International Journal of Current Pharmaceutical Research

ISSN- 0975-7066 Vol 3, Issue 3, 2011

Research Article

BENEFICIAL EFFECTS OF COSTUS IGNEUS AND DOSE-RESPONSE STUDIES IN STREPTOZOTOCIN-INDUCED DIABETIC RATS

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Received: 06 May 2011, Revised and Accepted: 27 May 2011

ABSTRACT

Herbal medicines could be the natural answer to some ailments and can often be readily available. For these reasons, they are growing in popularity in wealthy countries and their use remains widespread in developing regions. Botanical ingredients in dietary supplements and traditional herbal medicines contain bioactive constituents with potential health benefits. Here aqueous (AEC), methanolic (MEC) and ethanolic (EEC) extracts of leaves of the plant Costus igneus (C. igneus) were screened for their phytochemical constituents and free radical scavenging activity. Blood glucose lowering effect of three extracts were checked and also the activities of hexokinase and glucose-6-phosphate dehydrogenase were estimated. Concentration of MDA, GSH and activities of catalase, glutathione peroxidase and glutathione reductase were evaluated in diabetic treated rats. MEC being most potent extract among three, a dose dependent study was conducted with MEC (25, 50, and 100 mg/kg BW) on hypoglycemic and antioxidant activities and MEC at a dose of 100 mg/kg BW exerted maximum beneficial effects. MEC was tested for toxic effects also and MEC at a dose of 250 mg/kg BW exerted toxicity by elevating the levels of serum glutamate oxaloacete transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), acid phosphatase (ACP) and alkaline phosphatase (ALP).

Keywords: Costus igneus, AEC, MEC, EEC.

INTRODUCTION

Many herbal medicines possess antioxidant properties, which play an important role in therapeutics[1,2]. Recent studies have found that reactive oxygen species (ROS) have been incriminated in the pathogenesis of both acute and chronic heart disease. An imbalance between oxidative stress and antioxidative defense mechanisms in diabetics can result in cell and tissue damage and accelerate diabetic complications. Administration of appropriate antioxidants could prevent or retard diabetic complications to some extent[3].

Previous reports suggest that Costus igneus (C. igneus) leaf extract exerts antidiabetic and hypolipidemic effects in diabetes induced rats[4]. However, reports of patients experiencing negative health consequences caused by the use of herbal medicines are also on the rise. Hence it is inevitable to conduct dose-response studies to reveal the potency and efficacy of any drug or plant material before it can be treated as a remedy for any disease. As a first step towards exploring antidiabetic and antioxidant activities in the plant material, we used C. igneus leaves in water, methanol and ethanol each containing 100 mg/kg BW. Antidiabetic and antioxidant activities were studied to assess most beneficial of the three extracts. A dose response study was performed to determine the most effective dose. Toxicity studies were carried out by analyzing urea, uric acid and creatinine in blood and assaying SGOT, SGPT, ACP and ALP. Histopathology of liver and kidney was also studied at highest doses.

MATERIALS AND METHODS

Plant material

C. igneus was collected from Konni, Pathanamthitta, Kerala, India. The plant was identified and authenticated by Dr. G. Valsaladevi, Curator, Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram, India. A voucher specimen was deposited at Department of Botany, University of Kerala, Kariavattom, Thiruvananthapuram, India (Voucher No. KUBH 5791).

Preparation of plant extracts (Cold extraction)

Extraction and preparation of crude extracts was carried out by cold percolation method at room temperature and by solvent evaporation. This helps in protection of any heat labile metabolite present in it.

Aqueous extract: Fresh leaves of C. igneus were washed and the clean leaves were ground. The ground material was immersed in distilled water and kept in the cold room overnight. After one day, it was filtered through cheese cloth, the filtrate was centrifuged. The supernatant was lyophilized and the powdered material was used as aqueous extract which was light green in colour. Yield of AEC obtained from 100 g leaves was 0.78 g. The extract obtained was stored in a closed container in the refrigerator at 4^{o}C until required.

