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Chemical and Biological Assessment of African Olive Leaf Extract

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ABSTRACT

Since the leaves of the Mediterranean olive have been recommended in the literatures as a remedy for the treatment of diabetes and hypertension, as well as they also contain antioxidant agents, we decided to investigate the possible biological effects of chemically standardized African olive leaf extract (OLE) of *Olea europaea* subspecies africana growing wild in Saudi Arabia. Chemical standardization of oleuropein in African OLE using HPLC showed a high percentage reached up to 30%. Total phenols were 36 mg/g calculated as gallic acid equivalents (GAE) while the total amount of flavonoids was 1.35 mg/g calculated as rutin equivalents (RE). The in vitro antioxidant activity of African OLE showed an IC_{50} of 60.2 µg/ml using diphenylpicrylhydrazine (DPPH) assay. The biological analysis of OLE included screening for potential antihypertensive and hypoglycemic activity. Daily treatment for six weeks with African OLE (200 mg/kg) resulted in partial mitigation of L-N-nitroarginine methyl ester (L-NAME) induced hypertension relative to the untreated group. OLE was tested at three dose levels (100, 300, 500 mg/kg) for anti-hyperglycemic activity. OLE exhibited blood glucose-lowering activity against streptozotocin (STZ) induced hyperglycemia in rats. The highest dose of OLE (500 mg/kg) showed a significant decrease in blood-glucose level by 69.6% at the 4th week of treatment compared to the untreated diabetic group. Therefore, OLE showed protective effects against L-NAME-induced hypertension and STZ-induced diabetes. The mechanisms underlying the observed anti-hyperglycemic activity can be attributed, at least partly, to enhance skeletal muscle uptake of glucose, inhibition of hepatic gluconeogenesis, insulinomimetic effect and antioxidant activity.

Keywords: Olea africana, African olive, anti-hyperglycemic, antihypertensive, antioxidant, HPLC.

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INTRODUCTION

Mediterranean diet rich in olive drupes and olive oil is associated with the lower incidence of cardiovascular disease, cancer, inflammation and stroke [1,2]. Olive (*Olea europaea* L.) phenolics are known as powerful antioxidants, both in vitro and in vivo and it is known that olive oil represents a key healthy component of Mediterranean diet [3]. Not only olive oil, but olive leaf also has different beneficial effects on human health [4]. Compounds obtained from olive leaf extracts (OLE) have been subject to numerous investigations. OLE from Mediterranean olive has been commercialized as a food supplement, which can be consumed in the form of tea, syrup and capsules. OLE is well-known for its hypotensive effects [5] and its antioxidant properties [6], hypoglycemic [7], cardioprotective [8], antimicrobial [9], radioprotective [10], antiatherogenic [11], antitumoral [12], anti-inflammatory [13], hypocholosterolemic, hepatoprotective [14] and anti-viral properties [15]. The main constituent of the olive leaf is the iridoid glycoside oleuropein, which is metabolized in the body to calcium elenolate, which is apparently responsible for many of the pharmacological actions of the olive leaf. Furthermore, the olive leaf contains triterpenes (oleanolic and maslinic acid), flavonoids (luteolin, apigenine, rutin), and chalcones (olivin, olivin-diglucoside). It has been traditionally used in hypertonia, arteriosclerosis, rheumatism, gout, diabetes mellitus, and fever [16]. In South western regions of Saudi Arabia (Belgurashi governorate), another type of olive grows wild commonly known as African olive, *Olea europaea* subsp. africana (Mill) P.S. Green (O. europaea subsp. cuspidate (Wall. ex G. Don) Cif , O. chrysophylla Lam., O. africana Mill.).

The objective of the present study is the preparation of chemically, and biologically standardised olive leaf extracts (*O. europaea* subsp africana (Mill) P.S. Green) from the plant grows wild in huge amounts in southern areas of Saudi Arabia. Chemical analysis included determination of oleuropein content of the standardized olive leaf extract. Biological screening was conducted for the potential antihypertensive and antihyperglycemic activities of OLE extract. This is in addition to investigation of the potential underlying mechanisms for its proposed antihyperglycemic action.

MATERIAL AND METHODS

Chemicals

Ammonium molybdate, carboxymethylcellulose (CMC), α-D-glucose, HEPES buffer, Krebs' buffer, malachite green, methanol, phenylmethylsulphonyl fluoride, streptozotocin (STZ), L-N-nitroarginine methyl ester (L-NAME), and gliclazide were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA). Ultra-Sensitive Rat Insulin Enzyme-Linked Immunosorbent Assay Kit was purchased from Crystal Chem. Inc. (Downers Grove, IL, USA). All other chemicals were of analytical grade.

Animals

Male Sprague Dawley rats (150–200 g) were obtained from the animal facility of the National Research Center (Giza, Egypt). The animals were provided with standard pellet diet and water ad libitum. They were kept at standard living conditions (room temperature of 25± 2 ^{o}C, 45–55% relative humidity and 12 h dark/light cycle). Procedures involving animals and their care were conducted in conformity with the institutional guidelines of Ain Shams University (Cairo, Egypt).

Plant material

O. europaea subsp. africana (Mill) P.S. Green leaves were collected at Belgurashi governorate (Saudi Arabia) at April 2011. The plant was identified by the staff of the Department of Biological Science, Faculty of Science, King Abdulaziz University, Saudi Arabia. A Herbarium specimen was kept at the Herbarium of the Department of Natural Products and Alternative Medicine, Faculty of Pharmacy, King Abdulaziz University (#OE1125A).

Extraction

Powdered leaves (500 g) were extracted with 80 % methanol till exhaustion at 60 ^{o}C using IKA Ultra-Turrax homogenizer T 25 digital (Janke and Kunkel, IKA Labortechnik, Stauten, Germany) to give 160 g of African olive leaf extract (OLE).

HPLC analysis

The HPLC system consisted of an Alliance Waters separations module 2695, photodiode array detector model 2996, (Milford, MA, USA). Column heater was set to 25±2 ^{o}C. The control of HPLC system and data processing were performed by Empower software (Build 1154, Waters). Analytes were separated on Agilent Zorbax Eclipse XDB-C18 (250mm×4.6mm i.d., 5µm particle diameter) protected with Agilent Zorbax XDB-C18 pre-column (Agilent Technologies, Palo Alto, CA, USA). The mobile phase was programmed to deliver 25 % acetonitrile and 75 % ammonium acetate (0.02 M, pH 4.5 with acetic acid) with a flow rate of 1.0 mL min^{-1}. The UV detector was set at 280 nm.

