



RESEARCH ARTICLE

Polyherbal Formulation Ameliorates Diabetes Mellitus in Alloxan-Induced Diabetic Rats: Involvement of Pancreatic Genes Expression

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ABSTRACT

Medicinal plants and herbal formulations have been used as folk medicines for the treatment of hyperlipidemia, diabetes and related metabolic disorders in Asian counties. Current study was designed to evaluate the therapeutic potential of polyherbal formulation consisting of *Swertia chirata* (Chirata), *Artemisia absinthium* (Afsanteen boti), *Caesalpinia bonduc* (kranjwa magz), *Bunium persicum* (Kala zeera), *Gymnema sylvestra* (Ghurmar boti), *Citrullus colocynthis* (Kortumma), *Sphaeranthus indicus* (Mundi boti), and *Cuminum cyminum* (Safeed zeera) in alloxan induced diabetic rats. Rats were grouped into 6 groups and induced diabetes using 150mg/kg alloxan monohydrate except control group. Group I served as control, group II was diabetic group and group III was considered +ve control (glibenclamide treated). Group IV, V and VI were treated with 200, 400 and 600mg/kg of polyherbal formulation upto 8 weeks of experiment. Sampling for biochemical parameters and gene expression analysis was done at 8th week of experiment. Results have shown that polyherbal formulation produced anti-diabetic and antihyperlipidemic effects by reducing serum glucose, glycosylated hemoglobin, total cholesterol, triglycerides, LDL, VLDL levels and increasing serum insulin, glucokinase and HDL levels. Gene expression analysis has revealed that polyherbal formulation upregulated the mRNA expression of Ins-1 and Pdx-1 genes in alloxanized hyperglycemic rats. All these properties of polyherbal formulation make it a potential candidate for the treatment of diabetes. However, further research is required to elucidate its mechanism of action.

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INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by high blood glucose level because the beta (β) cells of pancreas do not produce adequate insulin, or insulin does not produce response at particular receptors (Riseru *et al.*, 2009). Diabetes mellitus is considered as third greatest "killer" after cancer and cardiovascular diseases (Hatware and Annapurna, 2014). The number of diabetic patients is increasing day by day in both rich and developing countries. According to statistics, 5% of all casualties in the world are caused by diabetes, a number which is expected to rise by 50% in next 10 years (Piya *et al.*, 2010). Chronic hyperglycemic effects of diabetes account for various types of abnormalities such as failure and dysfunction of the eyes, nerves, heart, kidneys and blood vessels (Si *et al.*, 2011).

As there is no specific remedy for diabetes, so the most important goal in the cure of all types of diabetes is preservation and even potential regeneration of pancreatic β - cells.

From past three decades, regardless of the huge advancement made in the treatment of diabetes, the patients are still suffering from complications (Dey *et al.*, 2002). Despite significant improvement made by synthetic drugs in the management and treatment of diabetes mellitus, the search for new natural anti-diabetic agents is going on due to least side effects associated with plants. According to an estimate made by world health organization (WHO), 80% of people particularly in developing countries are using natural medicines for treatment of different diseases (Almalki *et al.*, 2016). Many plants are used in folk medicines due to their anti-hyperglycemic potential. To date, more than 800 species of plants have been

investigated and their antidiabetic effects are reported (Mannan *et al.*, 2014).

Medicinal plants or combination of plants have therapeutic properties due to the presence of different complex substances known as secondary metabolites such as flavonoids, polyphenols and alkaloids (El-Abhar and Schaalán, 2014). Scientific research studies have also reported the use of polyherbal formulations for the treatment of diabetes. Polyherbal formulation considerably alters the pattern of glucose tolerance, lipids profile and other biochemical parameters in diabetes mellitus (Ghorbani, 2014). It is believed that herbal formulations containing multiple plant products produce synergistic antidiabetic effects and could enhance the desired actions. The polyherbalism improves the therapeutic efficiency of formulation and reduces unwanted adverse effects (Paoli *et al.*, 2015). So the present research work was designed to assess the potential of polyherbal formulation consisting of *Artemisia absinthium* (Afsanteen boti), *Sphaeranthus indicus* (Mundi boti), *Swertia chirata* (Chirata), *Gymnema sylvestra* (Ghurmar boti), *Caesalpinia bonduc* (kranjwa magz), *Bunium persicum* (Kala zeera), *Cuminum cyminum* (Safeed zeera) and *Citrullus colocynthis* (Kortumma) on glycemic levels, lipid profile and different genes involved in β cells regeneration and insulin secretion from β cells in alloxanized hyperglycemic rats.

