ANTI DIABETIC EFFECT OF *ACHYRANTHES RUBROFUSCA* LEAF EXTRACTS ON ALLOXAN INDUCED DIABETIC RATS

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ABSTRACT

The aqueous and ethanolic extracts of *Achyranthes rubrofuca* leaves (AR) were studied for their hypoglycemic activity. Thirty animals were taken and they were divided into five groups. First group acts as control, remaining 4 groups were induced diabetics by administering alloxan (120mg/kg i.p). Second group serves as diabetes control, third group treated with Glibenclamide (5mg/kg), fourth and fifth group were given aqueous and ethanolic extracts of leaves (200mg/kg/ body weight/day/po for 28 days) to rats. The anti hyperglycemic activity by AR was compared with the group treated with the standard oral hypoglycemic agent. Treatment with aqueous and ethanolic extract of AR caused a significant change when compared to the untreated animals with respect to body weight, blood glucose level, and lipid profile. Aqueous extract showed slightly better activity than ethanolic extract but it may not be statistically significant. There is significant increases in the pancreatic enzyme like SOD, CAT and Glutathione expression when compare with the untreated group. Decreases in LPO level is observed in the group treated with extracts when compare with control groups animals. The histopathological studies also show the regenerative effect of pancreas, supported the above activities of AR leaves.

Keywords: *Achyranthes rubrofusca* L., Hypolipidemic, Diabetic, Alloxan.

INTRODUCTION

Plants have been considered as sources of medicinal agents for the treatment of many diseases. Before the advent of insulin injections and other pharmaceutical preparation, healers relied heavily upon medicinal plants and herbs to treat diabetes. More than 1200 plants have been described to be experimentally or ethno pharmacologically used in the treatment of diabetes mellitus (Oliver 1980).

Achyranthes rubrofusca (AR) ***Linn***, belonging to the Amaranthaceae family known as "*Kadaladi"* is a native herb widely distributed through out the Western Ghats of South India. AR is widely used in the traditional treatment of diabetic mellitus. The plant is an erect, much branched suffructicose or diffused shrub up to 1cm height. AR which having many physiological affects and traditionally used in the treatment of various disorders like diabetes, liver ailments, cuts and boils (Aminuddin and Khan 1992). In our present study we have evaluate the anti diabetic property of *Achyranthes rubrofusca* (AR) using alloxan induced diabetic rat model compare with a standard drug Glibenclamide. In the present study we try to evaluate the metabolic disorder and the change in the expression of enzymes due to diabetic. This gives some idea about the anti diabetic property of the plant extracts.

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MATERIALS AND METHODS

Plant materials

Specimens of *Achyranthes rubrofusca* (Amaranthaceae) were collected from the Western Ghats region of South India during the month of Feb-March 2004, and air-dried. The plant was identified and authenticated by Dr. V. Chelladurai and a voucher specimen 2032 was deposited at the herbarium of Botanical survey of India, Coimbatore, India.

Preparation of Aqueous and Ethanol Extract

The dried plant leaves were powdered and extracted according to increase in polarity from n-hexane, ethyl acetate, ethanol and aqueous by continuous extraction by Soxhlet apparatus for 72 h. Aqueous and ethanol extract were selected forth study based on the preliminary phytochemical evaluation (Geetha *et al*., 2006).

The extract filtered and dried using vacuum pump. The extracts were administered orally daily to different groups of rat at a dose of 200mg/kg body weight. The dose was fixed according to the toxicity studies (OECD guidelines). In the toxicity studies no mortality observed up to 2000mg/kg, this dose was considered as the maximum tolerated dose. From this 1/10 of the dose was selected for further pharmacological studies.

