Vol. 39 No. 1 (pp. 1 - 44) 2010

DIABETOLOGIA CROATICA JOURNAL OF DIABETES, ENDOCRINOLOGY AND METABOLIC DISEASES

UDC 616.379-008.64.43

ISSN 0351-0042

CONTENTS

ORIGINAL RESEARCH ARTICLES

ANTIHYPERGLYCEMIC ACTIVITY OF METHANOLIC EXTRACT OF
MADHUCA LONGIFOLIA BARK
Akash P. Dahake, Chirantan S. Chakma, Rita C. Chakma, Prashant Bagherwal 3
METABOLIC RISK MARKERS IN WOMEN WITH POLYCYSTIC
OVARIAN MORPHOLOGY
M.Š. Alebić, L. Duvnjak
ANTIHYPERGLYCEMIC, ANTIHYPERLIPIDEMIC AND ANTIOXIDANT
EFFECTS AND THE PROBABLE MECHANISMS OF ACTION OF <i>RUTA</i>
GRAVEOLENS INFUSION AND RUTIN IN NICOTINAMIDE-
STREPTOZOTOCIN-INDUCED DIABETIC RATS
O.M. Ahmed, A.A. Moneim, I.A. Yazid, A.M. Mahmoud
ADVANTAGE OF PRANDIAL INSULIN AS A THERAPEUTIC
APPROACH IN INITIAL SECONDARY PANCREATIC β-CELL
EXHAUSTION IN TYPE 2 DIABETIC PATIENTS
S. Kokić, V. Kokić, M. Krnić, L. Mirić, Ž. Jovanović, Ž. Orlić-Crnčević

DAMA - DIABETOLOGY ALUMNI MEDICAL ASSOCIATION VUK VRHOVAC UNIVERSITY CLINIC, ZAGREB



DIABETOLOGIA CROATICA

MEDICAL SCIENTIFIC JOURNAL OF THE VUK VRHOVAC INSTITUTE UNIVERSITY CLINIC FOR DIABETES, ENDOCRINOLOGY AND METABOLIC DISEASES

SCHOOL OF MEDICINE, UNIVERSITY OF ZAGREB SOCIETY FOR DIABETES AND METABOLIC DISEASES OF THE CROATIAN MEDICAL ASSOCIATION

DIABETOLOGY ALUMNI MEDICAL ASSOCIATION VUK VRHOVAC UNIVERSITY CLINIC, ZAGREB

Referred in:
BIOLOGICAL ABSTRACTS (BIOSIS)
BIOSIS PREVIEWS
CABI Bibliographic Databases:
CAB Abstracts
Global Health
Abstracts on Hygiene and Communicable Diseases
Nutrition Abstracts and Reviews Series A:Human and
Experimental
Nutrition and Food Sciences Database
Review of Aromatic and Medicinal Plants
Tropical Diseases Bulletin
VetMed Resource
CIRRIE Database of International Rehabilitation Research
DOAJ: Directory of Open Access Journals
EBSCO Bibliographic Databases
ELSEVIER SCIENCE B.V. (SEDBASE)
EMBASE / Excerpta Medica
Open Science Directory
SCOPUS
ULRICH'S PERIODICALS DIRECTORY
VINITI Abstracts Journal (REFERATIVNYI ZHURNAL)

VOLUME 39, NUMBER 1, 2010



DIABETOLOGIA CROATICA

EDITORIAL BOARD OF MEDICAL - SCIENTIFIC JOURNAL DUGI DOL 4A, 10000 ZAGREB, CROATIA PHONE: (385-1) 235 38 00 • FAX: (385-1) 233 15 15 E-mail: Diabetologia.Croatica@idb.hr

EDITOR-IN-CHIEF

Željko Metelko

All manuscripts are to be forwarded to the Editorial Board's address:

DIABETOLOGIA CROATICA

Dugi dol 4a, 10000 Zagreb, Croatia Phone: (385-1) 23 53 800 Fax: (385-1) 23 31 515 E-mail: Diabetologia.Croatica@idb.hr Diabetologia Croatica is published quarterly

Annual subscription: 45 USD payable, as well as other reimbursements, to: ZAGREBAČKA BANKA ZAGREB Account No.: 30101-620-16-25731-840-3279073 Note: For Diabetologia Croatica

For Croatian subscribers: Annual subscription: HRK equivalent to 45 USD to be transferred to: Diabetologia Croatica VUK VRHOVAC Institute Dugi dol 4a, 10000 Zagreb Account No.: 30105-603-4975 (HRK) Note: For Diabetologia Croatica

Language editing: LinguaMed, Zagreb

Layout and technical editing: Saša Pepeonik

Printing house: TIPO ART d.o.o., Zagreb

Exempt from sales taxes according to decision No. 3716/1-1977 of the Ministry of Education and Sport of the Republic of Croatia

ASSISTANTS TO EDITOR-IN-CHIEF

Nikica Car Zdenko Škrabalo

EDITORIAL BOARD

Božica Mandić Ranko Mesić Lovorka Perković Lea Smirčić-Duvnjak

ADVISORY BOARD

Shigeaki Baba, Tokyo Želimir Beer, Varaždin Mirko Grujić, Sarajevo Aleksandar Plašeski, Skopje Ljiljana Jović-Paškvalin, Rijeka Juraj Romac, Split Manuel Serrano Rios, Madrid

The journal address on WWW is: http://www.idb.hr/diabetologia/index.html including full text articles. ¹ Departments of Pharmaceutical Chemistry,

² Pharmacology,

³ Pharmacognosy

B. Pharmacy College, Rampura, Kakanpur, Godhara, Dist. Panchmahal, Gujarat, India

ANTIHYPERGLYCEMIC ACTIVITY OF METHANOLIC EXTRACT OF MADHUCA LONGIFOLIA BARK

Akash P. Dahake¹, Chirantan S. Chakma², Rita C. Chakma¹, Prashant Bagherwal²

Key words: Madhuca longifolia, hypoglycemia, streptozotocin

SUMMARY

The study was carried out to assess the antihyperglycemic effects of methanolic extract of Madhuca longifolia bark in normal, glucose loaded and streptozotocin induced diabetic rats. All three animal groups were administered the methanolic extract of Madhuca longifolia at a dose of 100 and 200 mg/kg body weight (p.o.) and the standard drug glibenclamide at a dose of 500 µg/kg. Serum glucose level was determined on days 0, 7, 14 and 21 of treatment. The extract exhibited a dose dependent hypoglycemic activity in all three animal models as compared with the standard antidiabetic agent glibenclamide. The hypoglycemia produced by the extract may be due to the increased glucose uptake at the tissue level and/or an increase in pancreatic β -cell function, or due to inhibition of intestinal glucose absorption. The study indicated the methanolic extract

of Madhuca longifolia to be a potential antidiabetic agent, lending scientific support for its use in folk medicine.

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia, hypertriglyceridemia and hypercholesterolemia, resulting from defects in insulin secretion or action or both (1). Diabetes is crudely grouped into two types: insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM). Both types are associated with excessive morbidity and mortality(2).

Madhuca longifolia, synonym *M. indica*, belonging to the family Sapotaceae, is an important economic tree growing throughout India. Traditionally, *Madhuca longifolia* bark has been used against diabetes, rheumatism, ulcers, bleeding and tonsillitis (3).

The flowers, seeds and seed oil of madhuka have great medicinal value. Externally, the seed oil massage is very effective to alleviate pain. In skin diseases, the juice of flowers is rubbed for oleation. It is also beneficial as a nasya (nasal drops) in diseases of the head due to pitta, like sinusitis (4).

Corresponding author: Akash P. Dahake, M. Pharm., Department of Pharmaceutical Chemistry, B. Pharmacy College, Rampura, Kakanpur, Godhara, Dist. Panchamahal, Gujarat, India *E-mail*: akashpdahake@gmail.com

The aim of the present study was to demonstrate the antihyperglycemic effects of methanol bark extract of *Madhuca longifolia* in normal and streptozotocin (STZ) induced diabetic rats.

MATERIAL AND METHODS

Plant material

Bark samples were collected from Udupi, Karnataka, India, during January and February 2009 and authenticated by Dr. Gopalkrishna Bhat, a taxonomist from Department of Botany, Poornaprajna College, Udupi. The specimen sample No.14 is kept at the Department, B. Pharmacy College, Rampura, Kakanapur, Godhara.

Preparation of extract

Dried bark samples (7 kg) were ground in a Waring blender and sifted through a wire screen (mesh size 2 mm \times 2 mm). The powdered leaves (500 g) were exhaustively extracted with methanol. The extracts were filtered and evaporated to dryness at a temperature below 30 °C. The extract obtained with methanol was 28 g.

Animals

Wistar rats of both sexes (150-200 g) were maintained under standard animal house conditions, fed standard pellet diet (Hindustan Lever Ltd., Bombay) and allowed water *ad libitum*. Fasted animals were deprived of food for at least 16 h, but were allowed free access to water. The study was approved by the Institutional Animal Ethics Committee of B. Pharmacy College, Rampura, Godhara.

Acute toxicity and selection of doses

The acute toxicity studies were carried out in adult female albino rats weighing 150-200 g, by up and down method as *per* OECD 425 guidelines (5). Overnight fasted animals received test drug at a dose of 2000 mg/kg body weight orally. Then the animals were observed continuously once in half an hour for the next 4 hours and then after 24 hours for general behavioral, neurologic and autonomic profiles and to find out mortality. The extract was found safe to up to a dose of 2000 mg/kg body weight.

Oral glucose tolerance test

The oral glucose tolerance test was performed in overnight fasted normal animals (6). Rats divided into four groups (n=6) were administered 2% gum acacia solution, methanolic extract 100 mg/kg, 200 mg/kg and glibenclamide (500 μ g/kg). Glucose (2 g/kg) was fed 30 min after the administration of methanolic extract. Blood was withdrawn from the retro-orbital sinus at 0, 30, 60, 90 and 120 min of methanolic extract administration. Fasting serum glucose levels were estimated by the Radio Immuno Assay kit (BRAC, Mumbai).

Normoglycemic study

For normoglycemic study, rats were divided into five groups (n=6) and administered 2% gum acacia solution, methanolic extract 100 mg/kg, 200 mg/kg and glibenclamide (500 μ g/kg) (7). Blood glucose levels were estimated on days 0, 4, 8 and 12.

Determination of total protein, total cholesterol, creatinine and blood urea nitrogen

Total proteins were determined by the method described by Lowry *et al.* (8). Creatinine and blood urea nitrogen were determined by the kit supplied by Merck Specialties, Ltd. Total cholesterol was determined by the kit supplied by Beacon Diagnostics, Kabilpore, India.

Induction of experimental diabetes

Diabetes was induced by administering intraperitoneal injection of a freshly prepared STZ solution (60 mg/kg of body weight) in 0.1M cold citrate buffer to the overnight fasted rats. Because of the STZ instability in aqueous media, the solution is made using cold citrate buffer (pH 4.5) immediately before administration. Animals with blood glucose values above 250 mg/dL on day 3 of STZ injection were considered as diabetic rats. The treatment was started after day 5 of diabetes induction and was considered as day 1 of treatment.

Experimental design

The animals were divided into five groups of six animals, as follows: group I, normal healthy control; group II, diabetic control (STZ 60 mg/kg i.p.); group III, diabetic + methanolic extract (100 mg/kg body weight, orally); group IV, diabetic + methanolic extract (200 mg/kg body weight, orally); and group VI, diabetic + glibenclamide (500 μ g/kg body weight, orally).

Blood sampling

At the end of day 12, blood samples were collected from the inner canthus of the eye under light ether anesthesia using capillary tubes (Micro Hemocrit Capillaries, Mucaps). Blood was collected into fresh vials containing anticoagulant antiserum and separated in a centrifuge at 2000 rpm for 2 min. Serum insulin levels were estimated by the Radio Immuno Assay kit supplied by the Board of Radiation and Isotope Research, Bhaba Atomic Research Centre (BRAC), Mumbai, India.

Statistical analysis

Data were statistically evaluated by use of one-way ANOVA, followed by post hoc Scheffe's test using 7.5 version of the SPSS computer software. The values were considered significant at P<0.05.

RESULTS

Acute toxicity studies

Acute toxicity studies revealed the non-toxic nature of methanolic extract at the two dose levels tested. There were no morphological changes like distress, hair loss, restlessness, convulsions, laxative effect, coma, weight loss, etc. At the end of the treatment period, there was no lethality or toxic reaction at any of the doses selected.

Glucose tolerance test

In all groups except for glibenclamide, at 30 min of initiating glucose tolerance test, blood glucose concentration was higher than at zero time but decreased significantly from 30 min to 120 min (Table 1). Methanolic extracts were enhancing glucose utilization, thus the blood glucose level was significantly decreased in glucose loaded rats.

Normoglycemic study

In normoglycemic rats, the doses of 100 and 200 mg/kg reduced hyperglycemia on days 4, 8 and 12 of treatment (Table 2). A significant hypoglycemic activity was found on day 12 with 100 and 200 mg/kg doses.

Table 1. Effect of methanolic extract on serum glucose level (mg/dL) on glucose tolerance test in glucose loaded rats

Group	Treatment	0 min	30 min	60 min	90 min	120 min
1	Control (vehicle)	89.2±2.9	108.3±1.1	103.7± 2.1	100.2*±1.4	96.3*±1.6
2	Methanolic extract (100 mg/kg)	80.4±1.1	92.7±2.1	86.7±3.1	83.6±1.8	79.8±1.3
3	Methanolic extract (200 mg/kg)	89.3±1.7	97.4±2.2	90.5±2.5	84.2*±1.2	75.4*±1.3
4	Glibenclamide (500 μ g/kg)	83.4±1.5	79.5±1.1	80.1±2.7	75.1*±2.1	73.6±2.9

Values are expressed as mean ± SEM; n=6 in duplicate for each treatment; *statistically significant difference from the corresponding zero time value; P<0.05

Group	Dose	Day 0	Day 4	Day 8	Day 12
1	Control (Vehicle)	78.4±3.3	76.1±36	79.4±2.2	76.3±2.4
2	Methanolic extract (100 mg/kg)	82.1±2.6	80.3±3.3	79.8*±3.7	77.5*±1.3
3	Methanolic extract (200 mg/kg)	74.4±2.3	70.6±2.4	67.3±3.6	61.8*±1.3
4	Glibenclamide (500 µg/kg)	80.1±2.3	73.2±3.9	66.7*±3.2	59.6*±1.7

Values are expressed as mean ± SEM; n=6 in duplicate for each treatment; *statistically significant difference from the corresponding zero time value; P<0.05

No.	Group	Week 0	Week 1	Week 2	Week 3
1	Normal control	128.86±1.71	141.32±2.03	130.13*±3.52	117.37±1.16
2	Diabetic control	190.81±2.68	309.55±4.48	380.05±5.13	410±2.06
3	Methanolic extract (100 mg/kg)	390.13±3.08	332.39*±3.14	268.94±2.06	200.90±3.46
4	Methanolic extract (200 mg/kg)	411.91±5.02	341.13±4.6	200.52*±1.33	141.37*±4.63
5	Glibenclamide (500 μ g/kg)	385.5±2.02	302.8*±3.32	190.3±3.68	132.6±4.87

Table 3. Effect of methanolic bark extract of *Madhuca longifolia* on serum glucose level (mg/dL) in streptozotocin induced diabetic rats

Values are expressed as mean ± SEM; n=6 in duplicate for each treatment; *statistically significant difference from the corresponding zero time value; P<0.05

Table 4. Effect of methanolic bark extract of *Madhuca longifolia* on body weight (g) in streptozotocin induced diabetic rats

No.	Group	Initial body weight	Final body weight
1	Normal control	161.6±1.1	169.6±3.4
2	Diabetic control	150.8±2.3	114.0±2.8
3	Methanolic extract (100 mg/kg)	160.0±3.1	152.4±1.5
4	Methanolic extract (200 mg/kg)	158.9±4.1	169.5±0.7
5	Glibenclamide (500 µg/kg)	168.4±2.7	180.2±4.0

Values are expressed as mean ± SEM; n=6 in duplicate for each treatment

Table 5. Effect of methanolic bark extract of *Madhuca longifolia* on total protein, total cholesterol, serum creatinine, and blood urea nitrogen in streptozotocin induced diabetic rats

Group	Total Protein	Total cholesterol	Serum creatinine	Blood urea nitrogen
Normal control	7.638±0.29	62.660±1.22	0.51±0.04	46±1.36
Diabetic control	3.670*±0.20	80.333±2.01	0.78±0.08	75±3.44
Methanolic extract (100 mg/kg)	5.252±0.08	65.500*±1.64	0.71±0.03	62*±2.31
Methanolic extract (200 mg/kg)	6.598*±0.07	61.333±0.55	0.63±0.03	51±1.55
Glibenclamide (500 µg/kg)	7.239±0.14	60.039±0.79	0.54*±0.02	48±1.85

Values are expressed as mean ± SEM; number of animals (n) = 6-8; *statistically significant difference from the corresponding zero time value; P<0.05

STZ induced diabetes

After oral administration of 100 and 200 mg/kg of the methanolic extract of *Madhuca longifolia*, a significant reduction was observed in the blood glucose level of STZ induced diabetic rats. A dose dependent effect was seen with doses of 100 and 200 mg/kg of methanolic extract throughout the study period (Table 3). Blood sugar level was also determined before and after glibenclamide treatment. Glibenclamide, a known hypoglycemic agent, reduced blood sugar level.

