Review Article

Pharmaceutical BIOPROCESSING

Dipeptide synthesis and evaluation of anti-diabetic activity of 4-hydroxyisoleucine from fenugreek seeds

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Fenugreek is one of the oldest medicinal plants, originating in India and Northern Africa. The hypoglycemic effects of fenugreek have been attributed to several mechanisms. Under *In vitro* conditions, the amino acid 4-hydroxyisoleucine(4-OHIL) in fenugreek seeds increased glucose-induced insulin release in human and rat pancreatic islet cells. It was observed that 4-OHILextracted from fenugreek seeds has insulin tropic activity. This amino acid appeared to act only on pancreatic beta cells, since the levels of somatostatin and glucagon were not altered.

Experimental: In order to separate amino acids from methanol extract of fenugreek seeds, ion exchange chromatography method containing 225H cationic resin was used. Five dipeptides were prepared from 4-OHILnamely; GLY-L-4-OHIL, ALA-L-4-OHIL, SER-L-4-OHIL, VAL-L-4-OHIL, THR-L-4-OHIL using cbZ-Cl in protecting step, DCC in peptide forming step and HBr in acetic acid in deprotection step.

Results: Five dipeptides were confirmed by spectral data like IR, MASS, NMR and evaluated for anti-diabetic activity. The studies showed that only GLY-L-4-OHIL showed improved activity than the other derivatives and it is comparable with 4-OHIL.

Conclusion: Remarkable anti diabetic activity was observed when compared with standard drug and the parent compound 4-OHIL.

Keywords: fenugreek ▪ isoleucine ▪ somatostatin ▪ dipeptides

Introduction

In many developed and developing countries the rise of lifestyle disorders like metabolic disorders has been increasing with the rise in economic development. Although these metabolic disorders can be controlled by increasing the physical activity and following proper diet, but the major concern is changing one's usual life style. However, this problem may be addressed by taking functional foods which are having beneficial effects on several metabolic disorders. The use of functional foods is sometimes limited due to safety concerns [1].

Diabetes is a lifestyle disorder characterized by high levels of glucose in blood (hyperglycemia) [2]. This metabolic disorder is caused mainly due to insulin deficiency or insulin resistance or sometimes both [3].

Fenugreek is the one of the oldest medicinal plants, originating in India and Northern Africa. As an annual plant, fenugreek grows to an average height of two feet. This plant is locally used in diabetic and non-diabetic people for reducing blood lipids and it also has antioxidant and anti-bacterial activity. This plant is used in therapy of atherosclerosis, rheumatism, sugar lowering, blood lipids lowering and appetizer [4]. The mechanisms by which fenugreek may lower blood glucose levels have not been well established in humans. The effects of fenugreek seeds are mainly due to the gum fraction, but do not exclude a longer term effect of other fenugreek components on glycemia. A novel amino acid derivative extracted from fenugreek seeds, 4-hydroxyisoleucine, stimulated glucose-dependent insulin release in isolated rat and human pancreatic

islet cells. In a trial of acute effects in healthy volunteers, trigonelline reduced the early glucose response during an OGTT [5]. In diabetes subjects, fenugreek seeds acts as an anti-diabetic agent by bringing the glycemic control [3,6]. The amino acid present in this plant appear to act only on pancreatic beta-cells, since the levels of somatostatin and glucagon were not altered [4].

4-Hydroxyisoleucine

4-Hydroxyisoleucine (4-OHIL) is the most commonly found free amino acids in fenugreek seeds. It occurs in two isomeric forms. The major isomer has a (2S, 3R, 4S) configuration which gives 90% of it in the seeds. While the minor isomer has a (2R, 3R, 4S) configuration and it possess both hypoglycemic & insulin tropic properties *in vitro* or *in vivo*. Due to this reason, it has becomea potential candidate for treating diabetes [7].

