2. AIM OF THE WORK

The aim of this study is to evaluate the hypoglycemic effect and the probable underlying mechanisms of action of Bitter gourd extract in diabetic rats by studying the changes in insulin signaling pathway in different tissues.

Bitter Gourd

3. MATERIALS AND METHODS

3.1. Animals and diet

In the present study we used seventy local wistar derived strain of rats, obtained from animal house of Medical Research Institute. The rats were housed in cages at 23°C on a 12-hour light/dark cycle. The animals were fed and given tap water *ad libitum*. The design of this study was in accordance with the ethical guidelines prescribed by the MRI, Alexandria University, Alexandria.

3.1.1. Establishment of type 2 diabetic rat:

- Pregnancy was induced in healthy virgin female rats (we used animals from different litters in order to avoid gene interference) obtained from our rat colony established in the animal house unit in Medical Research Institute.
- All animals were maintained under normal diet throughout pregnancy and lactation.
- The day of delivery was recorded and after 4 days (day 5 post-natal) the neonates were injected intraperitoneally with streptozotacin (STZ) (100mg/Kg) for establishment of neonatal –STZ induced type-2 diabetes. This model has many advantages over other models of diabetes and is considered to be one of the suitable experimental animal models of type 2 diabetes mellitus.[129] In parallel some neonates injected with citrate buffer only as a healthy control rats.
- All rats were followed up for 8-12 weeks to establish diabetes which was confirmed by fasting blood glucose level more than 200 mg/dl.[130]
- After follow up, the male rats were divided into two main groups:

1. Control healthy group (control group, n=10)
2. Diabetic rats: the diabetic rats were subdivided into three groups:-
    a. Untreated diabetic rats (No Tx group, n=10):the rats received no treatment.
    b. Glibenclamide treated group (Gliben Tx group, n=10): the rats treated orally with Glibenclamide (0.1 mg/kg) daily for 30 days.
    c. Bitter gourd extract treated groups: the rats in this group was divided into 4 groups (10 rats each) received different doses of bitter gourd ethanolic extract:
        i. Tx 100: rats received orally 100 mg/kg of bitter gourd extract for 30 days.
        ii. Tx 200: rats received orally 200 mg/kg of bitter gourd extract for 30 days.
        iii. Tx 400: rats received orally 400 mg/kg of bitter gourd extract for 30 days.
        iv. Tx 600: rats received orally 600 mg/kg of bitter gourd extract for 30 days.

After the end of treatment periods the overnight fasting rats were sacrificied by cervical dislocation. The blood sample was obtained to separate serum and the animals were dissected out to obtain liver and muscle tissues for assessment of phospho-insulin receptor, insulin receptor substrate-1, PKC and Glut-4.

3.2. Preparation of Bitter gourd ethanolic extract:

3.2.1. Sample preparation:

Egypt variety of young, green Bitter gourd fruits was obtained from Research farmer of vegetable department in Abbies, Alexandria which were kindly supplied by Prof. Dr. Esam M. Said Abd El-Kader, Vegetable Department, Faculty of Agriculture, University of Alexandria.

The fruits of bitter melon were cleaned and cut into small pieces, and then oven dried at 50°C for a day. The dried sample was then pulverized into fine powder in a grinder, which was then stored at 4°C until use.[115]

3.2.2. Extraction of Bitter gourd:

Crude liquid extraction, from fruits of bitter melon was proposed using 67% ethanol, 76°C and a time of contact of 72 h.[131]

Procedure:

- About 100 g of powder were soaked in liter of water/ethanol solvent (67% ethanol) at 76°C for 72 hours.
- The mixture was filtrated then the residues of filtrated resoaked in 67% ethanol solvent for 72 h at 76°C.
- These extracts were evaporated under vacuum to remove water ethanol solvent.
- The extract was collected in sample collecting vials and stored at 4°C.
- Different concentration was prepared from the crude extract (100mg/kg, 200mg/kg, 400mg/kg and 600mg/kg).

3.3. Methods:

3.3.1. Determination of insulin in rat serum:

Rat insulin ELISA kit (Mercodia, sweden) is used for the non radioactive quantitative of insulin in rat sera .[132]

Principle:

This assay is a sandwich ELISA based on capture of insulin molecules from samples to the wells of a microtitre plate coated by a monoclonal mouse anti-rat insulin antibodies and the binding of biotinylated polyclonal antibodies to captured insulin, binding of horseradish peroxidase (HRP) to the immobilized biotinylated antibodies, and quantification of the immobilized antibody-enzyme conjugates by monitoring horseradish peroxidase activities in the presence of 3, 3', 5, 5'-tetramethylbenzidine. The reaction is stopped by adding acid to give colorimetric endpoint. The enzyme activity is measured spectrophotometrically by the increased absorbance at 450 nm.

Reagents:

- Assay buffer: (0.05 M phosphate buffered saline, pH 7.4, containing 0.025 M. EDTA, 0.08 % sodium azide and 1% BSA).
- Wash buffer: (50 mM Tris buffered saline containing Tween- 20).
- Insulin standards in assay buffer: (0.15, 0.40, 1, 3 and 5.5ug/L).
- Matrix solution: (charcoal Stripped pooled mouse serum).
- Insulin detection antibody: (biotinylated anti-insulin antibodies).
- Enzyme conjugate solution: (Streptavidin- horseradish peroxidase conjugates in assay buffer).
- TMB Substrate :(3, 3', 5, 5' - tetramethylbenzidine).
- Stop solution: (0.5 M H_2SO_4).

Procedure:

- 10 µl of rat insulin standards were added in duplicate in the order of ascending concentrations. 10 µl of negative and positive control were added to the appropriate wells.
- 10 µl of unknown serum were added to the remaining wells.
- 100 µl of enzyme conjugate were added to all wells and incubated at room temperature on plate shaker for 2 hours.
- Solutions were decanted from the plate and the plate was washed 6 times with 200 µl diluted wash buffer.
- 200 µl of substrate solution (TMB) were added to each well. The plate was covered and shake in the plate shaker for approximately 15 minutes.
- 50 µl of stop solution were added and shake for 5 seconds to ensure mixing then the absorbance was read at 450 nm in a plate reader within 30 minutes.

Calculation:

The reference curve (Figure:12) was constructed by plotting the difference absorbance unit at 450 nm against the concentration of rat insulin standards.

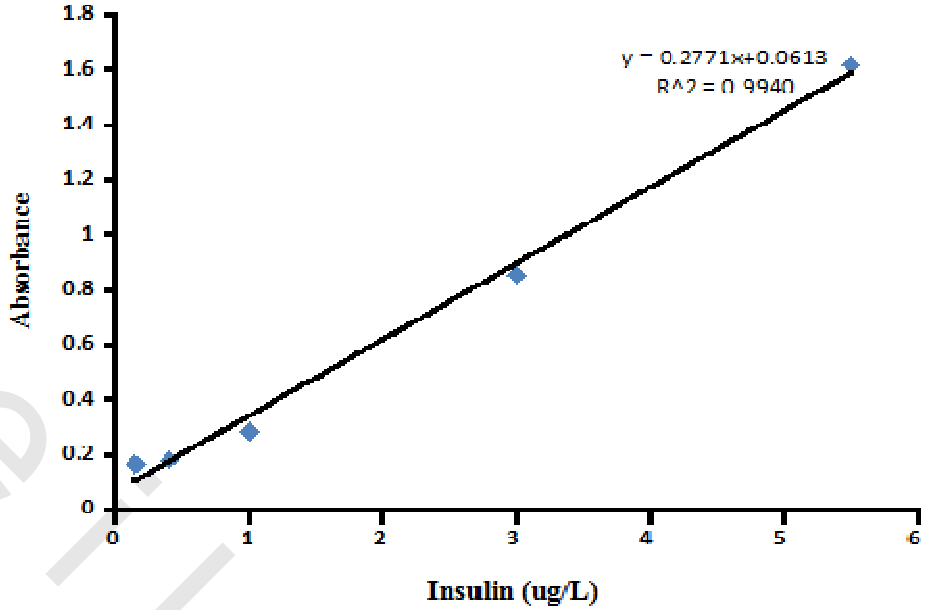

Figure (12): *Standard curve of insulin.*

3.3.2. Determination of fasting blood glucose:

Plasma glucose levels were determined according to an enzymatic calorimetric method which has been described by Trinder.[133]

Principle:

Glucose is oxidized in the presence of glucose oxidase. The hydrogen peroxide formed reacts under catalysis of peroxidase with phenol and 4–aminophenazone to a red-violet quinoneimine dye. The intensity of the color is proportional to glucose concentration.

$$Glucose + O_2 + H_2O \xrightarrow{Glucose\ oxidase} Gluconate + H_2O_2$$

$$H_2O_2 + phenol + 4\text{-}aminophenazone \xrightarrow{peroxidase} Quinoneimine + 4H_2O$$

Reagents:

Working reagent

- Phosphate buffer (pH 7.5) 0.1 mol/L
- 4 – Aminophenazone 0.25 mmol/L
- Phenol 0.75 mmol/L
- Glucose oxidase >15 KU/L
- Peroxidase >1.5 KU/L
- Standard**:** Glucose 100 mg/dl

Procedure:

- 1 ml of enzyme reagent was mixed in test tubes with 10 µl of plasma sample or glucose standard.
- The mixture was incubated at 37 °C for 5 minutes.
- Reagent blank was run through the same procedure.
- The absorbance of standard (ΔA standard) and the sample (ΔA sample) were measured against reagent blank at 540 nm.

Calculation:

$$\text{Glucose concentration (mg/d)} = 100 \times \frac{\Delta A \text{ sample}}{\Delta A \text{ standard}}$$

Where, concentration of standard was 100 mg/dL.

3.3.3. Calclation of HOMA-Insulin resistance index.

The insulin resistance index (IRI) was derived using the homeostasis model assessment (HOMA) as follows :[134]

IRI= fasting insulin ng (µU/ ml) × fasting glucose (mmol/L) /22.5

3.3.4. Lipid profile Parameters:

3.3.4.1. Determination of triglycerides:

The triglycerides level was determined by the enzymatic colorimetric method. [135]

Principle:

Glycerol and fatty acids are first formed by the action of lipase on the triglycerides. Glycerol is then phosphorelated by adenosine triphosphate (ATP) to produce glycerol -3-phosphate and ADP in a reaction catalyzed by glycerol kinase.
Glycerol- 3 - phosphate is oxidized by glycerol phosphate oxidase producing dihydroxy acetate phosphate (DAP) and hydrogen peroxide. The latter reacts with 4 aminoantipyrine and 4- chlorophenol under the catalytic influence of peroxidase to form quinoneimine.

$$\text{Triglycerides} \xrightarrow{\text{Lipase}} \text{Glycerol + Fatty acids.}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerol Kinase}} \text{Glycerol-3 phosphate + ADP.}$$

$$\text{Glycerol-3 phosphate + } O_2 \xrightarrow[\text{oxidase}]{\text{Glycerol phosphate}} \text{DAP + } H_2O_2.$$

$$H_2O_2\text{+ 4-aminoantipyrine+ 4-chlorophenol} \xrightarrow{\text{Peroxidase}} \text{Quinnoneimine+HCl + } H_2O.$$

Reagents:

Enzyme/buffer reagent:

❖ Piperazine-N, N′-bis (2-ethanesulfonic acid) buffer(pH 7.5)	50 mmol/L
❖ 4 - chloro phenol	5 mmol/L
❖ 4 – Aminoantipyrine	0.25 mmol /L
❖ Magnesium ions	4.5 mmol/L
❖ ATP	2 mmol/L
❖ Lipase	≥ 1.3 U/mL
❖ Peroxidase	≥ 0.5 U/mL
❖ Glycerol kinase	≥ 0.4 U/mL
❖ Glycerol -3 - phosphate oxidase	≥ 1.5 U/mL
❖ Standard: Triglycerides	200 mg/dl

Procedure:

- ❖ 1 ml of enzyme reagent was mixed with 10 µl of serum sample or triglycerides standard in test tubes.
- ❖ Reagent blank was run through the same procedure.
- ❖ All tubes were incubated at 37 C for 5 minutes.
- ❖ The absorbance of standard (ΔA standard) and the sample (ΔA sample) were measured against reagent blank at 546 nm.