Biochemical assay

The protein amount in diabetic control group was reduced as compared to normal control. The lowered levels of protein after treatment with methanolic extracts increased near to the control one. Total cholesterol level, which was increased in diabetic rats, was also reduced after treatment with methanolic extracts. Creatinine and blood urea nitrogen values were significantly increased in STZ induced diabetic rats. Oral administration of methanolic extracts for three weeks significantly lowered creatinine and blood urea nitrogen in STZ induced diabetic rats.

DISCUSSION

A wide range of synthetic oral antidiabetic drugs such as sulfonylureas and biguanides have been used for 50 years now in the treatment of diabetes. However, they have not been of much benefit in controlling the complications of the disease. Streptozotocin is a broad spectrum antibiotic obtained from *Streptomyces achromogenes*. STZ causes massive reduction in insulin release *via* destruction of β cells of the islets of Langerhans and thereby induces hyperglycemia (9).

In the present study, the antihyperglycemic activity of methanolic bark extract of Madhuca longifolia was assessed in normal and STZ induced diabetic rats. Oral administration of a single dose of methanolic bark extract of Madhuca longifolia caused a significant decrease in serum glucose level in normal rats. A dose of 200 mg/kg of methanolic extract produced maximum glucose lowering effect, whereas 100 mg/kg of ethanolic extract showed a significant hypoglycemic effect throughout the study period. In the oral glucose tolerance test, the Madhuca longifolia bark extract showed significant reduction of serum glucose levels and these effects were dose dependent. The extract of Madhuca longifolia bark displayed a significant hypoglycemic effect in normal rats. The main mechanism by which the extracts bring the hypoglycemic effects most probably involves stimulation of peripheral glucose consumption. Furthermore, the glycemia profile observed in the glibenclamide group indicates that the extract of Madhuca longifolia acts on the liver or on peripheral glucose consumption (10).

The glibenclamide effects on glucose can be attributed to the enhanced activity of the β cells of the pancreas, resulting in secretion of a large amount of insulin. These results have indicated that some drugs may also be effective in NIDDM. The significant hypoglycemic effects of *Madhuca longifolia* bark in diabetic rats indicate that this effect can be mediated by stimulation of glucose utilization by peripheral tissues.

Insulin deficiency or insulin resistance is associated with hypercholesterolemia (11). Administration of methanolic extract of *Madhuca longifolia* bark to diabetic rats lowered the total cholesterol level.

Renal disease is one of the most common and severe complications of diabetes. Insulin is a physiological factor, which plays an important role in the maintenance of protein balance (12). In addition, significant elevations in the urea and creatinine levels indicate an impaired renal function in diabetes. Methanolic extracts of *Madhuca longifolia* bark improve renal function that is generally impaired in diabetic rats.

The results of the present study clearly indicated the methanolic extract of *Madhuca longifolia* bark to have a hypoglycemic effect on STZ induced diabetic rats. The extract was highly effective in managing the complications associated with diabetes mellitus, such as hypercholesterolemia and impaired renal function. Therefore, the *Madhuca longifolia* bark extract showed a therapeutic action against the development and progression of diabetic complications mentioned above. Further studies are in progress to isolate the active principle(s) and elucidate the exact mechanism of action of *Madhuca longifolia* bark.

REFERENCES

- George P, Rudvid B. Lipids and diabetes. J Clin Bio Card 2000;3:159-162.
- Korashik A, Hottor M. Use of animal models in the study of diabetes. In: Wately BI, editor. Diabetes mellitus. 13th ed. New Delhi: National House, 1996; p. 317.
- Khare CP. Rational western therapy. Ayurvedic and other traditional usage. Encyclopedia of Indian medicinal plant. Botany. Springer, 2000; p. 292.
- http://www.herbalcureindia.com/herbs/madhukalongifolia.htm

- Organisation for Economic Cooperation and Development. OECD Guidelines for the testing of chemicals. OECD guideline 425: Acute oral toxicity: up and down procedure. Approved: June 1998.
- Boner-Weir S, Deery D, Leahy JL, Weir GC. Compensatory growth of pancreatic â cells in adult rats after short-term glucose infusion. Diabetes 1989;38:49-53.
- Rakesh B, Sanjay J, Deep Q, Amit J, Giriraj S, Ravi G. Antidiabetic activity of aqueous root extract of *Ichnocarpus frutescens* in streptozotocin-nicotinamide induced type-II diabetes in rats. Indian J Pharmacol 2008(40);1:19-22.
- 8. Kingsley G. The determination of serum total protein, albumin and globulin by the biuret reaction. J Biol Chem 1939;131:197-200.

- Rakieten N, Rakieten ML, Nadkarni MV. Studies on the diabetogenic action of streptozotocin. Cancer Chemother Rep 1963;29:91-98.
- Kavalali G, Tansali H, Goksel S, Hatemi H. Hypoglycemic activity of *Urtica pilurifera* in streptozotocin induced diabetic rats. J Ethnopharmacol 2002;84:241-245.
- 11. Sharma SR, Dwivedi SK, Swarup D. Hypoglycemic and hypolipidaemic effects of *Cinnamomum tamala* Nees leaves. Indian J Exp Biol 1996;34:372-374.
- Pathak A, Dhawan D. Effects of lithium on the levels of blood urea and creatinine in diabetic rats. Med Sci Res 1988;26:855-859.

METABOLIC RISK MARKERS IN WOMEN WITH POLYCYSTIC OVARIAN MORPHOLOGY

Miro Šimun Alebić, Lea Duvnjak

Key words: polycystic ovary syndrome, polycystic ovarian morphology, metabolic risk markers, hyperandrogenism

SUMMARY

Polycystic ovary syndrome (PCOS) is a common endocrine condition in women of reproductive age, which is associated with a range of metabolic implications. Data concerning metabolic features of patients with polycystic ovarian morphology (POM) without any other PCOS diagnostic criteria (nonPCOS-POM) are limited. In the present study, metabolic profile of 46 women with nonPCOS-POM was investigated in comparison with 36 women with PCOS D phenotype and control group (N=146). PCOS D phenotype was defined according to the European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM) criteria as irregular anovulatory periods and POM. NonPCOS-POM patients in comparison to PCOS-D phenotype group showed a significantly lower waist circumference (73.0; 17.0; 99.5; 11.0; P<0.001), a homeostatic

model assessment of insulin resistance (HOMA-IR) (1.1; 1.0; 12.8; 6.9; P < 0.001) and a significantly higher fasting glucose-insulin ratio (FGIR) (15.2; 13.0; 5.9; 3.4; P < 0.001). There was no significant difference in waist circumference, FGIR and HOMA-IR between the nonPCOS-PMO and control group. As neither PCOS-D phenotype nor nonPCOS-POM patients are characterized by hyperandrogenism, our data suggest that hyperandrogenism is not the only factor contributing to the increased metabolic risk in women with PCOS D phenotype. NonPCOS-POM patients share a similar metabolic risk profile with the control population and could not be considered as patients with an increased metabolic risk.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a common endocrine condition in women of reproductive age, with a prevalence estimated to 6.6% (1). It is associated with a range of reproductive, obstetric, psychological and metabolic features. In 2003, the European Society for Human Reproduction and Embryology/American Society for Reproductive Medicine (ESHRE/ASRM) defined PCOS as the presence of at least two of the following abnormalities: hyperandrogenism, polycystic ovarian morphology (POM) on ultrasound, and irregular anovulatory

Corresponding author: Miro Šimun Alebić, Vuk Vrhovac University Clinic, Dugi dol 4a, HR-10000 Zagreb, Croatia *E-mail:* malebic@idb.hr

periods (2,3). According to these criteria, four PCOS phenotypes have been identified: phenotype A including women with hyperandrogenism, POM and irregular anovulatory periods; phenotype B including women with hyperandrogenism and irregular anovulatory periods; phenotype C including women with hyperandrogenism and POM; and D phenotype including those with irregular anovulatory periods and POM. Phenotypes A, B and C represent the hyperandrogenic (PCOS-HA) subpopulation and D phenotype the non-hyperandrogenic subpopulation (PCOS-D). It remains unclear whether PCOS-D phenotype is associated with lower cardiovascular risk in comparison to PCOS-HA subpopulation. It has been suggested that an increased metabolic risk might be related to hyperandrogenism (4-11). However, in clinical practice, a certain percentage of women with POM do not meet the diagnostic criteria for PCOS. In view of the fact that this group of patients might represent a step between the PCOS-D subpopulation and women without PCOS and POM (nonPCOSnonPOM), it would be of special clinical interest to investigate the presence of metabolic risk in this subpopulation.

To determine if nonPCOS-POM condition is associated with an increased metabolic risk we aimed to characterize a large group of patients according to the ESHRE/ASRM criteria and to compare the nonPCOS-POM group with PCOS-D phenotype and control group (nonPCOS-nonPOM).

MATERIALS AND METHODS

Two hundred and seventy four patients were recruited from the Outpatient Department of Reproductive Medicine, Vuk Vrhovac University Clinic, during the period between January 2008 and December 2009. PCOS was diagnosed according to the ESHRE/ASRM criteria (2,3). The patients meeting the ESHRE/ASRM criteria were divided into PCOS-HA group (n=44) including patients with hyperandrogenism and PCOS-D group (n=36) including patients without hyperandrogenism. The patients that did not meet the ESHRE/ASRM criteria were divided into nonPCOS-POM group (n=46) including patients with POM and nonPCOS-nonPOM patients (n=148) as a control group.

On study entry, waist circumference (WC), body mass index (BMI), systolic and diastolic blood pressure, antral follicular count (AFC) and Ferriman-Gallwey score were determined in all patients.

The levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E_2), prolactin (PRL), thyroid-stimulating hormone (TSH), glucose, insulin, testosterone and dehydroepiandrosterone sulfate (DHEAS) were determined on day 3-5 of the next menstrual cycle. Fasting glucose-insulin ratio (FGIR) and homeostatic model assessment of insulin resistance (HOMA-IR) were calculated as markers of insulin resistance and increased metabolic risk. FGIR was calculated as fasting glucose (mmol/L)/fasting insulin (mIU/L), HOMA-IR as fasting glucose (mmol/L) x fasting insulin (mIU/L)/22.5 (2).

Statistical methods: normality of distribution was tested using Shapiro-Wilk W test. Between-group differences were analyzed using Mann-Whitney U and Kruskal-Wallis tests. Statistical significance was set at $P \le 0.05$ in all analyses, carried out using STATISTICA StatSoft, version 8.0.

RESULTS

Clinical and laboratory data of the study (nonPCOS-POM), PCOS-HA and PCOS-D groups are shown in Table 1. NonPCOS-POM patients showed significantly lower WC and HOMA-IR and significantly higher FGIR in comparison to PCOS-D phenotype and PCOS-HA group.

There was no between-group difference in systolic and diastolic blood pressure.

Clinical and laboratory data of the study (nonPCOS-POM) group and nonPCOS-nonPOM-control group are shown in Table 2. There was no significant difference between the nonPCOS-PMO and nonPCOS-nonPOM groups either in WC, FGIR and HOMA-IR as metabolic risk markers or in systolic and diastolic blood pressure. Comparison between the nonPCOS-PMO and nonPCOS-nonPOM age-matched groups is shown in Table 3. The age-matched nonPCOS-POM group had a significantly higher diastolic blood pressure as compared with the nonPCOS-nonPOM group.

Table 1. Clinical and laboratory data of the non polycystic ovary syndrome-polycystic ovarian morphology (nonPCOS-POM) study group, polycystic ovary syndrome-hyperandrogenic (PCOS-HA) group and polycystic ovary syndrome-D phenotype (PCOS-D) group

	nonPCOS-POM (study) aroup	PCOS HA group		PCOS D group	
	(n=46)	(n=44)	P-value	(n=36)	<i>P</i> -value
Body mass index	22.0;4.0	25.5;7.0	<0.01	23.0;4.0	0.93 (NS)
FGIR	15.2;13.0	11.3;8.9	0.02	5.9;3.4	<0.001
HOMA-IR	1.1;1.0	1.5;1.4	0.05	12.8;6.9	<0.001
Waist circumference (cm)	73.0;17.0	79.0;19.0	0.03	99.5;11.0	<0.001
Systolic blood pressure (mm Hg)	120.0;15.0	120.0;15.0	0.40 (NS)	120.0;10.0	0.41 (NS)
Diastolic blood pressure (mm Hg)	75.0;10.0	80.0;10.0	0.33 (NS)	80.0;10.0	0.89 (NS)
Age (yrs)	29.6;4.1	31.1;5.4	0.23 (NS)	30.3;4.6	0.51 (NS)

Data are shown as median; interquartile range; values of P<0.05 were considered to be statistically significant (Mann-Whitney U test); FGIR, fasting glucose-insulin ratio; HOMA-IR, homeostatic model assessment of insulin resistance

Table 2. Clinical and laboratory data of the non polycystic ovary syndrome-polycystic ovarian morphology (nonPCOS-POM) study group and non polycystic ovary syndrome-non polycystic ovarian morphology (nonPCOS-nonPOM) group

	nonPCOS-POM (study) group	nonPCOS- nonPOM group	
	(n=46)	(n=148)	P-value
Body mass index	22.0;4.0	23.0;4.0	0.78 (NS)
FGIR	15.2;13.0	14.5;9.7	0.34 (NS)
HOMA-IR	1.1;1.0	1.2;1.0	0.46 (NS)
Waist circumference (cm)	73.0;17.0	74.5;14.0	0.63 (NS)
Systolic blood pressure (mm Hg)	120.0;15.0	117.5;15.0	0.21 (NS)
Diastolic blood pressure (mm Hg)	75.0;10.0	70.0;10.0	0.14 (NS)
Age (yrs)	29.6;4.1	32.7;5.6	<0.001

Data are shown as median; interquartile range; values of P<0.05 were considered to be statistically significant (Mann-Whitney U test); FGIR, fasting glucose-insulin ratio; HOMA-IR, homeostatic model assessment of insulin resistance

Table 3. Age matched comparison between the non polycystic ovary syndrome-polycystic ovarian morphology (nonPCOS-POM) study group and non polycystic ovary syndrome-non polycystic ovarian morphology (nonPCOS-nonPOM) control group

	nonPCOS-POM (study) group	nonPCOS- nonPOM group	
	(n=46)	(n=77)	P-value
Body mass index	22.0;4.0	23.0;5.0	0.81 (NS)
FGIR	15.2;13.0	14.8;9.8	0.32 (NS)
HOMA-IR	1.1;1.0	1.1;0.8	0.61 (NS)
Waist circumference (cm)	73.0;17.0	73.0;12.0	0.90 (NS)
Systolic blood pressure (mm Hg)	120.0;15.0	115.0;15.0	0.13 (NS)
Diastolic blood pressure (mm Hg)	75.0;10.0	70.0;10.0	0.04
Age (yrs)	29.6;4.1	30.5;3.1	0.07 (NS)

Data are shown as median; interquartile range; values of P<0.05 were considered to be statistically significant (Mann-Whitney U test); FGIR, fasting glucose-insulin ratio; HOMA-IR, homeostatic model assessment of insulin resistance

DISCUSSION

Since the introduction of the ESHRE/ASRM criteria for the diagnosis of PCOS, metabolic implications of nonhyperandrogenic PCOS D phenotype are still a matter of controversy. Women with PCOS in general have a significantly higher rate of impaired glucose tolerance, ranging from 18% to 40%, with an increased prevalence of type 2 diabetes reported as high as 15% compared to 2.3% in the general population (12). In addition to the risk of diabetes mellitus, many other metabolic consequences have PCOS-affected been reported in females. Approximately 50% of women with PCOS present with an android pattern of obesity (13). Excess visceral or periomental fat seems to be predictive not only of the metabolic syndrome but also of cardiovascular disease (14,15).

