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In Vitro and *In Vivo* Anti-Diabetic Activity of a Nearly Threatened Woody Tree Species *Pterospermum Xylocarpum*

Hassanain Hataf Jaber[1], K. Rajyalakshmi[2], M. Kishore Babu[3], A. Krishna Satya[4]

[1, 2, 3, 4]Department of Biotechnology, Acharya Nagarjuna University, Nagarjuna Nagar, Guntur-522510 A.P. India

Abstract: ***In the present study Nearly Threatened Woody Tree Species Pterospermum Xylocarpum was selected for the screening of crude extracts [Hexane, Chloroform, Methanol and Aqueous (Water) extracts]for their anti-diabetic activity both in vitro and in vivo. In vitro anti-diabetic activity was done by both Alpha-Amylase inhibition assay and by Glucose uptake in Yeast Cells assay.The in-vitro analysis reveals that the Methanol extract inhibits the amylase enzyme along with the standard drug. In Vivostudies Male Sprague Dawley (150 – 180gms) rats were used. The animals were maintained under standard environmental conditions and were fed with standard pellet diet and water ad libitum. The experimental protocol was approved by the Institutional animal ethics committee. CPCSEA guidelines were adhered during the maintenance and experiment. Four extracts [Hexane, Chloroform, Methanol and Aqueous (Water)] have been tested for the acute hypoglycaemic activity and there was a significant change in the methanol extract administered rat models.***

Keywords: Anti-diabetic activity, in-vitro, in- vivo studies, Methanol extract

1. Introduction

Diabetes mellitus, one of the most common endocrine metabolic disorder has caused significant mortality due to micro vascular (retinopathy, neuropathy, and nephropathy) and macro vascular (heart attack, stroke and peripheral vascular disease) complications. Type 2 diabetes or noninsulin-dependent diabetes mellitus, is the most common form of the disease, accounting for 90% -95% of cases in having high blood glucose level as the body does not produce enough insulin. According to World Health Organization the diabetic population is likely to increase up to 300 million or more by the year 2025.

Recently, some medicinal plants have been reported to be useful in diabetes worldwide and have been used empirically in anti-diabetic and anti-hyperlipidemic remedies. Anti hyperglycemic activity of the plants is mainly due to altering the function of pancreatic tissues by causing an increase in insulin output or by inhibiting the intestinal absorption of glucose or may be due to the facilitation of metabolites in insulin dependent processes. More than 400 plant species having hypoglycemic activity have been available in literature, however, searching for new antidiabetic drugs from natural plants is still attractive because they contain substances which are alternative and safe on diabetes mellitus. Most of plants contain glycosides, alkaloids, terpenoids, flavonoids, carotenoids, etc., that are frequently reportedto have anti-diabetic effect.

In the present study anti-diabetic activity of a Nearly Threatened Woody Tree Species *Pterospermum Xylocarpum* (1) is screened both *in vitro* and *in vivo*.

2. Materials and Methods

Collection of plant material

The medicinal plant *Pterospermum Xylocarpum* leaves were collected from the Seshachalam forest area. The collected plant material was shade dried for two weeks, powdered, labelled and stored in a dry, cool place for further studies.

Chemicals and Reagents

All the chemicals, solvents and reagents used were of analytical grade and procured from Merck, Sigma, Sd-fine and SRL.

Soxhlet extraction

The dried plant material was packed into soxhlet apparatus, subjected to successive extraction with solvents such as Hexane, Chloroform, Methanol and Aqueous (Water). For this purpose, the plant material of 300gms was packed into the extractor and fitted with apparatus. The respective solvent was filled and apparatus was operated at 45°C until 35 cycles were run. The experiment was repeated several times with fresh dry powder until ample amount of crude extract was collected. After collection of extract, the remaining plant powder was removed, dried and once again loaded with another successive solvent *i.e.*,from non-polar to polar. The isolated fraction of each extract was labelled with their respective solvent names and subjected to further studies.

In vitro Antidiabetic activity

Antidiabetic activity by Alpha-Amylase inhibition assay

Reagents:

Potato starch-(1%w/v), Test extracts, Alpha amylase (1%w/v), Acetate Buffer (0.1M, 7.2pH)
Iodine-Iodine indicator – mix 635mg Iodine and 1gm potassium iodide in 250ml distilled water
Reference Standard- Acarbose.

Procedure: α- Amylase is an enzyme that hydrolyses alpha-bonds of large alpha-linked polysaccharides such as starch and glycogen, yielding glucose and maltose. It is the major form of amylase found in humans and other mammals.

