) - Diabetic Untreated Control
Group (Sita) - Diabetic received Sitagliptin 25 mg/kg s.c
Group (Lob-10)- Diabetic received Lobeline 10 mg/kg s.c /day
Group (Lob-25) - Diabetic received lobeline 25 mg/kg s.c /day

Lobeline and Sitagliptin were dissolved in physiological saline solution and to harmonize the experiment, saline was also given to normal and diabetic untreated control groups throughout the study.

2.2.4 Fasting Blood Glucose (FBG) measurement

FBG was measured at weekly bases for 3 weeks by using the glucometer strips and samples were obtained from tail veins by tail nipping.

2.2.5 Determination of inhibitory effect on DPP- IV activity

DPP IV assay was carried out following the modified colorimetric method of Al-Masri et al [49]. The chromogenic substrate Gly- Pro- pNA (hydrochloride), is cleaved by the enzyme DPP IV to release the yellow free Paranitroaniline (pNA) which is measured at 405 nm.

At the end of 21 days, mice were anesthetized by kitamine (100mg/kg i.p.) and blood samples were collected from venous pool from the eyes to heparinized tube. Samples were centrifuged

(3000 rpm for 10 minutes at 37º) for separation of the plasma and plasma was stored at -20° till measurement of absorbance. 10 µl plasma samples were mixed with 190 µl 0.5 mM Gly- Pro- pNA (diluted from a 100 mM stock solution in DMSO, stored at −20° C) in 50 mM Tris buffer pH 8.3 in a final volume of 200 µl.

Absorbance was determined at 405 nm in a 96-well plate reader after incubation for 30 minutes at 37° C and DPP IV inhibition for each group of mice was calculated as percentage relative to the negative control group. Plasma from diabetic control mice was used as negative control and blank was by using distilled water instead of the plasma.

The percentage of inhibition was calculated using the given formula [50]:

$$\text{Inhibition\%} = \frac{(\text{Absorbance of control} - \text{Absorbance of inhibitor})}{\text{Absorbance of control}}$$

2.3 Statistical Analysis

Statistical analysis was performed using SPSS software version 24 using one-way ANOVA with tukey post-test for comparison and the *P* value ≤0.01 was considered significant. Values are expressed as mean ± SEM.

3. RESULTS AND DISCUSSION

Hence in this Study, the efficacy of Loboline against DPP IV enzyme which is responsible for incretins degradation was assessed by two axes: first *in silico* then the results obtained was applied in vivo.

3.1 *In silico* Study

Molecular docking is a computer- aided tool that helps in saving time and money that it shifts experiments from laboratories to the computer. Binding affinity of the ligands into the target protein could be hypothesized.

Lobeline showed good affinity to DPP IV enzyme *in silico* compared to synthetic available inhibitors.

Table 2 shows the binding energy and all types of interactions of Lobeline and Sitagliptin as a reference with the residues of amino acids involved in the active site of DPP IV enzyme. Neither of compounds gave ionic interacions.

Fig. 6 illustrates the interactions within the binding site of the enzyme. Although Sitagliptin had stronger hydrogen bonds but Lobeline consumed less total binding energy with DPP IV than Sitagliptin -106.1 vs. -55.9 Kcal/mol due to the strong formed hydrophobic bonds.

This result suggested Lobeline as a potential DPP IV inhibitor which may be effective in treating T2DM.

3.2 *In vivo* Study

Results of the *in silico* study had to be confirmed by in vivo study. HFD with dose of 175 mg/kg alloxan were used to induce DM in mice. Drugs were administered daily for 21 days subcutaneously. FBG and DPP IV inhibition were assessed.

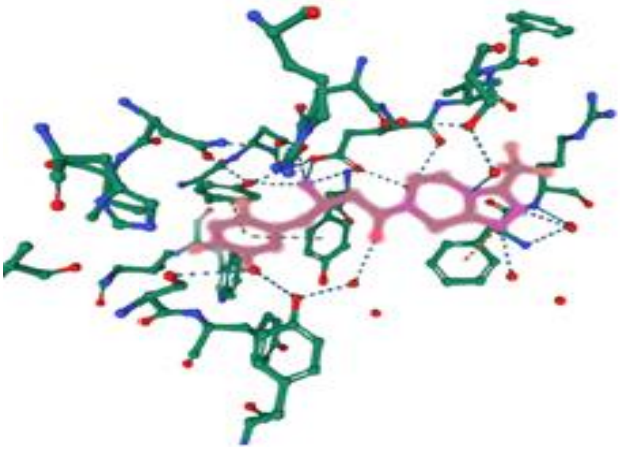

Fig. 5. Sitagliptin in the active site of DPP IV enzyme (PDB ID: 1X70)

