PERIODICUM BIOLOGORUM
VOL. 116, No 3, 313–318, 2014

UDC 57:61
CODEN PDBIAD
ISSN 0031-5362

Original scientific paper

Inhibitory effect of the plant proteins mixture on the specific sucrase activity in intestine of diabetic mice

ROBERTA PETLEVSKI[1]
MIRKO HADŽIJA[2]
ZLATKO SABOL[3]

[1]University of Zagreb, Faculty of Pharmacy and Biochemistry
Department of Medical Biochemistry and Haematology
Domagojeva 2, Zagreb, Croatia

[2]Ruđer Bošković Institute
Laboratory for Experimental Medicine
Bijenička 52, Zagreb, Croatia

[3]Polyclinic for Children·s diseases Dr Sabol
IV. Cvjetno naselje 21, Zagreb

Correspondence:
RobertaPetlevski, PhD
University of Zagreb
Faculty of Pharmacy and Biochemistry
Department of Medical Biochemistry and Hematology
A. Kovačića 1, HR-10 000 Zagreb, Croatia
Tel. + 385 1 6394784
Cell phone: +385 915669677
Fax + 385 1 4612 716
E-mail: rpetlevski@pharma.hr

Key words: diabetes mellitus, plant protein, α-glucosidase, sucrase inhibition

Abstract

Background and Purpose: *Increased activity of sucrase, one of the intestinal alpha-glucosidase founded in diabetes mellitus. Inhibition of sucrase activity, plays a major role in preventing rise in postprandial glucose level in diabetics.*

On peer-reviewed literature could be found regarding investigation the effect of mixture plant proteins, Mw 3 – 15 kDa (MPP), isolated from Astragali radix – Astragalus membranaceus Fisch., Foenugraeci semen – Trigonella foenum graecum L., Cichorii radix – Cichorium Intybus L. and Urticae radix and herba – Urtica dioica L. on sucrase activity. This plants are used in traditional medicine of treatment of diabetes mellitus. The aim of this study was to determine activity of sucrase in small intestinal homogenates of NOD diabetic mice on feeding with and without MPP in chow.

Materials and Methods: *In mice diabetes was induced by i.v. injection of aloxan-monohydrate (75 mg/kg b.m.) seven days before treatment with MPP.*

The proteins (Mw 3 – 15 kDa), were isolated from ethanol extract, each plants separately, by gel filtration method on Sephadex G-25 column. Eluted fraction which highest absorbance on 280 nm were pooled, dialyzed, lyophilized and mixed (MPP) and before treatment in mice solvent in sterile PBS. After seven days of treatment diabetic NOD mice with MPP (1,8 g/d), the small intestine was removed and divided into three segments, from pylorus to duodenum, and two equal lengths of the jejunum and ileum and homogenized in cold 0.14M KCl. Specific sucrase activity was determined using method of Dahlquist et. al., by sucrose as substrate.

Results and Conclusion: *We confirmed the increased specific sucrase activity in the intestine of diabetic NOD mice. Our results also indicate that MPP have strongly inhibitory potential on intestinal sucrase activity ($p<0.05$) in diabetic mice. Conclusions drawn from this study should be further supported and our future experiments will be focused on determining the amino acid sequence of each protein from MPP.*

Received September 10, 2014.

INTRODUCTION

In digestion of carbohydrates, dietary disaccharides such as sucrose, lactose, and maltose are hydrolyzed to their constituent – monosaccharides, by a series of specific disaccharidases: sucrase, maltase, and lactase, named α – glucosidases, which are attached to the small intestinal brush-border membrane *(7)*. These monosaccharides are absorbed across the wall of the duodenum and ileum by an active, energy-requir-

ing, carrier mediated transfer process *(27).* Diabetes mellitus is associated with the postprandial hyperglycemia which is considered to be a high-risk factor resulting in the development of chronic complications of diabetes mellitus. A series of reports have been showed that activities of disaccharidases including sucrase and isomaltase are abnormally high in small intestine of diabetic patients and experimental diabetic animals *(16).*

