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Attenuation of oxidative stress in Alloxan-induced diabetic rabbits by ethanolic extract of the fruit of *Lagenaria siceraria*

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ABSTRACT

Diabetes is a widespread and devastating disease. It has been estimated that the number of people affected with diabetes in the world will increase to 300 million by 2025. It is associated with several mechanisms, one of which is oxidative stress. Oxidative stress plays an important role in the pathogenesis and the complications of diabetes. Hyperglycemia results in overproduction of oxygen free radicals, which contributes to the progression of diabetes. Thus, the present study was investigated on antidiabetic activity and amelioration of oxidative stress by Lagenaria siceraria fruit extract in alloxan- induced diabetic rabbits. Diabetes was induced in rabbits by administration of alloxan monohydrate (150mg/kg, i.p). The ethanolic extract of the fruit of Lagenaria siceraria (EELS) at a dose of 100, 200 and 400mg/kg of body weight were administered at single dose per day to diabetes induced rabbits for a period of 14 days. The result of present study showed that significant antidiabetic activity against alloxan induced diabetes by decreasing the levels of fasting blood glucose in a dose dependent manner. Data also showed that EELS possessed strong antioxidant activity, which was conformed by decreasing the activities of lipid peroxidation and increased enzymic and non-enzymic antioxidants. From the above results, it is concluded that ethanolic extract of the fruit of Lagenaria siceraria possesses significant antidiabetic and antioxidant effects in alloxan induced diabetic rabbits.

Keywords: Diabetes mellitus, oxidative stress, hyperglycemia, free radicals, antioxidants, diabetic complications.

INTRODUCTION

Oxidative stress is a consequence of the imbalance between reactive oxygen species (Pro-oxidants) production and antioxidant capacity. This can occur as a result of either heightened ROS generation, impaired antioxidant system, or a combination of both. In the presence of oxidative stress, uncontained ROS attack, modify, and denature functional and structural molecules leading to tissue injury and dysfunction [1]. Oxidative stress has been associated with many diseases such as atherosclerosis, diabetes, chronic kidney disease, hypertension, rheumatoid arthritis, epilepsy, alzheimer's disease, Parkinsons' disease and cancer as well as aging [2, 3, 4, 5, 6, 7, 8, 9].

Diabetes mellitus is a metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both [10]. Hyperglycemia results in overproduction of oxygen free radicals, which contributes to the progression of diabetes. Free radicals may play an important role in the causation and complications of diabetes mellitus [11]. In diabetes mellitus, alterations in the endogenous free radical scavenging defense mechanisms may lead to ineffective scavenging of reactive oxygen species, resulting in oxidative damage and tissue injury. Oxidative stress is currently suggested as a mechanism underlying diabetes and diabetic complications [12].

Free radicals are continually produced in the body as a result of normal metabolic processes and interaction with environmental stimuli. Thus, to protect the cells and oxygen system of the body against reactive oxygen species, humans have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of

components, both endogenous and exogenous in origin, that function interactively and synergistically to utilize free radicals [13].

Enhanced oxidative stress and changes in antioxidant capacity, observed in both clinical and experimental diabetes mellitus, are thought to be the etiology of chronic diabetic complications [14]. The presence of diabetes mellitus confers increased risk of many devastating complications such as cardiovascular diseases (CVD), peripheral vascular disease (PVD), complications such as coronary artery disease (CAD), stroke, neuropathy, renal failure, retinopathy amputations and blindness [15, 16]. Several drugs such as oral hypoglycemic agents and insulin preparations are presently available to reduce hyperglycemia in diabetes mellitus. The main disadvantages of the currently available drugs are that, they have to be given throughout the life and produce side effects.

Resmi C.R et al., 2001 demonstrated Ayurvedic medicines are considered to be less toxic and free from side effects compared to synthetic drugs [17]. Since ages, medicinal plants and their bioactive constituents are used for the treatment of diabetes mellitus throughout the world [3]. Still more numbers of herbs are needed to be screened for their antioxidant property for the management of diabetes mellitus. One such plant is Lagenaria siceraria. Lagenaria siceraria (LS) has been used since ancient times in Indian folklore medicine for its many medicinal properties [18].

