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Evidence of stable insulin and its increased efficacy during oral administration with *Desmodium gangeticum* extract in rats

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ABSTRACT

Objective: To find the efficacy of administering insulin orally with *Desmodium gangeticum* (DG) extract in diabetes induced rats.
Methods: Diabetes was induced in Wistar male rats by streptozotocin and subsequently treated with the mixture of DG/insulin/DG + insulin and compared with diabetic rats. Standard glucose estimation assay described elsewhere was used to determine the diabetic levels. The effects of homeostatic factors (the effect of temperature, gastric juices, pH, trypsin, and mercaptoethanol) on insulin and insulin-DG were analyzed.
Results: The test results proved that the reported combination of insulin-DG was effective in maintaining glucose homeostasis. More interestingly, insulin-DG was stable against all the degrading parameters while insulin without DG suffered degradation. A molecular interaction was suggested between DG and insulin through nuclear magnetic resonance and Fourier transform infrared spectroscopy analysis.
Conclusions: Insulin-DG mix is a potent anti-diabetic agent and importantly, DG extract is protective to insulin when used in prescribed combination and delivered orally. This could be a possible solution for painful subcutaneous injections.

1. Introduction

Desmodium gangeticum (DG) belonging to the family Leguminosae is a fundamental part of Ayurvedic medicines (indigenous) for diabetes. The extracts obtained from this plant contained isoflavone glycosides[1] and it is also used as diuretics and digestion enhancers. More recently, DG extract was found to play a role in decreasing the blood glucose level in rats[2]. Insulin, a natural hormone secreted by pancreatic beta cells, regulates blood glucose level homeostasis. Type I diabetes mellitus results in destruction of insulin producing beta cells and hence such patients are prone to high blood glucose complications. Injection of insulin through subcutaneous route has become unavoidable as non-invasive route of delivery is still proven to be a challenge. The oral route is suitable and the desired route of drug delivery, especially when repeated or routine administration is necessary. But the effect of digestive enzymes and microenvironmental factors reduce the stability of the peptide hormone during absorption and when encountering digestive enzymes. There are other attempts of insulin delivery such as packing insulin in polymeric microspheres[3,4] or with polymer films but little is known about their side effects and feasibility[5,6]. Though, oral administration of insulin bound with vitamin B_{12} coated dextran nanoparticle promises good results[7], an attempt to transport insulin with herbal extract is a novel natural approach. Since DG was found to elicit basal glucose levels in rats as well as in Ayurvedic treatment of humans, its combinatorial role with peptide hormone would prove this extract a novel additive to insulin with protective functions.

In this study, the efficacy of DG extract along with insulin was tested in diabetes induced male Wistar rats and compared with its counterparts. The insulin-DG mix was subjected to various temperatures, gastric juices, pH, trypsin, and β-mercaptoethanol, respectively and its insulin protective capability was assessed through activity of intact insulin in maintaining blood glucose homeostasis. In addition, Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) analysis suggested an interaction between the extract and insulin molecule and hinted a

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secretagogue kind of activity by the DG extract.

2. Materials and methods

2.1. Animals

Healthy male Wistar rats weighing between 170 and 190 g were procured from Department of Experimental Medicine, Annamalai University, Chidambaram. The animals were sheltered under standard conditions: temperature (22 ± 3) ℃, relative humidity 30%–70% and 12:12 light: dark cycle. The animals were fed with standard pellet diet (Amrit Feeds Ltd., Bangalore) and water *ad libitum*. The Institutional Animal Ethics Committee approval was sorted for the study. The animals were acclimatized for one week before starting the experiment. Streptozotocin (65 mg/kg; Sigma Aldrich, USA) in 0.02 mol/L citrate saline buffer was administered intraperitoneally, as described earlier[8]. Blood glucose level higher than 200 mg/dL was considered diabetic.

2.2. Preparation of aqueous extract of the roots of DG

The plant was collected from the herbal garden maintained in the department and taxonomically identified at the Department of Botany, Saint Berchman's College, Mahatma Gandhi University, Kerala and the voucher specimen A/C no. 3908 was retained for future reference. Extracts were prepared as stated elsewhere[2].

