RESEARCH ARTICLE

Anti-Diabetic, Anti-Hyperlipidemic and Anti Oxidant activities of "ATH-2K13" in Streptozotocin induced Diabetic Rats

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ABSTRACT

Diabetes is the most widely seen metabolic disorder and it is not only a metabolic disorder but also induce some serious disorder like diabetic retinopathy, neuropathy, nephropathy, cardiovascular damage and cataracts etc. The main objective of the present work is to study the anti-diabetic, anti-hyperlipedimic and anti-oxidant activities "ATH-2K13"which was a poly herbal formulation. There are many methods used to induce the diabetes normally, but among all the methods one of the widely accepted method is chemical method of induction and it is easier to induce the diabetes also. As a part of induction we were choosing Streptozotocin as a diabetic control. The standard drug used in the present work was glibenclamide. The two test doses taken in the present study were 100mg/kg and 200mg/kg. Acute toxicity studies and OGTT tests were conducted before screening as safety evaluation of new formulation. The total duration of the study was 14days after 48 hours of diabetes induction to the rats. Total five groups of containing albino Wistar rats of either sex were taken for the evaluation. After 14 days of study blood was collected and serum was separated for the biochemical evaluation. Body weight and blood glucose levels were checked daily.

Keywords: ATH-2K13, Streptozotocin, anti-diabetic, anti-oxidant anti hyperlipedimic, acute toxicity studies, OGTT, 14days.

INTRODUCTION

Diabetes mellitus is a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both[1]. Globally by 2030 this number is estimated to rise up to 435 million[2]. There is a lot of progress in the treatment and management of the DM now a day. Many health organisations and social service organisations have been trying as good as they can. But it is not only sufficient but also peoples they also try to get awareness and take precautionary measurements to overcome this disorder, at least to reduce the victim count. Now a day along with allopathic medicine there is wide range of research is going on herbal formulation to find supplement. Herbal formulation is the safest type of dosage form to treat many disorders. Diabetes can be managed by diet, exercise, and chemotherapy. However, the pharmacological drugs are either too expensive or have undesirable side effects or contraindications[3]. In the present study of screening of anti-diabetic, anti hyperlipedimic and anti-oxidant activities we were using three herbals. So this is a poly herbal formulation and we named it as ATH-2K13.The major herbals present in this formulation were *Phyllanthus umbilicus, Curcuma longa, and honey*. Two test doses were prepared for screening called 100mg/kg and 200mg/kg. However among all type of formulations herbal formulations are safest and less toxic[4].

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MATERIALS AND METHODS

Procurement and identification of plant material

The dried rhizomes of *Curcuma longa*, fruits *Phyllanthus umbilicus* and honey were collected. The dried rhizomes were neatly cleaned and kept under shade drying and the fruits of Phyllanthus umbilicus were taken and cleaned and washed thoroughly and cut in to small pieces. These pieces were also kept under shade drying. Pure Honey is taken and preserved at cool place. *Curcuma longa* is

commonly called as turmeric and *Phyllanthus umbilicus* is commonly called as Amla.

Preparation of the extract

The rhizomes of *Curcuma longa* and dried fruits of *Phyllanthus umbilicus* were taken and powdered. The Fine powder was taken for extraction. The dried powder is submitted to successive extraction by soxhlet apparatus with 100%methanol at 72degrees temperature for 18hrs. All the extract was filtered through membrane filter and then the extract dried in room temperature. Then the suspension of herbals was prepared by adding equal quantities of methanolic extract of Amla and turmeric, Honey. In the preparation of this poly herbal formulation tween 80 is used as suspending agent. 100mg/kg body weight and 200mg/kg body weight doses were prepared[5].

Drugs and chemicals

Streptozotocin was used as a standard drug. Glibenclamide (Batch no: G080851) was a gifted sample from TABLETS INDIA LTD, Chennai. Standard Glucose estimation kits were procured from ROBONIK (INDIA) PVT.LTD, Mumbai. Enzymatic kits for the estimation of lipid profile were obtained from CHEMA DIAGNOSTICA (INDIA).