Determination of oleuropein content in OLE

About 56 mg of OLE was accurately weighed into 100 ml volumetric flask and dissolved in 80 % MeOH using sonication for 10 min at 60 ^{o}C. The volume was adjusted to 100 mL using the same solvent. Sample of 10 ml was filtered through Nylone membrane syringe filters (0.45 micron), and an injection volume of 10 uL was used for HPLC-DAD analysis. A stock solution of oleuropein, 2 mg/mL was prepared in 25% acetonitrile-water (v/v). Serial dilutions were prepared spanning the range 50 – 2000 ng/µL. A volume of 10 uL was injected for HPLC-DAD analysis.

In vitro antioxidant assay (DDPH assay)

In order to measure the antioxidant activity, 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical scavenging assay was used. The method was carried out as described by Braca et al. [17]. An aliquot of DPPH solution (4.5 mg in 100 ml MeOH) was prepared and 1.0 ml of this solution was added to 3.0 ml of OLE solution in methanol at different concentrations (20-300 µg/ml of alcohol extract). Thirty minutes later, the absorbance was measured at 517 nm. A blank was prepared without adding extract. Ascorbic acid 1mM was used as standard. Lowering the absorbance of the reaction mixture indicates high free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\%\text{Inhibition} = (A_{control}-A_{Test}) \times 100/A_{control}$$

Determination of total phenolic compounds

Total phenolics in the OLE were determined using Folin-Ciocalteu reagent according to the method described by Slinkard and Singleton [18] using gallic acid as a standard phenolic compound. Briefly, 7 ml distilled H_2O, 0.5 ml Folin-Ciocalteu reagent and 0.5 ml of OLE solution (conc. 500 µg/ml) was mixed together. After 3 min, 2 ml of 20% Na_2CO_3 were added and heated at 100°C for 1 min in a water bath. Gallic acid (0.03–0.46 mg /ml) was measured as reference standard. Absorbance was measured at 685 nm after cooling in darkness, and the results were expressed in mg of gallic acid equivalents (GAE) / g dry weight.

Determination of total flavonoids content

Total flavonoid content was measured by the aluminum chloride colorimetric assay described by Lamaison and Carnat [19] with slight modification of Elberry et al. [20]. An aliquot of 1 ml of OLE (3.0 mg/ml) or standard solution of rutin (0.04-0.66 mg/ml) was added to 1 ml of 2% methanolic $AlCl_3$, $6H_2O$. The absorbance was measured 10 min later at 430 nm. The total flavonoid content (mg/g) was determined from the calibration curve, and expressed as mg rutin equivalents (RE) per gram of dried extract. All the determinations were carried out in triplicate, and the mean values were calculated.

Determination of antihyperglycemic effect of OLE in STZ-diabetic rats

Experimental protocol

Type 2 diabetes was induced in overnight-fasted rats by a single intraperitoneal injection of freshly prepared STZ (40 mg/kg, dissolved in 0.1 M cold citrate buffer, pH 4.5) [21]. Rats were tested for successful induction of diabetes 2, 5 and 8 days after STZ injection by determining fasting blood glucose (FBG) levels. Only rats with blood-glucose levels >250–350 mg/dl were enrolled in the study. The OLE was tested for the antidiabetic activity in STZ-induced diabetic rats by; (I) studying the effect of three oral doses of OLE on FBG level and

serum insulin in STZ-induced diabetic rats, (II) testing the effect of the methanolic extract on the oral glucose tolerance test (OGTT) in STZ-induced diabetic rats, (III) testing the in vitro activity of the methanolic extract on glucose absorption, glucose uptake, hepatic microsomal activity of glucose-6-phosphatase (G-6-Pase) and total antioxidant capacity in pancreatic tissues.

In **experiment I**, six groups of rats (six animals each) were used. Animals in **group 1** were healthy (non-diabetic) rats and served as the control group. Animals in **groups 2** through 6 were STZ diabetic rats. **Group 2** served as diabetic-untreated control. **Groups 3, 4** and **5** were treated orally with OLE at 100, 300 or 500 mg/kg/day, respectively. **Group 6** received gliclazide (15 mg/kg/day).

Animals were given the assigned treatments for 4 weeks. OLE as well as gliclazide was suspended in CMC (0.1%). Control **groups 1** and **2** were administered the vehicle (CMC). Dosing volume for all groups was 10 ml/kg. FBG levels were determined weekly during the 4-week treatment period. At the end of the 4-week treatment period, animals were overnight fasted and subjected to OGTT.

In **experiment III**, two sets of in vitro mechanistic studies were carried out. These included intestinal glucose absorption using isolated rat gut preparation, glucose uptake by rat psoas muscle. Each experiment was done in six replicates.

Assessment of blood-glucose level

Blood samples were collected from the tail vein of the overnight (12–15 h) fasting rat for determination of blood-glucose level using One Touch Ultra (LifeScan/Johnson & Johnson, Milpitas, CA, USA).

Oral glucose tolerance test (OGTT)

In order to assess the effect of orally administered OLE on systemic glucose homeostasis, OGTT was carried out in conscious fasted rats after 4 weeks administration of the extract. STZ-induced diabetic rats were overnight fasted before being subjected to OGTT. The initial blood samples (at 0 min) were drawn from the tail vein for FBG. Rats were administered a single oral dose of the assigned treatment (CMC for control diabetic group, or 100, 300, and 500 mg/kg of OLE). After 2 h, all animals were given a glucose solution by intragastric gavage to achieve a glucose load of 2 g/kg. Blood samples were then collected from the tail vein at 30, 60, 90 and 120 min and blood glucose was determined as previously described. Total glycemic responses to OGTT were calculated from respective areas under the curve (AUC) of glycemia during the 120 min observation period [22].

Determination of serum insulin

Serum insulin levels were measured after 4-week of treatment in STZ-induced diabetic rats. Ultra-Sensitive Rat Insulin Enzyme-Linked immunosorbent Assay Kit (Crystal Chem. Inc., Downers Grove, IL, USA) was used for the determination of serum insulin.

