Study of Hypoglycemic Effect of *Juglans regia* Leaves and its Mechanism

Teimori M (M.Sc.)[1], Montasser Kouhsari S (Ph.D.)[2], Ghafarzadegan R (M.Sc.)[3*], Hajiaghaee R (Ph.D.)[3]

1- Department of Cellular and Molecular Biology, School of Biology, Faculty of Science, University of Tehran, Tehran, Iran
2- Department of Cellular and Molecular Biology, School of Biology, Faculty of Science, University of Tehran, Tehran, Iran
3- Department of Pharmacognosy and Pharmaceutics, Institute of Medicinal plants, ACECR, Karaj, Iran
*Corresponding author: Department of Pharmacognosy and Pharmaceutics, Institute of Medicinal plants, ACECR, Karaj, Iran
Tel: +98-261-4764010-19, Fax: +98-2614764021
Email: reza.ghafary@yahoo.com

Receive: 28 Oct. 2009 **Acceptance:** 1 March 2010

Abstract

Background: Hypoglycemic effect of *Juglans regia* leaves was reported in other previous researches.

Objective: To investigate the mechanism of hypoglycemic action of *Julans regia* Leaves Methanolic Extract (JRLME), we designed the following study.

Methods: Male wistar rats were classified in five groups, each of six, including alloxan-induced diabetic rats treated with JRLME at doses of 250 (D+JRLMEa), 500 mg/kg (D+JRLMEb), Acarbose at dose 20 mg/kg (D+Ac), vehicle alone (DC) and normal rats treated with vehicle alone (NC). The Postprandial blood glucose level was examined in a short time (after a single dose treatment) and a long time (after three weeks daily treatment) model. After three weeks of treatment, all groups' animals were killed to dissect pancreatic and myocardium for RNA extraction and RT-PCR, to assess insulin and glut-4 genes expression, respectively. In addition, other normal rats were killed to extract intestinal brush border membrane for α-glucosidase solution preparation. The effect of JRLMEa on α-glucosidase activity was evaluated by an *in vitro* method, compared to Acarbose as a reference drug.

Results: The plant extract had a significant hypoglycemic action in both short and long term models. Decrease in PBG level was the most at 8 hour after treatment and more in D+Ac (53%), D+JRLMEa (40%) and D+JRLMEb (29%) versus DC, respectively. There was also permanent PBG reduction in treated groups in comparison with the DC one during long term period.

Conclusion: We did not find change in the insulin and glut-4 genes expression. On the other hand, the *in vitro* assay of α-glucosidase activity displayed inhibitory action of JRLME, like Acarbose, but less effectively.

Keywords: *Juglans regia*, Blood Glucose, insulin, glut-4, α-Glucosidase

[Downloaded from jmp.ir on 2026-11-11]

[DOR: 20.1001.1.2717204.2010.9.33.25.1]

Introduction

Diabetes is possibly the world's fastest growing metabolic disease [1]. Chronic hyperglycemia is the most well-known symptoms of Non-Insulin Independent Diabetes Mellitus (NIDDM), which is important in development of diabetic complications [2]. Several reasons are reported for enhancement of glucose level in diabetic peoples. Decline in insulin secretion, Defect in glucose transport system (GLUT1, GLUT4) [3], and enhancement of hydrolytic activity (Sucraseisomaltase) of intestinal brush border membrane are three important causes of hyperglycemia in diabetic people [4].

Juglans regia L. has been widely used in Iranian traditional medicine for remedy of various ailments. Different parts of *juglans regia* L. such as kernel, leaves and septum have a significant hypoglycemic effects. For example, hydroalcoholic extract of *Juglans regia*'s leaves has anti hyperglycemic effect on diabetic rats and human [5, 6].

In the present study, at first, we have examined the effect of *Juglans regia* Leaves Methanolic Extract (JRLME) in blood glucose levels of the diabetic rats which were treated with JRLME for 25 days at dose of 250 mg/kg [12].

