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Full Length Research Paper

Wild Olive Leaf Extracts Up regulate Insulin-signaling Gene Expression in Diabetic mice

¹Afaf S. Altalhi; ^{1&2**}Aziza M. Hassan; ³Shatha Dhaifallah Al-Harhi and ³Fatima Sulaiman Al-harbi

¹Biotechnology Department, Faculty of Science-Taif University, Taif-KSA

²Cell Biology Department, National Research Center, Dokki, Giza- Egypt

³Biology Department, Faculty of Science-Taif University, Taif-KSA

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Regulation of insulin gene expression in response to increases in blood glucose levels is essential for maintaining normal glucose homeostasis; however, the exact mechanisms by which glucose stimulates insulin gene transcription are unclear. Olive leaves extracts(OLE) are reported to have beneficial effects on people with normal and impaired glucose tolerance, the metabolic syndrome, type 2 diabetes, and insulin resistance. However, clinical results are controversial and the molecular characterization of OLE effects is limited. This study investigated the effects of OLE, prepared from wild olive leaves, on gene expression of insulin and glucagon in mice tissues. Diabetes in mice was induced by intraperitoneal injections of alloxan. The alterations in gene expressions of pancreatic insulin and hepatic glucagon were evaluated throughout the RT-PCR analysis. The results indicated that diabetic mice showed significantly increased in the expression of pancreatic insulin gene followed by significantly decreased in the expression of hepatic glucagon gene. The administration of OLE at 0.33 g/kg for six days before alloxan treatment; up-regulated pancreatic insulin and modulated hepatic glucagon genes expression. Meanwhile, administration of OLE after induced diabetic mice showed a highly significant increase in gene expression of pancreatic insulin followed by significantly decreased in gene expression of hepatic glucagon. Results suggest that the OLE possess a potent anti-hyperglycemic effect, which may be due to the presence of antioxidants such as polyphenols, these antioxidant activities restraining the oxidative stress which is widely associated with diabetes pathologies and complications.

Keywords: Olive leaves, Insulin, Glucagon, Diabetes, Gene expression

INTRODUCTION

Diabetes mellitus is a common endocrine disease characterized by hyperglycemia and long-term complications affecting the eyes, kidneys, nerves, and

blood vessels (Kim et al., 2007 and Tuttle et al., 2007). At present, there are more than 194 million people with diabetes worldwide (Liao et al., 2015), and this number is estimated to increase to 333 million by 2025 (Zhu et al., 2010). The management of diabetes is considered a global problem, and a cure has yet been discovered. The β -cells in pancreatic tissue secrete insulin hormone that regulated

*Corresponding Author's Email: Dr_azizahassan@yahoo.com

blood glucose homeostasis. The harm and dysfunction of the β -cells participated in the pathogenesis of type 1 and type 2 diabetes (Khodabandehloo et al., 2016). The secretion of the insulin is attributed to control by hormonal, nutrients and by pharmacological factors. Amongst these; glucose remains the most affected regulator of the machinery of insulin secretion (Fu et al., 2013).

The therapeutic measurements include the use of insulin, analogs, and alpha glycosidases inhibitors like acarbose and biguanides for the treatment of hyperglycemia. However, these drugs are associated with many side effects including hypoglycemia at higher doses, liver problems, lactic acidosis, obesity, osteoporosis sodium retention and diarrhea (Hamza et al., 2010; Stades et al., 2004; Chiang et al., 2007). Currently, many herbal medicines therapeutic available been recommended for the treatment of diabetes. There are many natural plants have been investigated recently as possible treatments to prevent and improve diabetes (Ryu et al., 2005; Gupta et al., 2009 and Adiga et al., 2010). Herbal drugs are prescribed widely because of their effectiveness, less side effects and relatively low cost (Dixon et al. 2005). One of the major classes of bioactive compounds is plant polyphenols which are found in seeds, fruits, leaves, and quite existing in the diet, are potentially influential for human health (Prior and Gu 2005; Dixon et al. 2005).