Inhibition some of the intestinal disaccharidases could regulate the absorption of carbohydrate and these inhibitors could be used therapeutically in the treatment of the Type 2 diabetes mellitus. Acarbose is the first available alpha-glucosidase inhibitor *(28).* In patients with Type 2 diabetes mellitus, acarbose has been found to reduce postprandial glucose concentrations *(5).* The use of acarbose is associated with a high incidence of gastrointestinal symptoms such as flatulence, abdominal distension and diarrhea which result from the fermentation of unabsorbed carbohydrate *(30).* Several medicinal plants and their products have been used to control diabetes in the traditional medicine worldwide *(4, 12, 19, 23, 24).* Some of them are *Astragalus membranaceus* Fisch., *Trigonella foenum graecum* L., *Cichorium Intybus* L. and *Urtica dioica* L.

The anti-diabetic potential of *Astragalus membranaceus* Fisch. has been progressively studied in the recent past. Its polysaccharides, saponins and flavonoids and several isolated compounds exibited differential potentials of correcting the characteristic defects of inadequate insulin production, secretion, and action on target cells *(2).*

Trigonella foenum graecum L is used both in cooking and for the treatment of diabetes in many parts of the world, especially in China, Egypt, India and Middle Eastern countries. Active compounds of fenugreek included soluble fiber, saponins, trigonellin, diosgenin, and 4-hydroxyisoleucine *(21).* Some animal studies have shown that fenugreek seed extracts have the potential to slow enzymatic digestion of carbohydrates, reduce gastrointestinal absorption of glucose, and thus reduce postprandial glucose levels *(11).* Oral administration of 2 and 8 g/kg of plant extracts produces dose dependent decrease in the blood glucose levels in both normal as well as diabetic rats *(14).* In study of Pushparaj et al. 2007 and Petlevski et al, 2003, are described anti-diabetic effects of *Cichorium intybus* L.. A dose of 125 mg of plant extract/kg body weight exhibits the most potent hypoglycemic effect. Moreover, daily administration of *Cichorium intybus* (CIE) (125 mg/kg) for 14 days to diabetic rats attenuates serum glucose by 20 %, triglycerides by 91 % and total cholesterol by 16 %. In addition, hepatic glucose-6-phosphatase activity (G-6-P) markedly reduces by CIE *(25).*

Urtica dioica has also been studied for its medicinal effects. It has been shown that the water extract leaves of this plant had inhibitory effect on porcine pancreatic alpha-amilase and alpha-glucosidase *(22, 26).*

From ethanol extracts of selected four medicinal plants were isolated proteins, Mw 5 – 15 kDa, pooled, dialyzed, lyophilized and mixed (MPP). The purpose of this study is to examine this protein mixture (MPP) in a dose of 1,8 g/d, on sucrase activity in small intestine in normoglycemic and alloxan induced diabetic mice in order to determine one of the probable mechanism of MPP antihyperglycemic effect.

MATERIAL AND METHODS

2.1. Preparation of extracts

Plant material used in this study: Astragali radix – *Astragalus membranaceus* Fisch., Foenugraeci semen – *Trigonella foenum graecum* L., Cichorii radix – *Cichorium Intybus* L. and Urticae radix and herba – *Urtica dioica* L. were identified at the Ruđer Bošković Institute, Department of Molecular Medicine, Zagreb, by testing of the external matter of plants *(31).*

7 g of each dry plant material extracted with 100 mL 60 % ethanol, on room temperature. After seven days, the macerate was filtrated and ethanol evaporated on a rotator evaporator (Büchi R-144, Switzerland), at a temperature of 45° C and frozen on the –20° C until use.

2.2. Isolation and purification of the proteins from plant extract

a) Gel filtration

Gel filtration separates molecules according to differences in size as they pass through a gel filtration medium packed in a column. Sephadex G-25 (*Pharmacia, LKB, Uppsala, Sweden*) is recommended for the majority of group separations involving globular proteins. This medium is excellent for removing salt and other small contaminants away from molecules that are greater than Mr 5 000.

