Indian Journal of Traditional Knowledge
Vol 19 (1), January 2020, pp 137-144

Hypoglycemic and hypolipidemic effects of a poly herbal formulation in streptozotocin induced diabetic rats

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Received 26 September 2018; revised 18 October 2019

Diabetes mellitus is a chronic disease with several comorbidities. Currently used synthetic medicines have various side effects. Herbal medicines are being widely used for management of diabetes. The present study was conducted to evaluate the effect of poly herbal formulation (PHF) (mixture of *Citrullus colocynthis*, *Syzygium cumini* and *Holarrhena antidysenterica)* in diabetic rats. We evaluated enzyme inhibition activity of PHF by ELISA and anti-hyperglycemic action was assessed in streptozotocin induced diabetic rats. Animals were divided into 5 groups, i.e., blank, negative control, standard control and PHF 250 and 500 mg/kg dosage respectively. Blood glucose level assessed through one touch method blood glucose monitoring system. The statistical analysis was performed by using one-way ANOVA p value of ≤ 0.05 was considered significant. Results of study indicated that PHF sufficiently inhibited DPPH at 0.5 mg/mL and serial dilutions with IC_{50} of 197 µg/mL while α- glucosidase inhibition assay showed marked inhibition of PHF at 0.5 mg/mL and serial dilutions with IC_{50} of 235 µg/mL. Oral intake of PHF reduced blood glucose level, serum triglyceride and cholesterol levels in wistar albino rats in dose dependent manner. Considering the multidirectional effects of PHF, it is concluded that PHF may be a therapeutic option in management of diabetes.

Keywords: Antidiabetic, Antioxidant, Poly herbal formulation

IPC Code: Int. Cl.[20]: A61P 3/10, A61P 17/18, A61K 39/00

Diabetes Mellitus (DM) is a chronic disease having significant morbidity and mortality. Its risk is increasing tremendously all over the world[1]. DM is one of the most commonly found diseases affecting people of both civilized and non-civilized countries. It is caused by abnormal metabolism of carbohydrates, proteins and lipids that leads to severe complications[2]. It is presumed that DM will be the one of the largest non-contagious disease in the world by year 2025 with the major diabetic population in developing world[3]. Lacking of systematized health care system in developing countries causes patients of chronic diseases like DM among the worst sufferers in community. Moreover, very few people has access to the modern medicine and they rely mostly on medicinal flora of their native lands for management of diabetes and its complication[4].

Herbal medicines are being widely used for combating DM. Several individual herbs have reported anti-hyperglycemic effect in animal and human trials[5] and a number of marketed herbal preparation having anti-diabetic activity[6]. Herbal preparations are the basis for finished herbal products and may include comminuted or cut herbal materials, or extracts, tinctures and fatty oils of herbal materials. They are produced by extraction, fractionation, purification, concentration, or other physical or biological processes[7].

A herbal formulation having antioxidant, anti-hyperglycemic, hypolipidemic effects is the most reliable strategy to combat DM as it may be caused by reactive oxygen species such as hydroxyl radicals

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(OH) and superoxide anion radical (O_2), etc., and DM is mostly associated with hyperlipidemia[8].

Citrullus colocynthis, *Syzygium cumini* and *Holarrhena antidysenterica* are the plants with reported antihyperglycemic, hypolipidemic and antioxidant potential. Moreover, they are used traditionally to treat diabetes. (Table 1) *C. colocynthis* is among the most common traditional plants used as remedy against diabetes mellitus in the subcontinent since long. *C. colocynthis* fruit extract has reported in vitro antioxidant potential[9,10], *in vitro* α-glucosidase inhibition[11], in vivo anti-hyperglycemic effects[12,13]. A randomized clinical trial also proved *C. Colocynthis* fruit capsules (100 mg) as an effective and safe anti-diabetic intervention compared to placebo[14].