Table 1. The physiochemical properties of lobeline related to lipinski's and veber rules

Common Name	Molecular weight MW	Hydrogen Bond acceptors HBA	Hydrogen Bond Donors HBD	logP	Rotatable Bonds RB	Polar surface area PSA
Value to be according to Lipinski	500> g/mol	<10	<5	<5		
Value to be according to Veber		Sum ≤12			≤10	≤140 A°
Lobeline	337.463	2	2	4.236	6	41

Table 2. Binding energy and interactions with residues involved in the binding site

Predicted interactions with residues	Lobeline	Sitagliptin
H-S ARG 125	-1.2	0
H-M GLU 205	-2.1	-3.5
H-S GLU 206	-2.5	-3.5
H-S TYR 547	0	-9.3
H-S TYR 662	-1.8	0
H-S TYR 666	0	-3.4
V-S ARG 125	-5.1	0
V-M GLU 205	-3.5	0
V-S GLU 205	-4	0
V-M GLU 206	-0.2	-4.1
V-S GLU 206	-5.1	0
V-M VAL 207	-4.5	0
V-M PHE 357	-3.3	0
V-S PHE 357	-11.6	-10.7
V-M ARG 358	-1.4	0
V-S ARG 358	-5.1	-4.9
V-S TYR 547	-4.3	0
V-M SER 630	-3.2	0
V-S SER 630	-3	0
V-S TYR 631	-3.6	0
V-S TYR 662	-9	0
V-S TYR 666	-10.9	-4.3
V-M HIS 740	-1.9	0
Total binding energy (Kcal/mol)	-106.1	-55.9

H and V are for hydrogen and van der Waals bonding; M and S are for Main chain and Side chain

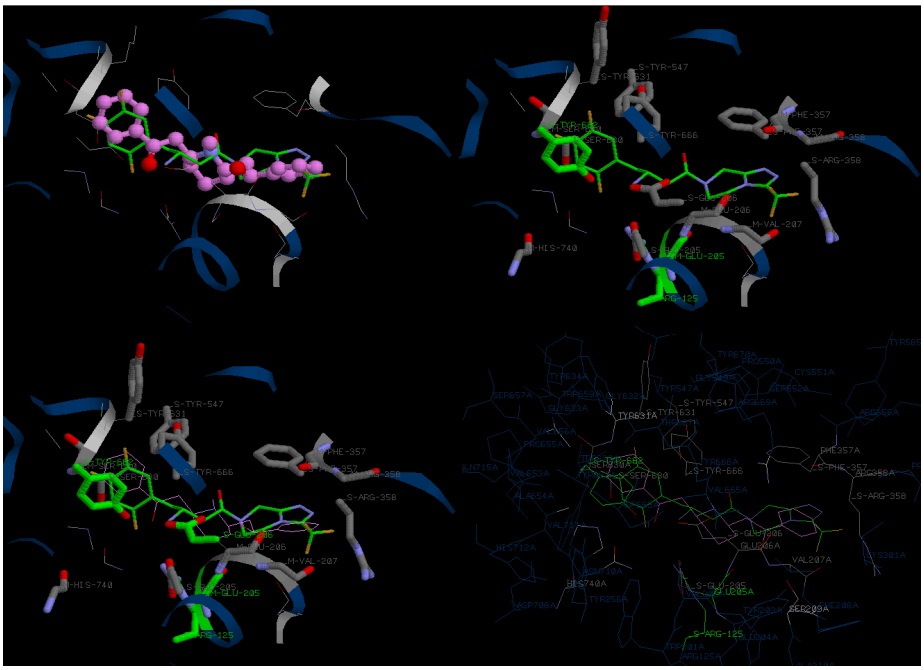

Fig. 6. Docking pose of lobeline within the active site of DPP IV enzyme (PDB ID-1X70)
Pink color represents Lobeline and green color represents Sitagliptin; Green and grey color represents the amino acids involved in hydrogen bonding and van der Waals interactions respectively

3.2.1 Fasting Blood Glucose (FBG) measurement

Table 3. shows the levels of fasting blood glucose (FBG) of the mice groups during the period of treatment.

HFD with Alloxan were able to induce diabetes in mice which led to a significant increase in blood glucose levels by an over 2-3 fold in all mice at day 0 of treatment duration as compared to the normal control group (NC) (P < .001) and remained high for 3 weeks in the untreated diabetic control group (DC). The results showed that both Sitagliptin (as positive control) and Lobeline administration had a hypoglycemic effect on diabetic mice, as they decreased the levels of blood glucose.