Freeze-dried plant material of each plant was dissolved in distilled water (1mg/mL), then loaded in a Sephadex G-25 gel filtration column, pre-equilibrated and eluted with Tris-HCl buffer. Eluted fractions (1 mL per tube) were collected. In eluted fractions, the proteins were measured by spectrofotometer at 280 nm. Fractions with the highest absorbantion at 280 nm were pooled **(Fig. 1a-1d).**

b) Dialysis and lyophilisation

Pooled fractions (No. 8, 9 and 10 or No. 9,10 and 11) of each plant were dialyzed using a 3.5 kDa *cut off* cellulose membrane (*Spectrum Medical Industries,* CA, USA) against destilled water at 4° C over night. The molecules less of 3.5 kDa can free move across membrane, but greater molecules remained inside.

After dialysis, isolated fractions were lyophilized (Wkf L-501), so as to stabilize and preserve the proteins.

2.3. Animals

Three-month old, male mice of NOD strain, body mass 23 – 35 g, devided in three groups, were used in the

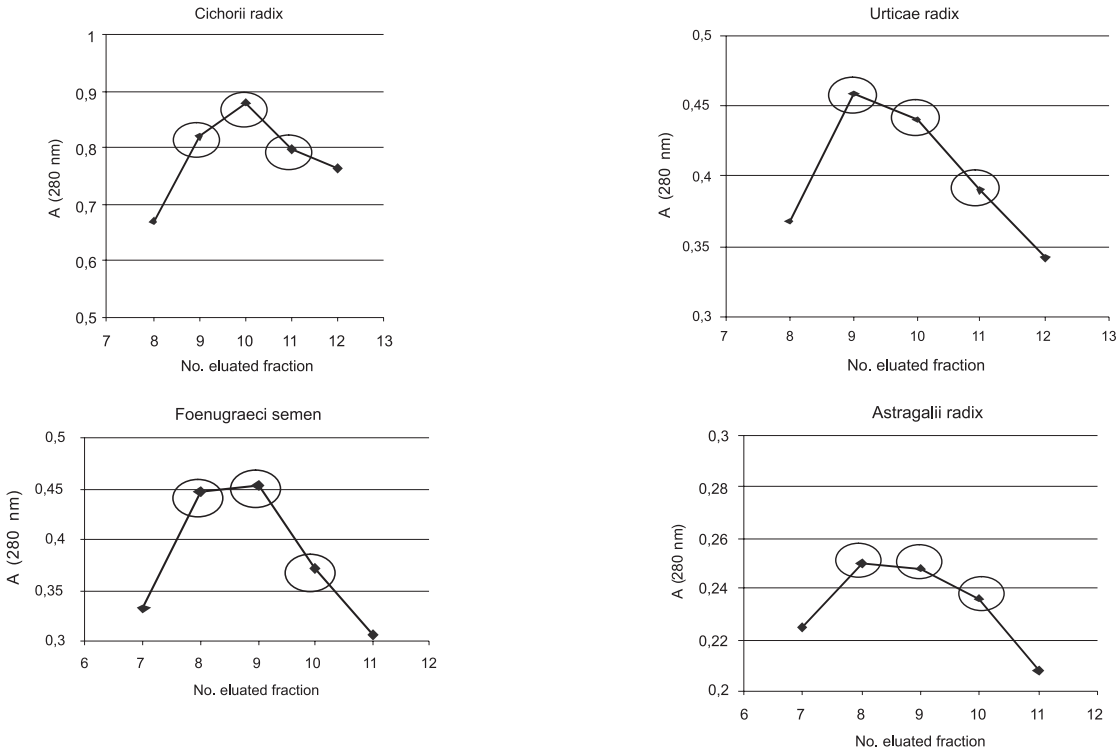

Figure 1a.–1.d. *Fractions of plant proteins from Sephadex G-25 column (No. 9,10,11 from Cichorii radix and Urticae radix and 8, 9, 10 from Foenuagraeci semen and Astragalii radix), with the highest absorbantion on 280 nm were pooled and dialysed.*

study (Animal department of the Laboratory of Molecular Medicine, Institute Ruđer Bošković, Zagreb, Croatia) The mice were housed in metabolic cages on a 12-h light/dark cycle at a temperature of 22–24°C. All mice were fed *ad libidum* with standard laboratory chow (Mucedola, Italy), and had free access to water. Diabetes was induced by *i.v.* injection of Alloxan monohydrate (Sigma, St Louis, MO, USA) in Hank's solution (pH=7.0) in a dose of 75 mg/kg body mass, 7 days before the treatment with MPP. There were three groups of NOD mice, six animals in each group: (1) control (C) mice; (2) diabetic (D) mice fed with standard chow, free of MPP; (3) diabetic (D/MPP) mice fed with standard chow containing MPP (1.8 g/d). They were fed for 7 days. Chows with MPP were prepared daily. Body weight was measured after 7 days of feeding. All experiments were carried out in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, under ether anesthesia, between 9:00 and 10:00 hours, without fasting.

