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Pharmacognostic Standardization and Hypoglycemic Evaluations of Novel Polyherbal Formulations

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ABSTRACT

The present investigation was carried out to focus on the hypoglycemic effect of newly developed polyherbal formulation (5EPHF) consisting of five medicinal plant extracts viz., *Aegle marmelos*, *Murraya koenigii*, *Aloe vera*, *Pongamia pinnata* and *Elaeodendron glaucum* in normal and alloxan induced diabetic rats. The formulation was subjected to pharmacognostic, physico-chemical and phytochemical evaluations which will assist in standardization for authenticity, quality and identification of the herbal products. Further, the results showed that the formulation treated group significantly altered the glucose level in normoglycemic and experimentally-induced diabetic animals. Treatment with 5EPHF at dose 200 mg/kg to diabetic rats resulted in significant reduction of serum glucose, glycosylated haemoglobin, total cholesterol, triglyceride, low density lipoprotein, creatinine, urea whereas significant increased level of insulin and high density lipoprotein was observed. The formulation treatment significantly inhibited lipid peroxidation and elevates the level of antioxidants enzymes in alloxanized rats. Histopathological studies on 21 day revealed that the regeneration effect of 5EPHF on damaged pancreatic islet cells was found almost comparable to that of standard tolbutamide drug. The present study clearly demonstrated that the developed novel phytomedicine is having potential hypoglycemic and antioxidant activities which may be beneficial for the management and treatment of diabetes mellitus.

Key words: Polyherbal formulation, pharmacognostic evaluations, hypoglycemic activity, lipid peroxidation, diabetes mellitus.

INTRODUCTION

The World Health Organization (WHO) released the guidelines to formulate traditional medicine and recommended to study its usefulness including evaluation, safety, and efficacy for public health care in developing nations [1]. In order to assess the quality control and quality assurance of herbal medicines, standardization of herbal formulations is required which can be achieved using modern sophisticated analytical techniques. As per traditional Indian system of medicines, the combination of herbal drugs is more preferable which enhances the desired pharmacological activities and considered to be less toxic and free from undesirable side effects other than synthetic ones [2,3].

Diabetes mellitus (DM) is endocrinal syndrome associated with depleted insulin secretions, damaged pancreatic β -cells with altered carbohydrate, lipid and protein metabolism and additionally increased risk of complications of various vascular diseases etc [4]. WHO predicted that Indian people are more genetically susceptible to diabetes accounting about 30 to 33 millions and would go up to 40 millions by the end of 2010 which further will reach to maximum of 74 millions by 2025 [5]. Worldwide the diabetes is third leading cause of death due to high incidence of morbidity and mortality after cancer and cardiovascular diseases. Complications such as renal failure, coronary artery disorder, cerebro-vascular disease, neurological complications, blindness, limb amputation, long term damage, dysfunctions and failure of various organs and eventually premature death are associated with chronic hyperglycemia [4].

In-house polyherbal formulation (5EPHF) was prepared consisted of five plants viz., *Aegel marmelos* (L.) Corr., (Family: Rutaceae; Hindi- Bael); *Murraya koenigii* (L.) Spreng (Family: Rutaceae; Hindi- Meethi neem); *Aloe vera* (L.) (Family: Liliaceae; Hindi: Ghikanvar); *Pongamia pinnata* (L.) (Family: Fabaceae; Hindi: Karanja) and *Elaeodendron glaucum* Pers. (Family: Celastraceae; Hindi-Jamrassi). Based on literature survey, all the selected plants presently used clinically to possess antidiabetic and other medicinal values [6-13]. Furthermore, in our previous studies we had reported the antidiabetic activities of *Aegel marmelos* (L.) Corr., *Murraya koenigii* (L.) Spreng, *Aloe vera* (L.), *Pongamia pinnata* (L.) and *Elaeodendron glaucum* Pers. plants [14-17] respectively in alloxan-induced rat model. Therefore, in our present study an attempt has been made to develop a novel polyherbal formulation (5EPHF) and the hypoglycemic activity is evaluated in alloxan-induced rat model.

MATERIALS AND METHODS

1.1. Drugs and chemicals

Tolbutamide (TBM) was supplied as gift sample by Hoechst Pharmaceuticals, Mumbai, India. Alloxan monohydrate was procured from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. Folin-Ciocalteu reagents were from Sigma-Aldrich Inc. (St. Louis, MO, USA); One Touch Glucometer (Accu-chek Sensor) and Diagnostic-kits were purchased from Roche Diagnostics GmbH, Mannheim, Germany. All other reagents and chemicals used were of analytical/ pharmacopoeial grade purchased from E. Merck India Ltd and Ranbaxy respectively.

1.2. Plant authentications and extraction

The laboratory prepared 5EPHF formulation consists of five plant extracts viz., *Aegel marmelos* (L.) Corr., (Rutaceae); *Murraya koenigii* (L.) Spreng (Rutaceae); *Aloe vera* (L.) (Liliaceae); *Pongamia pinnata* (L.) (Fabaceae) and *Elaeodendron glaucum* Pers. (Celastraceae) respectively. All the plants were harvested from local region which was identified and authenticated by an expert Dr N Shiddamallayya of Regional Research Institute (Ay.), Bangalore (India), where RRCBI/Mus/3-6 had been deposited in herbarium and one of the Voucher Specimen DOP/HB/459 was submitted at the institutional herbarium. Separately all the plant materials (1kg) was soxhlet extracted using analytical grade solvents (2.5 l, 72 h each) with methanol at temperature between 45-65°C. The obtained extract was dried under reduced pressure at 40°C in rotary evaporator and thereafter frozen and lyophilized which were finally refrigerated between 4-8°C up to further use in an airtight containers.

