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Investigation of the effect of addition of *Momordica charantia* to glibenclamide on amelioration of endothelial dysfunction in diabetic rats by activating Takeda G protein-coupled receptor 5

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Abstract: Endothelial dysfunction (ED) is a significant risk factor of blood vessel related diseases of diabetes and this study evaluate the effect of adding *Momordica charantia* (*Mc*) to glibenclamide (GLB) on ED markers in diabetic rats. Streptozotocin (STZ-40mg/kg b. w.) induced diabetic rats were randomly put into 3 groups with 10 rats/group; diabetic control [DC] group, glibenclamide treated group (GLB -2.5mg/kg) and GLB-*Mc* treated group (2.5mg/kg + 400mg/kg). Serum glucose was measured weekly for eight weeks whereas insulin, sVCAM-1, vWF-Ag and interleukin-6 [IL-6] were measured at week0 and week8. Luciferase assay was performed to determine luminescence. At week8, GLB and GLB-*Mc* groups revealed improvements in blood glucose and insulin concentrations ($P \leq 0.05$) when compared to corresponding baseline values with GLB-*Mc* group showing slightly greater improvements. GLB-*M c* group also revealed improvement ($P \leq 0.05$) in vWF-Ag, sVCAM-1 and IL-6 concentrations but was non-significant in GLB group when compared to corresponding baseline values. Comparison between GLB and GLB-*Mc* group showed significantly high concentration of sVCAM-1 in GLB group ($P \leq 0.05$) due to its minimal effect on TGR5 activation. We conclude that adding *M. charantia* to GLB may be a useful choice for modulating diabetes induced ED due to its stimulatory effect on TGR5 receptors.

Keywords: Diabetes mellitus, endothelial dysfunction, TGR5, *Momordica charantia*, glibenclamide.

INTRODUCTION

The description of mysterious disease associated with thirst and frequent urination (diabetes mellitus) dates back to 1500 BC by Hindu Scholars. The term diabetes mellitus (D.M) is a combination of two words, the word "diabetes" is Greek which means siphon-to pass through. The other word is "mellitus" which is a Latin one and means sweetness or honeyed. The term was first used in 15th centaury. It is a chronic endocrine disorder causing abnormalities of metabolic system of the body. The most common form of D.M is type 2 (T2) or "adult-onset diabetes" that is associated with increasing insulin deficiency due to impaired β-cell function and insulin resistance or both (Das and Shah 2011; Kaur *et al.,* 2018).

Diabetes causes endothelial dysfunction (ED); a disparity of vasoconstrictive and vasodilatative substances produced by endothelium and is one of the main steps in the causing vascular complications (Cristina *et al.,* 2018). Diabetes induces endothelial dysfunction through a series of unwanted intracellular events. There is a decline or loss of endothelial function such as vasodilation, reduced nitric oxide bioavailability, anticlumping, fibrinolysis, high concentrations of cytokines, chemokines and reactive oxygen species (Avogaro *et al.,* 2011; Gaiz *et al.,* 2017). Thus, ED is the basis for vascular complications of diabetes mellitus. Endothelium derived glycoprotein Von Wile brand factor (vWF: Ag) involved in cellular activation, platelets activation, coagulation and soluble vascular cell adhesion molecule-1 (sVSAM-1) associated with atherosclerosis are well known biomarkers and found raised in ED (Hegazey 2019).

Strategies for the management of T2 DM goes on changing from time to time in an attempt to develop optimal therapy. One such target is Takeda G protein (TGR5), a G-protein coupled receptor which is extensively expressed in almost all types of EC. TGR5 exert useful effects by regulating different other molecules that lead to diabetes induced ED. Indeed, some hypoglycemic drugs have shown improvement in the endothelial function but such treatments are very limited in number (Zelniker *et al.,* 2019; Briere *et al.,* 2015; Cai *et al.,* 2021).

The use of herbs in treatment of T2 DM has shown promising results previously (Sridharan *et al.,* 2011) and

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this study investigates the usefulness of *Momordica Charantia* (*M. charantia*) in diabetes linked ED. *M. charantia* is shown to exert multiple beneficial effects in diabetes (Bortolot *et al.,* 2019; Elekofehinti *et al.,* 2018; Liaw *et al.,* 2022; Deng *et al.,* 2022). Based on its diverse useful effects in DM, the present study attempts to investigate the effect of standardized fruit extract of *M. charantia* on TGR5 activation and possible improvement in ED.

