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Development of a Poly Herbal Capsule Utilizing Ethanolic Extracts of Withania Coagulans, Picrorhiza Kurroa, and Gymnema Sylvestre

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ABSTRACT: Diabetes mellitus, a high-morbidity endocrine disorder, is often managed with expensive synthetic hypoglycemic agents that possess significant adverse effects. Natural products such as Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre provide a cost-efficient, readily available, and environmentally sustainable option. The present research aims to investigate the antidiabetic efficacy of a herbal formulation (PHF) derived from Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre in streptozotocin-induced diabetic rats. To this end, five distinct groups of Wistar rats were formed, including one control group. Within the four diabetes cohorts, one group functioned as the diabetic control, while the other two groups received PHF at dosages of 200 mg/kg and 400 mg/kg, respectively, and the fifth group was treated with metformin for a duration of 28 days. On the last day of the experiment, all rats were euthanized, and samples were obtained for various analyses. The research results indicated that the administration of PHF to diabetic rats led to significant ($p < 0.001$) enhancements in fasting blood glucose (FBG) levels, glycated hemoglobin, and other biochemical indicators. The histological analysis demonstrated the preventive effect of PHF against diabetes-induced damage in the pancreas, kidneys, and liver. It also facilitates the regeneration of β-cells in the pancreas. These data indicate that PHF is a safer and more effective choice for diabetes management.

INTRODUCTION

Diabetes is an endocrine disorder that presents a significant risk to life, accompanied by substantial economic and social repercussions. A recent update from the IDF indicates that one in eight worldwide fatalities is attributable to diabetes [1, 2]. Diabetes mellitus (DM) is a complex form of diabetes, marked by elevated blood glucose levels and disruptions in the metabolism of glucose, lipids, and proteins. Complications related to diabetes mellitus include foot ulcers, cardiovascular disorders, diabetic nephropathy, and retinopathy [3]. In 2021, there were 6.7 million fatalities globally attributable to diabetes, and over 541 million individuals were diagnosed with impaired glucose tolerance. The figure is projected to increase from 541 million to 783.2 million adults by 2045 [4, 5]. Pakistan is among the most impacted nations, with an estimated 33 million individuals diagnosed with diabetes. Financially, global health costs for diabetes were estimated at 966 billion USD in 2021 and are anticipated to rise to 1054 billion USD by 2045. Furthermore, the incidence of diabetes is anticipated to rise in the forthcoming years inside emerging countries. This concerning tendency is intensified by insufficient methods for the investigation and treatment of these disorders [2]. Presently, existing medications just aim to reduce blood glucose levels, and their prolonged use induces significant adverse effects such as impaired vision, stomach discomfort, alopecia, rashes, blisters, and nausea. Synthetic medications like metformin and phenformin are restricted owing to their detrimental effects on diabetic patients. Consequently, there is an urgent want for more effective and safer treatment options to mitigate these adverse

effects and consequences. Natural products have been shown as an alternative therapy for diabetes mellitus due to their cost-effectiveness, safety, and good efficacy [6–8]. These options are used as adjuncts or substitutes for traditional therapies for diabetes, cardiovascular disease, and cancer. Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre have a longstanding and traditional history of medical use in Asian nations. Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre possess a variety of natural bioactive substances that may effectively treat diabetes. Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre are nutrient-rich and essential to our nutrition. Numerous studies have investigated the therapeutic properties of Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre in treating various conditions, particularly emphasizing their potential for diabetes management. This area of research is gaining prominence as scholars examine the underlying mechanisms and antidiabetic efficacy through both in vitro and in vivo methodologies [9]. Consequently, researchers are now concentrating on the unexamined metabolites of Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre for antidiabetic therapy. This research aims to evaluate the antidiabetic potential of a herbal formulation (PHF) based on Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre in an in vivo mouse model. The findings indicated that the polyherbal formulation regulates both hypoglycemia and hyperlipidemia.

METHODOLOGY

Collection and Identification of plants: Various specimens of Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre were gathered from diverse geographical locations.

Extract Preparation: All collected Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre were desiccated, stored in the shade, and pulverized into a coarse powder. Extracts were made using green extraction technique. To this end, 200 g of each Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre was placed in a beaker, along with 600 mL of 80% ethanol. The combination underwent microwave extraction at 400 W for 6 minutes, with the cycle repeated three times. The extracts were further processed using a rotary evaporator to get a concentrated extract, followed by lipolysis to eliminate water content up to 99%. All extracts were stored at 4°C for subsequent use [10].