Glucose absorption by rat gut preparation

The effect of OLE on glucose absorption by rat isolated intestine was determined by assessing the fall in glucose concentration in the incubation medium with time, according to the method of Al-Awadi et al. [23]. Briefly, an overnight-fasted rat was sacrificed under anesthesia, and the abdomen was rapidly opened. Small segments (each about 5 cm) close to the duodenum were rinsed with Krebs' solution by pushing the solution gently from the syringe. The segments were placed in well-oxygenated Krebs'-containing 11.1 mM glucose along with the used concentrations of OLE (100, 300 or 500 µg/ml) and Krebs' solution as control, incubated at 37 ^{o}C for 2 h in CO_2 incubator under 95% O_2 and 5% CO_2 atmosphere. Aliquots of 10 µl were removed from the incubation mixture at 0, 30, 60, 90 and 120 min, and change in glucose concentration in the medium was studied. Glucose concentration was determined by glucose oxidase methods using GLUCOSE-TR kits obtained from Spinreact S.A.U. (Carretera Santa Coloma, Spain).

Glucose uptake by rat isolated psoas muscle

Glucose uptake by rat isolated psoas muscle was determined by a slight modification of the method described by Gupta et al. [24]. Psoas muscle was isolated from two anesthetized adult rats (kindly identified by A. Nagy, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia) and placed immediately in Krebs' solution. Muscle tissue was cut into pieces of equal weight (500 mg) and incubated in a 7-ml incubation mixture of well-oxygenated Krebs' solution containing 11.1 mM glucose and insulin 50 mU/ml along with the different concentrations of OLE (100, 300 and 500 µg/ml) or Krebs' only as control. Incubations were kept at 37 ^{o}C for 2 h in CO_2 incubator under 95% O_2 and 5% CO_2 atmosphere. Aliquots (10 µl) were taken from the incubation mixtures at 0, 15, 30, 60, and 120 min, and changes in glucose concentration were measured using GLUCOSE-TR kits obtained from Spinreact S.A.U. (Carretera Santa Coloma, Spain).

Activity of G-6-Pase in rat isolated hepatic microsomes

The effect of OLE on G-6-Pase activity in rat hepatic microsomes was determined according to the method described by Baginsky et al. [25]. Overnight-fasted rats were euthanized by decapitation and livers were dissected out, placed in an ice-cold buffer at pH 7.4 containing 250 mM sucrose, 25 mM HEPES–KOH, 2.5 mM EDTA and 0.1 mM phenylmethylsulphonyl fluoride and homogenized. The homogenate was centrifuged at 12,000g for 10 min, and the resulting supernatant was centrifuged for 1 h at 100,000g. The

pellet was re-suspended at a protein concentration of 40 mg/ml in the homogenization buffer and G-6-Pase activity was measured by monitoring the release of phosphate from G-6-P. Microsomes (equivalent to 0.04 mg protein) were incubated at room temperature in 400 µl of a buffer at pH 7.2, containing 50 mM HEPES, 100 mM KCl, 2.5 mM EDTA, 2.5 mM $MgCl_2$, and 1.0 mM G-6-P. The released phosphate was measured by adding 600 µl of 1 N HCl containing 10 mg/ml ammonium molybdate and 0.38 mg/ml malachite green. After 15 min incubation at room temperature, the absorbance was measured at 620 nm [26]. Then OLE (100, 300 or 500 µg/ml) was added before the addition of the microsomes and the same amount of buffer was used as control.

In vivo total antioxidant capacity (TAC) in pancreatic tissues

The determination of the TAC is performed by the reaction of the antioxidants in the sample with a defined amount of hydrogen peroxide (H_2O_2). The antioxidants in the sample eliminate a certain amount of the provided H_2O_2. The residual H_2O_2 is determined colorimetrically by the enzymatic reaction which involves the conversion of 3,5, dichloro-2-hydroxybenzensulphonate to a colored product [27].

Total antioxidant capacity= $(A_B-A_S)*3.33$ mM H_2O_2
Where: A_B = the absorbance of blank, A_S = the absorbance of the sample

Assessment of blood pressure lowering activity of olive leaf extract

In this study, the blood pressure lowering effect of OLE in established hypertensive male Wistar rats using L-NAME was assessed using method reported by Khayyal et al [5]. To assess the anti-hypertensive effect of test compound, L-NAME induced hypertensive rats were used as previously described with minor modifications [28]. Briefly, male wister rats (weight 175 - 200 g) were obtained from the animal house colony of the National Research Center and allowed free access to water and standard food pellets. Animals were allocated into four different groups (n=8); normal animals were given vehicle only, positive control group, which was given L-NAME (50 mg/kg) daily p.o., low dose treated group was given L-NAME (50 mg/kg) and OLE (100 mg/kg) daily p.o., and high dose treated group was given L-NAME (50 mg/kg) and OLE (200 mg/kg) daily p.o. Systolic, and diastolic arterial blood pressure was measured every two-week non-invasively from tail artery.

Statistical analysis

Data are expressed as Means ± SEM. Data was statistically analyzed by using SPSS® software (SPSS Inc., USA). Means of different groups were compared using one-way ANOVA followed by Tukey Kramer as a post hoc test. P values less than 0.05 were considered as significant.

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RESULTS AND DISCUSSION

The plants of the flora of Kingdom of Saudi Arabia still need great investigations to detect their importance in the discovery of new sources for new molecules that can be used in treatment of different ailments or the economical use of the available flora used traditionally by Saudi communities. Olive is one of the blessed plants reported in holly Quran and is well known for its diverse biological activities. In recent years, natural olive leaf or olive leaf extract (OLE) have become popular as commercial herbal medicines, marketed as having anti-ageing, immunostimulant and antibiotic properties. South Western regions of Kingdom of Saudi Arabia are rich in another olive species, known as wild African olive (Olea europaea subsp. Africana). Therefore, it seems reasonable to consider to evaluate the leaves of O. europaea subsp. Africana chemically and biologically as a suitable alternative source to the common Mediterranean olive (O. europaea, subsp. Europaea) which does not grow in the Kingdom for the use in preparation based on OLE.