2.3. Oral glucose tolerance test (OGTT)

Normal and diabetic rats were fasted overnight for 18 h and were randomized into following groups (three per group); 40% solution glucose was given orally (1 g/kg body weight): Group 1 (healthy control), Group 2 (induced diabetic control), Group 3 (diabetic rats that are administered with insulin through intraperitoneal injection), Group 4 (insulin administered through oral route), Group 5 [insulin mixed DG (0.3 mL) given through oral route], and Group 6 [insulin mixed DG (0.3 mL) was administered through intraperitoneal route]. Phlebotomy was performed from the orbital plexus of each animal at 0, 30, 60, 90,120 and 180 min. The blood glucose levels and plasma insulin were calculated using standard kits (Qualigens, India).

2.4. Stability of insulin with DG extract

2.4.1. Physical and chemical treatments

The stability of insulin associated with DG root extract was studied through native polyacrylamide gel electrophoresis (PAGE). Insulin sample and insulin-DG mix were subjected to different temperatures, pH, various concentrations of proteases and detergents to assess the stability.

2.4.1.1. Temperature

Definite quantity of insulin, DG and insulin mixed DG was subjected to temperature of 100 ℃, whereas duplicates were kept at room temperature.

2.4.1.2. pH

Insulin, DG, insulin mixed DG samples were treated with phosphate buffer (0.1 mol/L) with pH ranging from 3 to 10. The pH ranges were taken to match the pH levels in the stomach and the intestines.

2.4.1.3. Trypsin and β-mercaptoethanol treatment

Proteolytic efficiency was determined on insulin and protective effect of insulin in DG mixed with insulin by treating it with trypsin and β-mercaptoethanol. The treated samples were incubated for 30 min. A volume of 20 μL of each (subjected to temperature, pH, trypsin and mercaptoethanol) sample were then loaded onto the gel wells and run at 125 V for 2–3 h.

2.5 Effect of gastric and intestinal juices on stability of insulin and insulin with DG extract

2.5.1. Preparation of gastric juice and intestinal juice from rat gut

The pyloric-ligated rat model described elsewhere was used[9]. Briefly, in anaesthetized animal (pentobarbitone, 35 mg/kg body weight) abdomen was opened and a thread was wound around the pyloric sphincter and ileum, ligated without disrupting blood supply. The stomach was replaced with care ensuring the abdomen wall was closed into two layers with interrupted sutures. After the animal recovered from the anaesthetic agent, it was starved for water in the post-operative period. After 4 h, the animal was sacrificed (cervical dislocation) and the oesophageal end was tied before the stomach was dissected out. The stomach and ileum were cut open along the greater curvature. The gastric juice and intestinal juice were collected into test tubes, respectively. The extracts were centrifuged (2 000 r/min for 10 min) and both the sediment and the supernatant were used for the estimation of various biochemical parameters.

2.5.2. Treatment with gastric juice

Definite quantity of insulin mixed DG was treated with definite quantity of gastric juice. The protein content of the sample was estimated by Lowry's method at an interval of 0, 15, 30, 60 min, and 17 h respectively.

2.5.3. Treatment with intestinal juice

Definite quantity of insulin mixed DG was treated with definite quantity of intestinal juice. The protein content of the sample was estimated by Lowry's method at an interval of 0min, 15min, 30min, 60min, and 17 h respectively.

2.5.4. Detection of interaction between insulin and DG extract

2.5.4.1. Spectrochemical analysis

Absorption spectra of insulin, DG and insulin mixed DG were recorded on a Shimadzu UV-2450 spectrophotometer at 275 nm. Change in absorption pattern was determined by scanning whole sample at a range of wavelength starting from 250 to 300 nm.

2.5.4.2. NMR studies

A proton NMR was taken on a Bruker 300 MHz and recorded using software Avance 2 TopSpin 1.3. Samples were dissolved in D_2O and then filtered through glass wool plug that eluted into the NMR tube.

2.5.4.3. FTIR analysis

IR-spectra were obtained using a Bomem IR-spectrometer (Bomem, Canada). The mixtures were lyophilized and mixed

with 300 mg of micronized potassium bromide. The mix was then compressed into discs at a force of 10 kN for 2 min using a manual tablet presser (Perkin Elmer, Norwalk, USA). A 256-scan interferogram was collected with a 4 cm^{-1} resolution in the mid-IR region for each spectrum at room temperature. Insulin spectra were recorded based on the double subtraction procedure[10] while, insulin-free systems and others were read under identical conditions with blank subtraction.