Animals

Healthy adult albino rats of Wister strain of either sex between the age of 2-3 months and weighing 200-300 grams were used for the present study. The animals were housed individually in polypropylene cages, maintained under standard conditions (12 hours light and12 hours dark cycle, 24±5°C and 40-60% humidity) [6]. They were fed with standard rat pellet diet (National Institute for Nutrition, Hyderabad) and provided water ad libitum. All the animals are collected from central animal house SICRA LABS PVT.LTD, KUKATPALLY, HYDERABAD and all experiments were conducted according to the ethical norms approved by CPCSEA, Ethical committee IAEC reg. no. (769/2011/CPCSEA).

EXPERIMENTAL

Phytochemical Screening:

A preliminary phytochemical screening of methanolic extracts was carried by using standard procedures. The phytochemicals presented in the extracts were shown in table 1 and 2.

Acute Oral Toxicity Studies

Acute oral toxicity studies[7] of the extracts were carried out as per the OECD guidelines, draft guidelines 423 adopted and received from Committee for the Purpose of Supervision and Control of Experiments on Animals (CPCSEA), Ministry of social justice and empowerment, Government of India. Albino wistar rats (n=30) were fasted for 16 to 18hrs and were divided into four groups of 6 animals each and the treatment protocol was described below. Before the drug administration, blood samples were collected by spinning the tail vein for the estimation of glucose levels at regular intervals.

Treatment protocol:

All the rats were randomized into five groups comprising of six animals in each group as given below.

Group I : normal rats received 100mg of ATH-2K13
Group II : normal rats received 200mg/kg ATH-2K13
Group III: normal rats received 500mg/kg ATH-2K13
Group IV: normal rats received 750mg/kg ATH-2K13
Group V: normal rats received 1000mg/kg ATH-2K13

ATH-2K13of various doses were administered orally using an intra-gastric tube and monitored for 48hrs.After the administration of single dose of ATH-2K13 no mortality rates are observed. It was shown in table no.1

ORAL GLUCOSE TOLERANCE TEST

Albino wistar rats (n=30) were fasted for 16 to 18hrs and were divided into four groups of 6 animals each and the treatment protocol was described below. Before the drug administration, blood samples were collected by spinning the tail vein for the estimation of glucose levels at regular intervals.

Treatment protocol

All the rats were randomized into five groups comprising of six animals in each group as given below.

Group I: normal rats received (10% acacia mucilage)

Group II: normal rats received glucose

Group III: normal rats received 5mg/kg glibenclamide.

Group IV: normal rats received 100mg/kg ATH-2K13

Group V: normal rats received 200mg/kg ATH-2K13

ATH-2K13(100mg/kg, 200mg/kg) and Glibenclamide (5mg/kg)[8] were administered orally using an intra-gastric tube. After 30mins of the administration of standard and poly herbal formulation ATH-2K13 orally 1% glucose is administered. Immediately blood glucose levels were monitored at 0hr, 1hr, 2hr, 4 hr. and6 hrs. By using "Glucocheck" blood glucose testing instrument after the administration of single dose of 1%glucose. It was shown in table no.2

Anti-diabetic activity Treatment protocol:

All the rats were randomized into five groups comprising of six animals in each group as given below.

Group I : normal rats received (10% acacia mucilage)

Group II : Diabetic control received 65mg/kg of Streptozotocin.

Group III: normal rats received 5mg/kg glibenclamide.

Group IV: normal rats received 100mg/kg ATH-2K13.

Group V: normal rats received 200mg/kg ATH-2K13.

Induction of experimental diabetes

It is a chemical method of diabetes induction. Diabetes was induced by using Streptozotocin. Streptozotocin (65 mg/kg) was dissolved in ice cold citrate buffer (pH 4.3) immediately before use. The Streptozotocin solution[10] was injected through I.P route in the dose of 65 mg/kg in rats. 5 % glucose solution was administered orally for 24 hrs. This administration was to prevent mortality due to initial hypoglycemia induced by Streptozotocin. After 48 hrs. Of STZ injection, fasting blood glucose levels were tested by using glucose oxidase-peroxidase reactive strips of Gluco-check digital blood sugar monitoring instrument. All the four groups Rats showing fasting blood glucose more than 250 mg/kg were considered diabetic and used for further study.