Chemical analysis of olive leaf extract

HPLC analysis

To determine the suitable solvent for extraction of plant material, a preliminary HPLC analysis of leaf samples extracted with different solvents viz. water, ethanol, methanol and 80 % MeOH and 80% ethanol at 60 ^{o}C using Ultraturrax homogenizer. The 80 % MeOH extract showed the highest amount of oleuropein (30%). Therefore, it was selected for extraction of olive leaf sample for biological study.

Linearity

The presented method exhibited strict linearity between the peak area and concentration covering the range from 0.1 – 1.4 mg/ml for Oleuropin. The linear regression equation was found to be y =10131 x + 522.08 with correlation coefficient (r = 0.993), where y is the spot area and x is the concentration in mg/ml.

Selectivity

Oleuropin was completely separated from other components of the samples which means that it could be easily estimated without any interference.

Detection and quantitation limits

The detection and quantitation limits were evaluated based on signal to noise ratio (S/N = 3 and S/N=10 respectively). Oleuropn demonstrated a detection limit of 0.01 mg/ml and quantitation limit of 0.04 mg/ml,

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Precision (repeatability)

Precision of the method was assessed by the determination of six different concentrations of oleropin, each applied as 1ml in triplicates (Table 1). Repeated analysis of a homogenous sample (sample 1) was performed using the same equipments, analytical procedure and the same plate. The % RSD was found to be 2.49 indicating precision of the method (Table 2).

Table 1: Effect of graded doses of olive leaf extract on blood glucose concentration in STZ-induced diabetic rats

	Blood glucose level (mmol/l)					
Time	Zero	1st Week	2nd Week	3rd Week	4th week	% reduction in B.G. after 4 weeks
Normal control	4.4±0.12	4.6±0.09	4.6±0.15	4.6±0.07	4.9±0.12	-10.9±5.9
Diabetic control	30.3[a]±4.81	31.7[a] ±4.3	30.9[a] ±4.23	30.9[a] ±4.71	30.7[a] ±3.9	-4.0±4
Gliclazide 15 mg/kg	18.5[a,b] ±1.87	12.7[a,b] ±2.27	9.3[a,b] ±1.9	7.0[a,b] ±0.8	5.3[a,b] ±0.23	70.331±2.6
Olive 100mg/kg	25[a,b] ±3.87	25.4[a,b] ±4.3	23.5[a,b] ±4.5	17.4[a,b] ±2.03	11.9[a,b] ±1.2	51.2±3.7
Olive 300 mg/kg	25.9[a,b] ±3.76	25.6[a,b] ±4.3	23.1[a,b] ±4.3	17.8[a,b] ±1.8	11.9[a,b] ±1.2	53.5±3.3
Olive 500 mg/kg	29.8[a,b] ±1.8	26.2[a,b] ±3.9	24[a,b] ±3.9	17.5[a,b] ±1.9	9.1[a,b] ±1.5	69.6±4.8

[a] significantly different from control at p<0.05
[b] significantly different from diabetic control at p <0.05

Table 2: Change in blood glucose levels after treatment with olive leaf extract (100, 300 and 500 mg/Kg) in STZ-diabetic rats after glucose loading during oral glucose tolerance test (OGTT)

	Blood glucose level (mmol/l)				
Time	Zero	30 min	60 min	90 min	120 min
Normal control	4.49±0.2	11.2 ±1.12	8.27 ±0.57	6.08 ±0.21	4.97 ±0.18
Diabetic Control	27.3[a]±5.22	47.7[a]±1.88	34.4[a] ±4.57	29.2[a] ±4.63	26[a] ±4.18
Gliclazide 15 mg/kg	27.3[a] ±5.22	32.4[a,b] ±5.39	27.2[a] ±4.89	24[a] ±4.51	23.2[a] ±4.15
Olive 100mg/kg	21.5[a] ±1.31	32.1[a,b] ±1.94	27[a] ±1.43	21.3[a] ±1.24	17.4[a] ±1.37
Olive 300mg/kg	20.4[a] ±1.69	29.3[a,b] ±1.83	26.9[a] ±1.88	18[a,b] ±1.83	15.1[a,b] ±1.16
Olive 500mg/kg	17.6[a,b] ±1.46	24.9[a,b] ±2.31	17[b] ±1.51	14.9[b] ±0.96	14.4[a,b] ±0.56

[a] significantly different from control at p<0.05
[b] significantly different from diabetic control at p <0.05

Accuracy

Accuracy was validated using the standard addition analysis method through the application of the proposed method on three sample solutions: sample 1 showing Concentration equals 0.32 mg/ml for Oleuropin. Besides, two fortified sample solutions (F1 and F2) were examined.

F1 was prepared by adding 1 ml of synthetic mixture containing 0.5 oleuropin to 1 ml solution of sample (1), similarly F2 was prepared by adding 1 ml solution containing 0.8 of

oleuropin to 1 ml solution of sample (1). The original sample and the two fortified samples were analyzed on the same plate under the same previously described conditions, and the data are presented in Table 3. The results were satisfactory regarding ICH guidelines. After application of the suggested method, concentrations of oleuropin were determined and the data are illustrated in Figure 3.

Table 3: Effect of different dose levels of olive leaf extract on glucose absorption by rat gut preparation

	Blood glucose level (mmol/l)					
Time	Zero	30 min	60 min	90 min	120 min	% change
Normal control	192.8±1.6	183.4±2.9	161.1±3.84	148.4±1.7	127.1±3.5	34.1
Gliclazide 15 mg/kg	192.8±1.6	179.7±2.83	168.9±2.5	159.8±2.3	150.7±2.1	21.9*
Olive 100mg/kg	185.2±8.4	189.2±4.8	170.8±4.7	161.6±4.0	125.1±4.4	32.5
Olive 300 mg/kg	183±8.2	172.2±8.3	154.7±2.4	149.2±2.9	121.5±7.4	33.6
Olive 500 mg/kg	190.9±3.7	183.7±1.13	142.9±16.3	139.1±14.8	128.7±5.1	32.6

* significantly different from control at $p<0.05$

Total Phenolics and total flavonoids

Phenolic substances and flavonoids have been shown to be responsible for the antioxidant activity of many of plants [29]. Therefore, the amount of total phenolics and total flavonoids present in standardized wild olive leaf extract was investigated. The content of total phenolics is expressed as mg gallic acid/g of extract (GAE) and calculated from the regression equation ($y=0.0057x - 0.0242$, $R^2=0.98$) and the content of total flavonoids is expressed as mg rutin/g of extract (RE), (regression equation: $y=0.0041x+0.0986$, $R^2=0.98$). The total phenol in OLE reached up to 36 mg GAE /g of dried olive leaf extract, while the total flavonoids reached up to 1.35 mg RE/g of dry olive leaf extract.