Methanolic extract: Extraction was performed in 80% methanol. It was kept in cold room overnight. After centrifugation at 3000 rpm for 15 min the extract was treated with petroleum ether. Methanol was then removed via rotavapour (Speed Vac) at 40-50^{0}C under reduced pressure. It was then dried in a desiccator to get a brown sticky compound. Extracted yields were determined from the original weight of the ground sample before extraction over the weight of extract following rotoevaporation. 100 g fresh leaves yielded 1.681 g extract.

Ethanolic extract: Washed leaves of C. igneus were ground with 100% ethanol and it was kept in cold room overnight. Filtered and centrifuged. From the supernatant lipid material was removed by hexane. The extract was then concentrated in a vacuum evaporator. After complete evaporation, the concentrated material was obtained which is dried in desiccators. Yield obtained from 100 g leaves was 1.03 g.

Screening of phytochemicals

1 g of the plant extract was dissolved in 100 ml of its own mother solvents to obtain a stock of concentration 1% (v/v). The extracts thus obtained were subjected to preliminary phytochemical screening based on methodos described previously[5,6].

Radical scavenging activity (in-vitro)

Radical scavenging activity of AEC, MEC and EEC was compared with BHT by in-vitro studies.

Chemicals

All the chemicals used were high quality analytical grade reagents. Streptozotocin was purchased from Sigma Aldrich Co., USA. One-touch glucometer was purchased from Bayer Diagnostics India Ltd. Solvents such as methanol, petroleum ether and ethanol were purchased from Merk, India. All other chemicals used for the study were of analytical grade.

Animals

Male albino rats of Sprague- Dawley strain, with identical age and comparable weight, (150-180 g) used for the experiment were

obtained from Animal House, Department of Biochemistry, University of Kerala, Thiruvananthapuram, India, were used for the experiment. Animals were housed in standard polypropylene cages. Cages were kept in an environment with controlled temperature (28-32°C), humidity (55–60%) and photoperiod (12:12 h) light–dark cycle and fed a standard diet supplied by Gold Mohur rat feed, Hindustan Lever Ltd and given water ad libitum.

Animal experimentation was conducted in accordance with the institutional ethical guidelines for the conduct of the experiments on laboratory animals as per CPCSEA rules [Sanction No: IAEC-KU-13/05-06-BC-AH (4)]. Animals were handled using the laboratory animal welfare guidelines[7]. They were weighed weekly and the food and water consumption were observed. The animals were also examined regularly for their physical activity.

Induction of diabetes: Rats were pretreated with streptozotocin (60 mg/kg, i.p.) to induce diabetes. Streptozotocin–induced hyperglycemia has been described as a useful experimental model to study the activity of hypoglycemic agents[8]. Streptozotocin selectively destroys the pancreatic insulin secreting β cells, leaving less active cells and resulting in a diabetic state[8,9].

Because streptozotocin is capable of producing fatal hypoglycemia as a result of massive pancreatic insulin release, rats were treated with 20% glucose solution (5-10 ml) orally after 6 hr. The rats were then kept for the next 24 hr with 5% glucose solution bottles in their cages to prevent hypoglycemia[10].

After specific time periods, experimental rats were sacrificed by euthanasia (intraperitoneal injection of ketamine at a dose of 60 mg/kg), and the blood and tissue samples were collected immediately.

Experimental design

Hypoglycemic & antioxidant activities

For evaluating the hypoglycemic and antioxidant activities of the most potent extract among AEC, MEC & EEC, rats were divided into 5 groups, with 12 rats in each group.

Group I: Normal control (NC)

Group II: Diabetic control

Groups III: Diabetic+AEC at a dose of 100 mg/kg BW (AEC_{100})

Group IV: Diabetic+ MEC at a dose of 100 mg/kg BW (MEC_{100})

Group V: Diabetic+ EEC at a dose of 100 mg/kg BW (EEC_{100})

The duration of the experiment was 30 days. Different extracts of C. igneus were dissolved in physiological saline and given orally.