MATERIALS AND METHODS

Source of standardized fruit powder of Momordica charantia

The standardized fruit powder of *Momordica charantia* (*M.charantia*) was purchased from Hamdard Laboratories (Pvt.) limited Karachi Pakistan. The percentage yield of the active ingredients is given in table 1.

The microbe free powder was stored in air tight container at room temperature and suspended in 0.5% CMC w/v suspension before use.

Table 1: Percentage yield of ingredients of standardized fruit powder of *Momordica charantia*

S. No.	Constituents	Yield (%)
1	Momordicoside L	2.5
2	Momordicoside X	2
3	Momordicoside D	1
4	Momordicoside K	4
5	Momordicine I	3
6	Momordicine II	4
7	Heavy metals	<8

Experimental animals

For the current study, albino rats of 8-10 weeks of age and 200-250 grams each were recruited randomly from animal house of the Gandhara College of Pharmacy, Gandhara University. The rats were kept as per standard protocol i.e., kept at room temperature giving them 12 hours dark and light cycle. The rats were acclimatized for a week. Approval for the study was sought from the Animal Ethical Committee of the Institute.

Dose selection

In order to select the dose, we performed the oral toxicity study in accordance with the guidelines of Organization for Economic Co-operation and Development OECD. A range of doses of standardized fruit powder of *M charantia* was administered starting from 100mg/kg to 4000mg/kg b. w. The animals were followed for 72hr. and observed closely for any change in behavior and/or other health conditions such as mild diarrhoea or weight loss.

Glucose Tolerance Test (GTT)

Injection of 2 grams of glucose per kilogram body weight was given to the overnight fasting rats. Blood was drawn at 8 intervals each with duration of 15 minutes (i.e., at 15, 30, 45, 60, 90, 120, 150 and 180 minutes). Glucose level was determined in these bold samples using Bayer glucometer.

Diabetes Induction

Intraperitoneal injection of streptozotocin about 40mg/kg body weight was used to induce Diabetes in rats. Exactly after 15 minutes of STZ injection, nicotinamide mixed in normal saline was administered at a dose of 110mg/kg. In less than 24-hour time period, 5% glucose solution was given to rats to overcome the chances of drug induced hypoglycemia. After two weeks of diabetes induction, the rats with fasting glucose of above 150mg/dl were to have diabetes mellitus and were recruited to the study (Ananda *et al.,* 2012).

Treatment was started after one week of STZ injection i.e., on 15^{th} day of diabetes induction and counted as the day 1 of therapy.

Study design

Study rats were recruited into 4 groups (10 rates each group). The study was conducted for a period of 8 weeks. The rats were fed twice a day throughout the study period. The normal control (NC) group was given vehicle alone. Diabetic control (DC) group received STZ-40mg/kg b. w. GLB treated group received CMC suspension of glibenclamide (2.5mg/kg b. w) by intragastric gavage whereas glibenclamide and *M. charantia* fruit extract treated group received CMC suspension of GLB-*M.c* (2.5mg/kg + 400mg/kg b. w) respectively.

The present study (Ref. No. 2454) was approved by the *Animal Ethical Committee* of Gandhara College of Pharmacy, Gandhara University, Peshawar.

Biochemical measurements

The blood samples were centrifuged at 3000rpm for 20 minutes. Serum was frozen at -80°C for later analysis.

Serum glucose and insulin levels were measured every two weeks by the methods described earlier (Kim *et al.,* 2011; Ahn *et al.,* 2011). sVCAM-1 and vWF-Ag are circulating markers of ED and were measured as described previously (Antonova *et al.,* 2013). These markers were assessed in start of study i.e., pre-treatment, and then in end of study i.e., week 8 (post- treatment). TGR5 luciferase assay was done as per Sato *et al.,* 2007. In brief, Chinese Hamster Ovary (CHO) cells obtained from Chozan→ Cell Line Engineering and Development, Sigma Aldric (Merck KGaA, Darmstadt, Germany) were kept in minimum essential medium alpha (a-MEM) supplied with 10% (v/v) fetal bovine serum (FBS), 100 lM non-essential amino acids (NEAA), 100U/mL penicillin, and 100 lg/mL streptomycin sulfate. The cells were transfected with 3.8 µg of TGR5 expression plasmid

(pCMVSPORT6/TGR5), 3.8µg of CRE-driven luciferase reporter plasmid (pCRE-Luc) and 0.4 µg of neomycin-resistant gene expression plasmid [pcDNA3.1(+)] using Lipofectamine 3000 (Thermo Fisher scientific, France) for obtaining a stable cell line. The transfected cells were selected with 400µg/mL G418 sulfate and single clones were grown in 96-well plate, independently. TGR5-expressing CHO cells were treated with 10µM lithocholic acid (LCA) or study drugs, followed by luciferase assay. Centro Microplate Luminometer LB 963 (Berthold Technologies, Germany) was used to determine luminescence.