Then, the extent of insulin and Glucose transporter-4 (glut-4) genes expressions have been inspected in the pancreatic and myocardial tissues, respectively. Finally,We examined *in vitro* effect of the JRLME in the disaccharidases activity (sucrase-isomaltase) of the intestinal brush border membrane.

Materials and methods

Chemicals and reagents

DEPC Water, Taq polymerase and RNA extraction kit purchased from CinnaGen (Tehran, Iran); Nase, Nase free kit from Fermentas (Ontario, Canada), RT kit and Primers from Bioneer (Daejon, Korea), dNTPs from BioFlux (Tokyo, Japan), The kits for determination of glucose were from ChemEnzyme (Tehran, Iran). All other chemicals and solvents were of the highest commercial grade from Merck (KGaA, Germany) or from Sigma (St Louis, MO, USA).

Plant Materials

Leaves of *Juglans regia* L. (Juglandaceae) were collected from Tehran province (Damavant Mountains) and were authenticated by Professor Ahmad Qahraman and voucher specimen (No. 33239) was deposited at the herbarium of University of Tehran, Tehran, Iran. The leaves were cleaned, shed dried at 25°C, and ground with a blender.

Methanolic Extract of *Juglans regia* Leaves (JRLME)

The powdered leaves of *J. regia* (200g) were defatted with light petroleum (b.r. 40-60°C) in continuous extraction apparatus. The marc was dried and re-extracted three times with fresh 70% methanol at room temperature for 24 h. The methanolic phases were pooled and the residue was removed by filtration. The methanolic extract concentrated at 40 °C by rotary evaporator and then lyophilized to get a powder (JRLME). The percentage yields based on the dried starting material was 17%. The powder was stored in the dark at 4 °C for subsequent experiments.

Animals

Male Wistar rats (*Rattus norvegicus*) weighing 200 – 250 g were used in this study (Pasteur Institute, Tehran, Iran), Animals were housed six per standard rat cage, in a room with a 12:12 h light/dark cycle (lights on 07:00

[Downloaded from jmp.ir on 2026-11-11]

[DOR: 20.1001.1.2717204.2010.9.33.25.1]

h) and controlled temperature (22 ± 1°C). Commercial rodent pellets and tap water were available *ad libitum*. They were allowed to adapt to the laboratory conditions for one week before the study. There were six rats per group in each experiment. The procedures were performed in accordance with institutional guidelines for animal care and use.

Preparation of alloxan-induced diabetic wistar rats

Diabetes was induced in overnight fasted rats by subcutaneous injection of Alloxan monohydrate (100 mg/kg, Sigma, St Louis, MO, USA), dissolved in citrate buffer (pH = 4.5), according to a previously described method [7, 8]. After one week of administration, survived rats with marked hyperglycemia (postprandial blood glucose > 250 mg/dl) were selected and used for this study.

Design

The rats were divided into five groups of six each. Group I (NC): Normal rats treated with distillated water alone; Group II (DC): Diabetic rats treated with distillated water alone and in other groups, extract waz dispersed in distillated water. Group III (D+JRLME): Diabetic rats treated with JRLME at the dose of 250 mg/kg; Group IV (D+JRLMEb): Diabetic rats treated with JRLME at the dose of 500 mg/kg. Group V (D+Ac): Diabetic rats treated with Acarbose (20 mg/kg).

Single dose of JRIME was administerad every day orally using intragastric tube for 21 days. Postprandinal plasma blood glucose levels were estimated in short time and long time models. For the estimation of insulin (insulin) and glucose transporter-4 (glut-4) mRNA expressions rats were killed at the end of 21 days of treatment, and the hearts and pancreas of NC, DC and D+JRLMEa groups were removed promptly. In a separate experiment the inhibitory effects of plant extract on intestinal α-Glucosidase (sucrase and maltase) were measured by an *in-vitro* method.

Oral administration of the plant extract

1 mL of each samples were administered orally at 11-12 a.m. using an intragastric tube.