Dose-response relationship of MEC

The relationship between the amount of drug administered and the magnitude of the desired response is referred to as dose-response relationship. Knowledge of the relationships among dose, drug-concentration in blood and clinical response (effectiveness and undesirable effects) is important for the safe and effective use of drugs in individual patients. This information can help identify an appropriate starting dose, the best way to adjust dosage to the needs of a particular patient, and a dose beyond which increases would be unlikely to provide added benefit or would produce unacceptable side effects. Historically, drugs have often been initially marketed at what were later recognized as excessive doses (i.e., doses well onto the plateau of the dose-response curve for the desired effect), sometimes with adverse consequences. This situation has been improved by attempts to find the smallest dose with a discernible useful effect or maximum dose beyond which no further beneficial effects is seen. Hence it was decided to carry out investigations with different doses of methanolic extract (MEC).

Experimental protocol

In order to assess the most effective dose of MEC, a study was conducted with 5 groups of 12 rats in each group to assess hypoglycemic and antioxidant activities.

Hypoglycemic & antioxidant activities

Group I: Diabetic control

Groups II: Diabetic+MEC_{25}

Group III: Diabetic+ MEC_{50}

Group IV: Diabetic+ MEC_{100}

Group V: Diabetic+ MEC_{250}

Datas were confirmed by conducting duplicate studies.

Toxicity assessment

Male albino (Sprague-Dawley) rats of 150-180g were allocated into 5 groups of 6 rats in each group. (Group I: Normal control (NC), Group II: NC + $MEC_{25,}$ Group III: NC + MEC_{50}, Group IV: NC + $MEC_{100,}$ Group V: NC + MEC_{250}).

Studies include the analysis of various enzymes like SGOT, SGPT, acid phosphatase and alkaline phosphatase.

Statistical analysis

The results were analyzed using a statistical program SPSS/PC+, Version 11.0 (SPSS Inc. Chicago, USA). One way ANOVA was employed for comparison among groups. Post-hoc multiple comparison tests of significant differences among groups were determined. Pair fed comparisons between the groups was made by Duncan's multiple range tests[11].

Correlation coefficient r (Pearson, Spearman) were used for statistical evaluation. All results were considered as statistically significant at $P \leq 0.05$.

RESULTS

Phytochemical constituents were determined qualitatively in each fraction (Table 3).

Table 5: Qualitative Phytochemical Analysis of AEC, MEC and EEC

Plant constituents	AEC	MEC	EEC
Carbohydrates	-	-	-
Steroids	+	+	+
Triterpenoids	+	+	+
Alkaloids	+	+	-
Tannins	+	+	+
Glycosides	+	+	+
Saponins	+	+	+
Flavonoids	+	+	+
Fixed oils	-	-	-

'+' indicates presence; '-' denotes absence

Scavenging of DPPH Radical by AEC, MEC and EEC compared to Butyrated Hydroxyl Toluene (BHT)

The concentration required to quench DPPH radical by 50% (IC_{50}) was found to be 0.240 mg/ml, 0.206 mg/ml, 0.084 mg/ml and 0.054 mg/ml for AEC, EEC, MEC and BHT respectively (Table 7).

Table 7: Scavenging of DPPH radical by MEC, EEC, AEC compared to butyrated hydroxyl toluene (BHT)

Conc (µg)	Percentage Inhibition			
	AEC	MEC	EEC	BHT
20	8	14	12	36
50	12	25	17	78
100	16	61	34	98
150	34	73	42	97
200	45	89	49	95
250	54	94	63	95
300	65	87	75	95

Values expressed as mean ± SEM, for n = 6

MEC exhibited highest free radical scavenging activity among the three extracts. Radical scavenging activity of MEC was found to be maximum at 250 µg. In the case of BHT, maximum activitiy was shown at 100 µg concentration and there was no further increase of activity on raising the concentration above it. It was 94 % in the case of methanolic extract of C.igneus at 250 µg concentration where as BHT showed a maximum activity of 98% at 100 µg.

Concentration of blood glucose

All the three extracts brought down the level of blood glucose in significant levels. AEC showed a reduction of 40% when MEC and EEC reduced blood glucose by 68% and 58% respectively (Figure 11). Maximum effect was shown by MEC (I- Control, II- Diabetic, III- D+AEC$_{100}$, IV- D+MEC$_{100}$ & V- D+EEC$_{100}$).