STATISTICAL ANALYSIS

Data are mean±SD of 6 rats per group. Data analysis was done by SPSS 25 and p vale of ≤0.05 was considered significant. The groups were compared using mean changes in levels of various parameters by applying Mann-Whitney U-test and Kruskal-Wallis one-way ANOVA test.

RESULTS

Standardized fruit powder of *M. charantia* did not kill the rats at 4000mg per kg body weight during acute toxicity study and this dose of 4000mg per kg body weight was taken as ALD_{50} cut off dose according to Globally Harmonized Classification System category 5 (safe dose). $1/10^{th}$ of the upper limit dose of *M.* i.e., 400mg/kg was chosen for further investigations.

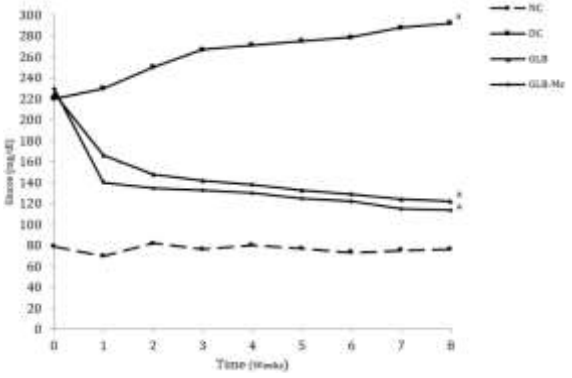

Fig. 1A: Changes in serum glucose concentration overtime
[a] p≤0.05, compared with baseline; [b] p≤0.05, GLB vs. GLB-*Mc*
NC: Normal control (negative); DC: Diabetic control (Positive); GLB: Glibenclamide;
GLB-Mc: Glibenclamide + *Momordica charantia*

Biochemical analysis

Hyperglycemia and insulin resistance are the main characteristics of T2 DM that activates ED many folds (Kaur *et al.*, 2018). In this study, serum glucose concentrations of all the study groups were recorded at the start of the treatment as baseline values, and then every week for a total period of eight weeks. At the end, DC group showed severe hyperglycemia when compared to corresponding baseline value. Both the treatment receiving groups i.e., GLB (2.5mg/Kg) and GLB-*M c* (2.5/400mg/Kg) revealed lower blood glucose levels (p=≤0.05) even from the 1st week of treatment when compared to their corresponding baseline values and continued throughout the study period with the GLB-*Mc* group showing slightly greater improvement. Comparison between treatment receiving groups revealed no significant difference at the end (fig. 1A). Significant improvement was also observed in serum insulin concentrations of GLB and/or GLB-*Mc* receiving groups at the end when compared to corresponding baseline values. No significant difference was observed between treatment receiving groups at the end (fig. 1B).

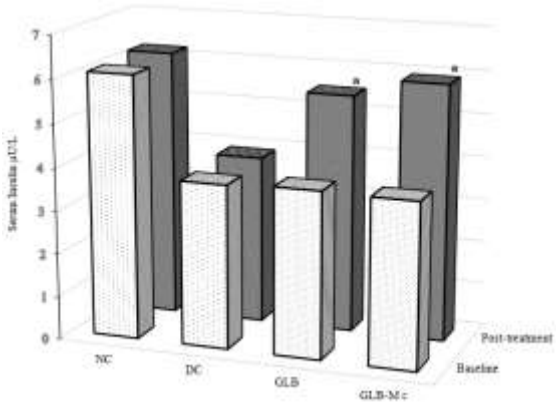

Fig. 1B: Changes in serum insulin concentration
[a] p≤0.05, compared with baseline; [b] p≤0.05, GLB vs. GLB-*Mc*
NC= Normal control (negative); DC= Diabetic control (Positive); GLB= Glibenclamide;
GLB-Mc= Glibenclamide + *Momordica charantia*

Biomarkers of ED

ED was assessed by determining the levels of vWF-Ag and sVCAM-1 measured before and after therapy. Elevated serum concentrations of vWF-Ag and sVCAM-1 were found in diabetic groups at baseline. The group receiving GLB showed improvement in serum concentrations of vWF-Ag and sVCAM-1 at the end but did not reach statistical significance in either case. On the other hand, the group receiving GLB-*M c* was associated with a improvement (p=≤0.05) in serum concentrations of vWF-Ag and sVCAM-1 at the end when compared to corresponding baseline values. Furthermore, comparison between treatment receiving groups revealed significantly high serum concentration of sVCAM-1 in GLB- group at the end (figs. 2A & 2B).

