

## ANTIHYPERGLYCAEMIC, ANTIHYPERLIPIDAEMIC AND ANTIHYPERTENSIVE EFFECT OF A POLYHERBAL FORMULATION IN ALLOXAN-INDUCED DIABETIC RATS

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### Abstract

Polyherbal formulation (POL-4) is popularly used by traditional practitioners to treat diabetes in district Badin, Sindh, Pakistan. This study validates the folk claim of POL-4 in diabetes and associated dysfunctions (hyperlipidaemia, endothelial dysfunction, hypertension) using OGTT and alloxan-challenged diabetic assays. Administration of POL-4 extract (300 mg/kg bw) markedly ( $p < 0.01$ ) lowered blood glucose levels compared to only glucose loaded (2 g/kg bw) rats. In alloxan (135 mg/kg bw) fed rats, chronic (21 days) administration of POL-4 powder (3% and 6% w/w in diet) significantly ( $p < 0.001$ ) prevented the rise in systolic blood pressure, heart rate, serum blood glucose and lipid parameters, while improved serum high density lipoprotein-cholesterol and endothelium-dependent aortic acetylcholine-mediated relaxation. POL-4 administration showed no signs of toxicity or lethality in mice at 1 - 5 g/kg bw doses. These findings attest antihyperglycaemic, anti-hyperlipidaemic, endothelium modulatory and antihypertensive effects of POL-4, thus providing evidence to the folk use of POL-4 in diabetes and related complications.

### Rezumat

În districtul Badin, Sindh, Pakistan, pentru tratamentul diabetului, este utilizată tradițional o formulare ce conține părți aeriene provenite de la mai multe plante (POL-4). Acest studiu validează folosirea POL-4 în diabet și disfuncții asociate (hiperlipidemie, disfuncție endotelială, hipertensiune), pe un model de diabet zaharat indus cu aloxan. Administrarea extractului POL-4 (300 mg/kgc) a scăzut semnificativ ( $p < 0,01$ ) nivelul glicemiei față de lotul control tratat cu glucoză (2 g/kgc). La șobolanii tratați cu aloxan (135 mg/kgc), administrarea cronică (21 zile) a POL-4 (3% și 6% m/m în dietă) a prevenit creșterea tensiunii arteriale sistolice, a ritmului cardiac, modificarea parametrilor serici glicemici și lipidici. POL-4 la dozele de 1 - 5 g/kgc nu a prezentat semne de toxicitate la șoareci.

**Keywords:** alloxan-induced diabetes, POL-4, antihyperglycaemic, antihyperlipidaemic, antihypertensive

### Introduction

Diabetes is an alarming health burden of the modern society, commonly prevailing as diabetes type 2 (more than 90%) [1]. Persistent hyperglycaemia in diabetes may damage and cause failure of diverse body organs like eyes, kidneys, peripheral neurons, blood vessels (micro and macro) and heart [2]. Diabetes has become pandemic affecting 387 million individuals globally, its predicted prevalence from 8.3% in 2014 will increase to 10% by the year 2030 [3]. Diabetes dependent mortality is reported around 80% or even more in developing countries. Pakistan is ranked at 7<sup>th</sup> position in the world with 6.9 million diabetic patients and expected to be at 4<sup>th</sup> position in 2025 with estimated 11.5 million diabetic patients [4]. Hyperlipidaemia is a known and a common co-morbid in diabetic patients

[5]. Around 70% to 97% cases develop endothelial dysfunction and hypertension in diabetic patients with hyperlipidaemia.

A diverse range of pharmacological agents are available for the treatment of diabetes such as oral (biguanides, sulphonylureas and alpha-glucosidase inhibitors) or injectable (insulin and its analogues) preparations, however, their use is associated with wide range of adverse effects including development of increased risk of hypoglycaemia, lactic acidosis, weight gain, gastrointestinal toxicities, liver injury, heart and kidney failures [6]. Hence, the global interest is turning towards search of newer drugs that could combat diabetes progression in an innovative, effective and safe manner. Among alternative measures to address diabetes, medicinal plants/herbs are playing an important role in the management of metabolic diseases and related

cardiovascular issues. It has been predicted that more than 25% of the prescription drugs in developed countries are derived from plants/herbs. World Health Organization has estimated that herbal remedies are being used in three quarters of the world population for their primary health care need [7]. Thus, medicinal herbs are preferred for the management of diabetes either as single herb [8] and/or polyherbal formulations (Diasulin and Cogent db) [9, 10]. Polyherbal formulation (POL-4) is comprised of *Nigella sativa*, *Gymnema sylvestre*, *Trigonella foenum graecum* and *Cichorium intybus*. It is used by traditional practitioners for the treatment of cardiovascular disorders and diabetes in interior parts of Sindh province, Pakistan [11, 12]. Description of the ingredients of POL-4, including

their common names, parts used, family, traditional uses and phytochemical composition, is presented in Table I. There are a few reports available supporting the effectiveness of the ingredients (*G. sylvestre*, *T. foenum graecum*, *N. sativa*) of POL-4 in diabetes [13-16], however, little is known about the usefulness of *C. intybus*, while no reports are available for the effectiveness of polyherbal formulation (POL-4) effectiveness in diabetes. POL-4 has also been studied for its cardiovascular and antihyperlipidaemic effects [11, 12]. The current study validates the folk claim of traditional practitioners for the effectiveness of POL-4 in diabetes using OGTT assay and alloxan-induced diabetic rats.

