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The mechanism underlying hyperglycemia in streptozotocin injected rats and the effect of a dietary supplement.

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Abstract : Oxidation stress and increased incretin concentration are among the factors involved in the occurrence of hyperglycemia in diabetic patients. The mechanism underlying these phenomena is not well understood. In the present study hyperglycemia was produced in rats by intraperitoneal injection of streptozotocin as 60 mg/kg B. wt and a trial was made to correct the resulting hyperglycemia with a dietary supplement composed of guar gum, chicory, cape gooseberry fruit, turmeric, blackberry, *Ginko biloba* leaves and sumac. Estimation of the antioxidant power of the ethanol and methanol extracts of this formula by five different methods of antioxidant assays (DPPH, TPTZ, LPIA, β -carotene and TEAC) proved the efficiency of this formula to scavenge different types of free radicals. This formula was able to prevent the shedding of DPP4 from cell membrane or inhibit its activity in streptozotocin injected rats, thus caused increased incretin which prolong insulin action. Thus, it can be seen that oxidation stress and release of DPP4 to circulation are among factors contributing to hyperglycemia due to either insulin insufficiency or resistance. The formula composed of the mentioned ingredients was able to correct hyperglycemia and deal with these complications to a great extent.

Keywords: Hyperglycemia, streptozotocin, DPP-4, incretin, antioxidant, dietary supplement, DPPH, TPTZ, LPIA, β -carotene, TEAC

Introduction

Free radicals and oxidant sources are multiple, they include O_2 , HO_2 , $HOCl$, $RO(O)^2$, $ONOO_2$, and $LO(O)^2$. These free radicals and antioxidants that can scavenge or destroy them do act by different chemical and physical characteristics. Natural antioxidant compounds present in fruits, vegetables or herbs are involved in the defense mechanism of the organism against chronic diseases caused by oxidation stress (1). Diseases such as, cancer, coronary heart disease, obesity, type 2 diabetes, hypertension and cataract are in part caused or affected by oxidation stress and in turn can benefit from antioxidant consumption. Vitamin A, vitamin C, β -carotene, vitamin E, flavonoids, proanthocyanidins, stilbenes, coumarins, lignans, and lignins are amongst the most important exogenous antioxidants present in plant sources and exert their benefits on consumers (2 & 3).

Therefore, multiplicity of methods for assessment of the antioxidant capacity of food sources seems to be of considerable importance to enable investigators to select suitable analytical procedure that reflect the true value of the antioxidant capacity for sources that can be used to protect or participate in treatment of diseases. In fact, it is difficult to find a single analytical method that can reflect the total antioxidant capacity of a particular sample or a composed dietary supplement. This is because it is needed to reflect lipophilic and hydrophobic

capacity, difference in both hydrogen atom transfer and electron transfer, various reactive oxygen species (O, OH & ONOO) (4). Therefore, it is preferable to use more than one method to get accurate measure for the antioxidant capacity of any source.

Although diabetes is the outcome of insulin insufficiency or resistance, yet multiple studies stress on oxidation stress as a factor contributing to the incidence of the disease and its complications (5 & 6). Not only oxidation stress, but also other factors may be involved. Dipeptidyl peptidase-4 inhibitors act directly on the incretin hormone system and exert a positive effect on pancreatic cell biology (7). In order to reach an effective and positive control to diabetes and its complications the mechanism of action of all factors contributing to the incidence of the disease has to be carefully understood.

The present investigation tries to throw light on the role of both oxidation stress and dipeptidyl peptidase-4 inhibitors' action on diabetes in streptozotocin injected rats. Also, to show how far a dietary supplement formed from some plant and herbal sources can participate in correction of the body chemistry and help to control diabetes.