Wild olive (*Olea europaea*) is grows over a very wide area, ranging from South Africa through to Ethiopia, and south-west Saudi Arabia. The olive tree specifically its leaves have been applied for the treatment of diabetes, fever, wounds, atherosclerosis, gout and hypertension since timeworn (Jänicke et al., 2003). Olive leaf has been traditionally applied for century to treat and prevent various diseases. It is used as antimicrobial, hypotensive, hypoglycemic, antiarrhythmic, vasodilator, anti-atherosclerotic, antihepatotoxic, anti-nephrotoxic and antioxidants to enhance the immune system (Jemai et al., 2009; Zari and Al-Attar 2011). Polyphenols such as hydroxytyrosol and oleuropein in olive leaf improve hepatic, cardiac, and metabolic parameters by decreasing oxidative stress and inflammation in fed rats with a high-fat diet; suggested to be useful in the prophylaxis in autoimmune diabetes (Cvjeti et al., 2010; Poudyal et al., 2010).

The objectives of this study were to investigate the effects of the aqueous crude extract of wild olive on up-regulating insulin signaling genes expression during hyperglycemia in mice.

MATERIALS AND METHODS

Olive Leaf Extract Preparation

Olive leaves were collected from a wild olive tree in Taif province (Western Saudi Arabia). Leaves were washed to remove impurities such as dust and then dried. The

aqueous extract of olive (AEO) was prepared by soaked 50 gm of the powder leaves in 500 ml of distal water and kept overnight in shaking incubator at room temperature (Sanarya et al., 2011). After 48 hours, the obtained solution was passed through a filter to remove insoluble particles. Finally, the extract was lyophilized with a freeze-dryer-cryodo (ALPHA, 1-2 LD plus).

Experimental Animals

Adult males Swiss albino mice (*Mus musculus*) MFI strain, 8-10 weeks old weighed 25-30g, were obtained from the animal house of King Fahad Medical Research Centre KAU. Animals were housed in polyplastic cages with steel wire tops in an air-conditioned room ($22\pm 2^\circ\text{C}$, 45-75 % relative humidity) maintained in a controlled atmosphere of 12h light / 12h dark cycles. The mice were kept in maintained on standard laboratory diet (20% crude protein 4% crude fat, 3, 50% crude fiber and energy 2850 k. cal/kg diet) and water was provided ad libitum and all the animals were fasted overnight before sacrificed and tissues collection. Total numbers of 40 male mice were used for control and four groups' treatments (8 mice in each treatment). The handling of the animals was approved by the local Ethical Committee for the Care and use of Laboratory Animals.

Model of alloxan diabetes

Alloxan-induced hyperglycemia (Nukatsuka et al. 1989) in male mice was used as the criterion for diabetes. Diabetes was induced in mice by a single intraperitoneal injection of freshly prepared alloxan solution in normal saline at a dose of 120 mg/kg body weight (b.w.)

Experimental Design and Animal Groups

After an acclimatization period of one week, the animals were randomly assigned to five groups (8 mice/group), depending on their treatment and housed in filter-top polycarbonate cages. The animal groups were divided as follows: Group 1, untreated control (fed on basal diet); Group2, treated daily with oral dose of AEO at 0.33g/kg, as referenced by Sanarya et al., (2011), for six days; Group3, diabetic mice (treated with a single intraperitoneal injection of alloxan at a dose of 120 mg/kg body weight (b.w.), Group 4, treated daily with oral dose of AEO for six days before alloxan treatment and Group5, treated with alloxan then orally treated with AEO for six days. At the end of the experimentation period, animals were killed by cervical decapitation and a part of liver and pancreas were immediately isolated for gene expression analysis.