Selection of animals

Animal were obtained from the PSG IMS&R, Coimbatore, India animal house the experimental

procedure were approved by the Institutional Animal Ethical Committee (158/99/CPSSEA). Male Albino Wistar rats (175-200 g) were used in the present study. The animals were housed under standard environmental conditions (23±1^{o}C) with relative humidity of 50±10% and maintain 12:12 dark and light cycle, maintained with free access to water and *ad libitum* standard laboratory diet (70% carbohydrates, 25% proteins, 5% lipids (Hindustan liver Bangalore). After randomization before the experiment, the rats were acclimatized for a period of two weeks. We selected the male animals for our studies since the females were reported, to be protected from lipid-Induced reduction in insulin action (Hevener *et al*., 2002).

Induction of Experimental diabetes mellitus

The diabetes was induced in Wistar rats as described by Trivedi *et al*. (2004) animals were allowed to fast for 24 hrs prior to injection with freshly prepared aqueous solution of alloxan monohydrate 120 mg/kg, i.p. The rats were kept on 5% glucose solution in the cages to prevent hypoglycemia (Daisy *et al*., 2009). After five days, the rats with fasting serum glucose levels more than 300 mg/dl were considered as diabetic and were used in the subsequent experimental procedures (Trivedi *et al*., 2004; Sasaki *et al*., 1972).

Experimental design

The rats were divided into two groups' normal animals and hyperglycemic induced animals. The hyperglycemic rats were divided into 4 groups consisting of six animals each.

Group 1 Normal control
Group 2 Diabetic control
Group 3 Diabetic rats treated with 5 mg/kg of Glibenclamide orally
Group 4 Diabetic rats treated with 200 mg/kg (body weight) aqueous extract.
Group 5 Diabetic rats treated with 200 mg/kg (body weight) ethanol extract.

Group 1 and 2 animals were fed with distilled water along with 5% carboxy methyl cellulose. After the induction of diabetes Group 3, 4 and 5 animals were kept overnight fasting. Group 4 and 5 animals were treated with aqueous and ethanol extract of plant drug dissolved in 5% carboxyl methylcellulose. Group 3 animals were treated with standard drug glibenclamide dissolved in 5% carboxyl methyl cellulose.

Blood glucose level and body weight were monitored at regular weekly intervals for four weeks. Animals were sacrificed after the 28th day by cervical dislocation. Blood was collected and serum was separated by centrifugation at 5000 rpm for 20 min. Collected serum was used for biochemical analysis. The pancreas were immediately removed and suspended in ice-cold saline for histopathological studies.

Biochemical Estimation

Estimation of blood glucose level

Fasting blood glucose level was measured on day 0, 7, 14, 21 and 28. Blood was collected from the tail vein and fasting blood glucose level was measured by O-Toludine method. The results were expressed in terms of milligram per deciliter of blood (Bopanna *et al*., 1997).

Estimation of lipid profile

The total cholesterol level was measured on 0 day and 28th day 9 serum triglycerides and HDL were estimated (Foster and Dunn 1973).

Estimation of Tissue lipid peroxides (LPO)

The pancreas were isolated, washed and homogenized (Remi motor, model No RQ-127) by using 0.15M ice cold KCL buffer and filtered, the filtrate were subjected to anti oxidant studies.

1.0ml of the tissue homogenate filtrate was mixed with 4.0 ml of 0.85 N sulphuric acids and mixed gently. Then 0.5 ml of phosphotungistic acid was added and stirred well. The contents were centrifuged for 10 minutes. The supernatant was discarded and the sediment was mixed with 2.0 ml of N/12 sulphuric acid and 0.3ml of phosphotungstic acid. The mixture was centrifuged for 10 minutes. The sediment was suspended in 4.0 ml of distilled water and Thiobarbituric acid (TBA) reagent. The tubes were kept in a boiling water bath for 30 minutes. After cooling, 5 ml of butanol was added to each tube and the colour extracted in butanol phase was read at 532 nm. The lipid peroxide content was expressed as nano moles of TBA reactants/ mg protein (Ohkawa *et al*., 1979; Lubec, *et al*., 1996).

