ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Antioxidative Role of *Costus pictus* D.DON in Hyperglycemic Rats administered with Alloxon

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Abstract

The present study focuses on the role of *Costus pictus* extract (CPE) against the oxidative stress caused by hypeglycemia in liver and kidney of alloxon administered rats.Hyperglycemic rats were administered with two dose levels(200 and 400mg/kg body weight) of CPE for 30 days.At the end of treatment,blood glucose,serum marker enzymes alanine amino transfrease(ALT),aspartate amino transferase(AST),lactate dehydrogenase (LDH) and alkaline phosphatase(ALP) were estimated.Antioxidant enzymes glutathione peroxidase(GPx),glutathione-S-transferase(GST),catalase(CAT),superoxide dismutase(SOD) and reduced glutathione(GSH) and lipid peroxidation product malondialdehyde(MDA) were estimated in the liver and kidney of rats.The level of serum marker enzymes were significantly elevated in alloxon treated hyperglycemic rats whereas a significant reduction of antioxidant enzymes and reduced glutathione in liver and kidney was noted in hyperglycemic rats. On the other hand, administration of *C. pictus* extract caused a considerable protection against the oxidative stress which was evident from the reversal of marker enzymes to control level.Similarly,antoxidant enzymes also exhibited marked recovery to the control level.Increased reduced glutathione and reduction in lipid peroxidation were also evident following administration of CPE.This clearly shows that CPE by virtue of its remarkable antioxidant potential protected the liver and kidney against the hyperglycemic oxidative stress.

Key words:*Costus pictus*,antioxidant enzymes,hyperglycemia,liver,kidney,oxidative stress.

INTRODUCTION

Diabetes mellitus is a major public health problem globlly.Recent report of WHO estimates that in 2030 the number of diabetic cases will rise from 366 million in 2011 to 552 million, if no immediate action is taken.(http://www.idf.org/media-events/press-releases/2011/diabetes-atlas-5th-edition) .India with 25 million diabetic incidences will touch 57 million by 2025, the highest number of diabetes in the world [1,2].The hall mark clinical feature of diabetes is elevated blood glucose level, a state of hyperglycemia which is due to reduced insulin production because of loss of insulin-producing pancreatic beta cells(Type I) or lack of responsiveness of target body muscles and adipose tissue to the insulin(Type II)[3].Both Type I and Type II diabetes exhibit severe chronic hyperglycemia in patients,resulting in increased oxidative stress which is a condition of excess formation of free radicals and insufficient removal of highly reactive molecules such as reactive oxygen species(ROS) and reactive nitrogen species(RNS)[4,5].Oxidative stress in hyperglycemia is induced by glucose autoxidation,formation of advanced glycation end products and activation of the polyol pathway.Increased free fatty acids and leptin also contribute to increased reactive species generation[6].This multiple generation of free radicals increases oxidative stress in vital organs[7].

Hyperglycemia caused oxidative stress primarily contribute to the development and progression of diabetes related health complications such as cardiovascular disease,cerebrovascular defect,renal failure,retinopathy,neurological complications and premature death[8].Patients with diabetes often show increased levels of markers of free radical induced damages, but also have reduced antioxidant defense[9-12].There are strong evidences that free radical induced oxidative stress is the major cause for the aggravation of diabetes and onset of diabetic related complications[13-15]. Hyperglycemia caused oxidative stress invariably affects hepatic and renal functions in multiple ways ,primarily by altering the antioxidant defence mechanisms [16-20]. Ameliorating oxidative stress through treatment with antioxidants might be effective strategy to reduce the intensity of the diabetic complications[7,21-23], besides normalizing hyperglycemia.

Drugs recommended for diabetic are known to cause side effects[24] and there has been a search for new class of compounds with potent hypoglycemic and antioxidant properties.Use of plant extracts and phytochemicals with known antioxidant,antidiabetic and antihyperlipidemic properties are of immense importance in the therapeutic treatment of diabetes and related complications[25-26].Ethnobotanical information indicates that more than 800 plants are used as traditional remedies for the treatment of diabetes[27].In the present study,*Costus pictus*,commonly known as insulin plant with antidiabetic activity has been chosen.Antidiabetic effect of *C.pictus* has been experimentally proved by several workers[28-30].However,studies on its antioxidative role in the protection against hyperglycemic oxidatative stress caused complications in vital organs are meager. The present study focuses on the antioxidative role of *C. pictus* to prevent hyperglycemic oxidative stress on liver and kidney.