The anti-diabetic activity of *S. cumini* has been studied since long for its anti-diabetic properties the pulp bark and seeds, have shown potent anti-diabetic actions[15,16]. Different extracts of seeds of *S. cumini* are the most studied and effective hypoglycemic agent reported in study of different experimental models[17-21]. *S. cumini* seeds are also said to prevent the secondary complications induced by diabetes like nephropathy, gastropathy, neuropathy and diabetic cataract[22]. *S. cumini* showed promising hypoglycemic effects when studied in human subjects[23]. Moreover, *S. cumini* seeds have radical scavenging properties against DPPH, superoxide and hydroxide scavenging assays[24,25]. *H. antidysenterica* seed extracts have significant anti-diabetic effects *in vivo*[26-28]. Hypoglycemic effect of *H. antidysenterica* seeds on streptozotocin induced diabetic rats. and inhibit α - glucosidase *in vitro*[29]. No metabolic toxicity was reported in kidneys and liver[28].

There is a need to search compounds having antioxidant and anti-diabetic potential from herbal sources to combat DM as it may be caused by reactive oxygen species such as hydroxyl radicals (OH) and superoxide anion radical (O_2), etc. Moreover, the present synthetic drugs have many side effects such as chronic tissue damage, hypoglycemia and weight gain[30].

Table 1 — Plants used in formulation

Sr.	Plants	Reported activities
1.	*Citrullus colocynthis*	Antioxidant[9,10], anti-hyperglycemic[12,13], antihyperlipidemic[47]
2.	*Syzygium cumini*	Antioxidant[24,25], anti-hyperglycemic[17-21], antihyperlipidemic[48]
3.	*Holarrhena antidy senterica*	Antioxidant[49], anti-hyperglycemic[26-28], antihyperlipidemic[28]

The primary objective of study was to formulate and validate an anti-diabetic poly herbal formulation (PHF) consisted of three valuable anti-diabetic plants namely *C. colocynthis* (Fruit), *S. cumini* and *H. antidysenterica* (seeds). Secondary objective of study was to check effect of PHF on triglyceride and cholesterol levels.

Methodology

Chemicals

Streptozotocin (Bio word), Glibenclamide (SANOFI, Pakistan, Limited), Galoxose D (Glaxo Smith Kline), Triglycerides liquicolor, Cholesterol liquicolor (Human Diagnostic, Germany), Ketamine (Budapest, Hungary) and Xylazine (Prix Pharmaceutica, Lahore), α-glucosidase, Acarbose, Ascorbic acid and DPPH (Sigma Aldrich).

Plant Material and Extraction

The plant materials were collected from nearby market of Bahawalpur. They were identified and authenticated by botanist from Department of Botany, The Islamia University of Bahawalpur (Voucher no 2201/L.S) and deposited in herbarium, IUB. The plant materials were dried in shade and screened carefully to remove any extraneous matter or adulteration. They were subjected to coarse grinding using grinder. Grounded plant materials were macerated with 80% methanol for 15 days with frequent stirring. After 15 days, soaked plant material was filtered first with muslin cloth followed by filtration through whatmann filter paper. The process was repeated thrice with interval of 15 days. The filtrate was subjected to evaporation at low temperature (30 to 40°C) and under reduced pressure using rotary evaporator (Heidolph Laborota 4000 efficient, Germany). A crude thick viscous paste of each plant was obtained. These were crude extracts of plants[31].

Poly Herbal Formulation (PHF)

PHF was prepared by taking extracts of *S. cumini* (seeds), *H. antidysenterica* (seeds), *C. collocynthis* (fruit) in specific ratio based on Pari L & Saravanan R methodology[32]. At last PHF was weighed, labeled and stored in air tight amber colored glass container for further *in vitro* and *in vivo* experiments. For *in vivo* experiment, the dose of PHF was selected by

performing an effective dose fixation study method with slight modification[33].