Estimation of Tissue Glutathione

To 0.5 ml of tissue homogenate filtrate, 20 % Trichloro citric acid (TCA) was added and precipitated. The contents were mixed well for complete precipitation of protein and centrifuged. To an aliquot of clear supernatant, 2.0 ml of 5,5'-Dithio-bis(2-NitroBenzoic acid (DTNB) reagent and 0.2M-phosphate buffer were added to make a final volume of 4.0 ml. The absorbance was read at 412 nm against blank containing TCA instead of sample. A series of standards treated in similar way to determine glutathione content. The amount of glutathione was expressed as nano moles of GSH oxidized/mg protein. Reduced glutathione was measured according to the method of Beutler *et al*. (1963).

Assay of Super Oxide Dismutase (SOD)

Required amount (100µl) of homogenate was added to tubes containing 0.5 ml of carbonate buffer and 0.5 of EDTA solution. The final volume was made up to 2.5 ml.

The reaction was initiated by the addition of 0.5ml of epinephrine and increase in absorbance at 480 nm was measured in a Systronics 119 UV spectrophotometer. 100% auto oxidation of epinephrine to adrenochrome was performed in a control tube without the enzyme.

The enzyme unit of activity was defined, as the enzyme required for 50 % inhibition of epinephrine auto oxidation. Super oxide dismutase was assayed by following the method (Misra and Frisovich 1979).

Assay of Catalase (CAT)
To 6.0ml phosphate buffer, 0.1ml sample and 0.4ml hydrogen peroxide was added. The reaction was stopped at 15, 30, 45 and 60 seconds by the addition of 2 ml dichromate acid reagent. The tubes were kept in boiling water bath for 10 minutes and the color developed was read at 620 nm. Standards in the range of 2-10nm were taken and preceded similar to the test with blank containing reagent alone.

The activities were expressed as nm of H_2O_2 consumed /minute/mg protein. Catalase was assayed (Sinha 1972).

Histopathological Studies
A small portion of, pancreas was fixed in 10% formalin for histopathological studies. Pancreas sections were taken with 5 µ thick, and stained with Alum hemotoxylin and eosin. Sections were observed under microscope for Histopathological changes as described previously (Galigher and Kozloff 1971).

STATISTICAL ANALYSIS

The values were expressed as mean ± SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey multiple comparison tests. P values < 0.05 were considered as significant

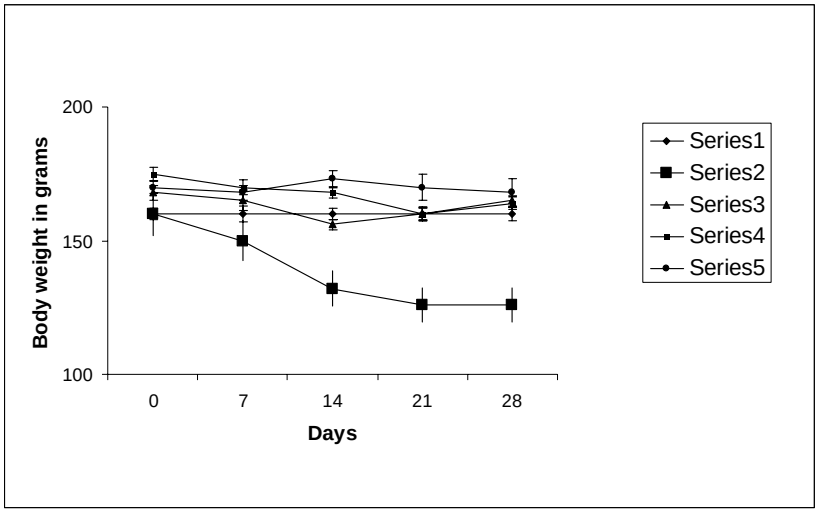
RESULTS

Body weight
There was a significant reduction in body weight of the animals in diabetic group in comparison to control. After administration of ethanol and aqueous extract of AR for 28days, the body weight was recovered significantly ($p>0.05$) with respect to the control (graph).

Fasting blood glucose level
Fasting blood glucose levels of all animals before treatment were within the normal levels. Fasting blood glucose level was significantly elevated after 5 days of Alloxan treatment with respect to control level. Treatment of ethanol and aqueous extracts of AR for 28 days resulted restoration of fasting blood glucose level near to normal $P<0.001$, when compared with diabetic control and normal control (table 1).