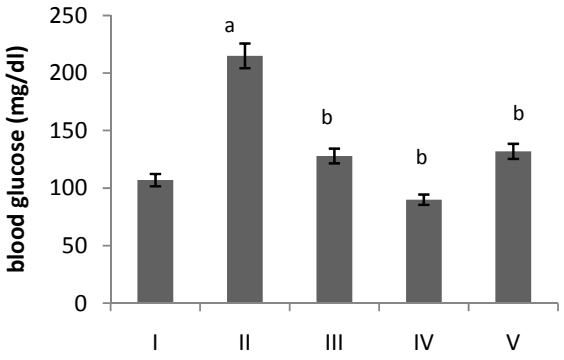

Fig. 11: Concentration of blood glucose

Values expressed as mean ± SEM, for n = 6. a-Statistically significant when compared to group I at p < 0.05. b-Statistically significant when compared to group II at p < 0.05

Activities of Hexokinase (HK) & Glucose-6-Phosphate Dehydrogenase (G-6-P Dhase)

Activities of hexokinase and glucose-6-phosphate dehydrogenase were decreased in the diabetic rats where as they were significantly increased in all treated rats. The effect was more pronounced in MEC treated rats (Table 6).

Table 6: Activities of hexokinase (HK) and glucose-6-phosphate dehydrogenase (G-6-P Dhase)

Groups	Hexokinase (Units$ /mg protein)	G-6-P Dhase (Units& /g protein)
I (NC)	0.26±.02	125.06±2.89
II (Diabetic)	0.06±.01[a]	85.28±1.97[a]
III (D+AEC$_{100}$)	0.10±.02[b]	98.06±3.27[b]
IV (D+MEC$_{100}$)	0.19±.02[b]	112.29±2.07[b]
V (D+EEC$_{100}$)	0.11±.02[b]	98.37±3.25[b]

Values expressed as mean ± SEM, for n = 6. a-Statistically significant when compared to group I at p < 0.05

b-Statistically significant when compared to group II at p < 0.05. $Activity expressed as mg glucose

phosphorylated/min. &Amount of enzyme which causes an increase in OD of 1.0/min

Concentration of MDA and GSH and activities of Catalase, Glutathione Peroxidase (GSH Pxase) and Glutathione Reductase (GSSG Rase)

Concentration of MDA, which was significantly increased in diabetic rats showed pronounced reduction in all the treated rats. Glutathione content was increased in AEC, MEC and EEC treated rats. Antioxidant enzymes (catalase, glutathione peroxidase and glutathione reductase) showed higher activities in the treated groups. In all the cases, maximum effect was observed in the group fed MEC (Table 8).

Table 8: Concentration of MDA and GSH and activities of catalase, glutathione peroxidase (GSH Pxase) and glutathione reductase (GSSG Rase)

Groups	MDA (mmol/100g wet tissue)	GSH (mmol/100g wet tissue)	Catalase (Units£/mg protein)	GSH Pxase (Units¥/mg protein)	GSSG Rase (Units§/mg protein)
I (NC)	3.24±0.09	56.23±2.13	67.26±1.57	2.63±0.14	7.57±0.16
II (Diabetic)	5.34±0.05[a]	42.37±0.89[a]	45.29±1.30[a]	1.14±0.08[a]	4.19±0.27[a]
III (D+AEC$_{100}$)	4.42±0.26[b]	48.01±2.53[b]	54.95±2.07[b]	1.68±0.08[b]	5.69±0.25[b]
IV (D+MEC$_{100}$)	3.98±0.36[b]	52.28±1.49[b]	63.18±1.64[b]	2.44±0.10[b]	6.29±0.18[b]
V (D+EEC$_{100}$)	4.27±0.16[b]	46.71±0.98[b]	57.62±2.40[b]	1.79±0.08[b]	5.97±0.26[b]

Values expressed as mean ± SEM, for n = 6. a-Statistically significant when compared to group I at p < 0.05

b-Statistically significant when compared to group II at p < 0.05. £Values x 10^{-3} units {(velocity constant/second)/ mg protein}. ¥Units (µmoles of NADPH oxidized/minute). §Values x 10^{-2} units (µmoles of NADPH oxidized/minute)

While assessing the biological activity of aqueous, methanolic and ethanolic extracts, it was found that MEC showed maximum beneficial action in diabetic rats by enhancing hypoglycemic and antioxidant effects. Hence further experiments were designed to determine the optimum dose of MEC with more beneficial action and less toxicity.