Calculation:

$$\text{Triglycerides concentration (mg/dl)} = 200 \times \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{Standard}}}$$

Where, concentration of standard was 200 mg/dL.

3.3.4.2. Determination of total cholesterol :

Serum total cholesterol level was determined on the basis of an enzymatic calorimetric method.[136]

Principle

Cholesterol esterase (CHE) hydrolyzes esters and H_2O_2 is formed in the subsequent enzymatic oxidation of cholesterol by cholesterol oxidase (CHO) according to the following reaction:

$$\text{Cholesterol ester} + H_2O_2 \xrightarrow{\text{CHE}} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{CHO}} \text{Cholesterol-3-one} + H_2O_2$$

$$2\,H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinonimine} + HCl + H_2O$$

Reagents:

Enzyme / buffer reagent

- Phosphate buffer (pH 6.5) 100 mmol/L
- 4 – Amino phenazone 0.3 mmol/L
- Phenol 5 mmol/L
- Peroxidase >5 KU/L
- Cholesterol esterase > 150 U/L
- Cholesterol oxidase > 100 U/L
- Standard: Cholesterol 200 mg/dl

Procedure:

1 ml of working reagent was mixed with 10 µl of serum sample or cholesterol standard in test tubes. Reagent blank was run though the same procedure. All the tubes were incubated at 37 °C for 5 minutes. The absorbance of standard (ΔA standard) and the sample (ΔA sample) were measured against reagent blank at 546 nm.

Calculation:

$$\text{Cholesterol concentration (mg/dl)} = 200 \times \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}}$$

Where, concentration of standard was 200 mg/dl.

3.3.4.3. Determination of HDL- Cholesterol (HDL-C):

High density lipoprotein (HDL–cholesterol) level was determined by precipitation method. [137]

Principle:

When serum is treated with phosphotungustic acid in the presence of magnesium ion, the low density lipoprotein (LDL), very low density lipoprotein (VLDL) and chylomicron are precipitated from serum. The HDL cholesterol remains dissolved in the supernatant. The supernatant then acts as a sample and assayed for cholesterol by an enzymatic method.

Reagents:

Precipitant

- Phosphotungstic acid 0.4 mmol/L
- Magnesium chloride 20.0 mmol/L
- Standard: HDL-cholesterol standard 15 mg/dL

Procedure:

Precipitation:

- 500 µl of precipitant was added to 200 µl of serum sample, mixed well and incubated for 10 minutes at room temperature.
- The mixture was then centrifuged for 10 minutes at 4000 rpm.
- After centrifugation the clear supernatant was separated from the precipitate.
- 1 ml of enzyme reagent of Cholesterol determination was mixed in test tubes with 100µl of sample supernatant, cholesterol standard, or distilled water (as blank).
- The mixture was incubated at 37^0C for 10 minutes.
- The absorbance of standard (ΔA standard) and the sample (ΔA sample) were measured against reagent blank at 546 nm.

Calculation:

HDL -Cholesterol concentration (mg/dl) = 52.5 x $\frac{\Delta A \text{ sample}}{}$

Where 52.5= Standard cholesterol concentration (15) x $\Delta A_{\text{standard}}$ tion factor (3.5)

3.3.4.4. Calculation of LDL- Cholesterol:

The low density lipoprotein cholesterol concentration (LDL–C) was calculated from the total cholesterol concentration (TC), the HDL cholesterol concentration (HDL–C) and the triglycerides concentration (TG) .[137]

$$\text{LDL-C} = \text{TC} - (\text{HDL-C}) - \text{TG}/5 \text{ mg/dl.}$$

3.3.5. Determination of total protein:

Principle:

A modification of the method of Lowry *et al.* was used for the determination of protein in the samples. The color produced is thought to be due to a complex between the alkaline copper- phenol reagent and tyrosine and tryptophan residues of the protein in the sample. The protein concentration in each sample was estimated by referring to a standard curve (Figure:16) which was constructed using bovine serum albumin.[138]

Reagents:

- Sodium hydroxide 0. 1M.
- Sodium carbonate (anhydrous) 2% in 0.1M NaOH.
- K/Na tartarate 2%.
- Copper sulphate 1%.
- Lowry C reagent: prepared immediately before use by mixing volumes of sodium carbonate, K/Na tartarate and copper sulphate reagent in a ratio: 100: 1: 1.

- Folin- Ciocalteau reagent. The working reagent was prepared by diluting the stock reagent 1: 1 (V/V) with distilled water immediately before use.
- Standard bovine serum albumin

Procedure:

The sample was diluted in distilled water (1: 10). Aliquots of 10µl of diluted samples were mixed with 2.5ml of Lowry C reagent. After incubation for 10 minutes at room temperature, 0.25ml of working Folin- Ciocalteau's reagent was added. The tubes were then mixed and incubated in a dark place for one hour at room temperature, after which the absorbance was read at 695 nm using spectronic 21 spectrophotometer.

A blank containing phosphate buffer saline instead of the sample was treated similarly.

The total protein amount was computed with reference to the protein standard curve (Figure:13) (2,4,6,8,10,20,30,40,50,60,70,80,90,100 µg protein).

Calculation:

$$\text{The total protein concentration (mg/ml)} = \frac{\text{The total protein amount (mg)}}{\text{The sample volume (ml)}}$$

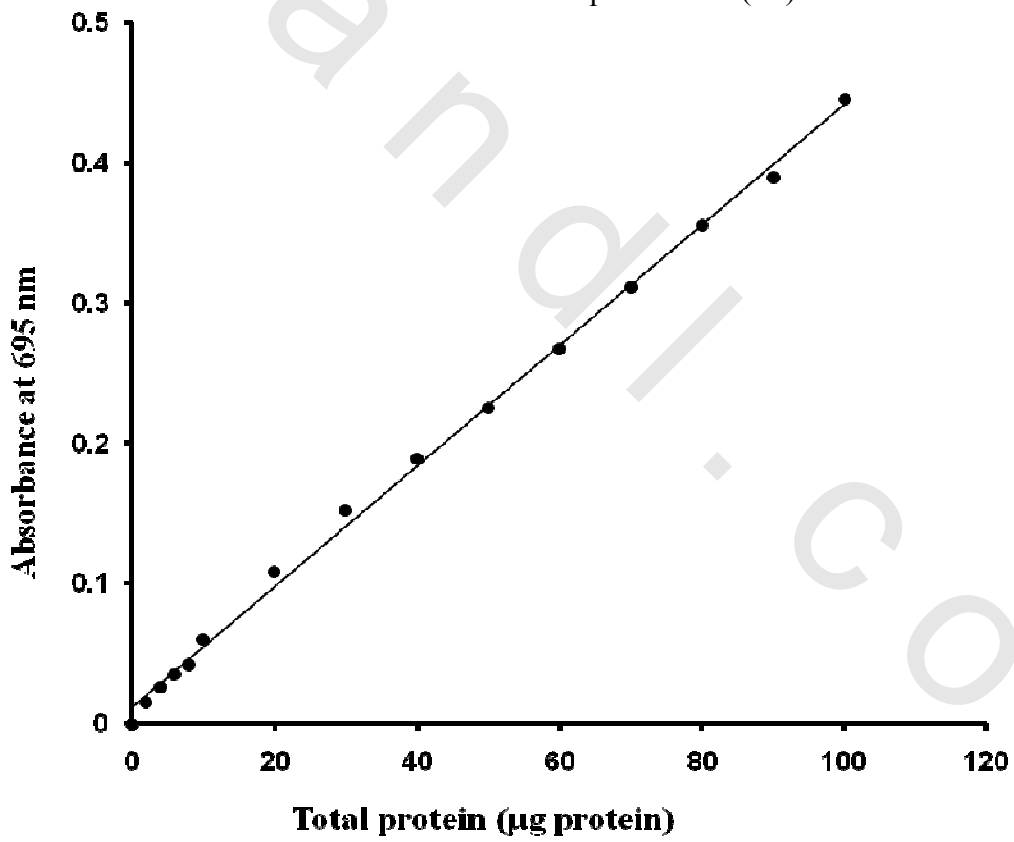

Figure (13): *Standard curve of total protein*

3.3.6. Determination of Tissue level of Phospho-Insulin Receptor-β (Tyr1150/1151).

Rat phospho-insulin receptor-β (Tyr1150/1151) ELISA kit (CSTs pathScan) is used for the non radioactive quantitative of Phospho-Insulin Receptor-β (Tyr1150/1151) in rat tissues.[139]

Principle:

This assay is a solid phase sandwich ELISA based on detected transfected Phospho-Insulin Receptor-β (Tyr1150/1151) protein. An insulin receptor β mouse mAb has been coated onto the microwells. After incubation with sample, both phospho- and non phospho-insulin receptor proteins are captured by the coated antibody. Following extensive washing, phospho-IGF-1 Receptor -β (Tyr1135/1136)/ Insulin Receptor-β (Tyr1150/1151) rabbit mAb is added to detect the captured Phospho-Insulin Receptor-β (Tyr1150/1151). Anti-rabbit IgG, HRP-Linked Antibody then used to recognize the bound detection antibody . HRP Substrate, TMB, is added to develop color. The optical density for this developed color is proportional to the quantity of Phospho-Insulin Receptor-β (Tyr1150/1151).

Reagents:

- An insulin receptor β mouse mAb coated microwells.
- Phospho-Insulin Receptor-β (Tyr1150/1151) detection Ab.
- Anti-rabbit IgG, HRP-Linked Ab.
- TMB Substrate: (3,3^{-},5,5^{-} -tetramethylbenzidine)
- Stop solution.
- Wash buffer.
- Sample diluent.

Procedure:

- 100 µl of sample diluent were added to microcentrifuge tube.
- 100 µl of sample were added to tube and vortex for a few seconds.
- 100 µl of diluted sample were added to the appropriate well then microwells were sealed with tape and pressed firmly.
- Microplate was incubated for 2 hours at 37 ^{o}C.
- Microplate was washed four times with 200 µl of wash buffer and 100 µl of detection antibody was added to each well then micro-wells were sealed with tape and the plate was incubated for one hour at 37 ^{o}C .
- Microplate was washed four times with 200 µl of wash buffer and 100 µl of HRP-linked secondary antibody was added to each well, the plate was sealed and incubated for 30 minutes at 37 ^{o}C.
- Microplate was washed four times with 200 µl wash buffer then 100 µl of TMB substrate was added and incubated for 10 minutes at 37 ^{o}C .

- 100 µl of stop solution was added to each well.
- The absorbance was read on a microplate reader at a wavelength of 450 nm.

Calculation:

Concentration of P-IR protein was calculated as a difference absorbance unit/mg protein content of the sample (AU/mg protein) at 450 nm.

3.3.7. Determination of tissue level of insulin receptor substrate-1 (IRS-1).

Rat insulin receptor substrate-1 (IRS-1) ELISA kit (WKEA MED SUPPLIES) is used for the non radioactive quantitative of insulin receptor substrate-1 in rat tissues.[140]

Principle:

Rat insulin receptor substrate-1 (IRS-1) present in the sample binds to antibodies adsorbed to the microwells. A conjugated anti-rat IRS-1antibody binds to rat IRS-1 captured by the first antibody. Following incubation unbound conjugated anti-rat IRS-1 antibody is removed during a wash step. HRP substrate is added to develop color, reaction is terminated by the addition of a sulphoric acid solution and the color change is measured. The optical density for this developed color is proportional to the quantity of IRS-1.

Reagents:

- Standard: 180 pg/ml.
- Standard diluent.
- Enzyme conjugate
- Sample diluent.
- Substrate A
- Substrate B
- Stop solution.
- Wash buffer solution.

