EFFECTS OF *ARNEBIA EUCHROMA* EXTRACT ON STREPTOZOTOCIN INDUCED DIABETES IN RATS: A STEREOLOGICAL STUDY

A. Noorafshan^{1,*}, S. Ebrahimi², E. Esmacilzadeh^{3,*}, H. Arabzadeh³, M. Bahmani-Jahromi³, S. Ashkani-Esfahani³

¹Histomorphometry and Stereology Research Center, ²Medical Ethics Department, ³Student Research Committee, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract

Background. Diabetes mellitus (DM) is considered as an important health confounder in our world, which necessitates its better management by new methods. In this study, we have evaluated the effects of oral *Arnebia Euchroma* (AE) extract on different stereological parameters of the pancreas as well as blood glucose in Streptozotocin (STZ)-induced diabetes in rats.

Methods. We divided 48 Wistar rats into 4 groups: C1 including normal rats, C2 not-treated diabetic rats, E1 with diabetic rats receiving 100 mg/kg AE extract orally, and E2 including diabetic rats treated with 300 mg/kg AE extract. Stereological study was done and the levels of blood glucose were also estimated and compared between experimental and control groups.

Results. There were significant differences in volumes of pancreatic islets, β cell populations, blood glucose levels in AE treated groups compared with not-treated diabetic group.

Conclusion. Although AE did not completely prevent or heal the pancreatic damage, its oral administration showed promising effects on maintaining the population of beta cells, the main insulin secreting cells, after STZ-induced injury and also lowered blood glucose levels compared to the not-treated diabetic group.

Key words: *Arnebia euchroma*, Diabetes, Streptozotocin, Stereology.

INTRODUCTION

Diabetes mellitus (DM) has become an important global issue (1). It has manifested as the prevalence of this disease that has been increasing globally since 1980 (2). Thus, many medical researchers seriously recommend planning for its prevention as well as early detection and management.

DM consists of a number of endocrine impairments, which are mostly associated with uncontrolled blood glucose levels. These conditions

have resulted from defects in insulin secretion mainly by pancreatic beta cells, insulin function, or both (3). Various etiologies can be found which were believed to be involved in DM's pathophysiology. Permanent destruction of the pancreas, specifically of insulin secreting beta cells, is considered as one of these etiologies, especially for DM type 1 (4). Pancreatic damage may be due to inflammatory processes such as pancreatitis, or may be induced mainly by autoimmune diseases, drugs or toxins, infections, starvation, low protein diet and high fat diet (5-7).

In order to perform the studies over DM, several animal models were designed. Among them, Streptozotocin (STZ) induced diabetes can be named as one of the most popular. STZ is a compound which has preferable toxicity for pancreatic beta cells (8). In a study by Deeds *et al.* STZ-induced DM animal models were introduced as a cost-benefit and efficient model which can be used in various strains of rodents in order to investigate the process involved in DM and design the related studies (9). In respect to the efficacy of this animal model, several studies can be found which used this experimental model for studying diabetes (10, 11). As an example, Lu *et al.* studied the role of phospholipase A2 and cyclooxygenase 2 in renal injury in Streptozotocin induced diabetic rats (12). Parmer *et al.* also applied the Streptozotocin diabetic model for evaluating the cardio protective effect of Lisinopril on diabetic rats (13).

For a better control of DM, several studies were done in order to introduce a new method of treatment. Among them, some traditional medications and practices are introduced as beneficial methods for prevention and even management of DM (14). *Arnebia Euchroma* (AE) (Jhonston) from the *Boraginaceae* family, *Magnoliopsida* class and *Tracheophyta* phylum can be found in different countries such as India, North Africa, Turkey, Himalaya and Iran especially in its west

*Correspondence to: Elmira Esmacilzadeh MD, MPH, Student Research Committee, Shiraz Medical School, Emam Hossein Square, Zand Street, Shiraz, Iran, E-mail: elmira69.es@gmail.com

and south west areas (15, 16). Various pharmacological effects have been found for this herbal medicine such as antioxidant, antimicrobial, antithrombotic, wound-healing, anti-inflammatory, and anticancer effects (15). Literatures shows that AE and its derivatives have anti-inflammatory effects on various diseases such as drug-induced colitis and rheumatoid arthritis (17, 18). The antimicrobial impact of AE is also declared by some studies (19). Antioxidant activity of AE was also mentioned in previous surveys (20).

Consequently, according to various effects of AE and their relationships with the mechanisms involved in pathophysiology of DM, we have aimed to investigate its possible protective effects against STZ-induced pancreatic injury by using stereological and histomorphometrical methods.