4-OHILis a branch amino acid extracted from the Fenugreek seeds which are having characteristic smell and bitter taste that of fenugreek seeds and leaves. The extracted natural active chemical is highly hygroscopic. The extract has several constituents. The active compound 4-OHIL is isolated by several purifications. The other constituent steroidal saponins (diosgenin, yamogenin, tigogenin, and neotigogenin) are also isolated separately from the extracts and mucilaginous fiber which are believed to be responsible for many of the beneficial effects fenugreek. The mucilage found in fenugreek does not dissolve but instead swells when mixed with fluids. Since the body cannot digest the mucilage from fenugreek it is believed to be an effective laxative. Medicinally, it was used for the treatment of wounds, abscesses, arthritis, bronchitis and digestive problems. The studies have confirmed the presence of 4-OHILin fenugreek seeds in two diastroisomers: the major one being the (2S, 3R and 4S) configuration, representing about 90% of the total content of 4-OHIL, and the minor one being the (2R, 3R and 4S) configuration. The major isomer is presently interesting with respect to experimental evidence indicating its ability to stimulate glucose-induced insulin secretion in micro molar concentrations. Some studies have also shown that the natural analogue of 4-OHIL is more effective as an anti-diabetic agent than a synthetic version [8,9]. The structure of 4-OHIL is depicted in **FIGURE 1**.

Biological uses of 4-OHIL

4-OHILin fenugreek extract plays a valuable role in insulin promotion and glucose regulation which may help to reduce body fat [10]. It has nutrient partitioning effect, which means it helps to shuttle nutrients to muscle cells preferentially over fat cells [11]. It appears to be glucose dependent. Higher is the blood glucose level, greater is the insulin promoting response elicited by 4-OHIL. By helping to regulate insulin needs, 4-OHIL works as an adaptogen. It is a non-protein amino acids isolated from fenugreek seeds. 4-OHIL will be more efficient to creatine in the muscle cells to enhance muscle strength and lean muscle mass and increase strength and size of muscle cells.It contains hormone precursors that can increase milk production in nursing mothers and it is widely used for insufficient lactation. 4-OHIL indirectly helps to lower the levels of triglycerides [12]. It has been used to treat bronchitis and asthma. It is also considered as a good herbal remedy for sore throat and coughs. It has been used to promote hair growth both in women and men [13]. It has been used for skin irritation, such as ulcers, boils, eczema, dandruff and cellulite.4-OHIL may help stimulate the secretion of insulin, reduce insulin resistance, and decrease blood sugar levels in diabetes patients [14].

Pharmacological uses of 4-OHIL

4-OHILshowed remarkable Anti diabetic activity, Antiplasmodic activity, Hypolipidemic activity, Immunological activity, Antibacterial activity, Anthelmintic activity, Anti-inflammatory, analgesic activity and Antioxidant activity [15-18].

Experimental

Materials and methods

The wavelength of the compounds was measured by UV-VIS spectrophotometer (UV--Pharma Spec 1700 Shimadzu) [19].

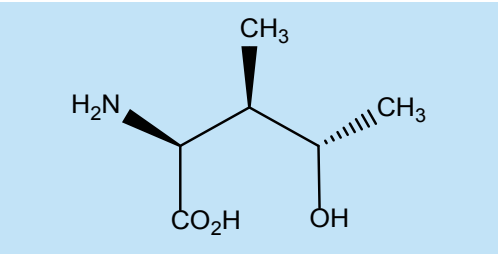


Figure 1. Structure of 4-hydroxyisoleucine

The HNMR Spectra were obtained on a Bruker DRX-400MHZ Spectrometer in $CDCl_3$ using TMS as internal standard and chemical shifts are expressed in δ scale [20].

FAB mass spectra were recorded on a JEOL SX 102/DA 6000 mass spectrometer using Argon/Xenon (6KV, 10Ma) as the FAB gas. EI mass spectra were recorded on JEOL JMS--D-300 spectrometer with the ionization potential of 70 eV and ES mass on Quantro--II, micromass instrument. Silica gel (60-120 mesh) for column chromatography was used. Room temperature mentioned were in the range between 20-400C unless stated otherwise [21-22].