The extracts were evaluated for alpha-glucosidase activity using the methods outlined in our prior research. The enzymes alpha-glucosidase (G5003-100UN) and alpha-amylase (A36250-2E) were acquired from Sigma Aldrich. The most potent Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre against both enzymes were chosen for the in vivo antidiabetic investigation. The chosen Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre were consumable and deemed safer for antidiabetic research.

Animal Selection: This investigation was conducted on three-month-old Wistar albino rats. The rats had a body weight of 150 ± 10 g and were acclimatized to laboratory settings for 15 days. All rats were allocated into five groups, with each group housed in individual cages (five rats per cage) at a room temperature of 25°C–28°C and subjected to a 12-hour light-dark cycle. The rats were given continuous access to sustenance and hydration. The experiment was conducted with authorization from the animal ethics committee of Government College University Faisalabad, as shown by ethical clearance letter number GCU/ERC/205. Laboratory Animal Care set procedures and rules that were adhered to throughout the experiment. The experiment used animals with fasting blood glucose (FBG) values of 75 ± 5 mg/dL [10].

ANTIDIABETIC EFFICACY OF PHF

Induction of Type 2 Diabetes in rats: Streptozotocine was widely used for the induction of both insulin dependent diabetes mellitus and non-insulin dependent diabetes mellitus (IDDM &NIDDM) in experimental animals. Mostly STZ is administered intravenously at a dose of 40-60mg/kg for IDDM, but they are also effective in intraperitoneal route of administration., Single dose below 40 mg/kg is not effective. Sometime STZ also can be given in multiple low doses, such doses are predominantly used in mice for induction of IDDM.

Administration of STZ causes alteration in blood insulin and glucose concentration. Hyperglycemia was observed after two hours of STZ administration with an associated drop in blood insulin. Later after six hours hypoglycemia occurred with high concentration of plasma insulin, gradually hyperglycemia developed and blood insulin level decreased

After acclimatization, the animals designated as controls were fed with a normal rat pellet diet and water ad libitum for four weeks, while the diabetic experimental animals were fed with a normal rat food and 30% fructose solution ad libitum for four weeks. After a 12 h fasting period, the experimental animals were injected intraperitoneally with 40 mg/kg of STZ dissolved

in cold citrate buffer (0.1 M, pH 4.5), while the control groups were injected with same quantity of citrate buffer. Three days later, Blood glucose was determined by taking blood, with a lancet, from the tail and measured using test strips (Accu-Chek glucometer) 10 days after the STZ injection. Rats with a fasting glucose level >250 mg dL−1 were classified as diabetic Animals with fasting blood glucose (FBG) level above 250 mg/dL with typical diabetic symptoms of polyuria, polydipsia and polyphagia were selected for the study. The animals were subsequently divided into the following groups (n =6)

Table 1: Experimental design for Antidiabetic activity

Normal Control	Control rats gavaged with normal saline
Diabetic Control	Diabetic control rats gavaged with normal saline.
Positive Control	Diabetic rat gavaged with Glibenclamide5 mg/kg b.w
PHF 100	Diabetic rat gavaged with polyherbal formulation **PHF3** 100 mg/kg
PHF 200	Diabetic rat gavaged with polyherbal formulation **PHF3** 200 mg/kg

All the groups received their treatment by oral route once a day. In addition, the blood glucose level, body weight, food and water intake were measured periodically throughout the treatment period.

Extract solutions were prepared by dissolving 5 g of PHF3 in 100ml of distilled water (50mg/ml of extract). Glibenclamide was used as a standard antidiabetic drug in STZ-induced diabetes to compare the antidiabetic properties of a variety of hypoglycemic compounds. A standard dug solution was prepared by glibenclamide 5mg/kg b.w.suspended in 100 ml distilled water, which will give 0.5mg/ml of glibenclamide solution as a standard.Antidiabetic study of polyherbal formulation was conducted by taking albino rats of wistar strain (180-250 g)

Overnight fasted animals were divide into groups to determine the antidiabetic activity of polyherbal formulation. The experimental rats were divided into groups consisting of six rats per group. The extract, Glibenclamide, and normal saline were orally administered once every day for eight weeks.