3. Results

3.1. OGTT in normal and diabetic rat

As expected, streptozotocin induced diabetic rats were hyperglycemic as compared to normal rats in OGTT analysis. Administration of insulin and insulin-DG mix through intraperitoneal and oral route, respectively reversed the blood glucose level to homeostasis after 90–120 min (Figures 1 and 2). Interestingly, higher hypoglycemic action of insulin mixed DG than insulin alone administered both intraperitoneal and oral route significantly (Figures 1 and 2).

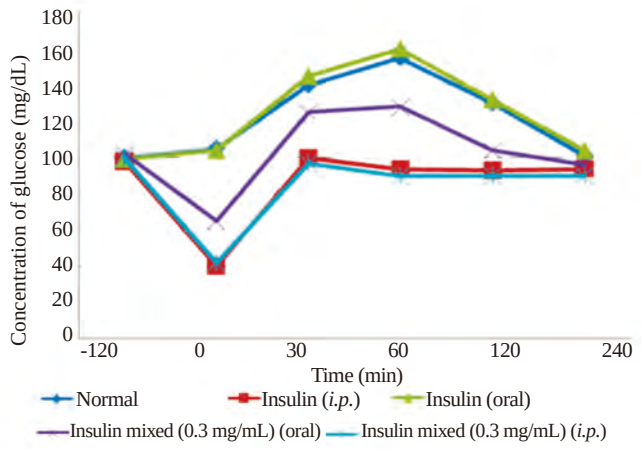

Figure 1. Glucose tolerance levels in normal rats.

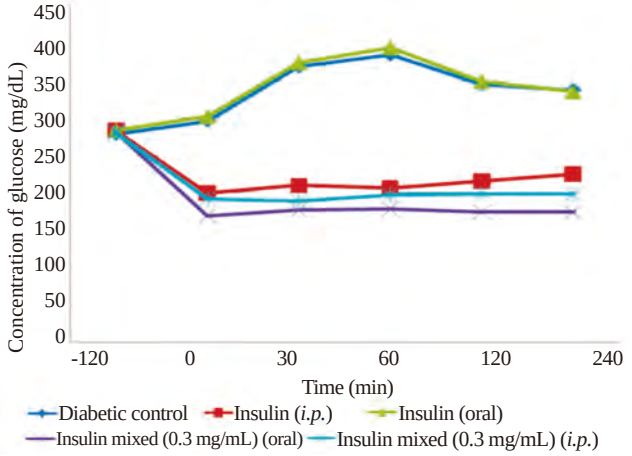

Figure 2. Glucose tolerance test in diabetic rats.

3.2. Stability of insulin with DG extract

3.2.1. Physical parameters

The stability of insulin and insulin-DG mix subjected to diverse temperature, pH, trypsin and β-mercaptoethanol were evaluated through PAGE analysis and results suggested insulin mixed with DG extract stayed integrated while insulin got degraded to such treatments (Figures 3–6). This clearly suggested the protective role of DG extract on insulin.

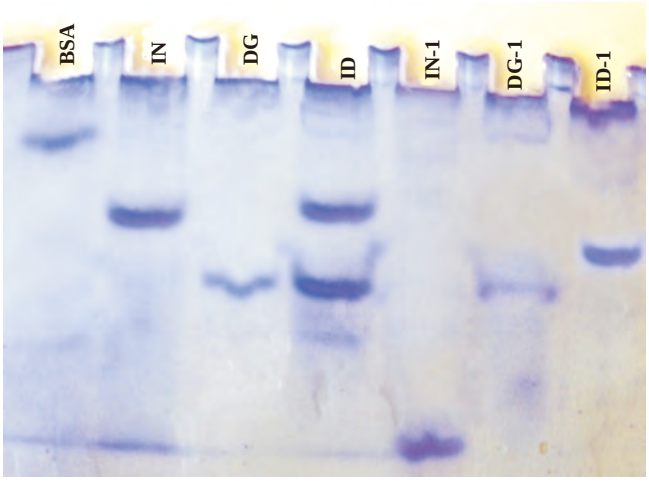

Figure 3. Protective effect of aqueous extract of DG on degradation of insulin by temperature.
BSA: Bovine serum albumin; IN: Insulin; ID: Insulin mixed DG; IN-1: Temperature treated insulin; DG-1: Temperature treated DG; ID-1: Temperature treated insulin mixed DG.