Gene Expression Analysis

RNA isolation and Reverse transcription (RT)

Total RNA was isolated from hepatic and pancreatic tissues using Trizol reagent (Invitrogen, Paisley, UK). RNA samples were subjected to DNase treatment to remove genomic DNA contamination in the presence of RNase inhibitor.

Quantity, purity, and quality of RNA

Quantitative estimation of RNA samples was done by a single beam UV-Spectrophotometer (GENESYS10uv) by measuring the RNA concentration at 260 nm and 280 nm. The purity of RNA was checked by means of absorbance ratios A260/A280 for protein and DNA contamination. Further, the samples were run on 1% agarose electrophoresis to check the quality of RNA (Sambrook et al., 1989).

Reverse transcription of RNA

The first-strand cDNA was prepared from the 5 µg of total RNA using Fermentas kits (Sigma, St. Louis, MO) as per the manufacturer's instructions. The reaction was carried out at 25°C for 10 min, followed by 1 h at 42°C, and finished with the denaturation step at 94°C for 5 min. The reaction tubes containing RT preparations were flash-cooled in an ice chamber until being used for cDNA amplification through polymerase chain reaction, (Brun et al., 2006; Hassan et al., 2012).

Polymerase chain reaction (PCR)

The first-strand cDNA from different mice samples was used as the template for amplification by the PCR with the following pairs of specific primers (from 5'to 3'): Insulin forward: (5'CAGAAACCATCAGCAAGCAGG-3' and Insulin reverse 5'-TTGACAAAAGCCTGGGTGGG-3'), Glucagon forward: 5'-AGAAGAAGTCGCCATTGCTG-3' and Glucagon reverse 5'CCGCAGAGATGTTGTGAAGA3'), to determine the relative expression as an internal control, primers for β-actin were designed 5'-TGGGACTATGGACTCCGTTC-3' and 5'-GCACCACATCCAAGACAGAG-3' that are taken from the literature (Prasadan, et al., 2002). β-actin, a house-keeping gene was used for normalizing mRNA levels of the target genes. PCR cycling parameters were one cycle of 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 70 °C for 40 s, and 72 °C for 5 min. The PCR products were electrophoresis onto ethidium bromide stained a 2.0% agarose gels. The ethidium bromide-stained gel bands were scanned and the signal intensities were quantified by the computerized Gel-Pro program.

Statistical analysis

All data were statistically analyzed using One-way analysis of variance (ANOVA) using the SPSS 11 program, followed by the Post-Hoc test for multiple comparisons. The significance of the differences among treatment groups was determined with the Waller–Duncan k-ratio (Waller and Duncan 1969). All statements of significance were based on a probability of $P < 0.05$.

RESULTS

Determination of gene expression

Analysis of the results of gene expression was based on quantifying the signal intensities in each band by using the computerized Gel-Pro image analyzer (Version 3.1 for Windows3). Bands produced from amplifying cDNA of pancreatic insulin, hepatic glucagon and the housekeeping gene β-actin as a control were analyzed. Results were expressed as the ratio between maximum optical density (max.OD) for each band of the target amplification product and the corresponding max. OD of β-actin. Expression of insulin mRNA in pancreas of the different groups of mice is summarized in **Table 1 & Figure 1** show the ratio of optical density of Insulin / β-actin expression in the pancreatic and hepatic tissues of controls and treated animals. Insulin expression was found to be significantly increased in alloxan treated group (0.50 ± 0.04) in compared to the control (0.21 ± 0.02) and OLE treated mice (0.37 ± 0.014). Our results indicated that, diabetic mice showed significantly increased in the expression of pancreatic insulin gene followed by significantly decreased in the expression of hepatic glucagon gene as shown in **Figure 2**. The administration of OLE at 0.33 g/kg for six days before alloxan treatment; up-regulated pancreatic insulin and modulated hepatic glucagon genes expression (insignificantly changed in compared to the control group). Meanwhile, administration of OLE after induced diabetes showed highly significant increased in gene expression of pancreatic insulin (0.57 ± 0.014) followed by significant decreased in gene expression of hepatic glucagon (0.28 ± 0.01). Our results suggest that the OLE possess a potent anti-hyperglycemic effect and its effects were more pronounced when it administrated as a therapeutic not as a protector.