2.4. Intestinal homogenates

Intestine was immediately excised, cut longitudinally, washed in ice-cold saline and then blotted on tissue paper. The small intestine was divided into three segments, from pylorus to duodenum, and two equal lengths of the jejunum and ileum. These tissues were homogenized (100 g/L) in cold 0.14 M KCl using a Teflone homogenizator (Janke and Kunkel, Staufen, Germany). Homogenates were centrifuged at 12 000 g for 30 min in a Mistral 2L refrigerated centrifuge (Measuring and Scientific Equipment Ltd, Crawley, UK). Supernatants were stored at – 20° C until analysis.

2.5. Sucrase activity

Sucrase activity was determined with 0.056 M sucrose in 0.1 M sodium maleate buffer, pH 6.0 *(9).* Protein concentration was determined by the method of Lowry *et. al.* using bovine serum albumin as standard *(17).* Sucrase activity was expressed as specific activity (U/g proteins).

2.6. Statistical analysis

Data are shown as mean ± standard deviation (S.D.). The significance of the effect of various treatments was assessed by use of Student's t-test. A value of $p < 0.05$ was considered statistically significant. SIGMASTAT program for Windows, version 3.0, Jandel Co. (San Rafael, CA, USA) was used for statistical analysis *(20).*

RESULT

3.1. Effect of MPP treatment on body weight and intestinal weight of NOD mice

Body weights of the experimental mice were measured after 7 days of feeding with standard chow or chow with

TABLE 1

Body weight (g) and intestine weight (g) of control (C), diabetic (D) and diabetic mice treated with mixture plant proteins, Mw 3 – 15 kDa (each group n=6) isolated from Astragali radix – *Astragalus membranaceus* Fisch., Foenugraeci semen – *Trigonella foenum graecum* L., Cichorii radix - *Cichorium Intybus* L. and Urticae radix and herba – *Urtica dioica* L. (D/MPP).

Group	No. of mice	Body weight (g) Mean ± S.D.	Intestine weight (g) Mean ± S.D.		
			Duodenum	Jejunum	Ileum
Control (C)	6	34.0 ± 0.80	0.30 ± 0.05	0.54 ± 0.07	0.41 ± 0.11
Diabetic (D)	6	29.0 ± 1.84*	0.37 ± 0.03	0.59 ± 0.05	0.49 ± 0.07
Diabetic group (D/MPP)	6	28.0 ± 1.50	0.35 ± 0.09	0.58 ± 0.10	0.45 ± 0.07

*P < 0.05 significantly different vs.C

MPP, (1,8 g/d) on a electric balance (SAC-62 ,0.1g Scaltec). Body weight of aloxan-induced diabetic mice (D) were significant decreased when compared with control group of animals (C) (p< 0.05) as a characteristic feature of diabetic status **(Table 1).**

Intestine weights of the control and diabetic mice on normal feeding as well as on feeding with MPP in chow, all measured immediately after excision, were not significantly different.

3.2. Effect of MPP treatment on sucrase activity in intestine of control and diabetic mice

It was foud that activity of sucrase in the interested intestinal regions (duodenum, jejunum, and ileum) of diabetic mice was significantly higher than sucrase activity in normal control mice (Fig.2.). Compared with normal, control mice, diabetes resulted in 2–3 fold increase in sucrase activity of duodenum, jejunum, and ileum, respectively. MPP treatment almost restored sucrase activity in intestinal regions of diabetic mice to the level near of normal, control mice. All these results demonstrated that diabetes induced significant increase in intestinal sucrase activity, and MPP treatment reversed the increase in sucrase activity under diabetic states.