**Table I**

Description of the components of polyherbal formulation (POL-4)

Components of POL-4 (herbs)	Common names	Parts used	Family name	Traditional uses	Phytochemical composition
<i>Nigella sativa</i> , L.	Kalonji/Black cumin	Seeds	<i>Ranunculaceae</i>	antidiabetic, antihyper-lipidaemic, diuretic, cardio- protective	Thymoquinone, volatile oil, saponins, flavonoids [13]
<i>Cichorium intybus</i> , L.	Kasni/ chicory	Seeds	<i>Asteraceae</i>	Cardiotonic, antidiabetic, cholesterol lowering effect	Caffeoylquininc acid, chlorogenic acid, flavonoids, phenolics, tannins [14]
<i>Trigonella foenum graecum</i> , L.	Methi-dana/ Fenugreek	Seeds	<i>Fabaceae</i>	Lipid lowering, cardio-protective, hypoglycaemic	Trigonelline, 4-hydroxyisoleucine, diosgenin, flavonoids [15]
<i>Gymnema sylvestre</i> , R.Br.	Gurmar-booti/ Ram's horn/ Periploca of the wood	Leaves	<i>Asclepiadaceae</i>	Antidiabetic, lipid lowering, cardio-effective, anti-obesity	Gymnemic acid, deacylgemnemic acid, saponins, gurmarin, flavonoids, phenolics [16]

## Materials and Methods

### Drugs, chemicals and kits used

Acetylcholine chloride, alloxan monohydrate, phenylephrine hydrochloride and glibenclamide were procured from Sigma (St. Louis, MO, USA). The kits for blood glucose, total cholesterol, triglycerides, low density lipoprotein-cholesterol and high density lipoprotein-cholesterol estimation were acquired from Roche Diagnostics and used Cobas c 111 for analysis. Anaesthetic agent (isoflurane) was received from Times Medicose Pharmacy outlet, Karachi, Pakistan. The various salts were mixed to prepare physiological solution which includes potassium chloride; potassium dihydrogen phosphate, sodium chloride, magnesium chloride, magnesium sulphate, sodium dihydrogen phosphate, calcium chloride, glucose and sodium bicarbonate were obtained from E. Merck, Darmstadt, Germany. Chemicals and reagents obtained for histological investigations containing formalin, buffer solution, ethanol, xylene, liquid paraffin, haematoxylin, eosin and DPX for mounting slides were available at "Aga Khan" University, Karachi, Pakistan. All the chemicals were of purity grade.

### Collection and identification of plants

Plants were purchased from herbal enterprise, Rehmania Pinsar Shop, Sargodha, Pakistan and validated by Taxonomist, Dr. Amin Shah, University of Sargodha,

Sargodha, Pakistan. The samples of ingredients of POL-4 were preserved in the herbarium under the following voucher numbers: Malik-632 (*N. sativa*), Malik-633 (*C. intybus*), Malik-634 (*T. foenum graecum*) and Malik-635 (*G. sylvestre*). The individual plant material was weighed, taken in equal part and combined ingredients of POL-4 were pulverized with herbal grinder and mixed thoroughly to get the compound herbal formulation, POL-4. The powdered materials were stored at 4°C in air tight jars.

### Preparation of the extract of polyherbal formulation (POL-4)

For the preparation of the aqueous-methanolic extract of POL-4, 500 g powdered material of POL-4 was soaked in defined proportion of aqueous-methanol (30:70) mixture for three days with occasional shaking. The initial filtrate obtained was subjected for filtration process applying a muslin cloth and passed through Whatman filter No.1. The method was repeated thrice and the resultant filtrates were mixed together and evaporated using rotary evaporator [17]. The obtained semi solid extract of POL-4 (POL-4.Cr) was air dried with resultant yield of 16% w/w.

### Phytochemical analysis

Qualitative phytochemical screening was carried out to determine alkaloids, anthraquinones, flavonoids, tannins, saponins, coumarins and terpenoids as constituents of POL-4 [18].

*Laboratory animals*

Sprague Dawley (SD) rats with weight ranges of 180 - 200 g and BALB/c mice (20 - 25 g) of both sexes were used in this study. Animals were sourced from Dow University of Health Sciences, Karachi and were bred at the animal house of "Aga Khan" University, Karachi, Pakistan. Animals were managed at  $23 \pm 2^\circ\text{C}$  and  $55 \pm 5\%$  relative humidity with twelve-hrs day/night cycles. There were kept in plastic cages containing sawdust which was replaced after every two days. Standard diet and drinkable water was given to all animals. The animals were acclimatized for 5 - 7 days prior to experimentation. The study scheme was approved (57-ECACUBBS-15) by Ethics Committee for Animal Care and Use (ECACU) of the "Aga Khan" University, Karachi, Pakistan, and was also in line with the rules of Institute of Laboratory Animal Resources, NRC (National Research Council) [19].