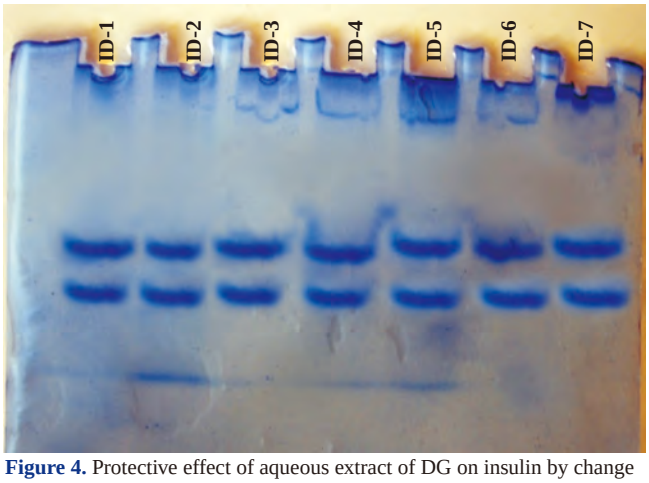

Figure 4. Protective effect of aqueous extract of DG on insulin by change in pH.
ID: Insulin mixed DG (pH 3–9).

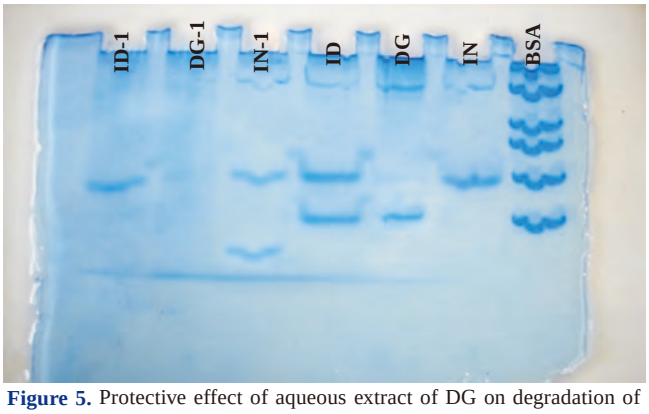

Figure 5. Protective effect of aqueous extract of DG on degradation of insulin by trypsin.
BSA: Bovine serum albumin; IN: Insulin; ID: Insulin mixed DG; IN-1: Trypsin treated insulin; DG-1: Trypsin treated DG; ID-1: Trypsin treated insulin mixed DG.

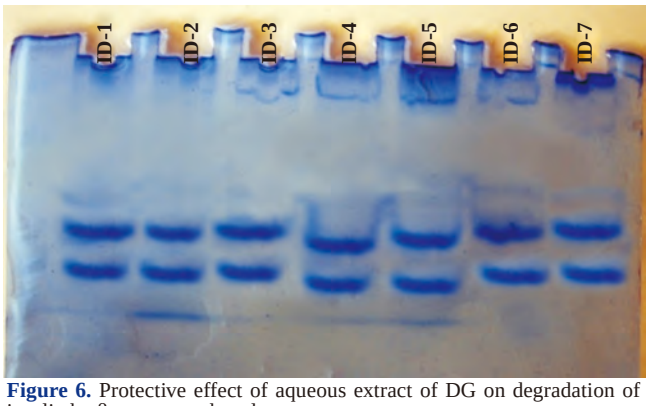

Figure 6. Protective effect of aqueous extract of DG on degradation of insulin by β-mercaptoethanol.
ID-1: Insulin-DG treated with 10 μg/L mercaptoethanol; ID-2: Insulin-DG treated with 20 μg/L mercaptoethanol; ID-3: Insulin-DG treated with 30 μg/L mercaptoethanol; ID-4: Insulin-DG treated with 40 μg/L mercaptoethanol; ID-5: Insulin-DG treated with 50 μg/L mercaptoethanol; ID-6: Insulin-DG treated with 60 μg/L mercaptoethanol; ID-7: Insulin-DG treated with 70 μg/L mercaptoethanol;

3.2.2. Effect of gastric and intestinal juices

Gastric and intestinal juices at varied concentrations (low to high dilutions) were tried on insulin and insulin-DG mix. Even at higher concentrations, the insulin-DG mix stayed stable while insulin was degraded (Figures 7 and 8). This proved to the stability of insulin-DG mix.