Procedure:

- 50 µl of rat IRS-1 standards were added after diluting with standard dilution in duplicate in the order of descending concentrations (120,80,40,20,10 pg/ml).
- Blank wells was set separately and all steps were the same but the sample and enzyme conjugate didn't add .
- 40 µl of sample dilution were added to the remaining sample wells, then 10 µl of sample was added and mix gently.
- Plate was covered and incubated for 30 minutes at 37 ^{o}C.
- Solutions were decanted from the plate and the plate was washed 5 times with 200 µl diluted wash buffer.
- 50 µl of enzyme conjugate were added to all wells, except blank well. Plate was covered and incubated for 30 minutes at 37 ^{o}C.

- Solutions were decanted from the plate and the plate was washed 5 times with 200 µl diluted wash buffer.
- 50 µl of substrate A and substrate B were added to all wells, covered and incubated for 15 minutes at 37 ^{o}C .
- 50 µl of stop solution were added to each well and mixed well then the absorbance was read at 450 nm in a plate reader within 15 minutes.

Calculation

The reference curve (Figure:14) was constructed by plotting the difference absorbance unit at 450 nm against the concentration of rat IRS-1standards.

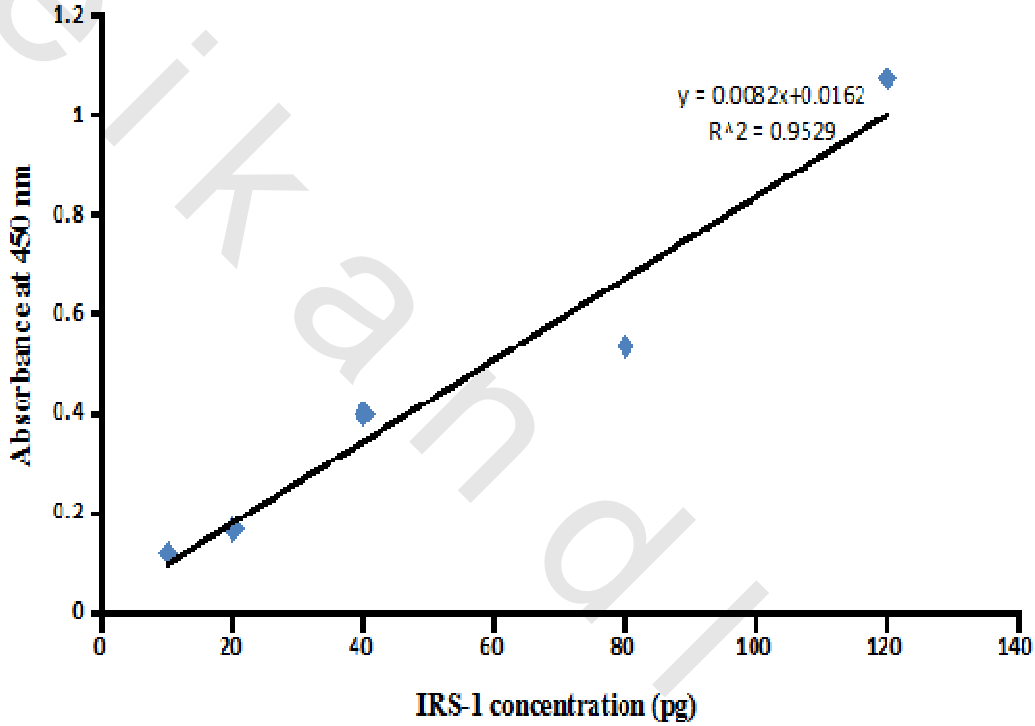

Figure (14): *Standard curve of IRS-1.*

3.3.8. Determination of tissue level of Protein Kinase C (PKC):

Rat protein kinase C (PKC) ELISA kit (WKEA MED SUPPLIES) is used for the non radioactive quantitative of PKC in rat tissues.[141]

Principle:

Rat protein kinase C (PKC) present in the sample binds to antibodies adsorbed to the microwells. A conjugated anti-rat PKC antibody binds to rat PKC captured by the first antibody. Following incubation unbound conjugated anti-rat PKC antibody is removed during a wash step. HRP substrate is added to develop color, reaction is terminated by the addition of a sulphoric acid solution and the color change is measured. The optical density for this developed color is proportional to the quantity of PKC.

Reagents:

- Standard: 900 pg/ml.
- Standard diluent.
- Enzyme conjugate
- Sample diluent.
- Substrate A
- Substrate B
- Stop solution.
- Wash buffer solution.

Procedure:

- 50 µl of rat PKC standards were added after diluting with standard dilution in duplicate in the order of descending concentrations (600, 400, 200, 100, 50 pg/ml).
- Blank wells was set separately and all steps were the same but the sample and enzyme conjugate didn't add .
- 40 µl of sample diluent were added to the remaining sample wells, then 10 µl of sample was added and mix gently.
- Plate was covered and incubated for 30 minutes at 37 ^{o}C.
- Solutions were decanted from the plate and the plate was washed 5 times with 200 µl diluted wash buffer.
- 50 µl of enzyme conjugate were added to all wells, except blank well. Plate was covered and incubated for 30 minutes at 37 ^{o}C.
- Solutions were decanted from the plate and the plate was washed 5 times with 200 µl diluted wash buffer.
- 50 µl of substrate A and substrate B were added to all wells, covered and incubated for 15 minutes at 37 ^{o}C.
- 50 µl of stop solution were added to each well and mixed well then the absorbance was read at 450 nm in a plate reader within 15 minutes.

Calculation

The reference curve (Figure:15) was constructed by plotting the difference absorbance unit at 450 nm against the concentration of rat PKC standards.

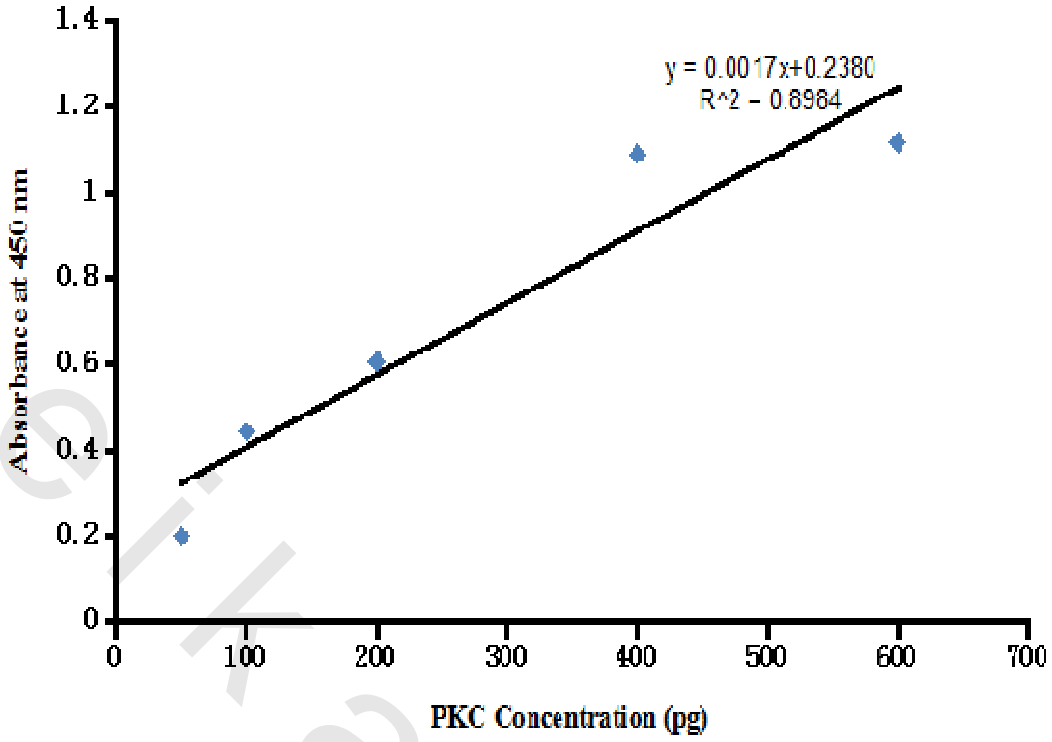

Figure (15): *Standard curve of PKC.*

3.3.9. Determination of tissue level Glucose Transporter-4 (Glut-4):

Rat glucose transporter-4 (Glut-4) ELISA kit (Uscn Life Science) is used for in vitro quantitative measurement of Glut-4 in rat tissues homogenates and other biological fluids.[140]

Principle:

The microtiter plate was pre-coated with an antibody specific to Glut-4. Standard or sample are then added to the appropriate microtiter plate wells with a biotin-conjugated antibody preparation specific for Glut-4. Avidin conjugated to HRP was added to each microtiter plate wells and incubated. After TMB substrate solution was added, only those wells that contain Glut-4, biotin-conjugated antibody and enzyme-conjugated avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of a sulphoric acid solution and the color change is measured at 450 nm. The optical density for this developed color is proportional to the quantity of Glut-4.

Reagents:

- Pre-coated, ready to use 96-well strip plate.
- Standard (lyophilized): 40 ng/ml.
- Standard diluent.
- Detection reagent A.
- Assay diluent A.

- Detection reagent B.
- Assay diluent B.
- TMB Substrate .
- Stop solution.
- Wash buffer solution.

Procedure:

- 100 µl of rat Glut-4 standards were added after diluting with standard dilution in duplicate in the order of descending concentrations (40,20,10,5,2.5,1.25,0.625 ng/ml).
- 100 µl of standard diluent was added to blank wells which was set separately .
- 100 µl of sample were added to the remaining sample wells, then plate was covered and incubated for 2 hours at 37 ^{o}C.
- 100 µl of prepared detection reagent A were added then plate was covered and incubated for one hour at 37 ^{o}C.
- Solutions were decanted from the plate and the plate was washed three times with 200 µl diluted wash buffer.
- 100 µl of prepared detection reagent B were added then plate was covered and incubated for 30 minutes at 37 ^{o}C.
- Microplate was washed five times with 200 µl of wash buffer and 90 µl of TMB substrate solution was added to each well and incubated for 25 minutes at 37 ^{o}C .
- 50 µl of stop solution were added to each well and mixed well then the absorbance was read at 450 nm in a plate reader immediately.

Calculation:

The standard curve (Figure:16) was constructed by plotting the difference absorbance unit at 450 nm against the concentration of rat Glut-4 standards.

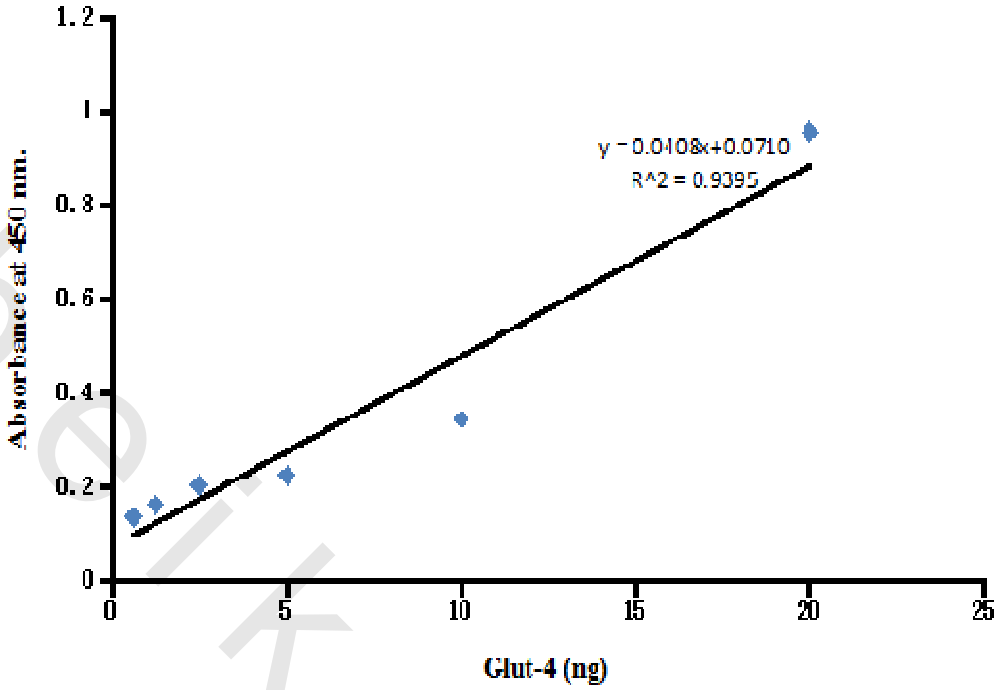

Figure (16): *Standard curve of Glut-4.*

4. RESULTS

This chapter contains some parameters of the data on the changes in glucose homeostasis and insulin signaling pathway parameters in different tissues to evaluate the hypoglycemic effect and the probable underlying mechanisms of action of Bitter gourd extract in diabetic rats.

