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Phytochemical and Antioxidant Activity of Polyherbal Hydroethanolic Extract in Streptozotocin Induced β-cell Destruction in Rats

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ABSTRACT

In the view of pathogenicity of diabetics, there are certain factors involved to produce oxidative stress over the pancreatic β-cells, that may be due glucose toxicity or due to involvement of free radical mechanisms. So, possible protection of β-cells against oxidative stress can be achieved by employing antioxidants rich herbs, which will not only acts as free radical scavengers and also for prevention of β-cells from further damage in Streptozotocin induced Type 1 diabetic rats. Preliminary phytochemical investigation of the polyherbal hydroethanolic extract (PHHE) supports the presence of active constituents like alkaloids, glycosides, flavonoids, terpenoids, etc for whose the anti oxidant property was assessed by β-carotene linoleate *in vitro* method of oxidation in TLC plate. To ascertain the antioxidant property *in vivo,* the enzyme level studies like SOD, CAT & GSH in liver and pancreas were made and also the deeper investigation of role of anti oxidants and possible protection over pancreatic β-cells were observed.

Keywords: Antioxidants, Type 1 Diabetes, Hydroethanolic, Polyherbal, Streptozotocin.

INTRODUCTION

A wide variety of pathological conditions, inclusive of diabetes, appears to have an etiological relation to the reactive oxygen species (ROS) induced and free radical mediated oxidation of biomolecules resulting in high state of oxidative stress and damage to the tissues. [1-2] Prevention of initial cellular damage caused by these species leads to the intense investigation and resulted in discovery of several potential antioxidants, as natural or synthetic. [3] Free radicals are atoms or groups of atoms that have at least one unpaired electron, which makes them highly reactive. Free radicals promote beneficial oxidation that produces energy and kills bacterial invaders. In excess, however, they produce harmful oxidation that can damage cell membranes and cell contents. They play an important role in the biological processes, but they can also have deleterious effects when produced in large quantity. [4] Oxygen free radicals are natural physiological products, but are also reactive species. Hyperglycemia alone does not cause complications resulted from chronic glucose toxicity. [5] Which is mediated and complicated through oxidative stress. [6] Free radicals generated *in vivo,* produce damage of everything found in living cells including of proteins, carbohydrates, DNA and other molecules in addition to lipids i.e. Oxidizable substrate. Hence, anti-oxidants are any substance that, when present at low concentrations compared to those of an oxidizable substrate, delays or prevents oxidation of that substrate. Anti-oxidant enzymes like GSH, SOD and CAT are important factors in the development of diabetic complications *in vivo*. Many other substances have been proposed to act as anti-oxidants *in vivo.* They include β-carotene, other carotenoids, xanthophylls, metallothioein, taurin and its precursors, polyamines, retinol, flavonoids and other phenolic compounds of plant origin. Diabetes mellitus is a metabolic disorder affecting carbohydrate, fat and protein metabolism. It represents a heterogeneous group of disorder having hyperglycemia, which is due to impaired carbohydrate (glucose) utilization resulting from a deficient insulin secretory response. To date there are different groups of oral hypoglycemic agents for chemical use, having characteristic profiles of side effects due to lack of informative over free radical scavenging. [7] Diabetic patients have been found to have higher levels of oxidative stress indices this may induce damage to the insulin producing β cells. Antioxidants therapy has been shown to protect such damage. [8] Such physiological conditions glucose may undergo auto-oxidation and contribute to ROS formation. [9] Indian traditional medicine is one of the richest medicinal systems among those available around the world. In accordance with the recommendations (WHO, 1980) an investigation of the

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antioxidant principles of plant origin used in the traditional medicine seems important. As our aim, is to deeply investigate the antioxidant activity of the selected herbs over damaged pancreatic β cells. Type-I diabetes is the small or complete lack of insulin production and Streptozotocin is the valuable and particular for induction of type-I diabetes and maximum number of β cell destruction was produced with less toxicity. [10]

MATERIALS AND METHODS

Chemicals and Reagents

Streptozotocin and other fine chemicals were purchased from Sigma Aldrich Pvt. Ltd, Mumbai, Maharashtra, India. Blood glucose concentration was analyzed by using One Touch glucometer (Ultra), Johnson & Johnson Co, India. Silica gel 'G' and 'H' grade as the stationary phase were utilized from the Institutional laboratory, School of Pharmaceutical Sciences, Vels University, Pallavaram, Chennai. All chemicals and reagents used were of analytical grade.