Alpha amylase inhibitory activity is based on the starch iodine protocol that was originally developed by (2). In alpha amylase inhibition method reaction tube contains 1ml substrate-potato starch, 1ml of test extract of five different concentrations such as 100, 200, 300, 400, and 500µg/ml respectively, 1ml of alpha amylase enzyme and 2ml of acetate buffer. The above mixture was incubated for 1hr. Then add 0.1ml iodine-iodine indicator to the reaction tube. Absorbance was taken at 565 nm in UV-Visible spectroscopy. All tests were performed in triplicate. % inhibition was calculated by following equation

% of inhibition = $[(A_0 – A_s) / A_0] \times 100$

Antidiabetic activity by Glucose uptake in Yeast Cells assay (3)

Reagents

1) Yeast cells
2) Glucose solution
3) Reference Standard: Metronidazole

Procedure

The commercial yeast in distilled water was subjected to repeated centrifugation (3000xg; 5min) until getting the clear supernatant solution and 10% (v/v) of the suspension was prepared in distilled water. Various concentrations of plant extracts 100, 200, 300, 400 and 500 µg/mL were prepared. 1ml of glucose solution (5, 10 and 25mM) was added to test extracts and incubated together for 10min at 37^0c. After incubation add 100 µL of yeast suspension followed by vortexing and further incubation at 37 °C for 60 min. After 60 min, the tubes were centrifuged (2,500×g) for 5 min and amount of glucose was estimated in the supernatant. Metronidazole was used as standard drug.The percentage increase in glucose uptake by yeast cells was calculated using previously published protocol. All the tests were performed in triplicate [4].

The percentage increase in glucose uptake by yeast cells was calculated using the following formula:

% of inhibition = $[(A_0 – A_s) / A_0] \times 100$

***In vivo* Acute Anti-diabetic analysis**

Chemicals and reagents

1) Streptozotocin (Sigma chemical co., U.S.A)
2) Glibenclamide (micro labs, India)
3) Glucose

Other chemicals and reagents used for the study were of analytical grade and procured from approved organizations.

Experimental animals

Male Sprague Dawley (150 – 180gms) rats were used for the present study. The animals were maintained under standard environmental conditions and were fed with standard pellet diet and water ad libitum. The experimental protocol was approved by the Institutional animal ethics committee. CPCSEA guidelines were adhered during the maintenance and experiment.

Evaluation of anti-diabetic activity

Treatment protocol

The animals were divided into five groups of three animals each as follows

Group I- Vehicle control, Normal saline (0.9 % w/v NaCl).
Group II- Diabetic control
Group III- Diabetes Extract 200 mg/ kg, p.o.
Group IV- Diabetes Extract 400 mg/ kg, p.o.
Group V- Diabetic standard treated, 0.5 mg/ kg of glibenclamide, p. o (micro labs)

The experiment was performed for the four extracts of Hexane, Acetone, Methanol and Aqueous with their two different concentrations of 200 and 400mg/kg bwt.Diabetes was induced in all groups except normal control by a single intraperitoneal injection of 60 mg/ kg of Streptozotocin (STZ) dissolved in a freshly prepared 0.1 M citrate buffer (pH 4.5) and 120 mg/kg of Nicotinamide (NA) dissolved in normal physiological saline. The animals in the vehicle control (Group I) received normal saline orally (0.9 % w/ v NaCl). The rats with blood glucose levels above 250 mg/ dl were considered as diabetic and used in this study(Liu et al., 2004). The four extracts were given two different concentrations of 200 & 400 each individual dose. The effective dosage calculated accordingly and changes in the blood glucose level in the subsequent hours for 24 hrs.

The oral glucose tolerance test was performed earlier to the diabetic assay with the same concentrations whether the rats were survived for the dosage and diabetic induction was calculated.

The Hexane, Acetone, Methanol and Aqueous extracts were administered orally to the diabetic induced rats about two concentrations and blood glucose levels were noticed for the activity.

3. Results and Conclusion

***In-vitro* Anti-diabetic Assay**

Alpha Amylase Inhibitory Anti-diabetic Assay

The *in-vitro* analysis of this assay mainly concentrated on the inhibition of amylase. Alpha amylase hydrolyses 1,4glycosidic linkage of glycogen and starch in to simple sugars. By the inhibition of alpha amylase enzyme in the track of digestive system in humans it controls diabetes by lowering the absorption of glucose from starch (Hara et al., 1990). The *in-vitro* analysis reveals that the Methanol extract inhibits the amylase enzyme along with the standard drug. The IC_{50} value of Methanol extractis 156.92 µ g/ml and remaining extracts have the high IC_{50} values thanMethanol extract.

