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ANTIDIABETIC EFFECT OF POLYHERBAL EXTRACT IN STREPTOZOTOCIN INDUCED DIABETIC RATS

Biju T. Francis* and [1]Sudha S.

*Department of Biotechnology, Karpagam University, Coimbatore-641 021, Tamilnadu, India.

[1]Department of Zoology, LRG Government Arts college for Women, Tirupur, Tamilnadu, India.

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***Corresponding Author'**
Biju T. Francis
Department of Biotechnology, Karpagam University, Coimbatore-641 021, Tamilnadu, India.

ABSTRACT

Diabetes mellitus is one of the most common metabolic disorder that continue to increase in numbers and its complications impose significant consequences on individuals, families, health systems and countries. There are several approaches to control the ill effects of diabetes and its complications; however herbal remedies are the preferred choice due to low cost and lesser side effects. Multifactorial metabolic diseases develop several complications like hyperlipidemia, hepatic toxicity, immunodeficiency etc. and multiple clinical failures observed in monotherapy treatment. Hence instead of mono-drug therapy, combinations of herbs are found to be a better option for the management of the disease. Present study focused on combined effects of seven well-known herbal medicinal plants used in the treatment of diabetes. The in-vitro and in-vivo antidiabetic effects for polyherbal hydro-alcoholic extracts of *Phyllanthus emblica* (fruit), *Phyllanthus Amarus* (whole plant), *Tinospora cordifolia* (whole plant), *Curcuma Longa* (rhizome), *Syzygium aromaticum* (flower), *Piper longum* (fruit) and *Moringa oleifera* (Leaf) have been evaluated. The antidiabetic effects of individual plants are well known and the synergistic effect is expected, but unclear. No sign of toxicity was observed in animals. The antidiabetic activity of polyherbal extracts were screened against streptozotocin-induced diabetes mellitus in rats and effects are comparable with that of glibenclamide.

KEYWORDS: Polyherb, antidiabetic, in-vitro, in-vivo, streptozotocin, diabetes mellitus.

INTRODUCTION

Diabetes is a chronic metabolic disorder characterizes by the high blood glucose level associated with the carbohydrate, fat and protein metabolism disturbance. Diabetes often debilitating and sometimes fatal disease in which either body cannot produce insulin insufficiently or cannot properly use the insulin produces by the body. In diabetes, high level of blood glucose may damage various organs especially the nerves and blood vessels.[1,2] Diabetes is sometimes called the "silent killer" because the signs of the disease are not always noticeable. If diabetes is not managed, it may impact not just the person with the disease but the whole family.[3] Diabetes mellitus is derived from the Greek word diabetes means siphon (lot of urine is excreted) and the mellitus, means honey as urine tasted sweet.[4]

The estimated global prevalence of diabetes was 9% in 2014.[5] An estimated 108 million people world-wide had diabetes in 1980 which was increased to 220 million by 2004 and had shot up to 387million people worldwide in 2014, which was further estimated to reach 592 million people (10% of adults) to have diabetes by 2035. Out of 387 million people, 46% were undiagnosed. Without urgent action, deaths rate will increase by more than 50% in the next 10 years. Additionally, 17% of all live births were associated with hyperglycaemia.[6,7] In 2012, 1.5 million deaths were noted due to diabetes and additional 2.2 million deaths was noted due increasing the risks of cardiovascular and other diseases. The percentage of deaths due to diabetes that occurs prior to age 70 is higher in low and middle income countries than in high-income countries.[8] The total healthcare expenditure for diabetes management is exceeded US$612 billion, as one in every nine dollars spend on diabetes. The majority of diabetes people (77%) are living in low-income and middle-income countries.[9]

Since ancient times, a number of herbal medicines have been used in the treatment of diabetes and many studies have been carried out in the search of a suitable plant drug that would be effective in Diabetes mellitus. This situation has led to the search for alternative therapies for diabetes from natural products and medicinal plants as these are commonly cheaper, less toxic, with fewer side effects and multi-target actions.[10] Streptozotocin induced diabetic rats are one of the commonly used animal models of insulin dependent diabetes mellitus characterized by high fasting blood glucose levels and drastic reduction in plasma insulin concentration.[11] The plants selected for antidiabetic investigations were taken from traditional literatures and has proven individual antidiabetic activity.

MATERIAL AND METHODS

Collection and Authentication of Plant Materials

The Plants were collected from different areas of Thrissur district (Kerala state) in the month of October. The plants were authenticated by Dr. M Kesavan M.S.A.M, (Chief Physician, Amala Ayurvedic Hospital and Research Centre, Amala Nagar, Thrissur, Kerala).