Statistical analysis were made to compare each parameter of the studied groups with the corresponding one in the healthy control group. The parameters in the four diabetic treated groups with bitter gourd extract (B.G) (Tx 100,Tx 200,Tx 400 and Tx 600) were compared to the untreated diabetic group (No Tx) and glibinclamide group.

4.1. Body weight changes:

The results of body weight are summarized in Tables (4) and Figure (17-18). The body weight of diabetic groups (untreated or treated with bitter gourd at doses of 100,200 and 400 mg/kg) were higher than control group of rats. The diabetic rats treated with 600 mg/kg bitter gourd showed no significant difference in the body weight compared to control rat. On the other hand, the glibinclamide treated group of rats showed non significant lower body weight compared to control rats. All bitter gourd treated groups of rats (with exception of Tx 600 group) showed higher body weight compared to the glibinclamide treated group (Table:4, Figures:17-18)

Table (4): Weight (gm) in healthy control and diabetic groups with and without treatment.

Healthy control	Diabetic Groups					
	No Tx	Tx Glibin	Tx 100	Tx 200	Tx 400	Tx 600
207.7±22.7	243.5±36.0 a	201.0±13.0 b	237.9±21.8 a, c	237.3±34.2 a, c	233.0±26.2 a, c	210.7±7.7 b

Data were presented as mean± SD.
a: Significant different from control by ANOVA-test (P<0.05)
b: Significant different from untreated diabetic group by ANOVA -test (P<0.05).
c: Significant different from Glibinamide treated group by ANOVA -test (P<0.05).

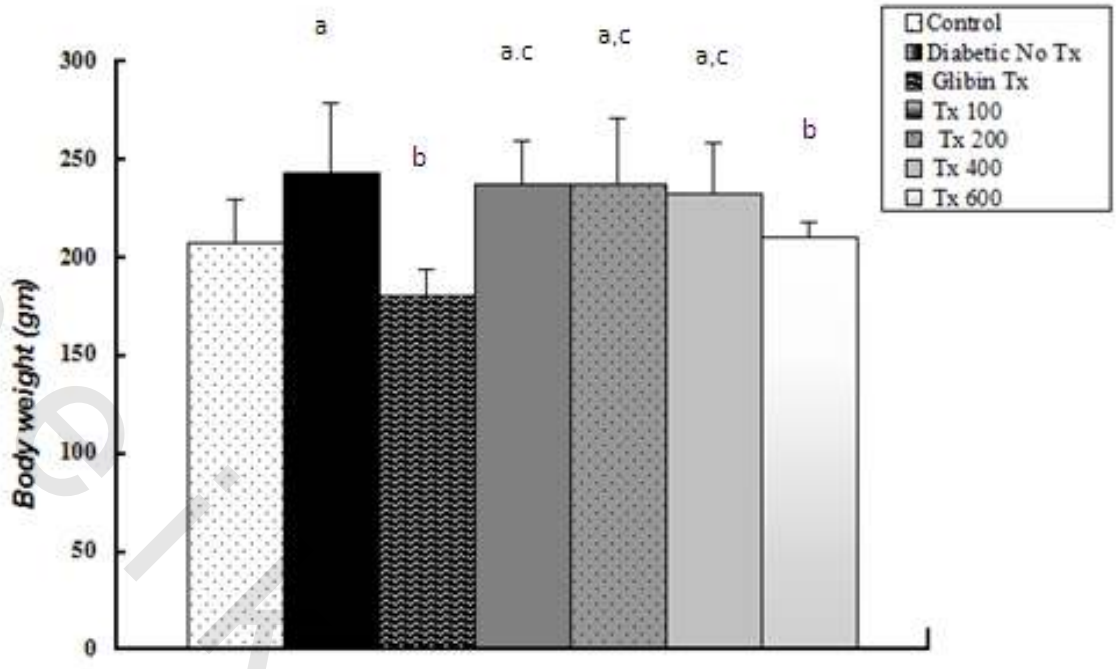

Figure (17): Weight (gm) in healthy control and diabetic groups with and without treatment. *(Data were presented as mean± SD, a: Significant different from control, b:Significant different from untreated diabetic group, c: Significant different from glibinamide treated group by ANOVA -test (P<0.05).*

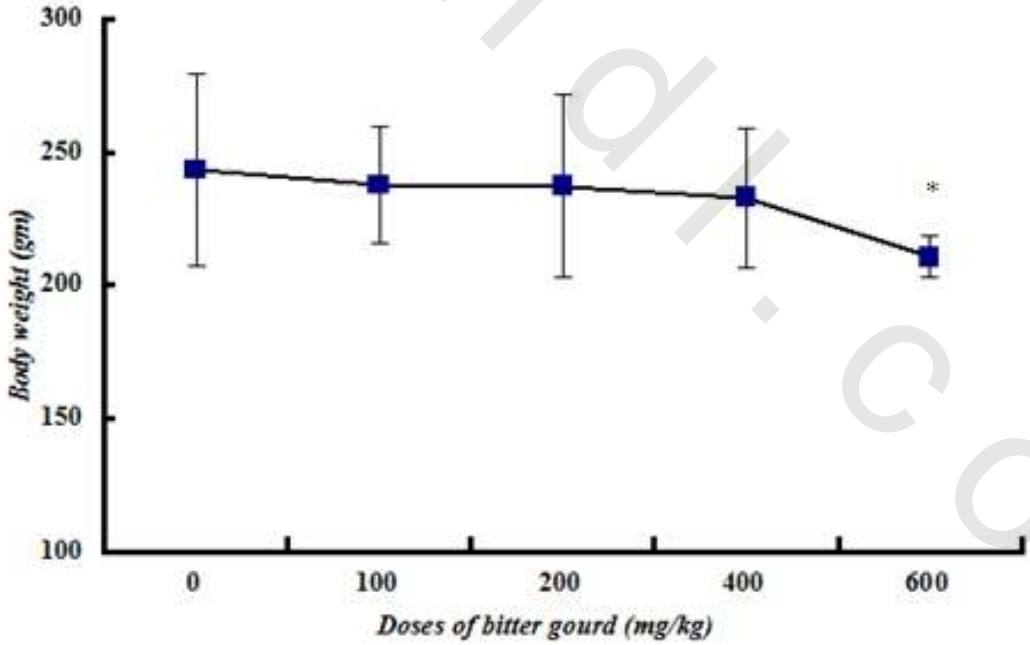

Figure (18): Dose-dependent effect of bitter gourd extract on body weight of diabetic rats. *(* Significant difference from untreated diabetic rats (0 concentration) by Anova-test (P<0.05).*

4.2. Glucose homeostasis parameters:

The metabolic inter-relationships between key tissues and organs (Liver and muscles) preserve glucose homeostasis and maintain blood glucose concentration within a narrow range.

The results of glucose homeostasis parameters; fasting plasma glucose, insulin and HOMA-IR are summarized in Tables (5) and Figures(19-22).

4.2.1. Fasting blood glucose level:

All diabetic groups of rats (with the exception of rats treated with 400 mg/kg bitter gourd) showed a significantly higher fasting blood glucose than control rats. Also, All the treated groups (with bitter gourd and glibinclamide) showed a significant lower fasting blood glucose than untreated diabetic rats.

There was a dose-dependent glucose lowering effect of bitter gourd at low doses (from 100 to 400 mg/kg) with the lowest blood glucose level observed in rats treated with 400 mg/kg after which the effect was lost causing elevation of glucose level (at dose 600 mg/kg) (Figures:19-20).

4.2.2. Fasting insulin level:

The untreated diabetic rats showed a significantly lower insulin level than control rats. Treatment with glibinclamide showed significant elevation of the insulin level (Table: 5, Figures: 21-22). Treatment of diabetic rats with bitter gourd showed a dose-dependent increase in fasting insulin level. Treatment with bitter gourd at a dose of 400 mg/kg produce insulin level that showed no significant difference between its and glibinclamide group while a dose of 600 mg/kg produce insulin level significantly higher than control rats. (Table:5, Figures:21-22).

4.2.3. Insulin resistance index assessed by HOMA (HOMA-IR):

The insulin resistance index (IRI) was calculated using HOMA model (HOMA-IR) using the level of fasting insulin and glucose. The untreated diabetic rats showed a higher level of insulin resistance level than control rats level. The treated diabetic groups of rats showed no significant changes in the insulin resistance status compared to control with the exception of the diabetic group treated with bitter gourd extract at a dose of 600 mg/kg which showed a high state of insulin resistance level (Table:5, Figures:21-22).

Table (5): Glucose homeostasis parameters:
The levels of FBS (mg/dl), Insulin (ug/ml) and HOMA-IR in healthy control and diabetic groups with and without treatments.

	Healthy control	Diabetic Groups					
		No Tx	Tx Glibin	Tx 100.0	Tx 200.0	Tx 400.0	Tx 600.0
FBS (mg/d l.)	94.8±5.7	226.0±20.7 a	118.4±11.1 a,b	140.1±3.4 a,b,c	138.9±40.5 a,b,c	108.1±24.1 b	173.7±8.9 a,b,c
Insulin (ug/ml)	0.40±0.13	0.25 ± 0.02 a	0.46±0.10 b	0.32±0.09 c	0.38±0.11 c	0.44±0.17 b	0.56±0.16 a,b
HOMA	2.27±0.72	3.40±0.54 a	3.23±0.77	2.68±0.72	3.07±1.05	2.94±2.01	5.84±1.85 a,b,c

Data were presented as mean± SD.
a: Significant different from control by ANOVA-test (P<0.05).
b: Significant different from untreated diabetic group by ANOVA -test (P<0.05).
c: Significant different from Glibinamide treated group by ANOVA -test (P<0.05).

