Modulation of hyperglycemia and oxidative stress by Ezetimibe in Sreptozotocin induce diabetes mellitus rats

Atheer Majid Rashid Al-Juhaishi

Department of Clinical Pharmacy, Collage of pharmacy/Karbala University.

Keyword: Ezetimibe, Hyperglycemia, Oxidative stress, DM, STZ.

(Received :March 2014, Accept : Jun 2014)

Abstract,

Diabetes mellitus (DM) is metabolic diseases characterized by chronic hyperglycemia due to reducing in insulin secretion, insulin function, or both. Ezetimibe is a drug that lowers plasma cholesterol levels. A total of 18 male adult albino rats were used in this study. The animals randomized into 3 groups (of 6 rats each). Rats in first group were injected with citrate buffer only and used as healthy control group. While the rats in other two groups were injected with streptozotocin (STZ) at a dose of 60 mg/kg I.P. and treated as following (for 12 weeks), diabetic control group rats received no treatment. Ezetimibe treated group rats received Ezetimibe 6 mg/kg orally once daily. Every 2 weeks, blood glucose level is measured. At the end of 12th weeks, blood samples were collected to measure the blood glucose level and superoxide dismutase activity, and then the animals were sacrificed. The pancreas was removed for histopathology assessment for the degree of islets damage. In result, Ezetimibe was significantly (P<0.05) lower blood glucose levels in compare with the diabetic controls and the activity of SOD was significantly (P<0.05) elevated in rats treated with Ezetimibe compared with the levels in the healthy and diabetic control rats. Histological studies of the pancreas of diabetic control group revealed moderate pancreas islets damage (atrophy of β-cells and cytoplasm rich by inflammatory cells) in compared to rats received Ezetimibe which have showed normal pancreas appearance to mild pancreas islets damage. In conclusions, the present results suggest that Ezetimibe exhibit hypoglycemic and antioxidant activity in diabetic rats.

تقليل ارتفاع السكر و الاجهاد التأكسدي بواسطة الازتيماب في مرض السكري المستحث بواسطة الستريبتوزوتوسين في الجرذان

أثير ماجد رشيد

جامعة كربلاء – كلية الصيدلة – فرع الصيدلة السريرية

مفتاح الكلمات: ازيتماب، ارتفاع السكر، مؤشر الاكسدة، داء السكري، ستريبتوزوتوسين.

الخلاصة

داء السكري هو مرض ايضي يمتاز بارتفاع سكر الدم المزمن بسبب نقصان افراز الانسولين، او وظيفة الانسولين، او كلاهما. ازيتماب هو دواء يستخدم لخفض مستوى الكولسترول في الدم. تم استخدام 18 ذكر من الجرذان البيضاء في هذه الدراسة. هذه الحيوانات قسمت بشكل عشوائي إلى 3 مجموعات (6 في كل مجموعة) الحيوانات في المجموعة الأولى حقنت بالستريت بفر واعتبرت المجموعة الصحية في حين حقنت بقية الحيوانات في المجموعتين الاخرى بعقار الستريبتوزوتوسين (60 ملغم لكل كغم تحت البطن) وعولجت على الشكل التالي, مجموعة السيطرة التي لم تعالج والمجموعة الثانية عولجة بازيتماب (6 ملغم لكل كغم عن طريق الفم) مرة واحدة يوميا. من ثم مستوى سكر الدم يقاس كل اسبوعين. وبعد انتهاء الأسبوع الثاني عشر من الدراسة، أخذت عينات

الدم لقياس مستوى سكر الدم ومؤشر الأكسدة المتمثل (سوبر اوكساید داسمتیز انزایم)، من بعد ذلك، الجرذان تقتل وتستخرج البنكرياس لفحص النسيجي. أظهرت النتائج قابلية الازيتماب لخفض مستوى سكر الدم معنويا ($P<0.05$) بالمقارنة مع مجموعة السيطرة وزيادة ملحوظة ($P<0.05$) في فعالية انزايم مضاد الاكسدة في الجرذان التي تعالجت بالازيتماب بالمقارنة مع مجموعتين السيطرة والصحية. الدراسة النسيجية لبنكرياس في مجموعة السيطرة اشارة الى تالف متوسط المستوى في جزر لنكرهانس متمثل بأضمحلال خلايا بيتا وتجمع الخلايا الالتهابية بالمقارنة مع مجموعة الازيتماب التي اظهرة شكلاً طبيعي الى تلف بسيط. أظهرت الدراسة ان الازيتماب ميتلك قابلية على خفض مستوى السكر في الدم ومضاد أكسدة في جرذان داء السكري.