Preparation of polyherbal extracts[12,13]

The selected part of plants was washed and dried under shade for 20 days; pulverized for further study. Soxhlet extraction method and cold maceration method has been used for the preparation of individual/polyherbal extracts of plants.

The powdered samples (14 g each) were extracted with 250 ml of methanol for 5 hours by using the Soxhlet apparatus followed by water using cold maceration method. Both the extracts were filtered through Whatmann filter paper No. 42 (125mm) to remove all unextractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent. The entire extracts were concentrated to dryness using a rotary evaporator under reduced pressure. The final dried samples were stored in labelled sterile bottles. This activity has been repeated upon requirement of additional samples.

a) In vitro Anti diabetic activity of test drugs in selected cell line[14,15]

Chemicals

3-(4, 5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Bovine Serum Albumin (BSA), D- glucose, Dulbecco's Modified Eagle's Medium (DMEM), Metformin and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Antibiotics from Hi-Media Laboratories Ltd., Mumbai. Insulin (Torrent Pharmaceuticals, 40IU/ml) was purchased from a drug store. Dimethyl Sulfoxide (DMSO), NaOH and Propanol from E.Merck Ltd., Mumbai, India.

Cell lines and Culture medium

L-6 (Rat, Skeletal muscle) cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of L-6 were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose

in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For *in vitro* studies, test substance dissolved in DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT Assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted at 24 h interval. After 24 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^o C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\text{Percentage growth inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \text{ X } 100 \right)$$

***In vitro* glucose uptake assay**[16,17]

Glucose uptake activity of test substance was determined in differentiated L6 cells. In brief, the 24 r cell cultures with 70-80% confluency in 40mm petri plates were allowed to differentiate by maintaining in DMEM with 2% FBS for 4-6 days. The extent of differentiation was established by observing multinucleation of cells. The differentiated cells were serum starved overnight and at the time of experiment cells were washed with HEPES buffered Krebs Ringer Phosphate solution (KRP buffer) once and incubated with KRP buffer

with 0.1% BSA for 30min at 37^0C. Cells were treated with different non-toxic concentrations of test and standard drugs for 30 min along with negative controls at 37^0C. 20µl of D-glucose solution was added simultaneously to each well and incubated at 37^0C for 30 min. After incubation, the uptake of the glucose was terminated by aspiration of solutions from wells and washing thrice with ice-cold KRP buffer solution. Cells were lysed with 0.1M NaOH solution and an aliquot of cell lysates were used to measure the cell-associated glucose. The glucose levels in cell lysates were measured using glucose assay kit (ERBA). Two independent experimental values in duplicates were taken to determine the percentage enhancement of glucose uptake over controls.

Evaluation of α-Glucosidase and α-Amylase Enzyme Inhibitory Activity.

α-Glucosidase Enzyme Inhibitory Activity[18]: Alpha-glucosidase inhibition was analysed using kinetic end point assay described by Pistia-Brueggeman with few modifications. Alpha-glucosidase inhibitory activity was performed following the modified method of Pistia-Brueggeman and Hollinsworth.

α-Amylase Enzyme Inhibitor Activity[19]: Alpha-amylase inhibitory activity of polyherbal extract was carried out according to the standard method with minor modification.In a 96-well plate, reaction mixture containing 50 µl phosphate buffer (100 mM, pH = 6.8), 20 µl of varying concentrations of extract and fractions (0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml) was preincubated at 37°C for 20 min. Then, the 20 µl of 1% soluble starch (100 mM phosphate buffer pH 6.8) was added as a substrate and incubated further at 37°C for 30 min; 100 µl of the DNS color reagent was then added and boiled for 10 min. The absorbance of the resulting mixture was measured at 540 nm using Multiplate Reader. The % inhibition has been obtained using the formula.

% inhibition = {Absorbance (Control)- Absorbance (Sample)}/ Absorbance (Control)

b) In-vivo evaluations of antihyperglycemic activity

Experimental design

Animals

Adult male albino Wistar rats (6 weeks), weighing 150 to 200 g were used in the study. The animals were housed in clean polypropylene cages and maintained in a well-ventilated temperature controlled animal house with a constant 12 h light/dark schedule. The animals were fed with standard rat pelleted diet and clean drinking water was made available *ad*

libitum. All animal procedures were performed after approval from the ethical committee and in accordance with the Organization for Economic Co-operation and Development (OECD) guidelines for the proper care and use of laboratory animals.