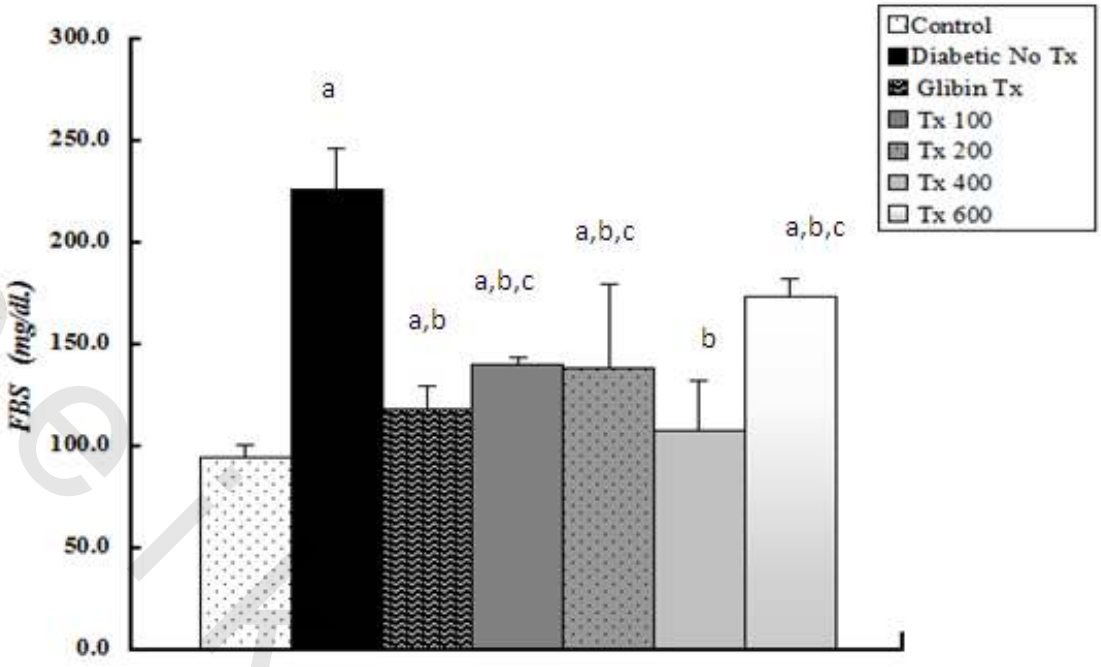

Figure (19): The levels of FBS (mgldl) in healthy control and diabetic groups with and without treatments. *(Data were presented as mean± SD, a: Significant different from control, b:Significant different from untreated diabetic group, c: Significant different from glibinamide treated group by ANOVA -test (P<0.05).*

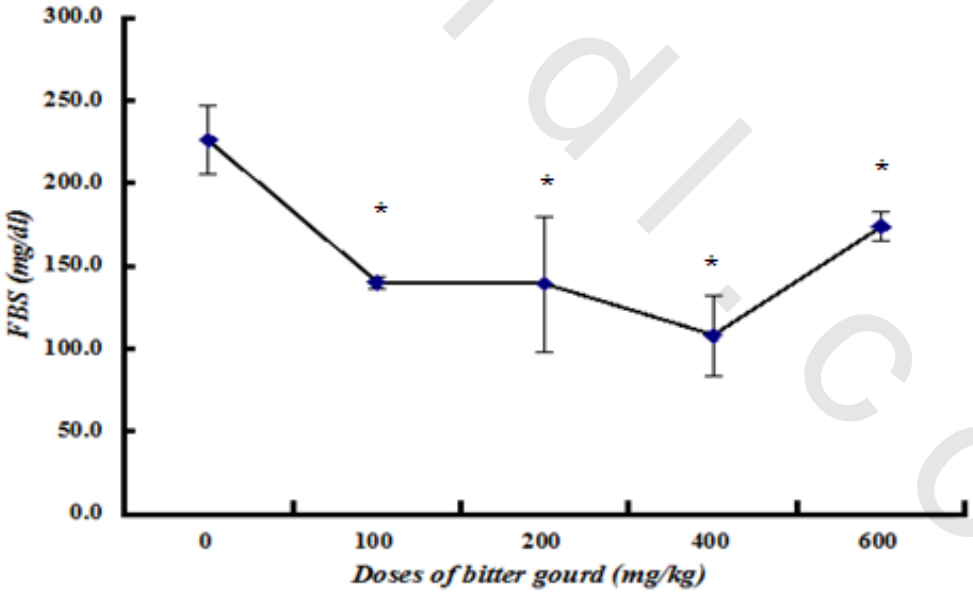

Figure (20): **Dose-dependent effect of bitter gourd extract on asting blood glucose of diabetic rats.** *(* Significant difference from untreated diabetic rats (0 concentration) by Anova-test (P<0.05).*

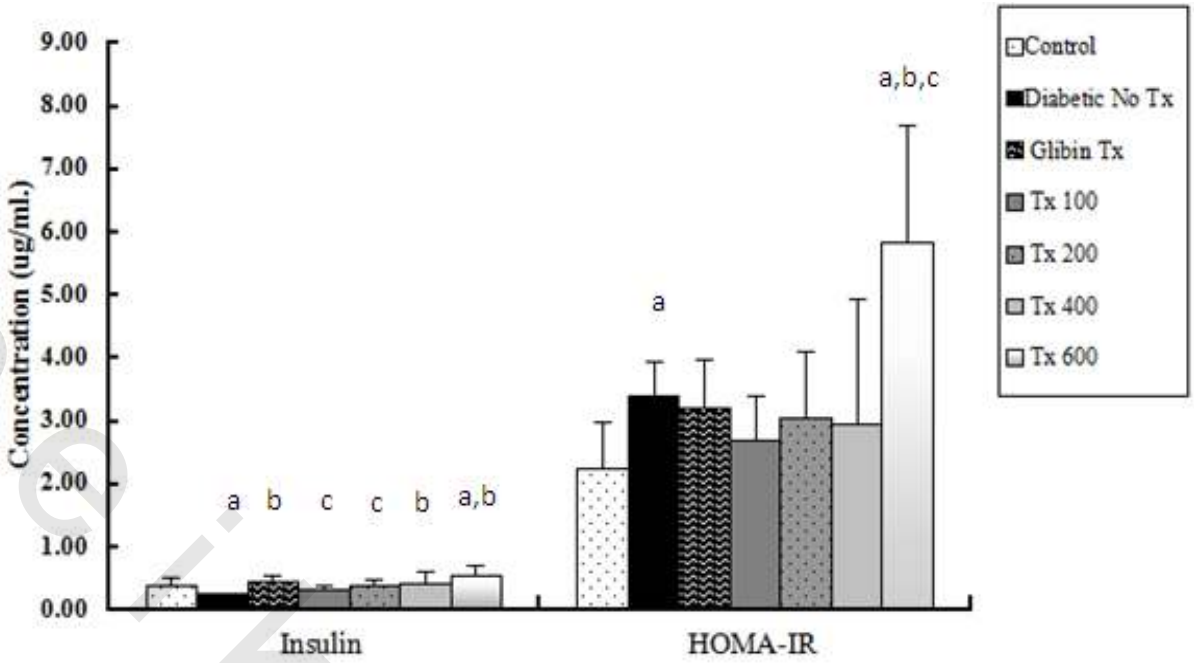

Figure (21): The levels of Insulin (ug/ml) and HOMA-IRin healthy control and diabetic groups with and without treatments. (*Data were presented as mean± SD, a: Significant different from control, b:Significant different from untreated diabetic group, c: Significant different from glibinamide treated group by ANOVA -test (P<0.05).*

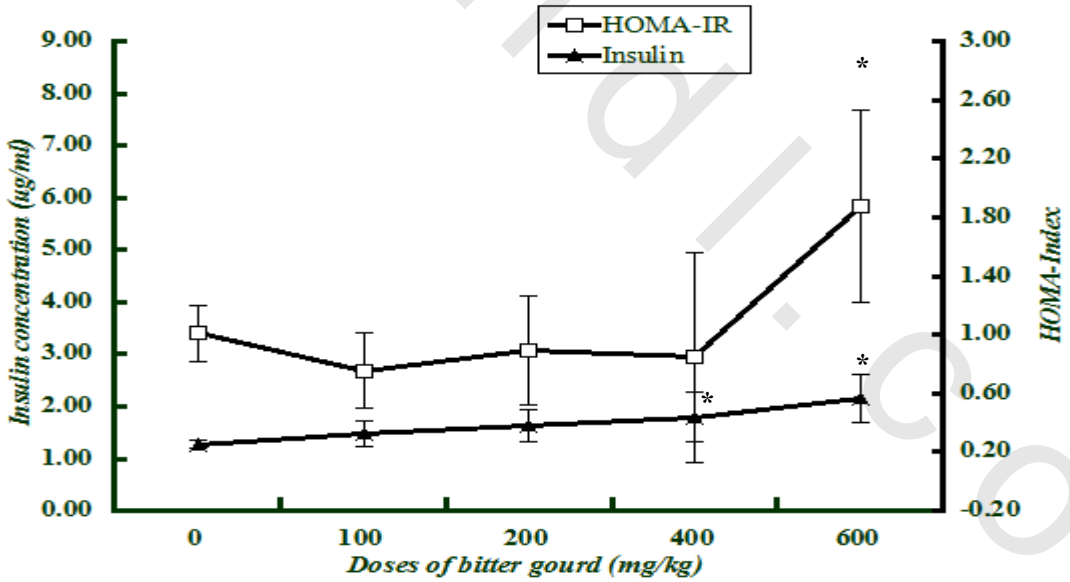

Figure (22): **Dose-dependent effect of bitter gourd extract on insulin and insulin HOMA-IR of diabetic rats**. *(*Significant difference from untreated diabetic rats (0 concentration) by Anova-test (P<0.05).*

4.3. Lipid profile:

The statistical analysis of serum triglycerides, total cholesterol, LDL-cholesterol and HDL-cholesterol are presented in Table (6) and Figures (23-26).

4.3.1. Triglycerides level:

Serum triglycerides level showed significantly higher level in the untreated diabetic rats compared to healthy control group. Glibinclamide treatment significantly decrease the triglycerides level compared to untreated diabetic group but still higher than control group.(Table 6 and Figures:23-24)

Treatment with bitter gourd at low doses (100 and 200 mg/kg) significantly decrease the triglycerides level compared to untreated diabetic group, while higher doses (400 and 600 mg/kg)showed no significant change in triglycerides level compared to untreated rats (Table 6 and Figures:23-24).

4.3.2. Total cholesterol level:

Untreated diabetic rats showed significantly higher serum total cholesterol level than control rats. Treatment with glibinclamide significantly corrected the cholesterol level.Treatment with bitter gourd showed no significant effect on the total cholesterol level except at highest dose **(**600 mg/kg) which showed significant decrease in the cholesterol level compared to untreated diabetic groups. (Table 6, Figures: 23-24).

4.3.3. LDL- cholesterol level:

Untreated diabetic rats showed a significantly higher LDL-cholesterol level than control rats. The treatment with glibinclamide significantly decreased LDL-cholesterol level to near control value.The diabetic rats treated with bitter gourd showed dose-dependent decline in the serum LDL-cholesterol level. The best results obtained with highest doses (400 and 600 mg/kg) (Table 6, Figures: 25 - 26).

4.3.4. HDL- cholesterol level:

In contrast with total and LDL-cholesterol, HDL-cholesterol showed significant decline in untreated diabetic rats compared to control rats. The treatment with glibinclamide significantly increased the level of HDL-cholesterol. The rats treated with bitter gourd showed dose-dependent increase in HDL until the dose 400 mg/kg after which HDL-cholesterol showed decreased value but still higher than untreated diabetic groups. (Table 6, figures: 25 - 26).

Table (6): Lipid profile
The Levels of Triglycerides (mg/dl), Total Cholesterol (mg/dl), LDL-Cholesterol (mg/dl) and HDL-Cholesterol(mg/dl) in healthy control and diabetic groups without and with Treatments.