On 0, 7th, 14th, 21stand 28th day of extract administration, the fasting glucose levels were determined. During the experimental tenure, weights of rats were taken daily and mean change was calculated. Blood samples were collected by tail prick method. Body weights and fasting blood glucose levels were measured on a weekly basis. At termination, the rats were fasted overnight and sacrificed by cervical dislocation.

Body weight assessment: The body weight of the normal rats before and after completion of experiment remained unchanged, but a significant reduction in body weight of STZ-induced diabetic rats was noted and maximum reduction was observed on 28th day.

Estimation of insulin level: After 28th day of treatment blood samples were withdrawn by in order to examine the insulin levels. Serum insulin was measured using a Glazyme Insulin-eia test. Insulin was estimated in plasma samples by radioimmunoassay kit (Estradiol RIA Kits,) according to method of The radioimmunoassay method is based upon the competition of unlabeled insulin in the standard or samples and radio iodinated (125I) insulin for the limited binding sites on a specific antibody. At the end of incubation, antibody bound and free insulin is separated by the second antibody and poly ethyleneglycol (PEG) aided separation method. Insulin concentration of samples is quantitated by measuring the radio activity associated with the bound fraction of sample and standards.

To 100 µl of serum sample, 0.3 ml of assay buffer was added. For standards, 100 µl of insulin standard of different concentrations (0, 12.5, 25, 50, 100, 200 µU/ml), 100 µl of insulin free serum was added. The volume was made up to 0.4 ml with assay buffer. To both samples and standards, 0.1 ml of insulin antiserum was added, mixed gently and incubated overnight at 4^{o}C. Next day, 0.1 ml of 125 I insulin was added to all tubes and incubated for 3 hours at room temperature. After incubation, 0.1 ml of second antibody was added followed by 1.0 ml of PEG and mixed gently. All the tubes were kept at room temperature for 20 minutes followed by centrifugation at 1500g for 20 minutes. The supernatant was carefully decanted without disturbing the pellet. Radioactivity was counted in the precipitate in a gamma counter (Perkin Elmer 1428,

Wizard 3, Shelton, U S A). Percentage binding of radiolabelled insulin antibody was calculated. Percentage of binding was calculated by taking binding of blank as 100%. Standard log-logit curve was plotted for percentage of binding of standards verses concentration of standards. Concentration of samples was calculated from the standard curve and represented as μU/ml of insulin. The decrease in serum insulin level indicates hyperglycemia in STZ-induced diabetic rats. The STZ-induced diabetic rats exhibited maximum decrease in serum insulin levels on 28th day.

Pancreatic weights assessment: After sacrifice pancreas, was dissected and rapidly stored in cold saline until further use. Pancreas was isolated and store in ice cold saline, blotted and weighted. There was a remarkable reduction in the pancreatic weights in diabetic control animals compared to Normal Control animals. Their were maximum decreased in the pancreatic weight in diabetic control animals.

Estimation of biochemical parameters: The animals were sacrificed by cervical dislocation on 28th day for determining various biochemical parameters. Various biochemical parameters like glycosylated hemoglobin, Total cholesterol, triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein (LDL). The kit used was kits of Triglycerides and Cholesterol (Minias Globe Diagnostic kit). Semi auto-analyzer was used for the analysis of sample. Theplasma profile for metabolic parameters like triglycerides and total cholesterol in theplasma was estimated in all the experimental groups. These estimations done by usingcommercially available biochemical kits procured from Accurex, Biomedical (Mumbai, India)

Estimation of glycosylated hemoglobin (HbA1c): Plasma 0.5ml was separated and cellswere washed twice (0.154 M saline) and stored at –200ºC. Oxalic acid (1 ml, 0.3 M) was added to an aliquot (2 ml) of adjusted hemolysate, mixed, and placed immediately in a boiling water bath at 100ºC for exactly 60 min. Evaporation was minimized by placing glass marbles on each test tube. After incubation samples were cooled in cold water for 2min and deproteinization carried out by the addition of1 mlof 40%w/v TCA (Trichloroacetic acid). The tubes were vortexed (30 sec) and then centrifuged (1500 g x 15 min). Thiobarbituric acid (0.5ml, 0.05 M) was added to the clear supernatant (2 ml), mixed, and incubated at 40ºC for60 min. The color developed was noted at 443 nm. Incubated in each assay were a blank using distilled water instead of hemolysate, aqueous standards of 5-HMF (0.01 mM/L - 0.05 mM/L), aqueous standards of fructose (1mM/L - 4 mM/L).