3.3. Determination of interaction between insulin and DG root extract

3.3.1. Spectrophotometric absorption of insulin and insulin mixed DG

The absorption spectra of insulin, insulin mixed with DG and aqueous extract of DG are shown in Figure 9. Addition of DG aqueous extract to the insulin solution resulted in increased absorption in UV spectra when compared to that of DG extract. This suggested a possibility of composite binding of insulin and DG.

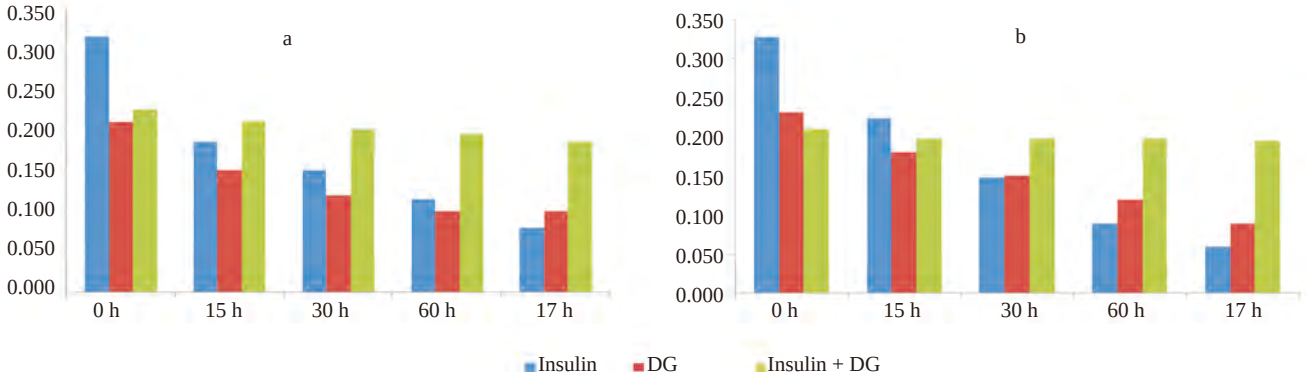

Figure 7. Effect of gastric juice on insulin degradation at lower concentration of insulin mixed DG (1:50 dilution) (a); effect of gastric juice on insulin degradation at higher concentration of insulin mixed DG (1:25 dilution) (b).

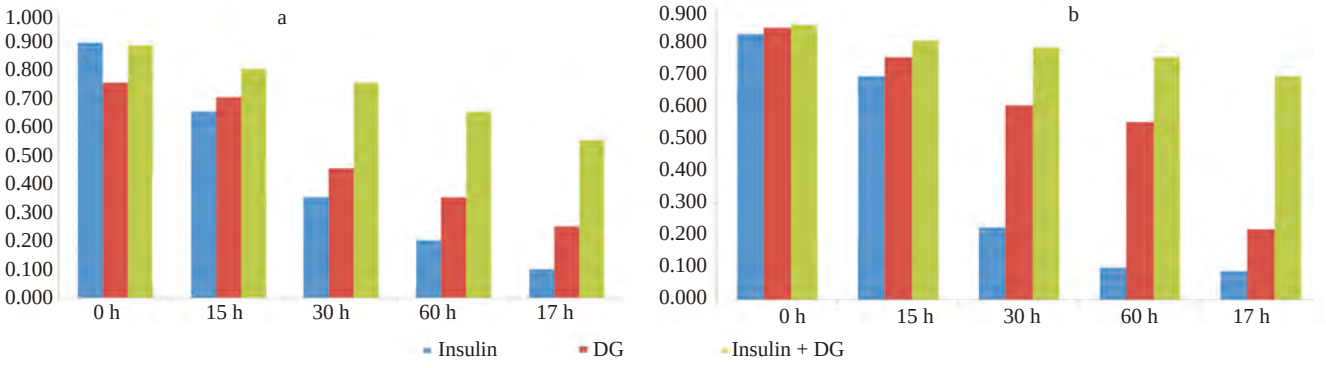

Figure 8. Effect of intestinal juice on insulin degradation at lower concentration of insulin mixed DG (1:50 dilution) (a); Effect of intestinal juice on insulin degradation at higher concentration of insulin mixed DG (1:25) (b).