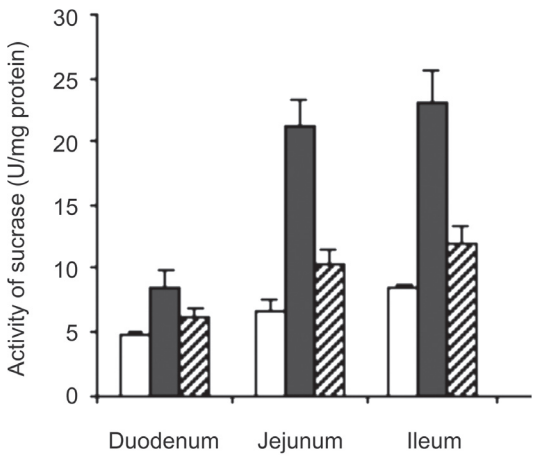

Figure 2. *Effects of treatment with mixture plant protein, Mw 3–15 kDa, (MPP) from plants isolated from Astragali radix - Astragalus membranaceus Fisch., Foenugraeci semen – Trigonella foenum graecum L., Cichorii radix - Cichorium Intybus L. and Urticae radix and herba – Urtica dioica L.. on specific sucrase activity in the small intestine of normal and aloxan-induced diabetic mice. Symbols represent as follows: white bar: normal, control mice (C), black bar: diabetic control mice, (D), diagonally striped bar: diabetic mice treated with MPP (1,8 g/d) (D/MPP). The results are expressed as mean ± S.D (n=6), *P < 0.05 significantly different vs.C; **P < 0.05 significantly different vs.D*

DISCUSSION

In the present study we investigated the activity of mixture plant proteins (3 – 15 kDa) (MPP) isolated by gel filtration method from given plants against mice intestinal sucrase.

Diabetes is possibly the world·s fastest growing metabolic disease, and as knowledge of the heterogenity of this disorder increases, so does the need for more appropriate therapies.

Carbohydrates are digested into glucose by intestinal alpha-glucosidase (one of them is sucase), which directly induces the increase in postprandial blood glucose levels. Structural and functional changes take place in the alimentary tract during diabetes, resulting in increased activities of intestinal disaccharidases *(6, 32).*

Finding effective mammalian alpha-glucosidase inhibitors from natural sources can be beneficial in the prevention and treatment of diabetes mellitus *(1, 13, 15).*

Astragalus Membranaceus Fisch. is used by practitioners of traditional Chinese Medicine to strengthen or tone the body·s overall vitality, improve digestion, and support the spleen. Astragalus is a good source of the essential trace

mineral selenium. Selenium represents a constituent part of biologically important proteins which are essential for effective functioning of antioxidant mechanisms in cells. Analysis shows that Astragalus contains polysaccharides, monosaccharides, flavonoid, alkaloid, including choline and betaine, folic acid, various amino acids, mucoitin, gum and cellulose *(8, 18).*

Another plant is *Trigonella foenum graecum* L. – Foenugraeci semen. The seeds of *Trigonella foenum graecum* L. have been reported to have antidiabetic and hypocholesterolaemic properties in both animal models and humans. Activity has been attributed largely to fenugreek's saponin and high fiber content. Antihyperglycaemic effects have been linked to delayed gastric emptying caused by the fiber content, and components that inhibit carbohydrate digestive enzymes *(3).* Authors Sauvaire *et al.* report about a new insulinotropic compound, 4-hydroisoleucine. This amino acid has been extracted and purified from fenugreek seeds *(29).*

Urtca dioica L. is a plant shown to reduce blood glucose levels upon oral ingestion. Authors Domola *et al*. were described one active fraction of Uricae extract, termed UD-1. Component UD-1 was due to one or more structurally related cyclical peptides that facilitate glucose uptake by forming unique glucose permeable pores *(10).* Onal et al., 2005 described inhibition of alpha-glucosidase by aqueus extracts of some potent antidiabetic medicinal herbs (including Urtica dioica) *(22).*

Hypogycemic and hypolipidemic properties of an ethanolic extract of *Cichorium intybus* L. was investigated from authors Pushparaj *et al*.. Authors concluded that administration of Cichorium extract produced a significant reduction in serum glucose, triglycerides and total cholesterol in STZ-induced diabetic rats without any effects on insulin secretion *(25).*

Increased specific activity of sucrase in the intestine, especially in jejunum and ileum of alloxan diabetic mice (Fig.2.), proved that experimental diabetes causes an increase in the digestive and absorptive functioning of sugars in the brush border membranes of intestinal epithelial cells *(13).*

Feeding diabetic mice with MPP (1.8 g/mice/day (D/MPP)) mixed with chow for 7 days led to significantly decreased ($p < 0.05$) specific activity of sucrase in duodenum, jejunum and ileum compared with diabetic mice on standard feeding (D) (Fig. 2). The decrease in the specific sucrase activities is the most prominent in jejunum and ileum.

