

ANTI-HYPERGLYCEMIC EFFECT OF DIFFERENT FRACTIONS OF *ANNONA RETICULATA* LEAF

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Received: 25 June 2016, Revised and Accepted: 02 July 2016

ABSTRACT

Context: Several species of the genus *Annona* were reported to have hypoglycemic properties and this makes *Annona reticulata* Linn. (Annonaceae) an interesting plant for investigating its anti-hyperglycemic potential.

Objective: Different fractions prepared from hydro-alcoholic extract of *A. reticulata* leave were investigated for their blood glucose lowering effect on Streptozotocin (STZ) induced hyperglycemic rats.

Methods: Ethyl acetate, methanol, and residual fractions (at dose level of 100 mg/kg by oral route) prepared from the hydro-alcoholic extract of *A. reticulata* leave were administered for 14 consecutive days to STZ induced hyperglycemic rats for evaluation of their anti-hyperglycemic potential. Anti-hyperglycemic potential was assessed by observation of a decrease in fasting blood glucose level.

Results: The studies revealed that ethyl acetate fraction decreased the blood glucose level of hyperglycemic rats from 447.67 to 234.17 mg/dL and is significant ($p < 0.001$) when compared with diabetic control group. The residual fraction and methanolic fraction decreased blood glucose level from 417.83 to 402.50 mg/dL and 432.33 to 371.67 mg/dL respectively but not significant when compared with the diabetic control group. Standard drug metformin (dose 300 mg/kg) reduced the blood glucose level from 447.33 to 219.50 mg/dL.

Discussion: Ethyl acetate fraction at tested dose level was capable not only to control the elevated blood glucose level but also able to attenuate certain secondary parameters associated with STZ induced hyperglycemia.

Conclusion: This study suggested that the ethyl acetate fraction prepared from hydro-alcoholic extract of *A. reticulata* leave exhibit potential anti-hyperglycemic property in the tested experimental models and should be investigated further.

Keywords: Streptozotocin, Diabetes, Dyslipidemia.

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INTRODUCTION

Annona reticulata Linn. (Annonaceae), commonly known as bullock's heart, is widely distributed in India; they are tall, with many branches, bearing nutritious fruits. Plants belonging to genus *Annona* such as *Annona muricata* Linn. (Annonaceae), *Annona glabra* Linn. (Annonaceae), and *Annona cherimola* Mill. (Annonaceae) have been reported to be used as hypoglycemic agents in Mexico [1]. Several species of the genus *Annona* such as *Annona squamosa* Linn. (Annonaceae) [2], *Annona cherimola* Mill. (Annonaceae) [3], *A. muricata* Linn. (Annonaceae) [4,5], *Annona macrophyllata* Donn. Sm. (Annonaceae) [6] had been reported for their anti-diabetic effect. The taxonomic profile of the plant and unavailability of sufficient scientific reports regarding the hypoglycemic potential of *A. reticulata* makes it an interesting plant of choice for investigating the hypoglycemic potential of the plant if any. Our previous studies had revealed that the hydro-alcoholic extract of leave of *A. reticulata* is having potential anti-hyperglycemic potential which is more corrective in nature [7] and made the plant more interesting to be investigated further. The phytochemical investigation by gas chromatography-mass spectrometry of different fractions made from hydro-alcoholic extract of leave of *A. reticulata* revealed the presence of several compounds which may be responsible for the observed anti-hyperglycemic activity of the extract [8]. The focus of the present investigation had been to evaluate the efficiency of different fractions prepared from hydro-alcoholic extract of *A. reticulata* leave in the regulation of blood glucose levels in streptozotocin (STZ) induced hyperglycemic rats so that it can help in identifying the compound/compounds those might be responsible for the observed anti-hyperglycemic activity of the extract.