1.3. Polyherbal formulation development

All the dried powdered extracts according to Table-1 were mixed in equal proportion (200 mg x 5/ 1g of formulation) or one part each and sieved in order to mix it properly [18]. Thereafter the formulation mixtures were suspended into freshly prepared carboxymethylcellulose (0.3% w/v in distilled water) solution and preserved till further experimentation.

Table 1: Composition of polyherbal formulation (5EPHF)

Plant Extracts	Parts used	Quantity (mg/ 1g of formulation)
<i>Aegel marmelos</i> (L.) Corr., (Family: Rutaceae; Hindi- Bael)	Root	200
<i>Murraya koenigii</i> (L.) Spreng (Family: Rutaceae; Hindi- Meethi neem)	Root	200
<i>Aloe vera</i> (L.) (Family: Liliaceae; Hindi: Ghikanvar)	Leaves	200
<i>Elaeodendron glaucum</i> Pers. (Family: Celastraceae; Hindi-Jamrassi)	Stem bark	200
<i>Pongamia pinnata</i> (L.) (Family: Fabaceae; Hindi: Karanja)	Leaves	200

2.4. Pharmacognostic evaluations (Standardization)

2.4.1. Organoleptic properties

Organoleptic properties of the laboratory developed 5E-PHF formulation were determined using the reported methods for color, odour, and taste [19, 20].

2.4.2. Phytochemical screening

In order to determine the presence of phytochemical in plant extract, standard identification tests for alkaloids, saponins and triterpenes, tannins, flavonoids, carbohydrates and sterols was performed [21].

2.4.3. Physico-chemical investigations

Total ash value was determined by taken accurately weighed 2 g of sample extract into ignited tared silica crucible where it was spread as a fine layer on the bottom. At the increasing temperature the sample was burnt up to red hot not exceeding 450°C until free from carbon. Then the crucible was cooled and resultant ash was weighed and thereupon percent total ash value was determined with reference to air-dried extract drug. The obtained ash during the above procedure was taken and boiled with 2N HCl (25 ml) for 5 min respectively for quantitative estimation of acid insoluble ash. Thereafter the insoluble ash was recovered on an ash less filter

paper and washed using hot water. Insoluble sample was transferred into a crucible which was again burnt for 20 min and weighed properly. In order to omit errors the whole step was repeated thrice and the percent acid insoluble ashes were determined with reference to air-dried drug. Water soluble ash was determined by using the recovered ash during the estimation for total ash was taken and boiled with H₂O (25 ml) for 5 min interval. Thereafter the insoluble ash was recovered on an ash less filter paper and washed using hot water. Insoluble sample was transferred into a crucible which was again burnt for 20 min and weighed properly. The whole step was repeated thrice in order to omit errors and the percent water soluble ashes were determined with reference to air-dried drug respectively. The pH of the sample formulations at concentration 1 and 10% respectively was determined using pH meter at room temperature. Loss on drying was carried out using the reported methods of [22].

2.4.4. Fluorescence analysis

Fluorescence test was conducted using methods reported by **Kokoski et al., 1958 [23]** and **Chase and Pratt, 1949 [24]**. Sample drug (1 mg) was treated separately with 1 ml each of NaOH (1N), NaOH (1N) in MeOH, NaOH (50%), Conc. H₂SO₄, H₂SO₄ (50%), Conc. HNO₃, HNO₃ (50%), CH₃COOH, Iodine (5%), HCl (1N), Ammonia solution (25%) and K₂Cr₂O₇ solutions respectively which was observed under Visible, UV-254 and UV-366 light.

2.4.5. Physical characteristics

Determination of physical characteristics of formulation for bulk density, tap density, angle of repose, Hausner ratio and Carr's index were carried out by following prescribed method of **Lachman et al., 1987 [25]** and **Aulton, 2002 [26]** respectively. Angle of repose (α) was determined by an equation $\alpha = \arctan H/R$, whereas, H is the height and R is the radius of the conical pile. Equation used for estimation of bulk density (D_b) was $D_b = M/V_b$ whereas, M is the mass of particles and V_b is the total volume of packing. Hausner ratio was measured by equation D_f/D_o Whereas, D_f is the tapped density and D_o is the bulk density. The equation used for measuring Carr's index (I) was $I = (D_f - D_o / D_f) \times 100$ whereas, D_f is the tapped density and D_o the bulk density.

2.5. Hypoglycemic evaluation

2.5.1 Experimental animals

Inbred adult male Charles-Foster (CF) albino rats (150-200 g), 2-3 months old, were obtained from School of Pharmacy, Chouksey Engineering College, Bilaspur and were used in the study. Before experimentation animals were acclimatized for a period of 5 days was housed in groups of six in polypropylene cages (34 × 47 × 18 cm³) lined soft wood shavings as bedding were renewed every 24 h intervals. They were maintained under standard environmental conditions of 12/12 h light/dark cycles, relative humidity 50-60% RH and at temperature 22±3°C, were fed with rat pellet diet (Gold Moher, Lipton India Ltd) and water *ad libitum* respectively. All the experimental procedures utilized were performed in accordance with the approval of the Institutional Animal Ethics Committee (169/ac/08/CPCSEA) under strict compliance of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines for the experimental studies.

