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Research Article

EVALUATION OF ANTIDIABETIC AND ANTIHYPERLIPIDEMIC ACTIVITY OF NEWLY FORMULATED POLYHERBAL ANTIDIABETIC TABLETS IN STREPTOZOCIN-INDUCED DIABETES MELLITUS IN RATS

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ABSTRACT

Objective: The study is concerned with the evaluation of the antidiabetic and antihyperlipidemic activity of the newly formulated polyherbal tablets.

Methods: In this study, rats were divided into five groups (n=6). Group I normal control, Group II received polyherbal antidiabetic tablet (PHADT) (400 mg/kg) b.w p.o, Group III streptozocin (STZ) (55 mg/kg b.w. p.o) treated diabetic rats, Group IV STZ+PHADT, Group V STZ+glibenclamide (10 mg/kg b.w. p.o). The treatment is made for 33 days. The body weight, feed intake was measured daily, blood sugar level measured every 5th day. Oral glucose tolerance test was estimated on the 33rd day. On 34th day, the blood was withdrawn from retro-orbital plexus and the serum was used for the lipid profile estimation. After scarification under overdose of ketamine, the liver and skeletal muscle glycogen were measured.

Results: The STZ-induced diabetic rats treated with PHADT, glibenclamide showed significantly increased body weight, decreased feed intake, decreased blood sugar level, high postprandial glucose clearance and decreased serum lipid profile, increased skeletal muscle glycogen content when compared to STZ-induced diabetic rats (Group III). A significant regeneration of pancreatic β cells was observed in diabetic rats treated with PHADT, glibenclamide.

Conclusion: From this study, it can be concluded that PHADT has antihyperglycemic activity as well as antihyperlipidemic activity.

Keywords: Streptozocin, Polyherbal antidiabetic tablet, Serum lipid profile, Glibenclamide.

INTRODUCTION

Diabetes mellitus (DM) consists of a group of disorders characterized by hyperglycemia; altered metabolism of lipids, carbohydrates, and proteins; and an increased risk of complications from vascular disease [1]. The prevalence of diabetes is increasing in Asian countries and it contributes to more than 60% of the world's diabetic population. Asians have large genetic and ethnic predeposition for diabetes hence develop diabetes at a younger age and at a lower body mass index and waist circumference when compared with the Western population. The present trend indicates that more than 60% of the world's diabetic population will be in Asia. The prevalence among adults aged 20-70 years is expected to rise from 285 million in 2010 to 438 million by the year 2030 [2].

At present, available therapies for DM include insulin and many oral hypoglycemic agents such as biguanides and sulfonylureas. However, these drugs used in the treatment of DM possess a number of limitations such as adverse effects and high rates of secondary failure. The plant kingdom holds great potential to meet this need. Many herbal medicines have been recommended for the treatment of diabetes [3]. However, scientific testing and validation of the efficacy of the most medicinal plants in alleviating DM1 and DM2 are rare. Thus, we have limited knowledge of their safety and efficacy, as the most of the data are based on information obtained from traditional medicinal plant practitioners [4]. The recent study shows that 30% of diabetic people use complementary and alternative method of treatment. Herbal medicine is the oldest known medical healthcare and is being used since centuries by many cultures. In this study, the antihyperglycemic and antihyperlipidemic activity of the polyherbal combination locally available was investigated and compared with the standard antihyperglycemic drug in rats.

METHODS

Animals

Adult male wistar rats (180-250 g) were procured from authenticated supplier, *In vivo* Bioscience, Bengaluru, Karnataka, India. All animals were housed under standard laboratory conditions, maintained on a 12 hrs light: 12 hrs dark cycle and food and water were provided *ad libitum*. Animals were acclimatized for 7 days to laboratory conditions before the test. The experimental protocols were approved by the Institutional Animal Ethics Committee (Ref No: PESCP/IAEC/06/2014. Date: 13-12-2014) and conducted according to CPCSEA guidelines (CPCSEA Reg. no: 600/PO/Ere/S/02/CPCSEA), Government of India. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Acute oral toxicity study

An acute oral toxicity study was performed for newly developed herbal formulation according to the toxic class method 425 as per organization for economic cooperation and development (OECD) guidelines. Female wistar rats (6) weighing between 150 and 180 g were used for acute toxicity study to determine lethal dose, 50% of extract. The rats were given dose p.o. up to 4000 mg/kg and no mortality was observed for this dose. The animals which were administered with the drug-treated animals were observed carefully for toxicity signs and mortality. 400 mg/kg was selected as the suitable dose for this study.