MATERIALS AND METHODS

Preparation of polyherbal formulation: For polyherbal formulation preparation, leaves of *Artemisia absinthium*, *Gymnema sylvestra*, *Sphaeranthus indicus* and *Swertia chirata*, fruits of *Caesalpinia bonduc* and *Citrullus colocynthis*, seeds of *Bunium persicum* and *Cuminum cyminum* were used. Samples were verified for taxonomy from Department of Botany and a voucher specimen of each plant was deposited in the herbarium of University of Agriculture, Faisalabad, (voucher specimen numbers: (*Gymnema sylvestra*:21144), (*Artemisia absinthium*: 21145), (*Sphaeranthus indicus*:21146), (*Swertia chirata*: 21147), (*Caesalpinia bonduc*:21148), (*Citrullus colocynthis*:21149), (*Bunium persicum*:21150), (*Cuminum cyminum*: 21151). For extract preparation, 100 grams of each plant were washed, shade dried at 25+2°C and powdered. The powders were decocted in boiling water (1:9) for 30 min, cooled and filtered. The filtrates were dried, mixed in equal ratio to prepare polyherbal formulation and stored at 2-8°C.

Qualitative phytochemical analysis: Phytochemical analysis of polyherbal formulation was performed for detection of alkaloids (Salehi-Surmaghi *et al.*, 1992), carbohydrates (Gupta *et al.*, 2013), tannins (Segelman *et al.*, 1969), phenolics and flavanoids by FeCl₃ test described by Mace (1963) and Raman (2006). Keller-kiliani test was performed for detection of glycosides (Ajaiyeobu *et al.*, 2002), stain test for fixed oil (Shabi *et al.*, 2014), frothing test for saponins (Kumar *et al.*, 2009) and Salkowski test for terpenoids (Evans, 1997).

Research study protocol and experimental induction of diabetes: We housed ninety (90) healthy young albino rats (180-200 g) in an animal facility at Institute of Pharmacy,

Physiology and Pharmacology, University of Agriculture, Faisalabad. Rats were divided into 6 groups, each group having 15 rats. Group I was control, group II was diabetic and group III was +ve control. Group IV, V and VI were Treated I, Treated II and Treated III. The experiment was conducted according to approved methods of Graduate Studies Research Board. Rats of group II, III, IV, V and VI were induced diabetes by using single intraperitoneal injection of alloxan monohydrate (150 mg/kg BW) dissolved in normal saline.

Administration of polyherbal formulation to alloxanized hyperglycemic rats and blood sampling:

After seven days of alloxan induction, group III was treated with 10 mg/kg of glibenclamide and group IV, V and VI were administered 200, 400 and 600 mg/kg of polyherbal formulation extract respectively through intragastric tube upto 8 weeks of experiment. After that, the rats were slaughtered by cervical dislocation and blood samples were collected to separate serum for further analysis. Their pancreata were dissected out and snap-frozen in liquid nitrogen for gene expression analysis.

Biochemical analysis: Serum glucose, serum insulin and Hb1Ac levels were determined by using commercially available kits. Hepatic glucokinase level was assayed spectrophotometrically by measuring absorbance of reaction cocktail consisting of tissue homogenate and glucose-6-phosphate dehydrogenase assay system at 340nm (Goward *et al.*, 1986).

Lipid Profile: Serum total cholesterol, triglycerides and high-density lipid (HDL) levels were determined by using commercially available kits (Dia Sys Diagnostic Systems USA reagent kit method). Low density lipoproteins (LDL) and very low-density lipoproteins (VLDL) concentrations were calculated by using following formulae (Friedewald *et al.*, 1972).

$$\text{LDL-Cholesterol} = \text{Cholesterol} - \left[\frac{\text{Triglyceride}}{5} + \text{HDL-Cholesterol} \right]$$

$$\text{VLDL-cholesterol} = \left[\frac{\text{Triglycerides}}{5} \right]$$

Gene expression analysis: RNA isolation was performed by using TRIzol method (ThermoFisher Scientific, Waltham, Massachusetts, USA) (Liu and Patel, 1995), quantified on nanodrop and subjected to cDNA synthesis with equal RNA concentration in each sample using the RevertAid cDNA synthesis kit (ThermoFisher Scientific). qRT-PCR was performed using Maxima SYBR Green/ROX qRT-PCR Master Mix (ThermoFisher Scientific). Expression of different genes involved in insulin signaling pathway (Pdx-1, IGF-1, Ins-1) was analyzed. The primer sequences of genes are given in Table 1. The cDNA was denatured for 15 seconds at 95°C for all genes. Then primers were annealed at 52°C for 25 seconds and extension time was 20 seconds at 72°C for 40 cycles. All these steps were accompanied by 95°C denaturation for 10 minutes at the start of a single cycle. Expression levels of genes were normalized to β -actin. The 2^{-*}(- $\Delta\Delta$ ct) method was used to analyze qRT-PCR data.