Assay of antioxidant enzymes in Pancreas After completion of the study (28 days), all the experimental group animals were sacrificed, pancreas was isolated and weights wererecorded. In the pancreatic tissue the oxidative stress marker like malondialdehyde (MDA) levels, antioxidant marker glutathione (GSH) levels and estimation of tissue nitrasative stress, Nitric oxide levels were measured for all experimental animal groups.

Results: A total of 20 distinct edible species, including Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre, were gathered for the manufacture of green extracts. All Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre were evaluated against two principal enzymes associated with hyperglycemia: α-glucosidase and α-amylase. Following preliminary screening, the most potent extracts of Withania coagulans, Picrorhiza kurroa, Gymnema sylvestre, Morchella esculenta, and Daedaleopsis nitida, exhibiting alpha-glucosidase and alpha-amylase inhibition exceeding 80%, were chosen to formulate a binomial herbal mixture (MF) in a 1:1 ratio, as detailed in Table 2.

Table 2: Effect of PHF on the blood glucose levels in diabetic rats

Animal Groups	**Fasting blood glucose levels (mg/dl)**			
	0 Day	**7 Day**	**14 Day**	**28 Day**
Normal Control	70.34±1.37	71.66±1.25	73.16±1.48	74.17±1.19
Diabetic Control	236.17±4.70	230.53±4.81	234.20±3.80	235.16±3.66
Positive control	244.10±3.06	218.84±3.91	180.42±3.12	99.5±2.13**
PHF 100 mg/kg	230.33±3.32	208.5±3.41	176.11±2.26	94.41±1.19**
PHF 200 mg/kg	248.46±2.72	207.32±2.28	169.44±2.56	91.2±3.12**

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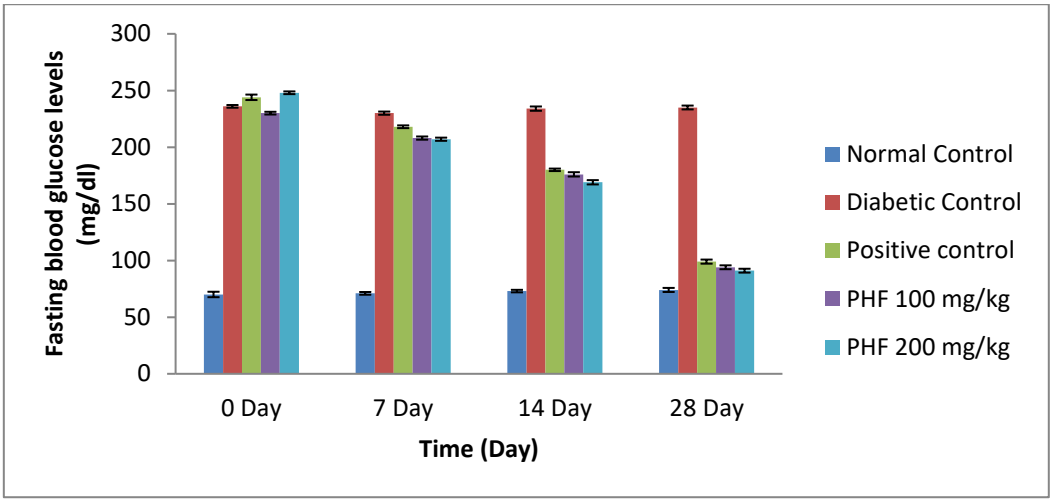

Figure 1: Effect of PHF on the blood glucose levels in diabetic rats

Body weight assessment: The body weight of the normal rats before and after completion of experiment remained unchanged, but a significant reduction in body weight of STZ-induced diabetic rats was noted and maximum reduction was observed on 28th day. The PHF and Glibenclamide treated rat showed no significant reduction in body weight on 28th day.

