

METHANOLIC FLOWER EXTRACT OF *PHLOGACANTHUS THYRSIFLORUS* NEES. ATTENUATES DIABETIC NEPHROPATHY IN ALLOXAN-INDUCED DIABETIC MICE

JUTISHNA BORA, DONKUPAR SYIEM, SURYA BHAN*

Department of Biochemistry, North Eastern Hill University, Shillong – 793 022, Meghalaya, India. Email: sry_bhan@yahoo.co.in

Received: 20 February 2017, Revised and Accepted: 28 March 2018

ABSTRACT

Objectives: The aim of the present study is to determine the effects of *Phlogacanthus thyrsoiflorus* on hyperglycemia and oxidative stress in kidneys of diabetic mice.

Methods: Aqueous flower extract (AFE) and methanolic flower extract (MFE) were prepared. Preliminary phytochemical screenings were carried out. Diabetic mice were prepared by administering alloxan at 150 mg/kg body weight (b.w). The effects of different doses (150–550 mg/kg b.w) of the MFE on hyperglycemia were examined for short-term duration in diabetic mice. Activities of antioxidant enzymes, superoxide dismutase (CuZn-SOD and Mn-SOD), catalase (CAT), and glutathione reductase (GR) were examined under normal, diabetic, and diabetic-treated groups. Histopathological studies were also carried out for the above groups.

Results: It has been found that the flavonoids and tannins were strongly present in MFE and further *in vivo* studies with MFE were carried out. It has been also observed that 250 mg/kg b.w dose of MFE showed significant reduction of blood glucose level. The activities of SOD, CAT, and GR increased in diabetic mice treated with MFE. Histological studies showed that MFE could further halt the damage caused by oxidative stress and reduced tissue injuries in diabetic mice.

Conclusion: MFE of *P. thyrsoiflorus* has positive effects in alloxan-induced diabetic mice and could be potential candidate for the detailed molecular studies and management of diabetes.

Keywords: Alloxan, Diabetes mellitus, Antihyperglycemic activity, Antioxidative activity, Metformin, *Phlogacanthus thyrsoiflorus*.

© 2018 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>) DOI: <http://dx.doi.org/10.22159/ajpcr.2018.v11i7.25393>

INTRODUCTION

Diabetes mellitus (DM) is a serious global health problem with 415 million population are under its grip worldwide in 2015 and this might extend to 642 million by 2040 [1]. Diabetes, also termed as DM, is a metabolic disorder characterized by chronic hyperglycemia. Deficiency of insulin secretion, insulin action, or both could lead to metabolic disturbance of carbohydrate, fat, and protein [2]. The prolonged hyperglycemia during DM stimulates increased production of free radicals through several mechanisms such as polyol pathway, glucose oxidation, and advanced glycation end product formation [3,4]. Therefore, there are enzymic and non-enzymic antioxidant defense systems in mammalian cells that minimize the cellular damage caused by free radicals. However, the relative overload of free radicals and lowering of antioxidant defense systems activities in DM added with the generation of more oxidative stress that results in pathogenesis and complications of diabetes [5,6].

The DM is considered to be a global problem and successful treatment has yet to be discovered. Although insulin therapy and oral hypoglycemic agents are available for the treatment of diabetes and are also effective in controlling hyperglycemia, they are associated with many side effects and failed to significantly alter the course of diabetic complications. Medicinal plants and their bioactive compound are used as an alternative method to treat the diabetes patient throughout the world since the 6th century [7]. A current research on free radicals has evaluated that foods which are rich in antioxidants possess an essential role in the prevention of adverse effects caused by diabetes. Resultantly, natural antioxidants, that are ubiquitous in fruits, leaves, and flowers, have received great attention and have been studied extensively, as they are effective free radical scavengers and are presumed to be less toxic than synthetic antioxidants [8]. Literature also reports about 800 plants which may possess antidiabetic potential [9].

Phlogacanthus thyrsoiflorus is an herb from a family of Acanthaceae, which has several common names such as titaaphul, chuhai, and tangkhul. *P. thyrsoiflorus* is one of the common medicinal plants used by local people of Assam to manage diabetes [10]. *P. thyrsoiflorus* produces a number of secondary metabolites such as alkaloids, flavonoids, steroids, triterpenoids, polyphenols, and glycosides. It has free radical scavenging property so it may prove as a very good medicinal herb [11]. In this regard, it makes great sense to evaluate the antidiabetic and antioxidative properties of flowers extract in alloxan-induced diabetic mice model.