Table 1: Effects of olive leaf extract on mRNA expression of Insulin and Glucagon in pancreatic and hepatic tissues of mice treated with Alloxan

Treatment	Pancreatic Tissue		Hepatic Tissue	
	Insulin	β-actin	Glucagon	β-actin
Control	0.21 ± 0.02 ^d	0.55 ± 0.017	0.45 ± 0.008 ^a	0.58 ± 0.06
Alloxan	0.50 ± 0.04 ^b	0.70 ± 0.01	0.25 ± 0.015 ^{bc}	0.84 ± 0.02
OLE	0.37 ± 0.014 ^c	0.56 ± 0.02	0.21 ± 0.015 ^c	0.67 ± 0.003
Alloxan + OLE	0.57 ± 0.014 ^a	0.55 ± 0.017	0.28 ± 0.01 ^b	1.04 ± 0.017
OLE + Alloxan	0.48 ± 0.010 ^b	0.59 ± 0.05	0.47 ± 0.012 ^a	1.30 ± 0.008

Within each column, means superscript with different letter are significantly different (P <0.05).

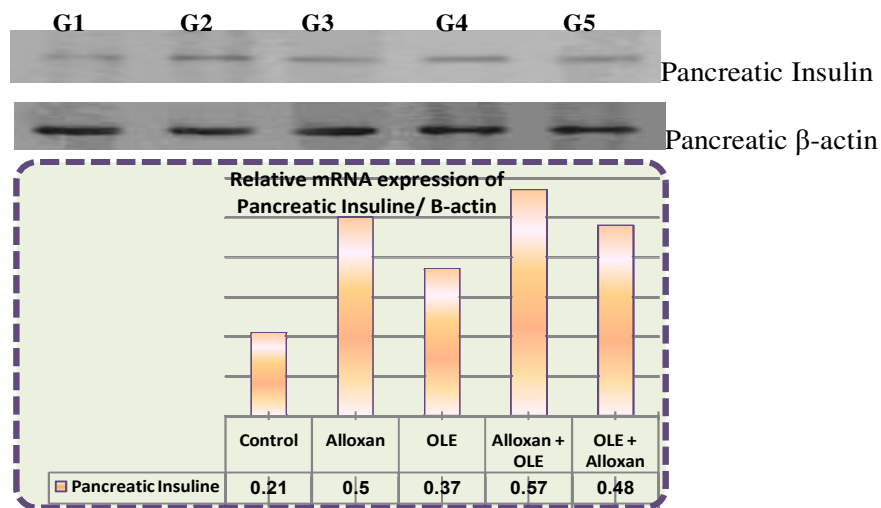


Figure 1: Histogram and photograph showing the effect of OLE Treatment on the relative transcript level of pancreatic insulin in Alloxan-treated mice. Results were obtained from three independent experiments and expressed as Mean ± SEM. Group 1: Control, Group 2: Alloxan, Group 3: OLE, Group 4: Alloxan + OLE and Group 5: OLE + Alloxan.