	Healthy control	Diabetic Groups					
		No Tx	Tx Glibin	Tx 100.0	Tx 200.0	Tx 400.0	Tx 600.0
Triglycerides (mg/dl.)	36.1±7.1	118.0±16.5 a	76.6±7.0 a,b	78.8±30.8 a,b	79.5 ± 43.1 a,b	102.3±21.1 a,c	103.4±15.2 a,c
Total Cholesterol (mg/dl)	142.1±11.9	185.7±12.9 a	155.7±5.7 b	173.0±22.1 a,c	176.0±27.1 a,c	177.2±21.0 a,c	157.0±14.1 b
LDL-Cholesterol (mg/dl)	90.8±12.5	134.4±13.2 a	101.9±4.8 b	121.1±17.4 a,c	117.1±36.3 a,b	105.3±11.3 b	97.2±15.9 b
HDL-Cholesterol (mg/dl)	44.1±4.5	27.7 ± 1.3 a	38.5±8.7 b	36.1±8.8	43.0±19.2 b	51.5±13.8 b,c	39.1±1.4 b

Data were presented as mean± SD.
a: Significant different from control by ANOVA-test (P<0.05).
b: Significant different from untreated diabetic group by ANOVA -test (P<0.05).
c: Significant different from Glibinamide treated group by ANOVA -test (P<0.05)

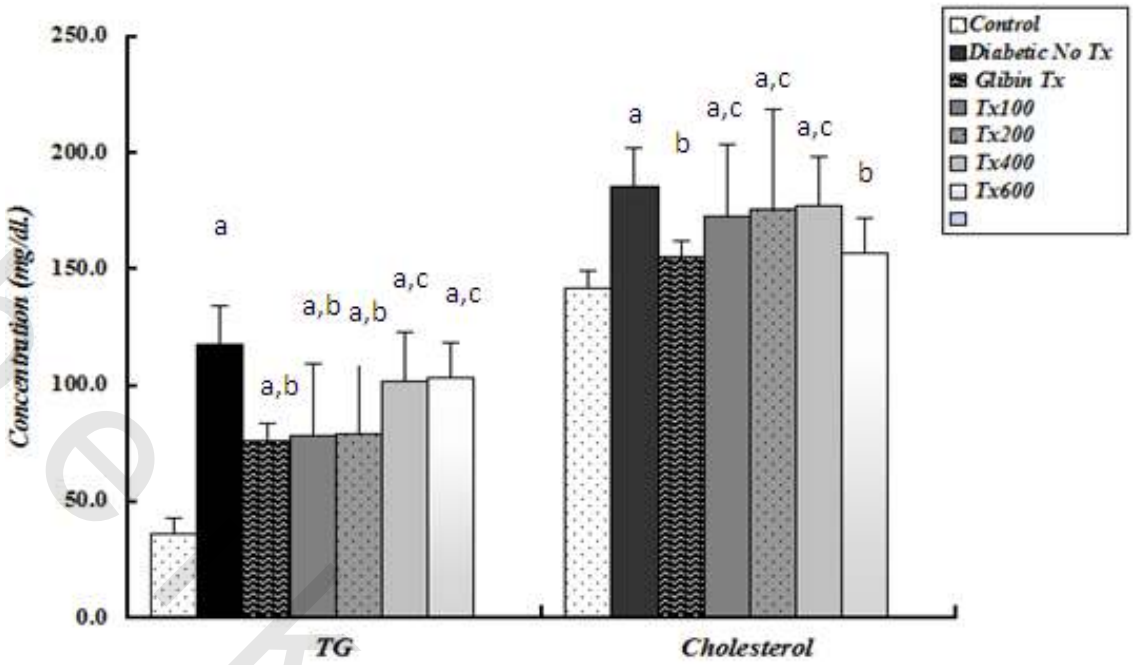

Figure (23): The Levels of Triglycerides (mg/dl),Total Cholesterol (mg/dl), (mg/dl) and in healthy control and diabetic groups without and with treatments. (*Data were presented as mean± SD, a: Significant different from control, b:Significant different from untreated diabetic group, c: Significant different from glibinamide treated group by ANOVA -test (P<0.05).*

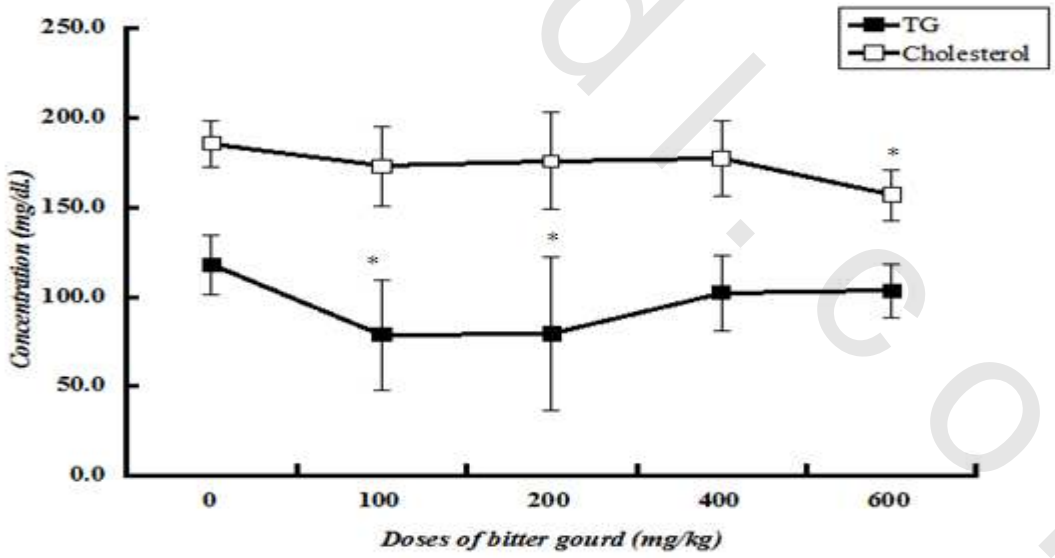

Figure (24): **Dose-dependent effect of bitter gourd extract on triglycerides and cholesterol of diabetic rats.** *(*Significant difference from untreated diabetic rats (0 concentration) by Anova-test (P<0.05).*

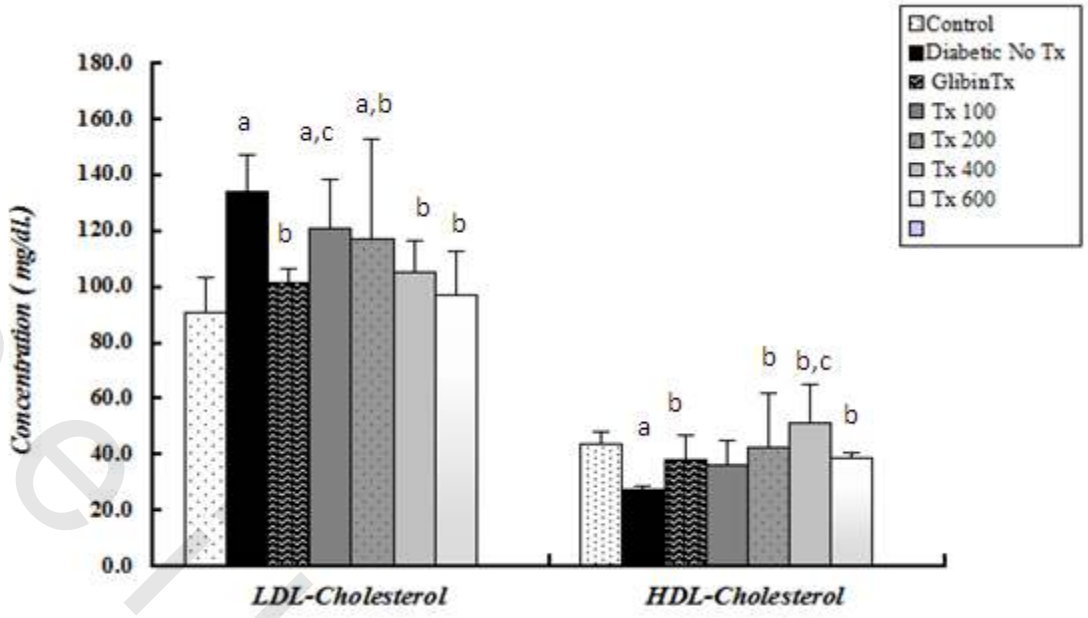

Figure (25): The Levels of LDL-Cholesterol (mg/dl) and HDL-Cholesterol(mg/dl) in healthy control and diabetic groups without and with treatments. *(Data were presented as mean± SD, a: Significant different from control, b:Significant different from untreated diabetic group, c: Significant different from glibinamide treated group by ANOVA -test (P<0.05).*

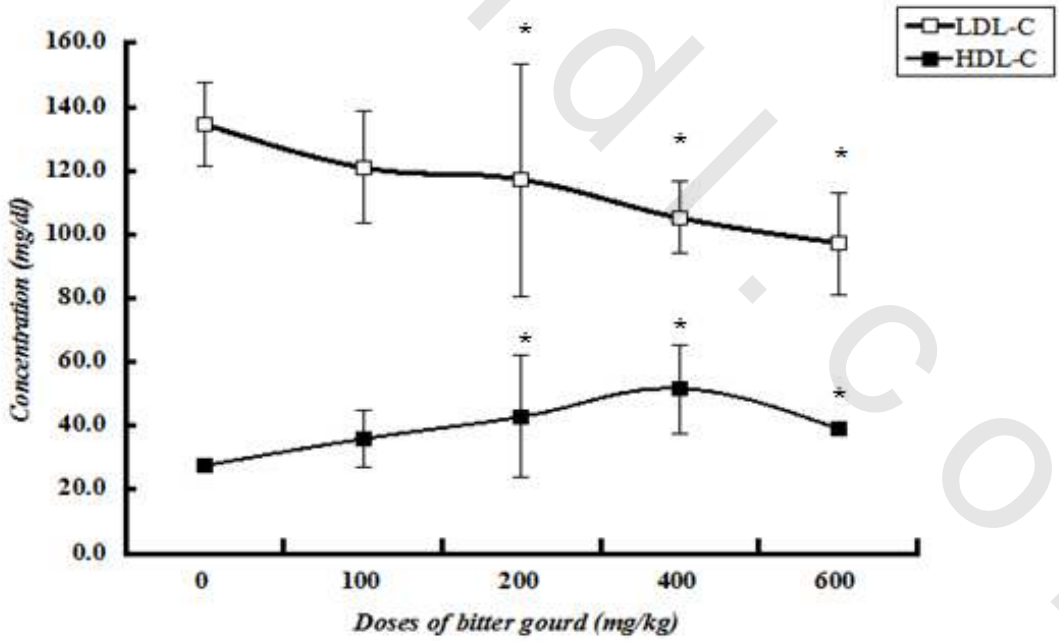

Figure (26): Dose-dependent effect of bitter gourd extract on LDL-cholesterol and HDL- cholesterol of diabetic rats. *(* Significant difference from untreated diabetic rats (0 concentration) by An*ova-test (P<0.05).

4.4. Insulin signaling parameters in the liver:

The results of liver insulin signal parameters;Phospho-IR, IRS-1 and PKC are summarized in (Table: 7 , Figures 27-30).

4.4.1. Phospho-insulin receptor (P-IR):

The hepatic tissue content of P-IR in the untreated diabetic rats showed lower level compared to control rats.The treatment with glibinclamide significantly increase P-IR level compared to untreated group. The treatment of diabetic rats with bitter gourd at dose (100 - 400 mg/kg) showed a dose-dependent increase in the liver content of P-IR while at higher dose (600 mg/kg) the P-IR level decline again to be at the same level as the untreated group. (Table 7, Figures27 -28).

4.4.2. Insulin receptor substrate-1 (IRS-1) :

The hepatic content of IRS-1 in untreated diabetic rats showed lower level compared to control group. The treatment with glibinclamide showed no change in the level of IRS-1. Treatment of diabetic rats with bitter gourd showed a slight non significant increase in IRS-1 with the first doses while 400 mg/kg dose showed a significant increase in the hepatic IRS-1content.The higher dose (600 mg/kg) showed a decline again in the level of hepatic IRS-1 to be lower than untreated group. (Table: 7, Figures: 27 - 28).

4.4.3. Protein kinase-C (PKC):

The hepatic content of PKC in the untreated diabetic group showed lower value compared to control group.The glibinclamide treatment significantly increase the hepatic PKC content compared to untreated group. The treated rats with bitter gourd showed that , the rats treated with lowest dose (100 mg/kg) have the highest hepatic content of PKC. The other rats treated with higher doses of bitter gourd showed a dose-dependent decrease in the hepatic content of PCK.(Table 7, Figure: 29 - 30)

Table (7): Liver insulin signal parameters.
The Levels of P-IR (pg/mg protein), IRS-1 (pg/mg protein) and PKC (pg/mg protein) in healthy control and diabetic groups without and with Treatments.