Collection of plant material

The fresh plants, viz., Gudmar and Amla were collected from Gandhi Krishi Vignan Kendra, University of Agricultural Science, Karnataka, India; Tulasi, Methi seeds, Karela, and Garlic were obtained from authorized supplier, Bengaluru in the month of December 2015. The plant was identified and authenticated by Dr. KP Sreenath, Taxonomist,

Table 1: Herbal powder mix composition

Serial number	Botanical name	Family	Parts used	Common name	Quantity (g)
1	Gymnemasylvestre	Asclepiadaceae	Leaves	Gudmar	30
2	Momordicacharantia	Cucurbitaceae	Seeds	Karela	10
3	Phyllanthusamarus	Euphorbiaceae	Fruits	Amla	10
4	Ocimum sanctum	Lamiaceae	Leaves	Tulasi	5
5	Trigonellafoenumgraecum	Fabaceae	Seeds	Methi	5
6	Allium sativum	Amaryllidaceae	Bulb	Garlic	5

Department Botany, Bangalore University, Bengaluru, Karnataka, India, and voucher specimen of the plant was kept in the college herbarium.

Tablet preparation and evaluation

All the individual herbal drugs were dried using hot air oven at 40°C for 24 hrs. The individual herbal drugs were then crushed using grinder and passed through mesh no. 40. The individual herbal drugs were then weighed as per the quantity required on digital precision balance. The herbal drugs were mixed geometrically using blender. The powder was tested and evaluated by pre-formulation studies: Angle of repose, bulk density, tapped density, compressibility index, and Hausner's ratio before tablet compression. Then, 400 mg of the mixed powder was weighed, and excipients were added and compressed into tablets using a single punch tableting machine. After that, the tablets were tested for the physical properties: Weight variation, friability, tablets thickness, tablets hardness, and disintegration time (Tables 1 and 2, Fig. 1).

Induction of diabetes in rats [5]

After 12 hrs of fasting in rats, stable DM was induced by single intraperitoneal injection of streptozocin (STZ) (55 mg/kg body weight) dissolved in 0.1 mol/L sodium citrate buffer (pH 4.5) to wistar rats; and control group rats received only citrate buffer. Rats then were given standard diet (20% glucose solution for 12 hrs to prevent initial drug-induced hypoglycemic mortality) and water *ad libitum* for 1 week. 1 week after STZ injection, fasted plasma glucose (FPG) level was measured using glucometer. Only rats with FPG level over 11.1 mMol (≥200 mg/dl) were considered to be qualified as diabetic rats and were used for the experiment.

Pharmacological activities

Animals were divided into five groups of six rats in each group. Group I (normal) received drinking water throughout the course till 33 days [5]. Group II received polyherbal antidiabetic tablet (PHADT) (400 mg/kg) p.o. for 33 days. Group III received STZ (55 mg/kg) i.p. 7 days before the study. Group IV received herbal tablet (400 mg/kg) p.o. for the duration of 33 days as well as STZ (55 mg/kg) i.p. 7 days before the study. Group V received STD (glibenclamide) 10 mg/kg p.o. [6] for 33 days as well as STZ (55 mg/kg) i.p. 7 days before the study. Fasting blood samples [5] were collected from the tail vein for blood glucose estimation on 0, 7th, 14th, and 30th day using one touch glucometer. The food and water intake was monitored daily for each rat, and the periodical body weight difference of the individual animals was also measured during 33 days of the experimental period. Oral glucose tolerance test (OGTT) was carried out after 33 days [5].

Biochemical analysis

At the end of the experimental period, overnight fasted animals were sacrificed by cervical decapitation under light ether anesthesia and blood was collected, serum was separated by centrifuging at 3000 rpm for 10 minutes. The serum was used for the assay of the biochemical parameters such as total cholesterol (TC) [7], high-density lipoprotein-cholesterol (HDL-C) [8], triglycerides [9] using the diagnostic kits. Low-density lipoprotein-cholesterol (LDL-C) [10] and cholesterol very low-density lipoprotein (VLDL) [11] were calculated using Friedewald's formula. After the biochemical estimations animal was euthanized by an overdose of ketamine anesthesia and liver and skeletal tissue was used for the glycogen level estimation using anthrone reagent [12].