Anti-Diabetes and Anti-hyperlipidemia studies

Collection of blood samples and estimation of Biochemical parameters

The total duration of the treatment of grouped animals with the standard extracts of ATH-2K13 was 14 days. After 48hrs of the induction of STZ in regular interval days the blood glucose was monitored for all the five groups' .On 1st day, 3rd day,5th day, 7th day, 14th day the blood glucose levels were monitored. On the 15th day, blood samples (Approximately 0.5ml) were collected from overnight fasted rats by puncturing the retro orbital sinus, under mild ether anaesthesia for biochemical estimations[11]. Blood samples were not allowed to clot for 30 min and serum was separated by centrifugation at 3000rpm and it is preserved for the estimation of total serum cholesterol, LDL-C, HDL-C, VLDL-C, SGOT, and SGPT. The obtained results were mentioned in table no.4.

Determination of serum lipid profile

Serum cholesterol, triglycerides, LDL-C, HDL-C, SGOT and SGPT were determined by using standard enzymatic kits obtained from ROBONIK (INDIA) PVT.LTD, Mumbai.

In-vivo Anti-oxidant Activity

After the14 days of screening of anti-diabetic and anti-hyperlipedimic activities on 15th day after the collection of blood from all groups animals by puncturing the retro orbital sinus the blood was collected and undergone for centrifugation at 3000rpm the serum was separated. The animals were then sacrificed and the liver was collected for the estimation of anti-oxidant activity of the test formulation. Liver was rapidly excised, rinsed in ice-cold saline. The 10%w/v homogenate was prepared by using 0.15M KCI, centrifuged at 800 g for 10 min at 4°C[12]. The supernatant fluid which is obtained after centrifugation was used for the estimation of

GSH, SOD, and Catalase. The obtained results were mentioned in table no.5

Glutathione

Glutathione (GSH) was estimated by using Elman's reagent (5, 5- di thio bis-(2-nitrobenzoic acid) [DTNB]). The sulfhydryl groups present in glutathione forms a colour complex with DTNB, which was measured by colorimeter at 412 nm. The amount of glutathione was determined using its molar extinction coefficient of 13600/m/cm and expressed in terms of µ mol/mg of protein[13].

Estimation of SOD

Estimation of SOD was done by auto oxidation of hydroxylamine at pH 10.2, which was accompanied by reduction of NBT, and the nitrite produced in the presence of EDTA was detected colorimetrically[14]. One enzymatic unit of SOD is the amount in the form of proteins present in 100 ìl of 10% liver homogenate required to inhibit the reduction of 24 mm NBT by 50% and is expressed as units per milligram of protein.

Estimation of catalase

Catalase activity was estimated by determining the decomposition of H_2O_2 at 240 nm in an assay mixture containing phosphate buffer[15]. One international unit of catalase utilized is that amount that catalyses the decomposition of 1 mm H_2O_2/min/mg of protein at 37°C.Catalase activity was calculated using the milli molar extinction coefficient of 0.07 and expressed in terms of micromole per minute per milligram of protein.

Statistical analysis*:*

All the values of body weight, fasting blood sugar, and biochemical estimations were expressed as mean ± standard error of mean (S.E.M.) and analysed for ONE WAY ANOVA and post hoc Dennett's t-test using computerized Graph Pad Prism In Stat version 5.0, Graph Pad software. Differences between groups were considered significant at *P<0.001* and very significant at *P < 0.0001* levels.

