Addendum

Based on the comments made by the 1st examiner, corrections / modifications are inserted in the addendum.

The thesis needs one page abstract which needs to be inserted in the beginning

Abstract

Diabetes mellitus is a disease involving metabolic disorders of carbohydrate, protein, and fat, associated with insulin deficiency and / or its action. Uncontrolled hyperglycemia, free radical and oxidative stress have been implicated as root causes in diabetes. The common market available synthetic antihyperglycemic drugs mostly develop side effects and / or drug resistance on prolong use. The current endeavor of the diabetes research is in search for appropriate drugs from herbal sources, which can alleviate the disease without causing any systemic toxicity. Steptozotocin (STZ)-induced diabetes in rats is an ideal in vivo model of diabetes.

In our study we have used bioflavonoid pelargonidin, the simplest anthocyanidin to find its antihyperglycemic potency in STZ-induced model diabetes. Previous in vitro studies from our laboratory indicated that the major glycated heme proteins, hemoglobin and myoglobin act as a source of free radicals and oxidative stress due to release of iron from heme pockets. Therefore, besides exploring the antidiabetic potential of pelargonidin the present study has been directed to understand the mechanism of oxidative stress with special emphasis on hemoglobin iron-mediated oxidative reactions in diabetic animal model. To encounter the problem of biodegradation in therapeutic approach of pelargonidin in diabetes as well as to enhance its efficacy, the present study has also used two modern drug delivery approaches, namely liposome encapsulated and PLGA-nanoparticle encapsulated pelargonidin therapy in STZ-induced diabetes rats. In vitro interactions of pelargonidin with RBC and hemoglobin have been done to get further insight into the action of the flavonoid.

The findings of the study demonstrated that pelargonidin is an effective antidiabetic agent and alleviates diabetic complications. Glycated hemoglobin is a source of iron and oxidative stress in diabetes. Pelargonidin counteracts hemoglobin-induced iron-mediated
oxidative stress reactions by lowering the glycation level (via enhanced insulin secretion) and free iron in hemoglobin and normalizing iron deposition in liver tissue. Pelargonidin treatment exerts hypolipidemic and antioxidative functions. Oxidative DNA damages encountered in diabetes are counteracted by this drug treatment. Compared to free pelargonidin, liposome and PLGA-nanoparticle encoded pelargonidin exhibit better therapeutic efficacy in STZ-induced diabetes. Pelargonidin interacts with RBC and hemoglobin. Interaction with RBC results in cell creation and lowering of membrane osmotic fluidity. Hemoglobin-pelargonidin interaction causes alteration of the secondary structure of the protein.

The thesis needs thorough revision with respect to language, grammatical and typological error.

Spelling mistakes and typing errors and grammatical mistakes:

- page 4 line 23 "adipokines" changed to adipokine.
- page 9 line 2 "Cortiosteroid" changed to Corticosteroid.
- page 9 line 18 "enviromental" changed to environmental.
- page 10 line 8 "artherosclerotic" changed to atherosclerotic.
- page 10 line 17 "complications" changed to complication.
- page 14 line 8 “use” changed to use.
- page 14 line 19 “kinace” changed to kinase.
- page 14 line 29 “increases” changed to increase.
- page 25 line 2 “multifaced” changed to multifaceted.
- Page 26 line 1 “Photochemical” changed to phytochemical.
- page 30 line 17 “ligands” changed to ligand.
- page 35 line 25 “consistant” changed to consistent.
- Page 36i line 10 “side effects and / or side drug resistance” changed to side effects and / or drug resistance.
- page 45 line 10 “colloidial” changed to colloidal.
- page 52 line 25 “dame” changed to damage.
- page 53 line 19 “acohol” changed to alcohol.
In page 54 after line 31 “after different concentration of NaCl. RBC osmotic fragility was determined by the method” following insertion is made “described by Harmening, D.M. (1997). 50 μl of RBC from control and pelargonidin-treated RBC was added to different test tubes containing different NaCl solutions (0.01

page 66 line 1 “diabetic” changed to diabetes.

page 87 line 6 “diferent” changed to different.

page 90 line 26 “cleavege” changed to cleavage.

P-1 there is large table showing timelines in the history of this disease. There is no table number. The table is not referred to in the text and imply inserted.

In page 1 after line 6 the following insertion is made.
The milestones in the history of diabetes research have been tabulated below.

Table 1: The milestones in diabetes research history.

And in page 34 Table 1 is numbered as Table 1A.

Some of the terminology used e.g. “diabeticity” (although used by some) are not well accepted. The author may consider using alternate terms.