Data analysis: Data were analyzed using one way-ANOVA. Duncan multiple range (DMR) test was applied in case of significant difference among experimental groups. $P < 0.05$ was considered statistically significant.

RESULTS

Phytochemical analysis of polyherbal formulation (Qualitative analysis): Crude aqueous extract of polyherbal formulation was subjected to qualitative analysis. The results of various chemical tests for the detection of phytochemicals are summarized in Table 2. Results have indicated the presence of alkaloids, phenolic compounds, flavonoids, glycosides, saponins, carbohydrates and terpenoids in polyherbal formulation.

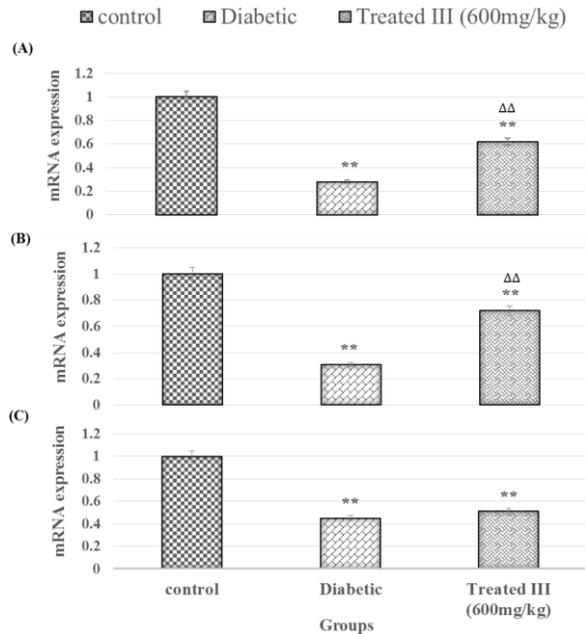


Fig. 1: Rat's pancreatic gene expression profile: (A) mRNA expression of Pdx-1 in control, diabetic and polyherbal formulation treated rat's pancreata (n=3) (B) mRNA expression of Ins-1 in control, diabetic and polyherbal formulation treated rat's pancreata (n=3) (C) mRNA expression of IGF-1 in control, diabetic and polyherbal formulation treated rat's pancreata (n=3). (** $P \leq 0.01$ shows significant difference between control and other groups. $\Delta\Delta P \leq 0.01$ indicates significant difference between diabetic and polyherbal formulation treated group III).

Table 1: Name and source of oligonucleotides (Primers). All oligonucleotides listed below were supplied by Invitrogen, Karlsruhe, Germany

Beta actin F	5'- CGAGTACAACCTTCTTGCAGC -3'
Beta actin R	5'- TATCGTCATCCATGGCGAACTG -3'
Pdx I F	5'- TCCCGAATGGAACCGAGACT -3'
Pdx I R	5'- TTCATCCACGGGAAAGGGAG -3'
Ins I F	5'- AGGCTCTGTACCTGGTGTGTG -3'
Ins I R	5'- CGGGTCCTCCACTTCACGAC -3'
IGF I F	5'- CGTACCAAATGAGCGCACC -3'
IGF I R	5'- CAAGCAGAGTGCCAGGTAGA -3'

Table 2: Qualitative analysis of polyherbal formulation

Phytochemical	Present/Absent
Phenolics	+
Alkaloids	+
Flavanoids	+
Saponins	+
Glycosides	+
Fixed oils	+
Tannins	-
Carbohydrates	+
Terpenoids	+

(+)= Present, (-)=Absent).

Biochemical analysis

Effect of polyherbal formulation on Serum Glucose, insulin and HbA1C: Serum glucose and glycosylated hemoglobin (HbA1c) levels were significantly ($P \leq 0.01$) increased in hyperglycemic rats. Glibenclamide produced significant reduction in serum glucose and HbA1C concentrations. However, treatment of diabetic rats with polyherbal formulation at 200, 400 and 600mg/kg upto 8 weeks significantly ($P \leq 0.01$) lowered the serum glucose and glycosylated hemoglobin levels in treated groups I, II and III respectively compared to diabetic group. Diabetic rats showed significant ($P \leq 0.01$) decrease in serum insulin level when compared to control group after induction of diabetes. This significant decrease was successfully reversed in the diabetic rats upon treatment with graded doses of polyherbal formulation extract for 8 weeks in dose dependent manner (Table 3).