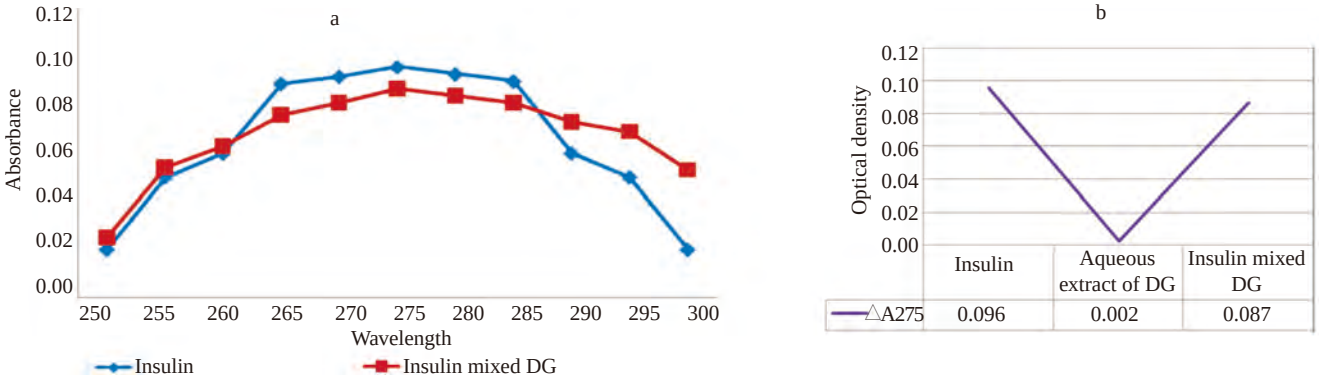

Figure 9. UV absorption maximum of insulin at varying wavelength (a); UV absorption of insulin, DG and insulin-DG at 275 nm (b).

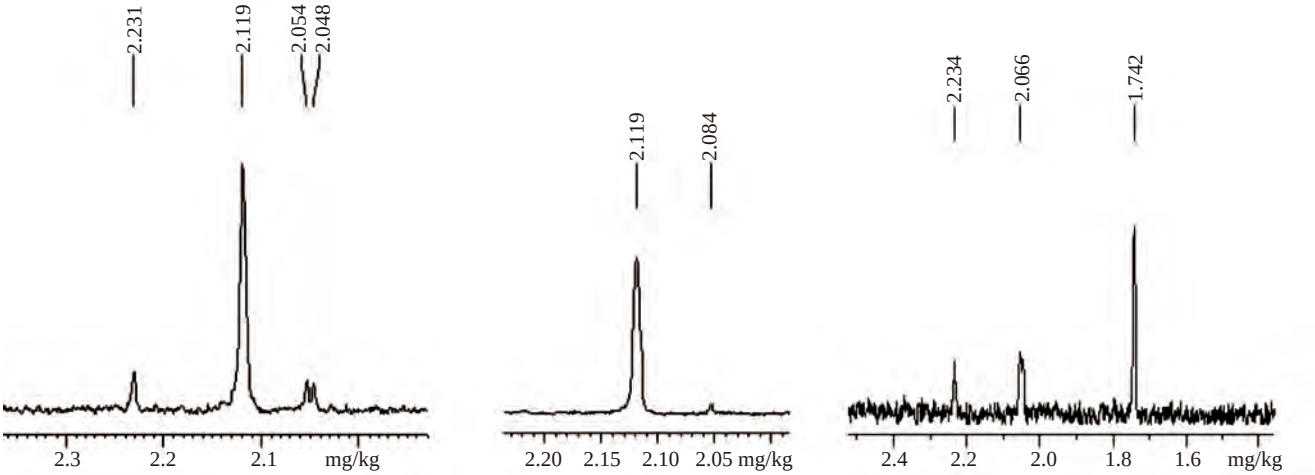

Figure 10. [1]H NMR spectra at 300.13 MHz of DG (a), [1]H NMR spectra at 300.13 MHz of insulin (b), and [1]H NMR spectra at 300.13 MHz of DG and insulin (c).