2.5.2 Acute toxicity studies and selection of doses

An acute oral toxicity study was performed according to "Organization for Environmental Control Development" guidelines (OECD: Guidelines 420; Fixed Dose Procedure). Overnight fasted albino rats (n=6) were used for the study. Experimental animals were administered with fixed methanolic extract of dose 5, 50, 300 mg kg⁻¹ and 2000 mg kg⁻¹ respectively was found tolerable. Three dose levels 200 mg/kg (One-tenth of the maximum lethal dose), 100 (half to that of one-tenth dose) and 50 mg/kg (quarter to that of one-tenth dose) were selected for assessment of possible antidiabetic activities. Then after dosing, animals were observed individually for first 30 min periodically and daily thereafter, till 14 days for any toxicity sign of gross changes in skin and fur, eyes and mucous membranes, circulatory, respiratory, and autonomic and central nervous systems, and behavior pattern if any.

2.5.3 Experimental Protocols

Diabetes was induced by a single dose intravenous injection of freshly prepared alloxan monohydrate (120 mg kg⁻¹ b.w.) in 0.9% w/v NaCl solution (normal saline) to overnight fasted albino rats. Blood glucose level was measured by using one-touch glucometer and diabetes was confirmed after 72 hr of alloxanisation. Rats with fasting blood glucose level more than 250 mg/dl were considered to be diabetic and were selected for further experimentation. The rats selected for studies were fasted over night and divided into 5 groups of 6 rats each as follows (n = 6) Group I served as normal control rats (non-alloxanized) that received vehicle (1 ml of 2.5% v/v Tween-80 in distilled water) only; Group II served as diabetic control rats (alloxanized); Group III diabetic rats received Tolbutamide (TBM; 250 mg kg⁻¹ b.w.) once only as reference drug and Group IV to VI rats were administered with test doses of 5EPHF (50, 100 and 200 mg kg⁻¹ b.w. day⁻¹) respectively. Quantitatively, plasma glucose was estimated by withdrawing blood samples from tail vein prior to dosing (day 0) and then at day 21 in all groups of animals. The effect of 5EPHF treatment on blood glucose level during three weeks in experimental rats was carried out by conducting normoglycemic and alloxan-induced diabetes studies. The final body weight, food and fluid intake of all groups of animals were monitored on a daily basis for 21 days at regular time. Fixed amount of rat chow and fluid was given to each rat and replenished the next day.

2.5.4 Blood sampling and biochemical estimations

At the end of 21th day, all the rats were euthanized by pentobarbitone sodium (60 mg/kg) and sacrificed by cervical dislocation. Blood sample was withdrawn from abdominal aorta into fresh centrifuge tubes and centrifuged at 2,500 rpm for 15 min to obtain serum and plasma. Serum samples were stored at -20°C until utilized for further uses. Thereafter, biochemical analysis was carried out by estimation of total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), glutamate oxaloacetate transaminases (SGOT), glutamate pyruvate transaminases (SGPT), alkaline phosphate (ALP), urea and creatinine, using Hitachi Auto analyzer. During analysis standard procedures were followed using commercial kit (Ecoline, E-Merck, India; Roche Diagnostics, GmbH, Germany) respectively. Plasma insulin was assayed by using commercial enzyme-linked immuno sorbent assay kit (ELISA, Boehringer Mannheim, Germany). The level of hemoglobin (Hb) and glycosylated hemoglobin (HbA_{1c}) was estimated using Drabkin reagent.

2.5.5 Assesment of oxidative stress related markers

Lipid peroxidation was estimated by assessing the plasma malondialdehyde (MDA) formation using the method described by Uchiyama and Mihara, 1978 [27] with minor modification by Sunderman *et al.*, 1985 [28] based on the thiobarbituric acid reactive substances (TBARS) test. Estimation of superoxide dismutase (SOD) activity a free radical scavenging enzyme, was performed at 470 nm (25°C) by the degree of inhibition of reaction using the method of Ukeda *et al.*, 1997 [29]. While catalase (CAT) activities were assayed by catalysis of hydrogen peroxide (H₂O₂) to H₂O in an incubation mixture and recorded at 240 nm for 30 s respectively following the method of Aebi, 1984 [30] respectively.

2.6 Histopathological studies

Histopathological studies were conducted in each group of normal, diabetic and formulation treated animals following methods of Jothivel *et al.*, 2007 [31] and Nagappa *et al.*, 2003 [32] with minor modifications. The animals were sacrificed after cervical dislocation and whole pancreas was excised, washed with normal saline to remove blood and then quickly fixed in 10% buffered formalin for 12 h periods. After that, specimens were dehydrated through the graded concentration of ethanol (50-100%), cleared in xylene and then embedded in paraffin wax. Using rotary microtome small sections of 5-µm thickness were cut down, mounted on glass slides, stained with hematoxylin-eosin and then examined for microscopy. The magnified photomicrographs were presented in Fig. 5(A-E) respectively.

2.7 Statistical analysis

The results are expressed as mean±S.E.M. The statistical significance was determined by One-Way Analysis of Variance (ANOVA) followed by *Post-hoc* Student Newman Keuls test. *P* < 0.05 was considered to be statistically significant.

2. Results

2.1. Pharmacognostic investigation of the 5EPHF formulation:

Results for physical characteristics like Bulk density (g/ml), Tap density (g/ml), Angle of repose (°), Hausner's ratio, Carr's index was estimated and found to be 0.46±0.03, 0.60±0.08, 41.61±0.97, 1.30±0.05 and 23.33±1.34 respectively as shown in Table 2.