Scheme for the extraction and isolation of amino acid (4-OHIL) from fenugreek seeds [23]:

Powdered seeds

+

50% methanol

+

Keep it for over night

+

Methanol layer is transferred to column having 225H cationic resin (ion exchange chromatography)

+

Wash with 2N Hcl, water, ammonium hydroxide

4-OHIL was identified by ninhydrin reagent using TLC spot test

Solid-phase dipeptide synthesis

Reagents and conditions of the above mentioned **scheme** are showed in **TABLE 1**.

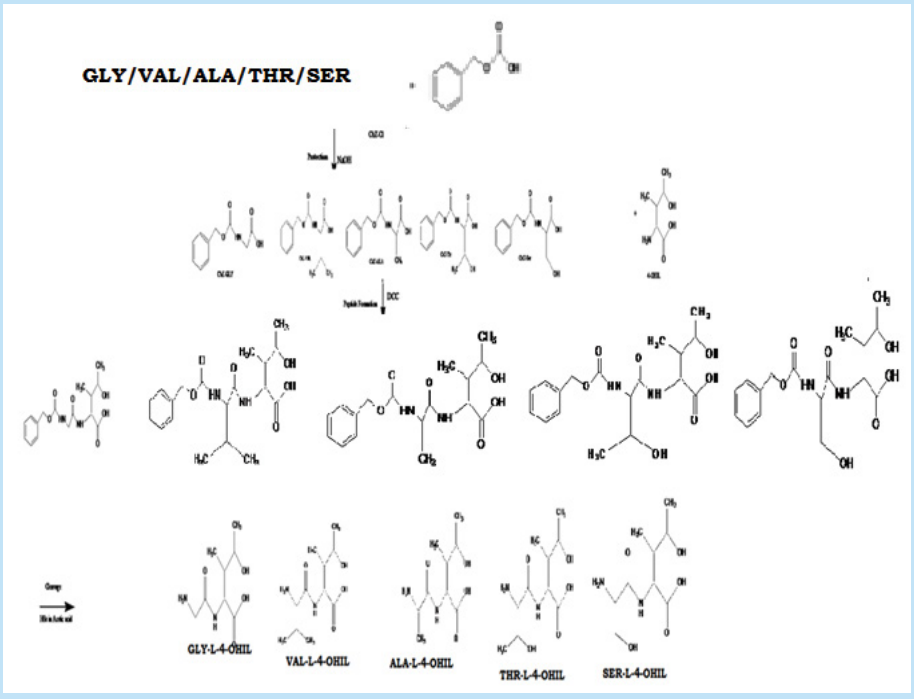
General procedure

Step I: protection

Gly/Val/Ser/Ala/Thr is dissolved in water and sodium bicarbonate (2eq) using a magnetic stirrer with heating and the mixture was cooled to 5°C and then Cbz-Cl (1.5eq) in para-dioxane was added slowly. The resulting mixture is stirred at 0°Cfor 1hour and allowed to warm at room temperature. Then water is added and aqueous layer is extracted 2 times with ethylacetate. The organic layer is back extracted twice with saturated sodium bicarbonate solution. The aqueous layers acidified to a pH of 1 with 10% HCl and extracted three times with ethylacetate and the organic layers were dried and concentrated in vaccum [24,25].

Step II

CarbobenzoxyGly/Val/Ser/Ala/Thrwas neutralized. A solution containing 2.34 gm of 4-OHILin 20 ml of di-methyl formamide was added and shaken for 10



Scheme: solid phase peptide synthesis of GLY-L-4-OH/VAL-L-4-OHIL/ALA-L-4-OHIL/THR-L-4-OHIL/SER-L-4-OHIL