Collection and Processing of herbs

Almost all the plant materials were collected from the forest region of Tamil Nadu and they were identified and authenticated by Dr. O. S. Vivekanandan, Botanist, School of Pharmaceutical Sciences, Vels University, Pallavaram. The parts proposed for this study were separated from the whole plant and kept for air drying under shadow (i.e. avoiding direct exposure to sunlight) and were subjected for size reduction.

Preparation of Poly Herbal Extract

A wide range of solvents with increasing polarity were chosen.

Step 1: In a 250ml round bottomed flask, weighed quantity of powdered drug were macerated with the respective solvents in the ratio of 1:2 (i.e. 50 g in 100ml) and kept with occasional shaking for a period of 72 hrs. After the maceration process, the active ingredients present in the supernatant solvent were collected in petri dishes and concentrated under reduced pressure.

Step 2: These extracts were labeled and its chemical constituents were identified, among the different extracts, the solvent carrying more number of active constituents and underlies in the category of Class 3: with low toxic potentials (ICH guidelines 1997) was selected and prepared for bulk extraction with 2 kg of coarsely powdered polyherbs, as similar as step 1.

Phytochemical screening of Poly Herbal Extract

Identification of chemical constituents were done for Alkaloids, Carbohydrates and Glycosides, fixed oils and fats, Saponin, Tannins, Phenolic compounds, Cardiac Glycosides, Reducing Sugar, Steroids, Flavonoids, etc. [11]

Detection of Chemical Constituents of hydroethanolic extract of Polyherbal formulation by TLC–UV and Spray reagent.

Thin layer chromatography is a technique in which separation of active fraction may be achieved on the basis of partition or on a combination of partition and adsorption depending on the particular type support, it preparation and its use with different solvent.

Preparation of the Plate

80gms of silica gel 'G' was weighed out and made into homogenous suspension with 85ml of distilled water to form slurry. The suspension was poured into a TLC applicator which was adjusted to 0.25mm thickness on a flat glass plate 20cm long. The coated plates were allowed to dry in air, followed by heating at 100°C-105°C for at least one hour. Cooled and protected from moisture. The plates were then stored in a dry atmosphere. Whenever required the plates were dried in hot air oven at 110°C for 30 minutes for activation.

Application of the substance

The polyherbal hydroethanolic extract of (1g approximately) was mixed well with 1ml of methanol and it was taken in a capillary tube and it was spotted on TLC plate, 2cm above its bottom of the end. Most solutions for application were between 0.1-1% strength. The starting points were equally sized as far as possible and had a diameter ranging from 2.5mm. The extract was subjected to TLC in different solvent systems to fix the suitable solvent system priorly and TLC was developed. Two distinct spots developed on the plate were identified and observed by means of U.V. Light and Spray reagents.

Anti-Oxidant evaluation of hydroethanolic extract of Polyherbal Formulation

Reagent Visualization was made by following the Standard method. [12]

The hydroethanolic extract of polyherbal formulation was distilled to remove alcohol by vacuum at 40°C and diluted to the original concentration by water. The extract was homogenized in the presence of ethanol (80ml) for 1 minute at room temperature and centrifuged at 4°C for 15 minutes. 4ml of extract was freeze-dried and solubilised in 1ml of methanol. This extract (25µl) was subjected to thin layer chromatography on 20/20 cm glass plates coated with 0.5mm silica gel 'H' and developed with Chloroform: Methanol (9:1 v/v). The separated compounds were located and identified by visualizing plates with folin cio calteu reagent (1:2 v/v folin cio calteu in H_2O) to detect phenolic groups and with a solution of β-carotene linoleate to detect anti-oxidant active compounds. Anti-oxidants protecting against the bleaching of β-carotene gave yellowish orange spots.