Lagenaria siceraria (Molina) Standley syn. L. leucantha Rusby; L. Vulgaris Ser. (Family: Cucurbitaceae) is commonly known as Bottle gourd (english) and lauki (Hindi), an excellent fruit in the nature having composition of all the essential constituents that are required for normal and good health of humans [19]. The plant is widely available throughout India. It is a climbing or trailing herb, with bottle- or dumb-bell shaped fruits. Both its aerial parts and fruits are commonly consumed as a vegetable. Traditionally, it is used as medicine in India, China, European countries, Brazil, Hawaiian island etc [20].

The plants contain tri terpenoid, cucurbitacins, flavones, C-glycosides, β-glycosides, vitamin-B, and ascorbic acid in fruits. It has been reported as cardio tonic, hepato protective, immunomodulatory, antihyperglycemic, antihyperlipidemic, analgesic and anti-inflammatory, antibacterial, diuretic, aphrosidiac, adaptogenic, antistress and anti anorectic [21, 22, 23, 24]. Keeping the above information in view, the present study was designed to investigate the antidiabetic potential and in-vivo antioxidant activity of ethanolic extract of the fruit of Lagenaria siceraria in alloxan induced diabetic rats.

EXPERIMENTAL SECTION

Drugs and Chemicals: All the chemicals and solvents were of analytical grade and were procured from S.D Fine-Chem Ltd, Mumbai, India. Alloxan monohydrate was procured from SRL Pvt. Ltd, Mumbai. Insulin was purchased from Hetero healthcare Ltd, India.

Plant materials: The fruit of Lagenaria siceraria was obtained from a local vegetable market in Udaipur. The samples were botanically authenticated. Voucher specimens were deposited for future reference.

Preparation of plant extract: The preparation of the extract of the fruit of Lagenaria siceraria was done in the Department of Pharmacology, Geetanjali Medical College, Udaipur. The dried, coarsely powdered plant material was extracted with 70% ethanol at 60°C for 24 hrs using a Soxhlet apparatus.

Preliminary Phytochemical screening: The freshly prepared ethanolic extract of the fruit of Lagenaria siceraria was subjected to preliminary phytochemical screening for detection of major chemical constituents [25].

Experimental animals:
Laboratory breed Newzealand white rabbits of either sex, weighing 1000-1500gm and Wistar albino rats weighing 150-180gm were used for the study. All the animals were procured from Animal House of Geetanjali Medical College, Udaipur. The animals were given free accesses to food and water. The protocol was approved by the IAEC, GMCH, Udaipur.

Acute Toxicity Study: Acute toxicity study of ethanolic extract of the fruit of Lagenaria siceraria (EELS) was determined in wistar albino rats (150-180 gm) according to the OECD guidelines No.425 [26].

Experimental design:
The rabbits were randomly divided into six groups of six animals in each. Out of 6 groups, five were made diabetic with a single dose of alloxan monohydrate (150mg/kg b.w.) infused via the ear vein of rabbit [27]. After three days

blood glucose level of surviving rabbits was measured and rabbits with blood glucose levels between 250-300 mg/dl were used for further study [28, 29].

Fasting blood glucose level and oxidative parameters of all the rabbits were determined before the start of the experiment. Rabbits were divided into the following groups –

Group 1: Normal untreated rabbits
Group 2: Diabetic control rabbits
Group 3: Diabetic rats treated with EELS [100 mg/kg/ once a day, daily]
Group 4: Diabetic rats treated with EELS [200 mg/kg/ once a day, daily]
Group 5: Diabetic rats treated with EELS [400 ml/kg/ once a day, daily]
Group 6: Diabetic rats treated with Insulin [5 Units/ kg/ once a day, daily]

The study of test compound was dissolved in distilled water and administered to rabbits by gastric oral tube and insulin was given subcutaneous [30]. The study period was carried out for 14 days, fasting blood glucose and oxidative parameters of the animals were determined on 1^{st}, 7^{th} and 14^{th} days. Blood was collected in heparinized vial and in plain vial for hemolysate preparation and for serum separation respectively.

ASSESSMENT OF ANTIDIABETIC ACTIVITY:
Blood glucose analysis: The rabbits were fasted overnight and the glucose level in the blood was measured by enzymatic GOD-POD method (Trinder P, 1969) by using glucose diagnostic kit (M/S Excel Diagnostics Pvt. Ltd.) [31].

DETERMINATION OF ANTIOXIDANT ACTIVITY:
1. Oxidative parameters
Estimation of MDA: The extent of lipid peroxidation was estimated by using the thiobarbituric acid method (Buege et al; 1978). The absorbance was measured using spectrophotometer at 532nm [32].