In vitro Antioxidant Assay

DPPH free radical scavenging Assay

The scavenging activity of herbal formulation was checked with 2, 2 diphenyl 1, picrylhydrazyl radical by a micro assay with some modifications[34]. Total assay volume was 100 µL. 90 µL of DPPH solution and 10 µL different serial dilutions of herbal formulation were placed in wells of a 96 well micro plate. The wells containing 90 µL DPPH and 10 µL of methanol was used as negative control. Ascorbic acid was taken as standard control. The whole process was done in triplicate. The reaction mixture was placed for 30 min at 37°C. The absorbance was measured at 517 nm by using Bio-tech ELISA micro plate reader. The percentage inhibition was taken as follows:

Scavenging activity % = 100 – (A*f*/ Ac) * 100

Where,

Ac = Absorbance of negative control

A*f*= Absorbance of herbal formulation

In vitro Anti-diabetic Assay

α-glucosidase Inhibition Assay

α-glucosidase inhibitory assay was performed with the method of[35] with some modification. Total assay volume was 100 µL. 10 µL of PHF (serial dilutions), 10 µL of α-glucosidase (0.5 unit/mL) and 70 µL of 0.1 M phosphate buffer pH 6.8 per well of 96 well plate were incubated for 15 min at 30°C. 10 µL of p-Nitrophenyl-α-D-glucopyranoside substrate solution was added and incubated for an additional 30 min. Absorbance was measured with HT BioTek microplate reader at 400 nm. The reaction system without sample (only methanol) was used as control and acarbose was used as standard control. Each experiment was conducted in triplicate. The enzyme inhibitory rates of samples were calculated as follows:

% inhibition= 100- (A*f*/ Ac) * 100

Where,

Ac = Absorbance of negative control

A*f*= Absorbance of herbal formulation

In vivo Anti- diabetic Activity

Experimental Animals

Male Wistar Albino rats weighing from 200-250 g were obtained from animal house of the Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur, Pakistan. All rats were acclimatized at room temperature of 20-22°C and at 45-55% relative humidity for 12 h, each of dark and light cycle. Rats were placed in polycarbonate cages (47×34×18 cm^{3}) with a maximum of 6 animals per cage. They were fed with a standard diet and water provided *ad libitum*. The experiment performed followed ethics declared by Animal Welfare Division of the Ministry of Environment & Forest, Council of International Organization of Medical Sciences. The whole manuscript complied according to the ARRIVE guidelines[36]. The experimental protocol was approved from Pharmacy Research Ethics committee (PREC), The Islamia University Bahawalpur Ref No. 37-2015/PREC date 08-09-2015 and is in accordance with the guidelines of committee for the purpose of experiments on animals.

Experimental Protocol

The animals were divided in to 5 different groups and each group contain 6 animals. Sample size was calculated through "resource equation" method[37].

Twelve hours before the experiment, the animals were fasted overnight but allowed for free access to water. Diabetes was induced by intra peritoneal injection of STZ to animals by the methodology of Zhang *et al*[38]. Normal rats were injected with the equivalent volume of citrate buffer. STZ-induced diabetic animals with diabetic status (Fasting Blood Glucose ≥ 250 mg/dL) for 2 weeks were taken for the study. After confirmation of diabetes, animals were allowed to stabilize for one week. The treatment was started on the same day considering it day one of the study. The vehicle, glibenclamide and poly herbal preparation were given orally through intra gastric tube for 14 days daily.

Blood Sampling and Biochemical Analysis

Blood samples were collected from the rat tail vein. The blood glucose (BG) levels of the rats were monitored daily by using one-touch blood glucose monitoring system. The hypoglycemic effect in blood glucose level was monitored on 0, 1st, 5th, 8th, 11th, and 14th day of induction. After 14th day of treatment, rats were fasted overnight. The blood for further biochemical screening was collected by cardiac puncture (anesthetized with 0.2 mL/100 g Ketamine/ Xylazine combination) and allowed to clot for 15 min. Serum was separated by centrifuging blood at 4000

rpm for 10 min. The animals were gently sacrificed by cervical decapitation.

Biochemical analysis for cholesterol and triglyceride was performed by using standard kits. The results were then obtained by running the respective samples, standards and blanks on micro lab 300 (Merck, Germany).