ANIMAL STUDIES

Animals

Animals were allotted and approved by the institutional ethical committee (Proposal no. 290/CPCSEA/ PHARMACOL-5/18.08.05) for the proposed study. Animals were acclimatized with food and water ad libitum for a period of 1 week in 12 hours day and night cycle, before starting the experiments.

Acute Toxicity

Acute study was performed following LD_{50} procedure. Wistar albino rats (200-250g) of either sex were selected and segregated in to 8 groups of 6 animals each. Single dose of hydroethanolic extract of polyherbal formulation, starting from the minimal dose of 50mg/kg up to 3000mg/kg administered orally. [13] The drug treated animals were observed carefully for its toxicity signs and mortality. From the maximum dose used, $1/5^{th}$ and $1/10^{th}$ of the concentration was considered as therapeutic dose for further study.

Induction of Experimental Diabetes

Male albino rats (200-250g) were fasted for 16 hours before the induction of diabetes with Streptozotocin (STZ), for induction of Type-1 Diabetes mellitus. Animals (n=45) were injected intraperitoneal with 0.22-0.25ml of freshly prepared solution of STZ (60 mg / ml in 0.01 m citrate buffer, pH 4.5) at a final dose of 60 mg / kg body wt. The diabetic state was assessed in STZ-treated rats by measuring the non-fasting

serum glucose concentration after 48 hours. Only rats with serum glucose levels greater than 300 mg / dl were selected and used in this experiment.

Thiobarbituric acid reactive substances (TBARS)

The TBARS levels measured as an index of malondialdehyde (MDA) production were determined. [14] MDA, an end product of lipid peroxidation reacts with thiobarbituric acid to form a red coloured complex. The measurement of MDA levels by thiobarbituric acid reactivity is the most widely used method for assessing lipid peroxidation. Briefly, 1g of the liver and kidney samples were homogenised in 4ml of 1.15% ice cold KCl using a homogeniser to form a 25% (w/v) homogenate. To 0.1ml of 25% homogenate, 0.2ml of 8.1% dodecyl sodium sulphate salt (SDS), 1.5ml of 1% phosphoric acid, 0.2ml of distilled water and 1.0ml of 0.6% 2-thiobarbituric acid were added. The mixture was heated in a boiling water bath for 45 minutes. Subsequently, the heated mixture was cooled in an ice bath, followed by an addition of 4.0ml of n-butanol to extract the cold thiobarbituric acid reactants. The optical density of the n-butanol layer was determined at 353nm after centrifugation at 1,000g for five minutes and expressed as nmol MDA/25mg wet weight.

Evaluation of Anti-oxidative properties

Superoxide dismutase (SOD)

SOD activity was measured based on the ability of the enzyme to inhibit the autoxidation process of pyrogallol. A modification of the procedure described was adopted for assay of SOD activity. Briefly, the tissues were homogenized in 50mmol/L phosphate buffer (pH 7.8) using a homogenizer. The homogenate was centrifuged at 1,600g for 15 minute. 20µl of 10 mmol/L of pyrogallol solution was added to various concentrations of the tissue supernatants and the rate of autoxidation was measured colorimetrically at 420nm. SOD activity is expressed as units of SOD/mg protein (1.0 U is defined as the amount of the enzyme, which causes 50% inhibition of pyrogallol autoxidation). [15]

Catalase (CAT)

CAT activity was measured based on the ability of the enzyme to break down H_2O_2. The tissues were homogenized in isotonic buffer (pH 7.4). The homogenate was centrifuged at 1,000g for 10 minutes. 20µl of 100-fold diluted tissue supernatant was added to 980µl of the assay mixture containing 900µl of 10mmol/L of H_2O_2, 50µl of Tris HCl buffer (pH8.0) and 30µl of distilled water. The rate of decomposition of H_2O_2 was monitored spectrophotometrically at 240nm. CAT activity is expressed as k/mg protein, where k is the first order rate constant. [16]