Introduction

Diabetes mellitus (DM) is metabolic diseases characterized by chronic hyperglycemia due to reducing in insulin secretion, insulin function, or both. This is lead to disturbance in metabolizing of carbohydrate, fat, and protein which related to deficiency of insulin effect on peripheral tissue. Insulin is a hormone synthesized by the beta cells of the Langerhans in pancreas, its function to utilize glucose from digested food as an energy source. The DM is classified according to patients' dependence on insulin into insulin-dependent DM which characterized by absolute insulin deficiency due to autoimmune mediate disease process and non-insulin-dependent DM which may be due to peripheral insulin resistance, defected insulin secretion or both. The complications of DM are microvascular and macrovascular including visual impairment, blindness, kidney disease, nerve damage, amputations, heart disease, and stroke [(1)]. The imbalance between the production of reactive oxygen species (ROS) like superoxide [O2.-], hydrogen peroxide [H2O2], and hydroxyl radical [OH.-] and antioxidative defense mechanisms such as superoxide dismutase, catalase, and glutathione peroxidase result in state of oxidative stress. Normally, ROS is continuously producing and in the same time, it is effectively elimination by antioxidant system. When the production of ROS is more than capacity of antioxidant defense system, result in alteration of essential biologic cellular proteins or DNA and cellular damage [(2)]. In DM, ROS production may be elevated via difference mechanisms, including increased flux though the polyol pathway [(3)], increased formation of advanced glycation end-product (AGEs) [(4)], mitochondrial superoxide overproduction [(5)], and activation of protein kinase-C PKC [(6)]. Ezetimibe is a drug that lowers plasma cholesterol levels. It acts by decreasing cholesterol absorption in the intestine. It may be used alone or together with statins, when statins alone do not control cholesterol [(7)]. The aim of the study is to assess the hypoglycemic and antioxidant effects of Ezetimibe.

Material and Methods

Animals

A total of 18 adult male albino Swiss rats were used in this study weighed 200-300 gm. They were obtained from Animal Resource Center, Al-Kafue University. The animals were apparently healthy and they were housed in individual cages, at temperature controlled environment (25±2°C) with ambient humidity. Lights were maintained on a 12-h light/dark cycle. The rats received standard chow diet with water.

Reagents and drugs

Streptozotocin (Sigma, USA), was dissolved in 1M citrate buffer (pH 4.5) [8]. Ezetimibe (Roche, Germany) 10 mg dissolving in 33 ml of corn oil [9], the reagents use in measurement of Superoxide Dismutase Activity including EDTA (Fluka, Switzerland), K2HPO4 (BDH, UK), KH2PO4 (BDH, UK), L-methionine (BDH, UK), Nitro Blue Tetrazolium chloride (Sigma-Aldrich, USA), Riboflavin (BDH, UK), Sodium cyanide (BDH, UK), Triton-X100 (Sigma-Aldrich, USA).

Animal model of diabetic mellitus

Diabetes mellitus was induced in the overnight fasted rats by a single intra peritoneal injection of STZ at a dose of 60 mg/ kg body weight [10] and freshly prepared before injection. One week after STZ treatment, glucose level measuring by using glucometer. Male rats with random blood glucose concentration more than 300 mg/ dl were considered diabetic and used for study [8].

Experimental Design

After one week acclimatization period, twelve diabetic rats fulfilling induced diabetes criteria were selected 6 rats were categorized into diabetic control and the rest of rats were placed in drug administered diabetic groups. Other 6 normoglycemic rats were considered as control group. Treatment of Ezetimibe started from the 7th day of injection period of streptozocin (STZ) and will be considered as 1st day of experiment. The treatment will continue for next 12 weeks.

Animal groups

A. Healthy control (non-diabetic) group, this group received single I.P. injection of citrate buffer only (same volume) at the time of STZ injection to the other animals for Diabetic induction.
B. The STZ-induced Diabetic rats, divided into,

1. Group I, Diabetic control received only corn oil by oral gavage for 12 weeks.
2. Group II, Diabetic treated with Ezetimibe in a dose of 6mg/kg daily by oral gavage for 12 weeks.