Effect of polyherbal formulation on Glucokinase enzyme: A significant decrease in glycolytic enzyme activity (glucokinase) was observed in diabetic, +ve control and polyherbal formulation treated groups I, II and III after administration of alloxan monohydrate compared to control group. Polyherbal formulation treatment of diabetic rats of treated groups I, II and III for 8 weeks showed significant ($P \leq 0.01$) elevation in hepatic glucokinase activity when compared to diabetic group (Table 3).

Effect of polyherbal formulation of lipid profile of alloxanized hyperglycemic rats: Significant change in serum total cholesterol, triglycerides, HDL-cholesterol, LDL and VLDL levels were observed in alloxanized hyperglycemic rats. Polyherbal formulation supplementation (200, 400 and 600 mg/kg) to diabetic rats of treated groups I, II and III up to 8 weeks showed significant ($P \leq 0.01$) decrease in serum total cholesterol, triglycerides, LDL and VLDL levels where as valuable elevation in HDL-cholesterol level was observed compared to diabetic group in dose dependent manner (Table 4). Results have also exhibited that highest dose (600 mg/kg) of polyherbal formulation was more effective in producing antihyperlipidemic effect compared to glibenclamide.

qRT-PCR based gene expression analysis (Effect on insulin signaling pathway):

The effect of polyherbal formulation on mRNA expression of Ins-1, Pdx-1 and IGF-1 genes in control, diabetic and highest dose (600 mg/kg) of polyherbal formulation treated group has been shown in Fig. 1a-1c. As the highest dose of herbal mixture extract was proved to be more effective compared to other doses according to biochemical results, that's why only highest dose treated group was compared with control and diabetic group in gene expression analysis. From this analysis, it was found that Ins-1, Pdx-1 and IGF-1 genes expressions were significantly ($P \leq 0.01$) reduced in diabetic and polyherbal formulation treated group after induction of diabetes compared to control group. Polyherbal formulation treatment of hyperglycemic rats significantly up regulated the mRNA expression of Ins-1 and Pdx-1 genes compared to diabetic group. However, mRNA expression of IGF-1 differ non-significantly between diabetic and 600mg/kg of polyherbal formulation treated group.

Table 3: Effect of graded doses of Polyherbal formulation and Glibenclamide on serum glucose, insulin, HbA1c and glucokinase level of alloxan induced diabetic rats

Treatment	Control	Diabetic	+ve control	Treated I	Treated II	Treated III
Serum Glucose (mg/dL)	109.80±4.28D	518.00±5.31A	138.20±2.65C	233.60±2.62B	224.20±3.38B	143.1±3.07BC
Serum Insulin (mg/dL)	17.95±0.69A	6.58±0.75D	17.65±0.73B	12.23±0.71C	14.65±0.67BC	15.78±0.64BC
HbA1c (%)	5.87±0.03E	13.92±0.07A	6.14±0.02D	7.70±0.03B	6.93±0.03C	6.84±0.03D
Glucokinase ($\mu\text{molG6PO}_4/\text{min/mg proteins}$)	198.6±1.30A	111.2±3.24D	181.6±1.23B	154.0±2.03C	164.4±1.67BC	176.2±1.84B

One-way ANOVA followed by DMR test. Values (mean±SE) with different superscripts within a row differ significantly ($P\leq 0.01$).

Table 4: Effect of graded doses of Polyherbal formulation and Glibenclamide on serum lipid profile of alloxan induced diabetic rats after 8 week treatment

Parameters	Control	Diabetic	+ve control	Treated I	Treated II	Treated III
Total Cholesterol (mg/dL)	120.20±3.62E	266.80±5.48A	140.20±3.25CD	142.00±2.47B	128.20±3.06BC	111.00±2.70D
Triglycerides (mg/dL)	69.20±1.28E	164.60±3.03A	91.00±1.95C	110.40±2.27B	109.40±2.69B	81.80±1.32D
HDL-C (mg/dL)	40.60±0.93A	17.80±1.83E	32.40±0.68CD	27.80±0.58D	31.40±0.51CD	34.20±0.73B
LDL (mg/dL)	65.12±1.60E	216.04±7.51A	89.60±0.51CD	92.12±0.73B	74.91±0.42BC	60.44±1.03D
VLDL (g/dL)	13.86±0.37E	32.96±0.83A	18.2±0.13C	22.08±0.27B	21.89±0.34B	16.36±0.20D

One-way ANOVA followed by DMR test. Values (mean±SE) with different superscripts within a row differ significantly ($P\leq 0.01$).