Materials and methods

Ingredients used to compose the formula were guar gum, chicory, cape gooseberry fruit, turmeric, blackberry, *Ginkgo biloba* and sumac. They were purchased from the local market. Most of the constituents used for preparation of the experimental diet were purchased from the local market except for casein which was obtained from Scerma Co., France. Chemicals used for the preparation of the vitamin and salt mixtures were obtained from Fluka (Germany) and BDH (England) Chemical Companies. Chemicals used for determination of antioxidant capacity of the prepared formula were of analytical degree. DPPH (1, 1-diphenyl-2-picrylhydrazyl), ABTS (2, 20-azinobis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt), trolox, TPTZ (2, 4, 6-Tris (2-pyridyl)-s-triazine) and β -carotene were obtained from Sigma-Aldrich Co., USA. Chemicals which were used for determination of lipid peroxide product in blood samples (malondialdehyde); trichloroacetic acid (TCA) and thiobarbituric acid (TBA) were obtained from BDH (England) and Merck (Germany) Companies, respectively. Kits used for the estimation of the biochemical parameters were obtained from different sources as follows; DPP-4 ELISA kit was obtained from Glory Co., USA. Kits used for determination of total cholesterol, HDL-cholesterol and triacylglycerols were obtained from Salucea Co., Netherlands. Kits used for determination of plasma catalase, RBCs superoxide dismutase (SOD) and RBCs glutathione peroxidase (GPx) were obtained from Biodiagnostic Co., Egypt.

The formula was prepared as mentioned in a previous publication (8). The samples were dried in a circulating air drying oven at 60 °C till complete dryness. The powdered formula was extracted twice once with 70% ethanol and the second with 80% methanol using an ultrasonic device (200 W, 59 kHz Shanghai Kudos Sonication Machine Company Ltd., China) for 60 min at room temperature. The mixture was then filtered through Whatman no. 4 filter paper and the filtrate was evaporated under reduced pressure at 30 °C until its volume was about 40 ml. The final volume of the extract was made to 50 ml with the extraction solvent and then it was taken out for the analysis (9).

The DPPH radical scavenging activity (DPPH) was determined according to the procedure of Hayat et al., (2010) (9). Trolox equivalent antioxidant capacity (TAEC) was estimated according to Ventura et al. (2013) (10). Ferric reducing power (FRAP) was assessed according to Barros et al. (2012) (11). β -carotene bleaching test was determined as described by Moulehi et al. (2012) (12) and lipid peroxidation inhibition activity was estimated according to Anup et al., (2006) (13).

The biological evaluation of the formula was assessed on experimental animals as mentioned in a previous study (8). Rats were rendered hyperglycemic by intraperitoneal injection with streptozotocin dissolved in citrate buffer (0.1 mol/L, pH 4.5) which was freshly prepared just before injection in a dose of 60 mg/kg B. wt. Those rats with fasting blood glucose of 200 mg/100 ml blood or above were included in the experiment. Then, diabetic rats were divided into two groups, one group given the standard control diet which was prepared as described by Reeves et al., (1993) (14), the other group was given the standard control diet + the formula as 100 g/Kg diet and compared with their respective controls (negative control given the standard diet and control given the standard diet + the formula as 100g/Kg diet). The experiment lasted for 4 weeks, then, biochemical parameters were determined as follows:

The antioxidant enzymes namely RBCs SOD, plasma catalase and RBCs GPx were estimated according to the methods of Nishikimi, et al. (1972) (15), Aebi, (1984) (16) and Paglia and Valentine (1967) (17), respectively. Plasma malondialdehyde (MDA) was measured as lipid peroxide product by the thiobarbituric acid assay (TBA) according to the method of Drapper and Hadley (1990) (18).

Lipid parameters were estimated as follows; plasma triacylglycerols was assessed as described by Chowdhury, et al., (1971) (19). Plasma cholesterol was determined as described by NCEP Expert Panel on Detection, (1988) (20). HDL-cholesterol was determined according to Lopes-Virella, et al (1977) (21). LDL-cholesterol and VLDL-cholesterol were determined according to Warnick, et al (1990) (22) as shown in the following equations:

$$\text{LDL-C} = \text{Total cholesterol} - (\text{HDL-C} + \text{VLDL-C})$$

$$\text{Where, VLDL-C} = \text{Triglyceride} / 5$$

Atherogenic index was calculated as described by Goh, et al., (2004) (23) according to the equation:

$$\text{Atherogenic index} = \log (\text{triglycerides} / \text{HDL-C}).$$

The concentration of plasma dipeptidyl peptidase-4 (DPP4) was estimated according to Longenecker et al., (2006) (24).