MATERIALS AND METHODS

Herbal Extract:Fresh *C. pictus* leaves collected from SASTRA University herbal garden were shade dried and pulverised .The powdered materials were extracted with ethanol solvent in a soxhlet apparatus for 24 hrs.The extract(CPE) was concentrated in rotary vacuum evaporator and refrigerated.A voucher specimen of the plant has been submitted with herbarium in Center for Advanced Research in Indian System of Medicine(CARISM), SASTRA University for future reference(CARISM: 102).

Chemicals: All the chemicals used were AR grade and purchased from Sigma Aldrich and Himedia Ltd,Mmubai .

Experimental Protocol: Experiments were conducted in the Central Animal Facility in SASTRA University.Male wistar rats weighing between 150 and 175 g were used. They were maintained in polythene cages and provided with pelleted feed and water *ad libitum*.All the experimental protocols were approved by the institutional ethical committee of CPCSEA.Rats were divided into 5 groups(I-V) with 6 animals per group as given below.

Group I:Control rats received only saline intraperitoneally and fed with pelleted diet.

Group II:Administered with 120mg/kg body weight of Alloxon in saline intraperitoneally.

Group III:Alloxon administered rats (6) received oral administration of Glibenclamide(5mg/kg body weight).

Group IV:Alloxon treated rats were given 200mg/kg body weight of CPE orally

Group V: Alloxon treated rats were administered with 400mg/kg body weight of CPE orally.

Rats exhibited hyperglycemia (275-300mg glucose/dl) after 72 hrs of alloxon administration were selected for herbal extract (CPE) administration. The treatment was continued for 30 days.At the end of the experiment, rats were anesthetized by CO_2 asphyxiation and blood samples were collected from retro orbital vein.Serum was separated for biochemical assays. Liver and kidney tissues were excised quickly, washed with physiological saline and frozen for use.Tissue homogenate was prepared in 0.1M Tris-HCl buffer(pH 7.4) and used for the determination of lipid peroxides (LPO), reduced glutathione (GSH), glutathione peroxidase(GPx),glutathione-S-tranferase (GST), catalase (CAT) and superoxide dismutase(SOD).

Serum biochemical assays: Serum was used for the estimation of cardio marker enzymes such as alanine aminotransferase(ALT),aspartate aminotransferase(AST), alkaline phosphatase(ALP),and lactate dehydrogenase(LDH) using Biosystem diagnostic kits(Barcelona,Spain).

Assay of lipid peroxidation: Tissue homogenate was used for the estimation of lipid peroxidation follwing the method described by Ohkawa *et al* [31] in which malondialdehyde(MDA) released was used as the index for lipid peroxidation. In brief, to 0.2 ml of tissue homogenate,0.2 ml of 8.1% SDS,1.5 ml of 20% acetic acid and 1.5 ml of 0.8% thiobarbituric acid(TBA) were added.The volume was made upto 4 ml with distilled water and incubated in a water bath at 95.5^0 C for an hour.The contents were cooled and 1 ml of water and 5 ml of n-butanol/pyridine mixture were added and shaken vigorously. The contents were centrifuged and the organic layer was separated for measurement of absorbance at 532 nm.The level of lipid peroxides was expressed as nmoles of MDA formed/mg of protein.

Reduced glutathione(GSH): GSH was estimated in the tissue homogenate[32].Briefly,0.1 ml of tissue homogenate was precipitated with 5% trichloro acetic acid and centrifuged to separate the protein.To 0.1 ml of the supernatant,0.2 ml of 0.6 mM DTNB{5,5 dithiobis(2-nitrobenzoic acid)} reagent and 0.2 M phospahate buffer(pH 8.0) were added to a final volume of 4.0 ml.The absorbance was measured at 412 nm.The amount of glutathione was expressed as nmoles/g tissue.