Effect of extracts on lipid profile
In alloxan diabetic rats, AR extracts decreased the plasma triglyceride level on 28th day ($P<0.01$). Daily administration of the extract or reference drug Glibenclamide showed a significant decrease in plasma cholesterol ($P<0.01$) when compared to diabetic control. Plasma HDL level in treated animals showed improvement $P<0.01$. On the other hand, Plasma cholesterol levels in diabetic control animals remained statistically unchanged through out the experiment (table 2).

Effect of extract on antioxidant enzymes
A significant decrease in SOD, CAT and glutathione



Graph: Effect of AR extracts on body weight in alloxan induced diabetic rats
N=6 animals in each group. Values are expressed as mean ± SEM.
$P^a < 0.01$; $P^b < 0.05$ Vs Initial body weight Data analyzed by one-way ANOVA followed by dunnett test

Table 1: Effect of AR leaf extract on fasting blood glucose level in alloxan induced diabetic rats.

Groups	Dose (mg/kg)	Blood Glucose Level (mg/dl)					
		Normal	Alloxan Induction	1 day	7 day	21 day	28 day
Normal	-	84 ± 4.2	87± 4.3	82 ± 3.8	84 ± 3.8	86 ± 3.0	80 ± 2.8
Diabetic Control	-	82 ± 3.8	240 ± 3.5[a]	236 ± 3.96 [a]	245 ± 4.16[a]	226 ± 4.08[a]	220±3.85[a]
Glibenclamide	5	86 ± 30	240± 3.75[a]	176± 2.33[a,b]	165± 1.87[a,b]	156± 1.92[a,b]	130±1.84[a,b]
Ethanol Extract	200	80 ± 2.8	230± 3.16[a]	178± 2.96[a,b]	154 ± 2.5[a,b]	130± 1.94[a,b]	106±1.75[a, b]
Aqueous extract	200	82 ± 3.8	226± 3.10[a]	190± 2.78[a,b]	160± 2.34[a, b]	132 ±1.98[a, b]	110±1.84[a, b]

n=6 animals in each group. Values are expressed as mean ± SEM
[a]P< 0.001 compared with Normal animals. [b]P < 0.001 compared with diabetic animals
Data analyzed by one-way ANOVA followed by Tukey multiple test.

Table 2: Effect of AR leaf extract on lipid profile in alloxan induced diabetic rats on 28th day

Treatment	Dose (mg/kg)	Total cholesterol mg/dl	Triglyceride mg/dl	HDL mg/dl	LDL mg/dl
Normal		70 ± 2.96	56.0 ± 1.45	24.5 ± 1.45	34.8 ± 1.45
Diabetic Control	-	104.7 ± 1.58[a]	146.67 ± 3.95[a]	18.82 ± 1.16[b]	56.82±1.16[b]
Glibenclamide	5	75 ± 2.66[d]	72.31 ± 2.10[a,d]	25.25 ± 1.10[f]	35.25±1.10[f]
Aqueous Extract	200	88.2 ± 3.7 [b, e]	95.01± 2.7[d,c]	23 ± 1.38	46 ± 1.38
Ethanol extract	200	84.43 ± 4.29 [c, d]	82.31 ± 2.64[a]	22.64 ± 1.25	44.6 ± 1.25

N=6 animals in each group. Values are expressed as mean ± SEM. [a]P< 0.001; [b]P< 0.01;
[c]P < 0.05 Vs normal control. [d]P< 0.001; [e]P< 0.01; [f]P < 0.05 Vs Diabetic control.

Table 3: Effect of *Achycanthes rubrofusca* on Antioxidants level in alloxan Induced Diabetic Rats on 28th day.