Table 2: Physical characteristics of the laboratory 5EPHF formulation

Parameters	Mean Value n=3
Bulk density	00.46±0.032
Tap density	00.60±0.083
Angle of repose	41.61±0.971
Hausner ratio	01.30±0.005
Carr's Index	23.33±1.340

All values are expressed as Mean±SD respectively

Physicochemical characteristics were determined and their percentage values are presented in Table 3. The percent total ash value, water soluble ash value and acid insoluble ash value was found to be 8.60±2.04, 1.09±0.03 and 1.30±0.05 respectively. Moisture content or loss on drying

for the sample was found to be 3.15 ± 1.52 %. The organoleptic parameters were reported in Table 4. Florescence analysis of formulation 5E-PHF was performed on treating with various chemicals and results were mention in Table 5.

Table 3: Physico-chemical characteristics of the laboratory 5EPHF formulation

Parameters	Mean Value (%)
Total ash value	8.60 ± 2.04
Water soluble ash value	1.09 ± 0.03
Acid insoluble ash value	1.30 ± 0.05
Water soluble extractive value	10.18 ± 2.56
Alcohol soluble extractive value	8.38 ± 3.12
pH of 1% w/v aqueous solution	6.05 ± 1.87
pH of 10% w/v aqueous solution	6.20 ± 2.29
Loss on drying	3.15 ± 1.52

All values are expressed as Mean $\pm$ SD respectively; n =3

Table 4: Organoleptic properties of the laboratory 5EPHF formulation

Appearance	Color	Taste	Odor
Moderately fine powder	Pale brown	Characteristics	Characteristics

Table 5: Powder fluorescence tests of the laboratory 5EPHF formulation

Treatment	Visible light	UV at $\lambda_{\max}$ 254 nm	UV at $\lambda_{\max}$ 366 nm
5E-PHF	Pale brown	Brown	Dark brown
5E-PHF+ NaOH (1N)	Yellowish green	Fluorescent green	Green
5E-PHF+ NaOH (1N) in MeOH	Yellowish green	Yellow	Green
5E-PHF+ NaOH (50%)	Yellow	Green	Dark Green
5E-PHF+ Conc. H_2SO_4	Yellow	Light brown	Brownish green
5E-PHF+ H_2SO_4 (50%)	Yellow	Green	Yellowish green
5E-PHF+ Conc. HNO_3	Brownish green	Yellow	Yellow
5E-PHF+ HNO_3 (50%)	Brown	Green	Light brown
5E-PHF+ CH_3COOH	Pale green	Green	Yellowish orange
5E-PHF+ Iodine (5%)	Yellowish brown	Dark brown	Black
5E-PHF+ HCl (1N)	Brown	Orange	Green
5E-PHF+ Ammonia solution (25%)	Yellowish brown	Light brown	Brown
5E-PHF+ $K_2Cr_2O_7$	Dark yellow	Yellowish brown	Blackish brown

2.2. Effect of 5EPHF on plasma glucose level in normoglycemic and diabetic rats:

Time dependant effect on the level of plasma glucose level in fasted normoglycemic rats is depicted in Fig-1(A). Statistical analysis by One-way ANOVA revealed that there was no significant difference among the groups at 0 day [F (4, 20) = 0.086, $P > 0.05$]. Similarly, statistical analysis at 21 day showed that there was significant difference among the groups [F (4, 20) = 3.82, $P < 0.05$]. Post-hoc test revealed that TBM (250 mg/kg) and 5EPHF (50, 100 and 200 mg/kg) showed significant decrease in the plasma sugar level compared to control. Further,

5EPHF (50 and 100 mg/kg) did not show any significant change, but 5EPHF (200 mg/kg) showed significant increase in glucose level compared to TBM (250 mg/kg).

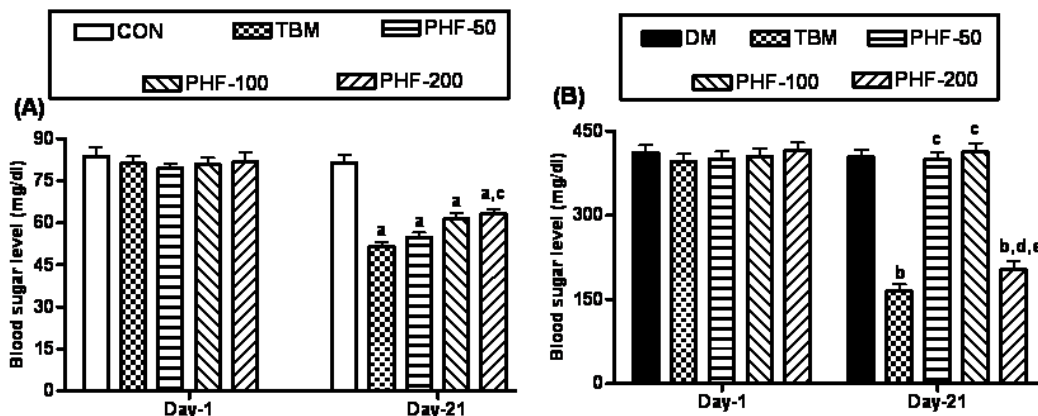


Fig-1. The hypoglycemic effect of the 5EPHF (50, 100 and 200 mg/kg) groups in fasted normoglycemic (A) and alloxanised (B) rats. All values are Mean $\pm$ SEM. ^aP<0.05 compared to CON, ^bP<0.05 compared to DM, ^cP<0.05 compared to TBM, ^dP<0.05 compared to 5EPHF (50 mg/kg) and ^eP<0.05 compared to 5EPHF (100 mg/kg) (One-way ANOVA followed by Student Newmann keuls test).