METHODS

Plant collection, preparation of extract and its fractions

The flowering twigs of *A. reticulata* were collected in the month of July-August 2010, from the rural area of Cuttack District, Odisha, India, and authenticated by Dr. Kshetra Mohan Das, Central Rice Research Institute, Cuttack, Odisha, India, and a specimen voucher (Specimen No. 10-11/SPS/SOAU) was kept in the University for future reference. After identification and authentication of the plant, leaves of the plant were collected for the experimental process. The leaves were shade dried, made into coarse powder and the powdered material was initially defatted with petroleum ether and then subjected to cold maceration process for 72-h using 1:1 mixture of methanol and water as solvent to prepare hydro-alcoholic extract of *Annona reticulata* leave (percentage yield 20.5% w/w with respect to dried powder). The extract was filtered and concentrated by rotary evaporator. For the preparation of different fractions method published by Sourav *et al.* (2012) was used [9]. The extract is mixed with water and methanol (1:1) and used for fractionation using chloroform, ethyl acetate, and methanol as solvents by the use of separating funnel. The different fractions prepared are chloroform fraction of hydro-alcoholic extract of *A. reticulata* (HAARC) (percentage yield 1.00% w/w with respect to concentrated extract), ethyl acetate fraction of hydro-alcoholic extract of *A. reticulata* (HAARE) (percentage yield 15.50% w/w with respect to concentrated extract), methanolic fraction of hydro-alcoholic extract of *A. reticulata* (HAARM) (percentage yield 12.80% w/w with respect to concentrated extract), and residual fraction of hydro-alcoholic extract of *A. reticulata* (HAARR) (Percentage yield 25.50% w/w with respect to concentrated extract). HAARC was not used for the experimental purpose as the yield volume of the fraction was very less, whereas all other fractions were used for

determination of their anti-hyperglycemic activity. The extract and all the fractions were stored at room temperature in desiccators till further use.

Preparation of interventions

The fractions and the hydro-alcoholic extracts were suspended in distilled water using 25% Tween 20 as suspending agent for the preparation of test interventions. The suspension of standard drug metformin was also prepared in a similar manner. The dose at which the test substances were administered was 100 mg/kg, and the standard drug was administered as a dose level of 300 mg/kg. Suspension of distilled water and 25% Tween 20 was used as solvent treatment throughout the study at a dose of 2 ml/kg. The solvent, test samples, and standard drugs were administered by oral route based on dose and corresponding weight of the animals.

Experimental animals

Healthy Wistar albino rats (180-250 g body weight) supplied by Central Animal House of School of Pharmaceutical Sciences, SOA University, Bhubaneswar, India, were used in the experiments. The animals were acclimatized to laboratory conditions for 1 week before commencement of the experiment. The study was approved by University Animals Ethics Committee (Regd. No. 1171/C/08/CPCSEA).

Induction of diabetes

Diabetes was induced as per the method published by Sachin *et al.* (2009) [10]. Hyperglycemia was induced by intraperitoneal administration of multiple low doses (40 mg/kg) of STZ to the overnight fasted rats for 5 consecutive days. STZ solution was prepared freshly in ice-cold citrate buffer (0.01 M, pH 4.5). After 12 days of STZ administration, the fasting blood glucose (FBG) levels were measured, and the rats with FBG level ≥ 250 mg/dL were considered as diabetic and used for experimental purpose. Blood glucose level was estimated by using Glucometer (One Touch Horizon, Lifescan, Johnson and Johnson Company).

Acute toxicity study

Healthy Wistar albino rats of either sex starved overnight were divided into five groups (n=4). Groups I-III animals were orally fed with different fractions with increasing dose levels of 0.25, 0.5, 0.75, and 1.0 g/kg. body weight; Animals of Group IV were treated with the extract with increasing dose levels of 0.25, 0.5, 0.75, and 1.0 g/kg body weight, while Group V (untreated) served as control. The animals were observed continuously for the first 2 hrs for any gross change in behavioral, neurologic and autonomic profiles or any other symptoms of toxicity and mortality if any, and intermittently for the next 6 hrs and then again at 24, 48 and 72 hrs for any lethality or death. One-tenth of the maximum safe dose of the fractions and extract tested for acute toxicity was selected for the experiment [11].

Multiple dose anti-hyperglycemic study

Multiple dose study was conducted as per methods published previously [12,13]. The apportionment of animals into different groups were done as follows: Group I - Normal rats (No Treatment), Group II - Diabetic rats (treated with solvent 2 ml/kg), Group III - Diabetic rats (hydro-alcoholic extract 100 mg/kg) (HAAR 100 mg/kg), Group IV - Diabetic rats (treated with ethyl acetate fraction 100 mg/kg) (HAARE 100 mg/kg), Group V - Diabetic rats (treated with methanolic fraction 100 mg/kg) (HAARM 100 mg/kg), Group VI - Diabetic rats (treated with residual fraction 100 mg/kg) (HAARR 100 mg/kg), and Group VII - Diabetic rats (treated with metformin 300 mg/kg). The treatment was given continuously for 14 days and fasting blood samples were collected on day 0, 1, 3, 6, 9, and 14 for estimating blood sugar.

Physical parameters such as body weight, water, and food intake were observed on weekly basis whereas biochemical parameters such as serum creatinine, urea, triglyceride and total cholesterol were measured at the end of the study (day 15). Blood was collected

for biochemical parameter observation after sacrificing the animals by decapitation. The biochemical parameters were measured using commercially available kits.