Table 2: Composition of each tablet

Serial number	Ingredients	Quantity taken (mg)
1	Herbal powder mix	400
2	Microcrystalline cellulose	380
3	Crospovidone	15
4	Magnesium stearate	2
5	Aerosil	3

Fig. 1: Polyherbal antidiabetic tablet

Histopathological investigations

The dissected samples of the pancreas from each group of diabetic animals were collected in 10% formalin-saline solution and stained with hematoxylin and eosin for the preparation of section using a microtome and histopathological studies were carried out.

Statistical analysis

Values reported are a mean±standard error. The statistical analysis was performed using analysis of variance, followed by Bonferroni method of Statistics using the Graph pad prism statistical program. With all analyses, an associated p value of less than 5% ($p<0.05$) was considered significant.

RESULTS

Acute toxicity studies

No toxic symptoms were observed after administration of different dose levels of extract up to a maximum of 4000 mg/kg p.o. according to OECD guideline 425. In addition to this, a dose of 5000 mg/kg dose was administered to a group of animals and symptoms like dyspnea were identified. Hence, the one-tenth of safe, tolerable dose was used as a therapeutic dose for further pharmacological study.

Pre-formulation parameters

The individual herbal samples were powdered and then the powder was analyzed for the pre-formulation parameters - Angle of repose, loose bulk density, tapped bulk density, Carr's index, Hausner's ratio and tabulated in Table 3.

Post-formulation parameters

Color and appearance: Tablets were light green in appearance with good shape and appearance (Table 4).

Body weight

The diabetic rats treated with PHADT tablets have shown a significant increase (190.3±8.49 g) in the body weight when compared to diabetic control (Group III) rats. The diabetic rats treated with glibenclamide have also shown a significant increase (214.7±5.97 g) in the body weight when it is compared to Group III (diabetic control rats) (Table 5).

Feed intake

While the diabetic control group during this study period of 30-day showed significant increase 14.5 g in feed intake from day 0 to 30th day. PHADT treated group has shown 8.67 g of change in the feed intake compared to diabetic control group during the entire study period. Glibenclamide (10 mg/kg) treated group has shown 8.87 g of change in the feed intake compared to diabetic control group during the entire study period (Table 6).

Antihyperglycemic activity of PHADT in experimentally induced diabetic rats

The Groups I and II rats have shown normal blood sugar level in the beginning day, and Groups III (diabetic control), IV, and V have shown increased blood sugar level. On 30th day, the diabetic control rats (Group III) have shown a significant increase (368.7 mg/dl) in blood sugar level when compared to normal control group (134.5 mg/dl). The Group II rats (139 mg/dl) have not shown any significant difference in blood sugar level when compared to Group I. Group IV diabetic rats treated with PHADT (260.2 mg/dl) have shown significant reduction in blood sugar level when compared to Group III diabetic rats. Group V diabetic rats administered with glibenclamide also showed a significant decrease (255.0 mg/dl) in blood sugar level when compared to Group III (diabetic control rat) (Table 7 and Graph 1).

Effect of PHADT on oral glucose tolerance

The rats of Group I showed significant rise in the blood sugar level at 1, 2 and 3 hrs after the oral glucose administration. From OGTT data, it was observed that rats treated with PHADT or glibenclamide showed significantly improved clearance of blood glucose at each time point monitored versus vehicle treated diabetic rat (Table 8 and Graph 2).

Effect of PHADT on lipid profile in STZ-induced diabetic rats

The diabetic rats have shown significant increase (221.0±7.991***) in total cholesterol level, significant increase (47.03±0.7969***) in triglycerides level, significant increase (213.9±8.201***) in LDL-cholesterol level, significant increase (1.189±0.09156***) in VLDL-cholesterol level and significant decrease (5.945±0.4578***) in HDL when compared to normal vehicle control. The increased lipid profile was brought back to near normal by the treatment with PHADT and glibenclamide. This observed restoration of the STZ evoked changes in the serum lipid profile shows the protective nature of PHADT (Table 9).

Effect of PHADT on glycogen level in STZ-induced diabetic rats

Table 10 shows glycogen levels of normal, PHADT treated, glibenclamide treated and diabetic rats.