Treatment	Dose (mg/kg)	SOD nm/ mg protein	Calatase nm of H_2O_2 consumed /minute/mg protein	Glutothione nm/mg protein	Lipid per Oxidation/mg protein
Normal	-	5.2 ± 0.34	46.0 ± 1.14	1.74 ± 0.01	4.5 ± 0.01
Diabetic Control	-	3.28 ± 0.2[a]	32.6 ± 3.95[a]	0.44 ± 0.01	6.18 ± 0.3[a]
Glibenclamide	5mg	4.98 ± 0.36[b]	44.8 ± 0.02[d]	1.38 ± 0.02[c,e]	4.12 ± 0.6[b]
Ethanol Extract	200	4.78 ± 0.54	43.8 ± 2.5[d]	0.98 ± 0.01[c,e]	4.78 ± 0.4
Aqueous extract	200	5.02 ± 0.44[b]	38.8 ± 27	0.89 ± 0.08[c,e]	4.93 ± 0.6[b]

N=6 animals in each group. Values are expressed as mean ± SEM. [a]P< 0.05; [e]P<0.001 Vs Normal
[b]P<0.05; [c]P<0.001; [f]P < 0.01 Vs Diabetic control. Data analyzed by one-way ANOVA followed by Dunnett test.

enzyme expression in animals treated with alloxan when compared to normal control group (Group I). Extract treated animals showed increase in SOD and CAT enzyme level but there is a decrease in LPO level (P< 0.001). The animal treated with Glibenclamide also produced significant (P< 0.001), increase in tissue glutathione and decrease in SOD and CAT enzyme level (table 3).

Super oxide dismutase has enhanced beta cell tolerance in the oxidative stress induced diabetes. Decrease in SOD activities may be responsible for the inadequate activities of Catalase and SOD is observed which may give rise to increased production of H_2O_2 and O_2. Extract treated rats showed increased activity off SOD and CAT preventing the accumulation of free radicals in liver and pancreas. Prevention of superoxide radical and H_2O_2 by SOD and CAT may ameliorate alloxan toxicity.

Histopathological observations

Alloxan induced animals showed necrosis and reduction in the number of islet cells and migration of WBC (fig. 2). Extract treated animals showed regeneration of these necrotic cells and increased number of islets (figs. 3-5).

DISCUSSION

In our present study, administration of AR to diabetic rats for 28 days caused significant reduction in blood glucose, triglycerides and cholesterol level and improvement in

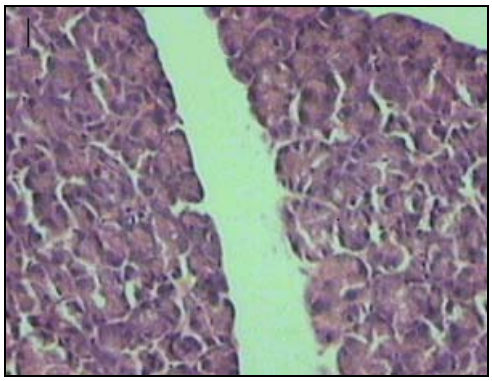


Fig. 1: Section of the pancreatic tissue of control animal showing Normal islets.

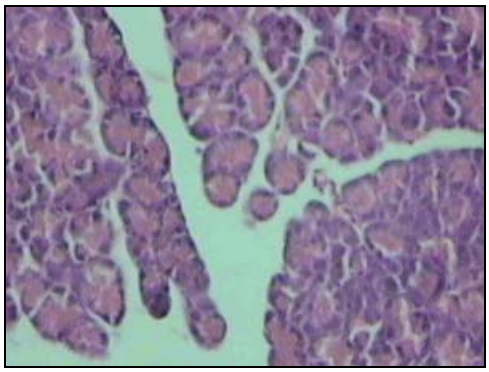


Fig. 2: Section of the Pancreatic tissue of alloxan 120 mg/ kg treated animal showing reduction and necrosis of islets (arrow marks)

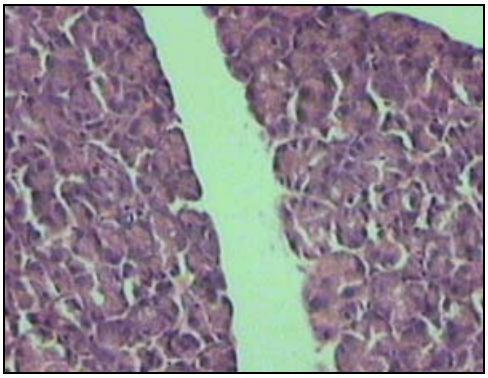


Fig. 3: Section of the pancreatic tissue of animals treated with glibenclamide 5mg/kg.