Statistical Analysis

All the results obtained were represented as Mean ± SEM. One way Analysis of variance for repeated measures (ANOVA) followed by LSD post hoc test was used to assess statistical significance. p value ≤ 0.05 was considered significant.

Results

A preliminary phytochemical analysis of PHF showed the presence of phenols, flavonoids, tannins, saponins and alkaloids. (Table 2)

Results of DPPH inhibition assay displayed that PHF was able to decolorize DPPH and therefore possess antioxidant potential by trapping free radical. DPPH is purple in color and contains an odd electron. When hydrogen from an antioxidant compound binds to odd electron of DPPH to form DPPH-H, DPPH turns to yellow[25]. PHF sufficiently inhibited DPPH at 0.5 mg/mL and serial dilutions with IC_{50} of 197 µg/mL. Results of *in vitro* α- glucosidase inhibition assay showed marked inhibition of PHF at 0.5 mg/mL and serial dilutions with IC_{50} of 235 µg/mL. (Fig. 1)

Dose fixation studies indicated the non-toxic nature of PHF in the dose range between 1 and 5 g/kg body weight in normal rats. There was no mortality or side effects in the rats treated with PHF at all the doses. 500 mg/kg was selected as the highest dose (1/10th of highest dose of 5 g/kg) for antihyperglycemic activity. The rats were also treated with 250 mg/kg dose of PHF.

The baseline characteristics of all the rats included for anti-diabetic study were same. (Table 3) Intra peritoneal injection of streptozotocin during *in vivo* experiment caused hyperglycemia in rats, as indicated by drastic increase in serum glucose levels. Initially serum glucose levels were insignificantly decreased after PHF administration but continuous administration for 11 and 14 days showed a marked significant decrease in serum glucose levels. PHF (250 mg/kg) showed a gradual decrease in blood glucose level from 355.3±3 mg/dL to 313.5±2 mg/dL, 281.8±42 mg/dL, 256.0 ± 4 mg/dL at 8th, 11th and 14th day of administration respectively. Moreover, 11 and 14 day treatment of PHF (250 mg/kg) was found to be as

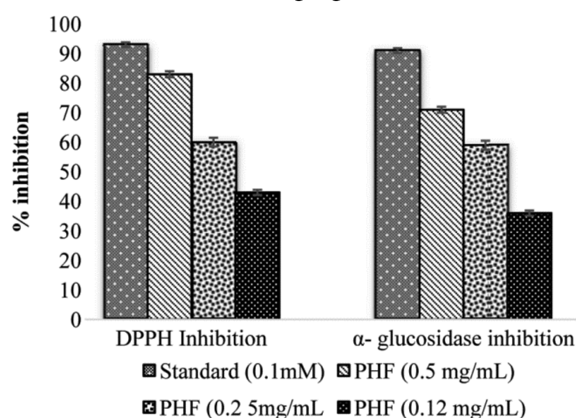

Fig. 1 — Antioxidant and α- glucosidase inhibitory potential of PHF (serial dilutions) and standards. DPPH Inhibition standard (ascorbic acid), α- glucosidase standard (Acarbose).

Table 2 — Preliminary phytochemical analysis

Sr. No	Chemical constituents	Tests Name	Result of sample
1	Saponin	Froath test	+
		Emulsifying properties	+
2	Anthraquinone glycosides	Borax test	+
		Bromine water test	+
		Nitric acid test	+
		Borntrager test	+
3	Phenolic glycosides	Erdmann's test	+
4	Alkaloids	Mayer's reagent	+
		Wagner's reagent	+
		Hager's reagent	+
		Dragendorff's reagent	+
5	Tannins	Ferric chloride test	+
		Bromine water	-
		Formalin test	-
		Sodium nitrite test	-
6	Flavonoids	Sodium hydroxide test	+

effective as glibenclamide (600 µgm/kg) which was taken as standard control of the study as shown in Table 4.