Liver glycogen level

The liver glycogen level has increased in STZ treated group of rats Group III (894.1±9.412) as compared to the liver glycogen level in a normal control group of rats (187.6±1.846). Group IV diabetic rats treated with PHADT tablet have shown significant decrease (420.8±24.99***) in liver glycogen level when compared to Group III diabetic control rats. The glibenclamide administered diabetic rats have shown significant decrease in liver glycogen level (278.7±14.39***) when compared to Group III diabetic control rats.

Muscle glycogen level

As per the data in the table 10, it is seen that the skeletal muscle glycogen level has decreased in STZ treated group of rats (21.65±2.024) as compared to the skeletal muscle liver glycogen level in the normal control group of rats (87.09±1.952). Group IV diabetic rats treated with PHADT have shown significant increase (40.48±1.092***) in skeletal muscle glycogen level when compared to Group III diabetic control rats. The glibenclamide administered diabetic rats have shown significant increase in skeletal muscle glycogen level (54.05±0.9524***) when compared to Group III diabetic control rats (Graph 3).

Histopathology of pancreas

Normal control rats showed normal distribution of acini, delicate collagen fibers around islands of Langerhans. Minimal vacuolar changes with islet cell degeneration, atrophy in the islands of Langerhans cells, dense collagen fibers around the acini was seen in STZ treated rats. Minimal islet cell regeneration, few collagen fibers around the islets was seen in PHADT and glibenclamide treated diabetic rats (Fig. 2a-e).

Table 3: The different pre-formulation parameters of the powder mix (PHADT)

Serial number	Parameters	Values
1	Angle of repose	29.94±0.93°
2	LBD	0.24±0.0066 g/ml
3	TBD	0.28±0.0057 g/ml
4	Carr's index	14.28%
5	Hausner's ratio	1.166

PHADT: Polyherbal antidiabetic tablet, LBD: Loose bulk density, TBD: Tapped bulk density

Table 4: The different post formulationparameters of the tablets

Serial number	Parameters	Values
1	% weight variation	0.859±0.148
2	Hardness of tablet	2.84±0.23
3	Friability test	0.362±0.028
4	Disintegration test	5.16±0.119

Table 5: Body weights of normal, PHADT treated, glibenclamide treated and diabetic rats

Group	Body weight (g)					
	0 Day	7th Day	14th Day	21th Day	28th Day	30th Day
1	180.7±2.76	169.7±5.98	179.0±4.46	189.3±5.94	190.3±5.64	197.0±3.98
2	170.0±3.87	165.0±4.12	173.3±6.68	179.3±6.88	187.0±6.78	188.7±4.80
3	178.3±4.01	144.7±3.71	141.0±3.85	143.7±4.91	134.7±6.25	139.3±7.49***^{a}
4	208.3±7.03	179.7±9.83	179.3±11.79	175.0±12.56	170.7±12.42	190.3±8.49***^{b}
5	196.7±9.54	198.7±7.07	204.0±4.73	208.7±5.07	211.7±5.57	214.7±5.97***^{b}

Each bar represents the mean±SEM (n=6) done by one-way ANOVA followed by Bonferroni method of statistics. ***^{a}p<0.001, compared with corresponding values for normal group; ***^{b}p<0.0001 compared with corresponding values for STZ treated group. PHADT: Polyherbal antidiabetic tablet, STZ: Streptozocin, SEM: Standard error of mean

DISCUSSION

STZ administration generally causes destruction of β cells after 3 days in rats 1. Pancreatic β cells are particularly sensitive to damage by nitric oxide and free radicals because of the low levels of free radical scavenging enzymes in the tissue. The results of this study indicate that the PHADT produced regeneration/proliferation of the pancreatic β-cells possibly due to the prevention of free radical formation induced by STZ. Since pancreas contains stable (quiescent) β cells which have regenerative capacity, after damage the surviving cells proliferate by replication to supplicate the lost cells [13].

Studies show that treatment with phytonutrients might be an effective strategy for reducing diabetes complications by influencing glucose metabolism and homeostasis by mechanisms such as modulation of glucose output from liver, inhibition of carbohydrate digestion and regulating the glucose metabolizing enzymes [13].