METHODS**Chemicals**

Alloxan, pyrogallol, and metformin were procured from Sigma-Aldrich Co. (St. Louis, MO, USA). Insulin was purchased from Gland Pharma Ltd. (Hyderabad, India). The other chemicals used were of analytical grade ordered from Merck Co. (Mumbai, India) and Sisco Research Laboratory.

Plant material

Flowers of *P. thyrsoiflorus* were collected from Assam, India (Voucher No: 12055). The specimen was submitted and identified by Dr. P.B. Gurung Curator herbarium, Department of Botany, NEHU, Shillong, Meghalaya.

Aqueous and methanolic extracts preparation

The flowers were separated, weighed, washed, and dried in the shade. It was then grinded to powder form using a grinder. Aqueous flower extract (AFE) was prepared by macerating with distilled water (10 x volumes) for 24 h at room temperature with continuous stirring. It was then filtered through Whatman paper number 1. The filtrate was transferred into lyophilized tube and freeze dried (lyophilization) for 1–2 days and stored in –20°C freezer for further use [12]. Similarly, methanolic flower extract (MFE) was prepared by extracting it with 10 x volume of methanol:aqueous

solution (4:1). After overnight stirring (extracting), the mixture was filtered and the filtrate evaporated to dryness at 40°C in a rotary evaporator [12]. The dried mass was stored and used for further investigations.

Phytochemical screening

Phytochemical screening were carried out on both AFE and MFE examining the chemical secondary metabolites of alkaloids, flavonoids, glycosides, tannins, terpenes, and saponins [13-15].

Test animals

Adult healthy male Swiss albino mice (Balb/C strain), 20–30 g in weight, were used for all the investigations. Procedure of all the experiments was reviewed and carried out in accordance with the institutional ethics committee guidelines (Animal models), 04-12-2014. Mice were housed in a polycarbonate cage under controlled temperature, i.e., at 22°C on a 12 h light/dark cycle and were fed with mice feed obtained from laboratory of Amrut, Pune, India, and water *ad libitum*.

Induction of DM in test animals

Alloxan monohydrate (150 mg/kg body weight [b.w]) dissolved in citrate buffer (0.1 M, pH 4.5) was intraperitoneally administered to overnight fasted test mice for induction of diabetes [16]. After 72 h of alloxan injection, mice with fasting blood glucose (FBG) above 200 mg/dl were considered as diabetic and included in the investigations.

Antihyperglycemic study

Alloxan-induced diabetic mice were divided into one control and five test groups comprising five mice in each group. Control group, consisting of diabetic mice, administered with only distilled water and diabetic mice in test groups were administered with increasing doses (150 mg/kg b.w–550 mg/kg b.w) of MFE intraperitoneally to carry out the antihyperglycemic study. After injection, FBG levels were monitored at 0.5 h, 1 h, 2 h, 4 h, and 6 h. After 6 h, the mice were fed and blood glucose levels were monitored at 24 h [17].

Antioxidant enzyme assays

Swiss albino mice were divided into four groups consisting of five mice in each group for the study. Doses were intraperitoneally administered in alternate days for a total of 21 days.

- Group 1: Normal mice administered with distilled water.
- Group 2: Diabetic mice administered with distilled water.
- Group 3: Ascorbic acid (50 mg/kg b.w) treated diabetic mice.
- Group 4: MFE (250 mg/kg b.w) treated diabetic mice.

Kidney tissues were extracted after sacrificing by decapitation on 21st days. Tissues were homogenized to make 10% (w/v) homogenate in ice cold 10 mM HEPES buffer, pH 7.4 containing 0.2 M mannitol, 50 mM sucrose, and 1 mM EDTA. Nuclei and cell debris were sedimented by centrifuging tissue homogenate for 10 min at 4°C at 1000 g. The supernatant collected was again centrifuged for 10 min at 4°C at 7500 g. Mitochondrial fraction was obtained with the resulting mitochondrial pellet from above procedure being washed gently by suspending in homogenate buffer and then resedimented at 7500 g for 10 min at 4°C. Cytosolic fraction was obtained by centrifuging the post-mitochondrial supernatant further for 10 min at 4°C at 1500 g.