MATERIALS AND METHOD

A. Extraction of ethanol plant material

The plant was gathered in summer time in Yasuj city, Kohgiluyeh and Boyer-Ahmad province, South-West of Iran. It was authenticated by the research center of agriculture and natural sources of Yasuj University of Medical Sciences, Yasuj, Iran (Voucher number: 157). Its leaves were dried in 50°C and 120 grams of it were turned into powder. The powder was extracted with 600 mL of alcoholic-water (7:3) mixture (with ethanol 80%) in Soxhlet apparatus for 72 hours. Then, the solvent was filtered and evaporated by Rota vapor for producing an extract with purification of 16%. Afterwards, the obtained AE hydroalcoholic extract was kept in -18°C until consumption.

B. STZ administration and detection of hyperglycemia

The rats received a single intra-peritoneal injection of STZ (Sigma-Aldrich®, Steinhelm, Germany) by a dosage of 60 mg/kg of the body weight after an overnight fasting. The dosage was used according to a pilot study in which this dose could bring the fasting blood glucose (FBS) to levels more than 300 mg/dL in 8 out of 10 rats.

After STZ injection, FBS was monitored daily by Glucometer (Rightest GM110, Bionime™; Switzerland). FBS higher than 300 mg/dL was considered as hyperglycemia (diabetes).

C. Animals and the experimental design

Forty-eight male mature normoglycemic rats Sprague-Dawley, which were separately housed in

cages (one rat per cage), weighing 220 ± 20 g were randomly divided into 4 groups ($n=12$). Standard conditions were prepared for them which were defined as free access to food and water as well as 12 hours dark/light cycles in rooms with a controlled temperature of $24 \pm 2^\circ\text{C}$. Furthermore, the study procedures ethical considerations were also approved according to the ethical considerations of research authorities of Shiraz University of Medical Sciences (Laboratory Animal Ethics Committee).

The first group was labeled as non-diabetic control (C1) and only received 1cc-distilled water orally every day from the first day with no other treatment. The second one named STZ-induced diabetics with no treatment control group (C2), which received distilled water from day one orally as well as 60 mg/Kg of body weight in 0.15 M NaCl with 100 mM sodium citrate buffer, pH 4.5 by a single intraperitoneal injection of streptozotocin on day 7. Another group, the first experimental group (E1), received 100 mg/kg body weight AE extract solved in distilled water (with a total volume of 1 mL) daily by gavage from the first day for 14 days besides a single dose of STZ on day 7. The second experimental group (E2) received 300 mg/kg AE extract solved in distilled water (1 mL) since the first day for 14 days as well as a single dose of STZ on day 7. Doses were chosen referring to a pilot study in which AE administration showed the best potential for reducing the blood glucose levels in STZ-induced diabetic rats.

D. The stereological study

At the end of the study, before sacrificing the rats, the body weights were measured to see if there would be any difference between studied groups. On day 14, rats were sacrificed by cervical dislocation under light ether anesthesia and their pancreas were extracted and cleaned of fatty and connective tissues by an expert in stereology research laboratory of Shiraz University of Medical Sciences, Shiraz, Iran. The weight of the pancreas was measured. Additionally, the primary volume of the pancreas (V_{primary}) was, also, estimated by immersion method (21). Then, they were kept in neutral buffered 4% formaldehyde solution (supplier methanol, pH 6.9) for 3 days. The shrinkage had to be determined since some tissue deformation may take place after fixation, tissue processing, and also staining, which will affect the stereological evaluations. The estimated shrinkage was also occupied for measurement of the final pancreas volume after tissue processing (22). Tissue shrinkage (dSh) was estimated

by using a previously used method by Noorafshan *et al.* The degree of shrinkage $d(sh)$ was calculated using the following formula: $d(sh) = 1 - (AA / AB) \times 1.5$ [AA and AB are the area of each circular piece of the pancreas after and before processing, sectioning, and staining, respectively (22)]. For assessing the shrinkage, limited numbers (about 10-13) of isotropic uniform random (IUR) slabs were prepared from each pancreas sample in the same block. Primarily, the pancreas tissue was cut in IUR directions; after punching a circle out of the tissue, all the pancreatic pieces and the circle were embedded in a paraffin cube. One section with 5 μm thickness as well as one 20 μm thickness slab were obtained from each sample and stained by Gomori's aldehyde fuchsin for microscopic study (22, 23).