Glutathione peroxidase(GPx): GPx estimation was done [33] by adding 0.2 ml of 0.8 mM EDTA ,0.2 ml of reduced glutathione, and 0.4 ml of 0.4 M phosphate buffer (pH 7.0) to 0.2 ml of tissue homogenate .The contents were incubated at 37^0 C for 10 minutes.The reaction was stopped by adding 0.5 ml of 10% TCA and centrifuged at 200 rpm.To the supernatant 3.0 ml of 0.3 mM disodium hydrogen phosphate and 1.0 ml of 0.04% DTNB were added.The color developed was measured at 420 nm immediately.The activity of GPx was expressed as nmoles of glutathione oxidised/min/mg protein.

Glutathione-S-transferase(GST): Assay of GST was done by the method of Habig *et al.,* [34].Briefly,0.1 ml of tissue homogenate was reacted with 1.0 ml of 0.3M phosphate buffer(pH 6.5),1.7 ml of water and 0.1 ml 30 mM CDNB(1-chloro-2,4-dinitrobenzene) by incubating at 37^0 C for 15 minutes. After incubation,0.1 ml of GSH was added and change in absorbance was measured at 340 nm for 3 minutes at an interval of 30 seconds.GST activity was expressed as units/min/mg protein.

Catalase(CAT): CAT activity was measured as follows[35].To 1.2 ml of 50Mm phosphate buffer(pH 7.0) 0.2 ml of the homogenate was added and the reaction was started adding 1.0 ml of 30 mM H_2O_2 solution.The decrease in absorbance was measured at 240 nm at 30 seconds intervals for 3 minutes.Enzyme activity was expressed as nmoles/min/mg protein.

Superoxide dismutase(SOD): To 1.0 ml of homogenate,0.75 ml of ethanol and 0.15 ml of chilled choloroform were added and centrifuged.To 0.5 ml of supernatant, 0.5 ml of 0.6 mM EDTA solution and 1 ml of 0.1 M carbonate –bicarbonate (pH 10.2) buffer were added.The reaction was initiated by adding 0.5 ml of 1.8 mM epinephrine and the increase in absorabance was read at 480 nm.One unit of the SOD activity was the amount of protein required to give 50% inhibition of epinephrine auoxidataion[36].

Statistical analysis: Statistical analysis of the results was done by one way analysis of variance (ANOVA) using GraphPad Prism 5 software ,followed by Dunnet's comparison test for significance .Significance was set at $p<0.05$ and $p<0.01$.Results are presented as Mean± S.E .

RESULTS AND DISCUSSION

Alloxon administration has caused significant elevation of glucose when compared to control rats.The elevation is over 301% than the normal rats,indicating severe hyperglycemia.Alloxon is known to destroy beta cells of the islets of Langerhan and responsible for reduced insulin secretion,thereby inducing hyperglycemia [37].In the present study,following alloxon treatment antioxidant enzymes,glutathione peroxidase,glutathione S-transferase,superoxide dismutase and catalase were reduced to significant level in the liver and kidney tissues when compare to normal rats(Table:1&2).. Alloxon caused hyperglycemia has resulted in profound alterations in the level of lipid peroxidation end product MDA and glutathione content in the liver and kidney. MDA level was significantly elevated with concomitant reduction in glutathione content in hyperglycemic rats(Table:3). Elevated lipid peroxidation and the drastic alterations in the antioxidant enzyme status and marker enzymes indicate the severity of oxidative stress caused by the prolonged hyperglycemia in the liver and kidney of alloxon treated rats.

On the other hand , groups which received extract of *C. pictus* exhibited considerable protection against the oxidative stress. Glucose level in the CPE treated rats were not significantly altered but showed reversal to normal level through the end of treatment period. The extent of glucose rise is also marginal with only 54% in the CPE(400mg) treated rats when compared to the diabetic rats .The study reveals a trend of recovery of the altered antioxidant status in the CPE treated rats.GSH level was also not significantly altered in both kidney and liver of CPE treated rats ,indicating protection from oxidative stress.MDA level which showed a drastic elevation in alloxon treated rats ,however, was less affected in the CPE administrated groups.Similar protective effect of CPE on antioxidant enzymes GPx,GST,SOD and CAT was noted in the kidney and liver tissues.