Statistical analysis

Results were expressed as a mean \pm standard error of the mean. The data were analyzed by one-way ANOVA followed by Turkey-Kramer multiple comparison test and $p < 0.05$ was considered as significant.

RESULTS

Effect of fractions and extract on blood glucose level of hyperglycemic rats

Effect of the fractions on FBG level of hyperglycemic rats showed in Table 1. At the end of the 14 days treatment, percentage decrease in FBG level of hyperglycemic rats observed to be 3.67%, 14.03%, 47.69%, and 50.93% with respect to treatment with residual fraction, methanolic fraction, ethyl acetate fraction and standard drug, respectively, when compared with the level at start of the study (day 0). However, it can also be observed that the significant ($p < 0.001$) reduction at the last day of observation found only in the groups treated with ethyl acetate fraction and standard drug when compared with diabetic control group. In other groups, no significant change was observed during the experimental period. The observations from a decrease in blood glucose level of hyperglycemic rats suggest that ethyl acetate fraction may be considered as a fraction with anti-hyperglycemic property and comparable with that of standard drug metformin.

Effect on body weight

The effect of the fractions against hyperglycemia induced loss of body weight of rats mentioned in Table 2. During the 1st week of observation (day 0 to day 7), decrease in mean body weight of the animals observed in all groups (except normal rats). The percentage decrease in mean body weight of animals in diabetic control group, extract treated group, residual fraction treated group, methanolic fraction treated group, ethyl acetate fraction treated group, and metformin-treated group during the 1st week of observation is 19.74%, 14.85%, 3.53%, 5.45%, 5.31%, and 20.27%, respectively. During this period, the animals treated with fractions had shown lesser percentage decrease in mean body weight and the mean body weight of animals in these groups is significantly different from the mean body weight of animals from the diabetic control group ($p < 0.001$). No significant difference observed between the mean body weights of animals treated with different fractions during the 1st week. During the 2nd week of observation (day 7 to day 14) decrease in body weight of the animals observed only in groups treated either with solvent or extract or metformin. In all other groups, there is an increase in body weight with respect to the weight observed during the previous week. The percentage decrease in mean body weight of animals in diabetic control group, extract treated group, and metformin-treated group during 2nd week of observation is 16.37%, 18.88%, and 12.26%, respectively. The percentage increase in mean body weight of animals in groups treated with residual fraction, methanolic fraction, and ethyl acetate fraction is 1.02, 3.18 and 3.57%, respectively. The mean body weight of the animals in the groups treated with residual fraction, methanolic fraction and ethyl acetate fraction is significantly ($p < 0.001$) different from the diabetic control group with no significant difference among themselves.

The observation of percentage change in mean body weight of animals of all groups during the study period (day 0 to day 14) revealed that decrease in mean body weight of animals in all groups (except normal rats) in range of 1.93-32.88. The highest percentage decrease was in diabetic control group (32.88) whereas the lowest decrease was in ethyl acetate fraction treated group (1.93). During the observation period, the animals treated with fractions had shown lesser percentage decrease in body weight and the mean body weight of animals in these groups is significantly ($p < 0.001$) different from mean body weight of animals from the diabetic control group, with no significant difference among themselves. The percentage decrease in mean body weight of

Table 1: Effect of extract, fractions and standard drug on blood glucose level of hyperglycemic rats

Multi dose STZ induced diabetic rat model (blood glucose, mg/dL)						
Treatment↓	Day 0	Day 1	Day 3	Day 6	Day 9	Day 14
Normal rat	101.33±5.110	101.33±4.499	100.50±4.515	102.00±4.973	101.17±5.205	100.83±4.527
Diabetic control	438.50±26.719	438.33±25.245	440.33±26.410	439.50±28.830	445.17±29.164	461.17±29.572
HAAR (100 mg/kg)	437.33±24.606	437.33±24.729	437.17±24.623	440.67±24.816	444.00±27.443	447.17±27.089
HAARR (100 mg/kg)	417.83±23.951	414.17±22.903	409.33±22.344	409.17±22.134	409.17±21.769	402.50±22.931
HAARM (100 mg/kg)	432.33±33.734	420.50±33.866	408.83±32.531	389.50±32.881	385.17±33.509	371.67±35.647
HAARE (100 mg/kg)	447.67±32.597	407.50±35.085	372.83±33.252	358.67±36.537	326.33±31.414 [#]	234.17±22.846 ^{***}
Metformin (300 mg/kg)	447.33±16.134	417.17±17.497	334.67±20.794	252.67±12.060 ^{***}	238.17±9.300 ^{###}	219.50±11.932 ^{***}