Glucose uptake by yeast cells Anti-diabetic Assay

In-vitro assay for the evaluation of anti-diabetic activity is done by evaluating the glucose uptake by yeast cells. Yeast is the eukaryotic single cell organism. The four plant extracts have shown the good hypoglycemic activity and it was inversely proportional to the concentration of the extract. Five concentrations were taken for the study and studies were effective at 500µ g/ml concentration. The IC_{50}value of methanol extracts is45.36µ g/ml followed by the aqueous extract 81.9µ g/ml.

Alpha Amylase Inhibitory Anti-diabetic Assay

S.No	Conc	hexane	IC_{50}	acetone	IC_{50}	methanol	IC_{50}	aqueous	IC_{50}	Acarbose	IC_{50}
1	100	16.9		26.06		34.51		26.29		38.24	
2	200	25.64	349.66	33.26	283.83	54.02	156.92	47.19	221.41	62.54	135
3	300	53.22	µg/ml	59.32	µg/ml	77.29	µg/ml	72.88	µg/ml	81.58	µg/ml
4	400	57.29		67.34		82.41		73.87		86.35	
5	500	64.04		69.66		87.08		76.97		92.78	

Glucose uptake by yeast cells Anti-diabetic Assay

S. No	Conc	Hexane	IC_{50}	Acetone	IC_{50}	methanol	IC_{50}	Aqueous	IC_{50}	Metronidazole	IC_{50}
1	100	20.16		35.88		49.85		46.86		55.98	
2	200	44.03	286.9	54.97	181.83	68.42	45.36	65.79	81.9	69.35	33.64
3	300	64.64	µg/ml	78.58	µg/ml	89.52	µg/ml	86.86	µg/ml	87.56	µg/ml
4	400	68.89		81.19		92.22		90.47		97.14	
5	500	72.08		84.26		94.91		93.53		99.87	

***In Vivo* Anti-diabetic Activity:**

Four extracts have been tested for the acute hypoglycaemic activity and there was a significant ($P<0.05$) change in the four extract and the methanol and aqueous extracts have more significance than the acetone and hexane extracts. The overall change was observed for the two extracts that P value was below 0.01.

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Oral glucose tolerance test on rats Hexane Extract

Group	0 min	30 min	60 min	90 min	120min
GrI Normal Control	84.36± 0.36	149.54± 0.12	128.68± 0.56	114.97± 0.54	108.58± 0.85
GrII He Ex 150	89.32± 0.26	140.68± 3.25	117.78± 1.94	111.54± 1.36	102.56± 1.25
GrIII He Ex 300	86.52± 0.12	135.29± 2.41	112.64± 2.34	108.96± 1.54	92.54± 2.25
GrIVGlibenclamide	87.67± 1.23	130.25± 1.54	105.64± 1.36	98.67± 1.25	83.86± 2.36

Oral glucose tolerance test on rats Acetone Extract

Group	0 min	30 min	60 min	90 min	120 min
GrI Normal Control	84.36± 0.36	149.54± 0.12	128.68± 0.56	114.97± 0.54	108.58± 0.85
GrII Ac Ex 150	85.74± 1.95	139.68± 2.36	120.87± 1.26	110.92± 2.58	104.54± 1.85
GrIII Ac Ex 300	84.65± 1.54	133.25± 2.24	109.58± 2.35	105.56± 3.65	91.58± 1.99
GrIVGlibenclamide	87.67± 1.23	130.25± 1.54	105.64± 1.36	98.67± 1.25	83.86± 2.36

Oral glucose tolerance test on rats Methanol Extract

Group	0 min	30 min	60 min	90 min	120 min
GrI Normal Control	84.36± 0.36	149.54± 0.12	128.68± 0.56	114.97± 0.54	108.58± 0.85
GrII Me Ex 150	82.54± 1.56	135.62± 0.68	115.87± 2.38	105.47± 1.69	90.25± 2.84
GrIII Me Ex 300	84.65± 1.03	130.85± 0.14	110.98± 1.35	100.85± 1.24	83.57± 2.35
GrIVGlibenclamide	87.67± 1.23	130.25± 1.54	105.64± 1.36	98.67± 1.25	83.86± 2.36