The protein concentration of cytosolic and mitochondria fractions was determined by Bradford method [18] using bovine serum albumin as a standard.

Both Mn-superoxide dismutase (SOD) and Cu/Zn-SOD activities were estimated according to the method of Marklund and Marklund [19]. The enzyme activities were expressed in units/mg of protein where one unit defines the amount of enzyme required to give 50% inhibition. The absorbance was read spectrophotometrically against reagent black at λ 470 nm.

Catalase (CAT) activities were determined by the method of Aebi [20]. Activities of CAT were calculated using the molar extinction coefficient of 43.6 cm⁻¹ and were expressed as nmoles of H₂O₂ decomposed/min/mg protein.

Glutathione reductase (GR) activities were determined by the method of Carlberg and Mannervik [21]. The GR activities were expressed as units/mg protein, and the absorbance was read at λ 340 nm following the oxidation of NADPH.

Histopathological studies

Kidney of normal, diabetic, and treated diabetic mice were excised after 21 days by sacrificing the mice by cervical dislocation and the histopathological studies were carried out by hematoxylin-eosin (HE) staining techniques [22]. After extractions, all the tissues were first fixed in formalin for 24 h then were dehydrated with ethanol gradients (70%–100%) before embedding in paraffin wax. The paraffin blocks were sectioned into 7 μ m sized using a rotary microtome. The slice sections were fixed on glass slides, deparaffinized, and stained with hematoxylin followed by eosin stains. Eventually, the sections on glass slides were covered by coverslip mounted with DPX and observed under Olympus BX51 light microscope.

Statistical analysis

Results of all the investigations were expressed as mean \pm standard error of the mean for each group consisting of five mice in every group. Data were analyzed using one-way ANOVA followed by Tukey's *post hoc* test.

RESULTS

Phytochemical screening

Screening results of AFE and MFE of *P. thyriflorus* showed different chemical constituents in Table 1. The flavonoids and tannins were found to be strongly present in MFE, and therefore, the further studies were carried out with MFE.

Antihyperglycemic study

The result of the effects of MFE on elevated FBG levels is shown in Fig. 1. There was minimal reduction (32.2%) of FBG level at the dose of 150 mg/kg b.w at 6 h. However, a significant reduction (31.3%) was observed at 250 mg/kg b.w at 2 h, 44.7% at 4 h, and 50% at 6 h from that of the control group. At 350 mg/kg b.w, the FBG levels were 45.5% at 2 h, 61.3% at 4 h, and 66.7% at 6 h when compared with the diabetic control group in the study. The higher doses of 450 mg/kg b.w and 550 mg/kg b.w showed drastic reduction of FBG level. At 450 mg/kg b.w, the FBG levels were 62% at 2 h, 68.2% at 6 h, and 73.3% at 6 h, while at 550 mg/kg b.w the FBG levels were 67.4% at 2 h, 75.6% at 4 h, and 79% at 6 h. Hence, 250 mg/kg b.w dose of MFE was considered to be the optimum dose for the study.

Kidney antioxidant enzyme assays

SOD (CuZn-SOD and Mn-SOD), CAT, and GR activities in kidney tissues of the experimental group are shown in Table 2. The SOD, CAT, and GR activities were reduced significantly in Group 2 (diabetic mice). However, Group 3 (ascorbic acid-treated diabetic mice) and Group 4 (extract-treated diabetic mice) showed a significant increase in antioxidant activity with increased in SOD, CAT, and GR activities in comparison to diabetic control group in the study.