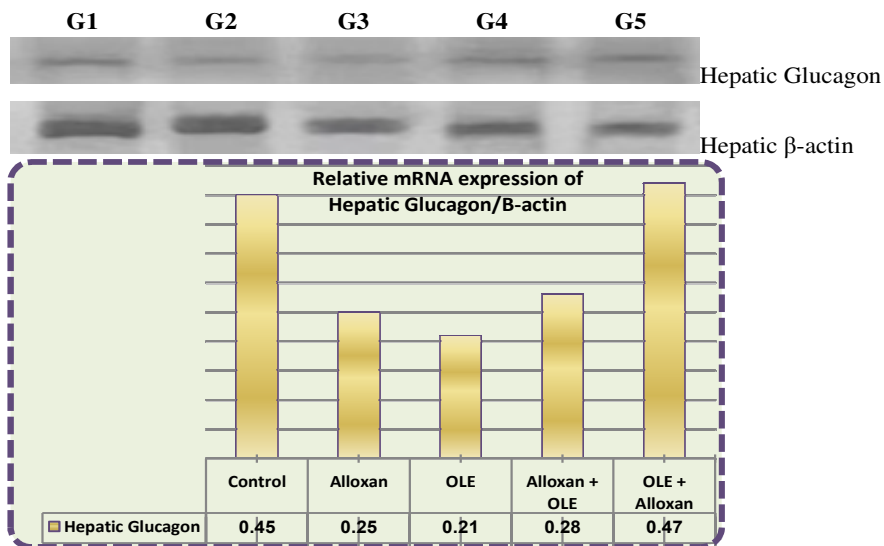


Figure 2: Histogram and photograph showing the effect of OLE Treatment on the relative transcript level of hepatic glucagon in Alloxan-treated mice. Results were obtained from three independent experiments and expressed as Mean ± SEM. Group 1: Control, Group 2: Alloxan, Group 3: OLE, Group 4: Alloxan +OLE and Group 5: OLE+ Alloxan.

DISCUSSION

Increasing the glucose levels in the blood stimulate insulin gene expression in the β -cells of the pancreatic tissues. Although different transcription factors have been involved in glucose-stimulated transcription of the insulin gene, the proper molecular mechanisms directing to up-regulation of insulin gene expression are obscure. Present studies investigated the effects of the aqueous extract of wild olive leaf on up-regulating insulin signaling genes expression during hyperglycemia in mice.

Diabetes can be induced in animals by streptozotocin and alloxan which produce active oxygen species accountable for diabetes complexity (Hamden et al., 2008). The present study employed such a diabetic animal model system to examine the hypoglycemic effect of wild olive leaf extracts. The results indicated that insulin expression was found to be significantly increased in the alloxan-treated group compared to the control followed by significantly decreased in glucagon expression in pancreatic and hepatic tissues. It is well known that hyperglycemia leads to the overproduction of free radicals and the non-enzymatic glycation of protein which exert deleterious effects on different organs acting in the glycemia regulation such pancreas and liver (Matough et al., 2012).

Latterly, considerably attentiveness has been focused on antioxidants in food that is prospect substance for preventing diseases caused by oxidative stress inclusive diabetes because of their low toxicity and distinguishes biological activity. In fact, previous studies reported that scavengers of oxygen radicals are effective in preventing diabetes in experimental animal models (Hamden et al., 2008). Olive is a well-known medicinal plant that has been used in traditional oriental medicine for several thousand years. However, one issue of concern regarding the use of olive as a therapeutic agent is its marginal anti-diabetic effects compared with commercially available oral hypoglycemics (Jemai et al., 2009; Zari and Al-Attar 2011).

The present results showed that OLE had significant hypoglycemic alloxan-induced diabetic mice via up-regulating insulin gene expression and down-regulated glucagon gene expression in the tissues of mice, Table 1. In agreement with the present results, Jemai et al., (2009), revealed that; the administration of oleuropein- and hydroxytyrosol-rich extracts significantly decreased the serum glucose and cholesterols (Dragana et al., 2011; Jouad et al., 2001). The eventual mechanism responsible for the hypoglycemic activity of OLE may result from a potentiating of glucose-induced insulin release or increased peripheral uptake of glucose (Eriko et al., 2003). So there have been two possible mechanisms suggested to explain the hypoglycemic effect of the olive leaf extract; to improve glucose-induced insulin release, and increased peripheral uptake of glucose (Karakaya, 2009). Olive leaves' oleuropein has been shown to fast the