Values expressed as mean±SEM; (n=6); One-way ANOVA followed by Turkey-Kramer multiple comparison test; [#]p<0.05, ^{**}p<0.01, ^{***}p<0.001 versus diabetic control group comparison of blood glucose level done on the day 6; [#]p<0.05, ^{**}p<0.01, ^{***}p<0.001 versus diabetic control group comparison of blood glucose level done on the day 9; [#]p<0.05, ^{**}p<0.01, ^{***}p<0.001 versus diabetic control group comparison of blood glucose level done on the day 14. STZ: Streptozotocin, HAAR: Hydro-alcoholic extract of *A. reticulata*, HAARR: Residual fraction of hydro-alcoholic extract of *A. reticulata*, HAARM: Methanolic fraction of hydro-alcoholic extract of *A. reticulata*, HAARE: Ethyl acetate fraction of hydro-alcoholic extract of *A. reticulata*, SEM: Standard error of the mean, *A. reticulata*: *Annona reticulata*

Table 2: Effect of test extract, fractions and standard drug on body weight of hyperglycemic rats

Treatment↓	Body weight (In g)			% change between day 0 and day 7	% change between day 7 and day 14	% change between day 0 and day 14
	Day 0	Day 7	Day 14			
Normal rat	201.00±4.844	209.17±3.506	215.67±2.679	4.06	3.11	7.30
Diabetic control	206.83±5.180	166.00±4.397	138.83±5.570	-19.74	-16.37	-32.88
HAAR (100 mg/kg)	205.33±4.177	174.83±3.563	141.83±6.570	-14.85	-18.88	-30.93
HAARR (100 mg/kg)	202.83±3.005	195.67±3.630 ^{***}	197.67±3.116 ^{***}	-3.53	1.02	-2.55
HAARM (100 mg/kg)	210.83±1.721	199.33±2.894 ^{***}	205.67±2.801 ^{***}	-5.45	3.18	-2.45
HAARE (100 mg/kg)	207.00±3.821	196.00±3.587 ^{***}	203.00±3.276 ^{***}	-5.31	3.57	-1.93
Metformin 300 mg/kg	205.50±3.149	165.83±6.896	152.83±6.447	-20.27	-12.26	-25.63

Values expressed as mean±SEM; (n=6); One-way ANOVA followed by Turkey-Kramer multiple comparison test; “-” sign represents decrease from the previous value observed for the parameter; [#]p<0.05, ^{**}p<0.01, ^{***}p<0.001 versus diabetic control group comparison of body weight done on the day 7; [#]p<0.05, ^{**}p<0.01, ^{***}p<0.001 versus diabetic control group comparison of body weight done on the day 14. HAAR: Hydro-alcoholic extract of *A. reticulata*, HAARR: Residual fraction of hydro-alcoholic extract of *A. reticulata*, HAARM: Methanolic fraction of hydro-alcoholic extract of *A. reticulata*, HAARE: Ethyl acetate fraction of hydro-alcoholic extract of *A. reticulata*, SEM: Standard error of the mean, *A. reticulata*: *Annona reticulata*

animals in extract treated and metformin-treated group is similar to that of diabetic control group.

Effect on water intake and food intake of hyperglycemic rats

The effects of the tests and standard substances on daily average water and food intake habit of hyperglycemic rats depicted in Tables 3 and 4, respectively. In all fraction treated groups the mean daily average water and food consumption lesser than that of the untreated diabetic animal group. In the case of daily average water intake observation; by end of week 2 the percentage decrease in the mean of daily average water intake of animals of the groups treated with methanolic fraction, ethyl acetate fraction, and metformin is 37.93, 50.43, and 49.79, respectively, and significantly (p<0.001) different from the diabetic control group. In other treatment groups, the mean average daily water intake has not decreased if compared with that of week 0 and difference with the diabetic control group is also not significant. In case of daily food intake habit observation by the end of week 2; percentage decrease in the mean of daily average food intake of the animals in groups treated with methanolic fraction, ethyl acetate fraction, and standard is 26.16, 33.73 and 31.95, respectively, and significantly (p<0.001) different from the mean of average daily food intake of animals in the diabetic control group. In case of residual fraction treated group the mean of average daily food intake is significantly (p<0.001) different from that of animals in diabetic control group when compared at end of week 2, but there is a percentage (26.26) increase from the value observed during week 0. In other treatment groups, neither percentage decrease from week 0 nor significant difference with diabetic control group is observed.