*Experimental design**Effect of POL-4 intervention on oral glucose tolerance test (OGTT) in rats*

SD rats were fasted for 12 - 14 hours and were divided randomly into five groups with 5 - 7 animals in each group for the OGTT assay. Group 1 served as normal control and received drinking water orally (10 mL/kg bw). Animals of group 2 were administered 2 g/kg bw of glucose load and served as OGTT control. Group 3 was administered glibenclamide (6 mg/kg bw, orally) dissolved in distilled water along with glucose load (2 g/kg bw). Group 4 and 5 were also administered hydro-alcoholic extract of polyherbal formulation (POL-4) at 150 and 300 mg/kg bw along with glucose load. After 30 min of oral glucose load (2 g/kg bw), blood samples were withdrawn from tail veins of rats at 0, 30, 60, 90 and 120 min intervals to estimate the blood glucose levels using Roche ACCU-Check glucometers.

*Effects of POL-4 intervention on alloxan-induced diabetic rats*

Freshly prepared alloxan monohydrate (135 mg/kg bw) was injected intraperitoneally once to SD rats on fasting (12 - 16 hrs), it is immediately transported to pancreas through glucose transporter 2, due to similarity with glucose, which imparts deleterious effects on the  $\beta$ -cells of the pancreas [20]. The animals were given free access to food and tap water during the experiment. Blood glucose was measured using ACCU Check glucometer and rats having glucose levels of  $> 250$  mg/dL were chosen in this study. The animals of group 1 ( $n = 6$ ) were administered normal saline only, while animals in group 2 - 5 ( $n = 6 - 7$  in each group) were administered the above mentioned amount of alloxan monohydrate at a single dose. The group 2 was kept as negative control, while group 3 was administered glibenclamide (6 mg/kg bw/day, orally), the standard oral hypoglycaemic agent. After fourth day of induction of diabetes in alloxanized

animals, the animals of group 4 and 5 were treated with POL-4 (3% and 6%) powder mixed in the diet for 21 days. Physiological parameters (food and water intake), biochemical parameters (glucose and lipids) were measured by using Cobas c-111 analyser at terminal day of the study.

*Measurement of endothelial reactivity in isolated rat aortae*

At the end of study, SD rats were kept fasted for 12 - 14 hrs, 2 - 5% v/w isoflurane was used as inhalational anaesthesia to achieve deep sedation and finally euthanization of these animals. Afterwards, dissection was quickly performed to remove carefully thoracic aorta. The descending thoracic aortic tube was isolated and kept in Krebs's solution for vascular tension assay to study the endothelial function. The aortic rings were fixed in a tissue bath assembly filled with Krebs's solution ( $37^\circ\text{C}$ ) and carbogen ( $\text{O}_2 = 95\%$  and  $\text{CO}_2 = 5\%$ ) bubbles. Force transducer (50-7905, Harvard Apparatus, USA) linked to a Trans-bridge (Model No. TBM4M, World Precision Instruments, UK) and PowerLab setup (ADInstruments, Australia) was used for the assessment of tissue responses. The aortic rings were stabilized to equilibrate at 1 - 2 g of resting tension for 45 min with change of buffer at every 15 min intervals. When rings got stabilized, acetylcholine (Ach;  $1 \times 10^{-8} - 10^{-4}$  mol/L) inhibitory response curves were constructed against the induced contractions of phenylephrine (P.E) concentration of  $1 \times 10^{-6}$  mol/L [21].

*Determination of blood pressure using non-invasive method*

Non-invasive blood pressure (BP) was measured at day 0 and at 2<sup>nd</sup> week of the study using plethysmography (Model No. 92, IITC Inc. USA) with tail cuff coupled with PowerLab data acquisition system attached to a computer with Lab Chart software (version 5.3) installed. Before starting the study protocol, SD rats were trained in the restrainers for the smooth BP measurements. When the animals became acclimatized to the training procedures, 3 - 5 readings of SBP (systolic blood pressure) within a time interval of 3 - 6 min were recorded in awaked rats and the readings were calculated according to mean values. Possible experimental variables such as body temperature ( $27^\circ\text{C}$ ) of the animals, respiration rate, body motions and intensity of noise were minimized [22].

*Collection of blood samples*

For terminal blood collection, the animals were starved for 12 - 16 hrs and anesthetized with inhalational isoflurane (2 - 5% v/w) in chambers until achievement of anaesthesia. By cardiac puncture procedure, blood was withdrawn from these animals in pre-labelled vacutainers. However, occasional blood samples for baseline levels were collected by puncturing tail vein of the rats. The serum was separated after centrifugation using Beckman centrifuge X-22 at 3000 revolution/min for 10 min. The pancreatic and aortic tissues were

isolated and from each animal and were preserved for histomorphological examination and endothelial reactivity studies, respectively.