Acute oral Toxicity

The acute oral toxicity effect of hydro alcoholic extracts of poly herbal formulation was evaluated in Wistar albino rats as mentioned in Organization for Economic Co-operation and Development (OECD) guidelines.[20] Animals were divided into 4 groups of six animals each. The Group1 was control, which received only 0.9% saline and the Group 2, 3, 4 are treated with polyherbal extracts (1:1:1) dissolved in 0.9% saline. The animals were kept fasting for overnight providing only water after which single oral administration of the hydro alcoholic extraction (herbal formulation) in a stepwise procedure at a fixed dose of 200 mg, 2000 mg and 5000 mg at the rate of 2 ml /100 mg bw. No food or water was given up to 4hrs after drug administration.

Animals were observed for toxicity and mortality every 30 minutes for first 24 hours and then daily for 14 days. If mortality was observed in animals, then the dose administered was assigned as toxic dose. If mortality was observed in a single animal, then the same dose was repeated again to confirm the toxic dose. If no mortality observed in a single animal, it is considered as non toxic and animal treated with next higher dose. The general observations are also observed continuously for every 30 minutes of the administration for first 24 hours and then daily for 14 days.

Experimental induction of diabetes mellitus[21,22]

The rat of 6weeks are divided into five groups (n=6) and kept for overnight fasting. Sterptozotocin was dissolved in citrate buffer (pH 4.5) and Nicotinamide was dissolved in normal physiological saline. Non-insulin dependent diabetes mellitus was induced in overnight fasted rats by a single intraperitoneal injection of 60 mg/kg Streptozotocin, 15 min after the i.p administration of 120 mg/kg of nicotinamide. Hyperglycemia was confirmed by the elevated levels of blood glucose with the glucose meter (Glucocard™ 01-mini, Arkray Factory, Inc., Japan) were determined at 72 h. The animals with blood glucose concentration more than 250mg/dl will be used for the study.

Study design

The experimental period was 28 days

Group 1; Control (Only normal saline)

Group 2: Only Streptozotocine 60 mg/kg/b.w.(IP) +Nicotinamide 120mg/kg (po)

Group 3: Streptozotocin (60 mg/kg) Nicotinamide 120mg/kg (po) rats treated Glibenclamide 20 mg/kg (po)

Group 4: Streptozotocin (60 mg/kg)+Nicotinamide 120mg/kg (po) rats treated with 250 mg/kg of extract.

Group 5: Streptozotocin (60 mg/kg) +Nicotinamide 120mg/kg (po) rats treated with 500 mg/kg. of extract.

The vehicle (normal saline), extract and glibenclamide were administered to the respective group animals for 28 days. Throughout the study period, extract and glibenclamide was freshly suspended in propylene glycol before administration. The fasting animal body weight, blood glucose level was estimated on 0, 7^{th}, 10^{th}, 15^{st} and 28^{th} day.

RESULTS

Statistical Analysis

All determinations were carried out in triplicates and data were analyzed by ANOVA followed by Tukey's multiple comparisons test for evaluation of significant differences. The values were considered significant at $p<0.05$.

Acute Toxic Effect

The acute toxicity tests on rats produced no deaths or toxic symptoms even at the highest dose of polyherbal extract (5000mg/Kg) as shown in Table 1. There was no visible change in the food intake and body weight when compared to control.

Table 1: Acute Toxic Effect of Herbal extract

Group	Body Weight			
	Control (Group 1)	200mg (Group 2)	2000mg (Group 3)	5000 mg (Group 4)
Toxic symptoms	-	-	-	-
Mortality	-	-	-	-
Food intake and Body weight	-	-	-	-

(-) represents the absence of Parameters, (+)represents the Presence of Parameters.

In vitro Anti diabetic activity of test drugs in selected cell line

The results for cytotoxic property studies and *In vitro* glucose uptake studies of polyherbal extract are presented in Tables 2,3 and 4. The polyherbal formulations extracts exhibited significant reduction in α- amylase and α- glucosidase activity.

Table 2: Cytotoxic properties of test drugs against L6 cell line.

Sl. No	Name of Test sample	Test Conc. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	RR 2580	1000	82.68±0.4	168.47±7.4
		500	81.15±1.0	
		250	80.36±1.2	
		125	33.64±3.8	
		62.5	15.75±5.1	

Table 3: *In vitro* glucose uptake studies for Test substances (N1) in L-6 cell line

Sl No.	Name of the Test substances	Test Conc. In mcg/ml	Protein content	Glucose uptake percentage (%)
1	Control	-	843.6	0.76±1.19
2	Std Rosiglutozone	100	842.4	115.26±7.72
3	RR 2580	100	826.7	34.67±4.25
		50	807.4	19.73±6.36

Table 4: *In vitro* glucose uptake studies for Test substances (N2) in L-6 cell line.