In conclusion, the present study showed that diabetes mellitus increased intestinal sucrase activity. MPP (3 – 15 kDa) treatment reversed the increase induced by diabetes while MPP suppressed specific sucrase activity ($p< 0.05$) in diabetic NOD mice. It is clear, that the study of traditional remedies for diabetes mellitus yields an excellent return in potential new sources of antidiabetic drugs.

REFERENCES

1. ADISAKWATTANA S, CHANTARASINLAPIN P, THAMMARATH, YIBCHOK-ANUN S 2009 A series of cinnamic acid derivatives and their inhibitory activity on intestinal ? – glucosidase. *J Enzym Inh & Med Chem 24(5)*: 1194–2000
2. AGYEMANG K, HAN L, LIU E, ZHANG Y, WANG T, GAO X 2013 Recent advances in *Astragalus membranaceus* anti-diabetic research: Pharmacological effects of its phytochemical constituents. *Evidence-Based Compl Altern Med Article* ID 654643: 9 pages
3. ALHABORI M, RAMAN A 1998 Antidiabetic and hypocholesterolaemic effects of Fenugreek. *Phytother Re 12 (4)*: 233–242
4. BAILEY C, DAY C 1989 Traditional plant medicines as treatments for diabetes. *Diab Care 12 (8)*: 553–564
5. BALFOUR J A, Mc TAVISH D 1993 Acarbose. *Drugs 46(6)*: 1025–1054
6. BHASKAR J J, MAHADEVAMMA S, VISHWANATHA S, SALIMATH PV 2010 Effect of Banana (Musa sp cultivar elakki bale) flower and stem on enzyeme activities of intestinal and renal disaccharidases in streptozotocin-induced diabetic rats. *J Food Biochem 34(3)*: 564–580
7. BROOM I 2005 Function of the gastrointestinal tract. *In:* Baynes J W, Marek H, Dominiczak H *(2 ed), Medicinal Biochemistry*. Publishing Inc., Philadelphia, p 113–126
8. CHU D 1998 Immunotherapy with Chinese medicinal herbs. Immune restoration of local xenogenic graft-versus host reaction in cancer patients by fractionated Astragalus membranaceus in vitro. *J Clin Labor& Immunol* 25: 119–123
9. DAHLQVIST A 1970 Assay of intestinal disaccharidases. *Enzymol Biol Clin 11*: 52–66
10. DOMOLA M S, VU V, ROBSON-DOUCETTE C A, SWEENEY G, WHEELER M B 2010 Insulin mimetics in Urtica dioica: structural and computational analyses of *Urtica dioica* extracts. *Phytother Res 24 (S2)*: 175–182
11. HANNAN J M, ALI R, ROKEYA B, KHALEQUE J, AKHTER M, FLATT P R, ABDEL-WAHAB Y H 2007 Soluble dietary fibre fraction of Trigonella foenum-graecum (fenugreek) seed improves glucose homeostasis in animal models of type 1 and type 2 diabetes by delaying carbohydrate digestion and absorption, and enhancing insulin action. *Br J Nutr 97*: 514–521
12. HANDA S S, CHAWLA A S 1989 Hypoglycemic plants – a rewiew. *Fitoterapia 60*: 195–224
13. JURETIĆ D, BERNIK Š, ČOP L, HADŽIJA M, PETLEVSKI R, LUKAČ-BAJALO J 2003 Short-term effect of acarbose on specific intestinal disaccharidase activities and hyperglycaemia in CBA diabetic mice. *J Anim Physiol Anim Nutr 87*: 263–268
14. KAVISHANKAR G B, LAKSHMIDEVI N, MAHADEVA MURTHY S, PRAKASH H S, NIRANJANA S R 2011 Diabetes and medicinal plants-A review. *Int J Pharm Biomed Sci 2(3)*: 65–80
15. KIM K Y, NAM K A, KURIHARA H, KIM S M 2008 Potent α – glucosidase inhibitors purified from the red alga *Grateloupia* elliptica. *Phytochemistry 69*: 2820–2825
16. LIU L, YU Y-L, LIU C, WANG X-T, LIU X-D, XIE L 2011 Insulin deficiency induces abnormal increase in intestinal disaccharidase activities and expression under diabetic states, evidences from in vivo and in vitro study *Biochemical Pharmacology 82*: 1963–1970
17. LOWRY O H, ROSEBROUGH N J, FARR A L, RANDALL R J 1951 Protein measurement with the Folin phenol reagent. *J Biol Chem 193*: 265–275
18. MA J, PENG A, LIN S 1998 Mechanism of the therapeutic effect of Astragalus membranaceus on sodium retention in experimental heart failure. *Clin Med J 111*: 17–23