Blood Glucose Levels and Activities of Hexokinase and Glucose-6-Phosphate Dehydrogenase

Even though all the 4 doses could exert hypoglycemic effect, most hypoglycemic activity was shown in the group given MEC$_{100}$ (68%). Activities of hexokinase and glucose-6-phosphate dehydrogenase were decreased in the diabetic rats where as they were significantly increased in all treated rats. The highest effect was shown in MEC$_{100}$ treated group (Table 9).

Table 9: Blood glucose levels and activities of hexokinase and glucose-6-phosphate dehydrogenase

Groups	Blood glucose (mg/dl)	Hexokinase (Units$/mg protein)	G-6-P Dhase (Units¤/g protein)
I (Diabetic)	308±2.78	0.06±.01	85.28±1.97
II (D+MEC$_{25}$)	146±1.05[a]	0.09±.01[a]	97.62±3.42[a]
III (D+MEC$_{50}$)	131±2.57[a]	0.11±.02[a]	102.43±1.61[a]
IV (D+MEC$_{100}$)	98±2.13[a]	0.19±.02[a]	112.29±2.07[a]
V (D+MEC$_{250}$)	105±2.45[a]	0.16±.01[a]	95.05±3.54[a]

Values expressed as mean ± SEM, for n = 6. a-significantly different when compared to group I at p < 0.05

$Activity expressed as µM of glucose phosphorylated/hour. ¤Amount of enzyme which causes an increase in OD of 1.0/min

Concentration of MDA and GSH and Activities of Catalase, Glutathione Peroxidase (GSH Pxase) and Glutathione Reductase (GSSG Rase)

Concentration of MDA, which was significantly increased in diabetic rats showed significant reduction in all the treated rats, but the effect was very much pronounced in MEC_{100} treated group. Glutathione content was increased in all groups but MEC_{100} showed much higher levels. Antioxidant enzymes (catalase, glutathione peroxidase and glutathione reductase) showed higher activities in all treated groups, but maximum effect was observed in the group fed MEC_{100} (Table 10).

Table 10: Concentration of MDA and GSH and activities of catalase, glutathione peroxidase and glutathione reductase

Groups	MDA (mmol/100g wet tissue)	GSH (mmol/100g wet tissue)	Catalase (Units£/mg protein)	GSH Pxase (Units¥/mg protein)	GSSG Rase (Units§/mg protein)
I (Diabetic)	5.34±0.05	42.37±0.89	45.29±1.30	1.14±0.08	4.19±0.27
II (D+MEC_{25})	5.21±.21	47.42±1.69[a]	57.22±0.96[a]	1.53±0.09[a]	5.01±0.35[a]
III (D+MEC_{50})	4.82±0.13	48.29±1.52[a]	58.25±2.20[a]	1.66±0.09[a]	5.64±0.13[a]
IV (D+MEC_{100})	3.98±0.36[a]	52.28±1.49[a]	63.18±1.64[a]	2.44±0.10[a]	6.29±0.18[a]
V (D+MEC_{250})	4.94±0.10	46.14±0.57[a]	49.37±1.48	1.37±0.17	5.24±0.32[a]

Values expressed as mean ± SEM, for n = 6. a-significantly different when group II-V are compared to group I at $p < 0.05$
£Values x 10^{-3} units (velocity constant/second). ¥Units (µmoles of NADPH oxidized/minute)
§Values x 10^{-2} units (µmoles of NADPH oxidized/minute)

Table 11: Activity of Serum glutamate oxaloacetate transaminase and Serum glutamate pyruvate transaminase

Groups	SGOT (µmol oxaloacetate lib/min/l)	SGPT (µmol pyruvate lib/min/l)
I (NC)	43.49±2.15	28.48±1.41
II (NC+MEC_{25})	43.61±2.21	29.36±1.45
III (NC+MEC_{50})	42.37±2.09	27.75±1.37
IV (NC+MEC_{100})	42.08±2.05	28.30±1.45
V (NC+MEC_{250})	51.45±2.58[a]	34.51±1.71[a]