Daily doses of Sitagliptin for 21 days significantly decreased blood glucose levels as compared to day 0 by 31.43, 33.96 and 43.27% respectively for three weeks (P < .001). The percentage of reduction of FBG levels compared to day 0 in Lob 10 were 6.56, 16.85 and 30.99% and were 18.62, 33.96 and 47.98% in Lob 25 treated mice as respectively on weekly basis and the differences between all treated groups compared to the DC group were significant (P < .001).

(Fig. 7) showed FBG changes in each group over treatment duration, glucose level in DC continued to rise while treatment administration led to a progress decline in FBG levels in all treated groups. (Fig. 8) illustrated the hypoglycemic effect in all study groups. Glucose levels in all treated groups were significantly less than the untreated diabetic control. (P <.001). So, Lobeline could be obviously considered as a hypoglycemic agent and this effect was more pronounced at a dose of 25 mg/kg than 10 mg/kg (P <.001) and yet this dose was selected for further tests to check the suggested mechanism of action related to DPP IV inhibition that may contribute in the glucose lowering effect due to enhancing the in cretin effect on insulin stimulation and glucagon inhibition.

Sitagliptin and Lobeline 25mg/kg groups were approximate with no significant difference (P = .464).

3.2.2 Determination the inhibitory effect on DPP- IV activity

Based on the hypoglycemic results and the *in silico* binding affinity of Lobeline to DPP IV enzyme, the inhibitory effect of Lobeline on the activity of DPP IV enzyme was checked after

treatment of mice with Lobeline (25 mg/kg) and Sitagliptin as positive control after 21 days of treatment. We measured the absorbance of the product pNa released from the chromogenic substrate and the Inhibition percentage was calculated as mentioned in materiel and method.

As shown in (Fig. 9), both Lobeline and Sitagliptin produced marked inhibitory effect on DPP IV enzyme compared to the untreated diabetic control group DC. Inhibition percentage values were 60.8 and 46.4 % respectively. This outcome was compatible with the *in silico* results where Lobeline consumed less energy to bind with DPP IV enzyme (-106.1 Kcal/mol) comparing with the reference inhibitor Sitagliptin (- 55.9 Kcal/mol), thus led to a higher inhibitory effect with Lobeline. Thereby it is clear that Lobeline takes the role of a competitive substrate on the active site of DPP IV enzyme and inhibits its activity which prolongs the half-lives of incretins.