In vitro antioxidant assay (DPPH assay)

The antioxidant activity of the OLE was examined by exploring scavenging activity of the stable 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical. The reduction capability of DPPH radical was determined by the decrease in absorbance at 517 nm induced by antioxidants. Ascorbic acid is commonly used as a reference standard. Olive leaf extract could reduce the stable radical DPPH to the yellow colored diphenylpicrylhydrazine. IC_{50} value is the effective concentration at which antioxidant activity is 50%. IC_{50} of OLE sample was 60.2 µg/ml. Standard ascorbic acid (1mM) was found to have 96.21% inhibition.

Determination of antihyperglycemic effect of OLE in STZ-diabetic rats

Assessment of blood-glucose level

OLE in a dose of 100 or 300 mg/kg/day for 4 weeks showed nearly the same reduction in FBG by 51.2 % and 53.5 % at the third and fourth weeks of treatment, respectively, as

compared to the untreated diabetic group. The highest dose of the extract (500 mg/kg) brought about a significant decrease by about 41.6% and 69.6% at the third and fourth weeks of treatment, respectively, as compared to the untreated diabetic group. Thus, the antihyperglycemic effect of O. europaea was time- and dose-related (Table 1). The serum glucose data obtained was clearly indicated that oral administration of wild olive leaf extract produced significant hypoglycemic effects in STZ-induced diabetic rats. The result of this study confirms the traditional use of wild African olive in treatment of diabetes and hypertension in south-eastern Morocco [30]. This is first report on the hypoglycemic activity of African wild olive leaf.

Oral glucose tolerance test (OGTT)

The three doses of the extract (100, 300 or 500 mg/kg) were evaluated for glucose tolerance in STZ-induced diabetic rats along with the standard drug gliclazide (15 mg/kg) by the end of the 4-week treatment. Rats were treated with the extract and changes in OGTT were assessed by comparing the AUC of each group with that of the untreated diabetic group. The total AUC of diabetic-untreated control rats was found to be 2526 mmol.min/l. The intermediate and highest doses of the methanolic extract, 300 and 500 mg/kg, reduced the AUC by 25.9 % and 18.2%, respectively, as compared to that of the untreated diabetic group (Table 2). Gliclazide, at a dose of 15 mg/kg, reduced the AUC by 15% as compared to that of the untreated diabetic group. However, the lowest dose of the methanolic extract, 100 mg/kg, reduced the AUC by 19.1% as compared to that of the untreated diabetic group. The hypoglycemic activity of Mediterranean olive leaf extract was previously shown to be attributed to the active constituent oleuropoeside [31].

Effect of OLE on glucose absorption by rat gut preparation

This experimental setup describes the active transfer of glucose across intestinal membranes [23]. The isolated intestinal segments were incubated with the methanolic extract and glucose absorption was determined by assessing the fall in glucose concentration in the medium with time. When no extract was added to the medium (control), there were 4.9%, 16.4%, 23 % and 34.1 % reduction in glucose concentration of the medium at 30, 60, 90, 120 min, respectively, denoting glucose absorption by the intestinal strip. There was no observable reduction in gut glucose absorption in the presence of extract at either 100, 300 or 500 µg/ml (Table 3).

Effect of OLE on glucose uptake by rat isolated psoas muscle

Since the observed hyperglycemia in diabetic rats might reflect reduced skeletal muscle glucose uptake [32], it was reasonable to examine the effect of the extract on rat psoas muscle uptake of glucose. The effect of African wild olive extract on glucose uptake by rat isolated psoas muscle was studied by measuring the decrease in glucose concentration in the incubation medium with time. Control incubations showed reductions in glucose concentrations by 4.9 %,

16.4 %, 23 % and 28.8% at 30, 60, 90, 120 min, respectively. The olive extract at different doses (100, 300 and 500 mg/ml) caused significant reduction in glucose concentration amounting to 24.3, 29.1 and 56.8 %, respectively, after 120 minutes (Table 4). This may reflect an increase in cellular concentration of glucose by uptake of glucose from media to cells. Direct effect in the absence of insulin indicates that the extract has either insulin-like effect on psoas muscle (skeletal muscle) or direct stimulatory effect on the enzymes involved in the metabolism of glucose.

Table 4: Effect of different dose levels of olive leaf extract on glucose uptake by rat isolated psoas muscle.

	Blood glucose level (mmol/l)					
Time	Zero	30	60	90	120	% change
Normal control	192.8±1.6	183.4±2.3	161.1±3.8	148.4±1.7	137.4±1.7	28.8
Gliclazide 15 mg/kg	192.8±1.6	179.7±2.8	168.9±2.5	148.2±10.4	109.2±4.1	43.4 [a]
Olive 100 mg/kg	192.9±4.3	189.5±4.3	176.5±3.3	150.3±4.3	145.9±4.1	24.3
Olive 300 mg/kg	190.6±0.9	193±1.63	178.7±3.4	172.1±14.7	135.1±2.3	29.1
Olive 500 mg/kg	191.7±4.1	190.3±4.1	169.9±7.4	152.9±5.4	82.79±2.9	56.8 [a,b]

[a] significantly different from control at p<0.05
[b] significantly different from gliclazide at p<0.05

Table 5: Effect of olive leaf extract on G-6-Pase activity in rat isolated hepatic microsomes

	G-6-Pase activity (% of control)	% inhibition of G-6-Pase activity
Control	100±9.8	
Gliclazide 15 mg/kg	16.7*±1.7	83.29±1.66
Olive- 100 mg/kg	78.9±0.82	21.1±0.82
Olive- 300 mg/kg	51.9*±2	48.1±2
Olive- 500 mg/kg	27.7*±1.0	72.3±1

* Significantly different from control at p<0.05

Table 6: Effect of daily oral administration of olive leaf extract on % increase in serum insulin in STZ diabetic rats

	Insulin (% increase relative to STZ)
Control	---
STZ	---
Gliclazide 15 mg/kg	48.0*± 1.92
Olive 100 mg/kg	16.0*±3.13
Olive 300 mg/kg	43.55*±2.04
Olive 500 mg/kg	58.2*±3.16

* significantly different from STZ- group at p<0.05

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Table 7: Effect of olive leaf extract on total antioxidants capacity status of pancreatic tissues.