Fig-1(B) showed time dependant effect on the level of plasma glucose level in alloxan treated rats. Statistical analysis by One-way ANOVA revealed that there was no significant difference among the groups at 0 day [F (4, 20) = 1.14, P>0.05]. Further, statistical analysis at 21 day showed that there was significant difference among the groups [F (4, 20) = 5.28, P<0.05]. Post-hoc test revealed that TBM (250 mg/kg) and 5EPHF (200 mg/kg) showed significant reduce in the plasma sugar level compared to diabetic control. Similarly, statistical analysis at 14 day showed that there was significant difference among the groups [F (7, 40) = 5.26, P<0.05]. Post-hoc test revealed that TBM (250 mg/kg) and ME (200 and 400 mg/kg) showed significant reduce in the plasma sugar level compared to diabetic control. Further, it has been observed that 5EPHF (100 and 200 mg/kg) showed significant increase in sugar level compared to TBM (250 mg/kg). Furthermore, 5EPHF-200 showed significant decrease in blood glucose level compared to DM, 5EPHF (50 and 100 mg/kg) group.

2.3. Effect on body weight, food and fluid intake:

The effect of 5EPHF on final body weight is illustrated in Fig-2 (A). Statistical analysis by One-way ANOVA revealed that there was significant difference among the groups for final body weight estimation [F (4, 20) = 0.78, P<0.05]. Post-hoc test revealed that DM, 5EPHF (50 mg/kg) showed significant decrease, and TBM (250 mg/kg) and 5EPHF (100 and 200 mg/kg) showed no change in body weight compared to control. Furthermore, there was significant increase in body weight in 5EPHF (100 and 200 mg/kg) compared to diabetic group.

The effect of different extracts of root of 5EPHF on food intake is illustrated in Fig-2 (B). Statistical analysis by One-way ANOVA revealed that there was significant difference among the groups [F (4, 20) = 3.11, P<0.05]. Post-hoc test revealed that only diabetic group showed significant increase in food intake compared to control. Fig-2 (C) illustrates the effect of 5EPHF on fluid intake. Statistical analysis by One-way ANOVA revealed that there was significant

difference among the groups [$F(4, 20) = 18.55, P < 0.05$]. Post-hoc test revealed that all the groups showed significant increase in food intake compared to control.

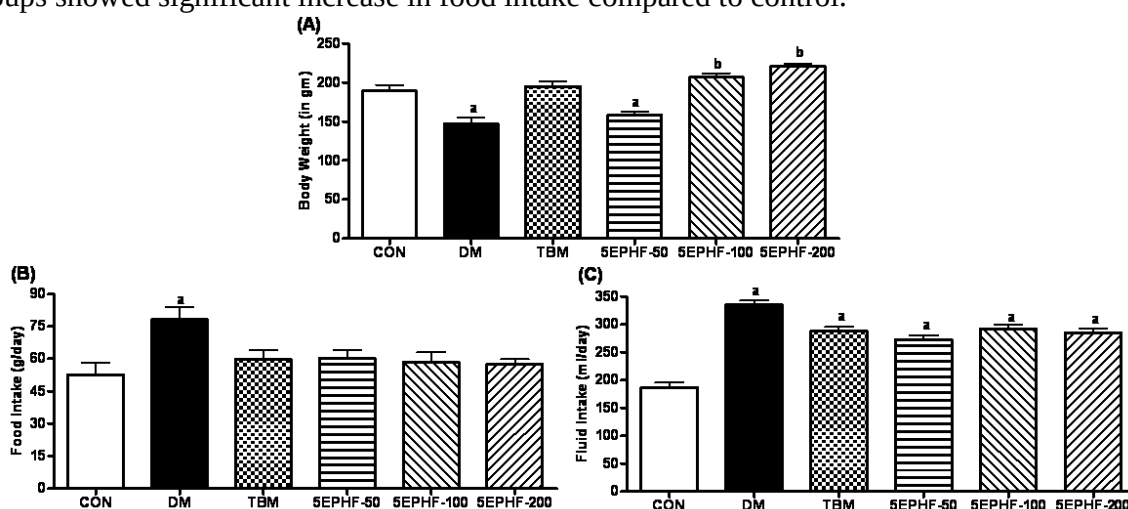


Fig-2. Effect of 5EPHF (50, 100 and 200 mg/kg) on final body weight (A), food intake (B) and fluid intake (C) levels. All values are Mean±SEM. ^aP<0.05 compared to CON, ^bP<0.05 compared to DM, ^cP<0.05 compared to TBM, ^dP<0.05 compared to 5EPHF (50 mg/kg) and ^eP<0.05 compared to 5EPHF (100 mg/kg) (One-way ANOVA followed by Student Newmann keuls test).

2.4. Effect on Biochemical parameters:

The effect of 5EPHF on plasma insulin is illustrated in Table-6. Statistical analysis by One-way ANOVA revealed that there was significant difference among the groups [$F(4, 20) = 2.35, P < 0.05$]. Post-hoc test revealed that diabetic, 5EPHF (50 and 100 mg/kg) showed significant decrease in plasma insulin level, however, there was no change in plasma insulin level in TBM (250 mg/kg) and 5EPHF (200 mg/kg) group compared to control. Further, there was significant increase in plasma insulin level in TBM (250 mg/kg) and 5EPHF (200 mg/kg) groups compared to diabetic group.