Sl No.	Name of the Test substances	Test Conc. In mcg/ml	Protein content	Glucose uptake percentage (%)
1	Control	-	876.6	0.49±1.18
2	Std Rosiglutozone	100	834.9	129.55±2.69
3	RR 2580	100	834.5	35.52±2.23
		50	779.1	18.06±1.71

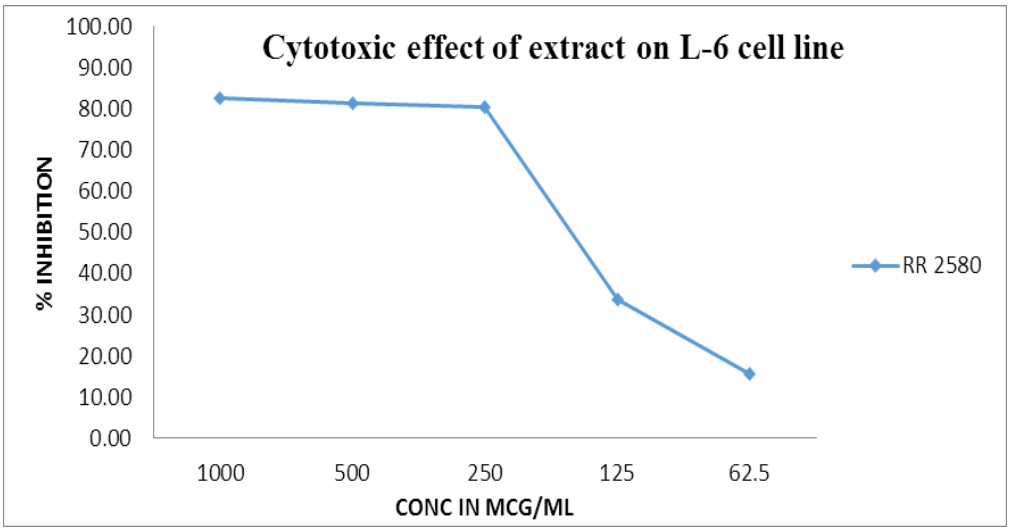

Figure 1: Cytotoxic effect of sample RR2580 on L-6 cell line.

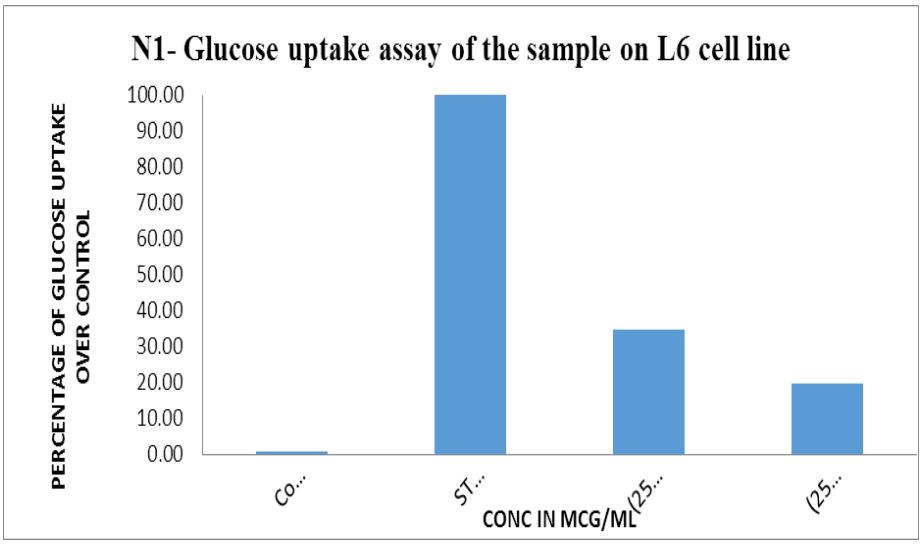

Figure 2: Glucose uptake assay of the sample RR 2342-RR 2344 (N1) on L6 cell line.