#### Estimation of biochemical parameters

The serum total cholesterol, low density lipoprotein-cholesterol, high density lipoprotein cholesterol, triglyceride and glucose were estimated by means of Cobas c-111 auto-analyser diagnostic system (Roche Diagnostics Switzerland).

#### Histomorphological studies of isolated tissue specimens

At last day of alloxan-induced diabetic experiment, the animals were anaesthetized with inhalation route by using isoflurane solution of 2 - 5% v/w in a closed jar and were perfused with intra-cardiac infusion (cannulation with aorta of heart) of saline to wash the blood from whole body including pancreas. The tissue specimens from the pancreas and the aortae of the control and the treated animals were harvested. Further, pancreatic tissues were preserved and perfused with neutral formalin (10%) and later embedded in paraffin for 24 - 48 hrs. The paraffin blocks were cut off into 5  $\mu$ m sections and procedure of haematoxylin/eosin (H/E) staining were carried out for microscopic examination using light microscope. For studying pancreatic architecture, system microscope (Olympus DX 43, Tokyo, Japan) and photographic digital camera were used for estimation of islet numbers while for the measurement of islet diameter, calibrated micrometre was used [23]. The observed histomorphological changes of pancreatic tissues of untreated and treated animals were noted by comparing with the corresponding tissue slides of the disease control animals.

#### Acute toxicity testing in mice

For acute toxicity, BALB/c mice (20 - 25 g) of both sexes were selected and were distributed randomly in different groups of ten mice/group. The increasing doses (1, 3 and 5 g/kg bw) of POL-4 extract were administered to overnight fasted mice. The animals of the control group were administered normal saline (orally, 10 mL/kg). All mice had free access to water and food and were kept under observation for any toxic effects like gastrointestinal spasm, anorexia, diarrhoea and/or mortality for 48 hrs. After 48 hrs, the survived animals were euthanized for organ necropsy to check whether the extract of POL-4 caused any damage to the vital organs including heart, liver, kidneys, pancreas and intestines as described by [24].

#### Statistical analysis

Data is displayed as mean  $\pm$  S.E.M. For comparison of two groups, unpaired Student's t-test was applied, while for comparison of multiple groups with control, One-way ANOVA (analysis of variance) was used tailed by Tukey's post-test and/or Dunnett's test. The P-value ( $p < 0.05$ ) was considered significant statistically. All graphs were constructed and statistical analysis was carried out using GraphPad Prism software (version 4.00).

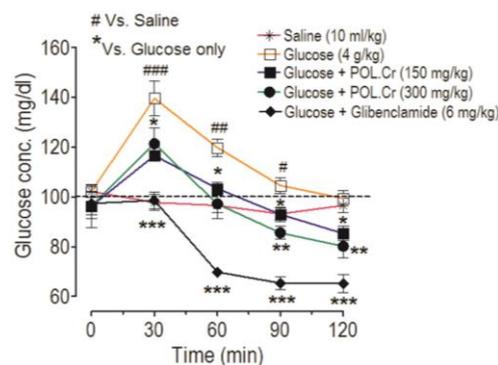
## Results and Discussion

### Phytochemical analysis

The qualitative phytochemical analysis of the aqueous-methanolic extract of POL-4 showed saponins, alkaloids, anthraquinones, flavonoids, tannins, coumarins and terpenoids as POL-4 constituents.

### Effect of POL-4 on oral glucose tolerance test in rats

The oral glucose tolerance test (OGTT) was carried out to assess the hypoglycaemic activity of the aqueous-methanolic extract of polyherbal formulation (POL-4.Cr). The solution of glucose (2 g/kg bw) was given to the normal rats. Glucose intake significantly ( $p < 0.001$ ) increased blood glucose levels in the animals at 30 min to 120 min after the glucose load when compared with untreated control group (Figure 1). The extract of POL-4 containing (150 and 300 mg/kg bw, orally) produced a significant ( $p < 0.05 - 0.01$ ) decline in glucose levels at 30 to 120 min intervals, with maximum effect at higher tested dose. Glibenclamide, an oral antidiabetic drug, administered at a dose of 6 mg/kg bw, also, showed marked ( $p < 0.001$ ) decrease at 30 to 120 min intervals when compared with glucose only administered animals at respective time intervals.



**Figure 1.**

Effect of POL-4 treatment on the oral glucose tolerance test (OGTT) in rats

Data is displayed as means  $\pm$  SEM. ns, non-significant, # $p < 0.05$ , ## $p < 0.01$  and ### $p < 0.001$  show a comparison of glucose-induced hyperglycaemic control with normal saline (Student's *t*-test), while \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  show a comparison of glucose-fed hyperglycaemic animals with animals of treatment groups (One way analysis of variance tailed by Dunnett's test). POL-4.Cr: the aqueous-methanolic extract of polyherbal formulation

### Effect of POL-4 treatment on physiological parameters in alloxan-challenged diabetic rats

When alloxan was administration to rats, it caused significant alterations in weight of the animals, systolic blood pressure (SBP), heart rate (HR), water and feed intake compared to data of animals fed normal diet (Table II). The POL-4 at 3 and 6% mixed in the normal diet produced significant ( $p < 0.01$ ) improvement in water/feed intake, body weight, SBP and HR with greater improvement at higher tested dose. A noticeable ( $p < 0.05$ ) increase in body weight was also observed

in the POL-4 treated animals compared to diabetic control animals (Table II).