Groups	Total antioxidant activity (residual H_2O_2 mM)	% reduction
Normal control	2.54* ± 0.02	
Diabetic control	2.91 ± 0.04	
Olive 100mg/kg	2.73 ± 0.04	6.2
Olive 300 mg/kg	2.57* ± 0.11	11.7
Olive 500 mg/kg	2.27* ± 0.02	22.0

Data are Means ±SD
* Significantly different from diabetic control at $p<0.05$

Effect of OLE on G-6-Pase activity in rat isolated hepatic microsomes

The liver is capable of releasing glucose from glycogen into circulation, where it serves as a fuel for other tissues that lack the ability to make glucose. The two metabolic pathways by which the liver can produce glucose are gluconeogenesis and glycogenolysis. A single enzyme, G-6-Pase, catalyses the final step of both of these pathways [33]. The increased hepatic glucose output is a major cause of the hyperglycemia that characterizes diabetes [34]. In our study, the activity of G-6-Pase was significantly decreased with doses 300 and 500 mg/kg by 51.9 and 27.7%, respectively, as well as in case of gliclazide (15 mg/kg) by 16.7 % compared to control group (Table 5). This finding indicated that the hypoglycemic effect of olive leaf extract may be mediated via suppressing important enzymatic step in liver glucose production.

Effect of OLE on the serum insulin level

STZ selectively destroys pancreatic insulin secreting β-cells [35], causing diabetes close to type-II in humans. Serum insulin levels were determined after 4 week of treatment with the methanolic extract of olive leaf in STZ-induced diabetic rats. Insulin was increased in a dose-dependent manner after administration of different doses of olive extract. Both the highest dose of the extract (500 mg/kg) and gliclazide significantly increased insulin release by 58.2 % and 48.0 %, respectively (Table 6). This observation indicates that olive extract enhances the insulin release from destroyed pancreatic beta cells, either by regenerating the partially destroyed pancreatic beta cells or by the release of insulin stored in the granules [36].

In vivo total antioxidant capacity (TAC)

The TAC of OLE was determined in pancreatic tissue homogenates of STZ-diabetic rats. The result (Fig. 1, Table 7) showed significant reduction in residual H_2O_2 concentration in groups treated with OLE 300 and 500 mg/kg doses compared to diabetic control, 11.7 and 22.0, respectively. These data highlight the antioxidant activity of OLE as a potential protective mechanism against STZ-induced oxidative damage of pancreatic tissues.

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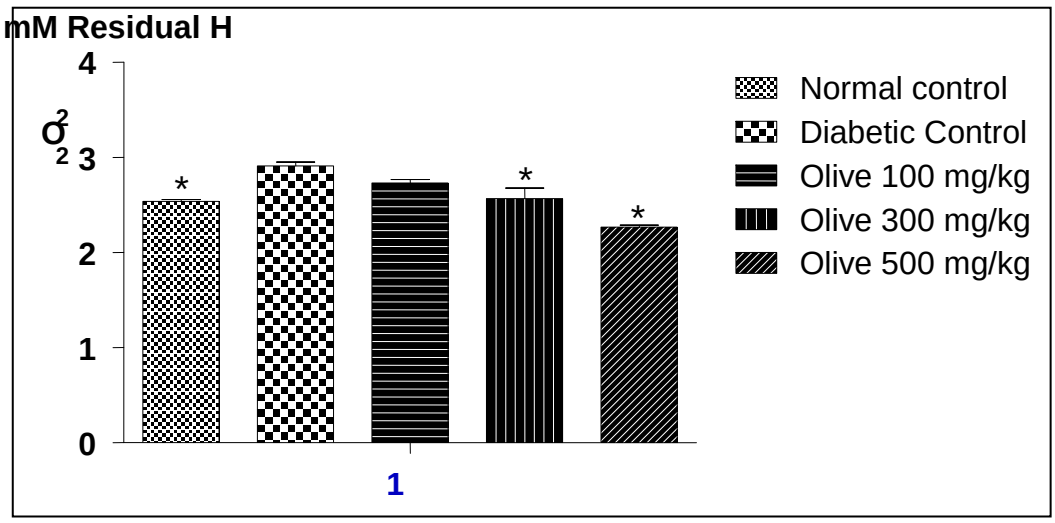

Fig. 1. Effect of treatment with different dose levels of OLE on total antioxidant capacity of pancreatic tissues. Data are expressed as mean ± SEM; n= 8.
*** Significantly different from untreated group at p< 0.05**

Assessment of blood pressure lowering activity of OLE

Rats that are chronically treated with the orally active nitric oxide synthase inhibitor, L-NAME, have been shown to develop a marked hypertension as a result of the chronic blockade of nitric oxide synthesis [5]. Chronic administration of L-NAME substantially increased systolic, diastolic and mean arterial blood pressure in the untreated group after two weeks. After four weeks, all L-NAME treated groups manifested significantly elevated blood pressure (systolic, diastolic and mean arterial blood pressure). Arterial blood pressure was recorded using noninvasive tail cuff method to assess the potential blood pressure lowering activity of extracts under investigation. Systolic blood pressure (A) and diastolic blood pressure (B) was recorded every two weeks after L-NAME first administration. Mean arterial blood pressure was calculated (C) afterward. After six weeks, the high dose of plant extract (200 mg/kg) partially aborted L-NAME induced hypertension showing blood pressure (systolic, diastolic and mean arterial blood pressure, Fig. 2-4) significantly lower than the untreated group. The results of wild olive extract were more significant over six weeks when compared to previously published data [5] over the same period on the Mediterranean standardized olive leaf extract.