Table 6: Effect of 5EPHF formulation treatment for 21 days on blood glucose and other biochemical features in alloxanised rats

Parameters	Control	DM	TBM	5EPHF-50	5EPHF-100	5EPHF-200
Insulin (μ U/ml)	15.6±1.51	6.4±0.46 ^a	14.3±1.87 ^b	6.2±2.52 ^a	8.2±2.06 ^a	15.6±2.48 ^b
TC (mg/dl)	63.4±2.46	95.7±4.03 ^a	69.2±3.84 ^b	62.5±2.70 ^b	69.8±2.63 ^b	64.7±4.25 ^b
TG (mg/dl)	84.6±5.36	120.2±4.57 ^a	82.7±3.65 ^b	86.3±5.27 ^b	93.1±6.31 ^b	87.9±4.94 ^b
LDL (mg/dl)	51.5±2.14	117.8±6.48 ^a	48.9±3.02 ^b	60.4±4.31 ^b	65.3±4.79 ^b	59.7±2.36 ^b
HDL (mg/dl)	34.4±1.62	20.8±2.43 ^a	37.3±1.78 ^b	39.5±3.26 ^b	32.7±2.45 ^b	38.1±3.14 ^b
Total Hb(mg/dl)	15.3±1.47	6.7±0.84 ^a	14.6±1.27 ^b	15.9±2.18 ^b	14.1±0.85 ^b	15.4±1.32 ^b
HbA1c (mg/dl)	3.6±0.57	10.7±0.64 ^a	8.4±0.46 ^a	7.5±0.54 ^a	8.2±0.81 ^a	5.1±0.75 ^{a,b,c,d,e}
SGOT (mg/dl)	55.2±3.54	103.3±6.93 ^a	64.1±4.48 ^b	65.5±3.08 ^b	70.4±2.24 ^b	67.8±2.10 ^b
SGPT(mg/dl)	42.7±4.37	105.6±7.25 ^a	54.8±2.64 ^b	58.2±2.49 ^b	57.5±5.80 ^b	60.9±5.32 ^b
ALP (U/L)	131.4±6.48	217.6±7.27 ^a	148.3±4.87 ^b	145.6±2.59 ^b	150.5±3.05 ^b	143.5±2.61 ^b
Urea (mg/dl)	26.3±2.53	35.2±4.19 ^a	31.7±1.49	33.5±1.84	38.2±3.50	34.3±2.66
Creatinine (mg/dl)	0.85±0.06	1.51±0.43 ^a	0.76±0.04	0.78±0.02	0.84±0.06	0.81±0.05

All values are Mean±SEM. ^aP<0.05 compared to control, ^bP<0.05 compared to Diabetic, ^cP<0.05 compared to TBM and ^dP<0.05 compared to 5EPHF (50 mg/kg) and ^eP<0.05 compared to 5EPHF (100 mg/kg) (One-way ANOVA followed by Student Newmann keuls test).

Table-6 showed the effect of 5EPHF on TC level. Statistical analysis by One-way ANOVA revealed that there was significant difference among the groups [F (4, 20) = 17.38, $P < 0.05$]. Post-hoc test revealed that only diabetic group showed significant increase in TC level compared to control. However, there was significant decrease in TC level in all treated groups compared to diabetic group. The similar trend was observed in TG [F (4, 20) = 1.98, $P < 0.05$], LDL [F (4, 20) = 4.16, $P < 0.05$], HDL [F (4, 20) = 27.51, $P < 0.05$], total Hb [F (4, 20) = 3.19, $P < 0.05$], SGOT [F (4, 20) = 22.18, $P < 0.05$], SGPT [F (4, 20) = 44.83, $P < 0.05$] and ALP [F (4, 20) = 73.82, $P < 0.05$].

The effect of 5EPHF on HbA1c level is shown in Table-6. Statistical analysis by One-way ANOVA revealed that there was significant difference among the groups [F (4, 20) = 17.38, $P < 0.05$]. Post-hoc test revealed that diabetic, TBM, 5EPHF (50, 100 and 200 mg/kg) groups showed significant increase in HbA1c level compared to control. Further, 5EPHF (200 mg/kg) showed significant decrease in HbA1c level compared to DM, TBM and 5EPHF (50 and 100 mg/kg) groups.

Table-6 showed the effect of 5EPHF on urea level. Statistical analysis by One-way ANOVA revealed that there was significant difference among the groups [F (4, 20) = 10.73, $P < 0.05$]. Post-hoc test revealed that only diabetic group showed significant increase in urea level compared to control. The similar trend was observed in Creatinine [F (4, 20) = 12.46, $P < 0.05$].

2.5. Effect on plasma antioxidant profile:

The effect of 5EPHF on LPO is illustrated in Fig-3 (A). Statistical analysis by One-way ANOVA revealed that there was significant difference among the groups [F (4, 20) = 9.03, $P < 0.05$]. Post-hoc test revealed that diabetic, 5EPHF (50 and 100 mg/kg) groups showed significant increase in LPO level compared to control. Further, TBM, 5EPHF (50, 100 and 200 mg/kg) groups showed significant decrease in LPO level compared to DM group. Furthermore, the 5EPHF (200 mg/kg) group showed significant decrease in LPO level compared to 5EPHF (50 and 100 mg/kg) groups.