Effect on observed Biochemical parameters

The effect of all fractions on biochemical parameters such as creatinine, urea, cholesterol, and triglyceride presented in Table 5. From the results it's evident that, effects of all the fractions on the observed biochemical parameters are more or less similar. By the end of week 2

in all fraction and standard treated groups the triglyceride level found to be significantly (p<0.001) lesser than the diabetic control group. The triglyceride levels of different groups at end of the experimental duration of 14 days were 318.973, 316.843, 116.20, 195.49, 198.32, and 97.33 mg/dL corresponding to diabetic control group, extract treated group, residual fraction treated group, methanolic fraction treated group, ethyl acetate fraction treated group, and metformin-treated group, respectively. In the case of total cholesterol level observation, the groups treated with residual fraction, ethyl acetate fraction, and metformin had shown significant (p<0.01-0.001) difference when compared with the diabetic control group, whereas the methanolic fraction treated group and extract treated group did not show any significant difference with the diabetic control group. The cholesterol levels of different groups were 138.485, 135.478, 78.90, 114.45, 61.65, and 33.59 mg/dL corresponding to diabetic control group, extract treated group, residual fraction treated group, methanolic fraction treated group, ethyl acetate fraction treated group, and metformin-treated group, respectively. In the case of creatinine level residual fraction, methanolic fraction and metformin had shown significant (p<0.001) difference when compared with the diabetic control group. Treatment with extract or ethyl acetate fraction did not bring any change in the creatinine level to be significantly different when compared with the creatinine level of the diabetic control group. In the case of urea only ethyl acetate fraction treated group has shown significant (p<0.01) decrease when compared with the diabetic control group. The metformin-treated group had also shown significant (p<0.001) difference with the diabetic control group when the urea level is compared on day 14, but the value is higher than that of the diabetic control group.

DISCUSSION

The current study focuses on the efficiency of different fractions prepared from hydro-alcoholic extract of leave of *A. reticulata* in the regulation of blood glucose levels in STZ induce dhyperglycemic rats.

Table 3: Effect of test extract, fractions and standard on average water intake habit of hyperglycemic rats

Treatment↓	Average water intake rat (ml/rat/day)			% change between week 0 and week 1	% change between week 1 and week 2	% change between week 0 and week 2
	During W0	During W1	During W2			
Normal rat	32.33±1.706	32.17±0.5426	31.67±1.542	-0.49	-1.55	-2.04
Diabetic control	76.83±0.6009	89.67±1.085	100.17±1.662	16.71	11.71	30.38
HAAR (100 mg/kg)	79.67±2.552	90.17±2.428	100.50±2.742	13.18	11.46	26.15
HAARR (100 mg/kg)	78.50±1.258	90.50±1.544	99.50±1.688	15.29	9.94	26.75
HAARM (100 mg/kg)	78.67±0.6667	66.67±2.092***	48.83±2.301***	-15.25	-26.67	-37.93
HAARE (100 mg/kg)	78.33±1.926	65.50±2.277***	38.83±0.6540***	-16.38	-40.72	-50.43
Metformin 300 mg/kg	79.33±0.9545	59.17±1.014***	39.83±1.302***	-25.41	-32.69	-49.79

Values are expressed as mean±SEM; (n=6); One-way ANOVA followed by Turkey-Kramer multiple comparison test; W0 is the observation during week before start of treatment; W1 observations done during 1st week of treatment; W2 observations done during 2nd week of treatment; “-” sign represents decrease from the previous value observed for the parameter; *p<0.05, **p<0.01, ***p<0.001 versus diabetic control group at end of week 1; *p<0.05, **p<0.01, ***p<0.001 versus diabetic control group at end of week 2. HAAR: Hydro-alcoholic extract of *A. reticulata*, HAARR: Residual fraction of hydro-alcoholic extract of *A. reticulata*, HAARM: Methanolic fraction of hydro-alcoholic extract of *A. reticulata*, HAARE: Ethyl acetate fraction of hydro-alcoholic extract of *A. reticulata*, SEM: Standard error of the mean, *A. reticulata*: *Annona reticulata*

Table 4: Effect of test extract, fractions and standard on average food intake habit of hyperglycemic rats