Table 1. Reagentsand conditions of dipeptide synthesis

Synthetic cycle	Reagents	Conditions
Protection step	Glycine,Alanine,Threonine,Serine,Valine,Cbz-Cl, dioxane	0-5°C,1hr
Peptide forming step	4-OHIL,DCC,DMF	25°C, 18hrs
De-protection step	HBr in acetic acid, NaOH	25°C,1hr

minutes for penetration of the reactant into solid. A solution of 2.31 gm of Di-cyclohexylcarbodimide in 4.6 ml of di-methyl formamide was then added and stirred for 18 hours at 25°C, then filtered and washed. Then 3 ml of acetic anhydride and 1 ml of tri-ethylamine in 20 ml of di-methyl formamide was added. After 2 hours, the resin was filtered and washed 4 times each with DMF, ethanol and acetic acid to remove excess reagents and bi-products. A hydrolysate of dried sample of the product showed CarbobenzoxyGly/Val/Ser/Ala/Thr4-OHIL [26].

Step III: The deprotection step (Gly/Val/Ser/Ala/Thr 4-OH Isoleucine)

CarbobenzoxyGly/Val/Ser/Ala/Thr4-OHILwas decarbobenzoxylated with HBr in acetic acid. The mixture was added to a round bottomed flask equipped with a stirrer and cooled in an ice bath during cleavage process. The peptide resin was slowly added under inert atmosphere andtemperature, it is allowed to proceed for room temperature with stirring for 3 hours. The mixture is filtered and it was saponified by shaking for 1 hour at 25°C in a solution of 40.5 ml of ethanol and 4.5 ml of 2N aqueous NaOH. The filtrate was neutralized with HCl and saponification step was repeated on the resin with more ethanolic sodium hydroxide for another hour. The resin was washed with ethanol and water and neutralized filtrates were combined. Finally Gly/Val/Ser/Ala/Thr4-OHILwas formed [27].

In vitro anti-diabetic activity

Non-enzymatic glycosylation of haemoglobin assay

Anti-diabetic activity of 4-OHIL and its dipeptides like Gly-L-4-OHIL, Val-L-4-OHIL, ALA-L-4-OHIL, Thr-L-4-OHIL, Ser-L-4-OHILwere investigated by estimating degree of non-enzymatic haemoglobin-glycosylation, measured colorimetrically at 520 nm. Glucose (2%), haemoglobin (0.06%) and Sodium azide (0.02%) solutions were prepared in phosphate buffer 0.01 M to get pH 7.4. 1 ml each of the above solution was mixed and 1 ml of each concentrations of 4-OHIL and dieptides were added to above mixture. Mixture was incubated in dark place at room temperature for 72 h [28-31]. The degree of glycosylation of haemoglobin was measured colorimetrically at 520 nm. Alpha-Tocopherol (Trolax) was used as a standard drug for assay. % inhibition was calculated by using the formulae (**FIGURE 2**).

$AT\text{-}A_0 / A_0 \times 100$

AT = Abs of test

A_0 = Abs of control

All the tests were performed in triplicate (n=3)

In vivo anti-diabetic activity of 4-ohil and gly-l-4-ohilin alloxan induced diabetic rats

Experimental design

Diabetes was induced by a single injection of Alloxan 60 mg/kg b.w. to rats fasting for at least 16 h through the tail vein in freshly prepared 10 mmol/l sodium citrate, pH 4.5. Blood glucose levels were measured daily 3 days prior and 7 days after alloxan administration [32]. Development of diabetes mellitus was proven by sustained hyperglycaemia and glycosuria (diabetic rats had glycaemia >250 mg/dl) [33,34].

Group separation

The rats were randomly divided into 5 groups (n = 6) as follows

Group I: Normal control animals I that received saline solution, i.p for 21 days.

Groups II: Diabetic control animals treated with alloxan(Sod CMC, i.p) for 3days and were left untreated for 21 days.

Group III: (Standard) diabetic rats treated with Standard drug Glipizide (5 mg/kg. P.o. daily) for 21 days.

Group IV: Diabetic rats treated with 4-OHIL 200 mg/kg p.o. for 21 days.

Group V: Diabetic rats treated with 4-OHIL 400 mg/kg p.o. for 21 days

Group VI: Diabetic rats treated with 200 mg/

kg of GLY-L-4-OHIL p.o. for 21 days.