Table 3: Effect of PHF in change on body weight of animals

Groups	Before induction	After induction	After treatment
Normal Control	181.7±1.11	182.9±1.12	184.12±1.18
Diabetic Control	180.16±2.08	171.73±1.12	165.15±1.13
Positive control	177.23±2.01	172.35±1.98	176.45±1.19
PHF 100 mg/kg	179.12±2.24	163.45±2.86	169.92±1.62
PHF 200 mg/kg	178.69±2.09	163.56±2.71	181.96±4.29

n=6 rats) ± SD, Values are statistically significant at *p<0.05, more significant at **p<0.01

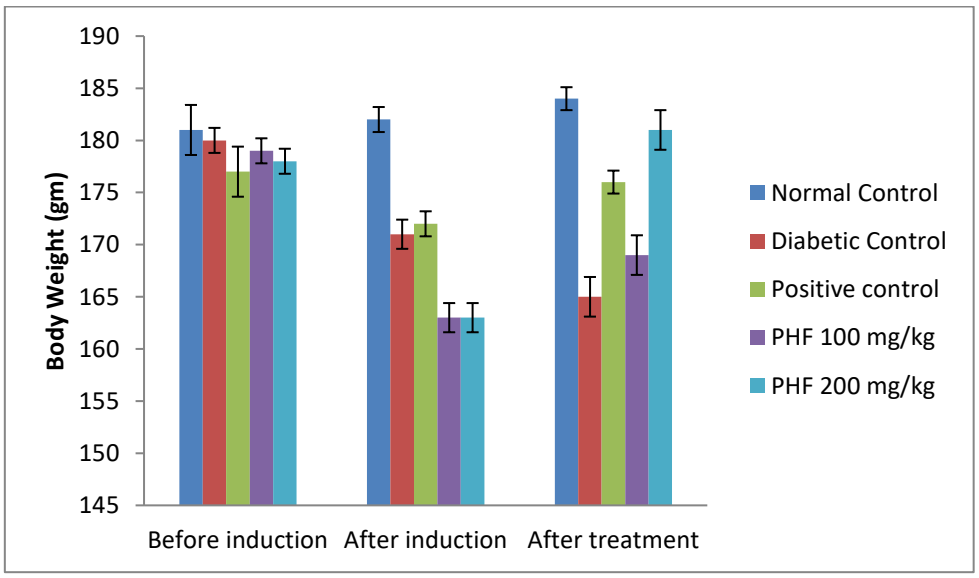

Figure 2: Effect of PHF in change on body weight of animals

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Estimation of insulin level: After 28th day of treatment blood samples were withdrawn by in order to examine the insulin levels. Serum insulin was measured

The decrease in serum insulin level indicates hyperglycemia in STZ-induced diabetic rats. The STZ-induced diabetic rats exhibited maximum decrease in serum insulin levels on 28th day. The administrations of PHF and Glibenclamide to diabetic rats for 28th day resulted in significant increase in serum insulin levels were observed.

Table 4: Effect of PHF on insulin level of animals

Groups	Insulin level (mg/dl)	
	Initial reading	Final reading
Normal Control	181.5±1.21	182.7±2.14
Diabetic Control	179.16±2.79	145.73±2.62
Positive control	178.26±1.26	172.56±2.71
PHF 100 mg/kg	170.18±2.24	152.65±2.45
PHF 200 mg/kg	178.41±1.38	171.56±1.97

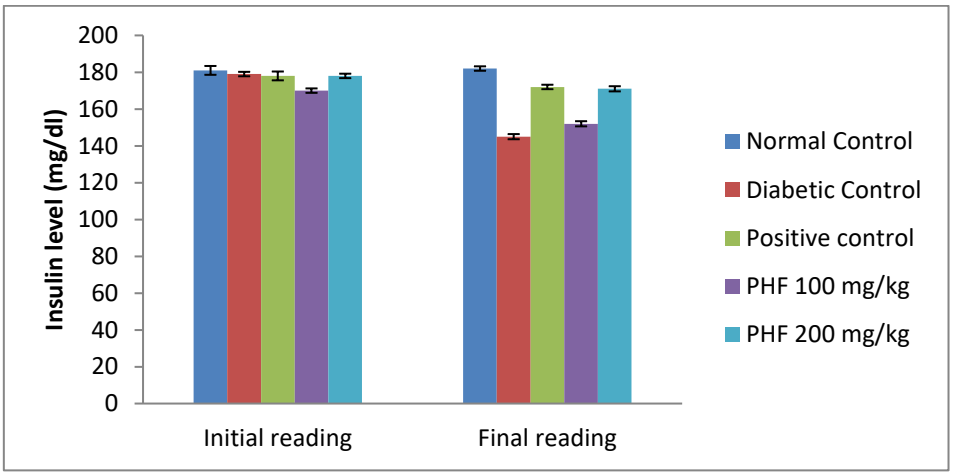

Figure 3: Effect of PHF on insulin level of animals

The decrease in serum insulin level indicates hyperglycemia in STZ-induced diabetic rats. The STZ-induced diabetic rats exhibited maximum decrease in serum insulin levels on 28th day. The administrations of PHF and Glibenclamide to diabetic rats for 28th day resulted in significant increase in serum insulin levels were observed.