In this study, the group of diabetic rats showed progressive and significant loss in the body weight throughout the study period. This may be due to insufficient insulin that prevents the body from getting glucose from the blood into the body's cells to use as energy [14]. When this occurs, the body starts burning fat and muscle for energy, causing a reduction in overall body weight. The diabetic rats treated with PHADT have shown significant increase in the body weight when compared to diabetic control (Group III) rats. This may be due to the antidiabetic activity of PHADT which may stimulate the insulin release in rats that leads to the utilization of glucose from blood and prevention of burning of fat and muscle.

In uncontrolled diabetes where blood glucose levels remain abnormally high (hyperglycemia), glucose from the blood cannot enter the cells - due to either a lack of insulin or insulin resistance - so the

Table 6: Feed intake of normal, PHADT treated, glibenclamide treated and diabetic rats

Group	Feed intake (g)						
	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
I	12.66	13.33	16	16.66	17.33	19	22**[a]
II	11.66	12.66	13.33	13.66	13.66	18.33	21.66**[a]
III	10	11.3	14.5	15.5	18.5	20.5	24.5*[a]
IV	12.66	13	13.66	14.33	17	18.33	21.33*[b]
V	11.66	13.33	15	19	19.5	19.8	20.53**[b]

Each bar represents the mean±SEM (n=6) done by one-way ANOVA followed by Bonferroni multiple comparison method of statistics. **[a]p<0.001, *[a]p<0.05 compared with corresponding values for normal group. **[b]p<0.001, *[b]p<0.05, compared with corresponding values for STZ treated group. PHADT: Polyherbal antidiabetic tablet, STZ: Streptozocin, SEM: Standard error of mean

Table 7: Blood sugar levels of normal, PHADT treated, glibenclamide treated and diabetic rats

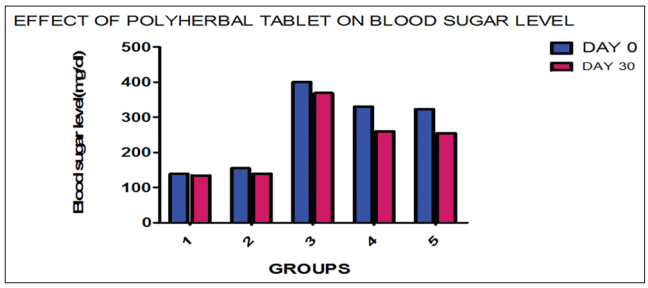
Group	Blood sugar level (mg/dl)			
	0 Day	7 Day	14 Day	30 Day
I	138.5±5.68	143.5±4.82	140.3±5.42	134.5±3.897
II	154.7±7.219	154.2±4.13	144.0±1.73	139.0±2.671
III	399.7±33.54***[a]	415.3±9.44	396.2±9.84	368.7±11.64***[a]
IV	330.2±18.99	381.0±17.36	278.2±26.07	260.2±26.47***[b]
V	322.5±32.37	355.8±30.80	292.7±24.85	255.0±22.83***[b]

Each bar represents the mean±SEM (n=6) done by one-way ANOVA followed by Bonferroni multiple comparison method of statistics. ***[a]p<0.001, compared with corresponding values for normal group; ***[b]p<0.001, compared with corresponding values for STZ treated group. PHADT: Polyherbal antidiabetic tablet, STZ: Streptozocin, SEM: Standard error of mean

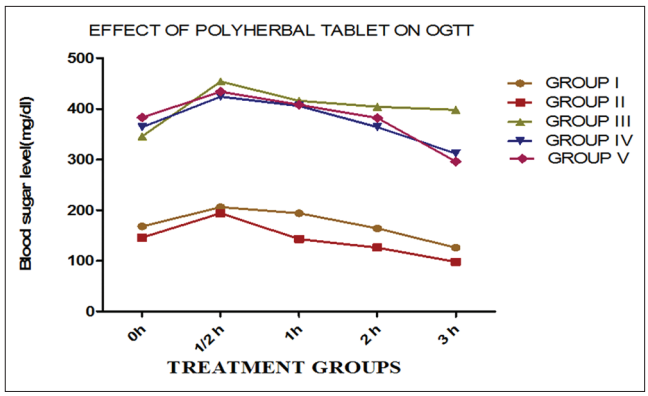
Table 8: Oral glucose level of normal, PHADT treated, glibenclamide treated and diabetic rats