Results

The values obtained for parameters of the antioxidant power of the composed formula are shown in table (1). These values are given for both ethanol and methanol extracts. As shown in the table, the antioxidant activity represented by the DPPH amounted to 72.94 ± 0.54 and 92.52 ± 0.12 percent scavenging activity for both ethanol and methanol extracts, respectively. The values obtained as determined by the ferric reducing power assay or the TPTZ amounted to 1101.25 ± 55.83 and 1307.39 ± 23.63 Mole TE /100g dry wt. for both extracts. The peroxide radical procedure gave values of 0.43 ± 0.006 and 0.88 ± 0.004 . Determination of the β -carotene percentage inhibition gave values of 72.12 ± 22.23 and 38.82 ± 7.64 . The ABTS method gave values of 0.26 ± 0.005 and 0.74 ± 0.062 $\mu\text{mol Trolox/gm dry wt}$.

Table (1): Antioxidant activity represented by DPPH (% scavenging activity), Ferric reducing power (FRAP) assay or TPTZ (1 moles Trolox equivalents/100 g of FW sample), lipid peroxidation inhibition activity (LPIA), β -carotene (as % inhibition) & ABTS ($\mu\text{mol Trolox/gm FW}$) for ethanolic and methanolic extracts of the prepared formula for diabetes.

Extract	DPPH (% Scavenging activity)	TPTZ (Mole TE/100g FW)	LPIA (% inhibition)	β -carotene (% inhibition)	ABTS ($\mu\text{mol T/gm FW}$)
Eth. extract	72.94 ± 0.54^a	1101.25 ± 55.83^a	0.43 ± 0.006^a	72.12 ± 22.23^a	0.26 ± 0.005^a
Meth. extract	92.52 ± 0.12^b	1307.39 ± 23.63^b	0.88 ± 0.004^b	38.82 ± 7.64^b	0.74 ± 0.062^b

*Values that share the same letter at the same column are not significant

*Values that share different letters at the same column are significant.

*The mean difference is significant at the 0.05 level.

The activities of the plasma or blood antioxidant enzymes of the rats injected with streptozotocin, either those fed on the standard diet or those fed on the diet containing the dietary supplement and the corresponding controls are presented in (table 2). The level of plasma malondialdehyde is also given. As shown in the table, injection with streptozotocin caused a non-significant decrease in the activity of superoxide dismutase. The control value was 730.32 ± 35.55 and changed to 660.22 ± 26.09 U/g Hb in diabetic rats. When diabetic rats were fed on the diet containing the formula the value changed to 732.79 ± 27.12 U/g Hb.

Table (2): Activity of RBCs superoxide dismutase (SOD), plasma catalase, RBCs glutathione peroxidase (GPx) & plasma malondialdehyde (MDA) of the control group and all other groups.

Group	SOD	Catalase	GPx	MDA
	U/g Hb	(U/L)	U/g Hb	(μ mol/ml)
Control negative Mean \pm SE	730.32 \pm 35.55 ^a	656.77 \pm 18.44 ^b	2323.33 \pm 353.87 ^c	1.62 \pm 0.16 ^a
Control+formula Mean \pm SE	739.17 \pm 20.77 ^a	645.10 \pm 24.47 ^b	1920.38 \pm 238.98 ^{bc}	1.77 \pm 0.09 ^a
Control diabetic Mean \pm SE	660.22 \pm 26.09 ^a	785.9 \pm 10.02 ^a	1050.50 \pm 73.81 ^a	1.95 \pm 0.18 ^a
Diabetic+formula Mean \pm SE	732.79 \pm 27.12 ^a	747.9 \pm 15.42 ^a	1353.72 \pm 160.08 ^{ab}	1.85 \pm 0.12 ^a

*Values that share the same letter at the same column are not significant.

*Values that share different letter at the same column are significant.