cellular uptake of glucose, directing to reduced plasma glucose (Sangi et al., 2015). Oleuropein is considered to be a glycoside, so it could potentially act as a transporter of glucose such as a sodium-dependent glucose transporter (SGLT1) found in the epithelial cells of the small intestine, thereby permitting its entry into the cells (Wolffram et al., 2002). Glucagon is the opposing hormone to insulin; its job is to raise blood levels of glucose and inhibits the insulin secretion. It is believed that long-term administration of the plant extract could provide a chance for regeneration of more insulin producing-Beta cells with consequent decrease of glucagon immune-expression and normalization of the blood glucose level and this finding confirmed our results (Sharma et al., 2015). Also Bock et al., reported that OLE supplementation was associated with a reduction in glucose and insulin excursion after oral glucose challenge, suggesting an improvement in both pancreatic b-cell function and insulin sensitivity (Bock et al., 2013). The present study, indicated that OLE was more effective when it administered after induced diabetes, as a therapeutic agent than a protector one. In these concerns, Bock et al., (2013) observed the effects of OLE supplementation and compared the results to common diabetic therapeutics (particularly metformin), and confirmed that OLE could have clinical significance for patients with type 2 diabetes, as the OLE improved insulin secretion to further aid glucose regulation, which does not occur with the use of metformin, suggesting an improvement in both pancreatic B-cells function and insulin sensitivity. Another study demonstrated a 28 % improvement in insulin sensitivity after treatment with 30 mg pioglitazone; however, it was 35 % improvement in insulin after treated with OLE (Miyazaki et al., 2002).

CONCLUSION

We demonstrate that wild olive leaf extracts, at 0.33 g/ kg for six days exhibited a pronounced hypoglycemia effects via up-regulates insulin expression gene and down-regulated glucagon gene expression in an experimental diabetic model. Olive leaf extract was more effective when it administered after induced diabetes, as the therapeutic agent than a protector one. These therapeutic effects highlighted on the olive leaves extract as a source of antioxidants able to prevent the frequency of oxidative stress-related metabolic diseases such as diabetes.