Estimation of hypoglycemic activity
Short-term experimental model

Blood samples were obtained from the tail vein and postprandial plasma glucose levels was estimated using a glucometer (On Call Now, San Diego, USA) after 1, 3, 5, 8 and 24 hours following administration of a single dose of JRLME and Acarbose samples to rats. The NC and DC groups were treated by the same volume of vehicle (1mL of distilled water).

Long-term experimental model

Two doses of JRLME were selected (250 and 500 mg/kg) for long term experimental model, but we preferred using 250 mg/kg instead of 500mg/kg as will be discussed in results. During the long-term treatment period with JRLMEa and Acarbose (20 mg/kg), the level of postprandial plasma glucose in all rats was estimated at the end of 1, 2 and 3 weeks of treatments using blood samples obtained from tail vein and a glocometer device (OnCall Now, San Diego, USA).

Gene expression analysis

To investigate the mechanism of JRLME atihyperglycemic action, at the end of 21 days of treatment, the animals in DC, NC and D+JRLME groups were analyzed for modulation at transcripts of glut-4 and insulin

[Downloaded from jmp.ir on 2026-11-11]

[DOR: 20.1001.1.2717204.2010.9.33.25.1]

genes by RT-PCR (Reverse Transcription Polymerase Chain Reaction).

The rats were anesthetized and killed, then Total RNA of pancreas and heart tissues of each rat were extracted by RNX-Plus kit. In brief, after homogenization of tissue samples (1mL per 50-100 mg tissue) with RNX-Plus kit, proteins were extracted with chloroform and total RNA was precipitated with isopropanol. The precipitated RNA was washed with 70% ethanol and resuspended in 50 µl of DEPC-treated water. Finally the DNA free RNA was prepared prior to RT-PCR using DNase I, RNase-free kit. Reverse transcription was carried out to obtain cDNA using AccuPower RT PreMix kit, 50 ng/µl template RNA and 25 ng/µl oligo dT18. The primers used were as follows: glut-4 F, 5′-AGG CAC CCT TAC CCT TTT-3′;glut-4 R 5′-GAC AGA AGG GCA ACA GAA GC-3′___ (318-bp); insulin F, 5′-TTC TTC TAC ACA CCC AAG-3′; insulin R, 5′-GCA GTA GTT CTC CAG TTG-3′___ (155-bp) and B-act F, 5′-AGC CAT GTA CGT AGC CAT CC-3′; B-act R, 5′-TCT CAG CTG TGG TGG TGA AG-3′___ (248-bp). For PCR reaction, 500 ng of the cDNA was added to a PCR reaction mix consisting of 10XPCR buffer (2.5µl), 50 MmMgCl2 (0.75 µl), 10 mM dNTP (0.5 µl), 10 pM of paired primers (0.5 µl of each), 0.25units of Taq polymerase and distilled water in a total volume of 25 µl. The reaction mixture wasoverlaid in a PCR thermal cycler for 35 cyclic reactions. PCR products were run on 1.5% agarose gels, stained with ethidium bromide and photographed. Images of radiographs were analyzed with TotalLab v1.10 using 1D analysis.

Inhibition assay for rat intestinal sucrase activity

α-Glucosidase activity was assayed using a literature method [9, 10] with a slight modification. Exactly 0.2 ml of 56 mM sucrose in 0.1 M potassium phosphate buffer (pH 7, 0.2 mL) was mixed with 0.1 mL of the plant extracts in 50% aqueous dimethyl sulfoxide (DMSO). After pre-incubation at 37 °C for 5 min, 0.2 mL of rat intestinal α-glucosidase solution prepared from intestine of normal rats (Kessler et al., 1978) was added. Instead of the plant extract, 0.1 mL DMSO was used for the blank sample. After thoroughly mixing, both sample and blank test tubes were incubated at 37 °C for 15 min and then the reaction was stopped by emerging test tubes in boiling water for 4 minute.