2. Antioxidant parameters
Superoxide dismutase activity: Superoxide Dismutase (SOD) activity was determined spectrophotometer by a method described by Misra and Fridovich (1972). The optical density was measured at 480nm [33].

Catalase: Erythrocyte catalase activity was assessed following Sinha (1972). This method is based on the decomposition of hydrogen peroxide by catalase. The decrease in absorbance at 620 nm was measured at room temperature. The catalase activity was expressed in micromoles of enzyme per milligram of haemoglobin [34].

Reduced glutathione: Reduced glutathione (GSH) concentration measurements were done according to Beutler and Kelly, 1963. Optical density was measured at 412 nm. Reduced glutathione values are expressed in mg/dl of whole blood [35].

STATISTICAL ANALYSIS:
The data was expressed as Mean ± S.D, (n=6). Data were analyzed using One way analysis of variance (ANOVA) followed by Dunnette's multiple comparisons test using SPSS computer package. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Preliminary phytochemical screening:
The phytochemical screening of Lagenaria siceraria showed that the presence of carbohydrates, proteins, alkaloids, amino acids, steroids, glycosides, flavonoids, saponins, terpenoids, tannins, vitamin C (Ascorbic acid), polyphenolic compounds.

The effects of Lagenaria siceraria on oxidative stress in alloxan-induced diabetic rabbits were evaluated by recording changes in serum glucose levels and in-vivo antioxidant levels.

Effect of EELS on blood glucose level:
A marked rise in fasting blood glucose level was observed in alloxan treated groups as compare to normal control. Whereas, treatment with EELS (100, 200 and 400mg/kg, p.o) caused significant reduction in the levels of fasting blood glucose in a dose dependent manner. The antihyperglycemic effect of ethanol extract at was found similar effective to the reference standard, Insulin (Table-1).

Table – 1: Effect of EELS on fasting blood glucose levels of alloxan-induced diabetic rabbits

Gr. No.	Group	Blood Glucose (mg %)		
		0' Day	7th Day	14th day
I	Control	91.83 ± 9.84	89.5 ± 6.95	91.33 ± 6.05
II	Diabetic control	264.3 ± 10.78	288.8 ± 13.92	292.22 ± 15.03
III	Std. (Insulin)	255.7 ± 17.44	118.5 ± 7.66**	96.17 ± 8.90***
IV	Alloxan + EELS (100mg/kg)	260 ± 17.85	161.2 ± 11.36**	112.3 ± 6.53***
V	Alloxan + EELS (200mg/kg)	282.5 ± 32.84	152.2 ± 40.13***	97.5 ± 6.22***
VI	Alloxan + EELS (400mg/kg)	253.7 ± 14.84	120.3 ± 8.61***	81.67 ± 9.81***

*Values are expressed as mean ±SD, (n=6). One way analysis of variance (ANOVA) followed by Dunnette's multiple comparisons test. * P<0.05, **P<0.01, ***P<0.001 when compared with positive control group.*

Table – 2: Effect of EELS on Malondialdehyde (MDA) or lipid peroxidation levels in alloxan-induced diabetic rabbits

Gr. No.	Group	MDA (nmol/ ml)		
		0' Day	7th Day	14th day
I	Control	2.93 ± 0.16	2.98 ± 0.14	2.96 ± 0.22
II	Diabetic control	5.63 ± 0.816	7.83 ± 0.78	8.75 ± 0.63
III	Std. (Insulin)	5.5 ± 0.33	6.38 ± 0.44**	7.38 ± 0.81*
IV	Alloxan + EELS (100mg/kg)	5.41 ± 0.35	6.81 ± 0.55*	4.85 ± 0.54***
V	Alloxan + EELS (200mg/kg)	5.67 ± 0.37	4.9 ± 0.48**	4.26 ± 0.58***
VI	Alloxan + EELS (400mg/kg)	5.2 ± 0.50	3.54 ± 0.56***	3.03 ± 0.36***

*Values are expressed as mean ±SD, (n=6). One way analysis of variance (ANOVA) followed by Dunnette's multiple comparisons test. * P<0.05, **P<0.01, ***P<0.001 when compared with positive control group.*

Table – 3: Effect of EELS on Superoxide dismutase (SOD) levels in alloxan-induced diabetic rabbits