Group VII: Diabetic rats treated with 400 mg/kg of GLY-L-4-OHILp.o. for 21 days.

The rats developed diabetes within 2 days after alloxanisation as evidenced by sustained hyperglycaemia and glycosuria. Only those animals with blood glucose level above 250 mg/dl were included in the study. Every week (from 1st week to 3rd week) on 1st, 7th, 14th, and 21st day, blood samples were collected by retro-orbital puncture under light ether anesthesia, then the serum was separated by centrifugation at 2000rpm for 15 min in a cooling centrifuge and blood glucose levels were measured [35,36]. On 21st day, glucose level was finally measured (**FIGURE 3**).

The values are expressed as Mean ± S.E.M and n=6 in each group. *P<0.05 (significant as compared with normal control), P<0.0001 for diabetic control vs normal control, P<0.05 for normal control vs standard, P<0.05 for normal control vs 4-OHIL, P<0.05 for normal control vs GLY-L-4-OHIL. All comparisons were made by One Way Anova followed by Dunnet's t test.

Streptozocin induced diabetes in rats

Experimental study

Diabetes was induced experimentally in rats by a single intraperitoneal injection of a freshly prepared solution of streptozotocin (STZ), at a dose of 80 mg/kg body weight in 0.1 m cold citrate buffer of pH 4.5. After 1, 7, 21 days blood was collected with aseptic precautions from the tail vein of the rats under ether anesthesia and blood glucose levels were determined using Glucometer [37]. Animals were considered to be diabetic if the blood glucose levels were above 250 mg/dl, and those animals only were used for the experimental analysis [38]. Diabetes was developed and stabilized in STZ-treated rats over a period of 7 days; control rats were given citrate buffer 4.5 [39,40]. The animals were followed up to 21 days to check the time required for 4-OHIL and GLY-L-4-OHIL