Luciferase assay was carried out for assessing TGR5 activation by glibenclamide alone and/or combining glibenclamide with *M. charantia* to explore the reasons for greater improvement in endothelial function. TGR5 expressing CHO cells treated either with 10 M lithocholic acid (LCA) and/or GLB, GLB-*M.c* were used for TGR5 agonistic activity that plays a major role by regulating the

molecules involved in diabetes-induced ED. GLB exerted a negligible effect whereas the activation of TGR5 by GLB-M c was comparable to positive control i.e., LCA (fig. 3).

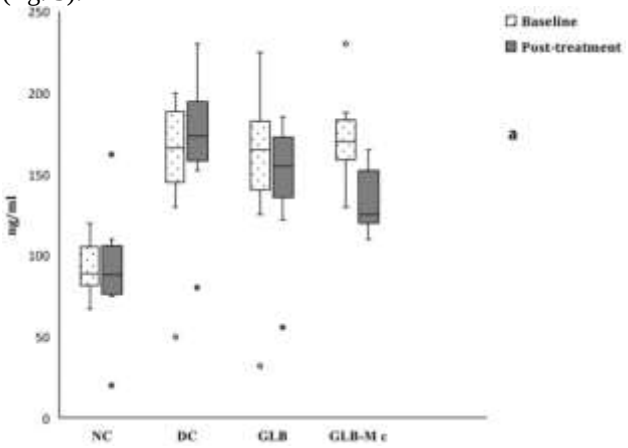

Fig. 2A: Changes in vWF-Ag concentration
[a] p≤0.05, compared with baseline; [b] p≤0.05, GLB vs. GLB-*Mc*
NC= Normal control (negative); DC= Diabetic control (Positive); GLB= Glibenclamide;
GLB-Mc= Glibenclamide + *Momordica charantia*

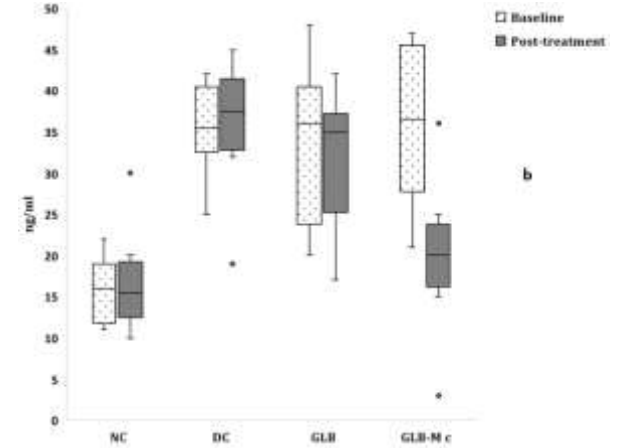

Fig. 2B: Changes in sVCAM-1concentration
[a] p≤0.05, compared with baseline; [b] p≤0.05, GLB vs. GLB-*Mc*
NC= Normal control (negative); DC= Diabetic control (Positive); GLB= Glibenclamide;
GLB-Mc= Glibenclamide + *Momordica charantia*

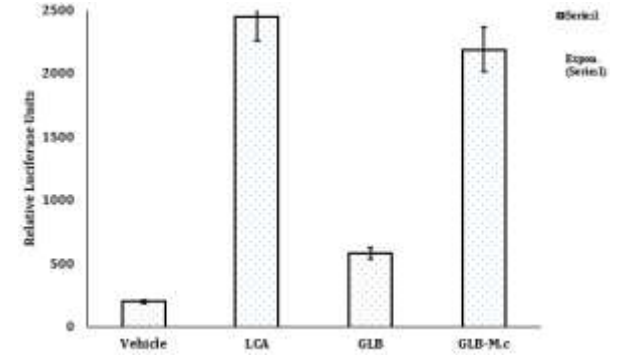

Fig. 3: Activation of TGR5
LCA= Lithocholic acid; GLB= Glibenclamide
GLB-Mc= Glibenclamide + *Momordica charantia*

IL-6 triggers the inflammatory response and is responsible for vascular diseases. Compared to baseline, the change (improvement) in IL-6 concentration of GLB receiving group was non-significant whereas significant (P≤0.05) in GLB-*M c* receiving group at the end. Comparison between treatment receiving groups showed no significant difference (fig. 4).