While some authors state that the nonhyperandrogenic PCOS D phenotype and PCOS-HA phenotypes share the same metabolic abnormalities, others have found similarities between PCOS D phenotype and non-PCOS population (4-11). However, ovarian morphology of nonPCOS population is not a uniform one and POM can be found in a respectable portion of this population. This subpopulation might be considered to represent a step between nonPCOS without POM (nonPCOSnonPOM) and D phenotype of PCOS. Literature data comparing these two subpopulations are lacking.

We embarked upon the present study to assess the metabolic status of nonPCOS-POM patients in comparison to nonPCOS-nonPOM and PCOS D phenotype. We used BMI, WC, FGIR, HOMA-IR, systolic and diastolic blood pressure on metabolic status assessment. As expected, our data confirmed an increased metabolic risk of PCOS-HA in comparison to nonPCOS-POM population. However, the PCOS D phenotype also showed significantly different metabolic markers compared to nonPCOS-POM population, suggesting an increased cardiovascular risk in PCOS-D group. On the other hand, there was no difference in the metabolic risk markers between the nonPCOS-POM and control (nonPCOS-nonPOM) groups. As shown by the age-matched comparison, the significant age difference between these two groups did not influence the conclusion significantly. The statistically significant difference in diastolic blood pressure between the two groups is unlikely to be of clinical importance.

As neither PCOS-D phenotype nor nonPCOS-POM patients are characterized by hyperandrogenism, our data suggest that hyperandrogenism may not be the only factor contributing to the increased metabolic risk in women with PCOS D phenotype. Women with POM that do not meet other PCOS criteria share similar metabolic risk markers with the control population. Our finding suggests that this population of women could not be considered as patients at an increased metabolic risk, which needs to be clarified in future follow up studies.

REFERENCES

- Azziz R, Woods KS, Reyna R, Key TJ, Knochenhauer ES, Yildiz BO. The prevalence and features of the polycystic ovary syndrome in an unselected population. J Clin Endocrinol Metab 2004;89:2745-2749.
- The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Consensus Statement: Revised 2003 consensus on diagnostic

criteria and long-term health risks related to polycystic ovary syndrome. Fertil Steril 2004;81:19-25.

 Balen AH, Laven JS, Tan SL, Dewailly D. Ultrasound assessment of the polycystic ovary: international consensus definitions. Hum Reprod Update 2003;9:505-514.

- Birdsall MA, Farquhar CM, White HD. Association between polycystic ovaries and extent of coronary artery disease in women having cardiac catheterization. Ann Int Med 1997;126:32-35.
- 5. Welt CK, Gudmundsson JA, Arason G, *et al.* Characterizing discrete subsets of polycystic ovary syndrome as defined by the Rotterdam criteria: the impact of weight on phenotype and metabolic features. J Clin Endocrinol Metab 2006;91:4842-4848.
- Barber TM, Wass JA, McCarthy MI, Franks S. Metabolic characteristics of women with polycystic ovaries and oligo-amenorrhoea but normal androgen levels: implications for the management of polycystic ovary syndrome. Clin Endocrinol (Oxf) 2007;66:513-517.
- Shroff R, Syrop CH, DavisW, Van Voorhis BJ, Dokras A. Risk of metabolic complications in the new PCOS phenotypes based on the Rotterdam criteria. Fertil Steril 2007;88:1389-1395.
- Kauffman RP, Baker TE, Baker VM, DiMarino P, Castracane VD. Endocrine and metabolic differences among phenotypic expressions of polycystic ovary syndrome according to the 2003 Rotterdam consensus criteria. Am J Obstet Gynecol 2008;198:670.e1-670.e7; discussion 670.e7-670.e10.

- 9. Norman RJ, Hague WM, Masters SC, Wang XJ. Subjects with polycystic ovaries without hyperandrogenaemia exhibit similar disturbances in insulin and lipid profiles as those with polycystic ovary syndrome. Hum Reprod 1995;10:2258-2261.
- 10. Teede H, Hutchison SK, Zoungas S. The management of insulin resistance in polycystic ovary syndrome. Trends Endocrinol Metab 2007;18:273-279.
- 11. Goverde AJ, van Koert AJ, Eijkemans MJ, Knauff EA, Westerveld HE, Fauser BC, Broekmans FJ. Indicators for metabolic disturbances in anovulatory women with polycystic ovary syndrome diagnosed according to the Rotterdam consensus criteria. Hum Reprod 2009;24:710-717.
- 12. Ehrman DA, Barnes R, Rosenfiled R, Cavaghan M. The prevalence of impaired glucose tolerance and diabetes in women with polycystic ovarian syndrome. Diabetes Care 1999;22:141.
- 13. Bhatia V. Insulin resistance in polycystic ovarian disease. South Med J 2005;98:902-909.
- 14. Sowers JR. Obesity and cardiovascular disease. Clin Chem 1998;44:1821-1825.
- 15. Grundy SM. Obesity, metabolic syndrome, and coronary atherosclerosis. Circulation 2002;105: 2696-2698.

ANTIHYPERGLYCEMIC, ANTIHYPERLIPIDEMIC AND ANTIOXIDANT EFFECTS AND THE PROBABLE MECHANISMS OF ACTION OF *RUTA GRAVEOLENS* INFUSION AND RUTIN IN NICOTINAMIDE-STREPTOZOTOCIN-INDUCED DIABETIC RATS

Osama Mohamed Ahmed¹, Adel Abdel Moneim¹, Ibrahim Abul Yazid², Ayman Moawad Mahmoud¹

Key words: Ruta graveolens infusion, rutin, nicotinamide-streptozotocin-induced diabetic rats

SUMMARY

This study was designed to evaluate and compare the efficacy of infusion of Ruta graveolens (R. graveolens) and its pharmacologically active constituent, rutin, on impaired glucose tolerance, lipid profile and oxidative stress in nicotinamide-streptozotocin-induced (type 2) diabetic albino rats. The study also suggested the probable physiological and molecular mechanisms of action of these treatments in this animal diabetic model. R. graveolens infusion and rutin were orally administered to diabetic rats at a dose of 125 and 50 mg/kg body weight/day, respectively, for 30 days. The results obtained revealed that both R. graveolens infusion and rutin led to significant amelioration of hyperglycemia, hyperlipidemia, serum insulin and Cpeptide concentrations, liver glycogen content and the activities of hexokinase, glucose-6-phosphatase and glycogen phosphorylase as well as oxidative stress in diabetic rats. On the other hand, the in vitro and in

E-mail: osamamoha@yahoo.com

situ studies indicated that R. graveolens infusion and rutin significantly enhanced insulin release from isolated islets of Langerhans, insulin binding to its receptors in rat diaphragm and peripheral glucose uptake by the rat diaphragm, whereas intestinal glucose and cholesterol absorption was significantly decreased. In addition, both treatment agents decreased resistin expression in adipose tissue, while rutin only was found to be effective in increasing adipose tissue peroxisome proliferator-activated receptor (PPAR) \Rightarrow expression. Based on these results, the study suggested both R. graveolens and rutin to exhibit antihyperglycemic and antihyperlipidemic properties via their insulinogenic effects, decreasing intestinal glucose and cholesterol absorption, improving peripheral insulin action, affecting mediators of insulin resistance, enhancing peripheral glucose uptake and decreasing hepatic glucose output in addition to the ameliorating effect on the antioxidant status in this condition.

INTRODUCTION

Diabetes mellitus (DM) has been classified into 3 categories, insulin dependent DM (IDDM), noninsulin dependent DM (NIDDM), and other specific types of diabetes (1). NIDDM is a much more prevalent form, responsible for 90% of the disease

¹ Physiology Division, Zoology Department, Faculty of Science, Beni-Suef University, Beni-Suef, P.O. 62514, Egypt

² Biological Application Department, Nuclear Research Centre, Atomic Energy Authority, Cairo, Egypt

Corresponding author: Assoc. Prof. Osama Mohamed Ahmed, Physiology Division, Zoology Department, Faculty of Science, Beni-Suef University, Beni-Suef, P.O. 62514, Egypt.

prevalence (1-3). It is a complex and heterogeneous disorder presently affecting more than 100 million people worldwide and causing serious socioeconomic problems (4).

Peroxisome proliferator-activated receptors (PPARs) have been identified as molecular focuses for drugs that target lipoprotein and glucose abnormalities associated with insulin resistance (5). There are three isoforms of PPAR: PPAR α , PPAR δ and PPAR \circledast (6). The expression of PPAR α is greatest in tissues with active metabolism, such as the liver, striated muscle and kidney, whereas PPAR δ has a very broad expression pattern that has made identifying its role more difficult. PPAR \circledast is highly expressed in fat, colon, placenta and macrophage (7,8). Two isoforms of PPAR \circledast) PPAR \circledast 1 and PPAR \circledast 2, can respond to the same signals and activate the same target genes: lipoprotein lipase, fatty acid binding protein, acyl-CoA-synthetase and CD36 (9,10).

Resistin, an adipocyte-derived protein, was originally found in a screen for genes that were induced during adipocyte differentiation, and it is found to be down-regulated in mature adipocytes exposed to thiazolinediones (11). It has been reported that resistin is expressed exclusively in adipocytes and is linked with the traits that are related to obesity and insulin resistance (12). The insulin-resistant effects of resistin are thought to account for the activation of glucose 6-phosphatase, which subsequently prevents glycogen synthesis and increases the rate of glucose production (13).

In recent years, there has been renewed interest in the treatment of different diseases using herbal drugs as the World Health Organization (WHO) has also recommended evaluation of the effectiveness of plants in conditions where we lack safe modern drugs (14). The treatment of diabetes with synthetic drugs is generally not preferred because of its high cost and the range of side effects thus caused; hence, the development of traditional or alternative medicine is needed (15). Several plants of *Rutaceae* family are used in traditional medicine worldwide (16). The most common medicinal plant of this family is *Ruta (R.) graveolens*, known as rue and native to Europe. The plant is now available all over the world, although

preferably grown in Mediterranean climate (16). This plant has been in medicinal use for various clinical conditions from ancient times, but the rationality of its use is still controversial. Rue contains various active compounds like flavonoids, coumarin derivatives, furoquinolines, volatile oils, undecanone and others (17). According to the available literature (18), *R. graveolens* plant contains approximately 2% of rutin.

Flavonoids, and particularly guercetin derivatives, have received special attention as dietary constituents in the last few years. Epidemiological studies have pointed out their possible role in preventing cardiovascular disease and cancer (19-23). This health-promoting activity seems to be related to the antioxidant (free-radical scavenging) activity to flavonoids (24). Quercetin (3,3',4',5,7-pentahydroxy flavone) is one of the most common native flavonoids occurring mainly in glycosidic forms such as rutin (5,7,3',4'-OH, 3-rutinose) (23,25). Rutin is abundantly present in onions, apples, tea and red wine (20). Rutin exhibits multiple pharmacological activities including antibacterial, antitumor, antiinflammatory, antidiarrheal, antiulcer, antimutagenic, myocardial protecting, vasodilator, immunomodulator and hepatoprotective activities (26). However, studies of the antidiabetic properties of R. graveolens and its pharmacologically active constituent, rutin, are scarce. Thus, this study aimed to assess the antidiabetic efficiency of the plant infusion and rutin in nicotinamide-streptozotocin (NA-STZ) diabetic rats and to suggest the probable mechanisms of action in this animal diabetic model.

MATERIALS AND METHODS

Experimental animals

White male albino rats (*Rattus norvegicus*) weighing about 150-200 g were used. They were obtained from the animal house of the National Research Center, El-Giza, Egypt. They were kept under observation for about 15 days before the onset of the experiment to exclude any intercurrent infection. The chosen animals were housed in plastic well aerated cages at normal atmospheric temperature (25 ± 5 °C) and normal 12hour light/dark cycle. Moreover, they had free access to water and were supplied daily with standard diet of known composition *ad libitum*. All animal procedures were in accordance with the recommendations of the Canadian Committee for Care and Use of Animals (Canadian Council on Animal Care) (27).

Preparation of plant extract

R. graveolens (sadab) was obtained from Experimental Station of Medical Plants (ESMP), Faculty of Pharmacy, Cairo University, Egypt. Its leaves were air dried and then powdered with an electric grinder. The infusion (water extract) was prepared according to the method described by Swanston-Flatt *et al.* (28). Powdered plant material was added to boiling water and infused for 15 minutes. The infusion was filtered and the filtrate was freshly used. The herb infusion was orally administered by gastric tube at a dose level of 125 mg/kg body weight (b.w.) *per* day for 30 days.

Preliminary phytochemical screening of *R. graveolens*

The air dried powder of *R. graveolens* was subjected to tests for detection of the presence of carbohydrates and/or glycosides, tannins, alkaloids and/or nitrogenous bases, flavonoids, saponins, unsaturated sterols and/or triterpenes and resins (29).

Rutin

Rutin in the form of rutin hydrate was purchased from Sigma Company, USA. It was suspended in distilled water and freshly prepared just before the administration. It was orally administered by gastric tube at a dose level of 50 mg/kg b. w./day (23) for 30 days.

Induction of diabetes mellitus

Type 2 DM was experimentally induced in animals fasted for 16 hours by intraperitoneal injection of 120 mg/kg b.w. nicotinamide dissolved in NaCl solution (0.9%) 30 minutes before intraperitoneal injection of 50 mg/kg b. w. STZ (Sigma Company) dissolved in citrate buffer (pH 4.5) (30). Ten days after STZ injection, rats were screened for serum glucose levels.

Experimental design

The rats were divided into 4 groups of 6 rats, as follows: group I consisting of normal control rats were orally administered an equivalent volume of vehicle (distilled water); group II was considered as diabetic control and orally given an equivalent volume of vehicle (distilled water); group III were orally treated with infusion of *R. graveolens* at a dose level of 125 mg/kg b.w.; and group IV were orally treated with rutin (50 mg/kg b.w.) dissolved in distilled water. All treatments were given daily for 30 days by gastric intubation. By the end of the experiment, animals were sacrificed and blood samples, visceral adipose tissue, pancreas and liver were obtained.

Histologic study

After sacrifice and dissection, pancreas was immediately excised from each animal, fixed in 10% neutral buffered formalin and transferred to Histopathology Department, Faculty of Veterinary Medicine, Beni-Suef University, Egypt, for preparation for blocking in paraffin wax and sectioning. Pancreas was stained with modified aldehyde fuchsin satin method (31).

Biochemical study

On the day before sacrifice, oral glucose tolerance test (OGTT) was performed in normal, diabetic control and diabetic rats treated with *R* graveolens infusion and rutin. Blood samples were obtained from lateral tail vein of rats deprived of food overnight (10-12 hours). Successive blood samples were then taken at 0, 30, 60, 90 and 120 minutes following the administration of glucose solution (3 g/kg b.w.) through gastric intubation. Blood samples were left to coagulate, centrifuged, and clear non-hemolyzed serum was obtained for determination of glucose concentration according to the method of Trinder (32), using reagent kit purchased from Spinreact Company (Spain).

Serum fructosamine was determined according to the method of Baker *et al.* (33) using reagent kit purchased from Reactivos Spinreact Company (Spain). Serum insulin and C-peptide were assayed in the Radioactive Isotopes Unit, Middle Eastern Regional Radioisotope Center (Dokki, Giza) by radioimmunoassay kits of DPC (Diagnostic Products Corporation, Los Angeles, USA) [coat-A-count] according to the methods of Marschner *et al.* (34) and Bonser and Garcia-Webb (35), respectively.