D1. Measuring the volume density of the pancreatic islets

In order to estimate the volume density (V_v) of the pancreatic islets, point-counting method was used on 5 μm -thickened slabs ($V_v = P(\text{islet}) / P(\text{reference})$; $P(\text{islet})$ and $P(\text{reference})$ were the number of the test points falling on the islet's profile and on the reference space, respectively). In this method, each prepared slide was analyzed by a video microscopy system made up of a microscope (E-200, Nikon, Tokyo, Japan) connected with a video camera, a computer, a flat monitor and a microcator (MT-12, Heidenhain, Traunreut, Germany). The software used for stereological studies is designed in Stereological Research Laboratory in Shiraz University of Medical Sciences, Shiraz, Iran. The stereological probes containing test points and unbiased counting frame were positioned on tissue image appearing on monitor screen (22). For conducting point-counting method, microscopic field of each slide was sampled in a systematic random manner and analyzed at a final magnification of 340 $\times$. The sampling started from the corner of the slide to outside of the tissue. Afterwards, the slide was moved at an equal distance in X and Y-axis directions. The average number of points hitting the pancreas and the islet were 260-300 and 2-4, respectively. The absolute volume (V) of the islets were estimated by using the following formula: $[V(\text{islets}) = V_v \times V(\text{primary}) \times (1 - d(sh))]$ ($V(\text{islets})$ = the absolute volume of islets, V_v = volume density of islets, $d(sh)$ = tissue shrinkage) (24).

D2. Numerical density and total number of Beta cells

On 20 μm slides, systematic random fields were prepared and by the "optical dissector" method at final magnification of 3400 $\times$ the numerical density (N_v) of the beta cells was estimated ($NV = (\Sigma Q / (h$

$\times a/f \times \Sigma p)) \times (t / BA)$; a/f is the area of the counting frame (here 184 μm^2), h is the height of the optical dissector (here 10 μm), ΣQ is the number of beta cells counted in all the dissectors, Σp is the total number of the counted frames, BA is the microtome block advance to cut the block (here it was 20 μm), and t is the mean of the final section thickness (17.4 μm on the average). In addition, t was measured using a microcator on a random field of each islet and mean value was calculated). In this method, the islands in pancreas were identified, randomly, at low magnification. The entire island was, then, evaluated at high magnification. In each field, the first 5 μm section of thickness was ignored to avoid bias counting. Cell counting was initiated from the next 10 μm portion of the slide, which is called "the height of dissector" or "h". The cells with nuclei in following optical scan of dissector height and the ones whose nuclei are partially or completely inside the counting frame were counted (25). For estimation of the total number ($N(\text{beta})$) of the beta cells the following formula was used: $N(\text{beta}) = N_v \times V(\text{islets})$.

E. Statistical analysis

The estimated volumes and counts were entered to SPSS (version 15.0). Descriptive analysis was used for calculating the mean and standard deviation (SD) of gathered data. One-way ANOVA test plus Tukey's post-hoc test were used for data analysis and $P\text{-value} \leq 0.05$ was considered as statistically significant.

RESULTS

According to our results, the rats' body weight, their pancreas weight as well as the primary and final volume of the pancreas showed no significant difference between 4 groups (Table 1).

Comparing the volume density and the total volume of islets, E1 and E2 were significantly different from C1 with $p\text{-value} < 0.001$; however, C2 did not differ noticeably in contrast with the experimental groups regarding these two parameters.

Furthermore, the numerical density and the total number of beta cells in E1 and E2 groups were significantly different in contrast with the C2 group giving a $p\text{-value} < 0.001$; moreover, although these two parameters showed higher rates in C1 group, the difference between the experimental groups and the normal rats was not considerable.

Levels of blood glucose were also shown to have a significant rise in C2 group in contrast with

Table 1. Effects of *Arnebia euchroma* (AE) on diabetic rats. Weights of the rats (g) and their pancreas (mg), primary (before making blocks) and final volume of the pancreas (mm³) and the shrinkage ratio (%), volume density (Vv; %) and total volume of the islets (mm³), numerical density (Nv; %) and total number of beta cells (×10⁶). The variables measured in each experimental group (C1 as non-diabetics, C2 as no-treatment diabetics, E1 as diabetics with 100mg/kg AE, and E2 as diabetic rats with 300 mg/kg AE) are shown as mean± SD (n=12)