DISCUSSION

Herbal therapies for treatment of diabetes are being used from decades both in insulin dependent and noninsulin dependent diabetic patients. Herbal products contain more than one active constituent compared to allopathetic treatment. These phyto constituents having different structure but with same curative potential act in synergistic way for treatment of different diseases (Mukherjee *et al.*, 2006). Results of phytochemical analysis of polyherbal formulation indicated the presence of alkaloids, flavonoids, phenols, glycosides and saponins in herbal mixture extract. These phytochemicals (alkaloids, saponins, polyphenols, flavonoids) may influence glucose metabolism by different mechanisms like stimulation of insulin release from the pancreatic β cells, activation of insulin receptors, glucose uptake in the insulin sensitive cells, increasing glucokinase activity and controlling glucose release from liver (Ramadan *et al.*, 2017).

The results have shown that alloxan monohydrate significantly ($P\leq 0.01$) raised serum glucose, glycosylated hemoglobin (HbA1c) and decreased serum insulin and glucokinase levels in all groups except control group. Interestingly, treatment of diabetic rats with polyherbal formulation resulted in significant glycemic control by lowering serum glucose and raising insulin secretion. Possible mechanism of action of antihyperglycemic activity of herbal mixture extract in hyperglycemic rats may be due to increased insulin discharge from existing β cells as well as increased transfer of glucose into peripheral tissues. qRT-PCR analysis also confirmed upregulation of Pdx-1 and Ins-1 genes which are directly related with pancreatic β cells regeneration and insulin secretion (Jie *et al.*, 2016).

Glucokinase (hexokinase IV) is present in the liver and pancreas of most vertebrates. It represents 95% of hexokinase activity in liver (Kawai *et al.*, 2005). In pancreatic β cells, glucokinase serves as a glucose sensor to modify insulin discharge. Its level is controlled by insulin, not glucose-6-phosphate. Due to this property, liver stores glucose-6-phosphate and converts it into glycogen for later use. Mutations in glucokinase have been associated with diabetes mellitus (Martin *et al.*, 2008). Treatment of diabetic rats with graded doses of polyherbal formulation significantly ($P\leq 0.01$) increased hepatic glucokinase activity resulting in increased utilization of glucose and formation of liver glycogen.

Hyperlipidaemia is a common complication of diabetes mellitus. The present study results identified significant ($P\leq 0.01$) change in lipid profile. Treatment of diabetic rats with different doses of polyherbal formulation extract resulted in gradual decrease in serum cholesterol, triglycerides, low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) levels with increase in HDL level. The lipid-lowering trend of aqueous polyherbal formulation extract may be due to the presence of flavonoids which are reported to lower the levels of cholesterol and triglycerides (Hossain *et al.*, 2016). HDL is known as good cholesterol and scavenger because it carries LDL away from arteries. Various research studies have reported that increase in HDL-cholesterol is linked with decline in coronary disease (Momo *et al.*, 2006).

qRT-PCR based gene expression analysis has been performed to analyze mRNA expression of different pancreatic genes involved in insulin signaling pathway like Pdx-1, Ins-1 and IGF-1. Results have shown that alloxan monohydrate significantly ($P\leq 0.01$) down regulated the Ins-1, Pdx-1 and IGF-1 mRNA expressions. The IGF-1 is a growth factor and has insulin-like effects on cell metabolism (Siddique and Awan, 2016). Pancreatic and duodenal homeobox factor-1 (Pdx-1) is expressed in the pancreas and duodenum and plays crucial role in pancreatic β -cells differentiation/ regeneration (Kubo *et al.*, 2011). Results of our research work have shown that there appeared non-significant ($P> 0.05$) difference in expression level of IGF-1 between diabetic and polyherbal formulation (600 mg/kg) treated groups. However, mRNA expression of Pdx-1 and Ins-1 were significantly ($P\leq 0.01$) reversed by the polyherbal formulation treatment. This finding further strengthens the role of polyherbal formulation in reversing hyperglycemic and diabetic conditions.

Conclusions: In conclusion, our research work has revealed that polyherbal formulation is an effective anti-diabetic agent as it can reduce hyperglycemia, improve lipid profile, prevent pancreatic β cells apoptosis and ameliorate insulin secretory capacity. However further research is required to elucidate its mechanism of action.

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