Liver glycogen content was determined according to the method of Seifter et al. (36). Liver hexokinase activity was determined according to the method of Branstrup et al. (37). Liver glucose-6-phosphatase and glycogen phosphorylase activities were determined according to the methods of Begum et al. (38) and Stallman and Hers (39), respectively. The liberated inorganic phosphate by glucose-6-phosphatase and glycogen phosphorylase was estimated according to the method of Munoz et al. (40) using reagent kits obtained from Biosystems, S.A (Spain). Serum total lipids (41), triglycerides (42), cholesterol (43), HDLcholesterol (44) and liver HMG-CoA reductase (45) were also estimated. Serum LDL-cholesterol level was calculated from Friedewald (46) formula (LDLcholesterol = total cholesterol - triglycerides/5 - HDLcholesterol). Serum vLDL-cholesterol concentration was calculated according to Nobert (47) formula (vLDL-cholesterol = triglycerides/5).

Liver lipid peroxidation, reduced glutathione, total thiols and catalase and peroxidase activities were also measured according to the methods of Preuss *et al.* (48), Beutler *et al.* (49), Koster *et al.* (50), Cohen *et al.* (51) and Kar and Mishra (52), respectively. Humalyzer 2000 Chemical Analyzer (Germany) was used for spectrophotometric measurements.

Physiologic techniques for the mechanism of action

Isolation of islets of Langerhans and incubation technique

Pancreatic islets were isolated from adult normal male albino rats (180-200 g) using the collagenase digestion technique of Howell and Taylor (53). Ten equal medium sized islets were picked in 0.35 mL Gey & Gey buffer (54) containing 2% bovine serum albumin using fine Pasteur pipette and stereo binocular microscope (American Optics, USA). Then, *R. graveolens* infusion or rutin was added to the isolated

islets to reach final concentrations of 0.2 and 1.0 mg/mL for each and incubation was carried out for 1 h at 37 °C. These two doses of *R. graveolens* infusion and rutin (0.2 and 1.0 mg/mL) were tested at two different concentrations of glucose (2mM and 8mM). Blanks were prepared by adding an equivalent volume of the vehicle. At the end of the incubation period, mixtures at a final volume 0.7 mL were centrifuged for 5 min at 3000 rpm and 0.2 mL of the supernatant was assayed for insulin concentration.

Intestinal glucose and cholesterol absorptions (in situ)

An intestinal perfusion technique (55) using variable flow mini-pump (Control Company, Texas, USA) was adopted to study the effect of R. graveolens infusion and rutin on intestinal glucose and cholesterol absorptions in normal rats fasted for 24 h and anesthetized with intraperitoneal sodium thiopental solution (50 mg/kg). Perfusion rate was 20 mL/h to a total volume of 20 mL via a thermoregulator set at 37 °C through 20-cm intestinal segment in situ of the anesthetized rats [with the addition of goat bile (1 mL/20 mL perfusion solution), in case of testing cholesterol absorption only]. The perfusing solution was composed of the following (in g/L): 7.37 g NaCl, 0.2 g KCl, 0.065 g NaH₂PO₄ . 2H₂O, 0.213 g MgCl₂.6H₂O, 1.02 g CaCl₂, 0.6 g NaHCO₂ and 1.0 g glucose or cholesterol, at pH 7.5. The results were expressed as percentage glucose or cholesterol absorption calculated from the amount of glucose or cholesterol in solution before and after perfusion with 62.5, 125, 250 mg/mL of R. graveolens and 25, 50, 100 mg/mL rutin in the solution.

Peripheral glucose consumption and insulin binding affinity (in vitro)

The peripheral glucose consumption was studied in rat diaphragm preparations from diabetic rats fasted for 24 h previous to sacrifice. Diaphragms were divided into two halves and incubated according to Zarzuelo *et al.* (55) at 37 °C, with constant oxygenation and shaking for 1 h. The nutrient solution was prepared with the following formula: 725 mL

1.3% NaHCO₃ was aerated for 3 min with carbogen, then added to 750 mL of saline solution. This saline solution was composed of the following (in g/L): 9.5 g NaCl, 0.4 g KCl, 0.3 g CaCl₂, 0.35 g NaHCO₃, 0.35 g MgCl₂.7H₂O, 0.2 g KH₂PO₄ and 1.0 g glucose. The resultant mixture was aerated with carbogen (95% O₂ $: 5\% \text{ CO}_2$) for 10 min before the incubation and at time intervals of 15 min during the incubation. The results were expressed as glucose consumption per 1 g diaphragm wet weight. The concentrations used were 0.2 and 1 mg/mL for R. graveolens and rutin in the presence and absence of insulin (25 µIU/mL). Stocks from samples incubated in the presence of insulin were taken in separate vials to estimate the non-binding insulin in the perfusion solution and the binding insulin was calculated from the formula (insulin binding affinity = insulin conc. before incubation non-binding insulin in the perfusing solution after incubation).

RNA extraction and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Visceral adipose tissue RNA was isolated according to the method of Otto *et al.* (56) by SV Total RNA Isolation System using reagent kit purchased from Promega Company (USA). RT-PCR for resistin and PPAR[®] was done by using Accupower RT/PCR PreMix (provided from Bioneer, Reno, USA). The kit contained 0.5 mL thin wall microtubes, each containing lyophilized reaction mixture of 10 mM dNTP, 10 U RNasin, 20 U M-MLV reverse transcriptase, IU thermostable DNA polymerase, precipitant and loading dye. The primers (obtained through Clinilab Company, Cairo, Egypt) used were as follows:

PPAR@; sense,

5'GGGTGAAACTCTGGAGATTTCC3', antisense, 5'TCAGCAACCATTGGGTCAGCTCT3' and resistin; sense,

5'GCTCAGTTCTCAATCAACCGTCC3', antisense, 5'CTGAGCTCTCTGCCACGTACT3'.

To each reaction tube, 1 μ g of RNA and 20 pmol of primer were added and the volume was made up to 50 μ L with distilled water. The lyophilized reaction mixture of each tube was dissolved by vortexing and

span-down briefly. The tubes were placed on a double heated led thermal cycler and the reaction series was performed as follows: 57 °C for 10 min, 42 °C for 60 min, 94 °C for 5 min, 35 cycles of 94 °C for 30 sec, 57 °C for 30 sec, 72 °C for 1 min.

RT-PCR samples were loaded in 1% agarose gel wells (Sigma, USA); 50V electrical power was applied (5 volts *per* 1 cm), then the samples were left to migrate for appropriate time. After migration, the cDNA bands were observed in the gel using UV illuminator. Gel images were scanned using a conventional computer scanner and then the images were analyzed using the computer software Pro-gel analysis v. 3.1.

Statistical analysis

The data were analyzed using the one-way analysis of variance (ANOVA) (57), followed by least significant difference (LSD) test to compare various groups with each other. Results were expressed as mean \pm standard error (SE) and values of *P*>0.05 were considered non-significantly different, while those of *P*<0.05 and *P*<0.01 were considered significant and highly significant, respectively. F-probability expresses the general effect between groups. The means that were not significantly different are followed by the same superscript symbol(s).

RESULTS

Qualitative phytochemical screening revealed the presence of carbohydrates and/or glycosides, tannins, alkaloids and/or nitrogenous bases, flavonoids, saponins, unsaturated sterols and/or triterpenes and resins in the tested plant (Table 1).

The oral glucose tolerance curve of diabetic rats showed a highly significant elevation at fasting state and 30, 60, 90 and 120 min after oral glucose loading as compared to normal animals. The treatment of diabetic animals with *R. graveolens* infusion and rutin induced a potential improvement of elevated values at all points of OGTT curve. However, while rutin seemed to be more potent at fasting state, the plant infusion appeared to be more effective at all other tested points after oral glucose loading (Fig. 1).

	Test	Result
	Molish's test	++ ve
Carbohydrates and/or glycosides	Fehling's test	++ ve
	Bendict's test	++ ve
	Mayer's test	+++ ve
Alkaloids and/or nitrogenous bases	Dragendorff's test	++ ve
	Wagner's test	++ ve
l la structura de stance la secol (se tritores secon	Libermann-Burchard test	++ ve
Unsaturated sterois and/or triterpenes	Salkwiski's test	+ ve
	Shinoda test	+++ ve
Flavonoids	NaOH test	++ ve
	Amyl alcohol test	++ ve
Concering	Froth test	+ ve
Saponins	Hemolysis test	+ ve
	Ferric chloride test	++ ve
Terreine	Match stick test	++ ve
Tannins	Vannilin HCl test	++ ve
	Phenazone test	++ ve
Resins		++ ve

Table 1. Preliminary phytochemical screening of R. graveolens

+ve, ++ve and +++ve = presence of active principles in low, moderate and high quantities, respectively

Table 2.	Serum	fructosamine,	insulin,	C-peptide	and	liver	glycogen	contents	of	normal,	diabetic	control	and
diabetic	treated	rats											

Parameter Group	Fructosamine (µmol/L)	Liver glycogen (mg/g tissue)	Insulin (μΙU/mL)	C-peptide (ng/mL)
Normal	163.046±2.61°	18.64±0.64 ^a	27.17±0.53ª	8.87±0.19 ^a
Diabetic control	233.046±3.99 ^a	8.80±0.54°	13.26±0.81 ^d	3.85±0.26 ^c
Diabetic treated with R. graveolens	194.371±4.76 ^b	10.17±0.87 ^{bc}	16.43±0.22 ^c	4.69±0.09 ^b
Diabetic treated with rutin	187.146±3.82 ^b	12.05±0.81 ^b	24.64±1.05 ^b	8.38±0.18 ^a
F-probability	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> <0.001
LSD at 5% level	11.44	2.15	2.13	0.57
LSD at 1% level	15.59	2.93	2.90	0.77

Data are expressed as mean \pm SE; there were six animals in each group; the means marked with the same superscript symbol(s) were not significantly different; the means with difference higher than the value of LSD at 5% level were significantly different (P<0.05) and those with difference higher than the value of LSD at 1% level were highly significantly different (P<0.01)

Table 3. Insulin release from isolated islets incubated for 1 h with two different doses of *R. graveolens* infusion and rutin in the presence of 2 and 8mM glucose *in vitro*

		Insulin release (µIU/islet/h)						
Glucose		R. gra	veolens	Ru	tin			
concentration	Control	0.2 mg/mL	1.0 mg/mL	0.2 mg/mL	1.0 mg/mL			
2mM	3.27±1.13 ^h	5.50±0.79 ^g	5.50±1.59 ^g	7.91± 1.13 ^f	9.29± 2.76 ^e			
8mM	17.48±1.23 ^d	22.31±0.55 ^c	23.28 ±0.45 ^b	29.10± 0.01 ^a	29.11± 2.02ª			

F-probability: P<0.001; LSD at 5% level: 4.40; LSD at 1% level: 6.26

As indicated in Table 2, the liver glycogen content and serum insulin and C-peptide exhibited significant decrease in diabetic rats. In contrast, serum fructosamine was remarkably elevated (P<0.01; LSD) in diabetic rats as compared to normal. Treatment of diabetic animals with the plant infusion and rutin induced profound amendment of these deteriorated changes. Rutin seemed to be more effective in improving these altered variables.

Both concentrations of both agents tested (Table 3) induced significant insulinotropic effects at 2 mM and 8 mM glucose as compared with the corresponding blanks; rutin appeared to be more potent than R. *graveolens* infusion.

Data shown in Table 4 indicated that the addition of *R. graveolens* extract as well as rutin to the incubation media caused a significant increase in glucose uptake by the rat diaphragm, in a dose dependent manner. The present data showed the effect of both agents to be potentiated in the presence of insulin. Moreover, rutin seemed to be more effective in enhancing peripheral glucose uptake in the presence and absence of insulin.

The addition of both rue infusion and rutin to the incubation media caused an increase of insulin binding to the rat diaphragm in a dose dependent manner. Both agents produced a significant increase in insulin binding with increasing concentrations as compared with the blank. Rutin seemed to be more effective in enhancing insulin binding to the rat diaphragm (Table 5).

Data on the effects of *R. graveolens* and rutin at varying concentrations on intestinal glucose absorption are given in Table 6. All the concentrations of *R. graveolens* and rutin tested produced marked decrease in the intestinal glucose absorption. The increasing concentration of both agents was found to be accompanied by detectable decreases in the intestinal glucose absorption. In addition, the concentrations of rutin tested seemed to be more effective than those of *R. graveolens* infusion in reducing intestinal glucose absorption. On the other hand, both *R. graveolens* infusion and rutin induced

Figure 1. Oral glucose tolerance curves of normal, diabetic control and diabetic rats treated with R. graveolens infusion and rutin. F-probability: p<0.001; LSD at 5% level: 4.40; LSD at 1% level: 6.26.



Table 4. Changes in peripheral glucose uptake (mg/g fresh diaphragm) of diabetic rat diaphragm with increasing doses of *R. graveolens* infusion and rutin in the presence and absence of insulin *in vitro*

		<i>R. graveolens</i> infusion Dose (mg/mL)		Rutin Dose (mg/mL)	
	Blank	0.2	1.0	0.2	1.0
In the absence of insulin	1.13±0.08 ^j	3.79 ± 0.07^{i}	4.13±0.04 ^h	4.54±0.09 ^g	5.49±0.05 ^f
In the presence of insulin (25 µIU/mL)	5.75±0.07 ^e	6.00±0.07 ^d	6.33±0.17 ^c	7.03±0.04 ^b	7.27±0.04 ^a

F-probability: P<0.001; LSD at 5% level: 0.235; LSD at 1% level: 0.316

Binding insulin concentration (µIU/100 mg fresh diaphragm/h)

Blank	R. grav	eolens	Rutin		
	0.2 mg/mL	1.0 mg/mL	0.2 mg/mL	1.0 mg/mL	
56.78±0.45 ^e	79.72±0.06 ^d	112.17±0.79 ^b	89.25±0.95 ^c	121.55±0.35 ^a	

F-probability: P<0.001; LSD at 5% level 1.78; LSD at 1% level 2.41

	R. gra	aveolens	R	Rutin
	Dose		Dose	
Blank	(mg/mL)	Absorption %	(mg/mL)	Absorption %
	62.5	28.97±0.56 ^b	25	16.40±0.94 ^e
45.55±1.42 ^a	125	27.23±0.31 ^b	50	14.30±0.34 ^e
	250	24.82±0.56 ^c	100	12.97±0.19 ^d

Table 6. Changes in intestinal glucose absorption with increasing dose of R. graveolens and rutin

F-probability: P< 0.001; LSD at 5% level: 2.891; LSD at 1% level: 3.894

Table 7. Changes in intestinal cholesterol absorption with increasing dose of *R. graveolens* infusion and rutin

	R. graveo	lens infusion	R	Rutin
	Dose		Dose	
Blank	(mg/mL)	Absorption %	(mg/mL)	Absorption %
	62.5	104.67±0.76°	25	110.00±1.21 ^b
131.50±1.75 ^a	125	95.33±0.76 ^e	50	102.00±0.52°
	250	94.50±0.88 ^e	100	98.83±0.54 ^d

F-probability: *P*<0.001; LSD at 5% level 2.89; LSD at 1% level 3.89

Table 8. Liver glucose-6-phosphatase, glycogen phosphorylase and hexokinase activities of normal, diabetic control and diabetic rats treated with *R. graveolens* infusion and rutin

Parameter	Glucose-6-phosphatase	Glycogen phosphorylase	Hexokinase (µg glucose
Group	(µg Pi liberated/100 mg tissue/h)	(µg Pi liberated/100 mg tissue/h)	phosphorylated/100 mg tissue/h)
Normal	2.00±0.08°	1.73±0.99 ^d	26.18±0.07 ^a
Diabetic control	15.95±0.21ª	12.65±0.02 ^a	3.82±2.04 ^c
Diabetic treated with R. graveolens	4.79±0.27 ^b	7.02±3.42 ^b	9.07±0.09 ^b
Diabetic treated with rutin	4.35±0.23 ^b	5.85±2.77°	8.17±3.89 ^b
F-probability	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> <0.001
LSD at 5% level	0.62	0.75	0.187
LSD at 1% level	0.84	1.02	0.255

Table 9. Serum lipid profile and liver HMG-CoA reductase activity of normal, diabetic control and diabetic rats treated with R. graveolens infusion and rutin

Paramete	r						HMG-CoA
Group	Total lipids (g/L)	CH (mg/dL)	TG (mg/dL)	HDL-CH (mg/dL)	LDL-CH (mg/dL)	VLDL-CH (mg/dL)	(HMG-CoA/ mevalonate)
Normal	2.44±0.11 ^c	57.00±0.56 ^d	45.85±0.71 ^d	34.23±0.24 ^a	13.60±0.39 ^d	9.17± 0.06 ^d	1.12±0.54 ^d
Diabetic control	4.81±0.27 ^a	89.58±0.18 ^a	151.36±2.45ª	15.43±0.45 ^d	43.85±0.69 ^a	30.29±0.50 ^a	1.42±0.76 ^a
Diabetic treated with <i>R. graveolens</i>	3.07±0.16 ^b	69.56±0.60 ^b	63.29±1.47 ^b	24.38±0.22 ^c	32.52±0.70 ^b	12.66± 0.29 ^b	1.31±0.73 ^b
Diabetic treated with rutin	3.05±0.15 ^b	66.69±0.49 ^c	58.03±1.42 ^c	26.60±0.18 ^b	28.66±0.34 ^c	11.61±0.28 ^c	1.25±0.01°
F-probability	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> <0.001
LSD at 5%	0.470	1.44	4.73	0.86	1.62	0.96	2.062
LSD at 1%	0.642	1.97	6.45	1.18	2.22	1.31	2.812

CH: cholesterol; TG: triglycerides; LDL-CH: low density lipoprotein cholesterol; HDL-CH: high density lipoprotein cholesterol; VLDL-CH: very low density lipoprotein cholesterol

potential decreases in intestinal cholesterol absorption in a dose dependent manner, with a more potent effect of *R. graveolens* (Table 7).