Blood Sample Collection

After anesthezing the animal with chloroform, (3-4ml) of blood was collected by intracardiac puncture and was put in test tubes (without anticoagulant) utmost care should be taken to avoid hemolysis, it was centrifuged at 3000 RPM for 15 minutes. The separated serum was taken by the aid of pasture pipette then transferred to another plastic test tube (1-2ml) of serum to be ready for biochemical examination.

Measurement of Blood Glucose Level

Blood glucose level (BGL) was monitored every 15 days (during treatment period) using a Glucometer and blood samples collected by tail tipping method.

Measurement of Superoxide Dismutase Activity

Superoxide dismutase activity is measured according to the method **Beyer Jr, W.F.; *et al.*, 1987**. Absorbance (R_1) of final solutions was measure immediately at 560nm using Spectrophotometer. All test tubes place under sunlight for 10 minutes then measure the absorbance (R_2) again by the same method then calculate the difference between two values [11].

Superoxide Dismutase activity = $R_2 - R_1$

Removal of Pancreas

Animals were sacrificed by drawing utmost all blood from heart and the pancreas of each rat were separated from the surrounding viscera using Dissect set. The pancreas were removed and washed with physiological saline solution. Then the pancreas immersed separately in 10% formalin solution.

Histological evaluation of pancreas lesions

Specimens of the islet of pancreas from each rat were fixed in 10% buffered formalin and processed in a paraffin tissue processing machine. After embedding, sections of the pancreas were made at a thickness of 7 mm and stained with Hematoxylin and Eosin for histological evaluation [12]. Each section was examined under a microscope (Humason, Gretchen), and the degree of islets damage was scored using a method similar to that illustrated by **Visser, J.; *et al.*, 1993**. They are scaled from 1-4 scores [13].

Ethics

The study was approved by the ethics Committee for animal experimentation, collage of pharmacy, Karbala University, Iraq. Throughout the experiments, all animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health.

Statistical analyses

Statistical analyses were performed using SPSS 18.0 for windows. Inc. The data were expressed as means±SEM and analyzed statistically using one-way analysis of variance (ANOVA) followed by LSD post-hoc test and Chi-square for discrete data. P values less than 0.05 were considered significant.

Result

Effect of Ezetimibe on blood glucose level

After induction of diabetic, the level of fasting blood glucose of diabetic rats in the range 300 -310 mg/dl were choice for this study. There is no significant difference ($P>0.05$) between the blood glucose levels of two diabetic experimental groups and both of them showed a significant difference ($P<0.05$) in compare with negative control. However, after 2 weeks, the blood glucose levels of the treated rats were significantly lower ($P<0.05$) than the positive controls as show in (table 1). In contrast, the blood glucose level of the untreated diabetic rat remained elevated throughout the experimental period which was clearly explained in (Figure 1).

Effect of Ezetimibe on superoxide dismutase activity

The results showed that the activity of SOD was significantly difference (P<0.05) between Negative and Positive control rats as clearly view in (Figure 2) and the activity of SOD was significantly elevated in rats treated with Ezetimibe (P<0.05) compared with the levels in the Negative and Positive control rats (Table 2).

Histopathological finding

The severity of pancreas islets damage of the three experimental groups were assessed at the end of study and the results are as follows: a cross section of islets of Langerhans of pancreas of Negative control group showed numerous beta cells with abundant basophilic cytoplasm. All rats in this group showed normal pancreas appearance (100%) as shown in Table (3) and Figure (3). There was a statistically significant difference between Positive and Negative control group (P<0.05) and the total score mean of the Positive control showed moderate pancreas islets damage (atrophy of β-cells and cytoplasm rich by inflammatory cells); and the total score mean of the Positive control showed mild to moderate pancreas islets damages (22.2% of the group had moderate damage and 11.1% had mild damage) as shown in Table (3) and Figure (3). Treatment of rats with Ezetimibe ameliorated the pancreas islets damages significantly (P<0.05) as compared with Positive control group and the total score mean of this group showed normal pancreas appearance to mild pancreas islets damages (22.2% of the group had normal pancreas appearance and 11.1% of the group had mild damage) as shown in Table (3) and Figure (3).