Kidney histopathological studies

As shown in Fig. 2, kidney of diabetic mice, the glomerulus has been shrunken which leads to widening of Bowman's capsule space, proximal convoluted tubule (PCT) with wide lumen, lined with high and deformed cuboidal cells, distal convoluted tubule (DCT)

Table 1: Phytochemical screening results of AFE and MFE

Screening	AFE	MFE
Alkaloids	Not present	Slightly present
Tannins	Slightly present	Strongly present
Glycosides	Slightly present	Strongly present
Flavonoids	Slightly present	Strongly present
Terpenes	Slightly present	Strongly present
Saponins	Strongly present	Slightly present

AFE: Aqueous flower extract, MFE: Methanolic flower extract

Table 2: Mean activities of SOD, CAT, and GR in kidney of different experimental groups

Groups	SOD (Units/mg protein)		CAT (Units/mg protein)	GR (Units/mg protein)
	CuZn-SOD	Mn-SOD	CAT	GR
Group 1	6.334±0.57	13.46±0.42	2.42±0.14	3.25±0.13
Group 2	3.4±0.15	8.9±0.46	1.54±0.10	2.30±0.15
Group 3	5.11±0.49***	11.6±0.26***	2.29±0.11***	3.01±0.12***
Group 4	5.02±0.18***	12.05±0.28***	2.24±0.09**	2.96±0.07***

Group 1: Normal mice, Group 2: Diabetic mice, Group 3: Diabetic mice treated with ascorbic acid, Group 4: Diabetic mice treated with MFE. Values are expressed as Mean±SEM, n=5. (**p<0.01, ***p<0.001 versus diabetic control group). SOD: Superoxide dismutase, CAT: Catalase, GR: Glutathione reductase

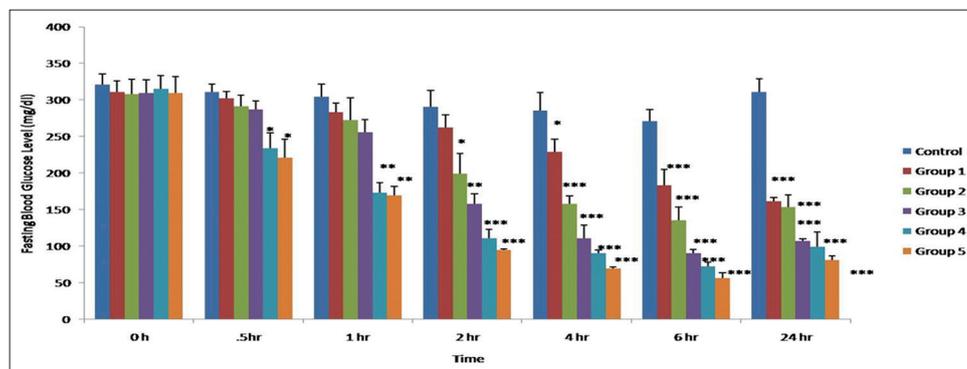


Fig. 1: Effects of methanolic flower extract on diabetic mice (short-term study) showing changes of fasting blood glucose level at different time interval for different experimental groups (150–550 mg/kg body weight dose). Values are expressed in mean ± standard error of the mean; n=5. (*p<0.05, **p<0.01, ***p<0.001)

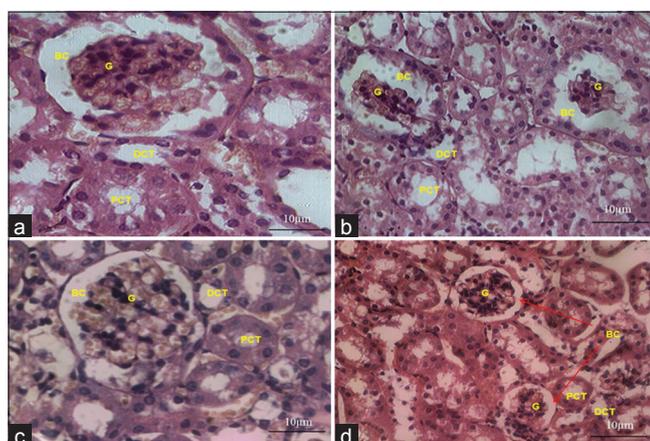


Fig. 2: Photomicrographs of section of kidney tissues showing histological changes. a: Normal mice, b: Diabetic mice, c: Diabetic mice treated with ascorbic acids, and d: Diabetic mice treated with methanolic flower extract. G: Glomerulus, BC: Bowman's capsule, PCT: Proximal convoluted tubule, DCT: Distal convoluted tubule. All images are under ×40 magnifications

with wide lumen and deformed cuboidal cells when compared with normal mice. The normal physiological structure of glomerulus without much widening of Bowman's capsule space and near normal PCT and DCT was seen in both ascorbic acid- and MFE-treated diabetic mice.