19. MARLES R J, FARNSWORTH N R 1995 Antidiabetic plants and their active constituents. *Phytomedicine 2(2)*: 137–189

20. MARUSTERI M, BACAREA V 2010 Comparing groups for statistical differences: how to choose the right statistical test? *Biochem Medica 20*: 15–32

21. NEELAKANTAN N, NARAYANAN M, de SOUZA R J, van DAM R 2014 Effect of fenugreek (*Trigonella foenum-graecum* L.) intake on glycemia: a meta-analysis of clinical trals. *Nutrition J 13*: 1–11

22. ONAL S, TIMUR S, OKUTUVN B, ZIHNIOGLU F 2005 Inhibition of alpha-glucosidase by aqueus extracts of some potent antidiabetic medicinal herbs. *Preparative Biochem and Biotechnol 35(1)*: 29–36

23. PETLEVSKI R, HADŽIJA M, SLIJEPČEVIĆ M, JURETIĆ D 2001 Effect of „antidiabetis" herbal preparation on serum glucose and fructosamine in NOD mice. *J Ethnopharm 75*: 181–184

24. PETLEVSKI R, HADŽIJA M, SLIJEPČEVIĆ M, JURETIĆ D 2003 Glutathione S-transferases and malondialdehyde in the liver of NOD mice on short-term treatment with plant mixture extract P-9801091. *Phytother Res 17*: 311–314

25. PUSHPARAJ P N, LOW H K, MANIKANDAN J, TAN B K H, TAN C H 2007 Anti-diabetic effects of *Cichorium intybus* in streptozotocin-induced diabetic rats. *J Ethnopharm 111*: 430–434

26. RAHIMZADEH M, JAHANSHAHI S, MOEIN S, MOEIN M R 2014 Evaluation of alpha-amylase inhibition by *Urtica dioica* and *Juglans regia* extracts. *Iran J Basic Med Sci 17*: 465–469

27. SACKS D B 2006 Carbohydrates. *In:* Burtis C A, Ashwood E R, Bruns D E *(4 ed),* Tietz *Textbook of Clinical Chemistry and Molecular Diagnostics.* Publishing Inc., Elsevier Saunders, p 840–7

28. SCHEEN A J, LEFEBVRE P J 1998 Oral antidiabetic agens. *Drugs 55(2)*: 225–36

29. SOUVAIRE Y, PETIT P, BROCA C, MANTEGHETTI M, BAISSAC Y, FERNANDEZALVAREZ J *et al.* 1998 4 – hydroisoleucine – a novel amino acid potentiator of insulin secretion. *Diabetes 47(2)*: 206–10

30. STEPHEN P, CLISSOLD P, EDWARDS C 1988 Acarbose. *Drugs 35*: 214–243

31. TUTIN T G, BURGES N A, CHATER A C, EDMONDSON J R, HEYWOOD V H, MOORE D M *(eds)* 1993 *Flora Europea*. Cambridge University Press, Cambridge 1–4, 2nd ed.

32. WANG H, DU Y, SONG H 2010 α-Glucosidase and α-amylase inhibitory activities of guava leaves. *Food Chem 123*: 6–13