Values expressed as mean ± SEM, for n = 6. a-significant when groups II-V are compared to group I at $p < 0.05$

Table 12: Activity of Acid phosphatase (ACP) and Alkaline phosphtatase (ALP)

Groups	ACP (µmol phenol lib/min/mg protein)	ALP (µmol phenol lib/min/mg protein)
I (NC)	9.47±0.47	1.54±0.07
II (NC+MEC_{25})	10.16±0.50	1.60±0.08
III (NC+MEC_{50})	9.85±0.49	1.48±0.07
IV (NC+MEC_{100})	9.40±0.47	1.39±0.06
V (NC+MEC_{250})	15.42±0.77[a]	1.94±0.09[a]

Values expressed as mean ± SEM, for n = 6. a- statistically significant when group II-V are compared to group I at $p < 0.05$

Activities of Serum Glutamate Oxaloacetate Transaminase (SGOT or AST) and Serum Glutamate Pyruvate Transaminase (SGPT or ALT)

The activities of serum glutamate oxaloacetate transaminase and serum glutamate pyruvate transaminase were normal in 25-100 mg treated rats, where as activities were elevated significantly in serum of MEC_{250} treated group (Table 11).

Activities of Acid Phosphatase (ACP) and Alkaline Phosphtatase (ALP)

Activities of acid phosphatase and alkaline phosphatase were increased significantly in the serum of high dose treated group where as their activities were normal in 25-100 mg treated rats (Table 12).

DISCUSSION

Beneficial activities of C. igneus were evaluated in diabetic rats by assessing the effects of 3 different extracts (AEC, MEC and EEC) at a dose of 100 mg/kg BW. Even though the 3 extracts were capable of exerting beneficial effects by reducing the concentrations of blood sugar and MDA, and increasing glutathione content and normalizing the activities of various enzymes such as hexokinase, glucose-6-phosphate dehydrogenase, catalase, glutathione peroxidase and glutathione reductase, the optimum benefits were obtained for the group that was given MEC at a dose of 100 mg/kg BW. Recently reports demonstrate the hypoglycemic activity of C. igneus[12-14] which are consistent with our findings. DPPH free radical was effectively quenched by three extracts and maximum efficiency was shown by MEC. DPPH is a relatively stable free radical that shows maximum absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance such as antioxidants, the radical functions as scavenger and the absorbance is reduced[15], when it reacts with the hydrogen donors in the antioxidant principles[16]. DPPH radicals react with suitable reducing agents, the electrons become paired off and the solution loses colour stoichiometrically depending on the number of electrons taken up[17].

Assessment of dose-response is an integral component of drug development, with studies designed to assess dose-response, an inherent part of establishing the safety and effectiveness of the drug. MEC_{100} exerted maximum beneficial effects by eliciting pronounced hypoglycemic and antioxidant activities. Our studies show some degrees of toxicity in rats treated with MEC_{250}. Presence of marked aminotransferase activities have been demonstrated in MEC_{250} treated group. Serum exhibited higher GOT activity. GPT activity was also higher in significant levels in the serum. Acid phosphatase and alkaline phosphatase levels were also raised significantly in the serum of 250 mg treated rats. The above findings indicate toxicity of 250 mg dose. The activities of these enzymes were normal when given MEC at doses ranging from 25mg- 100mg. Concentration of urea, uric acid and creatinine were also elevated in the 250 mg treated rats. From the above findings, it can be concluded that the most effective dose is MEC_{100}. 250 mg dose exhibited toxicity as evidenced by biochemical as well as histopathological studies.

Further toxicological studies are required for better understanding of the ill effects.

ACKNOWLEDGMENT

The authors thank Rajiv Gandhi National Fellowship for the financial assistance. Thanks are also due to Dr. Suja Mary Koshy, Veterinary Surgeon, Department of Biochemistry, University of Kerala, Thiruvananthapuram, India and Dr. G. Valsala Devi, Curator, Department of Botany, University of Kerala, Thiruvananthapuram, India.

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