	Healthy control	Diabetic Groups					
		No Tx	Tx Glibin	Tx 100.0	Tx 200.0	Tx 400.0	Tx 600.0
P-IR (pg/mg protein)	6.77±1.06	4.39±0.59 a	5.03±0.36 a,b	4.53± 0.53 a	4.77±0.43 a	5.45± 0.43 a,b	4.37±0.18 a,c
IRS-1 (pg/mg protein)	1.82±0.25	1.48±0.15 a	1.47± 0.05 a	1.53±0.06 a	1.51±0.05 a	1.66±0.05 a,b,c	1.38± 0.07 a
PKC (pg/mg protein)	52.3±9.8	29.1±5.7 a	46.8±17.2 b	55.7±3.0 b	45.2± 6.3 b	44±15.6 b	43.9±12.5 b

Data were presented as mean± SD.
a: Significant different from control by ANOVA-test (P<0.05).
b: Significant different from untreated diabetic group by ANOVA -test (P<0.05).
c: Significant different from Glibinamide treated group by ANOVA -test (P<0.05).

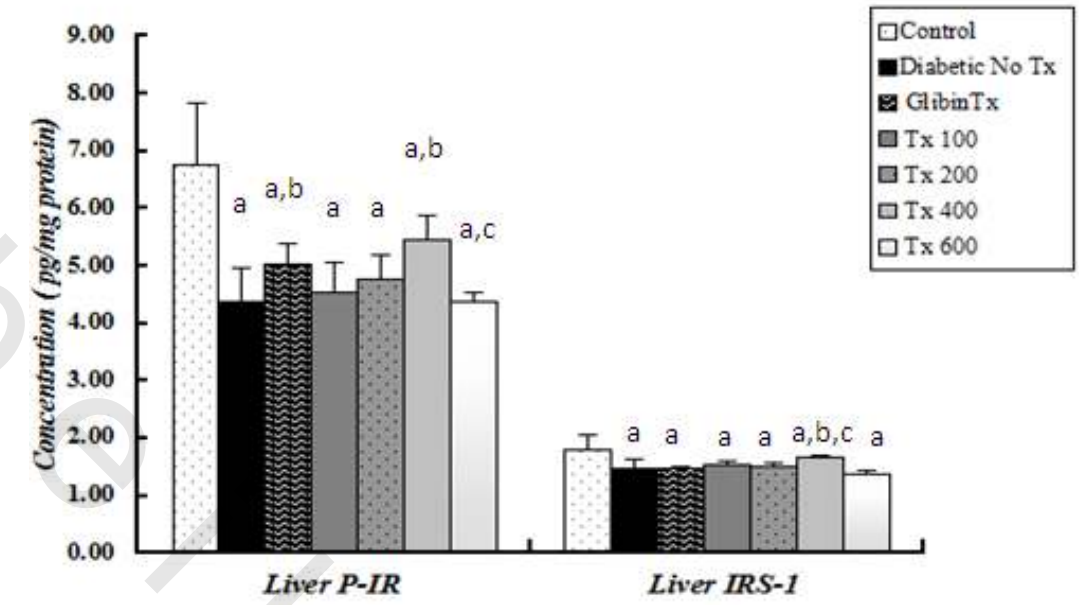

Figure (27): **The Levels of P-IR (pg/mg protein) and IRS-1 (pg/mg protein) in healthy control and diabetic groups without and with treatments. (***Data were presented as mean± SD, a: Significant different from control, b:Significant different from untreated diabetic group, c: Significant different from glibinamide treated group by ANOVA -test (P<0.05).*

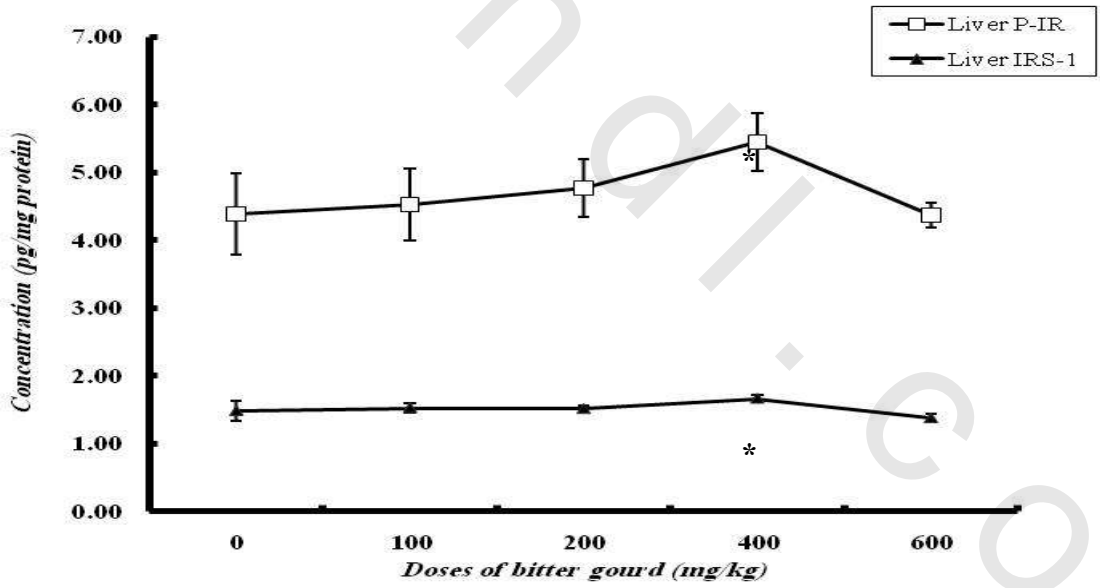

Figure (28): Dose-dependent effect of bitter gourd extract on liver P-IR and Liver IRS-1of diabetic rats (* Significant difference from untreated diabetic rats (0 concentration) by Anova-test (P<0.05).

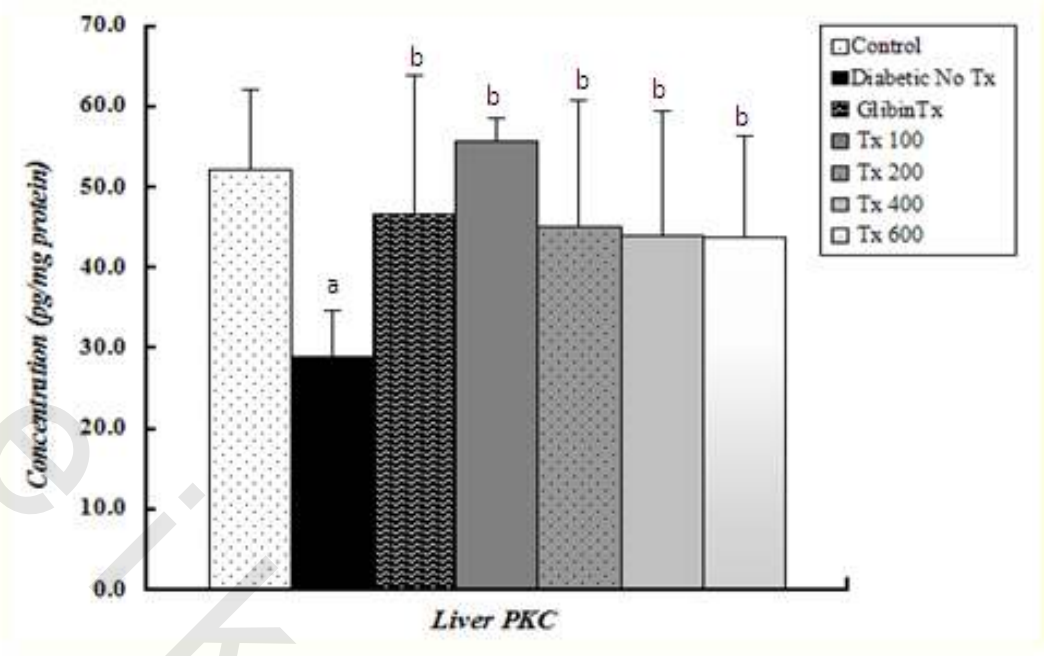

Figure (29): **The Levels of PKC (pg/mg protein) in healthy control and diabetic groups without and with treatments. (***Data were presented as mean± SD, a: Significant different from control, b:Significant different from untreated diabetic group, c: Significant different from glibinamide treated group by ANOVA -test (P<0.05).*

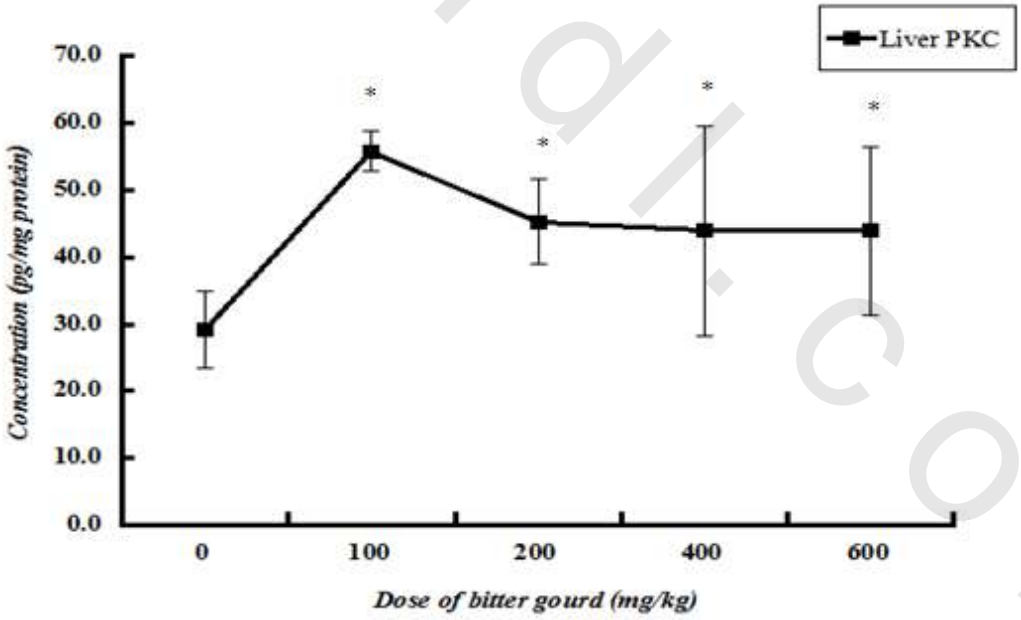

Figure (30): **Dose-dependent effect of bitter gourd extract on Liver PKC of diabetic rats**. *(* Significant difference from untreated diabetic rats (0 concentration) by Anova-test (P<0.05).*

4.5. Insulin signal parameters in muscle:

The results of muscle signal parameters;P-IR, IRS-1 and PKC are summarized in Tables 8 and Figures 31-34.

4.5.1. Phospho-insulin receptor (P-IR):

The muscle P-IR content in the untreated diabetic rats showed lower level than control rats. The treatment of diabetic rats with glibinclamide showed no significant correction of P-IR. While the diabetic rats treated with bitter gourd showed significant increase in the muscle P-IR content compared to untreated diabetic rats. The best result obtained with bitter gourd at a doses of 100 and 200 mg/kg. (Table: 8, Figures: 31-32).

4.5.2. Insulin receptor substrate-1 (IRS-1):

The muscle IRS-1 content in the untreated diabetic rats showed lower level than control group. Glibinclamide treated diabetic group showed near normal value level. The treatment with bitter gourd showed dose-dependent increase in IRS-1 in muscle but still lower than glibinclamide treated group and even untreated diabetic group. (Table: 8, Figures: 31- 32).

4.5.3. Protein kinase-C (PKC):

The muscle PKC content showed lower level in the untreated diabetic rats than control group. The treatment with glibinclamide resulted in significant increase in PKC. The bitter gourd treatment showed dose-dependent increase in the muscle PKC content from dose of 100 to 400 mg/kg after which its level declined again. (Table 8, Figures: 33 -34).

4.5.4. Glucose transporter-4 (Glut-4):

The muscle content of Glut-4 in the untreated diabetic rat showed a significantly lower level than control group. The treatment of diabetic rats with glibinclamide non significantly increase Glut-4 level in the muscle. The diabetic rats treated with bitter gourd showed dose-dependent increase in the muscle content of Glut-4 (Table 8, Figure 33-34).