Regarding liver glucose-6-phosphatase and glycogen phosphorylase activities, they were increased in diabetic rats, as illustrated in Table 8. The administration of both *R. graveolens* and rutin produced a profound improvement of these altered enzyme activities. In contrast, liver hexokinase activity (Table 8) showed a different pattern. The enzyme activity was enormously suppressed in diabetic control rats and profoundly increased in diabetic rats treated with *R. graveolens* and rutin. Furthermore, rutin appeared to be more potent in decreasing the activities of glucose-6-phosphatase and glycogen phosphorylase. On the other hand, treatment with *R. graveolens* produced a more potent effect than treatment with rutin in increasing hexokinase activity.

Data on the effect of *R. graveolens* and rutin on lipid profile of diabetic rats are presented in Table 9. Diabetic rats exhibited a highly significant increase (P<0.01; LSD) in serum total lipids, cholesterol, triglycerides, LDL- and vLDL-cholesterol and liver HMG-CoA reductase activity as compared with the non-diabetic group. Moreover, HDL-cholesterol was affected in an opposite manner, as it was decreased (P<0.01; LSD) in diabetic rats and significantly increased (P<0.01; LSD) in response to both treatment agents. The administration of both *R. graveolens* infusion and rutin led to marked amelioration of all parameters of the altered lipid profile. Liver lipid peroxidation, total thiols, reduced glutathione content and liver antioxidant enzymes variation as the result of treatment of diabetic rats with *R. graveolens* and rutin are presented in Table 10.

Figure 2. **PPAR** γ **cDNA** pattern in adipose tissue of normal control (Nc), diabetic control (Dc), diabetic treated with rutin (Rn) and diabetic treated with infusion of *R. graveolens* (Er).



Figure 3. Semiquantitative analysis of PPAR γ cDNA using scanning densitometry.



Parameter		Glutathione			
Group	Lipid peroxidation (nmol MDA/gm/h)	Total thiols (nmol/100 mg)	Reduced glutathione (nmol/100 mg)	Catalase (k x 10²)	peroxidase (U/g)
Normal	24.4±0.85 ^c	196.97±6.61 ^a	74.93±3.74 ^a	52.68±1.38 ^a	75.96± 1.17 ^a
Diabetic control	41.2± 2.15 ^a	89.93± 2.46 ^c	57.52±1.47 ^b	22.86±0.49 ^d	47.21±2.52 ^b
Diabetic treated with <i>R. graveolens</i>	29.9± 1.85 ^b	134.53±9.12 ^b	70.83±4.27 ^a	39.95±0.47°	62.97±3.86 ^c
Diabetic treated with rutin	29.3± 0.90 ^b	145.02±12.01 ^b	72.88± 4.43 ^a	44.10±1.84 ^b	68.66± 2.97 ^{ab}
F-probability	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> <0.001	<i>P</i> <0.001
LSD at 5%	4.59	24.58	10.83	3.55	8.27
LSD at 1%	6.26	33.48	14.78	4.84	11.28

Table 10. Liver lipid peroxidation, total thiols, reduced glutathione content, catalase and glutathione peroxidase activity in normal, diabetic control and diabetic rats treated with *R. graveolens* infusion and rutin

Liver malondialdehyde (MDA) content, which is an indicator of lipid peroxidation, was significantly increased in diabetic rats when compared to normal rats. On the other hand, treatment with both tested agents markedly ameliorated the increased liver MDA content. Meanwhile, the depleted liver total thiols and reduced glutathione contents of diabetic rats were significantly increased as the result of treatment with both *R. graveolens* and rutin.

Concerning the effect of *R. graveolens* infusion and rutin on PPAR® expression in visceral adipose tissue (Figures 2 and 3), densitometric analysis revealed a significant decrease of adipose tissue PPAR® cDNA in diabetic rats as compared with the normal control group. The treatment of diabetic animals with rutin produced a significant amelioration of PPAR® expression while treatment of diabetic rats with *R.* graveolens infusion did not show an increase. On the other hand, there was a significant increase in visceral adipose tissue resistin cDNA of diabetic rats as compared with the normal control ones, as illustrated in Figures 4 and 5. The administration of both *R.* graveolens and rutin produced a significant decrease in the visceral adipose tissue resistin expression. Figure 4. Resistin cDNA pattern in adipose tissue of normal control (Nc), diabetic control (Dc), diabetic treated with rutin (Rn) and diabetic treated with infusion of *R. graveolens* (Er).







Figure 6. Pancreatic tissues of normal male albino rats. The pancreas is subdivided by septa (s) into pancreatic lobules. The exocrine portion of the pancreas consists of pancreatic acini (pa), while endocrine portion consists of the islets of Langerhans (IL) which are scattered throughout the pancreas and contain alpha cells (a) at the periphery of islets, beta cells (b) in the core of islets and delta cells (d) of a relatively larger size. (X400)





As for the histologic study, the islets of Langerhans in the pancreas of diabetic rats exhibited marked degeneration of cells (Figure 7A and 7B) as compared with normal pancreata (Figure 6A and 6B). The islets of diabetic rats had vacuolations, many hydropic and necrotic cells, and pyknotic or irregular hyperchromatic nuclei. The treatment with either plant aqueous extract or rutin markedly succeeded to amend the disrupted islets of Langerhans of diabetic rats; the islet architecture and integrity were improved (Figures 8A, 8B, 9A and 9B). In spite of these ameliorations, there were still a few necrotic areas (n), pyknotic nuclei (pk) and vacuolations (v) in the islets of diabetic rats treated with the plant aqueous extract and rutin.

Figure 7. Pancreata of diabetic rats. Normal architecture of the islets is disrupted. Islets showing many hydropic cells, necrotic cells (n), pyknotic nuclei (pk), vacuolations (v) and irregular hyperchromatic nuclei (hcn). (X400)



Figure 8. Pancreata of diabetic rats treated with *R. graveolens* infusion. There are still few necrotic areas (n), pyknotic nuclei (pk) and vacuolations (v). The islet architecture is more organized and less disrupted as compared with that of diabetic control. Alpha cells (a) were noticed at the periphery of islets and many beta cells (b) in the core of the islets appeared intact. (X400)





Figure 9. Pancreata of diabetic rats treated with rutin showing its ameliorative effect on the islets of Langerhans. There are still few necrotic areas (n), pyknotic nuclei (pk) and vacuolations (v). Beta cells (b) in the core of islets appeared with improved integrity and were obviously increased in number. Alpha cells (a) and delta cells (d) at the periphery of islets were also noticed. (X400)





DISCUSSION

Study results revealed the treatment of diabetic rats with either R. graveolens infusion or its flavonoid, rutin, to lead to significant amelioration of glucose tolerance. A decrease in elevated serum glucose levels is in agreement with the results of Chakravarthy et al. (58,59), who demonstrated similar effects of the flavonoid, (-)-epicatechin, in alloxan diabetic rats, and also with those reported by Nuraliev and Avezov (60) on a hypoglycemic effect of quercetin in alloxan diabetic animals. Furthermore, the hypoglycemic effect of rutin in STZ diabetic animals was recorded by Vessal et al. (61) and Kamalakkannan and Prince (23). Thus, the hypoglycemic effect of R. graveolens may be due to the presence of flavonoids such as rutin. Moreover, the antihyperglycemic effect may also be attributed to the presence of substances other than flavonoids, i.e. glycosides, alkaloids, saponin, tannins, resins and triterpenes. Such compounds have been reported to be responsible for hypoglycemic action by several authors (61-65). The present study indicated these groups of compounds to be found in the tested plant.

In comparison with normal control rats, the present study revealed a profound decrease in fasting insulin level of STZ diabetic rats. This finding agrees with Akhani *et al.* (66) and may be ascribed to the diabetogenic effect of STZ, which leads to marked degenerative changes in β -cells, as indicated in the present study. Serum insulin concentration was increased markedly as a result of treating diabetic rats with both *R. graveolens* and rutin.

By its ability to scavenge free radicals and to inhibit lipid peroxidation (67), rutin prevents STZ-induced oxidative stress, protects β -cells resulting in increased insulin secretion, and decreases elevated blood glucose levels. In this context, a study by Vessal *et al.* (61) showed that quercetin, an aglycone of rutin, decreased elevated blood glucose concentration and increased insulin release in STZ-induced diabetic rats. Also, Coskun *et al.* (68) report that in STZ-induced diabetic rats, quercetin protected pancreatic β -cells by decreasing oxidative stress and preserving β -cell integrity. According to the work of Kamalakkannan and Prince (23), the increased insulin levels could also be due to the stimulatory effect of rutin, thereby potentiating insulin secretion from the existing β -cells of the islets of Langerhans in diabetic treated rats. Moreover, Hii and Howell (69) showed that exposure of isolated rat islets to certain flavonoids such as (-)epicatechin or quercetin enhanced insulin release. They suggest that such flavonoids may act on islet function, at least in part, *via* alteration in Ca²⁺ fluxes and in cyclic nucleotide metabolism.

In the present study, serum C-peptide was profoundly decreased in diabetic rats. The treatment with both *R*. *graveolens* infusion and rutin produced a marked increase in serum C-peptide level of diabetic rats. According to the results of Kamalakkannan and Prince (23), the increase in serum C-peptide levels concomitant with the increase of serum insulin levels in diabetic rats treated with rutin reflects the increase in insulin secretion.

According to our results, the marked increase in serum insulin and C-peptide levels after treatment of diabetic rats with *R. graveolens* infusion and rutin was due to the stimulatory effects of these agents on the insulin secretory response of the islets of Langerhans on the one hand, as indicated by the present *in vitro* study, in addition to the ameliorative effects of these agents on the integrity of β -cells as revealed by histologic study on the other hand.

While serum glucose concentration measurement is usually used to detect glycemic regulation in diabetes after treatment, fructosamine appeared more useful because it detects deterioration or improvement over a period of several days (1-3 weeks) after diabetes management. Serum fructosamine, a putative measure of glycosylated proteins, has been suggested by many authors to be of value as a screening test for diabetes mellitus (70,71). In the present study, serum fructosamine level was profoundly increased in diabetic rats at fasting state as compared with normal ones. On the other hand, treatment of diabetic rats with R. graveolens aqueous extract and rutin induced an obvious decrease of elevated fructosamine level. Elgawish et al. (72) report that agents with antioxidant or free radical scavenging power may inhibit oxidative reactions associated with protein glycation. Therefore, rutin with its free radical scavenging capability effectively reduced the formation of glycated proteins.

This explanation was supported by the present study, indicating that *R. graveolens* infusion and rutin have a strong antioxidant activity. A decrease in blood glucose levels may have also contributed to decreased levels of glycated proteins in *R. graveolens* and rutin treated diabetic rats. This finding is in accordance with the study by Kamalakkannan and Prince (23), which showed decreased levels of glycated hemoglobin in rutin-treated diabetic rats.

The liver glycogen level may be considered as the best marker to assess the anti-hyperglycemic activity of any drug (73). The increased hepatic glucose output in diabetes may be derived from glycogenolysis and/or gluconeogenesis, as reported by Raju et al. (74). Our results revealed an enormous depletion in hepatic glycogen content accompanied by a decreased hexokinase activity and profound elevation of hepatic glycogen phosphorylase activity and the gluconeogenic enzyme, glucose-6-phosphatase, as compared to that of normal control ones. These results are in accordance with those of Lavoie and Van de Werve (75), Ahmed (76) and Abdel-Moneim et al. (77), who found that STZ-induced diabetes reduced hepatic glycogen content and increased glucose-6-phosphatase activity in diabetic rats. These results are also in agreement with the work of Grover et al. (73), who demonstrated a decreased enzymatic activity of hexokinase in diabetic animals, resulting in depletion of liver glycogen. These changes may be due to insulin deficiency and/or insulin resistance, which in turn results in the activation of glycogenolytic and gluconeogenic pathways (77, 78).Moreover, deficiency of insulin secretion decreases hepatic tyrosine kinase responsible for the activation of glycogen synthase and as a result, glycogen breakdown prevails in diabetic animals (79). In the present study, the elevation of liver glycogen content after treatment with R. graveolens extract and rutin was due to amelioration of these altered enzyme activities secondary to the increase of insulin levels in the blood as well as improvement of insulin action. In addition, the enhanced peripheral glucose uptake and increased hepatic hexokinase activity as well as decreased glucose-6-phosphatase activity after treatment with the tested agents, as indicated in the

present *in vitro* and *in vivo* studies, lead to more intense formation of glucose-6-phosphate, which in turn inactivates glycogen phosphorylase and activates glycogen synthase in the liver and muscle (79,80). The increase in hexokinase activity and the decrease in glucose-6-phosphatase, in the present study, may have also reflected a decrease in hepatic glucose output and enhanced peripheral glucose uptake as the result of treatment.

The data obtained in the present study indicated that R. graveolens infusion and rutin produced a marked increase of peripheral glucose consumption in the presence and absence of insulin as compared with the corresponding controls. Both agents acted in a dose dependent manner with a more potent effect for rutin than rue infusion. The marked increase in the peripheral glucose uptake in the absence of insulin as a result of the tested agents suggests that they may have insulin mimetic action or non-insulin mediated effect. The enhanced rate of peripheral glucose uptake in the absence of insulin may involve an insulin independent increased protein expression of both GLUT1 and GLUT4 (81-83). Furthermore, all doses of both agents were able to potentiate the enhanced effect of insulin on peripheral glucose uptake in the presence of insulin. Such findings suggest that both an insulin-independent rise in glucose uptake per se as a direct result of the tested agents would possibly synergize with insulin to formulate concrete mechanisms contributing to the hypoglycemic activity observed herein. In addition, both R. graveolens and rutin increased the in vitro insulin binding affinity of rat diaphragm in a dose dependent manner. Thus, the insulin mediated effects of the tested agents in the present study may have included the increase in insulin binding affinity by these agents. In addition to the effect on hepatic glucose output and peripheral glucose uptake, the plant infusion and rutin induced a profound decrease in intestinal glucose absorption in a dose dependent manner.

Our results proved that both *R. graveolens* and rutin improved glucose tolerance and this amelioration seemed to be mediated *via* alleviation of the islet architecture, enhancement of insulin release, insulin binding affinity and peripheral glucose uptake and decreasing intestinal glucose absorption in addition to decreasing the activity of gluconeogenic and glycogenolytic enzymes.