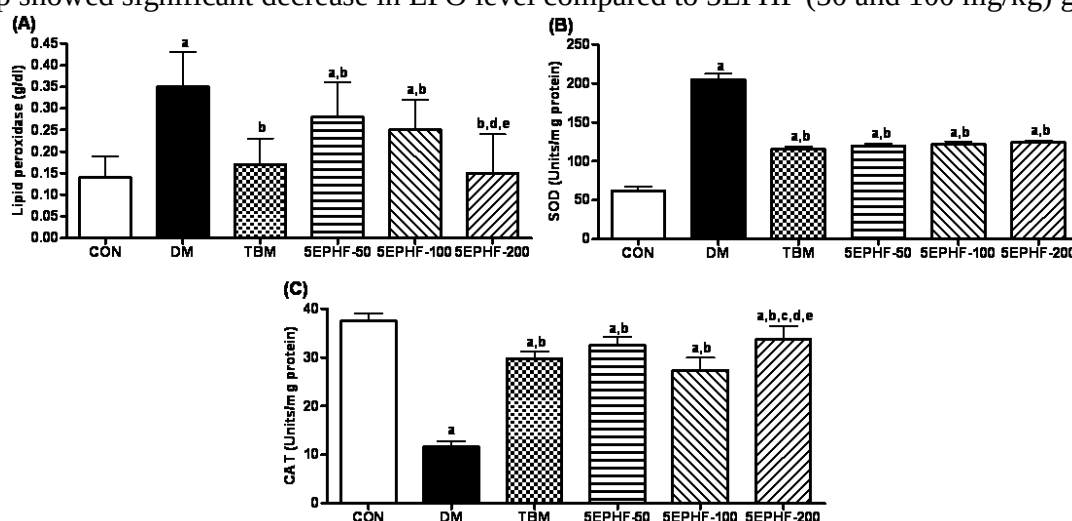


Fig-3. Effect of 5EPHF (50, 100 and 200 mg/kg) on LPO (A), SOD (B) and CAT (C) levels. All values are Mean $\pm$ SEM. ^a $P < 0.05$ compared to CON, ^b $P < 0.05$ compared to DM, ^c $P < 0.05$ compared to TBM, ^d $P < 0.05$ compared to 5EPHF (50 mg/kg) and ^e $P < 0.05$ compared to 5EPHF (100 mg/kg) (One-way ANOVA followed by Student Newmann keuls test).

Fig-3 (B) showed the effect of 5EPHF on SOD level. Statistical analysis by One-way ANOVA revealed that there was significant difference among the groups [$F(4, 20) = 21.26, P < 0.05$]. Post-hoc test revealed that diabetic, TBM (250 mg/kg), 5EPHF (50, 100 and 200 mg/kg) groups showed significant increase in SOD level compared to control. Further, there was significant decrease in SOD level in TBM (250 mg/kg) and 5EPHF (50, 100 and 200 mg/kg) groups compared to diabetic group.

The effect of 5EPHF on CAT is illustrated in Fig-3 (C). Statistical analysis by One-way ANOVA revealed that there was significant difference among the groups [$F(4, 20) = 16.89, P < 0.05$]. Post-hoc test revealed that diabetic, 5EPHF (50, 100 and 200 mg/kg) groups showed significant decrease in CAT level compared to control. Further, TBM, 5EPHF (50, 100 and 200 mg/kg) groups showed significant increase in CAT level compared to DM group. Furthermore, the 5EPHF (200 mg/kg) group showed significant increase in CAT level compared to TBM (250 mg/kg) and 5EPHF (50 and 100 mg/kg) groups.

2.6. Histopathological study

Photomicrograph (Fig. 4 A-F) depicts the islets of langerhans of pancreas belonging to different group of rats.

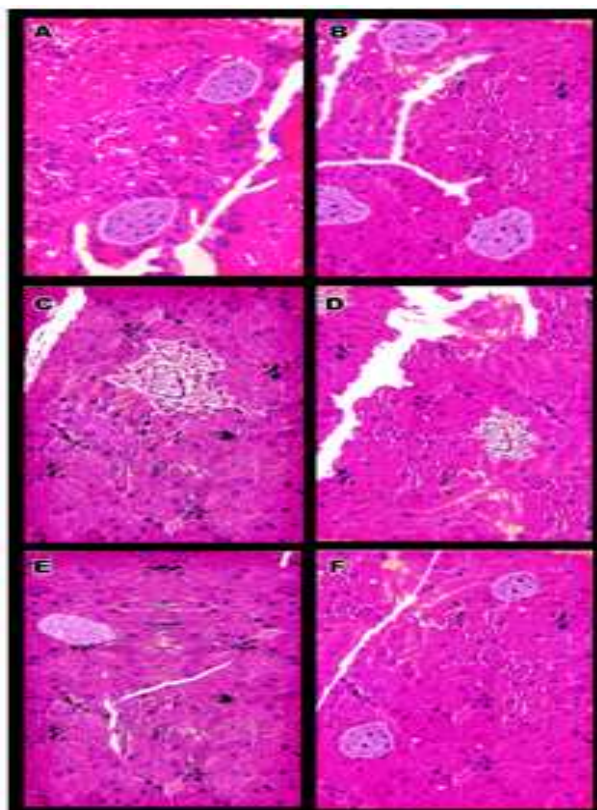


Fig. 4: Histopathological photomicrographs of pancreatic islets of Langerhans (magnification x 200) stained with hematoxylin-eosin (A & B) Normal untreated group showing well developed & granulated β -cells; (C & D) Diabetic group showing shrunken & atrophic changed β -cells and (E & F) Test group showing regenerated β -cells resembling normal cells when treated with tolbutamide (250 mg/ kg b.w.) and 5EPHF formulation (200 mg/ kg b.w.) respectively.