Gr. No.	Group	SOD (U/ ml)		
		0' Day	7th Day	14th day
I	Control	2.48 ± 0.39	2.56 ± 0.45	2.53 ± 0.32
II	Diabetic control	2.01 ± 0.16	1.45 ± 0.25	1.16 ± 0.23
III	Std. (Insulin)	2.09 ± 0.26	1.65 ± 0.21	1.41 ± 0.29
IV	Alloxan + EELS (100mg/kg)	2.05 ± 0.32	1.47 ± 0.45	1.65 ± 0.39*
V	Alloxan + EELS (200mg/kg)	2.11 ± 0.29	1.86 ± 0.31*	2.05 ± 0.23**
VI	Alloxan + EELS (400mg/kg)	2.31 ± 0.33	1.96 ± 0.28**	2.63 ± 0.34***

*Values are expressed as mean ±SD, (n=6). One way analysis of variance (ANOVA) followed by Dunnette's multiple comparisons test. * P<0.05, **P<0.01, ***P<0.001 when compared with positive control group.*

Table – 4: effect of EELS on Catalase (CAT) in alloxan-induced diabetic rabbits

Gr. No.	Group	CAT (µmol H2O2/ min/ mg)		
		0' Day	7th Day	14th day
I	Control	115.8 ± 9.17	112.7 ± 7.77	108.7 ± 8.86
II	Diabetic control	91.83 ± 6.90	60.25 ± 4.33	53.28 ± 6.72
III	Std. (Insulin)	94.33 ± 5.60	64.12 ± 3.73	58.06 ± 7.35
IV	Alloxan + EELS (100mg/kg)	97.17 ± 6.67	69.9 ± 7.09***	82.67 ± 8.64***
V	Alloxan + EELS (200mg/kg)	105 ± 5.83	78.5 ± 6.25**	94.67 ± 8.06***
VI	Alloxan + EELS (400mg/kg)	103.1 ± 8.31	90.83 ± 7.70***	117.3 ± 9.41***

*Values are expressed as mean ±SD, (n=6). One way analysis of variance (ANOVA) followed by Dunnette's multiple comparisons test. * P<0.05, **P<0.01, ***P<0.001 when compared with positive control group.*

Table – 5: Effect of EELS on Reduced glutathione (GSH) in alloxan-induced diabetic rabbits

Gr. No.	Group	GSH (mg/ dl)		
		0' Day	7th Day	14th day
I	Control	58.78 ± 3.99	56.9 ± 4.57	56.44 ± 3.30
II	Diabetic control	43.35 ± 3.72	32.13 ± 3.33	26.65 ± 1.72
III	Std. (Insulin)	45.17 ± 5.26	38.83 ± 6.55	30.26 ± 4.23
IV	Alloxan + EELS (100mg/kg)	40.5 ± 5.57	43.17 ± 5.03**	46.5 ± 5.50**
V	Alloxan + EELS (200mg/kg)	44.5 ± 3.48	46.25 ± 4.94**	51.88 ± 3.76***
VI	Alloxan + EELS (400mg/kg)	46.39 ± 6.48	50.27 ± 5.68***	57.47 ± 5.10***

*Values are expressed as mean ±SD, (n=6). One way analysis of variance (ANOVA) followed by Dunnette's multiple comparisons test. * P<0.05, **P<0.01, ***P<0.001 when compared with positive control group.*

Effect of EELS on Antioxidant activity:

Antioxidant status was estimated by determining the activities of superoxide dismustase (SOD), catalase (CAT), malondialdehyde (MDA) and reduced glutathione (GSH) levels. Oxidative stress was characterized by increased lipid peroxidation and/or altered non-enzymatic and enzymatic antioxidant systems. The erythrocyte antioxidant activity of SOD, CAT and GSH significantly decreased, while plasma MDA (lipid peroxidation) significantly increased in the alloxan-treated, group of rabbits. The control group of rabbits maintained optimal value activity of

the antioxidants studied. Administration of Lagenaria siceraria significantly decreased the elevated MDA, and also significantly increased the reduced enzymatic and non-enzymatic antioxidants activities. The diabetic animals treated with insulin alone showed no significant change in the levels of SOD, CAT, MDA and GSH. The results are shown in the Table - 2, 3, 4 and 5.

DISCUSSION

Alloxan (2,4,5,6-tetraoxypyrimidine; 2,4,5,6- pyrimidinetetrone) is an oxygenated pyrimidine derivative and was originally isolated in 1818 by Brugnatelli and got its name in 1838 by Friedrich Wöhler and Justus von Liebig [36]. This is one of the usual substances used for the induction of diabetes mellitus apart from streptozotocin.