The “diabeticity” term is changed to “diabetic condition” in page 94 line 6 and page 149 line 5.

Several figures (e.g. fig 2,3,4 and others) were taken form (adapted form) published papers by other authors without any acknowledgement.

Legends are modified with incorporation

Figure 2: Major diabetic complications. [Reproduced from wikipedia] (page 12).

Figure 3: Aldose reductase and the polyol pathway. Aldose reductase reduces aldehydes generated by reactive oxygen species (ROS) to inactive alcohols, and glucose to sorbitol, using NADPH as a co-factor. In cells where aldose reductase activity is sufficient to deplete reduced glutathione (GSH), oxidative stress is augmented. Sorbitol dehydrogenase (SDH) oxidizes sorbitol to fructose using NAD+ as a co-factor.

Figure 4: Consequences of hyperglycaemia-induced activation of protein kinase C (PKC). Hyperglycaemia increases diacylglycerol (DAG) content, which activates PKC, primarily the β- and δ-isofoms. Activation of PKC has a number of pathogenic consequences by affecting expression of endothelial nitric oxide synthetase (eNOS), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and plasminogen activator inhibitor-1 (PAI-1), and by activating NF-κB and NAD(P)H oxidases [Adapted from M Brownlee. Nature 414 : 813-820, 2001] (page 15).

Figure 5: The hexosamine pathway. The glycolytic intermediate fructose-6-phosphate (Fruc-6-P) is converted to glucosamine-6-phosphate by the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT). Intracellular glycosylation by the addition of N-acetylglucosamine (GlcNAc) to serine and threonine is catalysed by the enzyme O-GlcNAc transferase (OGT). Increased donation of GlcNAc moieties to serine and threonine residues of transcription factors such as Sp1, often at phosphorylation sites, increases the production of factors as PAI-1 and TGF-β1. AZA, azaserine; AS-GFAT, antisense to GFAT.[Adapted from M. Brownlee.Nature 414: 813-820.2001] (page 17).

Figure 7: Glucose and AGE formation pathways incorporating the polyol pathway and the AGE formation by the α-oxoaldehydes glyoxal, methyl glyoxal and 3-DG. 3-DG, 3-deoxyglucosone; MGO, methyl glyoxal; CML, CEL, , N-ε-(carboxyethyl) lysine; DOLD, deoxyglucosone-lysine dimmer; MOLD; methyl glyoxal-lysine dimmer; GOLD, tlyoxal – lysine dimmer. [Adapted from Brownlee et al. Diabetes Care. 11:73-9, 1988] (page 20).

Figure 9: The mechanism of STZ-induced toxic events in B cells of rat pancreas. MIT – mitochondria; XOD – xanthine oxidase.


Figure 13: Various types of nanoparticles used in biomedical research and drug.
P:8 I am not sure that the statement that in “type 1 primary symptoms involved nausea and vomiting” is correct. The primary symptoms are written above this paragraph.

page 4, line 5: “type 1 primary symptoms involved nausea and vomiting” is withdrawn.

Table 5(p96): DP group achieved lower blood glucose level compared to C however insulin level was approximately \( \frac{1}{2} \) of C. how can this be explained?
Similarly, FD group had a high glyco-Hb level compared to C in spite of a blood glucose level of C. These findings should be addressed in the discussion.

This is a good question raised by the examiner. However, it is not yet clear. Possible explanation is inserted as follows (page 101 line 30):
STZ destroys pancreatic \( \beta \)-cells. As a result, the insulin level goes down leading to diabetic condition. Pelargonidin treatment in diabetic animals may not act upon the already destroyed \( \beta \)-cells, but it may stimulate the still functional \( \beta \)-cells and/or stimulate regeneration of new cells to produce more insulin, which, in turn, reduces the glucose level. Return of blood glucose to normal level without full recovery of insulin level in DP group of rats is not yet clear. It is possible that in diabetic rats even small amount of insulin produced by pelargonidin treatment is more active in reducing blood glucose level, compared to that in normal animals.

Glucose level is reduced to almost normal level in case of FD group of rats, though it has high glyco-Hb level compared to C. It may be due to the fact that glyco-Hb is a stable product. It is reduced only when RBC breakdown occurs. As a result, lowering of glyco-Hb level requires longer time, compared to that of blood glucose level.