The reaction mixture was passed through a basic alumina column (6 mm × 35 mm h) to eliminate phenolic or acidic compounds. The amount of liberated glucose was determined by the glucose oxidase assay using a commercial test kit. The optical density (OD) of the wells was measured at 505 nm and the inhibitory activity was calculated using following formula: Inhibitory activity (%) = 100 * (1-[ODtest sample/ODcontrol])

Inhibition assay for rat intestinal maltase activity

Rat intestinal maltase inhibitory activity was determined using a literature method [9, 11] with a slight modification. The assay was carried out in the same manner as the assay of rat intestinal sucrose inhibitory activity, except for using 3.5 mM maltose in 0.1 M potassium phosphate buffer (pH 7, 0.35 mL) as a substrate.

Statistical analysis

All data are presented as means±S.D. for six rats in each group. Comparison between groups and between time points was made by one-way analysis of variance (ANOVA) followed by Duncan's test to analyze the difference. Differences were considered significant when P-values were less than 0.05.

[Downloaded from jmp.ir on 2026-11-11]

[DOR: 20.1001.1.2717204.2010.9.33.25.1]

All statistical analyses were performed using SPSS (SPSS Inc, Chicago, USA).

Results

Effects of JRLME on PBG in short term treatment

Figure 1 depicts antihyperglycemic effect of JRLME at different intervals of time after a single dose administration of plant extract in two different concentration and reference drug (Acarbose).

PBG level was decreased gradually from 3h to 8 h after treatment with JRLME and Acarbose, but thereafter a gradual rise occurred in PBG till 24h. The most reductions in PBG levels is at 8h, which are 31%, 18.5% and 44% for D+JRLMEa, D+JRLMEb and D+Ac groups, respectively, as compared to initial time ($p<0.0001$). In addition, the glucose levels reductions at 8h are 40%, 25% and 53% in comparison with DC groups, respectively.

Effect of JRLMEa on PBG in long term treatment

Figure 2 displays reduced PBG concentration in D+JRLMEa group, following daily JRLMEa administration, at the end of first, second and third weeks by 20, 20 and 27%, respectively, compared to the DC group ($p<0.0001$). In addition, the reduction of blood glucose levels in D+Ac group is 36, 36.5 and 43.7%, respectively, compared to DC group ($p<0.0001$).

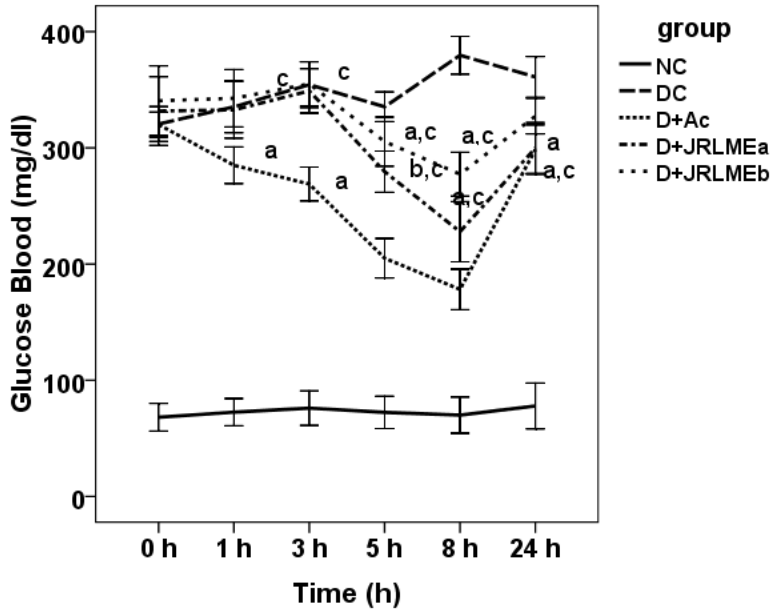


Fig. 1- Acute effect of the JRLME on PBG levels in alloxan-diabetics rats. NC indicates Normal Control; DC, Diabetic Control; D+JRLMEa and D+JRLMEb, Diabetic rats treated with *Juglans regia* Leaves Methanolic Extract at two doses of 250 and 500 mg/kg ; D+Ac, Diabetic rats treated with Acarbose. (data are mean ± S.D., *n*=9). P values are shown as ap<0.0001, bp<0.003 vs. DC; c p<0.0001 vs. D+Ac