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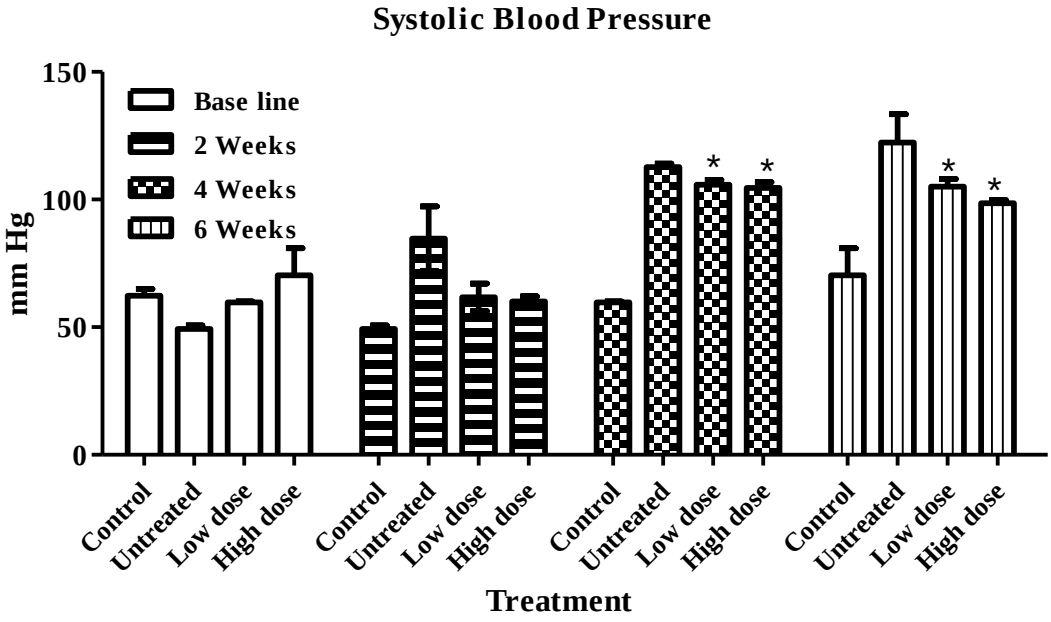

Fig. 2. Effect of treatment with different dose levels of OLE on systolic blood pressure in L-NAME hypertensive rats at different time points compared to control and untreated rats. Data are expressed as mean ± SEM; n= 8.

*** Significantly different from untreated group at p< 0.05.**

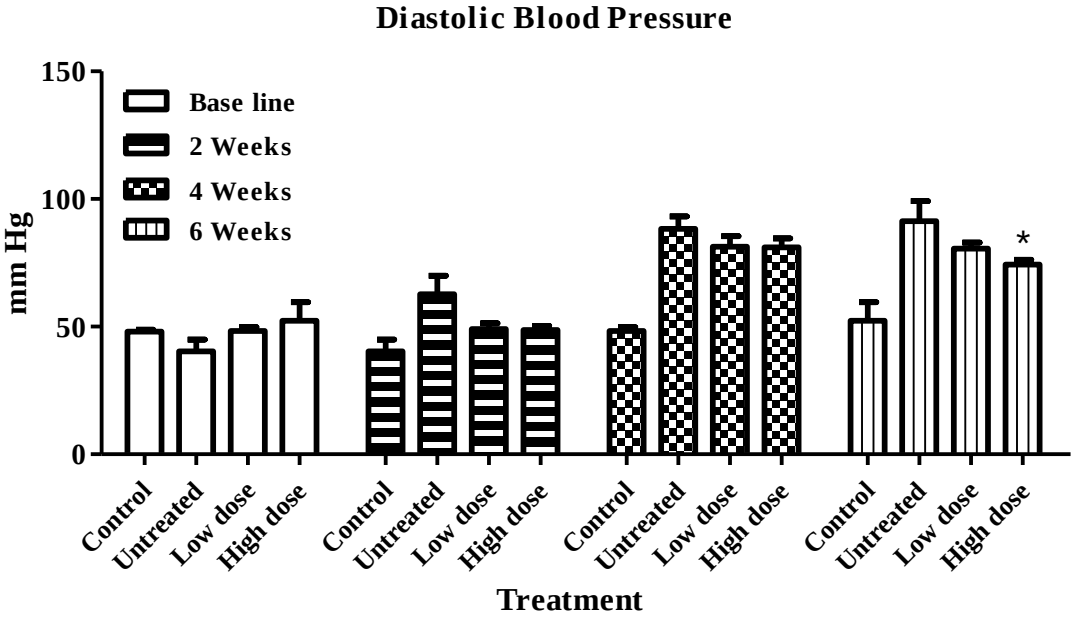

Fig. 3. Effect of treatment with different dose levels of OLE on diastolic blood pressure in L-NAME hypertensive rats at different time points compared to control and untreated rats. Data are expressed as mean ± SEM; n= 8.

*** Significantly different from untreated group at p< 0.05.**

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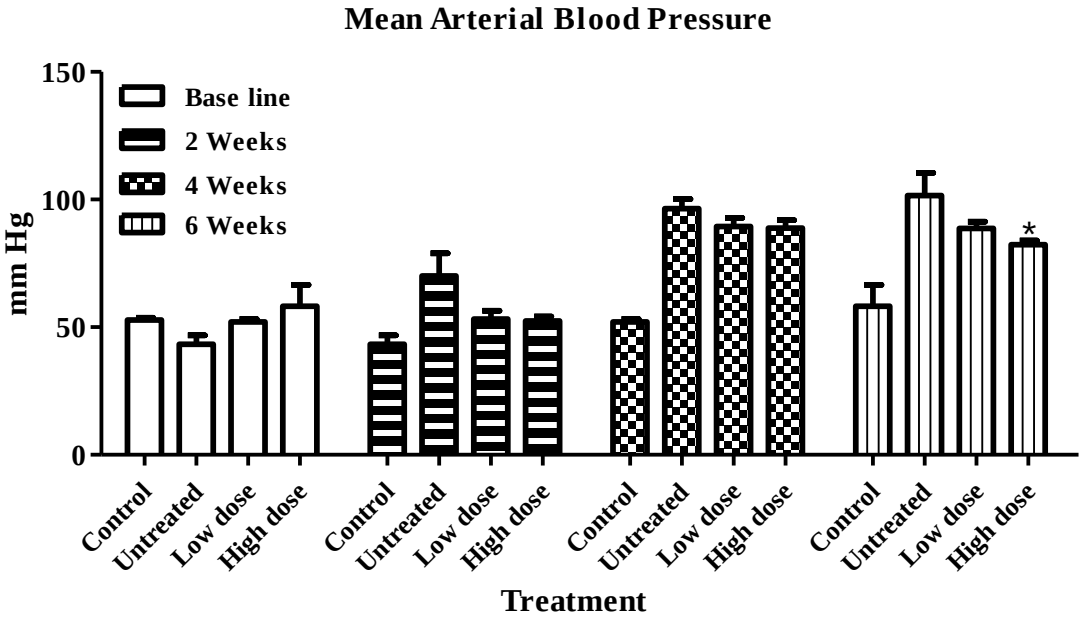