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REFERENCES

- Adiga S, Bairy KL, Meharban A, Punita IS (2010): Hypoglycemic effect of aqueous extract of *Trichosanthes dioica* in normal and diabetic rats. *Int J Diabetes Dev Ctries*; 30:38-42.
- Bock M, José G, Derraik B, Christine M, Brennan, Janene B, Biggs, Philip E, Morgan, Steven C, Hodgkinson, Paul L, Hofman, Wayne S, Cutfield (2013): Olive (*Olea europaea* L.) Leaf Polyphenols Improve Insulin Sensitivity in Middle-Aged Overweight Men: A Randomized, Placebo-Controlled, Crossover Trial. *plosone.org* vol.8 (3), e57622, 1 – 8
- Brun ME, Gasca S, Girard C, Bouton K, De Massy B, De Sario A (2006). Characterization and expression analysis during embryo development of the mouse ortholog of MLL3, *Gene* 371: 25–33.
- Chiang CK, Ho TI, Peng YS, Hsu SP, Pai MF, Yang SY, Hung KY, Wu KD (2007): Rosiglitazone in diabetes control in hemodialysis patients with and without viral hepatitis infection: effectiveness and side effects. *Diabetes Care*; 30: 3-7.
- Cvjeti-anin T, Mijlkovi D, Stojanovi I, Dekanski D, Stoši-Gujji S (2010): Dried leaf extract of *Olea europaea* ameliorates islet-directed autoimmunity in mice. *Br J Nutr*; 103: 1413-24.
- Dixon RA, Xie DY, Sharma SB (2005). Proanthocyanidins—a final frontier in flavonoid research? *New Phytol.* 165, 9–28.
- Dragana D, Slavica R, Nevena VR, Nataša DP, Aleksandar D, Dušan MM (2011): Olive leaf extract modulates cold restraint stress-induced oxidative changes in rat liver J. *Serb. Chem. Soc.* 76 (9) 1207– 1218.
- Eriko K, Shinya Y, Isafumi M, Mitsuhiro K, Kazuaki K, Yasuhiro O, Yoji T (2003): Identification of Anti- α -Amylase Components from Olive Leaf Extracts *Food Science and Technology Research*; Vol. 9 , No. 1 pp.35-39.
- Fu Z, Gilbert ER, Liu D (2013). Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. *Curr Diabetes Rev.* 2013 Jan 1;9(1):25-53. Review.
- Gupta S, Sharma SB, Bansal SK, Prabhu KM (2009): Antihyperglycemic and hypolipidemic activity of aqueous extract of *Cassia auriculata* L. leaves in experimental diabetes. *J Ethnopharmacol*; 123: 499-503.
- Hamden K, Carreau S, Boujbiha MA, Lajmi S, Aloulou D, Kchaou D, El feki A (2008): Hyperglycaemia, stress oxidant, liver dysfunction and histological changes in diabetic male rat pancreas and liver: Protective effect of 17 β - estradiol. *Steroids*, 73, 495–501.
- Hamza N, Berke B, Cheze C, Agli AN, Robinson P, Gin H, Moore N (2010): Prevention of type 2 diabetes induced by high fat diet in the C57BL/6J mouse by two medicinal plants used in traditional treatment of diabetes in the east of Algeria. *J Ethnopharmacol*; 128:513-518.
- Hassan AM, Abdel-Aziem SH, Abdel-Wahhab MA (2012): Modulation of DNA damage and alteration of gene expression during aflatoxicosis via dietary supplementation of *Spirulina* (*Arthrospira*) and whey protein concentrate. *Ecotoxicol Environ Saf* 79:294–300
- Jänicke J, Grünwald, Brendler T (2003): *Handbuch Phytotherapie*, Wissenschaftliche Verlagsgesellschaft, Stuttgart, Germany.
- Jemai H, El Feki A, Sayadi S (2009): Antidiabetic and antioxidant effects of hydroxytyrosol and oleuropein from olive leaves in alloxan-diabetic rats. *J Agric Food Chem*; 57: 8798-804.
- Jouad H, Haloui M, Rhiouani H, El Hilaly J, Eddouks M (2001): Ethnobotanical survey of medicinal plants used for the treatment of diabetes, cardiac and renal diseases in the North Center Region of Morocco (fez-Boulemane). *J. Ethnopharmacol*, 77, 175–182.
- Karakaya S (2009): Olive tree (*Olea europaea*) leaves: potential beneficial effects on human health. *Nutr Rev*; 67:632–638.
- Khodabandehloo H, Gorgani-Firuzjaee S, Panahi G, Meshkani R (2016). Molecular and cellular mechanisms linking inflammation to insulin resistance and β -cell dysfunction. *Translational Research* Volume 167, Issue 1, January 2016, Pages 228-256