Group	Weight (g)	Blood glucose level (mg/dl)				
		0 hr	½ hr	1 hr	2 hrs	3 hrs
I	180	168	206	194	164	126
II	130	146	194	143	126	98
III	204	346	454	416	404	398
IV	200	364	424	406	364	312
V	176	383	434	408	382	296

PHADT: Polyherbal antidiabetic tablet



Graph 1: Effect of polyherbal tablet on blood sugar level



Graph 2: Effect of polyherbal tablet on oral glucose tolerance test

Table 9: Lipid profile of normal, PHADT treated, glibenclamide treated and diabetic rats

Group	Total cholesterol (mg/dl)	HDL cholesterol (mg/dl)	Triglycerides (mg/dl)	VLDL cholesterol (mg/dl)	LDL cholesterol (mg/dl)
I	116.9±2.44	8.69±0.75	33.83±0.81	1.86±0.12	105.7±2.92
II	124.0±1.17	13.28±0.83**[a]	32.43±0.61	2.65±0.16*[a]	108.1±1.41
III	221.0±7.99***[a]	5.94±0.45	47.03±0.79***[a]	1.18±0.091	213.9±8.20***[a]
IV	182.5±2.88***[b]	21.57±1.04***[b]	38.73±0.67***[b]	4.31±0.20***[b]	156.7±3.63***[b]
V	159.3±2.97***[b]	33.22±1.40***[b]	26.45±0.63***[b]	6.64±0.28***[b]	119.5±2.73***[b]

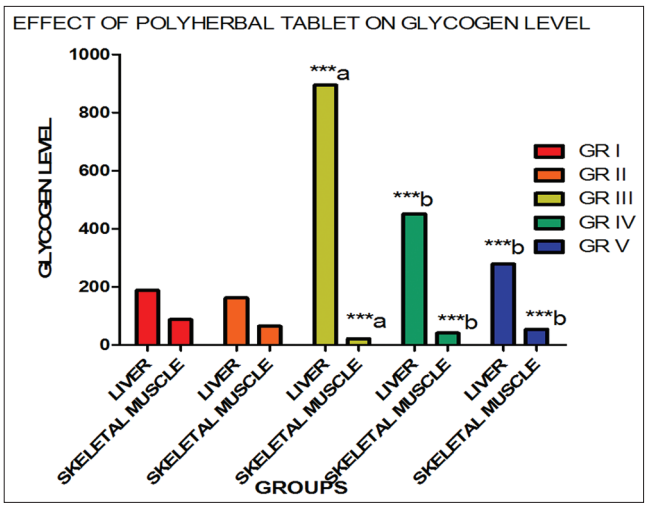
Each bar represents the mean±SEM (n=6) done by one-way ANOVA followed by Bonferroni multiple comparison method of statistics. *[a]p<0.05; ***[a]p<0.001, ***[a]p<0.0001; compared with corresponding values for control group (Group I). ***[b]p<0.0001 compared with corresponding values for diabetic control group (Group III). PHADT: Polyherbal antidiabetic tablet, SEM: Standard error of mean, HDL: High-density lipoprotein, LDL: Low-density lipoprotein, VLDL: Very low-density lipoprotein

body can't convert the food into energy. This lack of energy causes an increase in hunger [15]. In this study, the diabetic group of rats showed significant increase in the feed intake during the study period. Whereas, the PHADT, glibenclamide, and acarbose-treated rats showed a slight reduction in the amount of feed intake.

Table 10: Glycogen levels of normal, PHADT treated, glibenclamide treated and diabetic rats

Group	Treatment	Glycogen level (mg. of glycogen per g. of tissue)	
		Liver	Skeletal muscle
I	Normal	187.6±1.84	87.09±1.95
II	PHADT tablet treated	161.4±2.71	64.45±0.80***a
III	STZ treated	894.1±9.41***a	21.65±2.02***b
IV	STZ+PHADT tablet	420.8±24.99***b	40.48±1.09***a
V	STZ+Glibenclamide	278.7±14.39***b	54.05±0.95***b

Each bar represents the mean±SEM (n=6) done by one-way ANOVA followed by Bonferroni multiple comparison method of statistics. ***ap<0.0001; compared with corresponding values for control group (Group I). ***bp<0.0001, compared with corresponding values for diabetic control group (Group III). PHADT: Polyherbal antidiabetic tablet, STZ: Streptozocin, SEM: Standard error of mean



Graph 3: Effect of polyherbal tablet on glycogen level

Group IV diabetic rats treated with PHADT tablet have shown significant reduction in blood sugar level when compared to Group III diabetic rats. It could be speculated that PHADT might have exerted antihyperglycemic effect on diabetic rats by enhancing the insulin secretion. Plants in the PHADT may act on blood glucose through different mechanisms, some of them may have insulin-like substances, and others may increase β cells in the pancreas by activating the regeneration of these cells.