P119-Fig 38 and P99 fig 31 what is meant by “different forms of DNA”?
The different forms of DNA are form I (intact supercoiled DNA) and form II (nicked DNA).
The legends of Fig 31 and Fig 38 are changed as follows:
Fig 31. b: Hb-mediated DNA (plasmid) breakdown studied two weeks after pelargonidin encoded liposome treatment. Hb samples were isolated from different groups of rats (C,
D, DPN1, FD). Different forms of DNA [form I (intact supercoiled DNA) and form II (nicked DNA)] were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Similar results were obtained in three additional experiments. (page 99)

Fig 38. Hb-mediated DNA (plasmid) breakdown studied after two weeks of pelargonidin treatment. Hb samples were isolated from different groups of rats: a: (C, D, DPN1, DPN2, DPN3), b: C, D, DPN2, FD). Different forms of DNA [form I (intact supercoiled DNA) and form II (nicked DNA)] were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. Similar results were obtained in three additional experiments. (page 119)

P103: what has been shown in this thesis is that pelargonidin prevents glycation and associated oxidative stress not “glycation induced oxidative stress”. Hence such statement should be modified.

The statement in page 103 “pelargonidin and its role in ameliorating the hemoglobin glycation-induced oxidative stress” is modified as “pelargonidin prevents hemoglobin glycation and associated oxidative stress.”

Fig 26: it is not clear what a reader supposed to get out of this picture. I see black and red arrows the figure in the next mentioned ‘black arrows’ as “cells of pancreatic section”, does not mention red arrows. There are some magnification bars, without any mention of their sizes. The figure text should be rewritten.

Text of Fig 26 is modified as:


Black arrows indicate β-cells of pancreatic sections and red arrows indicate vacuolization. Bar length: 8 μm. (page 81)
**Fig 27:** Normal liver cells frequently (not always) show nucleoli. I am not sure what is meant by nucleus diffused with the nucleolus”, the point of this picture is not clear. There are some magnification bars, without any mention of their sizes. Better clarification is needed.

Legend of Fig 27 is modified as follows:

Fig 27. Liver sections stained with hematoxylin and eosin. a: normoglycemic rats (C), b: diabetic rats (D), c: pelargonidin-treated diabetic rats (DP), d: normoglycemic rats treated with pelargonidin (CP). Magnification: $\times$ 20. Black arrows indicate nucleus with a distinct nucleolus. White arrows indicate nucleus without distinct nucleolus. Bar length: 8 $\mu$m. (page 82)

**Fig 28.** Glomerular basement changes in this model can only be seen by ultrastructural morphology. For mesenchymal expansion a PAS stain is necessary. In this picture a black arrow points to an empty space. There are some magnification bar without mention of their sizes. As it stands now, this picture doesn’t have any message.

Several reports by different groups (B M. Jarrar et al. Saudi Journal of Biological Sciences 15: 307-313, 2008. C.T. Kumarappan and S. C. Mandal. Renal Failure, 30:307–322, 2008) suggest that the glomerular basement membrane morphology can be visualized by hematoxylin and eosin staining. However, it is be better seen by the ultrastructural morphology study or by using PAS stain. Fig. 28 is a representative slide of several slides. The black arrow, which the examiner has suggested a pointer to an empty space, indicates thickening of glomerulous membrane.

The text of Fig 28 is modified as follows:

Fig 28. Kidney sections stained with hematoxylin and eosin visualized at magnification 40X . a: normoglycemic rats (C), b: diabetic rats (D), c: pelargonidin-treated diabetic rats (DP). d: normoglycemic rats treated with pelargonidin (CP). Black arrow indicates enhanced glomerular basement membrane thickening and green arrows indicates diffused mesangium in the section of diabetic rats shown in b. Bar length: $4 \mu$m. (page 83)
The "summary" reiterates the results in point form without identifying any links or meaning of these finding.

The summary is presented in point form. After several points, the findings are briefly linked. (The examiner has probably overlooked it.) However, for better presentation, the summary is divided under different sections (followed by a conclusion) as mentioned below.

Page 148 after line 17: Action of Pelargonidin in STZ-induced diabetic rats
Page 150 after line 5: Action of liposome-encoded pelargonidin treatment in STZ-induced diabetic rats
Page 151 after line 7: Action of PLGA nanoparticle-encoded pelargonidin treatment in STZ-induced diabetic rats
Page 152 after line 5: Interaction study of pelargonidin with RBC and hemoglobin

It has been mentioned (p150) that the free drug treated animal's glycoHb didn't normalize at all. However, table 5 (p96) shows normalization.

In Page 96 (Table-5), the glycoHb levels (%) shown are 4.49±0.56 for control rats and 7.39±0.45 for FD group of rats. This clearly suggests that the glycoHb level is not fully normalized in treated rats (FD) and it has been discussed (page 150).