On the other hand, glibenclamide (6 mg/kg bw) administration to the animals through oral route

showed a normalized trend in water intake, food intake and slightly increasing trend in body weight was observed when compared to diabetic control animals as seen in Table II.

**Table II**

Effect of POL-4 administration on body weight, water and food intake, systolic blood pressure and heart rate in alloxan-induced diabetic rats

Parameters	Normal diet (ND) treated	Diabetic control rats (Di)	Diabetic + GB (6 mg/kg bw)	Di + POL-4 (3%)	Di + POL-4 (6%)
Body weight (g/gp/week)	220 ± 8.2	162 ± 7.5 <sup>###</sup>	190 ± 4.6 <sup>***</sup>	200 ± 6.4 <sup>***</sup>	210 ± 5.6 <sup>***</sup>
Food intake (g/gp/day)	140 ± 3.0	130 ± 2.6 <sup>ns</sup>	136 ± 3.4 <sup>ns</sup>	133 ± 5.0 <sup>ns</sup>	139 ± 8.1 <sup>ns</sup>
Water intake (mL/gp/day)	125 ± 2.0	150 ± 5.3 <sup>#</sup>	112 ± 6.5 <sup>***</sup>	120 ± 4.7 <sup>**</sup>	128 ± 3.7 <sup>**</sup>
Systolic Blood Pressure (mmHg)	118 ± 4.5	151 ± 2.6 <sup>###</sup>	147 ± 3.5 <sup>ns</sup>	140 ± 2.8 <sup>**</sup>	133 ± 6.4 <sup>***</sup>
Heart rate (beats/min)	322 ± 3.5	358 ± 5.3 <sup>###</sup>	346 ± 7.6 <sup>ns</sup>	339 ± 6.2 <sup>**</sup>	328 ± 5.9 <sup>***</sup>

<sup>##/\*\*</sup>p < 0.01; <sup>###/\*\*\*</sup>p < 0.001; ns = non-significant; # – show a comparison of diabetic control and normal saline (Student-t-test); \* – show a comparison of all treatment groups with Di (One way analysis of variance tailed by Dunnett's test); POL-4 = polyherbal formulation; ND = normal diet; diabetic control (Di); GB = glibenclamide; gp = group.

*Effect of POL-4 treatment on blood glucose and lipid parameters in alloxan-fed rats*

Administration of alloxan, in all animals except the animals of group one (to whom it was administered only the normal diet), induced hyperglycaemia and hyperlipidaemia as detailed in Table III. POL-4 administration (3% and 6%) mixed in normal feed caused significant reduction in the fasting blood glucose

(FBG) and serum total cholesterol (TC), low density lipoprotein cholesterol (LDL-C) and triglycerides (TG), while a mild increase was observed in high density lipoprotein cholesterol (HDL-C) compared to only alloxan administered diabetic rats. Similarly, administration of glibenclamide decreased significantly the blood glucose levels and lipid parameters compared to diabetic control group (Table III).

**Table III**

Effect of POL-4 administration on serum blood glucose and lipid profile in alloxan-induced-diabetic rats

Parameters (mg/dl)	Normal Diet (ND) treated	Diabetic control rats (Di)	Diabetic + GB (6 mg/kg bw)	Di + POL-4 (3%)	Di + POL-4 (6%)
Glucose	86.47 ± 5.6	276.4 ± 15.2 <sup>###</sup>	98.4 ± 7.30 <sup>***</sup>	106.8 ± 7.8 <sup>***</sup>	94.5 ± 6.9 <sup>***</sup>
TC	92.43 ± 5.3	143.6 ± 3.7 <sup>###</sup>	99.5 ± 8.1 <sup>**</sup>	102.8 ± 3.20 <sup>**</sup>	96.83 ± 5.3 <sup>***</sup>
HDL-C	34.58 ± 2.9	20.1 ± 3.6 <sup>#</sup>	30.77 ± 1.02 <sup>*</sup>	30.5 ± 2.3 <sup>*</sup>	35.71 ± 2.66 <sup>**</sup>
LDL-C	57.50 ± 3.4	190.11 ± 4.7 <sup>###</sup>	59.17 ± 6.5 <sup>*</sup>	62.4 ± 3.6 <sup>**</sup>	58.2 ± 3.14 <sup>***</sup>
TG	82.33 ± 4.7	179.85 ± 6.7 <sup>###</sup>	94.0 ± 4.4 <sup>*</sup>	98.51 ± 4.3 <sup>*</sup>	87.62 ± 3.1 <sup>***</sup>

\*p < 0.05; <sup>##/\*\*</sup>p < 0.01; <sup>###/\*\*\*</sup>p < 0.001, ns = non-significant; # – show a comparison of diabetic control with normal saline (Student-t-test), \* – show a comparison of all treatment groups with Diabetic control group (One way analysis of variance tailed by Dunnett's test). POL-4 = poly herbal formulation; ND = normal diet; Di = diabetic control; TC = total cholesterol; HDL-C = high density lipoprotein-cholesterol; LDL-C = low density lipoprotein-cholesterol; TG = triglycerides; GB = glibenclamide.