Repeated administration of PHADT significantly decreased hypertriglyceridemia and hypercholesterolemia. The observed antihyperlipidemic effect of PHADT may be due to decreased cholesterogenesis and fatty acid synthesis through inhibition of pancreatic cholesterol esterase and pancreatic lipase inhibition effect, respectively [16].

Glucose homeostasis is mainly regulated by the liver and skeletal muscle. Most glucose disposal occurs in the liver and skeletal muscle, with glycogen as the primary intracellular form of storable glucose (Saltiel, 2001). The glycogen levels in various tissues area are direct reflection of insulin sensitivity, as insulin promotes intracellular glycogen deposition. The skeletal muscle glycogen level in diabetic rats has decreased as compared to the normal rats. This is due to Insulin resistance in skeletal muscle which is manifested by decreased insulin-stimulated glucose uptake and results from impaired insulin signaling and multiple post-receptor intracellular defects including impaired glucose transport, glucose phosphorylation, and reduced glucose oxidation and glycogen synthesis [17]. The high blood glucose level may be the contributing factor for the high glycogen level in the liver of diabetic rats [18].

CONCLUSION

From this study, it can be concluded that PHADT has antihyperglycemic activity as it reduced blood glucose level, increased body weight, decreased feed intake in STZ-induced diabetic rats. In addition, PHADT also has shown significant reduction in the serum cholesterol, triglyceride, LDL, VLDL level and increased HDL level in diabetic rats which shows that PHADT also possesses antihyperlipidemic activity. Furthermore, the reduction in the blood sugar after glucose administration in OGTT test in PHADT treated rats showed that it acts as potent postprandial antihyperglycemic agent. However, the exact

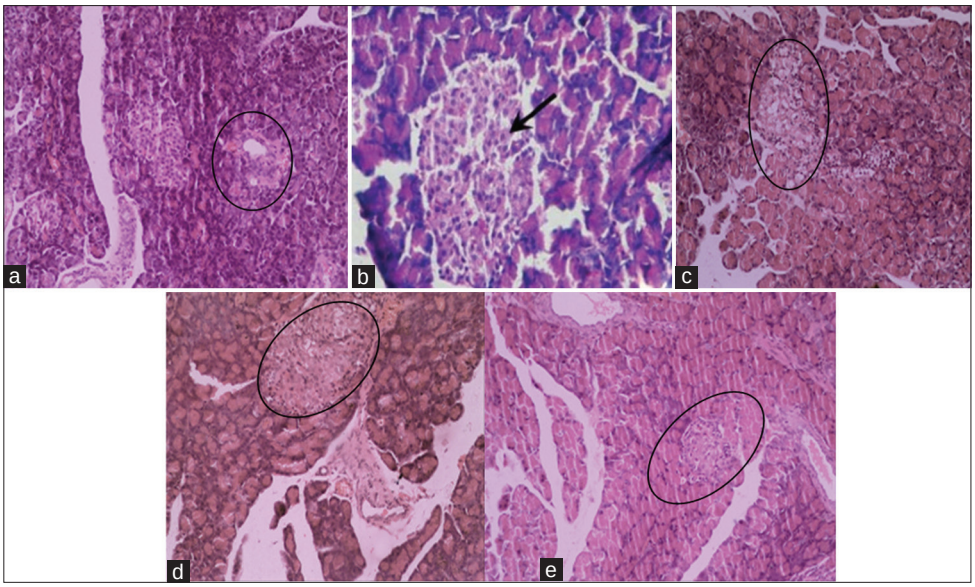


Fig. 2: (a) Normal control. (b) Polyherbal antidiabetic tablet (PHADT) treated. (c) Streptozocin (STZ) treated. (d) STZ+PHADT. (e) STZ+Glibenclamide treated

mechanism behind its activity is not well known. Hence, furthermore investigation is required for proper identification of mechanism involved.

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