Reduced glutathione (GSH)

Reduced glutathione levels were estimated based on the ability of the SH group to reduce 5, 5'-dithiobis-(2-nitrobenzoic acid) to form 1 mole of 2-nitro-5-mercaptobenzoic acid per mole of SH. The method was employed in the determination of GSH levels. Briefly, the tissues were homogenized in 50mmol/L Tris HCl buffer (pH 7.4). The homogenate was centrifuged for 20 minutes. To 0.5ml of tissue supernatant, 1.5ml of 0.2mol/L Tris HCl buffer (20mmol/L EDTA, pH 8.2). 0.1ml of 0.01mol/L of 5, 5'-dithiobis-(2-nitrobenzoic acid) and 7.9ml of methanol were added. The mixture was incubated at 37°C with occasional shaking for 30 minutes. The mixture was then centrifuged at 3,000g for 15 minutes and the absorbance of the supernatant was determined at 412nm. GSH levels are expressed as µmol/mg protein. [17]

Histopathological Studies

The dissected sample of pancreas from each group of diabetic animals were collected in 10% formalin solution and stained with hemotoxylin and eosin for preparation of section by using of microtome (Histopathology was carried out by Dr. C. J. Vijayalakshmi M. D, Pathology, in Vaishnave Clinic, Chennai-17). The histopathological studies were carried by the method described. [18]

Statistical Analysis

The results are expressed as mean ± S.E.M. Data were analyzed by using one way ANOVA followed by Tukey's multiple comparison test using Graph Pad prism Ver. 4.0. p values of <0.05 were considered as significant.

RESULTS

Phytochemical report and the yield

From the Table 1, it was found that most of the extracts were shown less conformity to active constituents like flavonoids, triterpenoids, etc which are highly required for availing antioxidant property. But, hydroethanolic and methanolic polyherbal extract was found to show positivity to maximum number of phytochemical constituents. Based on ICH guidelines, the bulk extraction was carried out with 75% hydroethanol. Around 2kg of grounded coarse powder of poly herbs were subjected for bulk extraction in 1:2 ratio, and the yield was found is 300gm.

Inference of Chemical tests by TLC –UV/Spray reagent and Antioxidant status by Reagent Visualization method

From the Fig.1 (a - d), it was observed that the TLC plate under UV region and Spray reagents application showed positive inference to the major components like alkaloids, glycosides, flavonoids, triterpenoids, etc. and bleaching with β-carotene linoleate, it was observed that formation of yellowish orange spot in the plate (Fig. 1.e.).

Toxicity study and dose fixing

From table 2 of acute study, the maximum dose of 3000mg/kg was found to show no signs of toxicity nor morbidity. Hence, 1/5th of the dose i.e., 600mg/kg and half of the same (300mg/kg) was also considered and preceded for animal studies.

Induction of Experimental Diabetes

The experimental data showed that, among 45 no. of animals, 40 nos. was found to have blood glucose levels greater than 300mg/dl after intraperitoneal injection with STZ (60mg/kg).

Table 1: Phytochemical report

Test	PH WE	PH ME	PHE E	PM BE	PHP E	PHHE (75%)
Alkaloids	+	+	+	-	-	+
Carbohydrates & Glycosides	+	+	+	-	+	+
Specific Glycosides	-	+	-	+	-	-
Cardiac Glycoside	-	-	+	-	-	+
Reducing sugars	+	+	+	+	+	+
Steroids	-	+	-	-	+	-
Saponins	-	+	-	+	-	-`
Tannins	-	+	+	+	-	-
Condensed tannins	-	+	-	-	-	-
Pseudo tannins	+	-	+	+	-	+
Flavonoids	-	+	+	-	-	+
Triterpenoids	-	+	+	-	+	+

PH-Poly Herbal:- WE-Water Extract; ME-Methanolic Extract; EE-Ethanolic Extract; BE-Benzene Extract; PE-Petroleum ether Extract; HE-Hydroethanolic Extract