Treatment↓	Average daily food intake (g/rat/day)			% change between week 0 and week 1	% change between week 1 and week 2	% change between week 0 and week 2
	During W0	During W1	During W2			
Normal rat	21.67±0.4944	22.17±0.4014	21.17±0.5426	2.31	-4.51	-2.31
Diabetic control	27.67±1.085	38.33±1.358	42.50±0.6191	38.53	10.88	53.59
HAAR (100 mg/kg)	26.67±0.7601	37.67±1.022	41.33±0.9545	41.24	9.72	54.97
HAARR (100 mg/kg)	29.17±0.4773	36.17±1.138	36.83±1.167***	23.99	1.82	26.26
HAARM (100 mg/kg)	28.67±0.7149	24.50±0.8466***	21.17±0.6009***	-14.54	-13.59	-26.16
HAARE (100 mg/kg)	29.17±0.6009	23.67±0.8819***	19.33±0.7601***	-18.86	-18.34	-33.73
Metformin 300 mg/kg	28.17±0.4773	19.17±0.8724***	19.17±0.6009***	-31.95	0	-31.95

Values are expressed as mean±SEM; (n=6); One-way ANOVA followed by Turkey-Kramer multiple comparison test; W0 is the observation during week before start of treatment; W1 observations done during 1st week of treatment; W2 observations done during 2nd week of treatment; “-” sign represents decrease from the previous value observed for the parameter; *p<0.05, **p<0.01, ***p<0.001 versus diabetic control group at end of week 1; *p<0.05, **p<0.01, ***p<0.001 versus diabetic control group at end of week 2. HAAR: Hydro-alcoholic extract of *A. reticulata*, HAARR: Residual fraction of hydro-alcoholic extract of *A. reticulata*, HAARM: Methanolic fraction of hydro-alcoholic extract of *A. reticulata*, HAARE: Ethyl acetate fraction of hydro-alcoholic extract of *A. reticulata*, SEM: Standard error of the mean, *A. reticulata*: *Annona reticulata*

Table 5: Effect of test extract, fractions and standard drug on biochemical parameters of hyperglycemic rats

Treatment↓	Urea (mg/dl)	Creatinine (mg/dl)	Cholesterol (mg/dl)	Triglyceride (mg/dl)
Normal rat	27.47±2.879	0.545±0.0376	49.252±5.496	89.177±6.219
Diabetic control	64.592±5.773	1.085±0.0762	138.485±13.426	318.973±24.561
HAAR (100 mg/kg)	62.492±5.283	0.958±0.0490	135.478±12.614	316.843±23.343
HAARR (100 mg/kg)	60.466±3.877	0.727±0.0491***	78.90±10.229**	116.20±5.342***
HAARM (100 mg/kg)	46.402±4.352	0.657±0.0619***	114.45±4.531	195.49±10.038***
HAARE (100 mg/kg)	37.78±2.877**	0.915±0.0664	61.65±5.255***	198.32±15.827***
Metformin 300 mg/kg	104.97±5.838***	0.638±0.0367***	33.59±1.550***	97.33±10.330***

Values are expressed as mean±SEM; (n=6); One-way ANOVA followed by Turkey-Kramer multiple comparison test; *p<0.05, **p<0.01, ***p<0.001 versus diabetic control group. HAAR: Hydro-alcoholic extract of *A. reticulata*, HAARR: Residual fraction of hydro-alcoholic extract of *A. reticulata*, HAARM: Methanolic fraction of hydro-alcoholic extract of *A. reticulata*, HAARE: Ethyl acetate fraction of hydro-alcoholic extract of *A. reticulata*, SEM: Standard error of the mean, *A. reticulata*: *Annona reticulata*

Table 1 represents the effect of the fractions on blood glucose level of STZ-induced diabetic rats. From the results, it is evident that only ethyl acetate fraction is capable to reduce the blood glucose level significantly (p<0.001) at the tested dose level, whereas the other fractions did not show any significant glucose lowering activity. Hence, the ethyl acetate fraction may be considered as the fraction having potential anti-hyperglycemic activity. The results observed may be attributed to the compounds present in ethyl acetate fraction. The major compounds 9-octadecenamide, (Z)-, ethyl isoallochololate and 9,10-secocholesta-5,7,10(19)-triene-3,24,25-triol, (3 α ,5Z,7E)-present in ethyl acetate fraction [8] may be considered as compounds responsible for the observed anti-hyperglycemic activity. The compound 9, 10-secocholesta-5, 7, 10(19)-triene-3,24,25-triol, (3 α ,5Z,7E)-is a hydroxyl derivative of vitamin D3 and vitamin D3 is known to have a positive effect in the management of diabetes [14-16]. Hence, the structural derivatives of vitamin D3 may be one of the responsible compounds for the observed anti-hyperglycemic activity.