Similar to the above results, a time-dependent decrease in blood glucose levels was also observed after administration of PHF (500 mg/kg). 8-day administration of PHF at 500 mg/kg insignificantly reduced blood glucose levels in rats, though still above the normal control level, while 11 or 14 day treatment with PHF at 500 mg/kg was effective enough for a thorough recovery, which was significantly lower than that after 8-day administration. Administration with glibenclamide at 600 µg//kg for 8 days reduced blood glucose levels markedly and further significant decrease in the levels was achieved when more treatments were given.

PHF lowered the serum triglyceride and cholesterol levels in diabetic rats significantly as compared to negative control. (Fig. 2 & Fig. 3)

Discussion

The present study reveals that the PHF possess antioxidant and α- glucosidase inhibition *in vitro* and was able to significantly reduce blood glucose levels in rats. Moreover, PHF has significant impact on serum cholesterol and triglycerides. The activities of the individual plant components of the PHF are well known for their antioxidant, antihyperglycemic and anti hyperlipidemic effects. (Table 1)

Preliminary phytochemical analysis of PHF showed presence of antioxidant phytochemicals as alkaloids and flavonoids. Previously reported study suggested that flavonoids are the main component in plant materials

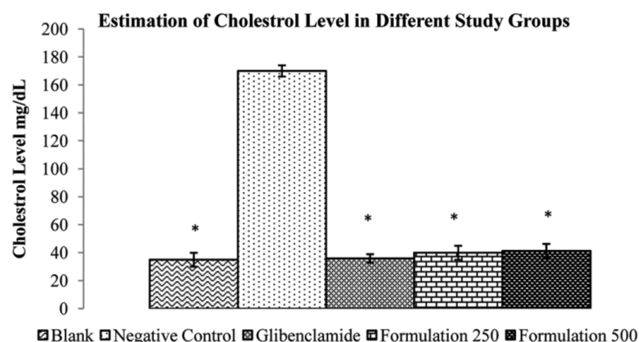

Fig. 2 — Effect of PHF on serum cholesterol levels. * p≤0.05 as compared to negative control

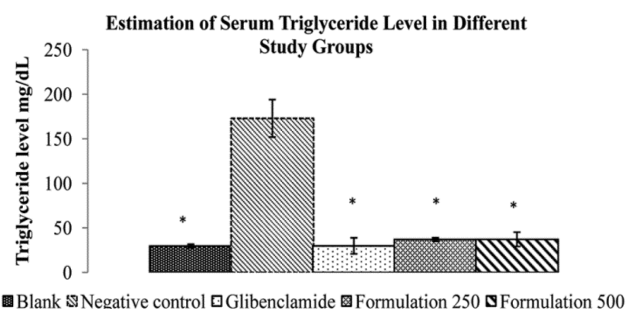

Fig. 3 — Effect of PHF on serum triglyceride levels. * p≤0.05 as compared to negative control

Table 3 — Baseline Characteristics of different groups

Baseline Characteristics	Baseline data of different groups.				
	Blank	Negative control	Standard control	PHF (250)	PHF (500)
Body weight (g) (Mean ±S.E.M)	203 ±5	201 ±8	203 ±5	204 ±4	202 ±3
Blood Sugar level (mg/dL) (Mean ± S.E.M)	118.6 ± 5	123.3 ± 3	126.0 ± 2	122.8 ± 2	120.6 ± 3

*There is no significant difference between any of the groups.

Table 4 — Time dependentEffect of Poly Herbal Formulation on blood glucose level

Groups	Before Treatment Blood Sugar level (mg/dL)		After Treatment Blood Sugar level (mg/dL)		
	0 Day	5 day	8th Day	11th Day	14th Day
Blank	118.6±5	117.8±4	114.1±2#	114.6±3#	118.6±4#
Streptozotocin	123.3±3	364.6±6	412.3±6	430.6 ±7	395.2±5
Glibenclamide	126.0±2	355.3±3	290.1±4*	271.8±2†	221.5±2#
PHF 250 mg/kg	122.8±2	350.1±5	313.5±2^	281.8±42†	256.0±4†
PHF 500 mg/kg	120.6±3	351.3±4	320.0±4^	283.5±3†	258.5±2†