Pancreatic weights assessment: After sacrifice pancreas, was dissected and rapidly stored in cold saline until further use. Pancreas was isolated and store in ice cold saline, blotted and weighted.

Table 5: Effect of PHF on Pancreas weight

Groups	Pancreas weight
Normal Control	7.90 ± 0.52
Diabetic Control	4.35 ± 0.21
Positive control	7.12 ± 0.47
PHF 100 mg/kg	6.15 ± 0.41
PHF 200 mg/kg	7.18 ± 0.11

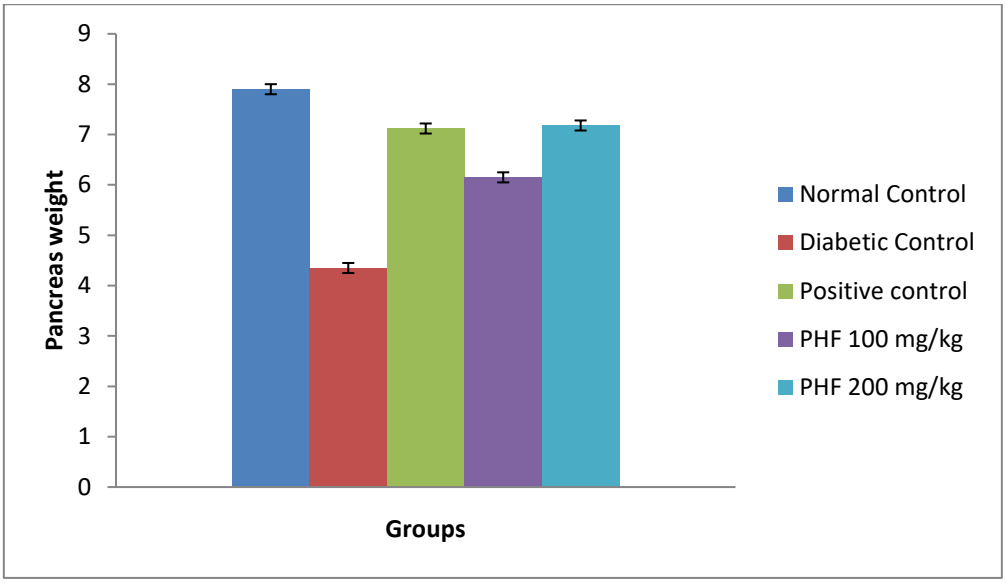

Figure 4: Effect of PHF on Pancreas weight

There was a remarkable reduction in the pancreatic weights in diabetic control animals compared to Normal Control animals. Their were maximum decreased in the pancreatic weight in diabetic control animals. Treatment with PHF (100 mg/kg, 200 mg/kg) showed increase in pancreatic weight dose dependently.

ESTIMATION OF BIOCHEMICAL PARAMETERS

Estimation of glycosylated hemoglobin (HbA1c): Diabetes is one of the most common diseases worldwide. This serious complication may result from increased glycation of healthy proteins as a consequence of associated chronic hyperglycemia. Most proteins (including hemoglobin) react with glucose and form covalent combinations without the requirement of enzymes. HbA1C is an inductor combination, which is reversible, but after the inner reformation of this combination, stable HbA1C is formed. When hemoglobin is glycated, its efficiency is reduced, which leads to a pathologic condition. In diabetic rats, there was a significant increase of HbA1c compared with normal control. Whereas, PHF at 100 and 200 restored the elevated HbA1c profile compared to diabetic control, which was as significant as positive control (standard treated).