*Effect of POL-4 treatment on endothelial dysfunction in alloxan-administered rats*

The animals in diabetic control group showed endothelial dysfunction which was evident in the development of endothelial resistance to the acetylcholine mediated inhibitory responses in the aortic rings of alloxan administered rats as seen in Figure 2. POL-4 administration at 3% and 6% in normal feed to alloxan-induced diabetic rats caused significant improvement in the endothelial dysfunction by enhancing acetylcholine (ACh)-induced relaxation to 55.24 ± 4.66 and 47.30 ± 3.4 vs. 22.91 ± 3.53%, (n = 4 - 6), respectively, at highest tested concentrations of ACh. The comparisons of concentration response curves of ACh in the aortae of various groups have been shown in Figure 2.

*Effect of POL-4 administration on histomorphometric characteristics of pancreatic tissues in alloxan-administered rats*

Haematoxylin/eosin (H/E) stained tissue sections of pancreas in alloxan-induced model, microscopic

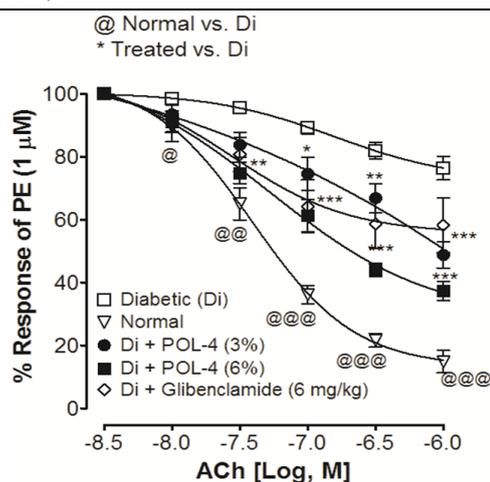
examination (for islet number using photographic digital camera, while for islet diameter measurement, calibrated micrometre was used) of alloxan treated group showed shrinkage of islets of Langerhans, beta cell degranulation, decreased numbers and size of islets of Langerhans compared to normal rats (having normal islet numbers, islet diameters, acinar cells, vacuolation and vascular supply) as seen in Figure 3. However, treatment with POL-4 (6%) and GB (6 mg/kg bw) showed revival and improvement in the numbers of islets of Langerhans and beta cells compared to only alloxan challenged animals as shown in Figure 3. When assessed for the effect on the number of islets of Langerhans, it revealed a significant reduction in POL-4 treatment group with respective value of 9.29 ± 2.58, n = 6 (p < 0.05 vs. only alloxan treated animals), as shown in Figure 4A. Similarly, mean values of islet diameter of all groups were measured. POL-4 administration showed a marked increase in islet diameter with respective mean value

of  $122 \pm 3.53 \mu\text{m}$ ,  $n = 5$  ( $p < 0.05$  vs. only alloxan treated animals) as seen in Figure 4B.

**Acute toxicity assay**

Oral incubation of POL-4 and its ingredients at 1, 3 and 5 g/kg bw in mice was devoid of any damage to vital organs, lethality or behavioural upsets such as writhing, palpitation or hyperventilation, lethargy, impaired motor actions mice within 48 hrs. POL-4 and its ingredients were found safe up to test dose of 5 g/kg.