The compound ethyl isoallochololate is an ester of a bile acid and is known to act as an emulsifying agent and helps in digestion of fats and oils by water-soluble digestive enzymes in the small intestines [17]. The compound 9-octadecenamide, (Z)- and its derivatives are known to be potent hypolipidemic agent. They have effect on peroxisome proliferator-activated receptor- α , tumor necrosis factor α , and potential antioxidant effect as well [18-20]. The presence of these compounds may be considered as responsible for the observed anti-hyperglycemic effect of the ethyl acetate fraction. The observation of chemical components of methanolic and residual fraction revealed glycerine as the major constituent [8]. Glycerine is capable of causing hyperglycemia in animals after oral or intraperitoneal administration [21]. The phytochemical analysis also revealed that methanolic fraction and residual fraction contains several other compounds such as D-glucose,6-O-a-D-galactopyranosyl-, α -D-glucopyranoside, O- α -D-glucopyranosyl-(1.fwdarw.3)- α -D-fructofuranosyl, α -D-Glucopyranose, 4-O- α -D-galactopyranosyl-, α -methyl-D-mannopyranoside which are

sugar like [8] in nature. Hence, the presence of glycerine and other sugar like compounds as major components may be the reason due to which the methanolic fraction and residual fraction had not been able to show any kind of anti-hyperglycemic effect at the tested dose level. Natural compounds like flavonoids and phenolics are known to have antioxidant property and prevent free radical chain reaction in biological systems and provide additional health benefits [22]. Flavonoids are known as excellent free radical scavenging agents and known to have properties for the management of diabetes and diabetic complications [23-25]. Flavonoids also lead to regeneration of pancreatic β -cells, reduce necrosis and degeneration of pancreatic β -cells and thus may be effective in treating hyperglycemia by preventing diabetic complications [26]. Flavonoids are also known to decrease triglyceride level [27]. Phenolic compounds are also known to decrease blood glucose in STZ-induced diabetic rats and increase in insulin secretion, which may be due to regeneration of β -cells brings by reduction in oxidative stress [28]. Phenolics tend to increase the glutathione levels and thus decreases levels of lipid peroxidation in diabetic rats, and contribute in the management of diabetes and diabetic complications [29]. Ethyl acetate fraction had been found to have a high content of flavonoids and phenolics in comparison to other tested fractions [30] and hence may be another reason for the observed anti-hyperglycemic potential.

Sreptozotocin induced hyperglycemia is associated with characteristic loss of body weight because of increased muscle wasting and loss of tissue proteins [31]. During the observation of the effect of the fractions on body weight loss, it is observed that all the fractions at the tested dose level are capable of arresting the loss of body weight significantly. Oxidative stress produced due to STZ administration is known to impair muscle repair and antioxidants are capable of improving oxidative imbalance and improve muscle repair [32]. The tested fractions had been identified to have antioxidant potential [30] and hence may be one of the reasons for prevention of loss of body weight by the fractions. Increase in body weight or prevention of loss of body weight in ethyl acetate fraction administered group may also be attributed to the components of the fraction which are capable of attenuating hyperglycemia condition. In the case of methanolic fraction and residual fraction administered group, the increase in body weight may also be due to the presence of sugar like compounds [33]. The extract at the tested dose level (100 mg/kg) did not show any protective effective against body weight loss. The extract had exhibited an increase in body weight of STZ induced hyperglycemic rats when administered at a dose level of 200-400 mg/kg [7], hence the diminished effect of the extract may be attributed to the decreased dose level. The standard drug metformin did not provide any protection to body weight loss. Metformin is known to reduce body weight due to decreased calorie intake [34] and hence the observation is in line with the property of the standard drug.

Sreptozotocin induced hyperglycemia is always accompanied by polydipsia and polyphagia [35]. In this study, significant ($p < 0.001$) decrease in water intake can be observed in the groups treated with methanolic fraction, ethyl acetate fraction, and metformin in comparison to diabetic control group. In the case of food intake habit, the groups treated with all fractions and metformin had shown significant ($p < 0.001$) decrease in food intake as compared to the diabetic control group. The significant decrease in water and food intake in case of ethyl acetate treated group may be due to the observed anti-hyperglycemic activity of the fraction. The decrease in water and food intake in case of metformin-treated group may be due to anti-hyperglycemic activity of the drug substance. Hence, the positive effect of ethyl acetate fraction on the secondary parameters like food and water intake supports the anti-hyperglycemic of the fraction.