- Kim YM, Namkoong S, Yun YG, Hong HD, Lee YC, Ha KS, Lee H, Kwon HJ, Kwon YG, Kim YM. 2007: Water extract of Korean red ginseng stimulates angiogenesis by activating the PI3K/Akt-dependent ERK1/2 and eNOS pathways in human umbilical vein endothelial cells. *Biol Pharm Bull*; 30:1674-1679.
- Liao Z, Chen X, Wu M (2015): Antidiabetic effect of flavones from *Cirsium japonicum* DC in diabetic rats. *Arch Pharm Res*; 33:353-362.
- Matough FA; Budin SB, Hamid ZA, Alwahaibi N, Mohamed J (2012). The Role of Oxidative Stress and Antioxidants in Diabetic Complications. *Sultan Qaboos Univ Med J.* 2012 Feb; 12(1): 5–18
- Miyazaki Y, Matsuda M, DeFronzo RA (2002): Dose-response effect of pioglitazone on insulin sensitivity and insulin secretion in type 2 diabetes. *Diabetes Care* 25: 517–523.
- Nukatsuka M, Sakurai H, Kawada J (1989): Generation of alloxan free radicals in chemical and biological systems: implication in the diabetogenic action of alloxan. *Biochem. Biophys. Res. Commun.* 165, 278|283
- Poudyal H, Campbell F, Brown L (2010): Olive leaf extract attenuates cardiac, hepatic, and metabolic changes in carbohydrate-, high fat-fed rats. *J Nutr* 140: 946-53.
- Prasadan K, Daume E, Preuett B, Spilde T, Bhatia A, Kobayashi H, Hembree, M, Manna P, Gittes GK (2002): Glucagon Is Required for Early Insulin-Positive Differentiation in the Developing Mouse Pancreas. *Diabetesvol. 51 no. 11 3229-3236*
- Prior RL, Gu L (2005). Occurrence and biological significance of roanthocyanidins in the American diet. *Phytochemistry* 66, 2264–2280.
- Ryu JK, Lee T, Kim DJ, Park IS, Yoon SM, Lee HS, Song SU, Suh JK (2005): Free radical-scavenging activity of Korean red ginseng for erectile dysfunction in non-insulin-dependent diabetes mellitus rats. *Urology*; 65: 611-615.
- Sambrook J, Fritsch EF, Maniatis T (1989). *Molecular Cloning. A Laboratory.* Cold Spring Harbor Laboratory Press, New York.
- Sanarya T, Al-Badri, Imad M, Al-Ani, Karim A, Al-Jashamy (2011): The Effect of Aqueous Olive Leaves Extract on the Pancreatic Islets of Streptozotocin Induced Diabetes Mellitus in Mice. *ANNALS OF MICROSCOPY* Vol 11, 4 – 11
- Sangi SMA, Sulaiman MI, Abd El-wahab MF, Ahmedani EI, Ali SS (2015). Antihyperglycemic effect of thymoquinone and oleuropein, on streptozotocin-induced diabetes mellitus in experimental animals. *Pharmacogn Mag.* 2015 Oct; 11(Suppl 2): S251–S257
- Sharma R, Amin H, Pradeep G, Prajapati G (2015). Antidiabetic claims of *Tinospora cordifolia* (Willd.) Miers: critical appraisal and role in therapy. *Asian Pac J Trop Biomed*, 5(1): 68-78.
- Stades AM, Heikens JT, Erkelens DW, Holleman F, Hoekstra JB (2004): Metformin and lactic acidosis: cause or coincidence? A review of case reports. *J Intern Med*; 255: 179-187.
- Tuttle KR, McGill JB, Haney DJ, Lin TE, Anderson PW, PKC-DRS, PKC-DMES, PKC-DRS (2007): Kidney outcomes in long-term studies of ruboxistaurin for diabetic eye disease. *Clin J Am Soc. Nephrol*; 2:631-636.
- Waller RA, Duncan DB (1969): A Bayes rule for the symmetric multiple comparison problems, *J. Am. Stat. Assoc.* 64 1484–1503.
- Wolffram S, Bločck, M, Ader P (2002): Quercetin-3-glucoside is transported by the glucose carrier SGLT1 across the brush border membrane of rat small intestine. *J Nutr*; 132: 630–635.
- Zari TA, Al-Attar AM (2011): “Therapeutic effects of olive leaves extract on rats treated with a sublethal concentration of carbendazim,” *European Review for Medical and Pharmacological Sciences*, vol. 15, no. 4, pp. 413–426.
- Zhu CF, Peng HB, Liu GQ, Zhang F, Li Y (2010): Beneficial effects of oligopeptides from marine salmon skin in a rat model of type 2 diabetes. *Nutrition*; 26:1014-1020.