In addition, marker enzymes such as ASL,ALT,ALP and LDH were greatly elevated in the serum of hyperglycemic rats However, these enzymes(ASL,ALT,ALP and LDH) did not show significantl alterations in CPE administered rats.(Table:4).The findings indicate that simultaneous administration of CPE and alloxon didn't cause significant alteration in the antioxidant status ,an evidence for the protection offered by the phytoconstituents present in the *C. pictus* leaves.Non-significant change in the glucose level in CPE (400mg) treated rats is an evidence for hypoglycemic effect of the *C.pictus*

Chronic hyperglycemia has been associated with damage and dysfunction of many vital organs such as kidneys,liver,retina,heart,peripheral and central nervous system[38].Hyperglycemia caused oxidative stress as evident from increased lipid peroxidation,reduced glutathione and antioxidant enzymes has been widely reported in diabetic patients and experimental diabetic animals[39-40].As observed in the present study,diabetic hyperglycemia caused alterations in antioxidant status in liver and kidney were reported earlier[41,42].

Table 1: Shows the changes of antioxidant enzymes level in the liver of rats.

TREATMENT GROUPS	GPx	SOD	CAT	GST
GROUP I	5.87±1.07	6.26±1.78	75.17±2.77	7.53±1.23
GROUP II	3.87±1.11**	3.78±0.78**	44.17±2.57**	4.25±0.82**
GROUP III	4.65±0.98	5.45±0.78	59.78±1.96*	6.54±0.95
GROUP IV	4.27±1.23*	4.96±1.31*	61.33±2.54*	5.93±0.78 *
GROUP V	5.28±0.16	6.13±0.78	65.67±2.42	6.57±0.68

Values are Mean±SE(Number=6);Significance at ** p<0.05,*p<0.01;Group I Vs Groups II,III,IV&V;Values are expressed as GSH(nmol GSH oxidized/min/mg protein),SOD(mg/protein),CAT(nm H_2O_2 oxidised/min/mg protein),GST(U/mg protein/min)

Table 2: Shows the changes of antioxidant enzymes level in the kidney of rats

TREATMENT GROUPS	GPx	SOD	CAT	GST
GROUP I	5.56±0.26	9.12±0.49	66.17±0.57	5.96±0.85
GROUP II	3.48±0.31**	4.68±0.94 **	31.32±1.58**	3.27±0.56**
GROUP III	4.81±0.98*	7.43±1.21	49.26±1.15**	4.53±0.71 *
GROUP IV	4.11±0.55	6.72±0.76 *	47.37±1.43**	4.13±0.54*
GROUP V	4.46±0.17	7.56±1.76	52.27±1.49*	4.86±0.34

Values are Mean±SE(Number=6);Significance at ** p<0.05,*p<0.01;Group I Vs Groups II,III,IV&V;Values are expressed as GSH(nmol GSH oxidized/min/mg protein),SOD(mg/protein),CAT(nm H_2O_2 oxidised/min/mg protein),GST(U/mg protein/min)

Table 3:Shows the changes in the levels of GSH and LPO in liver and kidney

GROUP I	GROUP II	GROUP III	GROUP IV	GROUP V
LIVER				
GSH	38.83±1.32	20.83±1.95**	32.67±1.97*	30.33±2.78	33.18±2.31
LPO	1.53±0.35	4.83±0.87**	3.56±0.65**	3.92±0.88*	2.29±0.43
KIDNEY				
GSH	12.15±0.15	7.73±0.54**	10.32±1.21	9.29±1.76**	10.60±1.40
LPO	1.75±0.11	4.60±0.43**	3.56±0.76**	3.51±0.43**	2.95±0.15

Values are Mean±SE(Number=6);Significance at ** p<0.05,*p<0.01;Group I vs Groups II,III,IV&V;Values are expressed as GSH(nm GSH oxidized/min/mg protein) and LPO (nm MDA/mg protein).

Table 4:Shows the changes in marker enzymes level in the serum

TREATMENT GROUPS	AST	ALT	LDH	ALP
	---------- U/L ----------			
GROUP I	64.50±2.26	43.50±1.33	496.21±6.25	112.08±2.63
GROUP II	116.70±2.17**	71.57±3.42**	572.36±11.37**	172.62±1.93**
GROUP III	88.56±2.63**	59.25±2.22 **	473.53±8.43	131.47±2.12*
GROUP IV	95.67±1.89 *	61.33±1.76*	512.27±10.42*	134.43±1.87*
GROUP V	82.67±2.26*	52.50±1.70	493.23±9.12	128.32±2.15

Values are Mean±SE(Number=6);Significance at ** p<0.05,*p<0.01;Group I vs Groups II,III,IV&V

Liver, the major metabolic organ involved in maintenance of circulating glucose homeostasis was subjected to severe oxidative stress in diabetic rats which is evident from the alteration in the marker and antioxidant enzymes.Hepatocellular injury, cell death and liver fibrosis occur when ROS and RNS are generated in excess[43].ALT is a gluconeogenic enzyme and its alteration indicates impaired insulin signaling in diabetics[44].High levels of AST,ALT ,ALP and LDH might be due to leakage from damaged liver and kidney cytosol and mitochondria[45].