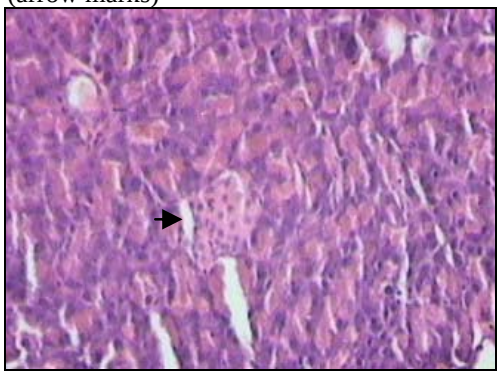


Fig. 4: Section of the pancreatic tissue of animals treated with Ethanol 300mg/kg extracts (arrow marks).

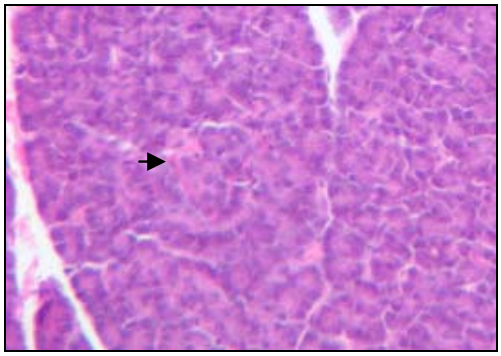


Fig. 5: Section of the pancreatic tissue of animals treated with aqueous extract 300mg/kg (arrow marks).

HDL. Damage produced by Alloxan to the islet cells has an impact on the insulin secretion (Dunn *et al*., 1943) through a direct effect on the pancreas. The alloxan is considered to be suitable compound for inducing experimental diabetes with symptoms such as body weight loss, polydypsia, polyuria, ketonuria, hyperglycemia and ketonaemia followed by hepatic glucose overproduction (Milagro and Martínez 2000; Lenzen and Panten 1988) leads to further pancreatic cell death. Alloxan inhibited glucokinase enzymes, glucose phosphorlyating enzymes in the pancreatic beta cells as well as in the liver. Alloxan is also considered as the primary target, which is responsible for the inhibition of glucose induced secretion and for toxicity of pancreatic beta cells.

In diabetic rats, decreased body weights were observed. This indicates the polyphagic condition and loss of weight due to excessive breakdown of tissue proteins (Kamalakkannan and Prince 2006) the decrease in body weight in diabetic rats could be due to dehydration and catabolism of fats and proteins (Hakim *et al*., 1997). Increased catabolic reactions leading to muscle wasting might also be the reason for the reduced body weight in diabetic rats (Rajkumar *et al*., 1991). Oral administration of AR extracts for consecutive 28 days to diabetic rats

improves the body weight. This could be due to a better control of hyperglycemic state in the diabetic rats and decreased fasting blood glucose level could improve body weight in alloxan-induced diabetic rats (Nagarajan *et al*., 2005; Pari and Saravanan 2004).

Diabetes mellitus is also associated with hyperlipidaemia with profound alteration in the concentration and composition of lipid (Odetola *et al*., 2006). Changes in the concentrations of the lipid with diabetes mellitus contribute to the development of vascular disease (Nikkilä and Kekki 1973; Howard *et al*., 1978). Fatty acids, an important component of cell membranes, are eicosanoid precursors and are therefore required for both the structure and function of every cell in the body (Rajasekaran *et al*., 2006). In type II diabetic condition the glucose itself acts as a toxic substance and produce number of Reactive oxygen species, which may cause damage to the islet cells. The islet is the least endowed tissue in terms of intrinsic anti-oxidant enzyme expression, including SOD-1, SOD-2, Catalase and glutathione peroxides (Tiedge *et al*., 1997; Grankvist *et al*., 1981).