Dyslipidemia is known as one of the major risk factors associated with diabetes, characterized by increased plasma triglyceride level, decreased high-density lipoprotein (HDL) cholesterol level, and increased low-density lipoprotein (LDL)-cholesterol level. The changes

in lipid profile of diabetic patients are associated with increased free fatty acid flux and secondary to insulin resistance [36,37]. Standard drug metformin is known to have a beneficial effect in decreasing the level of triglycerides and LDL-cholesterol by 10-15% along with slight increase in HDL levels [38]. Hence, the observed effect of metformin on lipid profile of diabetic rats is in agreement with the property of the drug. As the results suggest ethyl acetate fraction is capable to control, the level of triglyceride and cholesterol significantly ($p < 0.001$) in hyperglycemic rats. The residual fraction is also been able to reduce the cholesterol ($p < 0.001$) and triglyceride ($p < 0.01$) level significantly when compared with the diabetic control group. The treatment with methanolic fraction is capable to reduce only triglyceride level significantly ($p < 0.001$) with no significant effect on the cholesterol level. Oxidative stress has been shown to play a role in the causation of diabetes [39]. STZ produces oxygen radicals in the body, which cause pancreatic injury and could be responsible for increased blood sugar seen in animals [40]. Increase in systemic oxidative stress is considered as one of the factors that may contribute to the development of obesity, atherosclerosis, and other cardiovascular risk associated with which are associated with diabetes and another metabolic syndrome [41,42]. The fractions used in the current experiment also had potential antioxidant activity [30] and may be one of the reasons for controlling cholesterol and/or triglyceride level of diabetic rats. Under normal circumstances, insulin activates enzyme lipoprotein lipase and hydrolyses triglycerides. Insulin deficiency results in failure to activate the enzymes thereby causing hypertriglyceridemia and hypercholesterolemia [43]. The ethyl acetate fraction has shown significant anti-hyperglycemic potential and hence increase insulin secretion and/or insulin sensitization may be proposed to be another reason for the control of hypertriglyceridemia and hypercholesterolemia by the ethyl acetate fraction.

Increase in level of urea and creatinine is indicators of impaired renal function [44] and may not be seen all diabetic patients [45]. Induction of experimental diabetes by injecting STZ may alter kidney morphology and kidney function leading to diabetic nephropathy [46,47]. In this study, increase in the level of creatinine and urea was observed in all groups of diabetic rats and remained elevated in comparison with the normal rats till end of the experimental period. The treatment with residual fraction, methanolic fraction, and metformin has decreased the level of creatinine significantly ($p < 0.001$) when compared with the diabetic control group. Ethyl acetate fraction had not shown any kind of controlling effect on increased creatinine level. The urea level is controlled significantly ($p < 0.001$) only in ethyl acetate fraction treated group in comparison to diabetic control group. In metformin-treated group, there was a significant ($p < 0.001$) increase in urea level. In STZ induced diabetic models oxidative stress and reactive oxygen species play major roles in glucose-induced renal injury [48-51]. The effect of the fractions on the renal function of diabetic rats had been partial in providing protection either against increased creatinine level and/or against urea level and may be attributed to the antioxidant property of the fractions [30]. Effect of metformin in diabetic nephropathy is not clear and it is contraindicated in case of renal impairment and should not be used until renal function is normal [43]. However, some studies suggest that metformin may have some renoprotective activity [52]. Metformin is suggested to provide protection against STZ induced nephropathy by restoration of biochemical alterations and modulation of oxidative stress [53]. Hence, the effect of metformin on decreasing creatinine level may be due to restoration of biochemical alterations and/or modulation of oxidative stress. Metformin is known to be associated with a very rare, preventable but fatal adverse effect lactic acidosis [43], which may be associated with increase in urea level [54,55]. Hence, the increase in urea level may be due to metformin-induced lactic acidosis.

From the results of the study, it is evident that among the tested fractions ethyl acetate fraction is having better anti-hyperglycemic potential. Studies suggest that that the extracts of leaves of *A. reticulata* have the potential to inhibit the action of α -amylase and α -glucosidase [56]. Thus, the observed anti-hyperglycemic activity of ethyl acetate

fraction may be attributed to the antioxidant property of the fraction and/or due to inhibition of action of α -amylase and/or α -glucosidase and/or insulin secretion and/or insulin sensitization. The observed anti-hyperglycemic effect of the fraction may be due to one or more compounds present in the fraction. Further experimental observations are warranted to understand the mechanism/process involved.

CONCLUSION

Ethyl acetate fraction prepared from hydro-alcoholic extract of *A. reticulata* leave is having anti-hyperglycemic potential and the observed glucose lowering activity may be due to the presence of one or more pharmacologically active compounds in the fraction. Further studies are recommended to comprehend the exact mechanism of action and the compounds responsible for the action.

ACKNOWLEDGMENT

The authors are grateful to the president and vice-chancellor, S 'O' A University, Bhubaneswar, Odisha, India for providing necessary facilities to carry out the research work in the faculty of pharmacy, S 'O' A University.

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