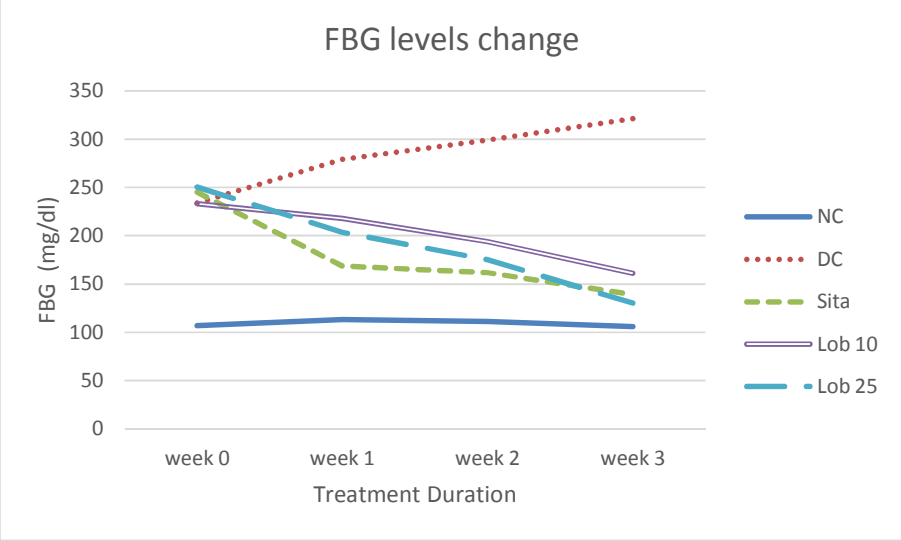

Fig. 7. FBG levels changes over treatment duration

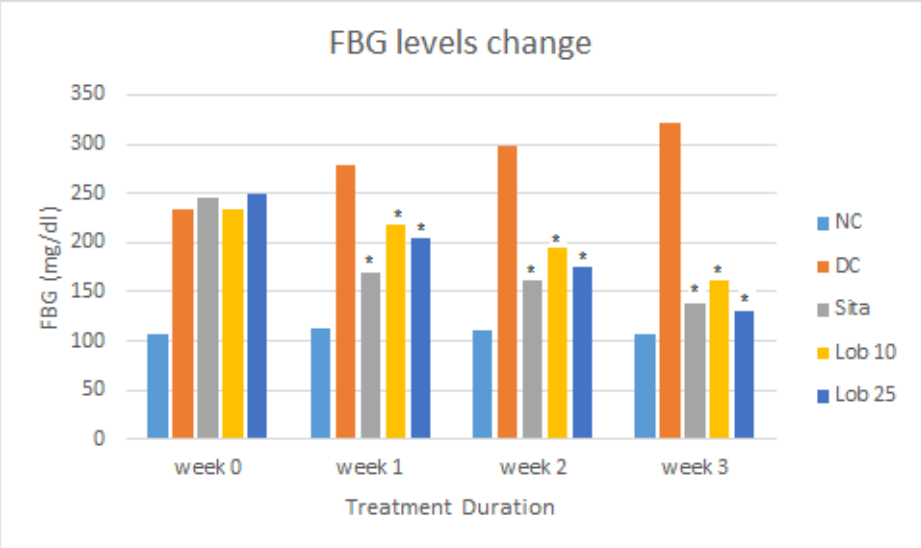

Fig. 8. Effects of Lobeline and Sitagliptin on fasting blood glucose levels

** indicates P value< 0.01 compared to DC group*

Table 3. Fasting blood glucose (FBG) levels and the percentage of reduction of FBG levels during the period of treatment

Period of treatment	0 days	7 days		14 days		21 days	
Group	FBG± SD (mg/dl)	FBG± SD (mg/dl)	↓FBG	FBG± SD (mg/dl)	↓FBG	FBG± SD (mg/dl)	↓FBG
NC	106.7±10.3	113±4.4	-	111±1.6	-	106±3.4	-
DC	234±2.4	279±2.5	-	299±3.6	-	321.2±6	-
Sita	245±4.3	168.5±1.4	31.43%	161.8±2.2	33.96%	139±2.4	43.27%
Lob 10	233.3±4.7	218±1.8	6.56%	194±6.5	16.85%	161±3.1	30. 99%
Lob 25	250.3±4.9	203.7±6.6	18.62%	175.2±7.1	30%	130.2±2.6	47.98%

↓= the percentage of reduction of FBG as compared to FBG at day 0

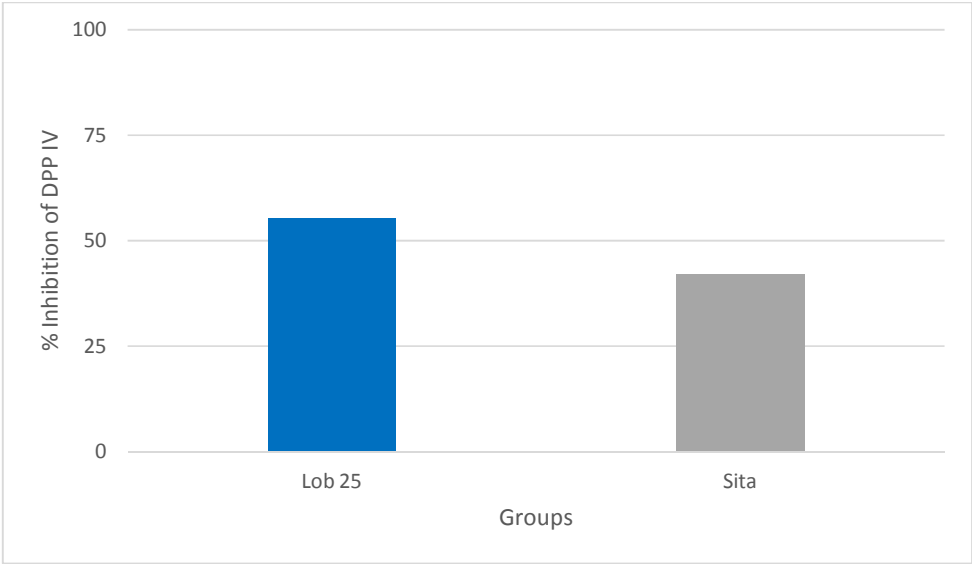

Fig. 9. Inhibition of DPP IV enzyme

4. CONCLUSION

The results obtained from the *in silico* and in vivo studies revealed that Lobeline alkaloid might to be a candidate as an antidiabetic agent in the future. The suggested mechanism is related to its effectiveness in inhibiting DPP IV enzyme and subsequently enhancing the postprandial effect of incretin on insulin augmentation and glucagon suppression.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was funded by Aleppo University- faculty of pharmacy as an academic research for master degree.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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