Groups	Weight		Pancreas volume			Islets		Beta cells		Blood glucose
	Body	Pancreas	Primary	Final	Shrink	Vv	Total volume	Nv	Total number	
C1	213.3 ±11.6	864.3 ±90.3	818.6±90.8	702.8 ±36.4	17.1 ±0.5	0.96 ±0.91*	6.1 ±0.6*	947.7 ±75.5*	5.5± 0.9*	170.6 ±27.3*
C2	211.3 ±28.6	902.5 ±170.6	870.1±2.1	675.7 ±120.4	22.5 ±0.9	0.39 ±0.73	2.1 ±0.5	127.8 ±84.6	2.5± 1.3	718.7 ±8.3
E1	174.5 ±9.4	858.6 ±20.4	812.8±19.8	601.4 ±45.1	23.1 ±0.7	0.66 ±0.14	3.9 ±1.6	568.3 ±44.6*	4.6± 0.5*	638.7 ±71.4*
E2	182.1 ±11.2	846.3 ±28.8	802.5±27.1	626.9 ±60.6	21.8 ±0.8	0.58 ±0.13	3.6 ±0.9	450.9 ±101.3*	4.2± 0.7*	595.9 ±79.6*

* P≤0.05 vs. C2 group.

the C1 group (P=0.009); however, E1 and E2 groups although had increase in levels of blood glucose, which was noticeable compared to the normal rats (C1), their increase was not higher than that of the untreated C2 group (P=0.006).

DISCUSSION

During the past few decades, diabetes mellitus has become a certain medical concern in both management of the disease and its complications as well as its prevention and additional treatments. A major process playing the role in pathophysiology of diabetes is destruction and loss of beta cells of the pancreas caused by infections, toxins, drugs or diseases. For instance, in a survey, Larry *et al.* detected that impaired circadian rhythm can accelerate DM type 2 development through loss of function and mass of the beta cells (26). Moreover, Talchai *et al.* reached to this point that defect in differentiation of beta cells may contribute to their pathologic effect in diabetes (27).

Several studies were conducted in order to achieve a more effective treatment method for this disorder. Many of them were recently focused on natural products because of the increasing tendency of the population in many regions in these types of medications as well as recent literature on their undeniable efficacy besides their fewer complications and side effects. For example, Tyagi *et al.* declared that aqueous root extract of *Anacyclus pyrethrum* can reduce elevated glucose level in Alloxan induced diabetic rats (14). Another study conducted by Hasan *et al.* manifested that ethanol extract of the herb, *Swietenia mahagoni*, could reduce the levels of blood glucose in diabetic rats (28). Moreover, Manik *et al.*

concluded that Allopolyherbal formulation (APHF) which consists of three popular herbal medications (*Trigonella foenum graceum*, *Momordica charantia*, *Aegle marmelos*) can be more effective than anti-hyperglycemic agents used for DM; furthermore, it can also minimize the hyperlipidemia states as well as cardiovascular risk factors of this endocrine disorder (29). Another survey conducted on Alloxan induced diabetic rats revealed that *Pterocarpus marsupium* heartwood, which is extracted by ultrasound-assisted extraction (UAE) methods, could have anti-diabetic effects (30).

In this study, we introduced AE as a potent agent, which can be used in the treatment of diabetes.

Several studies can be found in over-viewing the effects of this herbal agent. A number of them were focused on its anti-inflammatory as well as anti-microbial and antioxidant effects (17-19, 31). Nonetheless, there was no previous study on protective effects of AE on diabetes and specifically pancreatic beta cells; however, according to our previous experiments it was assumed that AE might be a helpful agent in this matter.

In our study, it can be seen that comparing the two groups which were treated with AE 100 mg/kg (E1) and 300 mg/kg (E2) with normal rats (C1) showed significant differences in volume densities and total volume of the pancreatic islets; volume densities and total numbers of the beta cells in AE-treated rats also showed considerable difference in contrast with the untreated diabetic ones. The blood glucose of the rats was also noticeably lower in the AE-treated group. However, these amounts were still not as normal as the ones in C1 group. Since the protective effects of AE were considered for evaluation in this study, we

started administrating it 1 week before induction of the diabetes. However, the lifespan of the rats after diabetes induction was a limitation for following them more than one week after STZ-induced diabetes because the mortality rate of the animals was very high after one week. Another limitation of this study was measurement of the blood glucose levels on which the amount of stress on the rats during the blood-taking procedures as well as resistance to STZ in genetically obese rats might have some impact. Nevertheless, we tried to lower the effect of these factors as much as possible by giving the blood sample taking to experts in animal laboratory of Shiraz University of Medical Sciences, Shiraz, Iran, to lower the time of sampling and by measuring the body weight of rats at the beginning of the study and normally distribute them in four groups.