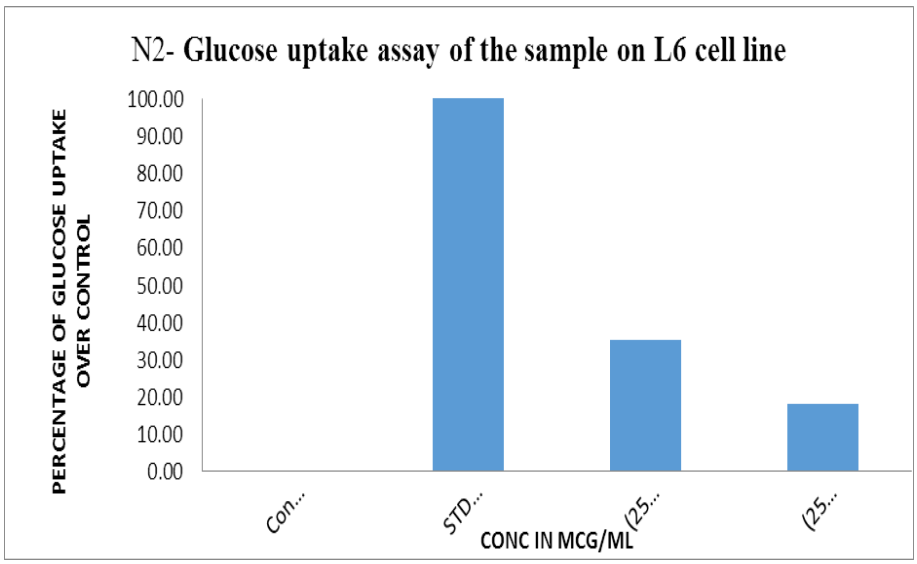

Figure 3: Glucose uptake assay of the sample RR 2342-RR 2344 (N2) on L6 cell line.

Table 4: *In vitro*- α- amylase and - α- glucosidase enzyme inhibitory activity.

Conc (µg/ml)	Absorbance	
Test	α- amylase	α- glucosidase
10µg	0.1115	0.1134
20µg	0.2122	0.1227
30µg	0.2345	0.1886
40µg	0.2138	0.2245
50µg	0.3286	0.3288
Blank	0.1264	0.1386
	IC50 = 46.00	**IC50 = 36.00**

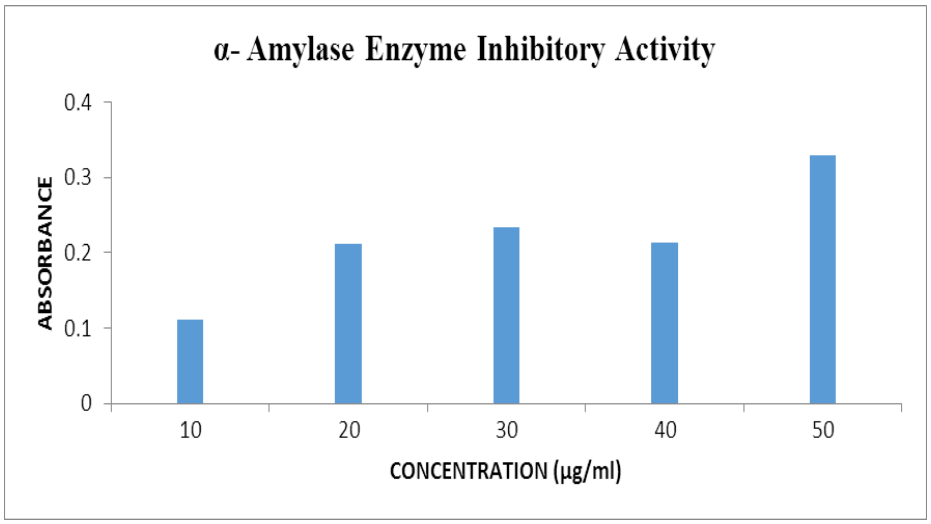

Figure 4: α- amylase enzyme inhibitory activity.

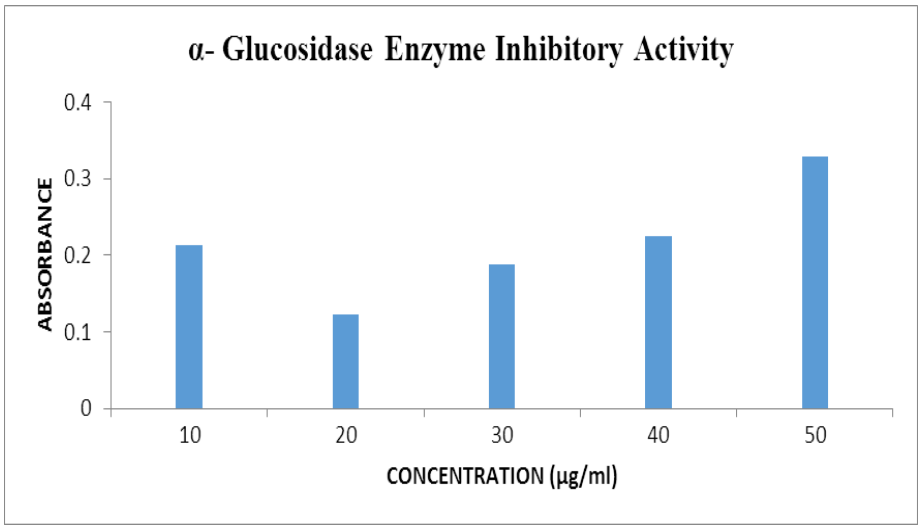

Figure 5: α- glucosidase enzyme inhibitory activity.