In view of the lipid profile, diabetic rats exhibited marked elevation of serum total lipids, triglycerides, total cholesterol, LDL-cholesterol, VLDL-cholesterol concentrations and hepatic HMG-CoA reductase activity.

The treatment of STZ diabetic rats with *R*. graveolens extract or rutin produced potential improvement of these altered serum lipid variables. These results are in agreement with the work of Nuraliev and Avezov (60), who demonstrated the decreasing effects of quercetin on decreasing the level of cholesterol and LDL-cholesterol.

The ability of quercetin to reduce plasma cholesterol and triglycerides in diabetic animals could be explained by the insulin releasing capacity of quercetin, which is a metabolite of rutin in isolated rat islets of Langerhans (69). The reduction of intestinal cholesterol absorption might have a role in the mechanism of action to augment the hypolipidemic activity of the tested materials. This assumption is supported by the present results which indicated that both plant extract and rutin successfully decreased the intestinal cholesterol absorption in situ. Furthermore, as evidenced in this study, the hypolipidemic activity of both treatments may also be mediated, at least in part, via inactivation of hepatic HMG-CoA reductase, a key enzyme, in cholesterol synthesis. In concurrence with this attribution, Raz et al. (84) state that inhibitors of hepatic HMG-CoA reductase are well established drugs for the treatment of hypercholesterolemia and decrease the incidence of dyslipidemia in diabetic subjects. This also coincides well with the work of Jung et al. (85), who state that flavonoids decrease liver HMG-CoA reductase activity in type 2 diabetic mice. Moreover, rutin has been reported to lower hepatic and blood cholesterol levels, as stated by Park et al. (86).

Taken together, it can be concluded that the ameliorative effect of *R. graveolens* extract or rutin on serum lipid variables may be attributed to their insulin

releasing capacity and insulin binding affinity and decreasing intestinal cholesterol absorption and activity of hepatic HMG-CoA reductase.

In the present study, amelioration of the glycemic and lipidemic states of diabetic rats in response to treatment with rutin may also be attributed to the increased expression of PPAR[®]. Rutin showed a potential effect on the expression of PPAR \otimes , but R. graveolens infusion had no effect on PPAR® expression; this may explain why rutin had more effective hypoglycemic and hypolipidemic effects than *R. graveolens* infusion. The effect of PPAR[®] on lipid and glucose control may be explained according to Lee et al. (87), Staels and Fruchart (88), Feige et al. (89) and Lefebvre et al. (90), who state that PPAR® promotes pre-adipocyte differentiation, stimulates the storage of fatty acids (FAs) in adipocytes and enhances insulin sensitivity. The action of PPAR[®] on insulin sensitivity results from its ability to channel FAs into adipose tissue. thus decreasing plasma FA concentration and alleviating lipotoxicity in skeletal muscle, liver and pancreas. Also, PPAR@ activation has been reported to improve insulin resistance by lowering the hepatic triglyceride content (91,92), activating hepatic glucokinase expression (93) and exhibiting an antiatherogenic effect synergistic with an HMG-CoA reductase inhibitory effect in rabbits (94). In addition, PPAR[®] can affect insulin sensitivity by regulating adipocyte hormones, cytokines and proteins that are involved in insulin resistance. Indeed, PPAR® downregulates the expression of genes encoding resistin and TNF α , whereas it induces adiponectin expression, which increases FA oxidation by activation of the AMP-activated protein kinase pathway (89,90). Moreover, Willson et al. (95) demonstrated the activation of PPAR® to improve insulin sensitivity and lower circulating levels of glucose, triglycerides and FFAs without stimulating insulin secretion in rodent models of type 2 diabetes.

Also, Hevener *et al.* (96) state that PPAR@ agonists increase glucose uptake in adipose tissue and skeletal muscle. Our results coincide with the work of Jung *et al.* (85), who found an increased expression of PPAR@ by the flavonoids hesperidin and naringin, and that of Anandharajan *et al.* (97), who showed an increased PPAR@ expression by *Pterocarpus marsupium* isoflavone on L6 myoblasts and myotubes.

The data obtained demonstrated that the treatment of diabetic rats with either R. graveolens extract or rutin caused a marked decrease of resistin mRNA transcripts that greatly increased in STZ diabetic rats. According to Rajala et al. (13), the insulin resistant effects of resistin are thought to account for the activation of glucose-6-phosphatase, which subsequently prevents glycogen synthesis and increases the rate of glucose production. The present results supported this suggestion since the increase in resistin expression was associated with profound elevation of glucose-6-phosphatase activity. On the other hand, Antuna-Puente et al. (98) postulated that the absence of resistin could allow for the activation of activated mitogen pyruvate kinase (AMPK) and reduce gene expression encoding for hepatic gluconeogenesis enzymes. However, little is known concerning the control of resistin gene expression. Steppan et al. (99) demonstrated that resistin mRNA and protein were downregulated during fasting and upregulated upon refeeding. Thus, these results reflect the ameliorative effect of R. graveolens and rutin on the glycemic and lipidemic states of diabetic rats.

This study revealed that both study agents had antihyperglycemic and antihyperlipidemic efficacy which may be mediated *via* pancreatic and extrapancreatic effects. However, further clinical studies are required to assess the efficacy and safety of *R. graveolens* and rutin in diabetic humans.

REFERENCES

- World Health Organization. Definition, diagnosis and classification of diabetes mellitus and its complications. In: Report of a WHO Consultation, Part 1: Diagnosis and classification of diabetes mellitus. WHO/NCD/ NCS/99.2, Geneva, 1999.
- Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004;27:1047-1053.
- 3. Cheng D. Prevalence, predisposition and prevention of type II diabetes. Nutr Metab 2005;2:2-29.
- Srinivasan K, Ramarao P. Animal models in type 2 diabetes research: an overview. Indian J Med Res 2007;125:451-472.
- Rosenson RS. Effects of peroxisome proliferator activated receptors on lipoprotein metabolism and glucose control in type 2 diabetes mellitus. Am J Cardiol 2007;99(Suppl):96B-104B.
- Bookout AL, Jeong Y, Downes M, Yu RT, Evans RM, Mangelsdorf DJ. Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. Cell 2006;126(4):789-799.
- Rosen ED, Spiegelman BM. Molecular regulation of adipogenesis. Annu Rev Cell Dev Biol 2000;16:145-171.
- Barish GD, Downes M, Alaynick WA, Yu RT, Ocampo CB, Bookout AL, Mangelsdorf DJ, Evans RM. A nuclear receptor atlas: macrophage activation. Mol Endocrinol 2005;19(10):2466-2477.
- Rosen ED, Spiegelman BM. PPARgamma: a nuclear regulator of metabolism, differentiation, and cell growth. J Biol Chem 2001;276(41):37731-37734.

- Allen T, Zhang F, Moodie SA, Clemens LE, Smith A, Gregoire F, Bell A, Muscat GE, Gustafson TA. Halofenate is a selective peroxisome proliferatoractivated receptor gamma modulator with antidiabetic activity. Diabetes 2006;55(9):2523-2533.
- Holcomb IN, Kabakoff RC, Chan B, Baker TW, Gurney A, Henzel W. FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. EMBO J 2000;19:4046-4055.
- Kim KH, Lee K, Moon YS, Sul HS. A cysteinerich adipose tissue-specific secretory factor inhibits adipocyte differentiation. J Biol Chem 2001;276:11252-11256.
- Rajala MW, Obici S, Scherer PE, Rossetti L. Adipose-derived resistin and gut-derived resistinlike molecule-beta selectively impair insulin action on glucose production. J Clin Invest 2003;111:225-230.
- Ayyanar M, Sankarasivaraman K, Ignacimuthu S. Traditional herbal medicines used for the treatment of diabetes among two major tribal groups in South Tamil Nadu, India. Ethnobotanical Leaflets 2008;12:276-280.
- 15. Arulrayan N, Rangasamy S, James E, Pitchai D. A database for medicinal plants used in the treatment of diabetes and its secondary complications. Bioinformation 2007;2(1):22-23.
- Raghav SK, Gupta B, Agrawal C, Goswami K, Das HR. Anti-inflammatory effect of *R.* graveolens L. in murine macrophage cells. J Ethnopharmacol 2006;104:234-239.
- 17. Pathak S, Multani AS, Banerji P, Banerji P. *Ruta* selectively induces cell death in brain cancer cells but proliferation in normal peripheral blood lymphocytes: a novel treatment for human brain cancer. Int J Oncol 2003;23:975-982.

- CSIR. In: Chadha YR, editor. The Wealth of India

 A dictionary of Indian raw materials and industrial products, Vol. IX. Publications and Information Directorate, Council of Scienti?c and Industrial Research, India, 1988; pp. 95-96.
- Hertog MG, Hollman PC, Katan MB. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in The Netherlands. J Agric Food Chemist 1992;40:2379-2383.
- Hertog MG, Feskens EJ, Hollmann PC, Katan MB, Kronthout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen elderly study. Lancet 1993;342:1007-1011.
- Hertog MG, Hollman PC, Katan MB, Kromhout D. Intake of potentially anticarcinogenic flavonoids and their determinants in adults in The Netherlands. Nutr Cancer 1993;20:21-29.
- Chu YH, Chang CL, Hsu HF. Flavonoid content of several vegetables and their antioxidant activity. J Sci Food Agric 2000;80:561-566.
- 23. Kamalakkannan N, Prince SM. Antihyperglycemic and antioxidant effect of rutin, a polyphenolic flavonoid, in streptozotocin-induced diabetic Wistar rats. Basic Clin Pharmacol Toxicol 2006;98(1):97-103.
- 24. Murota K, Terao J. Antioxidative flavonoid quercetin: implication of its intestinal absorption and metabolism. Arch Biochem Biophys 2003;417:12-17.
- 25. Havsteen B. Flavonoids, a class of natural products of high pharmacological potency. Biochem Pharmacol 1983;32:1141-1148.
- Janbaz KH, Saeed SA, Gilani AH. Protective effect of rutin on paracetamol- and CCl4-induced hepatotoxicity in rodents. Fitoterapia 2002;73:557-563.
- Canadian Council on Animal Care. Guide to the care and use of experimental animals. Vol. 2, CCAC, Ottawa, Ontario, Canada, 1993.

- 28. Swanston-Flatt SK, Day C, Flatt PR, Bailey CJ. Evaluation of antihyperglycemic properties of traditional plant treatment for diabetes in streptozotocin diabetic and db/db mice. In: Shafrir E, editor. Frontiers in diabetes research. Lessons from animal diabetes. Vol. 3. London: Smith-Gordon, 1995; pp. 286-293.
- 29. Claus FD. Phamacognosy, 4th edition. London: Henry Kumpton, 1961.
- 30. Punitha IS, Shirwaikar A, Shirwaikar A. Antidiabetic activity of benzyl tetra isoquinoline alkaloid berberine in streptozotocin-nicotinamide induced type 2 diabetic rats. Diabetol Croat 2005;34(4):117-128.
- Bancroft JD, Stevens A. Theory and practice of histological techniques, 2nd edition. Edinburgh: Churchill Livingstone, 1982; pp. 374-375.
- 32. Trinder P. Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Ann Clin Biochem 1969;6:24-27.
- 33. Baker JR, Metcalf PA, Holdway I, Johnson RN. Serum fructosamine concentration as a measure of blood glucose control in type 1 (insulin dependent) diabetes. Br Med J 1985;290:352-355.
- 34. Marschner I, Bottermann P, Erhardt F, Linke R, Maier V, Schwandt P, Vogt W, Scriba PC. Group experiments on the radio-immunological insulin determination. Horm Metab Res 1974;6:293-296.
- 35. Bonser A, Garcia-Webb P. C-peptide measurement: methods and clinical utility. CRC Crit Rev Clin Lab Sci 1984;19:279-352.
- 36. Seifter S, Dayton S, Novic B, Muntwyler E. The estimation of glycogen with anthrone reagent. Arch Biochem 1950;25:191-200.
- Branstrup N, Kirk JE, Bruni C. The hexokinase and phosphoglucoisomerase activities of aortic and pulmonary artery tissues in individuals of various ages. J Gerontol 1963;238:3280.

- Begum N, Sam GP, Shanmugasundaram KR. Serum enzymes in human and experimental diabetes mellitus. Indian J Med Res 1978;68:774-784.
- 39. Stallman W, Hers HG. The stimulation of liver phosphorylase b by AMP, fluoride and sulfate. A technical note of the specific determination of the a and b forms of liver glycogen phosphorylase. Eur J Biochem 1975;54:341-350.
- Munoz MA, Balon M, Fernandez C. Direct determination of inorganic phosphorus in serum with a single reagent. Clin Chem 1983;29:372-374.
- 41. Frings CS, Frendley TW, Dunn RT, Queen CA. Improved determination of serum total lipids by the sulpho-phospho-vanillin reaction. Clin Chem 1972;18(7):673-674.
- 42. Fossati P, Prencipe L. Serum triglycerides determined colorimetrically with an enzyme that produces hydrogen peroxide. Clin Chem 1982;28(1):2077-2080.
- 43. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem 1974;20(4):470-475.
- 44. Burnstein M, Selvenick HR, Morfin R. Rapid method for isolation of lipoprotein from human serum with polyanions. J Lipid Res 1970;11:583-395.
- 45. Venugopala Rao A, Ramakrishan S. Indirect assessment of hydroxymethylglutaryl CoA reductase (NADPH) activity in liver tissue. Clin Chem 1975;21(10):1523-1525.
- 46. Friedewald WT. Estimation of concentration of low-density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. Clin Chem 1972;18(7):499-502.
- Nobert WT. Clinical guide to laboratory tests, 3rd edition. Philadelphia: W.B. Saunders Company, 1995.

- 48. Preuss HG, Jarrel ST, Scheckenbach R, Lieberman S, Anderson RA. Comparative effects of chromium, vanadium and gymnema sylvestre on sugar-induced blood pressure elevations in SHR. J Am Coll Nutr 1998;17(2):116-123.
- Beutler E, Duron O, Kelly BM. Improved method for determination of blood glutathione. J Lab Clin Med 1963;61:882-888.
- 50. Koster JF, Biermond P, Swaak AJG. Intracellular and extracellular sulph-hydryl levels in rheumatoid arthritis. Ann Rheum Dis 1986;45:44-46.
- Cohen G, Dembiec D, Marcus J. Measurement of catalase activity in tissue extracts. Anal Biochem 1970;34:30-38.
- 52. Kar M, Mishra D. Catalase, peroxidase and polyphenoloxidase activities during rice leaf senescence. Plant Physiol 1976;57:315-319.
- 53. Howell SL, Taylor KW. Potassium ions and the secretion of insulin by islets of Langerhans incubated *in vitro*. Biochem J 1968;108:17-24.
- 54. Morgan NG, Montague W. Stimulation of insulin secretion from isolated rat islets of Langerhans by melittin. Biosci Rep 1984;4:665-671.
- 55. Zarzuelo A, Risco S, Gamez MJ, Jimenez J, Camara M, Martinez MA. Hypoglycemic action of *Salvia lavandulifolia* vahl. ssp. Oxyodon: a contribution to studies on the mechanism of action. Life Sci 1990;47:909-915.
- 56. Otto P, Kephart D, Bitner R, Huber S, Volkerding K. Separate isolation of genomic DNA and total RNA from single samples using the SV Total RNA Isolation System. Promega Notes 1998;69:19-23.
- 57. Roa M, Blane K, Zonneberg M. One way analysis of variance. Version IA (C). PC-STAT, Program coded by University of Georgia, USA, 1985.
- 58. Chakravarthy BK, Gupta S, Gode KD. Antidiabetic effect of (-)-epicatechin. Lancet 1982;1:272-273.