Oral glucose tolerance test on rats Aqueous Extract

Group	0 min	30 min	60 min	90 min	120 min
GrI Normal Control	84.36± 0.36	149.54± 0.12	128.68± 0.56	114.97± 0.54	108.58± 0.85
GrII AQ Ex 150	85.63± 0.25	139.64± 1.03	114.35± 1.82	105.32± 1.54	94.54± 1.24
GrIII AQ Ex 300	86.97± 1.09	131.65± 2.04	115.69± 2.57	101.65± 0.08	82.87± 1.36
GrIVGlibenclamide	87.67± 1.23	130.25± 1.54	105.64± 1.36	98.67± 1.25	83.86± 2.36

Effect of Hexane extracton Blood glucose level in Acute Model

Group	0 hr	1 hr	2 hr	4 hr	6 hr	8 hr
GrI Normal Control	84.84± 0.55	86.65± 0.65	84.52± 0.80	84.35± 0.38	84 ± 0.54	86.45± 0.65
GrII Diabetic Control	391.54± 1.95	385.65± 1.75	389.85± 1.23	385.45± 1.50	384.56± 1.03	390.75± 1.54
GrIII He Ex 150	386.65± 2.65	363.25± 2.39	345.68± 3.02	300± 1.54	275.56± 1.54	245.36± 1.39
GrIVHe Ex 300	389.85± 1.64	362.65± 1.35	349.89± 2.58	291.36± 2.00	284.69± 1.89	252.21± 2.01
GrVGlibenclamide	390.25± 1.32	360.52± 1.50	340.58± 2.65	320.54± 3.20	290.55± 2.25	270.95± 1.54

Effect of Acetone extract on Blood glucose level in Acute Model

group	0 hr	1 hr	2 hr	4 hr	6 hr	8 hr
GrI Normal Control	84.84±0.55	86.65±0.65	84.52±0.80	84.35±0.38	84 ±0.54	86.45±0.65
GrII Diabetic Control	390.54±1.95	384.65±1.75	388.85±1.23	388.45±1.50	388.56±1.03	390.75±1.54
GrIII Ac Ex 150	391.25± 1.47	361.69± 2.05	337.56± 1.55	290.3± 0.50	285.36± 1.25	250.54± 2.50
GrIVAc Ex 300	392.65± 1.23	366.65± 1.35	347.24± 1.20	299.65± 0.64	275.26± 1.68	233.26± 1.90
GrVGlibenclamide	390.25±1.32	350.52±1.50	340.58±2.65	310.54±3.20	290.55±2.25	270.95±1.54

Effect of Methanol extraction Blood glucose level in Acute Model:

group	0 hr	1 hr	2 hr	4 hr	6 hr	8 hr
GrI Normal Control	84.84 ±0.55	86.65 ±0.65	84.52 ±0.80	84.35 ±0.38	84 ±0.54	86.45 ±0.65
GrII Diabetic Control	391.54 ±1.95	385.65 ±1.75	389.85 ±1.23	385.45 ±1.50	384.56 ±1.03	390.75 ±1.54
GrIII Me ExI 150	386.96± 2.00	346.65± 1.30	325.96± 0.93	286.35± 1.54	275± 1.54	245.64± 1.56
GrIVMe Ex 300	391.26± 0.50	355.5± 1.40	330.25± 0.50	302.54± 1.65	259.62± 2.36	222.35± 1.85
GrVGlibenclamide	390.25 ±1.32	360.52 ±1.50	340.58 ±2.65	320.54 ±3.20	290.55 ±2.25	270.95 ±1.54

Effect of Aqueous extract on Blood glucose level in Acute Model

group	0 hr	1 hr	2 hr	4 hr	6 hr	8 hr
GrI Normal Control	84.84±0.55	86.65±0.65	84.52±0.80	84.35±0.38	84 ±0.54	86.45±0.65
GrII Diabetic Control	391.54±1.95	385.65±1.75	389.85±1.23	385.45±1.50	384.56±1.03	390.75±1.54
GrIII AQEx 150	392.32± 1.20	342.69± 2.45	325± 1.58	291.65± 1.57	271.56± 1.95	250.69± 1.29
GrIVAQEx 300	387.59± 1.36	362± 2.35	331.26± 1.47	305.96± 1.69	259.3± 1.85	232.54± 1.27
GrVGlibenclamide	390.25±1.32	360.52±1.50	340.58±2.65	320.54±3.20	290.55±2.25	270.95±1.54