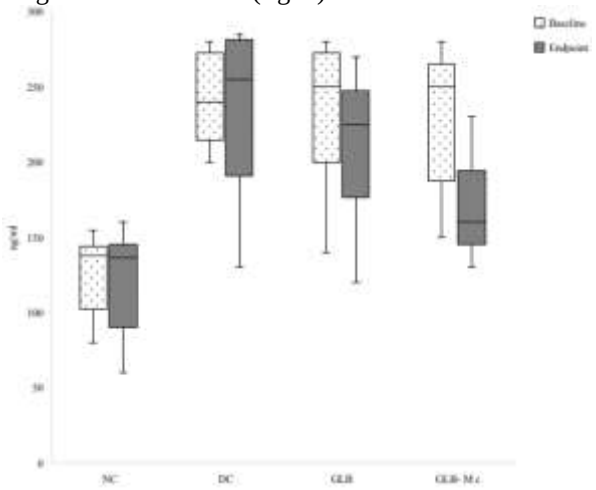

Fig. 4: Changes in IL-6
[a] p≤0.05, compared with baseline; [b] p≤0.05, GLB vs. GLB-*Mc*
NC= Normal control (negative); DC= Diabetic control (Positive); GLB= Glibenclamide
GLB-Mc= Glibenclamide + *Momordica charantia*

DISCUSSION

Much is known about the benefits of using *M. charantia* in D.M but the literature lacks its usefulness in diabetes induced ED which is the main cause of early development of vascular complications. The multifactorial pathogenicity of diabetes presents a great hurdle in achieving the optimum therapy and present study is the continuation of such attempts to achieve best treatment for diabetes.

The present study indicates that the addition of standardized extract of *M. charantia* to GLB augment beneficial effects in terms of glycemic control and insulin secretion and these findings are well supported by previous studies of Poonam *et al.,* 2013 and Nivitabishekam *et al.,* 2009 which revealed that *M. charantia* exert synergistic effect when used in adjunct with oral hypoglycemic drugs. The presence of triterpenoids i.e., momordicosides and momordicines in standardized fruit powder is the direct result of improvement in glucose control and insulin secretion. It has been shown that triterpenoids stimulate glucose uptake, regulate insulin secretion and inhibit α-glucosidase (Rehab *et al.,* 2019; Sun *et al.,* 2021) and these support the findings of the present study.

D M and ED are considered as the two sides of the coin. Diabetes induces ED through multiple mechanisms involving hyperglycemia directed reduced nitic oxide

(NO) production, reactive oxygen radicals and modulation of inflammatory responses (Wang *et al.*, 2022). ED is the basic cause of vascular complications of diabetes and should remain the principal focus in treating diabetes (Takeda *et al.*, 2020; Legeay *et al.*, 2020). Statistically significant declines were observed in serum levels of endothelial markers i.e., vWF-Ag and sVCAM-1 in GLB-*M.c* treated group suggesting improvement in endothelial dysfunction. Momordicosides exert agonistic activity on TGR5 receptors that are widely expressed in endothelial cells of brown adipose tissue, liver, intestine and spleen. Activated TGR5 receptors in turn, induces GLP-1 production thereby stimulating β-cell differentiation, proliferation, survival and secretion of insulin in a systematic manner (Cai *et al.*, 2021; Omotuyi *et al.*, 2018).

Elevated level of IL-6 is main risk factor of vascular complications. IL-6 regulates various events of vascular homeostasis by exerting direct effects on vascular endothelial cells (Su *et al.*, 2021). In current research, we observed a fall in level of IL-6 in GLB-*M.c* treated group indicating a substantial decrease in inflammatory responses. The degree of inflammation is directly related to the progression of diabetic complications and a decrease in serum level of IL-6 is indicative of improvement in endothelial dysfunction Indeed, IL-6 is becoming a favorite target for treating vascular diseases because of its important role in the progression of inflammation (Zhu *et al.*, 2020; Kim HR *et al.*, 2022).

CONCLUSION

Seeking optimum therapy for diabetes has always been the focus of research for the scientists and our findings suggest that adding *M. charantia* to glibenclamide not only improves glucose metabolism but also ameliorates endothelial dysfunction in D.M through activation of TGR5 receptors and lowering of inflammatory response. These effects are indicative of improvement in vascular complications of diabetes which may be helpful in delaying the long-term diabetic complications. Although, *M. charantia* exert diverse favorable affects in D.M, it is equally important to investigate its unwanted effects when used for a longer time period.

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