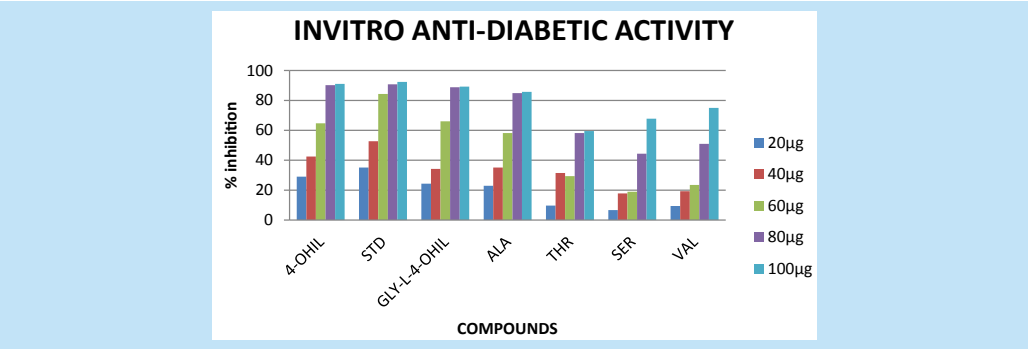


Figure 2. Non-enzymatic glycosylation of haemoglobin assay

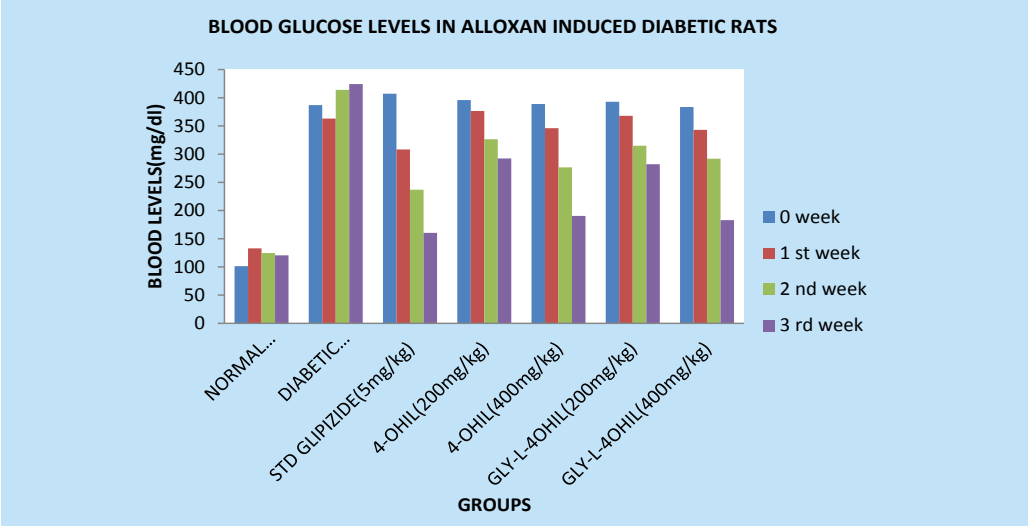


Figure 3. Blood glucose in alloxan induced diabetic rats

to produce peak hypoglycemic activity (**FIGURE 4**).

Group separation

The rats were randomly divided into 5 groups (n = 6) as follows

Group I : Normal control animals I that received citrate buffer (4.5), i.p for 21 days [41,42].

Groups II: Diabetic control animals treated with Streptozocin (Sod CMC, i.p) for 3 days and were left untreated for 21 days.

Group III: (Standard) diabetic rats treated with Standard drug Glibenclamide (25 mg/kg. P.o. daily) for 21 days.

Group IV: Diabetic rats treated with 4-OHIL 200 mg/kg p.o. for 21 days.

Group V: Diabetic rats treated with 4-OHIL 400 mg/kg p.o. for 21 days

Group VI: Diabetic rats treated with 200 mg/kg of GLY-L-4-OHIL p.o. for 21 days.

Group VII: Diabetic rats treated with 400 mg/kg of GLY-L-4-OHIL p.o. for 21 days.

The values are expressed as Mean ± S.E.M and n=6 in each group. *P<0.05 (significant as compared with normal control), P<0.05 for diabetic control vs normal control, P<0.05 for diabetic control vs standard, P<0.05 for diabetic control vs 4-OHIL, P<0.05 for Diabetic control vs GLY-L-4-OHIL. All comparisons were made by One Way Anova followed by Dunnet's t test.

Results

Physical and chromatographic data of Isoleucine derivatives are mentioned in **TABLE 2** and their spectral data is mentioned below [43-47].

Spectral data

4-OHIL

IR(KBr): 1641cm^{-1}(COOH), 3290cm^{-1}(OH), 3074 cm^{-1}(N-H)

HNMR(200MZ) (solvent: $CDCl_3$) δppm:11.084(d,1H,COOH), 1.41 (d,3H,CH-CH_3), 1.06(d,3H,CH-CH_3), 2.31(d,2H,NH_2), 2.07(d,1H,OH), 3.48(m,1H,CH-CH_3), 3.68(m,1H,CH-NH_2), 2.54(m,1H,CH-CH_3).

GLY-L-4-OHIL

IR(KBr): 3315.60cm^{-1}(N-H), 1742.39cm^{-1}(C=O), 1660cm^{-1}(C=O), 2960.77cm^{-1}(OH), 1365.19cm^{-1}(C-O), 1460.25cm^{-1}(C-H bending).