Discuss

In this study, Ezetimibe showed a significant reduction (P<0.05) in fasting blood glucose level of diabetic rats in compare with positive control group. **Yang, S. J. *et al.*, 2011** & **Deushi, M. *et al.*, 2007** demonstrated that, in hyperlipidemic diabetes mice, treatment with Ezetimibe for long-term could increase the number of β cells and the content of cytoplasmic insulin [14]. **Yong, Z. *et al.*, (2012)** revealed that Ezetimibe can directly inhibit the cholesterol absorption, reduce the FFA, improve the insulin resistance, and reduce blood glucose [15].

In this study, Ezetimibe showed a significant antioxidant activity by elevated SOD activity in compare with both positive and negative control group. **Masaya, F. *et al.*, 2010** demonstrated that, ezetimibe also reduced vascular superoxide levels in db/db mice, accompanied by the attenuation of NADPH oxidase subunit gp91phox and Nox4 and the prevention of down-regulation of Cu/Zn-superoxide dismutase (SOD) and extracellular SOD [16]. **Kuhlencordt, P.J. *et al.*, 2009** demonstrated that Ezetimibe is significantly enhanced vascular superoxide dismutase activity in rats and decrease ROS level reflecting antioxidant activity of Ezetimibe [17]. This result controversial with result mention by **Pandya, N. *et al.*, 2006** who refer to a significant decrease in SOD and increase in CAT activity, along with insignificant decrease in MDA and GSH levels [18].

Table (1): Mean blood glucose level (mg/dl) of the three experimental groups during period of experiment (N=6 in each group).

Group	Blood glucose level (mean ± SD)						
	Before treated	**After 2 weeks**	**After 4 weeks**	**After 6 weeks**	**After 8 weeks**	**After 10 weeks**	**After 12 weeks**
Negative Control	**111±4.83**	**102±3.57**	**104±2.87**	**108±5.39**	**107±3.55**	**108±3.74**	**105±4.9**
Positive Control	**303± 9.42****	**312±9.8**	**323±7.95**	**330±8**	**346±6.21**	**359±3.25**	**373± 2.07**
Ezetimibe Treated	**301±6.65 ****	**285±7.06**	**255±5.74**	**232±5.97**	**214±8.68**	**190±3.81**	**179±2.73 ***

*: Significant vs. Positive control group and Negative Control group.

**: significant vs. Negative control group.

Table (2): The mean SOD concentration (mg/L) of rats the three experimental groups during period of experiment (N=6 in each group).

Groups	SOD activity (mean±SD)	*P* value
Negative Control	208.7±3.5	
Positive Control	289.6±9.4	< 0.05*
Ezetimibe Treated	407.7±11.9	<0.05**

*: Significant vs. Negative Control group.

**: Significant vs. Negative and Positive Control groups.

Table (2): The differences in scores of pancreas islets damages and frequency among three experimental groups (N=18) using Chi-sequre (*P*<0.05).

Pancreas Islets Damage Scores	Study groups			Total
	Negative Control	Positive Control	Ezetimibe Treated	
	No. (%)	No. (%)	No. (%)	No. (%)
1	6 (33.4%)	0 (0%)	4 (22.2%)	10(55.6%)
2	0 (0%)	2 (11.1%)	2 (11.1%)	4 (22.2%)
3	0 (0%)	4 (22.2%)	0 (0)	4 (22.2%)
4	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	6 (33.4%)	6 (33.3%)	6 (33.3%)	18 (100%)