*The mean difference is significant at the 0.05 level

The activity of plasma catalase increased significantly due to injection with streptozotocin. The obtained value for the control negative was 656.77 \pm 18.44 and became 785.9 \pm 10.02 U/L for the streptozotocin injected rats. Addition of the formula caused a non-significant decrease in the value of the catalase activity (747.9 \pm 15.42 U/L) when being compared to the control diabetic (785.9 \pm 10.02 U/L). Glutathione peroxidase activity was significantly decreased due to streptozotocin injection. The control negative value was 2323.33 \pm 353.87 and became 1050.50 \pm 73.81 U/g Hb. Addition of the formula to the diet caused a relative increase in the activity of the enzyme (1353.72 \pm 160.08 U /g Hb). The plasma malondialdehyde showed a non-significant increase as a result of streptozotocin injection and the value was relatively slightly decreased when the formula was given with the diet (table 2).

The plasma lipid parameters of rats either the control or those injected with streptozotocin given the formula with the diet or without are shown in tables (3 & 4). The total cholesterol of the injected rats (67.90 \pm 4.40 mg/dl) was significantly lower than the control negative (102.74 \pm 7.35 mg/dl). Addition of the formula to the diet caused partial relative increase in cholesterol. The LDL-C, HDL-C and VLDL-C showed more or less a similar pattern to that of cholesterol. Triglycerides also behaved similarly, the control value was 112.17 \pm 2.94 mg/dl and the diabetic value was 62.79 \pm 3.63 mg/dl. This value was increased (91.94 \pm 14.25 mg/dl) when the formula was included with the diet. The calculated atherogenic index was low in streptozotocin injected rats (0.28 \pm 0.04) and slightly increased when the formula was included (0.31 \pm 0.06). However, the differences were all non-significant.

Table (3): Concentration of plasma cholesterol, plasma triglycerides & plasma VLDL-C of the control group and all other groups.

Group	Cholesterol (mg/dl)	Triglycerides (mg/dl)	VLDL-C (mg/dl)
Control negative Mean \pm SE	102.74 \pm 7.35 ^b	112.17 \pm 2.94 ^b	22.35 \pm 0.55 ^c
Control+formula Mean \pm SE	96.73 \pm 7.04 ^b	84.55 \pm 14.44 ^{ab}	13.46 \pm 0.67 ^{ab}
Control diabetic Mean \pm SE	67.90 \pm 4.40 ^a	62.79 \pm 3.63 ^a	12.59 \pm 0.73 ^a
Diabetic+formula Mean \pm SE	86.22 \pm 10.49 ^{ab}	91.94 \pm 14.25 ^{ab}	16.04 \pm 1.42 ^b

*Values that share the same letter at the same column are not significant.

*Values that share different letter at the same column are significant.

*The mean difference is significant at the 0.05 level.

Table (4): Concentration of plasma LDL-cholesterol, plasma HDL-cholesterol & atherogenic index of the control group and all other groups.

Group	LDL-C (mg/dl)	HDL-C (mg/dl)	A.I log (TG/HDL-C)
Control negative Mean± SE	29.15 ± 3.23 ^a	51.84 ± 4.02 ^c	0.34 ± 0.03 ^a
Control+formula Mean± SE	32.45 ± 5.59 ^a	50.80 ± 1.96 ^c	0.31 ± 0.03 ^a
Control diabetic Mean± SE	22.37 ± 3.95 ^a	32.94 ± 1.41 ^a	0.28 ± 0.04 ^a
Diabetic+formula Mean± SE	34.72 ± 5.22 ^a	43.02 ± 0.52 ^b	0.31 ± 0.06 ^a

*Values that share the same letter at the same column are not significant.

*Values that share different letter at the same column are significant.

*The mean difference is significant at the 0.05 level.

The concentration of plasma dipeptidyl peptidase-4 (table 5) showed a significant increase in streptozotocin injected rats (1.1 ± 0.18 ng/ml) relative to a negative control value of 0.5 ± 0.11 ng/ml. The value returned to near normal (0.50 ± 0.13 ng/ml) when the formula was included.

Table (5): Concentration of dipeptidyl peptidase-4 (DPP-4) of the control group and other groups.

Group	DPP-4 (ng/ml)
Control negative Mean± SE	0.52 ± 0.11 ^a
Control+formula Mean± SE	0.33 ± 0.07 ^a
Control diabetic Mean± SE	1.10± 0.18 ^a
Diabetic+formula Mean± SE	0.50 ± 0.13 ^b

* Values that share the same letter at the same column are not significant.

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*The mean difference is significant at the 0.05 level.