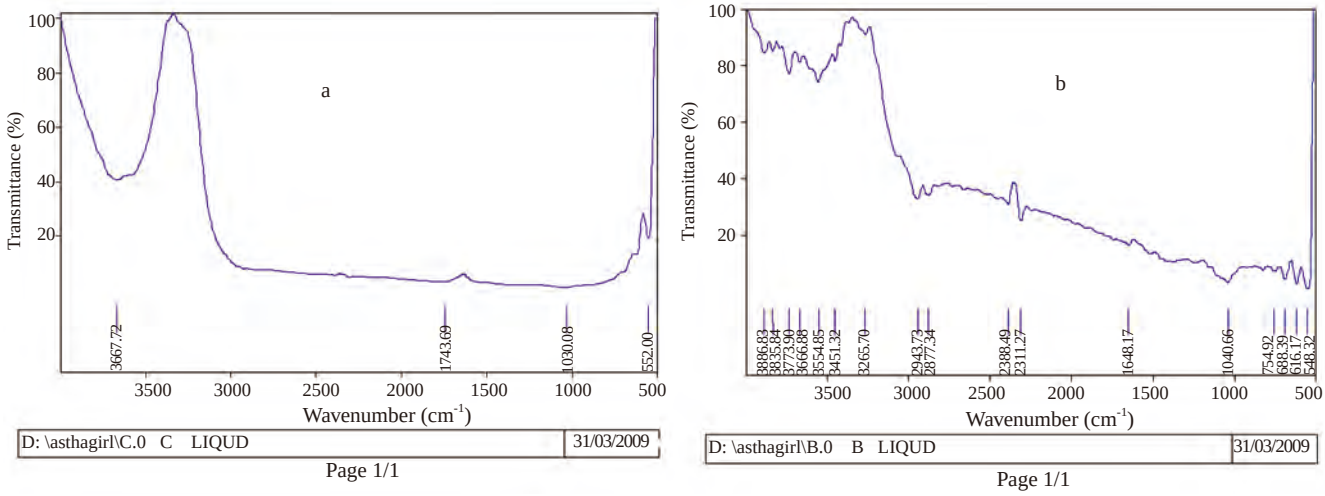

Figure 11. Determination of IR spectrum of extract of DG (a), and determination of IR spectrum of extract of insulin (b).

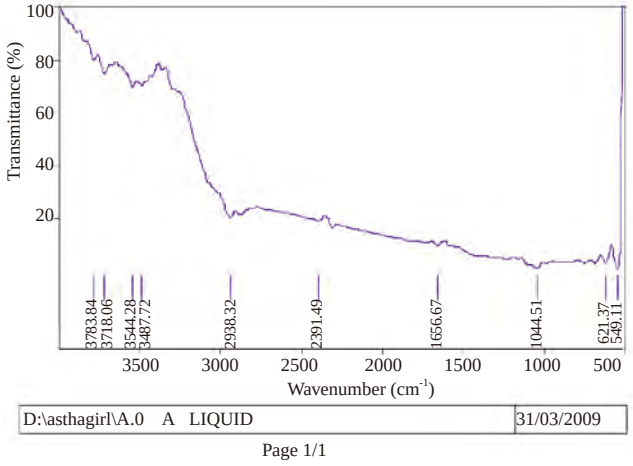

Figure 12. Determination of IR spectrum of extract of DG and insulin mixture.

3.3.2. NMR

Chemical shifts, changes in line width and decreased intensity of compound signals suggested an interaction between protein and small ligand. In [1]H NMR of aqueous DG extract, there were four distinct peaks in the chromatogram each at 2.23 mg/kg, 2.115 mg/kg, 2.034 mg/kg and 2.046 mg/kg, respectively and 2.115 was the maximum. And in case of insulin, they were at 2.119 mg/kg and 2.034 mg/kg in which 2.119 was the highest. To our surprise, the major significant peaks were lost and there has been a chemical shift to 2.234 mg/kg, 2.056 mg/kg and 1.742 mg/kg, respectively in the case of insulin-DG mix. This strongly suggests a complex formation between insulin with DG through chemical shift displacements of assignable aromatic protons of specific amino acids upon the addition of the DG (Figure 10).