In conclusion, overall, from this survey, it was achieved that *Arnebia euchroma* (AE) can be beneficial for DM by protecting the pancreatic beta cells from being destructed, here by STZ as a cytotoxic agent. Moreover, AE also can reduce blood glucose levels to some extent. However, more studies are needed, especially clinical trials, in order to evaluate AE's benefits on human body and introduce it as a supplementary therapy for patients at risk of DM specifically in those by beta cell malfunction or reduction.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

This study was financially supported by Shiraz University of Medical Sciences, Shiraz, Iran and registered as the dissertation of Medical Science Courses by Elmira Esmailzadeh (Code: 93-01-21-7818). Hereby, the authors would like to thank Dr. M. Vahedi, Mr. Sarayi, and all the personnel of Stem Cell and Transgenic Technology Research Center, also Ms. Keshavarzi from the Histomorphometry and Stereology Research Center of Shiraz University of Medical Sciences, Shiraz, Iran.

References

- Whiting DR, Guariguata L, Weil C, Shaw J. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes Res Clin Pract* 2011;94(3):311-321.
- Danaei G, Finucane MM, Lu Y, Singh GM, Cowan MJ, Paciorek CJ, Lin JK, Farzadfar F, Khang YH, Stevens GA, Rao M, Ali MK, Riley LM, Robinson CA, Ezzati M; Global Burden of Metabolic Risk Factors of Chronic Diseases Collaborating Group (Blood Glucose). National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *Lancet* 2011;378(9785):31-40.
- Association tAD. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 2012;35(1):64-71.
- Godfrey KJ, Mathew B, Bulman JC, Shah O, Clement S, Gallicano GI. Stem cell-based treatments for Type 1 diabetes mellitus: bone marrow, embryonic, hepatic, pancreatic and induced pluripotent stem cells. *Diabet Med* 2012;29(1):14-23.
- Garland PB, Newsholme EA, Randle PJ. Regulation of glucose uptake by muscle. 9. Effects of fatty acids and ketone bodies, and of alloxan-diabetes and starvation, on pyruvate metabolism and on lactate-pyruvate and L-glycerol 3-phosphate-dihydroxyacetone phosphate concentration ratios in rat heart and rat diaphragm muscles. *Biochem J* 1964;93(3):665-678.
- Das SL, Singh PP, Phillips AR, Murphy R, Windsor JA, Petrov MS. Newly diagnosed diabetes mellitus after acute pancreatitis: a systematic review and meta-analysis. *Gut* 2014;63(5):818-831.
- B.A. H, Martinez C. Experimental Diabetes and Diet. *Science* 1947;105(2734):548-549.
- Graham ML, Janeczek JL, Kittredge JA, Hering BJ, Schuurman HJ. The streptozotocin-induced diabetic nude mouse model: differences between animals from different sources. *Comp Med* 2011;61(4):356-360.
- Deeds MC, Anderson JM, Armstrong AS, Gastineau DA, Hiddinga HJ, Jahangir A, Eberhardt NL, Kudva YC. Single dose streptozotocin-induced diabetes: considerations for study design in islet transplantation models. *Lab Anim* 2011;45(3):131-140.
- Akkoc H, Kelle I, Erdinc M, Nergiz Y. Effects of Ethyl Pyruvate on Testicular Damage in Rats with Streptozotocin-Induced Diabetes. *Acta Endocrinol-Buc* 2012;8(1):35-45.
- Darwish I, Ismail C, Geumei A, Abdelbary A. Role of Targeting Asymmetric Dimethylarginine in Streptozotocin-Induced Diabetic Nephropathy in Rats: Effects of Taurine and Rosiglitazone. *Acta Endocrinol-Buc* 2015;11(4):449-456.
- Lu YL, Ye L, Wu H, Xia FZ, Yu J, Yang LZ. The Up-Regulated Expression of Both Phospholipase A2 and Cyclooxygenase-2 Is Involved in Renal Injury in Streptozotocin-Induced Diabetic Rats. *Acta Endocrinol-Buc* 2013;9(1):23-32.
- Parmer K, Prajapati A, Patel M, Patel B. Cardioprotective Effects of Lisinopril in Streptozotocin Induced Type-II Diabetic Rats. *Acta Endocrinol-Buc* 2012;8(2):177-188.
- Tyagi S, Mansoori MH, Kumar Singh N, Kumar Shivhare M, Bhardwaj P, Kumar Singh R. Antidiabetic Effect of Anacyclus pyrethrum DC in Alloxan Induced Diabetic Rats. *Eur J Biol Res* 2011;3(4):117-120.
- Liu T, Ma C, Yang L, Wang W, Sui X, Zhao C, Zu Y. Optimization of shikonin homogenate extraction from *Arnebia euchroma* using response surface methodology. *Molecules* 2013;18(1):466-481.
- Nasiri E, Hosseini-mehr SJ, Azadbakht M, Akbari J, Enayati-Fard R, Azizi S, Azadbakht M. The Healing Effect of *Arnebia Euchroma* Ointment versus Silver Sulfadiazine on Burn Wounds in Rat. *World J Plast Surg* 2015;4(2):134-144.
- Fan HY, Zhang ZL, Liu K, Yang MY, Lv WH, Che X, Xu H, Song WW. Effectiveness of a hydroxynaphthoquinone fraction from *Arnebia euchroma* in rats with experimental colitis. *World J Gastroenterol* 2013;19(48):9318-9327.
- Fan H, Yang M, Che X, Zhang Z, Xu H, Liu K, Meng Q. Activity study of a hydroxynaphthoquinone fraction from *Arnebia euchroma* in experimental arthritis. *Fitoterapia* 2012;83(7):1226-1237.
- Li HM, Tang YL, Zhang ZH, Liu CJ, Li HZ, Li RT, Xia XS. Compounds from *Arnebia euchroma* and their related anti-HCV and antibacterial activities. *Planta medica* 2012;78(1):39-45.
- Chang MJ, Huang GJ, Ho YL, Lin IH, Huang SS, Chang TN. Study on the antioxidant activities of crude extracts from the roots of *Arnebia euchroma* and *Lithospermum erythrorhizon*. *Extraction* 2008;7(6.58):10-56.