DISCUSSION

Alloxan, referred to as alloxan monohydrate (2,4,5,6-tetraoxypyrimidine; 5, 6-dioxyuracil), is a known diabetogenic agent and used to induce diabetes in laboratory rats and mice. It is a toxic glucose analog that destroys pancreatic beta-cells (insulin-producing cells) [23]. Therefore, alloxan administration in mice shows a significant increase in blood glucose level in the study.

Although the AFE had a good effect on diabetes, the effect of MFE was quite better than the former. The MFE reflected the good effects due to the presence of flavonoids and tannins compound which showed in phytochemical screening of MFE, while the other extract did not have much flavonoids and tannins. Flavonoids are highly effective free radical scavengers that could scavenge oxidized molecules, including singlet oxygen, and various other free radicals implicated in several diseases [24]. Flavonoids suppress the formation of reactive oxygen, chelate trace elements involved in free radical production, scavenge reactive species, and upregulate and protect antioxidant defenses [25]. Flavonoids might have the potential of secreting insulin from existing pancreatic β -cell [26]. There might be another possibility of flavonoids that is to protect the DNA from the oxidative damage, and hence, it could resist the changes in β -cell [27]. Similarly, tannins confer oxidative stress tolerance on plants. The short-term antihyperglycemic study has revealed that 250 mg/kg b.w of MFE was able to reduce FBG level in more significant way. Hence, 250 mg/kg b.w dose of extract was considered to be an optimum dose for the study.

SOD (Mn-SOD and CuZn-SOD) presents in mitochondria and cytosol is an antioxidant enzyme that defends against reactive oxygen species. They eliminate superoxide anion to hydrogen peroxide [28]. CAT enzyme, which is also an antioxidant enzyme, neutralized hydrogen peroxide to water and oxygen [29]. GR antioxidant enzyme in cytoplasm functions through converting oxidized glutathione to reduce form GSH and helps in maintaining a balanced intracellular environment. In this study, it was found that treatment with extract restores and increases the activities of antioxidants enzymes when compared with untreated diabetic mice. Therefore, it could be said that MFE of *P. thyriflorus* protects the kidney tissue from further oxidative stress under diabetic condition.

From histological study, it is confirmed that MFE of *P. thyriflorus* has the ability to protect and restore oxidative stress injury induced by diabetes. It is also confirmed from the study that extract is able to suppress oxidative stress in the tissue and enhancing the activity antioxidant enzyme, thus allowing tissue to recover and halt from further damage.

CONCLUSION

The findings of the present study signified that MFE of *P. thyrsoiflorus* has antihyperglycemic potential and treatment with extract may provide beneficial effects against oxidative stress. However, the exact mechanism is yet to be elucidated and further investigation is underway.

ACKNOWLEDGMENT

The authors would like to thank the Department of Science and Technology (DST), New Delhi, India, for providing INSPIRE fellowship, DRS programme, for providing infrastructural support to the Department of Biochemistry, NEHU, Shillong.

CONFLICTS OF INTEREST

There are no conflicts of interest. All authors contributed immensely for this work and prepared the manuscript.