Alloxan acts as a cytotoxin for beta-cells of the islet of langerhans, causes diabetes by inducing cell necrosis [37, 38]. The Reactive Oxygen Species (ROS) mediates the cytotoxic action with the increase in cytosolic calcium concentration, leading to rapid beta-cells destruction [39]. This results into decreased insulin secretion and elevated blood glucose level [40]. This causes an insulin-dependent diabetes mellitus (called "Alloxan Diabetes") in these animals, with characteristics similar to type-1 diabetes in humans [36].

Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS) which are formed under normal physiological conditions but become deleterious when not being quenched by the antioxidant systems [41]. There are convincing experimental and clinical evidences that the generation of reactive oxygen species is increased in both types of diabetes and that the onset of diabetes is closely associated with oxidative stress [42, 43].

Free radicals are formed disproportionately in diabetes by glucose autoxidation, polyol pathway and non-enzymatic glycation of proteins [44]. Abnormally high levels of free radicals and simultaneous decline of antioxidant defense systems can lead to the damage of cellular organelles and enzymes, increased lipid peroxidation and development of complications of diabetes mellitus [45].

The increase in the level of ROS in diabetes could be due to their increased production and/ or decreased destruction by nonenzymic and enzymic catalase (CAT), reduced glutathione (GSH), and superoxide dismutase (SOD) antioxidants. The level of these antioxidant enzymes critically influences the susceptibility of various tissues to oxidative stress and is associated with the development of complications in diabetes [46]. In the present study, ethanolic extract of the fruit of Lagenaria siceraria has been evaluated for antidiabetic and antioxidant activity in rabbits.

From the results obtained, it is evident that the diabetic rabbits had much higher levels of fasting blood glucose, malondialdehyde and decreased levels of superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) when compared with normal control rabbits. These adverse changes were reversed to near normal values in ethanolic extract of the fruit of Lagenaria siceraria treated. The possible mechanism by which ethanol extract brings about its hyperglycemic action may be by induction of pancreatic insulin secretion from β cells of islets of langerhans or due to enhanced transport of blood glucose to peripheral tissue [47]. Earlier many plants have been studied for their hypoglycemic and insulin release stimulatory effects [48, 49, 50, 51, 52, 53, 54, 55]. It is well known that CAT, SOD and GSH play an important role as protective enzymes against free radical formation in tissues [56].

Enzymatic antioxidant such as SOD and CAT are considered primary enzymes since they are involved in the direct elimination of ROS [57]. SOD is an important defense enzyme and scavengers O_2 anion from H_2O_2 and hence diminishes the toxic effects due to this radical or other free radicals derived from secondary reaction [58]. CAT is a haemoprotein, which catalyzes the reduction of hydrogen peroxides [59]. The antioxidant enzymes such as SOD and CAT are known to be inhibited in diabetes mellitus as a result of non- enzymatic glycosylation and oxidation [60].

GSH is by far the most important antioxidant in most mammalian cells. This ubiquitous tripeptide, γ-Glu-Cys-Gly, performs many cellular functions. In particular, the thiol containing moiety is a potent reducing agent [61]. GSH has the important function of destroying reactive oxygen intermediates and free radicals that are constantly formed in metabolism [62].

In the present study, the activities of SOD, CAT and GSH decreased in diabetic rabbits as reported earlier, which could be due to inactivation caused by alloxan generated ROS [63]. The ethanol extract of Lagenaria siceraria had reversed the activities of these enzymatic and non-enzymatic antioxidants, which might be due to decreased oxidative stress as evidenced by decreased lipid peroxidation. The preliminary phytochemical investigation of the

EELS revealed the presence of alkaloids, flavonoids, saponins, cardiac glycosides, triterpenoids, phenolic compounds and tannins. However, glycosides, flavonoids, tannins, organic sulphur compounds, catechol and alkaloids are active ingredients of hypoglycemic plant [64]. Flavonoids are reported to regenerate the damaged pancreatic beta cells [65]. Phenols have found to be effective antihyperglycemic agents [66]. Hence, it is worthwhile to isolate the bioactive principles which are responsible for theses activities, which is in progress in our laboratory.

CONCLUSION

In conclusion, the present study has shown that the ethanolic extract of the fruit of Lagenaria siceraria have antidiabetic and antioxidant effects. Also, data of this study indicates that Lagenaria siceraria can either increase antioxidant power or reduce the oxidative stress or both. The active principle (s) in the extracts may have better performance isolated and purified form.

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