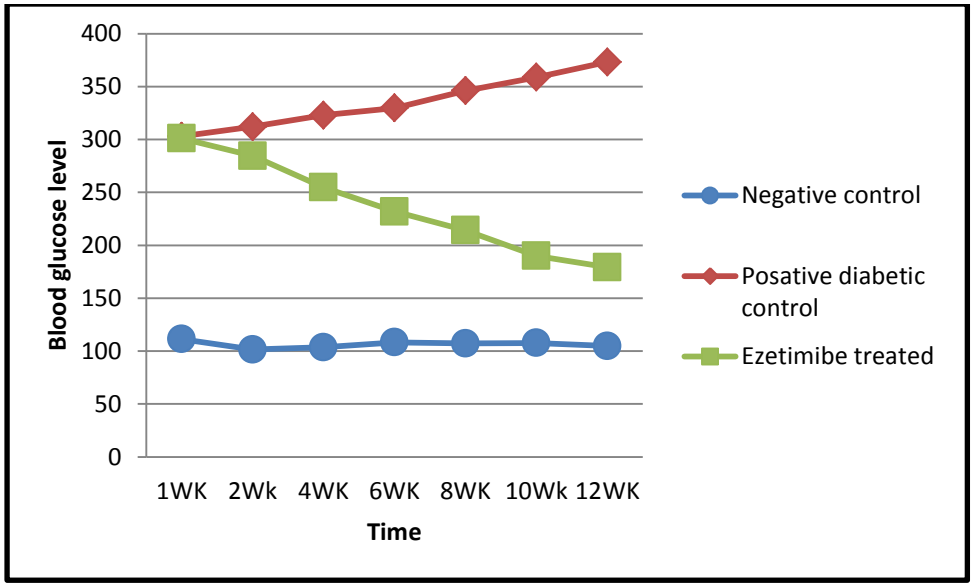

Figure (1): The mean blood glucose level (mg/dl) of rats in the three experimental groups during period of experiment.

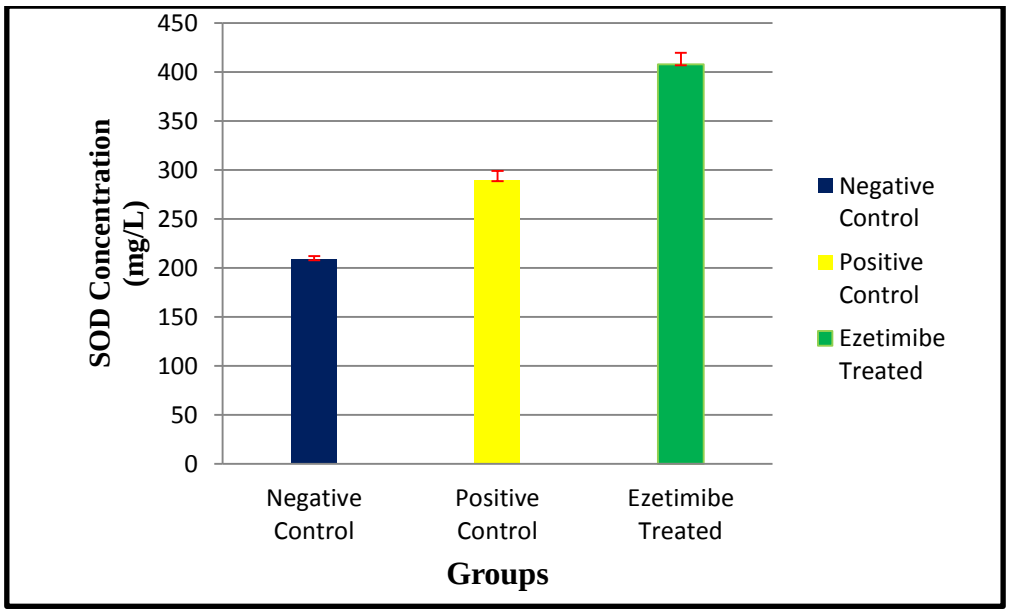

Figure (2): The mean SOD concentration (mg/L) of rats in the three experimental groups during period of experiment.