- 59. Chakravarthy BK, Gupta S, Gode KD. Functional beta cell regeneration in the islets of pancreas in alloxan induced diabetic rats by (-)-epicatechin. Life Sci 1982;31:2693-2697.
- 60. Nuraliev IN, Avezov GA. The efficacy of quercetin in alloxan diabetes. Exp Clin Pharmacol 1992;55:42-44.
- 61. Vessal M, Zal F, Vasi M. Effects of *Teucrium polium* on oral glucose tolerance test, regeneration of pancreatic islets and activity of hepatic glucokinase in diabetic rats. Arch Iran Med 2003;6:36-39.
- 62. Atta-Ur-Rahman, Zaman K. Medicinal plants with hypoglycemic activity. J Ethnopharmacol 1989;26:1-55.
- 63. Chattopadhyay RR, Sarkar SK, Ganguly S, Banerjee RN, Basu TK. Hypoglycemic and antihyperglycemic effect of leaves of *Vinca rosea*. Indian J Physiol Pharmacol 1991;35(3):145-151.
- 64. Marles RJ, Farnsworth NR. Antidiabetic plants and their active constituents. Phytomedicine 1995;2(2):137-189.
- 65. Essawy GS, Sobbhy HM, El-Banna HA. The hypoglycemic effect of volatile oil of some Egyptian plants. Vet Med J (Giza) 1995;43(2):167-172.
- 66. Akhani SP, Vishwakarma SL, Goyal RK. Antidiabetic activity of *Zingiber officinale* in streptozotocin-induced type 1 diabetic rats. J Pharm Pharmacol 2004;56(1):101-105.
- 67. Liao K, Yin M. Individual and combined antioxidant effects of seven phenolic agents in human erythrocyte membrane ghosts and phosphatidylcholine liposome systems: importance of the partition coefficient. J Agric Food Chem 2000;48:2266-2270.
- 68. Coskun OM, Kanter A, Korkmaz, SO. Quercetin, a flavonoid antoxidant, prevents and protects streptozotocin induced oxidative stress and â-cell damage in rat pancreas. Pharmacol Res 2005;51:117-123.

- 69. Hii CST, Howell SL. Effects of flavonoids on insulin secretion and Ca+2 handling in rat islets of Langerhans. J Endocrinol 1985;107:1-8.
- Hindle EJ, Rostron GM, Clarck SA, Catt JA. Serum fructosamine and glycated hemoglobin measurements in diabetic control. Arch Dis Childhood 1986;61:113-117.
- Donnelly JG. Carbohydrates and alterations in glucose metabolism. In: Bishop ML, Fody EP, editors. Clinical chemistry (principles, procedures, correlations). Philadelphia: Lippincott, 1996; pp. 308-309.
- 72. Elgawish A, Glomb M, Freelander M, Monnier VM. Involvement of hydrogen peroxide in collagen cross-linking by high glucose *in vitro* and *in vivo*. J Biol Chem 1996;271:12964-12971.
- 73. Grover JK, Vats V, Rathi SS. Antihyperglycemic effect of *Eugenia jambolana* and *Tinospora cordifolia* in experimental diabetes and their effects on key metabolic enzymes involved in carbohydrate metabolism. J Ethnopharmacol 2000;73:461-470.
- 74. Raju J, Gupta D, Rao AR, Yadava PK, Baquer NZ. *Trigonella foenum graecum* (fenugreek) seed powder improves glucose homeostasis in alloxan diabetic rat tissues by reserving the altered glycolytic, gluconeogenic and lipogenic enzymes. Mol Cell Biochem 2001;224(1-2):45-51.
- 75. Lavoie L, Van de Werve G. Hormone-stimulated glucose production from glycogen in hepatocytes from streptozotocin diabetic rats. Metabolism 1991;40(10):1031-1036.
- 76. Ahmed OM. The hypoglycemic effect of curcumin and esculetin and their probable mechanisms of action in streptozotocin diabetic albino rats. J Egypt Ger Soc Zool 2005;46(A):351-375.
- 77. Abdel-Moneim A, Ahmed OM, Rawi SM, Semmler M. Studies on the hypoglycemic and hypolipidemic effects of glimepiride and some antidiabetic plants on streptozotocin diabetic rats. J Egypt Ger Soc Zool 2001;34(A):175-206.

- 78. Ahmed OM. Evaluation of the antihyperglycemic, antihyperlipidemic and myocardial enhancing properties of pioglitazone in diabetic and hyperthyroid rats. J Egypt Ger Soc Zool 2006;51(A):253-278.
- Bollen M, Keppens S, Stalmans W. Specific features of glycogen metabolism in the liver. Biochem J 1998;336:19-31.
- Cadefau J, Bollen M, Stalmans W. Glucoseinduced glycogenesis in the liver involves the glucose-6-phosphate-dependent phosphorylation of glycogen synthase. Biochem J 1997;322(3):745-750.
- 81. Bähr M, Von Holty M, Muller G, Eckel J. Direct stimulation of myocardial glucose transport and glucose transporter-1 (GLUT1) and GLUT4 protein expression by the sulfonylurea glimepiride. Endocrinology 1995;136:2547-2553.
- Müller G, Satoh Y, Geisen K. Extrapancreatic effects of sulfonylureas – a comparison between glimepiride and conventional sulfonylureas. Diabetes Res Clin Pract 1995;28(Suppl):115-137.
- 83. Alpert E, Gruzman A, Totary H, Kaiser N, Reich R, Sasson S. A natural protective mechanism against hyperglycemia in vascular endothelial and smooth muscle cells: role of glucose and 12-hydroxyeicosatetraenoic acid. Biochem J 2002;362:413-422.
- 84. Raz I, Eldor R, Cernea S, Shafrir E. Diabetes, insulin resistance and derangements in lipid metabolism. Cure through intervention in fat transport and storage. Diabetes Metab Res Rev 2005;21:3-14.
- 85. Jung UJ, Lee MK, Park YB, Kang MA., Choia MS. Effect of citrus ?avonoids on lipid metabolism and glucose-regulating enzyme mRNA levels in type-2 diabetic mice. Int J Biochem Cell Biol 2006;38:1134-1145.
- 86. Park SY, Bok SH, Jeon SM, Park YB, Lee SJ, Jeong TS, Choi MS. Effect of rutin and tannic acid supplements on cholesterol metabolism in rats. Nutr Res 2002;22:283-295.

- Lee CH, Olson P, Evans RM. Minireview: lipid metabolism, metabolic diseases, and peroxisome proliferator-activated receptors. Endocrinology 2003;144:2201-2207.
- Staels B, Fruchart JC. Therapeutic roles of peroxisome proliferator-activated receptor agonists. Diabetes 2005;54:2460-2470.
- 89. Feige JN, Gelman L, Michalik L, Desvergne B, Wahli W. From molecular action to physiological outputs: peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. Prog Lipid Res 2006;45:120-159.
- 90. Lefebvre P, Chinetti G, Fruchart JC, Staels B. Sorting out the roles of PPAR alpha in energy metabolism and vascular homeostasis. J Clin Invest 2006;116:571-580.
- 91. Fulgencio JP, Kohl C, Girard J, Pegorier JP. Troglitazone inhibits fatty acid oxidation and esterification, and gluconeogenesis in isolated hepatocytes from starved rats. Diabetes 1995;45:1556-1562.
- 92. Yamauchi T, Kamon J, Waki H, Murakami K, Motojima K, Komeda K. The mechanisms by which both heterozygous peroxisome proliferatoractivated receptor gamma (PPAR[®]) deficiency and PPAR[®] agonist improve insulin resistance. J Biol Chem 2001;276:41245-41254.
- 93. Kim SY, Kim HI, Park SK, Im SS, Li T, Cheon HG. Liver glucokinase can be activated by peroxisome proliferator-activated receptor-*∞*. Diabetes 2004;53:S66-S70.
- 94. Shiomi M, Ito T, Tsukada T, Tsujita Y, Horikoshi H. Combination treatment with troglitazone, an insulin action enhancer, and pravastatin, an inhibitor of HMG-CoA reductase, shows a synergistic effect on atherosclerosis in WHHL rabbits. Atherosclerosis 1999;142:345-353.
- 95. Willson TM, Brown PJ, Sternbach DD, Henke BR. The PPARs: from orphan receptors to drug discovery. J Med Chem 2000;43:527-550.

- 96. Hevener AL, He W, Barak Y, Le J, Bandyopadhyay G, Olson P, Wilkes J, Evans RM, Olefsky J. Muscle-specific PPAR[®] deletion causes insulin resistance. Nat Med 2003;9:1491-1497.
- 97. Anandharajan R, Pathmanathan K, Shankernarayanan NP, Ram AV, Arun B. Upregulation of GLUT-4 and PPAR[®] by an isoflavone from *Pterocarpus marsupium* on L6 myotubes: a possible mechanism of action. J Ethnopharmacol 2005;97:253-260.
- 98. Antuna-Puente B, Feve B, Fellahi S, Bastard JP. Adipokines: the missing link between insulin resistance and obesity. Diab Metabol 2008;34:2-11.
- 99. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM. The hormone resistin links obesity to diabetes. Nature 2001;409:307-312.

- ¹ Department of Endocrinology, University Department of Medicine, Split University Hospital Center, Split, Croatia
- ² Emergency Medicine Service, Split, Croatia
- ³ University Department of Dermatology, Split University Hospital Center, Split, Croatia
- ⁴ Department of Pharmacology, School of Medicine, Josip Juraj Strossmayer University, Osijek, Croatia
- ⁵ Department of Endocrinology, University Department of Medicine, Rijeka University Hospital Center, Rijeka, Croatia

ADVANTAGE OF PRANDIAL INSULIN AS A THERAPEUTIC APPROACH IN INITIAL SECONDARY PANCREATIC β-CELL EXHAUSTION IN TYPE 2 DIABETIC PATIENTS

Slaven Kokić¹, Višnja Kokić², Mladen Krnić¹, Lina Mirić³, Željko Jovanović⁴, Željka Orlić-Crnčević⁵

Key words: type 2 diabetes mellitus, postprandial glycemia, blood glucose regulation, glucotriade, comparison of therapy

SUMMARY

The aim of this study was to compare prandial insulin therapy with standard therapeutic options available at that time (2000-2005). Two hundred and thirty seven type 2 diabetic patients were randomly selected and divided equally into three treatment groups: glimepiride + metformin + acarbose (3) OHA); two doses of biphasic insulin and at bed time with NPH insulin (2Mix + NPH); and two doses of metformin and three doses of lispro insulin before meals (3L + 2Met). During the 6-month treatment period, fasting plasma glucose levels (FPG) and postprandial glucose (PPG) 2 hours after breakfast were determined and HbA_{1c} was measured three times: on study entry (BL), at three months (3M) and at six months (6M). The 3L + 2 Met group showed greater reduction in FPG, PPG and HbA_{1c} as compared with

the other two groups (P < 0.05), and this reduction was already evident at 3 months of therapy, supporting the prandial insulin therapeutic approach.

INTRODUCTION

Type 2 diabetes mellitus, due to its high incidence and prevalence, is one of the most widespread chronic diseases, causing disability (mainly due to the development of microand macrovascular complications), enormous material costs for both patients and society (1), and, ultimately increased mortality (2,3). Type 2 diabetes mellitus accounts for 85% of all forms of diabetes, with a prevalence of 5%-7% of general population in western countries. During the last 3 decades, substantial evidence has been collected by epidemiological, cohort and animal studies that correlate appropriate regulation of glycemia with the prevention and delay of chronic complications of diabetes (4-6).

In order to achieve efficient prevention and delay of chronic micro- and macrovascular complications of diabetes, rigorous treatment targets for regulation of all components of the 'glucotriade' (fasting glucose $\leq 6.5 \text{ mmol/L}$, postprandial glucose $\leq 7.8 \text{ mmol/L}$ and HbA_{1c} $\leq 6.5\%$) have been established. Values of HbA_{1c} above 7.5% are now considered unacceptable. If there

Corresponding author: Asst. Prof. Slaven Kokić, MD, PhD, Department of Endocrinology, University Department of Medicine, Split University Hospital Center, Šoltanska 1, HR-21000 Split, Croatia *E-mail:* kokic.slaven@gmail.com

is no improvement/normalization of HbA_{1c} values after 3 to 6 months of therapy with 2 to 3 oral hypoglycemic drugs combined with appropriate lifestyle modifications (diet and exercise), then it is necessary to start insulin therapy. These circumstances represent the 'secondary exhaustion' of pancreatic βcells and any delay in initiating insulin therapy would be potentially dangerous for the patient, due to the rapid development of severe macrovascular complications. Currently, modern diabetology considers 4 different therapeutic approaches regarding 'secondary exhaustion' of pancreatic β -cells. In western countries, especially the USA, the basal-oral therapy (BOT) is very popular. BOT consists of a long-acting insulin analogue, glargin or detemir, at bedtime, with continuation of oral hypoglycemic drug therapy. Second approach is prandial insulin therapy plus metformin, which consists of a fast-acting insulin analogue, lispro, glulisine or aspart, before meal 3

Table 1. Anthropometric characteristics of study patients

		Experimental group				
		3 OHA	2Mix + NPH	3L + 2Met	Р	
	Male	38	27	32	0.2054	
Sex (n)	Female	41	52	47	0.205	
	Total	79	79	79		
Age (yrs)		64.1±10.3	66.0±12.7	64.2±8.4	0.721 ^B	
Body mass index (kg/m ²)		28.9±4.3	28.5±3.5	28.9±3.5	0.814 ^B	
Diabetes duration	(yrs)	8.14±4.67	10.03±6.2	9.45±3.6	0.005 ^B	

Data expressed as mean ± standard deviation, unless otherwise indicated; 3 OHA, glimepiride + metformin + acarbose; 2Mix + NPH, two doses of biphasic insulin (regular/NPH 30/70) and at bed time with NPH insulin; 3L + 2Met, two doses of metformin and three doses of lispro insulin before meals; ^A?2-test; ^BANOVA.

Table 2. Therapy-induced changes of 'glucotriade'

	Experimental group				
		3 OHA	2Mix + NPH	3L + 2Met	Р
	BL	9.1±2.8	11.2±3.7	11.1±3.4	<0.001
FPG (mmol/L)	3M	8.6±2.0	9.3±2.7	8.5±2.0	0.043
	6M	7.9±0.9	8.2±2.0	7.7±1.3	0.033
	Ρ	0.014	<0.0001	<0.0001	
	BL	10.7±3.11	12.6±3.3	11.9±3.8	0.003
PPG (mmol/L)	3M	9.9±2.8	9.9±2.7	8.3±2.3	< 0.001
	6M	9.4±1.7	9.3±1.8	7.4±1.5	< 0.001
	Р	0.0009	<0.0001	<0.0001	
	BL	8.9±1.9	10.2±2.1	9.5±2.0	<0.001
HbA _{1c} (%)	3M	8.0±1.4	8.5±1.3	7.5±1.1	<0.001
	6M	7.6±0.9	8.0±0.9	6.9±0.7	<0.001
	Р	<0.0001	<0.0001	<0.0001	

Data expressed as mean ± standard deviation; 3 OHA, glimepiride + metformin + acarbose; 2Mix + NPH, two doses of biphasic insulin (regular/NPH 30/70) and at bed time with NPH insulin; 3L + 2Met, two doses of metformin and three doses of lispro insulin before meals; FPG, fasting glucose; PPG, postprandial glucose; BL, start of the study; 3M, at three months; 6M, at six months.

Table 3. Normalized therapy-induced changes of 'glucotriade'

		Experimental group			
		3 OHA	2Mix + NPH	3L + 2Met	Р
FPG (mmol/L)	BL-3M	0.5±2.7	1.9±4.1	2.6±4.0	0.007
	BL-6M	1.0±3.1	2.9±3.7	3.5±3.5	<0.001
PPG (mmol/L)	BL-3M	0.8±3.3	2.7±3.5	3.6±4.5	<0.001
	BL-6M	1.3±3.3	3.2±3.4	4.6±3.9	<0.001
HbA _{1c} (%)	BL-3M	1.0±1.3	1.7±1.8	2.1±1.7	<0.001
	BL-6M	1.3±1.8	2.2±2.1	2.7±1.7	<0.001

Data expressed as mean ± standard deviation; 3 OHA, glimepiride + metformin + acarbose; 2Mix + NPH, two doses of biphasic insulin (regular/NPH 30/70) and at bed time with NPH insulin; 3L + 2Met, two doses of metformin and three doses of lispro insulin before meals; FPG, fasting glucose; PPG, postprandial glucose; BL-3M change from baseline at three months; BL-6M, change from baseline at six months.

times daily, plus 2 to 3 doses of metformin. Third approach is basal-bolus therapy or intensified insulin therapy, which consists of a fast-acting insulin analogue, lispro, glulisine or aspart, before meal 3 times daily, with 2 to 3 doses of a long-acting insulin analogue, glargin or detemir. For those that cannot implement this therapy, there is the possibility to use premixed insulin analogues in 2 to 3 doses (lispro + NPH or aspart + NPH) as a 'replacement' for intensified insulin therapy.