In-vivo evaluations of antihyperglycemic activity.

The antidiabetic effects of polyherbal formulation in streptozotocin induced diabetic rats have been evaluated and the results are tabulated in the following tables. No significant changes in body weights of rats were observed when compared with the standard glibenclamide formulation as shown in Table 6. The in-vivo results shown no significant changes in blood glucose levels of rats in control group while significant reduction was observed in both test groups (250mg/Kg and 500mg/Kg). The results of both test groups (250mg/Kg and 500mg/Kg) are also comparable with the marketed formulation (Glibenclamide) and more similar profile was observed for test group with highest drug concentration (500mg/Kg) when compared with standard formulation.

Table 6: Effect of extract on Body weight.

Group	Body Weight				
	0	7	14	21	28
Normal	150.7±3.72	151.8±2.48	156.5±1.51	158.2±2.13	162.5±2.16
Control	153.0±2.82	106.8±3.18***	113.2±3.25***	117.5±3.01***	124.3±2.50***
Standard	155.2±2.04	135.5±2.07***	138.3±2.33***	140.8±3.65***	144.7±2.65***
Ext-250	154.2±2.78	123.5±3.20***	124.7±2.50***	128.8±2.04***	135.0±3.28***
Ext-500	155.7±2.73	130.5±1.87***	126.7±2.73***	130.2±3.37***	143.8±2.99***

****P< 0.05 were considered significant.*

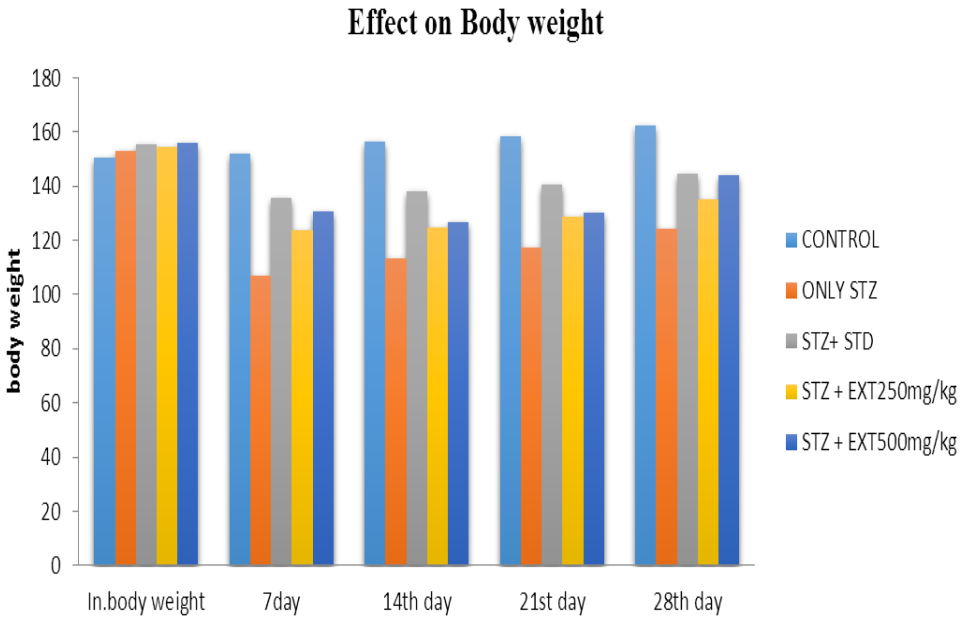

Figure 6: Effect of extract on Body weight.

Table 7: Normal blood sugar, stz induced blood sugar level and blood sugar level after treated with extract in stz induced diabetes mellitus.

Group	Body Weight				
	0	72h	10th day	15th day	28th day
Normal	73.50±2.42	74.33±2.65	75.00±2.53	74.67±1.63	75.17±2.13
Control	73.00±1.41	406.2±9.39***	377.8±9.60***	233.3±7.55***	152.2±4.16***
Standard	74.83±2.40	395.3±3.44***	214.5±5.71***	128.2±2.78***	85.83±2.78***
Ext-250	73.50±1.64	399.2±6.49***	252.7±7.99***	155.2±2.99***	97.67±2.73***
Ext-500	73.33±2.06	396.0±9.09***	241.3±9.20***	141.2±3.12***	89.50±2.88***