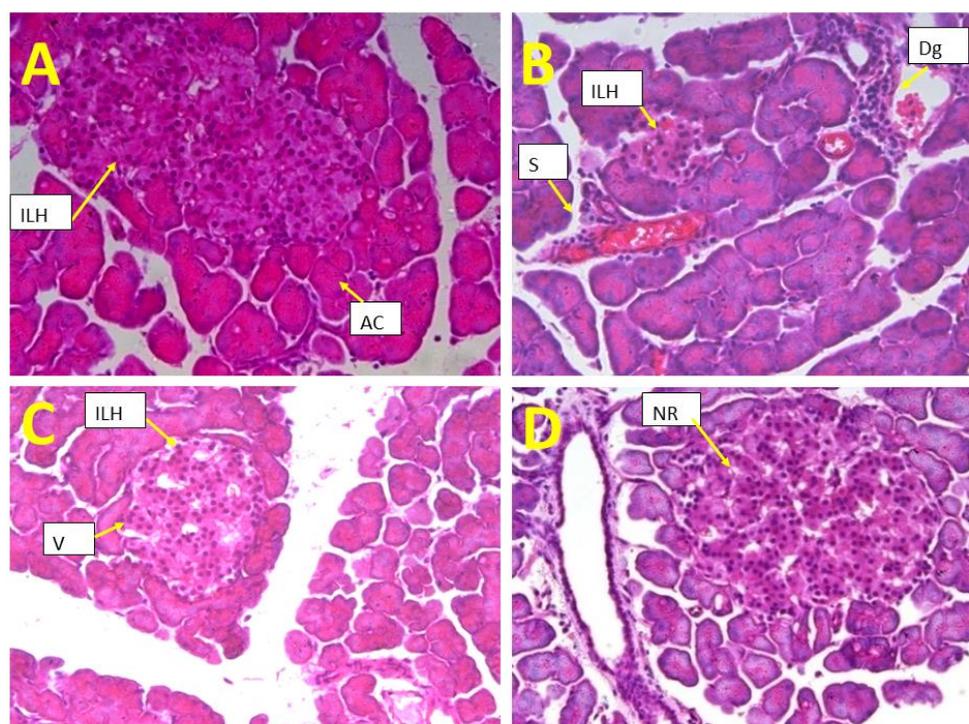
Diabetes a commonly prevailing endocrine disorder labelled with sustained hyperglycaemia develops due to defect in insulin secretion and/or in its action. Increased hepatic gluconeogenesis and/or insulin resistance due to its less peripheral utilization are known to promote progression of diabetes. It is a major risk factor for cardiovascular disorders (CVDs), a common cause of mortality and morbidity among diabetic patients. Diabetes is associated with multiple complications like hyperlipidaemia, retinopathy, nephropathy, neuropathy, coronary artery and cerebrovascular diseases, thus posing a major health burden in societies across the globe [25].



**Figure 2.**

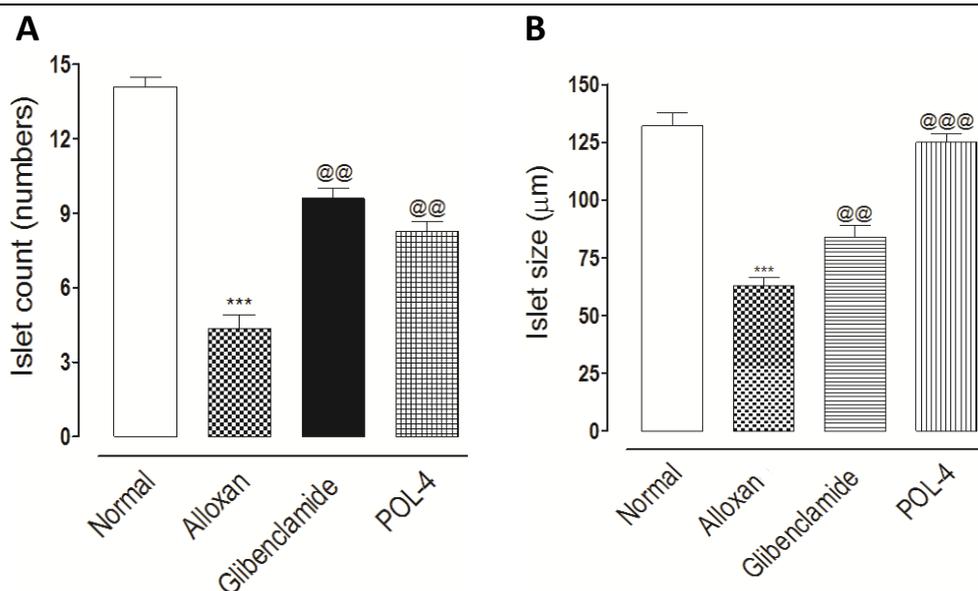
Effect of POL-4 intervention on endothelial dysfunction in alloxan-induced diabetic rats

Data depict means  $\pm$  S.E.M of 4 to 6 sets of experiments; @ $p < 0.05$ , @@ $p < 0.01$  and @@@ $p < 0.001$  show a comparison of diabetic control (Di) with normal diet administered animals (Student's *t*-test). \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  show a comparison of all treatment groups with diabetic control (One way analysis of variance tailed by Tukey's post-test). POL-4 = polyherbal formulation-4, Di = diabetic control, GB = Glibenclamide



**Figure 3.**

Effect of POL-4 (6%) administration on haematoxylin/eosin (H/E) stained pancreatic tissues observed at 400X showed the clusters of beta cells on central, while alpha cells in periphery  
Arrows show islets of Langerhans (ILE), shrinkage (s), acinar cells (Ac), normal revived/restored (NR) beta cells, degranulation (Dg) and vacuolation (V)  
(A) Pancreatic tissues of animals on normal diet (B) Pancreatic tissues of alloxan- induced diabetic animals (C) Pancreatic tissues of alloxan- fed + glibenclamide (6 mg/kg) treated animals (D) Pancreatic tissues of alloxan-fed + POL4 (6%) administered rats



**Figure 4.**

Effect of POL-4 (6%) intervention on (A) size ( $\mu\text{m}$ ) and (B) number ( $\text{N}/10 \text{ mm}^2$ ) of islets of Langerhans in alloxan-induced diabetic rats