Discussion

There are several methods for measuring the antioxidant capacity of any source. This is because no single assay can determine with satisfactory accuracy the radical sources present in a complex system such as in plants, herbs or animal sources. Therefore, in order to have reliable antioxidant profile for any source, it is preferable to use multiple assays encompassing reactivity towards either aqueous or organic radicals. Furthermore, the response of antioxidants may differ towards radicals or oxidant sources. Example, it has been reported that carotenoids are exceptional in quenching singlet oxygen, while phenolics and other antioxidants are better for quenching hydroxyl radicals (25). Based on this, the formula used in this study was assessed by different methods that can reflect its antioxidant capacity. It is clear that the combination of plant sources namely guar gum, chicory, cape gooseberry fruit, turmeric, black berry, *Ginkgo biloba* and sumac possess marked antioxidant capacity as determined by five different analytical methods. It can be observed that the methanol extract is more effective as antioxidant relative to the ethanol extract. The values obtained from the different analytical procedures are always higher in case of the methanol extract except in estimation of β -carotene % inhibition. This shows that whatever

the type of free radical generated, the ingredients used in the applied formula contain antioxidant compounds that operate and exert their antioxidant function. This also means that whatever the source of the oxidation stress that might occur in the body, the formula can be operative. It is important to note that testing the absorbance profiles of DPPH in methanol, ethanol and buffered methanol showed that the order of absorbance was highest in buffered methanolic solution, followed by methanolic and ethanolic solutions. This may explain the higher readings of absorbance in case of methanol extract (26).

Considering the mode of action of the different analytical procedure used for determination of the antioxidant capacity of the formula used in this study, it can be noticed that scavenging of DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical is the basis of a common antioxidant assay (27). TPTZ (2,4,6-Tris(2-pyridyl)-s-triazine) is usually used for determination of iron and phenols in different sources (28).

Peroxide radicals include a group of natural chemicals called reactive oxygen species (ROS) (29). ABTS (2, 20-azinobis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt) is a compound used for the estimation of the antioxidant activity of lipophilic and hydrophilic antioxidants, including flavonoids, hydroxyl cinnamates, carotenoids, and plasma antioxidants (30). This diversity of the analytical procedures enabled us to show how far the formula can deal with different types of free radicals formed under different conditions. It can be easily stated that the used formula deal with a wide range of free radicals generated and in turn is able to scavenge most of them. In a previous published article, it was shown that experimental animals injected with a dose of streptozotocin and treated with this formula, the hyperglycemia produced in animals decreased (8). It has been proved that streptozotocin injection caused the generation of free radicals and most probably this was behind the hyperglycemia that occurred either due to degeneration of pancreatic cells or due to direct action on insulin function causing insulin resistance. The reported values for the different analytical methods used confirm the formation of diverse types of radicals and the ability of the formula to deal with and ameliorate their destructive action. The ability of the formula to perform this function is mainly due to the bioactive compounds present in the different ingredients composing the formula. Among these is guar gum, the sulfation of which caused a sharp decrease in molecular weight and enhanced its antioxidant activities (31). Inulin present in chicory was found to decrease malondialdehyde levels in a clinical study of women with type 2 diabetes, which indicates its ability to minimize oxidation overload (32). Curcumin or turmeric is amongst the herbs that possess a marked antioxidant power (33). Blackberry contains a large number of phytochemicals including flavonoids, tannins, ellagitannins, anthocyanins, quercetin, cyanidins, salicylic acid, gallic acid and ellagic acid (34-36). The rich dark color in blackberry is due to anthocyanins. Thus, because of their dense content of polyphenols, blackberry is a strong antioxidant. They are ranked as fruits with high in vitro antioxidant strength. *Ginkgo bilboa* was reported to protect from oxidative cell damage (37). Sumac has been found to be a good source of antioxidants; it contains polyphenols as phenolic acids, flavonoids and anthocyanins (38).