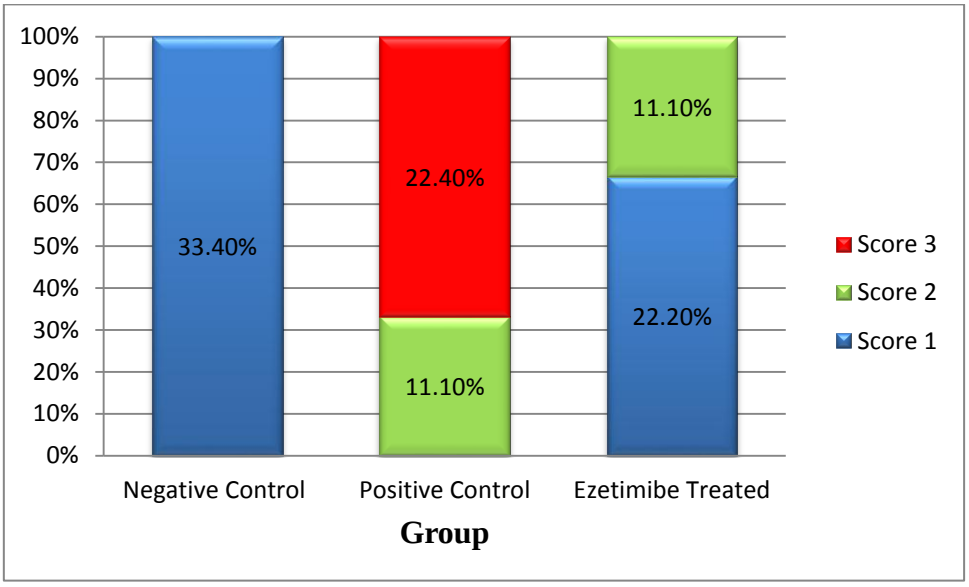

Figure (3): The relative frequency of pancreas islets damages scores and frequency among three experimental groups.

Reference

1. **Vats, v.; Yadav, S.P.; and Grover, J.K.,** *Ethanopharmacol*, **20**, 60-155, (2003).
2. **Cai, H.; and Harrison, D.G.,** *Circ. Res.* **87**,840-844, (2000).
3. **Dunlop, M.**, *Kid. Int. Suppl.* **77**, S3-12, (2000).
4. **Baynes, J.W.; and Thorpe, S.R.**, *J. Diab.* **48**(1), 1-9, (1999).
5. **Nishikawa, T.; Edelstein, D.; Du, X. L.; Yamaqishi, S.; Matsumura, T.; Kaneda, Y.; Yorek, M. A.; Beebe, D.; Oates, P. J.; Hammes, H. P.; Giardinol, I.; and Brownlee, M.,** *Nature*. **404** (6779), 787-90, (2000).
6. **Inoguchi, T.; Li, P.; Umeda, F.; Yu, H. Y.; Kakimoto, M.; Imamura, M.; Aoki, T.; Etoh, T.; Hashimoto, T.; Naruse, M.; Sano, H.; Utsumi, H.; and Nawata, H.,** *J. Diab.* **49** (11), 1939-45, (2000).
7. **Mitka, M.**, *JAMA.* **299** (19), 2266, (2008).
8. **Rafiq, K.; Sherajee, S. J.; Nishiyama, A.; Sufiun, M. A.; and Mostofa, M.**, *Afr. J, Pharm. and Pharmacol.* **3**(12), 636-642, (2009).
9. **Davis, H.R.; Pula, K.; Alto, K.B.; Burrier, R.E.; and Watkins, R.W.,** *Metabol*. **50**, 1234-41, (2001).
10. **Abo-Salem, O. M.; El-Edel, R. H.; Harisa, G. E.; El-Halawany, N.; and Ghonaim, M. M.,** *J. Pharm. Sci.* **22**(2), 205-210, (2009).
11. **Beyer Jr, W. F.; and Fridovich, I.,** *Analytical Biochemistry*. **161**, 559-566, (1987).
12. **Luna, L.G.,** Institute of Pathology. Landon, 1-31, (1996).
13. **Visser, J.; Klatter, F.; Vis, L.; Groen, H.; Strubbe, J.; and Rozing, J.,** *Metabolism, Clinical and Experimental* **53**,333–337, (2003).
14. **Yang, S. J.; Choi, J. M.; and Kim, L. et al.**, *Bioch. Biophys. Res. Commun.* **407**(1), 153–157, (2011).
15. **Yong, Z.; Jun, W.; Ping, G.; Jiaqing S.; Bin, L.; and Shisen, J.,** *Exp. Diabetes. Res.* **2012**, 1-6, (2012).
16. **Masaya, F.; Taishi, N.; Keiichiro, K.; Hisato, N.; Yoshiko, T.; Yi-Fei, D.; Osamu, Y.; Hisao, O.; and Shokei, K.,** *J. pharmco. Exp. Therap.* **335**(1), 70-75, (2010).
17. **Kuhlencordt, P.J.; Padmapriya, P.; Rutzel, S.; Schödel, J.; Hu, K.; Schafer, A.; Huang P.L.; Ertl, G.; and Bauersachs, J.,** *Atherosclerosis.* **202**(1), 48-57, (2009).
18. **Pandya, N.; Santani, D.; and Jain, S.,** *India. J. pharmaco.* **38**(3), 205-206, (2006).