3.3.3. FTIR

Insulin IR spectrum showed a characteristic band (2 936.77) between 3 100 and 2 850 cm^{-1} C-H absorption and this indicated aromatic ring in the spectrum and this was confirmed by the peaks having the wave number 1 655.75 and the substitutes pattern below 900 cm^{-1} (*i.e.*, 688.42, 621.91 and 547.29 cm^{-1}) to give C-H out of plane bending. The wave number 3 486.87 and 3 544.51 in the spectrum indicated the presence of N-H containing amine. Thus the characteristic absorption spectrum of aromatic amino acid in insulin was identified by the IR spectrum. On mixing with DG extract, the insulin spectrum had a characteristic change in their absorption pattern with wave number 2 936.77

changed to 2 943.73 cm^{-1}. Similarly, a new band was observed at 2 877.34 cm^{-1} indicating a shift in C-H absorption. A prominent new band at 3 263.70 cm^{-1} also indicated the presence of O-H and N-H absorption. This induced to suspect a change in the nature and structure of insulin which allowed its interaction with DG. In the same way, very less shift in the absorption pattern at 3 544.57 and 3 486.87 from insulin spectrum suggested that DG interacted with insulin in other functional group baring N-H. Finally, it is evident that DG interacts with insulin without affecting its peptide bond (Figures 11 and 12).

4. Discussion

Oral route of insulin administration eliminates invasive techniques and painful repeated injections. We have shown that insulin mixed with DG extract is a potent combination to bring down hyperglycemic conditions to homeostatic levels and it is significantly better than insulin injected through peritoneal route. This is advantageous in two ways: one avoiding intraperitoneal injections; second, it increases the efficacy of the insulin itself. This has been proved through OGTT. Though, we are focusing here on the combinatorial effect of insulin-DG mix, there have been reports that DG extract by itself was responsible for anti-diabetic effect. This raises doubts whether DG extract by itself is capable of inducing the other cells of Islets of Langerhans to produce insulin though we have not performed the absolute insulin levels after treatment.

The effect of insulin mixed with DG extract had a prolonged effect in maintaining the homeostatic glycemic levels. This was a hint about the stability of the insulin-DG extract. Subjection of insulin and insulin-DG extract to various physical parameters such as temperature, pH, trypsin digestion and beta mercaptoethanol treatment did not alter the latter but insulin was highly degraded which was evident in PAGE analysis. This means that, the complex is stable and resistant to normal microenvironmental changes. Next, in order to prove this mixture is resistant to harsh intestinal conditions, this was treated with intestinal and gastric juices. Interestingly, the insulin-DG mix was resistant and stable even after 17 h, comparatively. Though, microspherical and vitamin B_{12} coated delivery of insulin have proven their efficacy, this prolonged resistance have an edge over the existing technologies in oral delivery.

Studying insulin's interaction with DG extract was inevitable after the combination bestowed good stability and remained intact. In spectroscopic analysis, insulin solution has maximum absorption at 275 nm with the molar absorptivity of around 3.6×10^3 $mol^{-1}cm^{-1}$. Change in absorption of insulin mixed DG indicates the interaction between insulin and DG.

The confirmation was obtained from NMR chemical shifts and FTIR analyses. The FTIR spectra of pure insulin, DG, and insulin mixed DG were taken respectively. In insulin mixed DG sample, amide bond in the IR spectrum shifted by few cm^{-1} after complexation. Observed changes of amide band 1 655.73 to 1 648.17 in the present study can be attributed to an ionic interaction between DG and the amino group of insulin. Similarly, the absorption band showed by IR spectrum of insulin at 1 043.81, 2 311.72, 2 389.67, 2 936.77, 3 486.87, 3 544.51, 3 720.38 and 3 765.00 changed to 1 040.66, 2 311.27, 2 388.49, 2 943.73, 3 451.32, 3 554.85, 3 733.90 and 3 835.00 respectively. There were reports that the insulin monomer has six amino acid residues that can bind a positive moiety and ten amino acid residues that can scavenge a negative species[11]. We believe that these properties possibly are the DG extract binding stem in the insulin that did not alter the activity of the insulin though crystallization studies are necessary to confirm such claims. Hence, with this study we have a proof that insulin complexes with DG extract in turn get protected from harsh intestinal acids and enzymes. We have also observed a synergistic action of insulin and DG extract in reducing hyperglycemic condition in diabetic rats rather than normal rats.

To our knowledge, this is the first study that states an interaction between insulin and an extract of an Ayurvedic formulation that not only elicits protective function to insulin but also enhances and prolongs the activity of the hormone.

Conflict of interest statement

We declare that we have no conflict of interest.

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