Table 2: Acute study and its sign of toxicity

Treatment	Dose level	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
I	**50**	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	-
II	**100**	+	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	+	-
III	**150**	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-
IV	**300**	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-
V	**600**	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	+	-
VI	**1000**	+	+	+	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	+	-
VII	**1500**	+	+	+	-	-	+	+	-	-	-	-	+	+	+	-	-	-	-	+	-
VIII	**3000**	+	-	+	-	+	+	+	-	-	-	-	+	+	+	-	-	-	-	+	-

1. Alertness, 2. Aggressiveness, 3. Pile erection, 4. Grooming, 5. Gripping, 6. Touch Response, 7. Increased Motor Activity, 8. Tremors, 9. Convulsions, 10. Muscle Spasm, 11. Catatonia, 12. Muscle relaxant, 13. Hypnosis, 14. Analgesia, 15.Lacrimation, 16. Exophthalmos, 17. Diarrhoea, 18. Writhing, 19. Respiration, 20. Number of Deaths (Mortality).

Table 3: Fasting blood glucose concentration is normal and STZ-induced diabetic rats

Treatment and Dose	Fasting Blood Glucose concentration (mg/dl) at a regular interval days		
	IV	VIII	XV
Normal Control (0.5% Normal saline)	71.33 ± 3.13	71.83± 2.81	73.00± 2.39
Diabetic Control (0.5 % Normal saline)	151.30± 1.94	151.70 ± 2.78	152.00 ± 2.00
Test I (PHHE 600mg/kg)	145.30± 1.49^{ans}	136.30 ± 1.80$^{b\#}$	117.00 ± 2.00^{c*}
Test II (PHHE 300mg/kg)	124.20± 1.62^{a*}	110.00 ± 2.00^{b*}	74.50 ± 2.18^{c*,dns}
STD I(Glibenclamide/ Metformin 0.5:40mg/kg)	130.80± 1.85^{a*}	135.00± 1.00$^{b\#}$	138.00± 7.00$^{c@}$
STD II (Zinc Insulin 4 U)	125.00± 2.14^{a*}	133.00 ± 2.16^{b*}	137.70 ± 1.43$^{c@}$

n=6; Statistical analysis were done using One way ANOVA followed by Tukey's Multiple comparison method. ; Values are expressed as mean ± S.E.M; a, b & c (Day IV, VIII & XV respectively): Diabetic control vs Test I & II, STD I & II ; d = Day XV: Normal control vs Test I & II; * = P<0.001; # = P<0.01; @ = P<0.05; ns = P>0.05.

Table 4: Malondialdehyde content in liver of normal and STZ-induced diabetic rats

Treatment and Dose	Dose	TBARS Value (nmol MDA/25 mg wet. wt)
Normal Control (0.5% Normal saline)	0.5% of Normal Saline	5.160 ± 0.027
Diabetic Control (0.5 % Normal saline)	0.5% of Normal Saline	10.67 ± 0.156^{a*}
Test I (PHHE 600mg/kg)	PHHE –I (600mg/kg)	6.300 ± 0.047$^{a*, b*}$
Test II (PHHE 300mg/kg)	PHHE –II (300mg/kg)	5.330 ± 0.075$^{ans, b*}$
STD I (Glibenclamide/Metformin 0.5:40mg/kg)	Glibenclamide/Metformin (0.5:40mg/kg)	5.443 ± 0.052$^{ans, b*, cns}$
STD II (Zinc Insulin 4 U)	Zinc Insulin (4 U)	5.350 ± 0.040^{ans,b*,cns}

n=6; Statistical analysis were done using One Way ANOVA following Tukey's multiple comparison method. ; Values are expressed as mean ± S.E.M.; a = Normal control Vs Diabetic, Test I & II and STD I & II; b = Diabetic Control Vs Test I & II and STD I & II; c = Test II Vs STD I & II; * = P<0.001; # = P<0.01; @ = P<0.05**;** ns = P>0.05