[Downloaded from jmp.ir on 2026-11-11]

[DOR: 20.1001.1.2717204.2010.9.33.25.1]

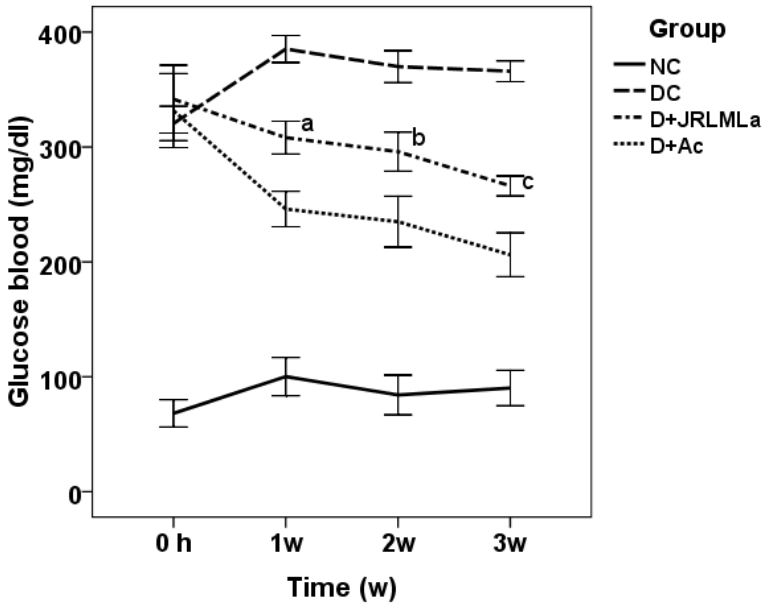


Fig. 2- Glycemic control by JRLMEa in alloxan-diabetic rats after 3 weeks treatment. PBG was measured at the end of first, second and third weeks of the experimental period. NC indicates Normal Control; DC, Diabetic Control; D+JRLMEa, Diabetic rats treated with *Juglans regia* Leaves Methanolic Extract (250 mg/kg); and D+Ac, Diabetic rats treated with Acarbose (20 mg/kg). (data are mean ± S.D., *n*=9).
P values are shown as $_a$p<0.008, $_b$p<0.002, $_c$p<0.001 vs. time (0h)

Effect of *Juglans regia* leaves methanolic Extract on insulin and glut-4 genes expression

Electrophoresis gel images revealed unchanged expression of both insulin (Fig. 4.A) and glut-4 (Fig. 4.B) transcripts by JRLME. β-Actin was used as internal control. Densitometric scanning also revealed no change in insulin (p>0.987) and glut-4 (p>0.843) genes transcripts, by JRLME, respectively as compared to DC group.

Effect of *Juglans regia* leaves methanolic Extract on rat intestinal sucrase and maltase activities

The JRLMEa showed strong inhibitory activity for both sucrase and maltase by 16.7 (±4.7) and 24.7% (±4.6), respectively (The data represent the average of three experiments). In addition, enzymatic inhibition for sucrase and maltase by acarbose (20 mg/kg) was 29.7 (±3.1) and 39.7% (±1.3). Further investigation is needed to isolate and identified the structure of active principle (s) of *Juglans regia* leaves against α-glucosidase activities and its (their) IC50.