Note: *p≤0.05, †p≤0.01, #p≤0.001, ^ insignificant compared to negative control. Values are expressed as mean ± SEM (n=6), 0 Day reading is normal sugar level of rats before streptozotocin administration, before treatment 5 day reading is after hyperglycemia induction reading. 8th to 14th day readings are after medicine administration.

for amelioration of diabetes mellitus and other pathophysiologic disorders that are caused by oxidative stress[39]. Diabetes is associated with much comorbidity including increased superoxide production, lipid peroxidation, glycation of the lipoproteins and oxidative DNA damage. Antioxidants are helpful in management of diabetes and many complications of diabetes can be tackled by appropriate antioxidants administration along with anti-diabetics[40].

α-glucosidase inhibitors are used as a therapeutic approach for combating hyperglycemia as it is the key enzyme in biosynthesis of glycoproteins and cleavage of glycosidic bond[11]. PHF exhibited the strongest inhibition of α-glucosidase. This is depicted by low IC_{50} generated however higher than acarbose. The previous studies mentioned that an ideal anti-diabetic agent should have a strong inhibition of α-glucosidase[30]. α- glucosidases from yeast are maltooligosaccharides hydrolyzes heterogeneous substrates such as sucrose. α-glucosidase inhibitors decreased postprandial hyperglycemia[11].

In vivo experimentation showed a drastic increase of blood glucose levels as compared to baseline after streptozotocin administration. Low-dose STZ induces a mild impairment of insulin secretion and hence causes hyperglycemia and symptoms similar to Type 2 diabetes[38].

Oral administration of PHF was able to significantly reduce blood glucose levels of diabetic rats in a time-dependent manner. However, the onset of hypoglycemic action of PHF appeared to be slower than that of glibenclamide. Glibenclamide elicit its hypoglycemic action on 8th day and decreased the elevated blood glucose levels to the significant extent. After 11 days or more of PHF administration, there was really no significant difference between PHF and glibenclamide in lowering blood glucose levels in diabetic rats. The mechanism of action of PHF in diabetes is not fully understood. The mechanism of individual plants was somewhat described in literature. *S. cumini* may have antihyperglycemic effects by insulinase inhibition[19]. A study indicated the presence of mycaminose ($C_8H_{17}NO_4$) in *S. cumini* as an anti-diabetic agent[41]. Beta-pyrazol-1-ylalanine is an insulin secretagogue present in *C. colocynthis*[42]. Saponins and glycosidic components in *C. colocynthis* are responsible for its antihyperglycemic action[19]. So it can be postulated that PHF may act as secretagogue and insulinase inhibitor as individual plants of PHF act through this mechanism. Moreover, PHF also contain saponins and glycosides (Table 2), that might be responsible for its antihyperglycemic action.

Diabetes is mostly associated with hypertriglyceridemia[43]. Hormone sensitive lipoprotein lipase that is responsible for hydrolysis of triglyceride is stimulated by insulin under normal circumstances. Increase in level of triglyceride occurs when deficiency of insulin leads to deactivate the lipoprotein lipase[44,45]. Hypertriglyceridemia and hypercholestrolemia is accompanied with high risk of coronary heart diseases, myocardial infarction, atherosclerosis and stroke[46]. PHF showed marked reduction of serum triglycerides and cholesterol in addition to hypoglycemia.

Conclusion

PHF consisted of *S. cumini* (seeds), *H. antidysenterica* (seeds), *C. colocynthis* (fruit) has significant antioxidant, antihyperglycemic action and hypolipidemic potential. So, this herbal formulation can be used as an effective treatment option for management of diabetes and its related complications. However, firm conclusion regarding use of this PHF as therapeutic agent can't be made due to caveat of small sample size in current study.

Acknowledgement

We are very grateful to Dr Qaiser Jabeen Associate Professor Department of Pharmacy, Faculty of Pharmacy and Alternative Medicine for providing animals and facilities to conduct this study.

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