****P< 0.05 were considered significant.*

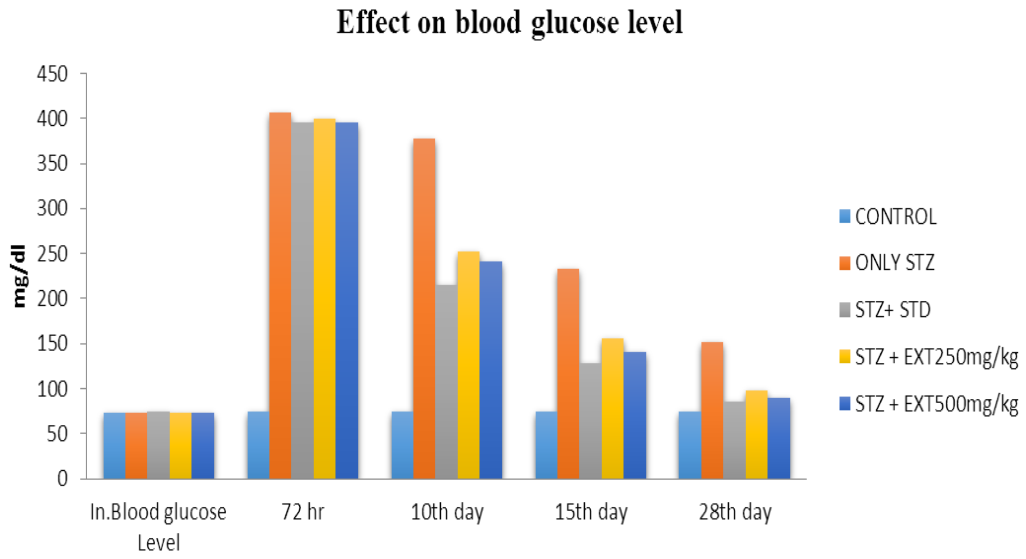

Figure7: Normal blood sugar, stz induced blood sugar level and blood sugar level after treated with extract in stz induced diabetes mellitus

DISCUSSION

Management of diabetes is still a challenge to the allopathic medicine systems as the availability of synthetic medicines are with high side effects. Though insulin and other oral antidiabetic medicines are available for the treatment of diabetes mellitus, herbal formulations are the preferred choice due lesser side effects. [23] During diabetes mellitus the increased blood sugar levels might be due to either insulin resistance of the body cells or decreased secretion of insulin from beta cells manifest in the decreased serum insulin levels.[24] The polyherbal formulations are proved effective for the treatment of various disorders then the single drug treatment. polyherbal therapies have the synergistic, potentiative, agonistic/antagonistic pharmacological agents within themselves that work together in a dynamic way to produce therapeutic efficacy with minimum side effects.[25,26]

The present study was performed to evaluate the antidiabetic activity of the polyherbal extract against diabetic rats. The polyherbal extracts exhibited significant reduction in α-amylase and α- glucosidase activity. The acute toxicity study of hydro alcoholic extract of herbal formulation in rats demonstrates that no major toxic symptoms and mortality were observed up to the dose of 5000 mg/kg bw during the 14 days of the observation. There was no visible change in the food intake and body weight when compared to control. The in-vivo results showed that the polyherbal extract (250mg/Kg and 500mg/Kg) have got antidiabetic activity that is comparable to the standard drug Glibenclamide and more similar profile was

observed for test group with highest drug concentration (500mg/Kg) when compared with standard formulation.

CONCLUSION

The present study was performed to evaluate the antidiabetic activity of the polyherbal extract against diabetic rats. The results showed that the polyherbal extract have got antidiabetic activity which is comparable to the standard drug Glibenclamide and no sign of toxicity were observed in animals. The study concludes that the hydro-alcoholic polyherbal extract has disclosed good antidiabetic activity against hyperglycemia condition. A combination of plants is used to get the enhanced desired activity and deep insight into these herbs may lead to the development of more potent, long acting and better antidiabetic drug.