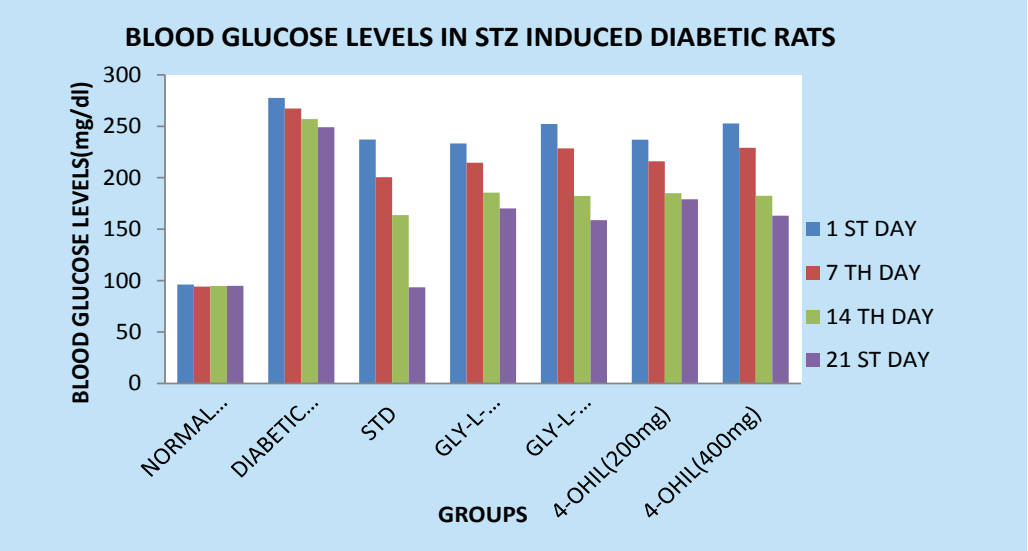


Figure 4. Blood glucose levels in Streptazocin induced diabetic rats

Table 2. Physical and thin layer chromatography data of dipeptides

S. No	Compound	Melting point	%yield	Rf Value
1.	4-OHIL	52°C	68%	0.8
2.	GLY-4-OHIL	54°C	72%	0.7
3.	Val–L-4-OHIL	52°C	69%	0.9
4.	Ala–L-4-OHIL	58°C	62%	0.8
5.	Thr–L-4-OHIL	61°C	59%	0.9
6.	Ser–L-4-OHIL	59°C	66%	0.9

HNMR:(200MZ)(solvent:$CDCl_3$) δppm:1.21(d,3H,CH-CH_3), 1.06(d,3H,CH-CH_3)2.01 (d, 1H, NH),2.01 (d, 1H, OH), 3.38(m,1H,CH-CH_3),3.54 (t,2H,$COCH_2NH_2$),8.0(d,1H, CONH), 2.60 (m,1H,CH-CH_3),11.00 (s,1H,COOH),4.45 (m, 1H,CHNH).

Mass (m/z): 202.

VAL-L-4-OHIL

IR(KBr): 3315.60cm^{-1}(N-H), 1742.39cm^{-1}(C=O), 1660cm^{-1} (C=O), 2978cm^{-1}(CH_3), 2960.77 cm^{-1}(OH),1365.19cm^{-1}(C-O), 1460.25cm^{-1}(C-H bending).

HNMR(200MZ)(solvent:$CDCl_3$)δppm:8.1(-d,1H,CONH),11.1(s,1H,COOH),0.96(-d,3H,1XCH_3), 1.21(d,3H,1XCH_3), 1.413(d,3H, CH_3),1.594(d,3H,1XCH_3), 2.412(d, 2H, NH_2), 2.17(d, 1H, OH), 2.746(m, 1H, CH), 3.74(m, 1H, 1XCH), 3.32(m, 1H, 1XCH), 3.66(d, 1H, 1XCH), 4.384 (t,1H,CH).

MASS (m/z): 246 [M+1].

ALA-L-4-OHIL

IR(KBr): 3315.60cm^{-1}(N-H), 1718.39cm^{-1}(C=O),1660 cm^{-1} (C=O), 2978 cm^{-1}(CH_3), 2960.77 cm^{-1}(OH), 1365.19cm^{-1}(C-O), 1460.25cm^{-1}(C-H bending).