Discussion

The hypoglycemic activity of walnut leaves was reported by previous studies [12]. In the present study, we displays that *Juglans regia* Leaves Methanolic Extract (JRLME) decreases the PBG levels in both short term and long term models. The administration of JRLME at dose of 250 (mg/kg) attenuates PBG more effectively than at dose of 500 (mg/kg) at 8h. Complex behavior of the plant extract may be originated from the competition between different compounds, so that dose-independent substances suppress dose-dependent effects, responsible for PBG levels reduction [12]. In long term model, the JRLMEa maintains the reduced PBG levels permanently, demonstrating therapeutics properties of the plant extract.

[Downloaded from jmp.ir on 2026-11-11]
[DOR: 20.1001.1.2717204.2010.9.33.25.1]

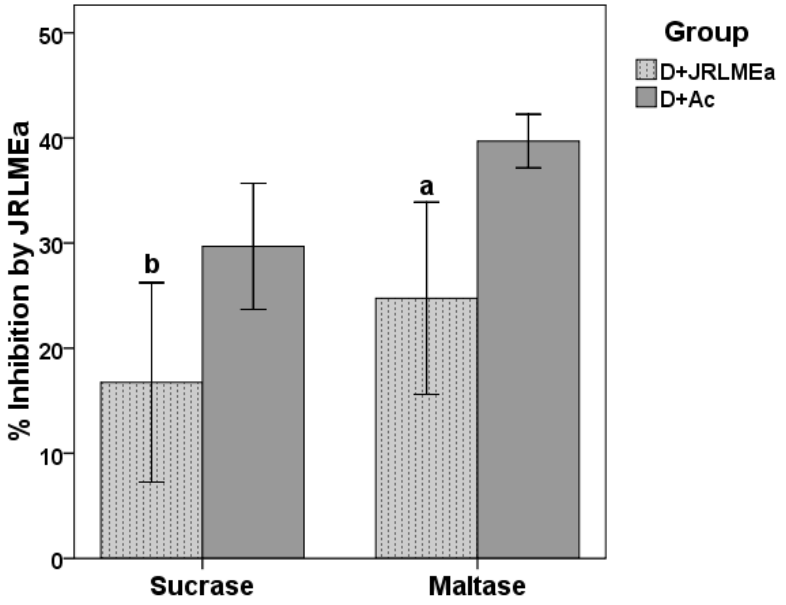


Fig. 3- *In vitro* inhibitory action of JRLMEa and Ac in intestinal α-glucosidase activity. D+JRLMEa indicates Diabetic rats treated with *Juglans regia* Leaves Methanolic Extract (250 mg/kg) and D+Ac, Diabetic rats treated with Acarbose (20 mg/kg). (data are mean ± S.D., *n*=9). P values are shown as ap<0.002, bp>0.315