Data show the means  $\pm$  S.E.M of 5 to 6 tissues from different animals; \*\*\* $p < 0.001$  show a comparison of alloxan administered rats with normal rats (Student's *t*-test), while @@ $p < 0.01$  and @@@ $p < 0.001$  show a comparison of treatments with only alloxan-fed animals (One way analysis of variance tailed by Dunnett's test). POL-4 = polyherbal formulation-4

To address this multifactorial endocrine dysfunction, among multiple treatment modalities, the use of natural products has gained popularity for its therapeutic success and wide acceptance in different cultures. It has been widely attested that folk or ethno-medical information on medicinal herbs show the presence of biological constituent(s) which represent 'leads' that could shortcut the discovery of modern medicines. It has also been estimated that  $> 80\%$  of people, equally in developed and emerging countries depend on traditional medicine to meet health care needs. Owing to lack of fully developed health care systems in developing countries, people with chronic diseases including diabetes, are among the worst victims [26]. In majority of traditional systems, diabetes is better managed by the herb combinations (poly herbal formulation) instead of prescribing a single herb, perhaps because of synergistic effectiveness and relatively lesser adverse effects [27].

Based on the folk use of POL-4 by traditional practitioners to treat diabetes in district Badin, Sindh, Pakistan, this study has been designed to provide an evidence to the medicinal use of POL-4 in diabetes and associated dysfunctions such as hyperlipidaemia, endothelial dysfunction and hypertension. Its antihyperglycaemic effects were observed through oral glucose tolerance test (OGTT) in rats, where the aqueous-methanolic extract of POL-4 (150 and 300 mg/kg bw) displayed prominent attenuation in the blood glucose levels similar to the effect of glibenclamide, a standard hypoglycaemic agent.

Administration of alloxan is well known in experimental animals to develop hyperglycaemia, hyperlipidaemia, endothelial dysfunction and hypertension. It is also known to charge histopathological changes in pancreatic tissues [28]. Alloxan is analogous to glucose which enters *via* glucose transporter-2 ( $\text{GLUT}_2$ ) and accumulates in beta cells of pancreas. It has been used as diabetogenic agent to induce experimental diabetic animal models for the evaluation of synthetic and natural compounds. In addition, alloxan is converted into more toxic compound dialuric acid which further generates toxic free radicals like hydrogen peroxide, superoxide radicals and iron-hydroxyl radicals, which are responsible for beta cells necrosis and also lower the antioxidant defence mechanism that results in development of diabetes [29].

Administration of POL-4 powder (3% and 6% mixed in diet) to alloxan-fed diabetic rats, showed significant protection against raised blood pressure, heart rate, serum glucose and lipid parameters except high density lipoprotein cholesterol, which was slightly increased. The polyherbal intervention has also improved endothelial dysfunction and histomorphological alteration in pancreatic tissues. These results are similar to Jackson and Bressler [30] who observed that sulfonylureas have an effect not only on insulin release but also a positive impact on pancreatic tissue architecture. These findings on the part of POL-4 may add possible mechanism(s) to its therapeutic potential in diabetes. Moreover, insulin is well known to regulate lipid metabolism as well by inhibiting lipolysis through hormone-sensitive lipases in adipose tissues [31].

Superoxide and hydrogen peroxide is enhanced in diabetes through oxidation of glucose, glycation (non-enzymatic) of proteins and oxidative degradation of glycated proteins molecules. Imbalance between enhanced production of free radicals and a resultant decline in the antioxidant defensive mechanisms leads to cellular and enzymatic deterioration, while sustained increased lipid peroxidation mediates onset of insulin resistance and/or diabetes. These pathways of oxidative stress equally contribute in establishment of diabetes and related complications [32].

The observed antihyperglycaemic, antihyperlipidaemic, endothelial modulating and antihypertensive properties of POL-4 might be mediated by the known presence of antioxidant constituents of *N. sativa*, *G. sylvestre*, *T. foenum graecum* and *C. intybus* which have also shown their effectiveness in diabetes when studied using different models either as individual herb or in combination with other herbs [13, 15, 33-38]. Similarly, hydroxyisoleucine, the chemical constituents of *T. foenum graecum* is known to block inflammatory pathways and reduces symptoms of underlying disease [39]. This study may attest the selected combination of constituents of POL-4 has promising protective effects against diabetes; hence this formulation may further be subjected for chemical standardization and clinical trials to optimize its clinical efficacy in diabetes.

## Conclusions

This study shows that polyherbal formulation (POL-4) possesses antihyperglycaemic, antihyperlipidaemic, endothelial modulating and antihypertensive properties possibly owned because of abundant presence of antioxidant constituents in the ingredients of POL-4, and or its inhibitory potential either on intestinal lipid absorption or lipid molecule synthesis. Thus, this study provides experimental evidence to the folk medicinal utility of POL-4 in diabetes and associated disorders.

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## Conflict of interest

The authors declare no conflict of interest.

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