Increased MDA, an aldehyde product of lipid peroxidation in liver and kidney is another indication of hyperglycemia caused oxidative stress.MDA is more cytotoxic and stable than reactive oxygen species[46].Increased lipid peroxidation in the membrane of liver[42] and kidney[47] has been reported in diabetic cases.The pathogenesis of diabetic nephropathy has been attributed to the formationof AGEs and the mitochondrial production of ROS,leading to cell death and kidney dysfunction[48,49].

Reduced levels of ascorbate,glutathione and superoxide dismutase are a common feature in diabetics[50]. GSH is invloved in the conversion of H_2O_2 to water and in the reduction of lipid peroxides.Intracellular GSH is derived mainly from synthesis .The hepatocytes are highly specialized to synthesis GSH from its precursors or to recycle it from GSSH[51]. Glutathione besides functioning as direct free –radical scavenger acts as co-substrates for glutathione peroxidase and for many enzymes, and forms conjugates in endo –and xenobiotic reactions[52,53]. Glutathione peroxidase is important in cellular defense against wide variety of hydroperoxides[54].Liver and kidney are abundant in both selenium dependent GSH-Px and independent Gpx.GST is rich in liver microsomes.The defective antioxidant defense in this study might be due to free radical caused damages to the liver and kidney.

Therefore, it is highly imperative to intervene the free radical generation process and minimizing the oxidative stress either by increasing the endogenous antioxidant mechanism or by supplementing natural antioxidants. The primary defence against oxidative stress in the cell rests with antioxidants which act by directly scavenging the radicals,sustaining the activity of antioxidant enzymes or inhibiting the activity of oxidizing enzymes[55].

Imbalance in free radicals and antioxidant defense mechanism is usually resulting in disease development. A better option to prevent this adverse condition is to supplement the diet with natural herbal antioxidants[56].In recent years, antioxidant therapy is gaining popularity to combat oxidative stress in diabetic patients.Numerous studies have demonstrated that antioxidant vitamins and supplements can minimize the oxidative stress and lipid peroxidation in patients and experimental rats.Antidiabetic herbal plants have been extensively documented and reviewed[57].

Natural compounds such as terpenoids, alkaloids, flavanoids, phenolics ,etc have been shown to have antidiabetic potential[58].Medicinal plants with promising antioxidant potential were also reported extensively[59].Phytochemicals with antioxidant effects include cinnamic aacids, coumarins, diterpenes, flavonoids, lignans, monoterpenes, phenylprpanoids, tannins and triterpens[60].

Hypoglycemic role could also be the reason for reduced oxidative stress resulting in insignificant alteration of the antioxidant enzymes.Phytochemical studies(GC-MS) of the ethanol extracts of the leaves(unpublished data) showed the presence of high level of Vitamin E(16%) and delta tocopherol (18%)which are well known antioxidants. Several

studies reported Vitamin E as the major antioxidant involved in protecting diabetic complications and is more potent oxidative stress reducer. Baydas[61] reported reversal of lipid peroxidation and GPx in liver and kidney of diabetic rats administered with Vitamin E .The protection offered by the CPE against the oxidative hepato and renal toxicity in hyperglycemic rats can be attributed to the hypoglycemic potential associated with rich antioxidant properties.

CONCLUSION

The presents study sheds light on the potential role of *C.pictus* in ameliorating the hyperglycemic oxidative damage in the vital organs liver and kidney by virtue of its hypoglycemic and antioxidant potential.The herb, besides being an effective antidiabetic medicine may also play a major role in preventing oxidative diabetic complications.

ACKNOWLEDGEMENT

The authors wish to thank the Dean and Associate Dean ,School of Chemical and Biotechnology, SASTRA University,for the facility.

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