Fasting Blood Glucose and Antioxidant status of PHHE

From the Table 3, it was observed that there was a gradual reduction in the fasting blood glucose level in both Tests I & II treated diabetic rats as compared with that of standard groups. Furthermore, on the 15th day end of the study the FBG level of Test I (PHHE – 600mg/kg/p.o.) treated diabetic rats was found to be reduced near to the fasting values of normal control (P>0.05). There was only a less significant difference in fasted blood glucose values of the standards (P<0.05) as compared with that of Test I treated diabetic rats. It was observed that from the Table 4, there is an increase in the level of TBARS value of diabetic control as compared with that of normal control and other groups (P<0.001). There is more significant increase (P<0.001) in the level of antioxidants like SOD, CAT and GSH of Test I & II treated groups as compared to that of diabetic control and also shown significant difference with standard drug treated groups (Table 5).

Table 5: Antioxidant status of Pancreas in normal and STZ-induced diabetic rats

Treatment and Dose	Antioxidant Parameters- Status of Enzyme levels		
	SOD (U/mg protein)	CAT (k/mg Protein)	GSH (µmol/mg protein)
Normal Control (0.5% Normal saline)	23.15 ± 0.240	0.474 ± 0.011	0.176 ± 0.003
Diabetic Control (0.5 % Normal saline)	12.50 ± 0.236^{a*}	0.248 ± 0.005^{a*}	0.067 ± 0.001^{a*}
Test I (PHHE 600mg/kg)	22.09 ± 0.407$^{ans, b*}$	0.434 ± 0.005$^{a\#,b*}$	0.179 ± 0.003 ^{ans,b*}
Test II (PHHE 300mg/kg)	21.09 ± 0.341$^{a*, b*}$	0.352 ± 0.010$^{a*,b@}$	0.147 ± 0.010 ^{a*,b*}
STD I (Glibenclamide/Metformin 0.5:40mg/kg)	13.93 ± 0.145$^{a*,b@,c*}$	0.295±0.004$^{a*,b\#,c*}$	0.121 ± 0.003^{a*,b*,*}
STD II (Zinc Insulin 4 U)	15.93 ± 0.236^{a*,b*,c*}	0.331±0.001^{a*,b*,c*}	0.129 ± 0.002^{a*,b*,*}

n=6; Statistical analysis were done using One Way ANOVA following Tukey's multiple comparison method. ; Values are expressed as mean ± S.E.M; a = Normal Vs Diabetic control, TEST I & II, STD I & II.; b = Diabetic control Vs TEST I & II, STD I & II.; c = TEST I Vs STD I & II.; * = P<0.001; # = P<0.01; @ = P<0.05; ns = P>0.05.

Histopathology report

The microanatomical study showed the hyperplasia with destroyed β-cells in diabetic control as compared with the normal. On viewing, Test I & II (600 & 300 mg/kg/p.o.) treated animals it was observed that there was a positive impregnation over destructed β-cells (reduced hyperplasia) and shown possible β-cell regeneration which was not observed in standard drug treated groups (Fig.2. a - f).

DISCUSSION

Phytochemical report and the yield

Based on the results obtained from the chemical tests, though it was shown that both the extracts of hydroethanol and methanol possessing maximum number of active principles, and on the behalf of the ICH guidelines (1997) for the safety use of solvents, ethanol & water was found to fall under category 3 of low toxicity potentials. And hence for the bulk extraction, 75% hydroethanolic solvent used was found to be quite worthy.