In our environment, at the time this study was carried out (from 2000 to 2005), there was no possibility to implement either BOT or intensified insulin therapy. The aim of the study was to compare prandial insulin therapy (lispro before meals) plus metformin (twice daily) with standard therapeutic options at that time, i.e. a combination of 3 oral hypoglycemic drugs, sulfonylurea (glimepiride) + metformin + acarbose; and two doses of premixed human insulin (regular/NPH in two doses plus NPH insulin at bedtime). The reason why we report on this study now is the fact that the German Diabetes Association (7) and also the Croatian Diabetes Association have accepted prandial insulin therapy (lispro before meals) plus metformin, along with the aforementioned BOT, as the first choice therapy of secondary exhaustion of pancreatic β -cells. Therefore, the results of this study may provide useful information for diabetologists.

PATIENTS AND METHODS

Two hundred and thirty seven type 2 diabetic patients, treated at the Department of Endocrinology, Split University Hospital Center, were randomly selected and divided equally into three treatment groups: glimepiride + metformin + acarbose (3 OHA); two doses of biphasic insulin (regular/NPH 30/70) and at bedtime with NPH insulin (2Mix + NPH); and two doses of metformin and three doses of lispro insulin before meals (3L + 2Met). Patient characteristics are summarized in Table 1. Only patients with HbA_{1c} \geq 7.5 %, body mass index (BMI) \geq 23, diabetes duration >5 <15 years, without any detectable renal or liver dysfunction, were considered eligible for the study. During the 6-month treatment period, blood glucose levels (fasting glucose (FPG) and postprandial glucose

(PPG) 2 hours after breakfast) were determined and HbA_{1c} was measured three times: on study entry (BL), at three months (3M) and at six months (6M). Anthropometric and laboratory (HDL, LDL, triglycerides, ALT, AST, GGT) measurements were also performed.

Statistical analysis included descriptive statistics, with data expressed as mean \pm standard deviation, unless otherwise indicated. The χ^2 -test, t-test and nonparametric Mann-Whitney and Kruskal-Wallis tests were used to assess statistical differences between two or more groups. For repeated measures ANOVA test was used to assess differences among three measurement times (BL, 3M and 6M) in the same group. *P* value less than 0.05 was considered statistically significant. All statistical analyses were performed with the StatPac Gold Statistical Analysis package.

RESULTS

There were no significant between group differences according to sex, age or BMI (Table 1), whereas the duration of diabetes was slightly shorter in 3 OHA group in comparison to other groups; however, we believe that this difference could not affect study results in any way.

After six months of therapy, there were no significant changes in BMI in any group, suggesting that none of the therapeutic modalities induced weight gain or weight loss. The number of hypoglycemic events during six months was lowest in 3L+2 Met group (10 events), in comparison to 3 OHA (18 events) and 2Mix + NPH (25 events) groups.

All three therapeutic modalities induced significant reduction in FPG, PPG 2 hours after breakfast and in HbA_{1c} (Table 2). However, as the baseline values of these parameters differed among the groups (lower values in 3 OHA group), there was a need for 'normalization' of the induced changes from the baseline values (Table 3). Only after this normalization from the baseline value, it became evident that the 3L + 2 Met induced greater reduction in FPG, PPG and HbA_{1c} in comparison to other groups, and that this reduction was already evident at 3 months of therapy.

	Studies			
Main outcomes	Kokic (this study)	Altuntas (9)	4-T study (11)	APOLLO study (12)
HbA _{1c} (%)	·			
at baseline	9.5	10.1	8.7	8.6
at endpoint	6.9	7.4	6.8	7.2
Δ (change)	2.6	2.7	1.9	1.4
Responder rate (% pts achieving HbA _{1c} target)				
≤ 7.0%	83.5	NA	69	49
$\leq 6.5\%$	25.3	NA	38	24
Δ FPG (mmol/L)	3.5	0.9	1.7	1.3
Δ PPG (mmol/L)	4.6	2.2	NA	4.6
No. of overall hypoglycemic events/patient/year	4.8	5.8	24.0	12.0
Change in body weight (kg)	-0.2	+1.4	+3.5	+5.7

Table 4. Comparison of key studies with prandial insulin therapy

Data expressed as mean; FPG, fasting glucose; PPG, postprandial glucose; NA, not available.

Furthermore, when we compared the percentage of patients that achieved the desired HbA_{1c} level (<6.5% by IDF criteria and <7% by ADA criteria) at six months of therapy, the advantage of the 3L + 2 Met group became remarkable. The responder rate of achieving IDF criteria for HbA_{1c} level was 3.8%, 1.3% and 25.3% for 3 OHA, 2Mix + NPH and 3L + 2 Met, respectively. The percentage of patients that achieved ADA criteria for HbA_{1c} level was 51.9%, 36.7% and 83.5% for 3 OHA, 2Mix + NPH and 3L + 2 Met, respectively.

DISCUSSION

The aim of the study was to establish the most efficient therapeutic approach for regulation of the 'glucotriade', in order to prevent or delay the development of chronic complications of diabetes. We confirmed our hypothesis on the prandial therapeutic approach, 3L + 2 Met group, to be most efficient in regulating all components of the 'glucotriade' (FPG, PPG 2 hours after breakfast and HbA_{1c}). This is in accordance with our previous pilot study (8) and subsequent reports by other authors (9-12). All these reports confirm the efficiency of the prandial therapeutic approach.

In May 2002, our pilot study was the first published report that introduced the therapeutic approach with 2 to 3 doses of fast-acting insulin analogue lispro at meals plus 2 to 3 doses of metformin (8). In May 2003, Altuntas *et al.* published a study with 60 diabetic patients (type 2), randomized in 3 therapeutic groups of 20 patients: lispro + metformin; lispro + NPH insulin; and intensified insulin therapy, regular + NPH insulin (9). After six months of therapy, their findings were similar to ours, as the combination of lispro + metformin proved to be superior to other therapeutic approaches.

The recently published articles by Holman *et al.* (10,11), and Bertzel *et al.* (12,13) show that the prandial therapeutic approach is very up-to-date in treating type 2 diabetes, especially due to 'secondary exhaustion' of pancreatic β -cells.

In the 4-T study (Treating To Target in Type 2 diabetes) by Holman *et al.*, 708 patients were randomized in 3 therapeutic groups (plus oral hypoglycemic drugs): biphasic, prandial and basal insulin therapy (10,11). The most efficient reduction of HbA_{1c} was accomplished by prandial insulin therapy (reduction by 1.4%). Furthermore, the percentage of patients reaching the HbA_{1c} reference values according to both ADA and IDF criteria was highest in the prandial insulin group (22.7% and 10.8%, respectively).

The results from the APOLLO study support these findings (12,13). The net of reduction of HbA_{1c} obtained by prandial insulin therapy was 1.9%. Moreover, the percentage of patients treated by

prandial insulin that reached the HbA_{1c} reference values was 69% according to ADA and 38% according to IDF criteria. For easy comparison, major results from the key studies of prandial insulin are presented in Table 4, along with this one.

There are some discrepancies between the results obtained in this study and in the 4-T and APOLLO trials (10-13). In our study, we did not record any weight gain, which is in contrast with the results from 4-T and APOLLO trials, where there was a significant increase in body weight after prandial insulin therapy. On the other hand, our results are consistent with the findings reported by Altuntas et al., as they did not observe any significant weight gain after prandial insulin therapy either (9). Another difference between the studies is the number of hypoglycemic events/patient/year. The number of hypoglycemic events was much higher in the 4-T and APOLLO trials in comparison to our findings and the study by Altuntas et al. (Table 4). These discrepancies may be explained by the fact that we used lispro insulin (2

hours of maximal efficiency), in contrast to aspart insulin in the 4-T trial (3 hours of maximal efficiency) (10,11).

We suggest that the advantage of prandial insulin therapy is to use fast-acting analogue, like lispro, which acts rapidly and in shortly fashion (up to 2 hours), without the hazard of hyperinsulinemia, potential hypoglycemic events and the need for extra meals that is responsible for weight gain. The addition of metformin suppresses the glyconeogenesis, and drives in the glucose in muscle and fat cells even without insulin, excluding the possible hyperinsulinemia and hypoglycemia.

The prandial insulin therapeutic approach is a feasible choice in controlling postprandial glycemia, although new specialized therapies are emerging, like GLP-1 analogues.

REFERENCES

- MacLeod KM, Tooke JE. Direct and indirect costs of cardiovascular and cerebrovascular complications of type II diabetes. Pharmacoeconomics 1995;8 Suppl 1:46-51.
- Neil HA, Gatling W, Mather HM, Thompson AV, Thorogood M, Fowler GH, *et al.* The Oxford Community Diabetes Study: evidence for an increase in the prevalence of known diabetes in Great Britain. Diabet Med 1987;4:539-543.
- Zimmet P. Type 2 (non-insulin-dependent) diabetes - an epidemiological overview. Diabetologia 1982;22:399-411.
- Panzram G. Mortality and survival in type 2 (noninsulin-dependent) diabetes mellitus. Diabetologia 1987;30:123-131.

- The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993;329:977-986.
- Reichard P, Nilsson BY, Rosenqvist U. The effect of long-term intensified insulin treatment on the development of microvascular complications of diabetes mellitus. N Engl J Med 1993;329:304-309.
- Matthaei S, Bierwirth R, Fritsche A, Gallwitz B, Haring HU, Joost HG, *et al.* Medical antihyperglycaemic treatment of type 2 diabetes mellitus: update of the evidence-based guideline of the German Diabetes Association. Exp Clin Endocrinol Diabetes 2009;117:522-557.

- Kokic S, Radman M, Capkun V, Dovzak-Kokic D, Tesanovic S. Comparative assessment of the treatment of type 2 diabetes mellitus. Ann Saudi Med 2002;22:163-166.
- 9. Altuntas Y, Ozen B, Ozturk B, Sengul A, Ucak S, Ersoy O, *et al.* Comparison of additional metformin or NPH insulin to mealtime insulin lispro therapy with mealtime human insulin therapy in secondary OAD failure. Diabetes Obes Metab 2003;5:371-378.
- Holman RR, Farmer AJ, Davies MJ, Levy JC, Darbyshire JL, Keenan JF, *et al.* Three-year efficacy of complex insulin regimens in type 2 diabetes. N Engl J Med 2009;361:1736-1747.
- Holman RR, Thorne KI, Farmer AJ, Davies MJ, Keenan JF, Paul S, *et al.* Addition of biphasic, prandial, or basal insulin to oral therapy in type 2 diabetes. N Engl J Med 2007;357:1716-1730.

- 12. Bretzel RG, Nuber U, Landgraf W, Owens DR, Bradley C, Linn T. Once-daily basal insulin glargine *versus* thrice-daily prandial insulin lispro in people with type 2 diabetes on oral hypoglycaemic agents (APOLLO): an open randomised controlled trial. Lancet 2008;371:1073-1084.
- 13. Bretzel RG, Eckhard M, Landgraf W, Owens DR, Linn T. Initiating insulin therapy in type 2 diabetic patients failing on oral hypoglycemic agents: basal or prandial insulin? The APOLLO trial and beyond. Diabetes Care 2009;32 Suppl 2:S260-265.

INSTRUCTIONS TO AUTHORS

DIABETOLOGIA CROATICA publishes original articles from the fields of diabetology, endocrinology and metabolic disturbances, as well as papers intended for the education of medical personnel in the areas. It is printed in English.

ARTICLES submitted for publication should contain original information not previously published or submitted for publication elsewhere.

MANUSCRIPTS. Original manuscripts in English should be typewritten, double-spaced and pages numbered consecutively in the following order:

Title page containing the title of the article, the name(s) of the author(s) including the first name and highest academic degree(s), the name of department and institution in which the work was done, and the address for correspondence, including telephone and/or fax number and e-mail address.

Summary and Key Words should be written on a separate page.

Text should be divided into following sections:

- **Summary:** should not contain more than 150 words, stating the purpose of the study, basic procedures, main findings with specific data and their statistical significance, and the principal conclusions. Summary should be followed by 3 to 10 key words or short phrases based on Medical Subject Headings list from Index Medicus whenever possible.
- **Introduction:** concisely indicate the importance of the work, its relation to previous work and (if it is a method) reasons why it is preferable to other methods.
- Materials and methods: describe the selection of subjects. Identify the methods, apparatus (including manufacturer's name and address) and procedures.
- **Results:** present your results in concise language and logical sequence, using tables and illustrations if necessary.
- **Discussion:** discuss significance of the results and emphasize conclusions that follow from them. Results and conclusions should be compared and contrasted with those of comparison methods or previous studies.
- References:

REFERENCES should be numbered consecutively as they are cited in the text. They must be indicated by Arabic numerals. References cited only in tables or figure legends should be numbered in accordance with the sequence established in the text. Journal abbreviations should be those of the List of

Journals Indexed for MEDLINE. List the first six authors followed by *"et al."* when there are seven or more. Examples of references:

Examples of references:

- Journals Pepeonik-Rogulja Z, Pinter S, Metelko Z. Can the age at diagnosis of type 1 diabetes mellitus affect the course of diabetic retinopathy. Diabetologia Croatica 2004;33:97-101.
- **Books** International Diabetes Federation. Diabetes Atlas, 3rd ed. Brussels: International Diabetes Federation; 2006.
- Chapter in Book Reaven GM. Insulin resistance and its consequences: type 2 diabetes mellitus and the insulin resistance syndrome. In: LeRoith D, Taylor SI, Olefsky JM, editors. Diabetes mellitus: a fundamental and clinical text. 3rd ed. Philadelphia: Lippincott Williams & Wilkins; 2004. pp. 899-915.
- Electronic Material Gazzaruso C, Garzaniti A, Giordanetti S, Falcone C, Fratino P. Silent coronary artery disease in type 2 diabetes mellitus: the role of lipoprotein(a), homocysteine and apo(a) polymorphism. Cardiovasc Diabetol [Internet] 2002 Nov [accessed 2002 Dec 9];1(5). Available from: URL: http://www.cardiab.com/content/1/1/5

TABLES should be typed double spaced on separate sheets. Each should be provided with a brief title. Each column should have a heading with clearly defined units. All non-standard abbreviations should be explained in footnotes.

ILLUSTRATIONS. Figures should be professionally drawn and photographed. Glossy black-and-white prints are requested. Each figure should have a label on its back indicating the number of the figure, the names of authors and the top of the figure. Figures should be cited in the text in consecutive order. Illustration legends should be typewritten on a separate sheet of paper.

REVIEW AND PUBLICATION. The manuscripts are reviewed anonymously. If the article is not accepted for publication or certain alternations or additions are suggested, a copy of the review, without the name of the reviewer, will be sent to the author for final decision. The editorial board is not obliged to publish articles in the order they are received. Manuscripts are not returned and all printed supplements become the property of the medical-scientific journal Diabetologia Croatica.

Submission of Manuscripts in Electronic Format

Diabetologia Croatica welcomes (and prefers) the submission of manuscripts in electronic format. The text stored as electronic file will be used directly for typesetting, which will improve the efficiency and speed of journal production. **Manuscripts should be submitted as e-mail attachments or files on diskettes or CD-ROMs.** Figures and legends should be sent as separate files.

Either 3.5 diskette or CD-ROM can be used, and any major word processing program is acceptable that works on IBM or IBMcompatible computers (WordPerfect, Word, ASCII code, etc.).

Diskettes must be labelled with the following information: 1) author's name(s), 2) article title, and 3) software used.

All manuscripts are to be forwarded to the Editorial Board's address:

DIABETOLOGIA CROATICA	Tel:(385-1) 2 35 38 00
Dugi dol 4a	Fax:(385-1) 2 33 15 15
10000 Zagreb	E-mail: Diabetologia.Croatica@idb.hr
Croatia	http://www.idb.hr/diabetologia/index.html