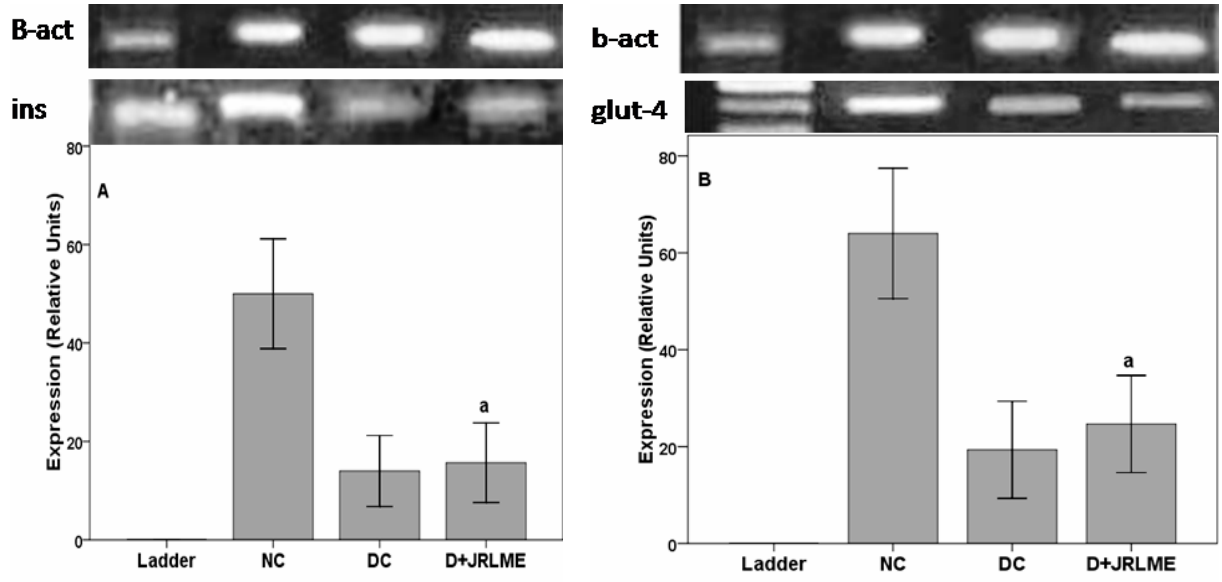


Fig. 4- Changes in insulin and cardiac glucose transporter-4 mRNA expression profile in rats. JRLME extract (250 mg/kg) was orally administered once daily for 3 weeks to D+JRLME group and also vehicle to NC and DC groups. Total mRNAs were separately prepared from the individual pancreas (insulin) and left ventricle (glut-4) of the rats. The relative levels of specific mRNAs were assessed by RT-PCR and images of radiographs were analyzed with TotalLab v1.10 from Phoretix using 1D analysis. Background was subtracted using the rolling disc method with a radius of 200, and density was measured as pixel intensity. (a) Analysis of insulin **transcripts (186 bp) in pancreas tissue in D+ JRLME group showed no elevated levels of** insulin **transcripts compared with DC rats (p>0.987). (b) Analysis of glut-4 transcripts (449 bp) in heart tissue in D+ JRLME group showed no elevated levels of glut-4 transcripts compared with DC rats (p>0.843). The data represent the average of three or four samples (only one image was shown here). NC: Normal Control; DC: Diabetic Control; D+JRLME: Diabetic rats treated with *Juglans regia* Leaves Methanolic Extract**

[Downloaded from jmp.ir on 2026-11-11]

[DOR: 20.1001.1.2717204.2010.9.33.25.1]

These effects might have been due to the increased release of insulin from remnant β-cells and/or regenerated β-cells [13, 14], restored insulin sensitivity [15], interference on absorption of dietary carbohydrates as well as disaccharides in small intestine [16] or facilitate utilization of glucose by peripheral tissues mediated by GLUT-4, a insulin dependent glucose transporter [17]. To understand the mechanisms by which *Juglans regia* leaves extract decrease PBG in short-term and long-term test, *in vitro* α-glucosidase inhibitory activity of the crude extract and modulation of insulin and glut-4 transcript levels were investigated.

The plant extract significantly inhibited α-glucosidase activity *in vitro* for both maltase and sucrase enzymes. However our results showed no changes in the insulin and glut-4 genes expression. The key compound responsible for inhibitory action of the plant extract may be phenolic substance, such as galic acid and caffeoylquinic acid [6, 18]. In conclusion, our data determine the use of the *Juglans regia* leaves was effective for glucose control of diabetic rats,witch may be correlated to inhibitory action of effective compounds of the plant extract.

References

1. Zimmet PZ. Diabetes epidemiology as a tool to trigger diabetes research and care. *J. diabeto.*1999; 42: 499 – 516.

2. Vessby J, Basu S, Mohsen R, Berne C,Vessby B. Oxidative stress and antioxidant status in type 1 diabetes mellitus. *J Intern Med*. 2002; 251: 69 – 76.

3. DeFronzo RA. Pathogenesis of type 2 diabetes: Metabolic and molecular implications for identifying diabetes genes. *J. Diabetes Rev.* 1997; 5: 177 – 269.