Confirmation of Chemical Constituents by TLC – UV/Spray reagent and Antioxidant potential by Reagent Visualization method

The colour changes as per from the Fig. 1 (a - d), it was confirmed the presence of alkaloids, glycosides, flavonoids

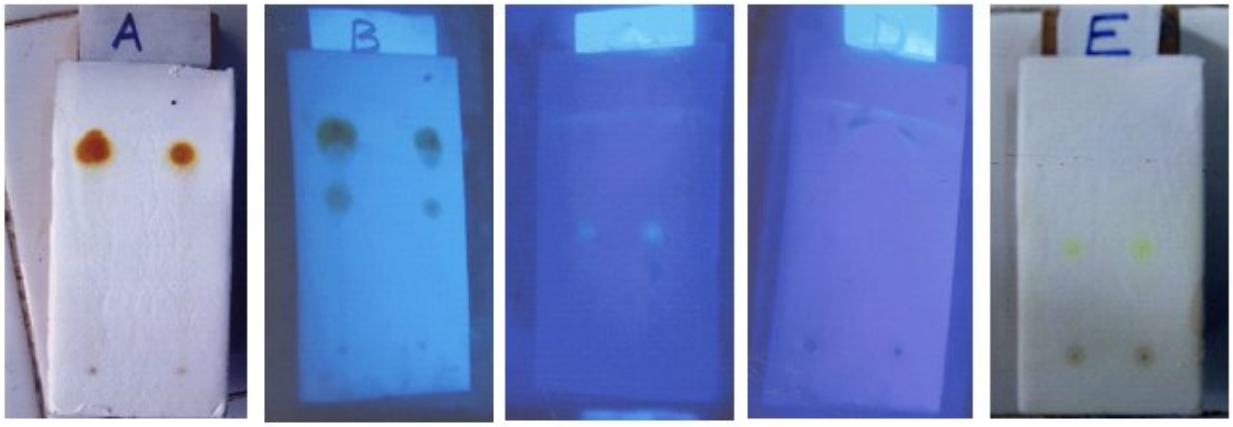

Fig. 1: A. showing reddish brown spots for alkaloids with Dragendroff Spray reagent, B. showing yellowish red fluorescence for glycosides in the UV-long wave region (after Sbcl2 and 100ºC), C. showing intense yellow fluorescence for flavonoids in the UV- long wave region, D. showing yellow fluorescence for triterpenoids in the UV-long wave region, E. showing yellow fluorescence for antioxidant activity using β–carotene linoleate oxidation method.