RESULTS

In the present study we have employed the screening of anti-diabetic, anti-hyperlipedimic and anti-oxidant activities of ATH-2K13 formulation in Streptopzotocin induced diabetic rats. It is a chemical method of induction. To induce the type-1 diabetes mellitus 65mg/kg body weight of Streptozotocin is used. This dose is given by dissolve in normal saline solution and administered through intraperitoneal route. Before the major screening is employed acute oral toxicity studies and oral glucose tolerance test was conducted and the results were shown in table no-3and 4.The blood glucose levels were gradually decreased from day 1 to day 14.The regular interval blood glucose levels were shown in table no-5. The in-vivo anti-oxidant data was mentioned in table no-6. The total biochemical parameters of blood serum were mentioned in table no.7.The obtained data is biochemical estimations were expressed as mean ± standard error of mean (S.E.M.) and analysed for ONE WAY ANOVA and post hoc Dennett's t-test using computerized Graph Pad Prism In Stat version 5.0, Graph Pad software. Differences between groups were considered significant at P<0.001 and very significant at P < 0.0001 levels.

DISCUSSION

After conducting the phytochemical screening from table no-1&2 active chemical constituents like alkaloids, proteins, carbohydrates, tannins, phenols etc., were observed. From the table no-3 there was no mortality. So, the poly-herbal formulation ATH-2K13 was safe. From the table no-4 there was significant oral glucose tolerance observed. Till the completion of screening the blood glucose levels of all groups of rats are recorded and tabulated. From the table no-5 significant changes in the blood glucose levels were observed after the statistical analysis. Diabetic control group blood glucose level is decreased from 400.1±5.20 to 225.4±7.8.The standard group blood glucose level is decreased from 151.8±8.4 to 75.02±4.3; the test group 1 (100mg/kg) blood glucose level is decreased from

385.72±10.4 to128.14±3.2. The test group 2 (200mg/kg) blood glucose level is decreased from 378.82±9.3 to108.22±2.50.. From the table no-6significant changes takes place in the anti-oxidant properties of enzymes GSH, SOD and Catalase. From the table no-7 significant changes takes place in the serum profile after the treatment of Streptozotocin groups with standard and test drug doses.

CONCLUSION

ATH-2K13 is a combination methanolic extract of dried fruits of Amla, dried rhizomes of turmeric. Honey itself acts as a vehicle. It had shown some effect in the treatment of Diabetes mellitus, hyperlipidaemia and it was a good anti-oxidant too. The significant p-value <0.0001 in Streptozotocin induced diabetic rats. The main mechanism of action of poly herbal formulation is to stimulates the b-cells of pancreas there by it reduces hyperglycaemic levels of blood glucose and it also efficiently acts on the lipid profile also. So we the are suggested for further research.

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Table 1: Phytochemicals constituents of *Curcuma longa*

Phyto constituents	**Curcuma longa**
Alkaloids	+
Carbohydrates	+
Glycosides	+
Tannins	+
Proteins and amino	+
acids	+
Saponins	+
Steroids	+
Flavonoids	+

Table 2: Phytochemicals constituents of *Phyllanthus umbilicus*

Phyto constituents	Embilica officinalis
Alkaloids	+
Carbohydrates	+
Glycosides	+
Tannins	+
Proteins and amino acids	+
Saponins	-
Flavonoids	+
Phenols	+

Table 3: Acute Oral Toxicity Studies of ATH-2K13

DOSE	NO OF RATS/NO.OF MORATLITY			
	6Hh	12hr	24hr	48hr
100mg/kg	6/0	6/0	6/0	6/0
200mg/kg	6/0	6/0	6/0	6/0
500mg/kg	6/0	6/0	6/0	6/0
750mg/kg	6/0	6/0	6/0	6/0
1000mg/kg	6/0	6/0	6/0	6/0

Table 4: Effect of ATH-2K13 on blood glucose level (mg/dl) in Streptozotocin diabetic rats on day 1 of the treatment (OGTT)

TREATMENT	FASTING BLOOD GLUCOSE LEVEL (mg/dl)					
	0hr	1h	2hr	4hr	6hr	24hr
Normal	94±4.15	82±2.45	78.7±4.1	84.8±2.5	92.3±4.0	98.5±2.5
Control (Streptozotocin 65mg/kg)	88.5±1.81	75±3.70	74.4±3.15	85±2.8	91.2±2.2	97.4±3.0
DC + Glibenclamid (5mg/kg)	86.5±2.51	75.8±1.71	68±3.57	61±2.2***	72.4±2.7***	82.5±1.4***
DC+ATH-2K13 (100mg/kg)	82±2.8	77.5±2.8	70.1±3.6	72.6±3.2***	80.5±2.5***	91.2±3.0***
DC+ATH-2K13 (200mg/kg)	92.5±3.12	82.7±3.6	78±2.80	82.2±3.5***	90.5±3.03***	95.5±3.21**