Table (8): Muscle insulin signal parameters.
The Levels of P-IR (pg/mg protein), IRS-1(pg/mg protein) , PKC (pg/mg protein) and Glut-4 (ng/mg protein) in healthy control and diabetic groups without and with Treatments.

	Healthy control	Diabetic Groups					
		No Tx	Tx Glibin	Tx 100.0	Tx 200.0	Tx 400.0	Tx 600.0
P-IR (pg/mg protein)	6.06 ±0.92	3.95±0.33 a	4.12±0.08 a	4.72±0.33 a,b,c	4.74±0.17 a,b,c	4.38±0.18 a,b	4.27±0.35 a
IRS-1 (pg/mg protein)	1.69±0.18	1.55±0.04 a	1.62 ±0.08	1.44±0.06 a,c	1.46±0.09 a,c	1.46±0.03 a,c	1.5± 0.25 a
PKC (pg/mg protein)	55.97±12.61	35.55±7.89 a	48.10±2.56 a,b	42.38±3.9 a,b	43.55±4.99 a,b	46.97±3.79 a,b	38.64±5.65 a,c
Glut-4 (pg/mg protein)	104±44.4	46.0±3.2 a	57.1±5.3 a	59.2±16.5 a	60.2±38.3 a	62.8±13.8 a	65.4±5.9 a

Data were presented as mean± SD.
a: Significant different from control by ANOVA-test (P<0.05).
b: Significant different from untreated diabetic group by ANOVA -test (P<0.05).
c: Significant different from Glibinamide treated group by ANOVA -test (P<0.05).

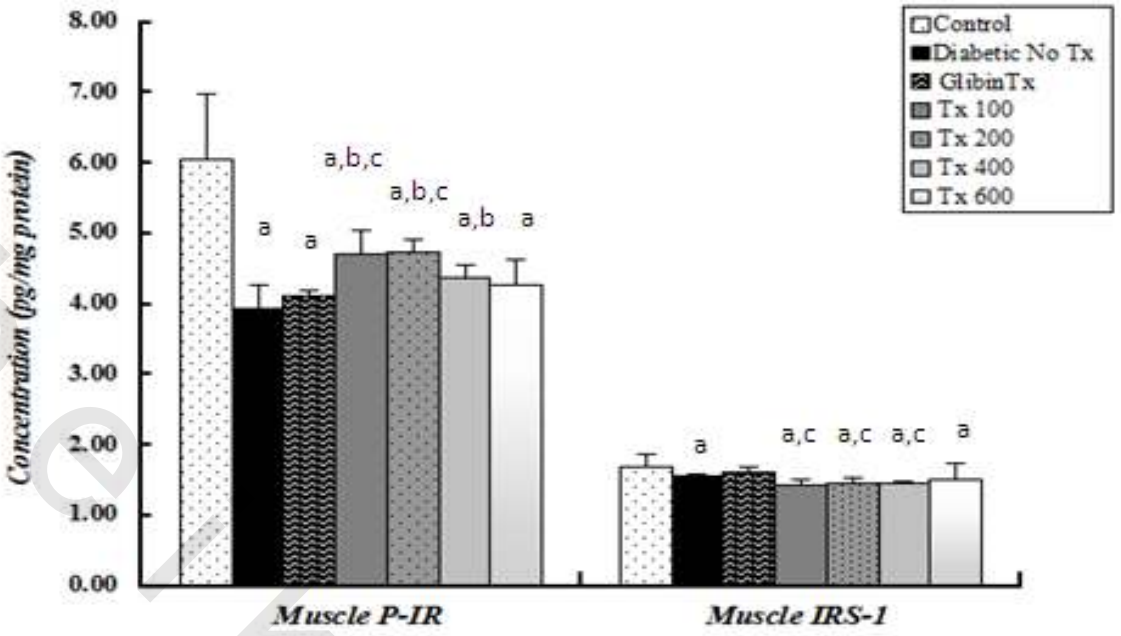

Figure (31): **The Levels of P-IR (pg/mg protein) and IRS-1(pg/mg protein) in healthy control and diabetic groups without and with treatments.** *(Data were presented as mean± SD, a: Significant different from control, b:Significant different from untreated diabetic group, c: Significant different from glibinamide treated group by ANOVA -test (P<0.05).*

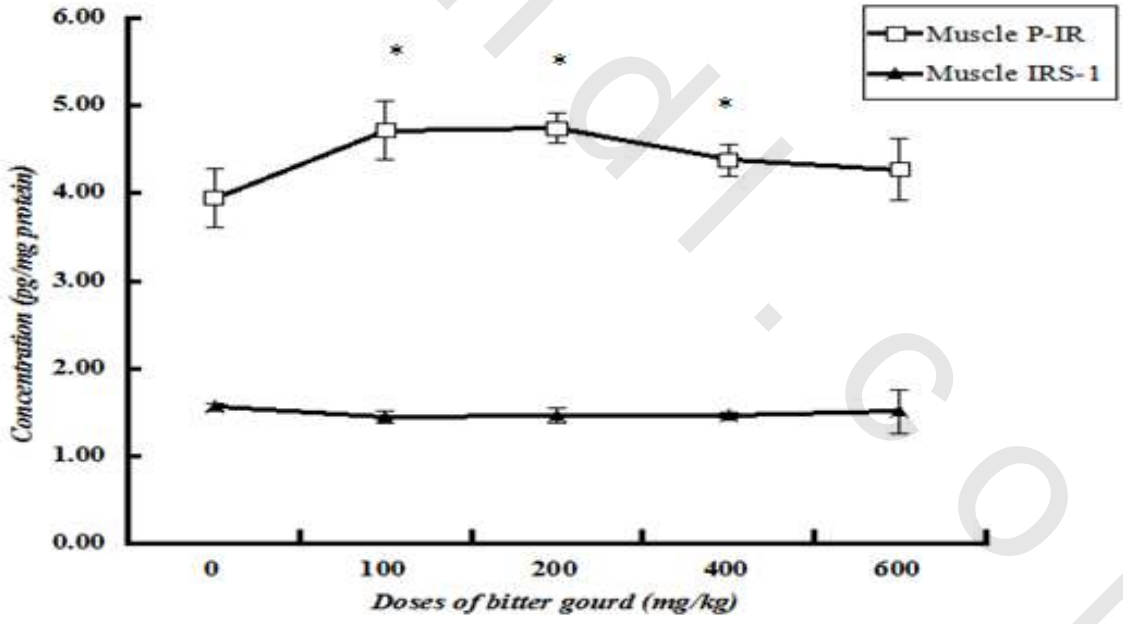

Figure (32): **Dose-dependent effect of bitter gourd extract on muscle P-IR and muscle IRS-1 of diabetic rats**. *(*Significant difference from untreated diabetic rats (0 concentration) by Anova-test (P<0.05).*

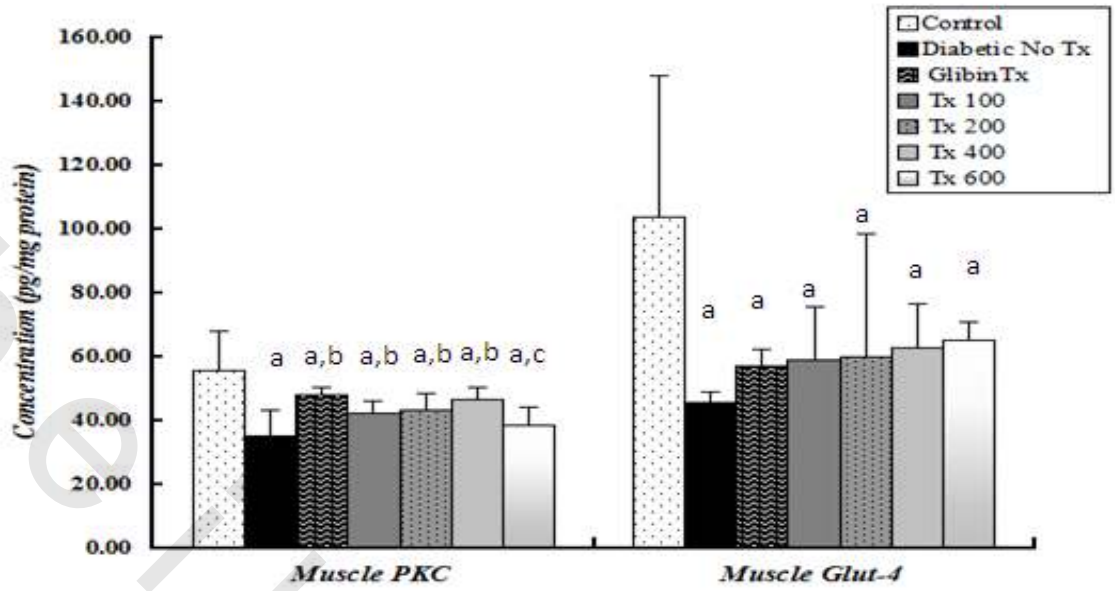

Figure (33): **The Levels of PKC (pg/mg protein) and Glut-4 (pg/mg protein) in healthy control and diabetic groups without and with Treatments.** *(Data were presented as mean± SD, a: Significant different from control, b:Significant different from untreated diabetic group, c: Significant different from glibinamide treated group by ANOVA -test (P<0.05).*

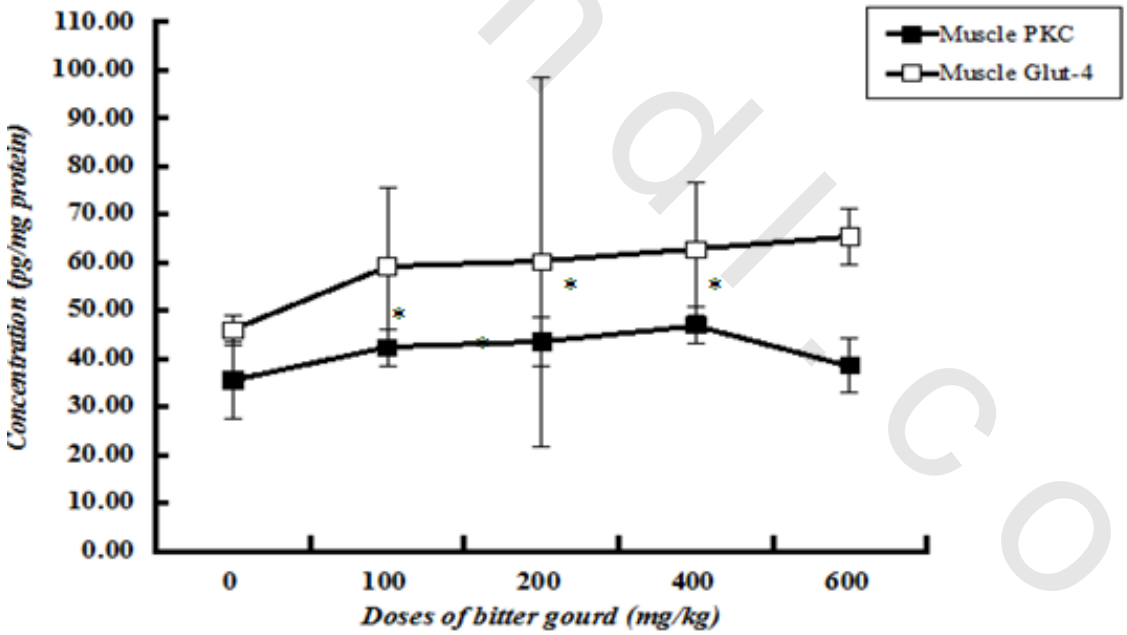

Figure (34): **Dose-dependent effect of bitter gourd extract on muscle PKC and Glut-4 of diabetic rats.** *(*Significant difference from untreated diabetic rats (0 concentration) by Anova-test (P<0.05).*