Fig. 4. Effect of treatment with different dose levels of OLE on mean arterial blood pressure in L-NAME hypertensive rats at different time points compared to control and untreated rats. Data are expressed as mean ± SEM; n= 8.
*** Significantly different from untreated group at p< 0.05.**

CONCLUSION

OLE showed protective effects against L-NAME-induced hypertension and STZ-induced diabetes. The mechanisms underlying the observed antihyperglycemic activity can be attributed, at least partly, to enhance skeletal muscle uptake of glucose, inhibition of hepatic gluconeogenesis, insulinomimetic and antioxidant activity. species.

Competing interests:

All authors declare no conflicts of interest in this work.

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REFERENCES

[1] Trichopoulou A, Critselis E. Eur J Cancer Prev 2004; 13:453-456.
[2] Fung TT, Rexrode KM, Mantzoros CS, Manson JE, Willett WC, Hu FB. Circulation 2008; 119:1093-1100.
[3] Fito M, De la Torre R, Covas MI. Mol Nutr Food Res 2007; 51:1215-1224.

[4] El SN, Karakaya S. Nutr Rev 2009; 67:632-638.
[5] Khayyal MT, El-Ghazaly MA, Abdallah DM, Nasser NN, Okpanyi SN, Kreuter MH. Arzneimittelforschung 2002; 52:797-802.
[6] Bouaziz M, Sayadi SEur J Lipid Sci Tech 2005; 107:497-504.
[7] Komaki E, Yamaguchi S, Maru I, Kinoshita M, Kakehi K, Ohta Y, Tsukada Y. Food Sci Tech Res 2003; 9:35-39.
[8] Somova LI, Shode FO, Mipando M. Phytomed 2004; 11: 121-129.
[9] Bisignano G, Tomaino A, Lo-Cascio R, Crisafi G, Uccella N, Saija A. J Pharm Pharmacol 1999; 51:971-974.
[10] Benavente-Garcia O, Castillo J, Lorente J, Alcaraz M. J Med Food 2002; 5:125-135.
[11] Wang L, Geng C, Jiang L, Gong D, Liu D, Yoshimura H, Zhong L. Eur J Nutr 2008; 47:235-243.
[12] Grawish ME, Zyada MM, Zaher AR. Med Oncol 2011; 28:1163-8.
[13] Bitler CM, Viale TM, Damaj B. J Nutr 2005; 135: 1475-1479.
[14] Poudyal H, Campbell F, Brown L. J Nutr 2010; 140: 946-953.
[15] Micol V, Caturla N, Perez-Fons L, Mas V, Perez L, Estepa A. Antiviral Res 2005; 66:129-136.
[16] Fleming T. Physician's Desk References for Herbal Medicine, second ed. New Jersey, Montvale: Medical Economics Company 2000; 556-557.
[17] Braca A, Nunziatina D, Lorenzo DB, Cosimo P, Mateo P, Ivano M. J Nat Prod 2001; 64: 892-895.
[18] Slinkard K, Singleton VL. Am J Enol Vitic 1977; 28:49-55.
[19] Lamaison JL, Carnat A. Pharm Acta Helv 1990; 65:315-320.
[20] Elberry AA, Harraz FM, Ghareib SA, Nagy AA, Gabr SA, Abdel-Sattar EA. Inter J Diab Mell 2011. (doi:10.1016/j.ijdm.2011.01.004).
[21] Yoshino G, Iwai M, Kazumi T, Matsushita M, Morita M, Matsub AK, Iwatani I, Baba S. Atherosclerosis 1989; 79, 41–46.
[22] Matthews JN, Altman DG, Campbell MJ, Royston P. Br Med J 1990; 300:230-235.
[23] Al-Awadi FM, Khattar MA, Gumma KA. Diabetologia 1985; 28:432-434.
[24] Gupta RK, Kesari AN, Watal G, Murthy PS, Chandra R, Maithal K, Tandon V. Curr Sci 2005; 88:1244-1254.
[25] Baginsky ES, Foa PP, Zad B. Glucose-6-Phosphatase. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis. New York: Academic Press 1974; 788-792.
[26] Parker JC, VanVolkenburg MA, Levy CB, Martin WH, Burk SH, Kwon Y, Giragossian C, Gant TG, Carpino PA, McPherson PK, Vestergaard P, Treadway JL. Diabetes 1998; 47:1630-1636.
[27] Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V. J Clin Pathol 2001; 54: 356-361.
[28] Morton JJ, Beattie EC, Speirs A, Gulliver F. J Hypertens 1993; 11: 1083-8.
[29] Agrawal PK. Carbon-13 NMR of flavonoids. New York: Elsevier 1989.
[30] Tahraoui A, El-Hilaly J, Israili ZH, Lyoussi B. J Ethnopharmacol 2007; 110:105-117.
[31] Gonzalez M, Zarzuelo A, Gamez MJ, Utrilla MP, Jimenez J, Osuna I. Planta Med 1992; 58: 513-515.

[32] Chaikenz RL, Banerji MA, Huey H, Lebovitz HE. Diabetes 1993; 42:444-449.
[33] Nordlie RC, Foster JD, Lange AJ. Annu Rev Nutr 1999; 19:379-406.
[34] Holman RR, Turner RC. In: Pickup J, Wiliams G, editors. Oral agents and insulin in the treatment of NIDDM. Textbook of Diabetes. Oxford: Blackwell 1990; 407-469.
[35] Hofteizer V. Diabetologia 1973; 9:178-184.
[36] Ivora MD. Arch Int Pharmacodyn Ther 1988; 296:224–231.