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REFERENCES

1. Type of diabetes and WHO, Definition, diagnosis and classification of diabetes mellitus and its complications. World health Organization, Geneva, 1999; 2-7.
2. Canadian diabetes association, Type of diabetes, http://www.diabetes.ca/about-diabetes/types-of-diabetes.
3. International diabetes federation, IDF WPR guidelines, Bangkok, 25 October 2005, http://www.idf.org/idf-wpr-type-2-diabetes-pratical-targets-and-treatments.
4. Diane L Huber, Disease Management: A Guide for Case Managers., 2005; 81.
5. Global status report on non-communicable diseases, World Health Organization, Geneva, 2014; 14.
6. International Diabetes Federation, Annual report, 2013; 14.
7. WHO Diabetes, NMH Fact Sheet, February 2010, http://www.who.int/nmh/publications/fact_sheet_diabetes_en.pdf.
8. Global report on diabetes. World Health Organization, Geneva, 2016; 6-9.
9. International International Diabetes Federation, annual report, 2014; 10.
10. Burcelin R, Eddouks M, Maury J, Kande J, Assan R, Girard J. Excessive glucose production rather than insulin resistance, accounts for hyperglycemia in recent-onset streptozotocin diabetic rats. Diabetologia, 1995; 38: 283-290.
11. Ozsoy-SacanO, Karabulut-Bulan O, Bolkent S, Yanardag R, Ozgey Y. Effects of Chard

(Beta vulgaris L. varcicla) on the Liver of the Diabetic Rats: A Morphological and Biochemical Study. Biosci. Biotechnol. Biochem, 2004; 68(8): 1640-1648.

12. Manjunatha BK, Vidya SM, Rashmi KV, Mankani KL, Shilpa HJ, Jagadeesh S. Evaluation of wound healing potency of Vernoniaarborea HK. Indian J. Pharmacol, 2005; 37: 223-6.
13. Harborne JB. Phytochemical Methods: a guide to modern techniques of plant analysis. 3rd Ed. Spingerinternational Publication., 1998; 4-7.
14. Francis D and Rita L. Rapid colorimetric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of Immunological Methods., 1986; 89: 271-7.
15. Angeline Yap, Shin Nishiumi, Ken-ichi Yoshida, and Hitoshi Ashida. Rat L6 myotubes as an in vitro model system to study GLUT4-dependent glucose uptake stimulated by inositol derivatives. Cytotechnology, 2007; 55(2-3): 103–8.
16. Hisako Takigawa-Imamura, Takumi Sekine, Mitsuo Murata, Kiyoshi Takayama, Kiyoshi Nakazawa, Junichi Nakagawa.Stimulation of glucose uptake in muscle cells by prolonged treatment with scriptide, a histone deacetylase inhibitor. Bioscience, Biotechnology, and Biochemistry, 2003; 67: 1499–1506.
17. M.A Bhutkar and S.B Bhise.In-vitro assay of alpha amylase inhibitory activity of some indigenous plants.Int. J. Chem. Sci., 2012; 10(1): 457-462.
18. Pistia-Brueggeman G, Hollingsworth, R. I. The preparation and screening strategy for glycosides inhibitors. Tetrahedron, 2001; 57(42): 8773-8.
19. Shai LJ, Magano SR, Lebelo SL, Mogale AM. Inhibitory effects of five medicinal plants on rat alpha-glucosidase: Comparison with their effects on yeast alpha-glucosidase. J. Med. Plant Res, 2011; 5: 2863-7.
20. Organization for Economic Co-operation and Development (OECD) guidelines., 2001; 423: 1-14.
21. Lorraine Jadeski, Jennifer M. Mataraza, Ha-Won Jeong, ZhigangLi, David B. Sacks. IQGAP1 Stimulates Proliferation and Enhances Tumorigenesis of Human Breast Epithelial Cells, The journal of biological chemistry, 2008; 283(2): 1008–17.
22. Pellegrino Masiello, Christophe Broca, Rene Gross, Michele Roye, Michele Manteghetti, Dominique Hillaire-Buys, Michela Novelli, Gerard Ribes. Experimental NIDDM-Development of a New Model in Adult Rats Administered Streptozotocin and Nicotinamide. Diabetes, 1998; 47: 224-9.

23. Ajay Mandal, P. Jagan Mohan Reddy. Invitro alpha amylase inhibitory activity of ethanol and hot water extract of polyherbal formulation., 2016; 5(1): 968-978.
24. Gohil T, Pathak N,Jivani N, Devmurari V, Patel J. Treatment with extracts of *Eugenia Jambolana* seed and *Aegle marmelos* leaf extracts prevents hyperglycemia and hyperlipidemia in alloxan induced diabetic rats. Afr J Pharm Pharmacol, 2010; 4: 270-275.
25. Raghavendra HG, Sen S, Reddy YSR, Sridhar C, Chakraborty R, Suchitra B, Nagaiah B, Asha Jyothi B, Ashok Babu B. Synergistic activity of *Tribulusterrestris* and *Annona squamosal* extracts against alloxan induced diabetes and hyperlipidemia in rats. Pharm Sc Mon, 2011; 2: 12-22.
26. Tiwari AK, Rao JM. Diabetes mellitus and multiple therapeutic approaches of phytochemicals: Present status and future prospects. Curr Sci, 2002; 83: 30-37.