In diabetic condition, hypoinsulinaemia increases the activity of fatty acyl coenzyme, an oxidase that indicates beta-oxidation of fatty acids, resulting in lipid peroxidation. Increased lipid peroxidation resulted in generation of harmful, free radical, which impairs membrane function, by decreasing the levels of membrane bound enzymes and receptors. This leads to cell injuries and complications such as atherosclerosis, brain and kidney damage. Diabetic animals treated with plant extracts showed marked decrease in LPO level. This may be due to the scavenging activity of the plant drug.

Super oxide dismutase has enhanced beta cell tolerance in the oxidative stress induced diabetes. Decrease in SOD activities may be responsible for the inadequate antioxidant defense in combating ROS mediated damage. During diabetes the decreased activities of Catalase and SOD is observed which may give rise to increased production of hydrogen peroxide and oxygen. Extract treated rats showed increased activity of SOD and catalase there by increases antioxidant defense in combating ROS mediated damage and preventing the accumulation of free radicals in pancreas. Which is supported by the histopathological studies .Prevention of superoxide radical and hydrogen peroxide by SOD and catalase may ameliorate alloxan toxicity. In diabetic animals, high glucose level decreases the gamma glutamyl cysteine ligase enzyme, which is the rate-limiting step for synthesis of glutathione. The plant extract treated diabetic animals showed marked increase in the glutathione synthesis, which may be due to the role played by the extract with over expression of glutamyl cysteine ligase.

The possible underlying mechanism by which AR can exert its lipid lowering activities is not elucidated. At this stage of the study, several fundamental mechanisms could be proposed to explain our results. AR act by decreasing the cholesterol biosynthesis especially by decreasing the 3- hydroxyl- 3- methyl - glutaryl co enzyme. A reductase (HMG-CoA reductase) activity (Key enzyme of cholesterol biosynthesis) and by reducing the NADPH required for fatty acids and cholesterol synthesis. In addition, AR leaves may improve hypercholesterolemia by modifying lipoprotein metabolism, enhanced uptake of LDL by increasing LDL receptor and by increasing the Lecithin - cholesterol acyl transferase (LCAT) activity. This may contribute to the regulation of blood lipids LCAT plays a key role in incorporating free cholesterol into HDL and transferring VLDL or IDL which is taken back by the liver cells. The repeated oral administration of Ethanol and aqueous extract for 28 days produced a significant decrease in plasma triglycerides in both the extract treated rats. The observed hypotriglyceridemic effect may be due to a decrease of fatty acids synthesis (enhanced catabolism of LDL, activation of LCAT and tissue lipases or inhibition of acetyl Co-A carboxylases such as acetyl -Co A and glycerol phosphate.

It is well known that level of glycemic control is the major determinant of plasma VLDL and triglycerides levels. The islet is the least endowed tissue in terms of intrinsic anti-oxidant enzyme expression, including SOD-1, SOD- 2, Catalse and Glutothione peroxidases.

In diabetic condition, hypo insulinaemia increases the activity of fatty acyl coenzymes an oxidase that indicates beta- oxidation of fatty acids resulting in lipid peroxidation resulting in generation of harmful free radical, which impairs membrane function, by decreasing the levels of membrane bound enzymes and receptors. This leads to cell injuries and complications such as atherosclerosis, brain and kidney damage. Diabetic animals treated with plant extracts showed marked decrease in LPO level. This may be due to the scavenging activity of the plant drug.

In histopathological study alloxan, induced animals showed necrosis and reduction in the number of islets cells. The reduction and necrosis in pancreatic cell may be due to the decrease in the antioxidant defense in combating ROS mediated damage. Extract treated animals showed regeneration of some the necrotic cells and decrease the cellular necrosis in the pancreas.

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