The oxidative state in the body is determined by the availability of bioactive compounds with antioxidant characters and the antioxidant enzymes produced inside the body. Concerning antioxidant enzymes, there was no significant change in the activity of superoxide dismutase. However, there was a significant increase in the activity of or catalase and a significant drop in the activity of glutathione peroxidase due to streptozotocin injection with a higher but not significant level of plasma malondialdehyde. Treatment with the formula caused a non-significant rise of superoxide dismutase, a relatively low increase in the activity of catalase and a non-significant rise in the activity of glutathione peroxidase, which means that the formula could correct to certain extent the derangement effect of streptozotocin and hyperglycemia. This means that not all antioxidant enzymes are affected the same way either due to injection of streptozotocin or consumption of the supplement formula. The obtained results partially agree with that obtained by Zemestani, et al., (2016) (39) in diabetic patients.

Contrary to other findings, (40) streptozotocin injection caused a drop in plasma total cholesterol, VLDL-cholesterol and triglyacylglycerols. The streptozotocin dose used by these authors amounted to 40 mg/kg body weight intra peritoneal while that used in this study was 60 mg/kg body weight. This relatively high dose was used to insure hyperglycemia in the majority of the injected rats. The food intake and the body weight of these injected rats were markedly decreased relative to controls [8]. It is assumed that the low food consumption and the drop in body weight of the animals are the reasons why these animals did not have high lipid parameters usually reported in diabetes. Another important finding is that the effect of streptozotocin on insulin activity was not so marked in spite of the hyperglycemia evidenced in injected animals (8). This means that insulin insufficiency may not be the main contributing factor in this case but most probably insulin resistance is the operating factor.

Dipeptidyl peptidase-4 (DPP-4) is a glycoprotein usually expressed on the surface of most cell types attached to the membrane and it is encoded by the *DPP4* gene (41). It is released to circulation by a process called shedding. Its action is related to immune regulation, signal transduction and apoptosis. Also, DPP-4 has a major role in glucose metabolism. DPP-4 inhibitors or gliptins, are a class of oral hypoglycemics that block DPP-4 (DPP-IV) and used to treat diabetes mellitus type 2 (42). It is known that increased incretin level caused by DPP-4 inhibitors such as gliptins can prolong the postprandial insulin action. Incretin which is secreted from the gut following meals induce secretion of insulin. This occurs through binding to certain receptors on the pancreas causing stimulation of insulin secretion and suppression of glucagon. Glucagon increases blood glucose levels, and DPP-4 inhibitors reduce glucagon and blood glucose levels. The mechanism of DPP-4 inhibitors is to increase incretin levels (glucagon like peptide-1 "GLP-1" and glucose-dependent insulinotropic polypeptide "GIP"), which inhibits glucagon release, which in turn increases insulin secretion, decreases gastric emptying, and decreases blood glucose levels (43-45). Improved glucose tolerance together with increased glucagon like peptide-1 and leptin was reported by Stphan et al., (2011) (46) in DPP-4 depleted Dark Agvuti rats with diet induced obesity.

It is thus clear that injection of streptozotocin caused the release of DPP-4 into circulation and raised its value significantly from 0.51 ± 0.11 ng/ml in negative control to 1.10 ± 0.18 ng/ml in streptozotocin injected rats. The enzyme; DPP-4 is expected to inhibit incretin level thus reduce insulin secretion and participate in hyperglycemia. When the formula was given with the diet, the level of DPP-4 returned to near normal and the insulin action was markedly improved. It is worth mentioning the adding the formula to the diet of even control animals lowered the level of DPP-4 however non-significantly. This shows that certain compound or compounds present in any of the items composing the formula succeeded to inhibit the release of DPP-4 into circulation thus lowered its concentration and inhibited its action thus improved insulin secretion and action and in turn lowered blood glucose level as reported previously from the same experiment (8).

It is thus concluded that the composed formula is characterized by a strong antioxidant characters evidenced by the various analytical procedures and that this antioxidant power is effective in reducing the degenerative action of streptozotocin on pancreatic cells. The degeneration may either cause insulin insufficiency or the formed insulin may be defected causing insulin resistance. Among the factors contributing to the resulting hyperglycemia is the glycoprotein enzyme DPP-4 which is released into circulation due to streptozotocin injection. Introducing the formula in the diet of the streptozotocin injected rats inhibit the secretion of DPP-4, giving a chance to incretin to inhibit glucagon release, which in turn increases insulin secretion and prolong insulin action.

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