Table 6: Effect of PHF on glycosylated hemoglobin levels in animals

Groups	**Glycosylated hemoglobin (HbA1c %)**
Normal Control	4.61±0.19
Diabetic Control	10.98±0.12
Positive control	4.67±0.07**
PHF 100 mg/kg	5.73±0.13*
PHF 200 mg/kg	4.92±0.09**

(n=6 rats) ± SD, Values are statistically significant at *p<0.05, more significant at **p<0.01

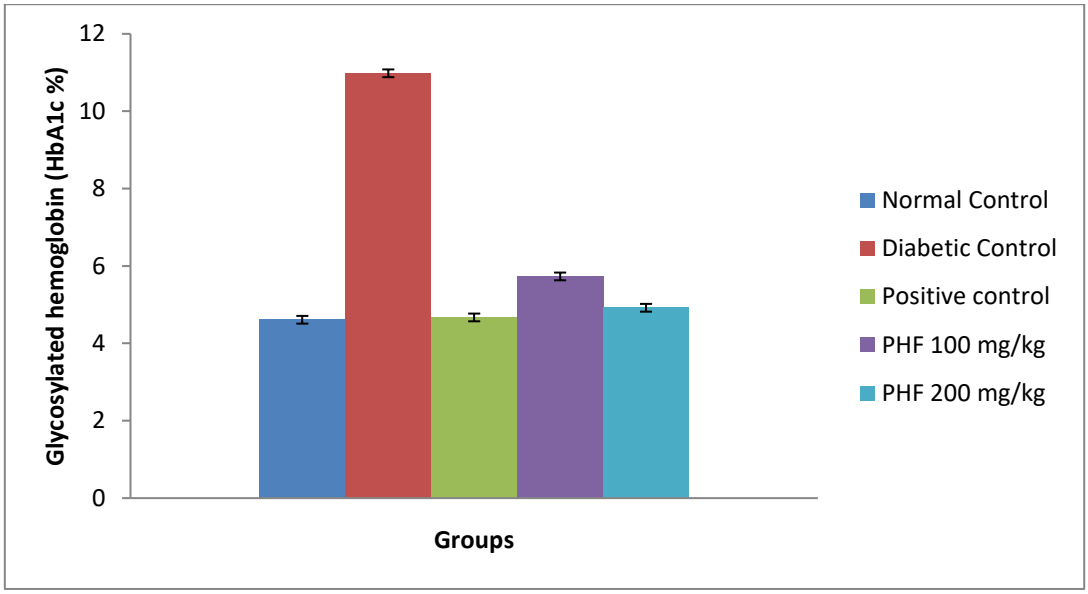

Figure 5: Effect of PHF on glycosylated hemoglobin levels in animals

Discussion: This research is the inaugural investigation that disclosed the possible antidiabetic benefits of a newly developed formulation, PHF, using extracts from Withania coagulans, Picrorhiza kurroa, and Gymnema sylvestre. The personal evaluation by in vitro enzyme inhibition experiment targeting alpha-glucosidase and alpha-amylase. Through this experimentation, Morchella esculenta and Daedaleopsis nitida were discovered as the most effective antidiabetic mushrooms. Their combination formulation demonstrated very substantial results in the in vitro testing. To examine the in vivo antidiabetic efficacy of this mushroom formulation, we utilized a streptozotocin-induced high-fat diet diabetic rat model. Following treatment with the mushroom blend, the results highlight the significant antidiabetic attributes of Morchella esculenta and Daedaleopsis nitida, reinforcing its potential as a therapeutic intervention for diabetes.

Conclusive Remarks: This research demonstrated the antidiabetic effectiveness of the novel PHF, which includes extracts from Daedaleopsis nitida and Morchella esculenta. The administration of PHF to STZ-induced diabetic rats resulted in a significant reduction in hyperglycemia and insulin resistance. The decrease in blood glucose levels may be ascribed to the inhibitory action of active phytochemicals from both mushrooms on important enzymes, particularly alpha-amylase and alpha-glucosidase, which are significant in hyperglycemia. Furthermore, histological examination of principal organs such as the pancreas, liver, and kidneys demonstrated the protective impact of PHF, ameliorating diabetes-induced damage in these organs and promoting the regeneration of β-cells in the pancreas. The reduction in cholesterol and triglycerides in PHF-treated groups underscores the potential of PHF in mitigating diabetes consequences. This research is the first investigation that underscores the potential of PHF as a natural and safe resource for the formulation of antidiabetic medications. Mushrooms, nutritionally dense and integral to our diet, possess considerable potential to treat diabetes. Nonetheless, thorough evaluation and clinical trials are advised before to administration to diabetic patients.

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