Fig. 4(A and B) shows histology of normal control group having normal and healthy cellular populations in islets of langerhans evenly distributed throughout the cytoplasm whereas, Fig. 4(C and D) shown severe atrophy (degeneration) with reduced size islet cells and irregular appearance in case of alloxan-monohydrate treated diabetic rats. However, tolbutamide treated diabetic rats in Fig. 4(E) shown moderate size expansion and restoration of normal islet cells with hyperplasia conditions. Treatment of animal groups with 5EPHF at dose 50 mg/ kg shows more restoration and enlarged islet cells were comparable to normal cells as shown in Fig. 4(F) respectively.

DISCUSSION

In the present study, the continuous post-treatment for 21 days with the 5EPHF showed potential hypoglycemic activity in normoglycemic rats and antidiabetic activity in alloxan-induced rat model in terms of significant hypolipidemic and antioxidant activity.

The physical characteristics and organoleptic evaluation of the drug are important parameters for determining adulteration with other related species or for authentication, purity and efficacy when compared with standard values. The total ash value was found higher due to presence of high content of carbonates, phosphates, silicates and silica which is useful for determining authenticity and purity of drug formulation. The less value of moisture content could prevent from growth of bacteria, fungus, yeast and other microorganisms, and hence may prolong stability of prepared formulation. Various chemical constituents present in plant material exhibit fluorescence which is an important pharmacognostical evaluation parameter. On applying different reagents, the substance if they were not fluorescent may be converted into fluorescent derivatives. Preliminary phytochemical screening revealed the presence of alkaloids, saponins, triterpenes, tannins, flavonoids, carbohydrates and sterols.

It has been suggested that diabetes is divided into two categories out of which one is genetically based and other as a result of dietary indiscretion [33]. In the present experiment we have found that 5EPHF significantly reduced the blood sugar level in hyperglycemic rats in dose-dependent manner. However, all the doses of 5EPHF showed similar significant lowering of blood glucose level in normoglycemic rats. In the present study we have found that the final body weight decreased and, increased food and fluid intake in experimentally-induced diabetic animals. Further, there was significant decrease in total Hb, plasma insulin and albumin levels, however, there was significant increase in HbA1c, urea and creatinine levels. The above findings are found to be similar with the previous published results of **Patel et al., 2009** [34]. It has been reported that protein glycation during hyperglycemia mostly leads to production of HbA1c. Hence, HbA1c level is used as the most reliable indicator for assessment of retrospective glycaemic control in the management of diabetes [35]. The reduction of HbA1c levels in diabetic animals indicated that there was less protein glycation, possibly resulting from the decrease in blood glucose levels observed in these animals. In consistency with the above findings observed, the 5EPHF showed significant mitigation effect in the HbA1c levels.

It has been suggested that the levels of serum lipids are usually elevated in diabetes mellitus, representing a high risk factor for coronary heart disease [36]. This abnormal high level of serum

lipids is mainly due to the uninhibited actions of lipolytic hormones on the fat depots. It has been observed earlier that the hypercholesterolemia and hypertriglyceridemia occurs in streptozotocine-induced diabetic animals. Under normal conditions, insulin activates the enzyme lipoprotein lipase, which hydrolyses triglycerides [37]. However, in diabetic state lipoprotein lipase is not activated due to insulin deficiency resulting in hypertriglyceridemia. Since lipid abnormalities accompanying with premature atherosclerosis, is the major cause of cardiovascular diseases in diabetic patient therefore ideal treatment for diabetes, in addition to glycemic control, should have a favorable effect on lipid profile. It has been systematically reported that cardiovascular diseases are listed as the cause of death in 65% people suffering from diabetes [38].

Impaired insulin secretions due to loss of β -cells in islet of langerhans were directly linked with the progression of diabetes mellitus (type II) diseases. The plausible mechanism of pancreatic β -cells damage may be due to generation of free radicals initiated by alloxan-monohydrate [39-41]. The regeneration and stabilization of total number of functional islet cell is important to recover from the diabetic disease condition. Various animal models were studied showing regeneration of β -cells in islet of langerhans while followed alloxan-treatment [42, 32]. Diabetes-induced animals treated with plant dose extracts of *Epicatechin* [43], *Vinca rosea* [44], Ephedrine [45] and *Gymnema sylvestre* [46] shown for regenerated β -cells in islet of langerhans reported earlier. Also, the saffron extract [47] were reported to exhibit significant antioxidant as well radical scavenging activities in alloxan-induced diabetic rats confirmed the possibilities for protection of pancreatic cells. Our histopathological findings for the novel 5EPHF were also in agreement with Mandlik et al., 2008 [48]. In our present study, the alloxan-induced rats showed atrophy of islet cells of pancreas while TBM treated group showed regeneration of cells. However, the 5EPHF (200 mg/ kg) showed significant mitigation in regeneration of islet cells of ruptured pancreas similar to control group which may be due to protective and potential healing effect or free radical scavenging activities of the novel developed phytomedicine.

CONCLUSION

The 5EPHF found to be potential antidiabetic formulation in alloxan-induced diabetic model through mitigating oxidative damage and modulating antioxidant enzymes. Further, isolation and establishment of exact mechanism of action of specific compound from 5EPHF is to be carried out in the future. Moreover, a study has to be carried out to establish the additive and/or synergistic property.

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