HNMR(200MZ)(solvent:$CDCl_3$)δppm:8.1(-d,1H,CONH),11.1(s,1H,COOH),1.06(d,3H,1X-CH_3), 1.21(d,3H,1XCH_3), 1.413(d,3H, CH_3), 1.594(d,3H,1XCH_3), 2.17(d, 1H, OH), 2.68(m, 1H, CH), 3.74(m, 1H, 1XCH), 3.38(d, 1H, 1XCH), 4.45 (t,1H,CH),2.0(d, 2H, NH_2), 1.28(d,3H,1X-CH_3).

MASS (m/z): 218[M+1].

Thr-L-4-OHIL

IR(KBr): 3315.60cm^{-1}(N-H), 1715.39cm^{-1}(C=O),1660 cm^{-1} (C=O), 2978 cm^{-1}(CH_3)

2960.77 cm^{-1}(OH),1365.19cm^{-1}(C-O), 1460.25cm^{-1}(C-H bending).

HNMR(200MZ): (solvent:$CDCl_3$) δppm:8.2(d,1H,CONH),11.1(s,1H,COOH), 0.91(d,3H,1XCH_3), 1.21(d,3H,1XCH_3), 1.3(d,3H, CH_3), 2.1(m, 1H, CH), 2.4(d, 1H, OH), 2.5(d, 1H, OH), 2.7(d, 2H,NH_2), 3.6(t, 1H, 1XCH), 3.38(m, 1H, 1XCH), 3.8(m, 1H, 1XCH), 4.45 (t,1H,CH).

MASS (m/z): 248[M+1].

SER-L-4-OHIL

IR(KBr): 3315.60cm^{-1}(N-H), 1742.39cm^{-1}(C=O),1660 cm^{-1} (C=O), 2978 cm^{-1}(CH_3), 2960.77 cm^{-1}(OH), 1365.19cm^{-1}(C-O), 1460.25cm^{-1}(C-H bending).

HNMR(200MZ)(solvent:$CDCl_3$)δppm:8.0(-d,1H,CONH),11.0(s,1H,COOH),0.96(d,3H,1X-CH_3), 1.21(d,3H,1XCH_3), 2.412(d, 2H, NH_2), 2.0(d, 1H, OH), 2.0(d, 1H, OH), 2.68(m, 1H, CH), 3.65(m, 1H, 1XCH), 3.38(m, 1H, 1XCH), 4.384 (t,1H,CH), 4.03 (t,2H,CH_2).

Mass (m/z): 333[M+2].

Discussion

Six Dipeptides from 4-OHILwere synthesized by fallowing the scheme; they are 4-OHIL, GLY-4-OHIL, Val–L-4-OHIL, Ala–L-4-OHIL, Thr–L-4-OHIL, Ser–L-4-OHIL [48-50].

The purified compounds were undergone with physical and spectral analysis and their structural configuration is conformed with Infra-red spectroscopy, Nuclear Magnetic Resonance spectroscopy and Mass spectroscopy.

The evaluation of Isoleucine derivatives were done with *in vitro* and *in vivo* anti diabetic activity [51], they are

i. Non-enzymatic glycosylation of haemoglobin assay
ii. Alloxan induced diabetic rats
iii. Streptozocin - induced diabetic rats

Statistical analysis reveals that all the experimental results were expressed as MEAN ± S.D where n=6 the results were subjected to one way ANOVA followed by Dunnetts test for multiple comparisons. The statistical significance of different values with $P<0.05$ was considered significant.

Out of all the derivatives prepared GLY-L-4-OHIL showed prominent anti diabetic activity when compared with standard and control.

Conclusion

Six dipeptides were synthesized from 4-OH Isoleucine with glycine, Threonine, Serine and Valine using Cbz chloride as protecting agent, DCC and DMF are used in peptide forming step and Hbr in acetic acid is used in deprotection step. Finally formed dipeptides were confirmed by spectral data (IR, MASS and HNMR). The formed dipeptides were evaluated for Anti-diabetic activity in different animal models. *In vitro* studies revealed that compared to all other dipeptides GLY-L-4-OHIL showed improved activity, then it was evaluated for *in vivo* studies which showed

remarkable anti diabetic activity when compared with standard drug and the parent compound 4-OH Isoleucine.

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