4. Ward A.O., Korsmo H. The Intestinal Brush Border Membrane in Diabetes. *J. Clin. Invest*. 1977; 60; 181 – 8.

5. Sarahroodi S, Rasekh HR, Kamalinejad M, Mahboubi S, Taheri Shalmani S and Nouri M. Glucose lowering effect of the water extract of septum of *Juglans regia L.* (Persian Walnut) fruit in male rats. Phcog. Mag. 2008; 14: 109 – 13.

6. Pereira JA, Oliveira I, Sousa A, Valenta˜ob P, Andrade PB, Ferreira ICFR, Ferreres F, Bento A. Walnut (*Juglans regia* L.) leaves: Phenolic compounds, antibacterial activity and antioxidant potential of different cultivars. *J. F. Chem. Tox*. 2007; 45: 2287 – 95.

7. Kumar S, Kumar D, Deshmukh RR, Lokhande PD, More SN, Rangari VD. Antidiabetic potential of *Phyllanthus reticulatus* in alloxan-induced diabetic mice. *J. Fitoter*. 2008; 79: 21 - 3.

8. Kameswara RB, Kesavulu MM, Giri R., Appa Rao C. Antidiabetic and hypolipidemic effects of *Momordica cymbalaria* Hook. fruit powder in alloxan-diabetic rats. *J. Ethnophar*. 1999; 67: 103 - 9.

9. Bhandaria MR, Jong-Anurakkuna N, Honga G, Kawabata J. α-Glucosidase and α-amylase inhibitory activities of Nepalese medicinal herb Pakhanbhed (Bergenia ciliata, Haw.). *J. Food Chem*. 2008; 106: 247 - 52.

10. Nishioka T, Kawabata J, Aoyama Y. Baicalein, α-glucosidase inhibitor from *Scutellaria baicalensis*. *J. Nat. Prod*. 1998; 61: 1413 – 5.

[Downloaded from jmp.ir on 2026-11-11]

[DOR: 20.1001.1.2717204.2010.9.33.25.1]

11. Toda M, Kawabata J, Kasai T. α-Glucosidase inhibitor from Clove (Syzgium aromaticum). *J. Biosc. Biotech. Biochem*. 2000; 64: 294 – 6.

12. Fathiazad F, Garjani A, Motavallian naini A. Study of hypoglycemic activity of the hydroalcoholic extract of *Juglans regia* in normal and diabetic rats. *J. Pharm. Sci*. 2006; 2: 13 – 7.

13. Esmaeili MA, Yazdanparas R. Hypoglycemic effect of *Teucrium polium*: studies with rat pancreatic islets. *J. Ethno*. 2004; 95: 27 – 30.

14. Sharma SB, Nasir A, Prabhu KM, Murthy PS. Antihyperglycemic effect of the fruit-pulp of *Eugenia jambolana* in experimental diabetes mellitus. *J. Ethno*. 2006; 104: 367 – 73.

15. Yolanda BL, Adriana GC. Effects of dietary polyunsaturated n−3 fatty acids on dyslipidemia and insulin resistance in rodents and humans. *The J. of Nutritional Biochemistry*. 2006; 17: 1–13.

16. Ortiz-Andrade RR, Garcia-Jimenez S, Castillo-Espana P, Ramirez-A G, Villalobos-Molina R, Estrada-Soto S. α-Glucosidase inhibitory activity of the methanolic extract from Tournefortia hartwegiana: an anti-hyperglycemic agent. *J. of Ethnopharmacol.* 2007; 109: 48 – 53.

17. Obatomi DK, Bikomo EO, Temple VJ. Antidiabetic properties of the African mistletoe in streptozotocin-induced diabetic rats. *J. Ethno*. 1994; 43: 13 – 7.

18. Amaral JS, Seabra RM, Andrade PB, Valent~ao P, Pereira JA, Ferreres F. Phenolic profile in the quality control of walnut (*Juglans regia* L.) leaves. *J. Food Chem*. 2004; 88: 373 – 9.

[Downloaded from jmp.ir on 2026-11-11]

[DOR: 20.1001.1.2717204.2010.9.33.25.1]