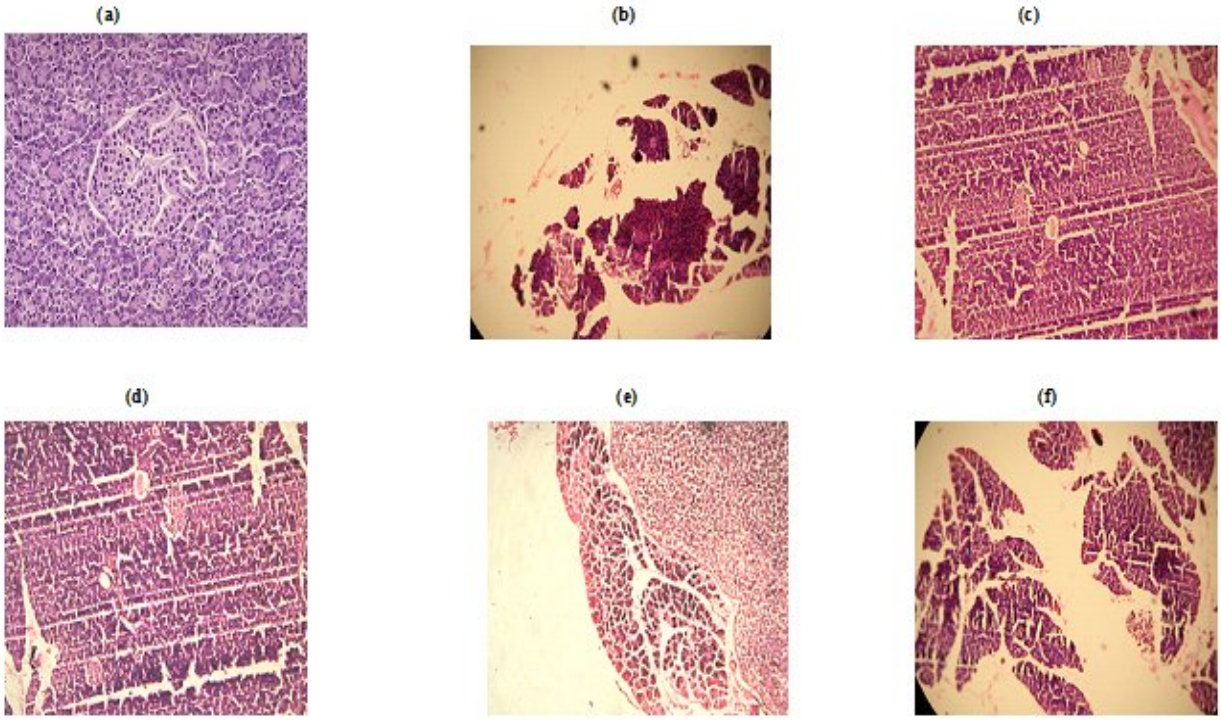

Fig. 2: Showing transverse section of pancreas: (a). Non diabetic control with normal acini with islets of β –cells; (b). Diabetic control with atrophic acini and reduction of β-cells size; (c). Test drug PHHE I (600mg/kg) with markedly normal regenerated and preserved cell; (d). Test drug PHHE II (300mg/kg) with marked proliferated and regenerated β-cells; (e). Std glibenclamide/metformin (0.5/0.4 mg/kg) with hyperplastic condition; (f). Std zinc insulin (40 I.U) with marked increased hyperplastic β-cells.

and triterpenoids and from the reagent visualization method, it was observed that the antioxidant property of PHHE was found to protect the bleaching of β-carotene and thereby gave yellowish orange colouration. This method confirms the presence of flavonoid and its free phenolic compounds. Flavonoids & free phenolics and triterpenoids have been reported to have multiple biological effects such as anti-oxidant, anti-inflammatory, analgesic, anti-pyretic and anti-diabetic activity. [19]

Toxicity studies and dose fixing

As per LD50 study, 1/5th from the maximum nonlethal dose (3000mk/kg/p.o.), i.e., 600mg/kg/p.o., from was considered and half of the same 300mg/kg/p.o., also studied, so as to compare the effectiveness of the previous one, for the antioxidant study and thereby glucose lowering effect on STZ induced Type-1 diabetic rats.

Type 1 diabetes

From the above result, it was observed that streptozotocin (60mg/kg/i.p.) induction destroyed the pancreatic β-cells and by this it's proved that STZ is a valuable agent for induction of type-1 diabetes in rats.

Fasting blood glucose and antioxidant status

The reduction in the blood glucose level till up to 15th day of study, replicates the positive impregnation of polyherbal formulation over β cells of pancreas. This might be due to the stimulation of regenerated β cells, by the antioxidant potentials of PHHE as evidenced from its recovery of antioxidant enzymes (Table 5). [20]

The decrease in the activities of antioxidant enzymes in diabetes may be due to low levels of insulin [21] and it was evidenced by the increased level of MDA thereby TBARS [22] in liver of diabetised rats. Although the precise mechanism of both standard drugs for its antioxidant effects by availability of plasma insulin were reported. [23-24] there is only a negligible reduction in fasted blood glucose levels as compared to Test I treated diabetic rats. This might be due the time lag between prior administration and the fasting blood collection and or due to the lack of sufficient insulin neither to produce antihyperglycemic action nor to scavenge free radicals generated *in vivo*.

Micro anatomical Changes

Furthermore, the micro anatomical study on pancreas, strongly lead to an evidence for the protective role of PHHE over free radical mediated β-cell destruction.

Based on the above results, we conclude that the new polyherbal formulation PHHE 600>300mg/kg/p.o., (based on activity) was an effective free radical scavenger on dose dependent manner with positive regeneration and stimulation of pancreatic β cells thereby produces antihyperglycemic action. The anti-oxidant property of extract may be because of the presence of flavonoids and its polyphenols.

Further the precise mechanism and the active constituents of PHHE responsible for its anti-oxidant and related pharmacological activities are still to be determined and further toxicological studies are to be established.

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