Values are expressed as Mean ± SEM; *P<0.01; **P<0.001; ***P<0.0001

Table 5: Effect ATH-2K13 on blood glucose level (mg/dl) in Streptozotocin induced diabetic rats

TREATMENT	FASTING BLOOD GLUCOSE LEVELS (mg/dl)				
	1st day	3rd day	5th day	7th day	14th day
Normal	100.32±3.0	85.7±1.1	90±1.4	105.12±1	80.14±3
D.Control(Streptozotocin 65mg/kg)	400.1±5.20	385.4±4.20	405.25±5.2	402.7±8.21	225.4±7.8
DC+Glibenclamide(5mg/kg)	151.8±8.4***	127.7±4.2***	95.4±4.14**	85.4±2.4***	75.02±4.3***
DC+ATH-2K13(100mg/kg)	385.72±10.4***	318.62±8.20***	246.62±7.5***	157.1±2.4***	128.14±3.2***
DC+ATH-2K13(200mg/kg)	378.82±9.3***	289.2±3.15***	178.2±6.3***	146.8±3.33***	108.22±2.50***

Values are expressed as Mean ± SEM; *P<0.01 **P<0.001; ***P<0.0001

Table 6: Effect of ATH-2K13 on liver homogenate in Streptozotocin induced diabetic rats after 14 days treatment. (In-vivo Anti-oxidant activity of ATH-2K13)

GROUP	GSH (mg/100g tissue)	SOD (Units/mg protein)	CAT (U/mg protein)
Normal	64.22±4.32	5.8 ± 1.1	90.24±2.7
D.Control(Streptozotocin 65mg/kg)	34.12 ±3.11**	3.11 ±0.1*	65.01±4.9**
DC + Glibenclamide(5mg/kg)	52.8 ±3.1**	4.92 ±1.4*	82.12 ±3.15*
ATH-2K13(100mg/kg)	48.3 ±3.9**	4.24 ±0.14**	77.21 ±4.2**
ATH-2K13(200mg/kg)	56.70 ±5.16**	4.9 ±1.1*	80.48 ±5.26*

Table 7: Effect of ATH-2K13 on serum lipid profile in Streptozotocin induced diabetic rats after 14 days treatment

TREATMENT	TC(mg/dl)	HDL-C	LDL-C	TG(mg/dl)	SGOT(U/L)	SGPT(U/L)	ALP(U/L)
Normal	105.6±1.3	44.2±3.00	34.24±1.2	78.30±2.3	88.54±2.2	33.14±1.5	120.4±1.0
D.Control (Streptozotocin 65mg/kg)	137.6±2.5	25.65±1.9	64.14±2.22	165.8±1.92	278.0±1.7	96.91±0.45	266.8±1.1
DC + Glibenclamide (5mg/kg)	117.6±1.9***	51.3±2.62**	42.35±2.8***	100.6±2.9***	154.7±2.5***	44.14±1.9***	129.7±2.03***
DC+ATH-2K13 (100mg/kg)	150.9±2.03***	39.10±1.4	51.4±2.8***	129.2±2.4***	185.5±2.25**	52.9±1.1**	148.0±1.0**
DC+ATH-2K13 (200mg/kg)	135.6±2.13***	50.7±1.9**	44.32±2.11***	108.9±3.45***	165.4±1.20***	48.4±1.0***	134.6±2.24**

TC= total cholesterol; TG= triglycerides; HDL= high density lipoproteins; LDL= low density lipoproteins; and ALP. All the values are expressed as Mean ± SEM and are very significant at*P<0.01, **P<0.001, ***P<0.0001.

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