REFERENCES

1. WHO. Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia. Geneva: WHO; 2006. p. 1-50.
2. Zhang Y, Wu L, Ma Z, Cheng J, Liu J. Anti-diabetic, anti-oxidant and anti-hyperlipidemic activities of flavonoids from corn silk on STZ-induced diabetic mice. *Molecules* 2015;21:1-11.
3. Inoguchi T, Sonta T, Tsubouchi H, Etoh T, Kakimoto M, Sonoda N, et al. Protein kinase C-dependent increase in ROS production in vascular tissues of diabetes: Role of vascular NAD(P)H oxidase. *J Am Soc Nephrol* 2003;14 8 Suppl 3:227-32.
4. Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, et al. Hyperglycemic pseudo hypoxia and diabetic complications. *Diabetes* 1993;42:801-13.
5. Baynes JW, Thorpe SR. Role of oxidative stress in diabetic complications: A new perspective on an old paradigm. *Diabetes* 1999;48:1-9.
6. Ceriello A. Oxidative stress and glycemic regulation. *Metabolism* 2000;49:27-9.
7. Kashikar VS, Tejaswita K. Indigenous remedies for diabetes mellitus. *Int J Pharm Pharm Sci* 2011;3:22-9.
8. Dakshayini PN, Basha PM. Phytochemical screening and *in vitro* antioxidant potential of *Tribulus terrestris* fruit and *Mesua ferrea* flower extracts: A comparative study. *Int J Pharm Pharm Sci* 2018;10:70-5.
9. Alarcon-Aguilara FJ, Roman-Ramos R, Perer-Gutierrez S, Aguilar-Contreras A, Contreras-Weber CC, Flores-Saenz JL. Study of the anti-hyperglycemic effect of plants used as antidiabetics. *J Ethnopharmacol* 1998;61:101-10.
10. Jaiswal V. Culture and ethnobotany of Jaintia tribal community of Meghalaya, Northeast India-a mini review. *Indian J Trad Knowl* 2010;9:38-44.
11. Kshirsagar R, Upadhyay S. Free radical scavenging activity screening of medicinal plants from Tripura, Northeast India. *Natl Prod Radiance* 2009;8:117-22.
12. Harborne JB. *Phytochemical Methods*. 3rd ed. London (NY): Chapman & Hall Publishers; 1998.
13. Rumanti RM, Nainggolan M, Harahap U. Phytochemical screening and antidiabetic activity of different leaf extracts from lotus (*Nelumbo nucifera* gaertn.) in streptozotocin induced mice. *Asian J Pharm Clin Res* 2017;10:190-92.
14. Farnsworth NR. Biological and phytochemical screening of plants. *J Pharm Sci* 1996;55:225-76.
15. Harbone JB. *Metode Fitokimia*. Vol. 2. Bandung: ITB; 1987. p. 49.
16. Syiem D, Gareth S, Khup PZ, Khongwir BS, Kharbuli B, Kayang H. Hypoglycemic effect of *Potentilla fulgens* L. in normal and alloxan-induced diabetic mice. *J Ethnopharmacol* 2002;83:55-61.
17. Patar AK, Bhan S, Syiem D. Effect of chlorophyllin, an semi-synthetic chlorophyll molecule on hyperglycemia and hyperlipidemia in streptozotocin induced diabetic mice. *Int J Pharm Pharm Sci* 2016;8:293-6.
18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248-54.
19. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem* 1974;47:469-74.
20. Aebi H. *CAT in vitro*. In: Packer L, editor. *Methods Enzymol*. Vol. 105. New York: Academic Press; 1984. p. 121-6.
21. Carlberg I, Mannervik B. *Gutathione reductase assay*. In: Packer L, editor. *Methods Enzymol*. Orlando (FL): Academic Press; 1985. p. 484-90.
22. Syiem D, Khup PZ. Evaluation of *Flemingia macrophylla* L.: A traditionally used plant of the north eastern region of India for hypoglycemic and anti-hyperglycemic effect on mice. *Pharmacol Online* 2007;2:355-66.
23. Szkudelski T. The mechanism of alloxan and streptozotocin action in B Cells of the rat pancreas. *Physiol Res* 2001;50:537-46.
24. Bravo L. Polyphenols: Chemistry, dietary sources, metabolism and nutritional significance. *Nutr Rev* 1998;56:317-33.
25. Agati G, Azzarello E, Pollastri S, Tattini M. Flavonoids as antioxidants in plants: Location and functional significance. *Plant Sci* 2012;196:67-76.
26. Kar MK, Swain TR, Mishra SK. Antidiabetic activity of *Clerodendrum serratum* L. moon leaves in streptozotocin-induced diabetic rats. *Asian J Pharm Clin Res* 2014;7:260-3.
27. Mujic A, Grdovic N, Mujic I, Mihailovic M, Zivkovic J, Poznanovic G, et al. Anti-oxidative effects of phenolic extracts from chestnut leaves, catkins and spiny burs in streptozotocin-treated rat pancreatic β -cells. *Food Chem* 2011;125:841-9.
28. Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Are oxidative stress-activated signalling pathways mediators of insulin resistance and β -cell dysfunction? *Diabetes* 2003;52:1-8.
29. Johansen JS, Harris AK, Rychly DJ, Ergula A. Oxidative stress and the use of antioxidants in diabetes: Linking basic science to clinical practice. *Cardiovasc Diabetol* 2005;4:5.