21. Scherle W. A simple method for volumetry of organs in quantitative stereology. *Mikroskopie* 1970;26(1):57-60.
22. Noorafshan A, Hoseini L, Karbalay-Doust S, Nadimi E. A simple stereological method for estimating the number and the volume of the pancreatic beta cells. *JOP* 2012;13(4):427-432.
23. Bangle R, Jr. Factors influencing the staining of beta-cell granules in pancreatic islets with various basic dyes, including paraldehyde-fuchsin. *Am J Pathol* 1956;32(2):349-362.
24. Bock T, Svenstrup K, Pakkenberg B, Buschard K. Unbiased estimation of total beta-cell number and mean beta-cell volume in rodent pancreas. *APMIS* 1999;107(8):791-799.
25. Gundersen HJ, Bagger P, Bendtsen TF, Evans SM, Korbo L, Marcussen N, Møller A, Nielsen K, Nyengaard JR, Pakkenberg B. The new stereological tools: disector, fractionator, nucleator and point sampled intercepts and their use in pathological research and diagnosis. *APMIS* 1988;96(10):857-881.
26. Gale JE, Cox HI, Qian J, Block GD, Colwell CS, Matveyenko AV. Disruption of circadian rhythms accelerates development of diabetes through pancreatic beta-cell loss and dysfunction. *J Biol Rhythms* 2011;26(5):423-433.
27. Talchai C, Xuan S, Lin HV, Sussel L, Accili D. Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. *Cell* 2012;150(6):1223-1234.
28. Hasan SM, Khan MI, Umar BU, Sadeque MZ. Comparative study of the effect of ethanolic extract of *Swietenia mahagoni* seeds with rosiglitazone on experimentally induced diabetes mellitus in rats. *Bangladesh Med Res Counc Bull* 2013;39(1):6-10.
29. Manik S, Gauttam V, Kalia AN. Anti-diabetic and antihyperlipidemic effect of allopolyherbal formulation in OGTT and STZ-induced diabetic rat model. *Indian J Exp Biol* 2013;51(9):702-708.
30. Devgan M, Nanda A, Ansari SH. Comparative evaluation of the anti-diabetic activity of *Pterocarpus marsupium* Roxb. heartwood in alloxan induced diabetic rats using extracts obtained by optimized conventional and non conventional extraction methods. *Pak J Pharm Sci* 2013;26(5):973-976.
31. Singh LK, Maheshwari DK, Shukla S. Antibacterial effect of butyryl alkannin from *Arnebia euchroma* against vancomycin-resistant pathogens of *Enterococcus faecalis* causing urinary tract infections. *Nat Prod Res* 2015;29(24):2299-2301.