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ORIGINAL ARTICLE

In vitro alpha-amylase inhibition and *in vivo* antioxidant potential of *Amaranthus spinosus* in alloxan-induced oxidative stress in diabetic rats

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Abstract *Amaranthus spinosus* Linn. (Amaranthaceae), commonly known as "Mulluharivesoppu" in Kannada, is used in the Indian traditional system of medicine for the treatment of diabetes. The present study deals with the scientific evaluation of alpha amylase and the antioxidant potential of methanol extract of *A. spinosus* (MEAS). The aim of this study was to investigate *in vitro* alpha-amylase enzyme inhibition by CNPG3 (2-chloro-4-nitrophenol α-D-maltotrioside) and *in vivo* anti-oxidant potential of malondialdehyde (MDA), glutathione (GSH), catalase (CAT) and total thiols (TT) in alloxan-induced diabetic rats of a methanolic extract of *A. spinosus*. Blood sugar was also determined in MEAS-treated alloxan-induced diabetic rats. MEAS showed significant inhibition of alpha-amylase activity and IC_{50} 46.02 μg/ml. Oral administration of MEAS (200 and 400 mg/kg) for 15 days showed significant reduction in the elevated blood glucose, MDA and restores GSH, CAT and TT levels as compared with a diabetic control. The present study provides evidence that

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the methanolic extract of *A. spinosus* has potent alpha amylase, anti-diabetic and antioxidant activities.

1. Introduction

Amaranthus spinosus Linn. (Amaranthaceae), commonly known as ''Mulluharivesoppu'' in Kannada, is an annual or perennial herb, native to tropical America and is found throughout India as a weed in cultivated as well as fallow lands (Anon, 1988). In Indian traditional system of medicine (Ayurveda) the plant is used as anti-diabetic, antipyretic, laxative, diuretic, digestible, anti-snake venum, antileprotic, in blood diseases, bronchitis, piles and anti-gonorrheal (Vaidyaratanam PS Varier's, 1996; William D'ymock, 1976; Kirtikar and Basu, 1987). Some tribes in India apply *A. spinosus* to induce abortion (Grubben and Denton, 2004).

The juice of *A. spinosus* was used by tribals of Kerala to prevent swelling around the stomach, while the leaves are boiled without salt and consumed for 2–3 days to cure jaundice (Hema et al., 2006). The Plant has a high concentration of antioxidant components (Odhavo et al., 2007; Cao et al., 1996; Gil et al., 1999; Vinson et al., 1998) and high nutritive values due to the presence of fibre, proteins and a high concentration of essential amino acids, especially lysine (Teutonico and Knorr, 1985).

A. spinosus is reported for its anti-inflammatory (Olumayokun et al., 2004), antimalarial (Hilou et al., 2006), antiandrogenic (Murgan et al., 1993a), immunomodulatory (Tatiya et al., 2007), anti-diabetic, anti-hyperlipidemic and spermatogenic activities (Sangameswaran and Jayakar, 2008), effect on hematology (Olufemi et al., 2003) and Biochemical changes in Epididymis (Murgan et al., 1993b). The betalains in stem bark of *A. spinosus* were identified as amaranthin, isoamaranthine, hydroxycinnamates, rutin, quercetin and kaempferol glycosides (Srinivasan et al., 2003; Ibewuike et al., 1997; Rastogi and Mehrotra, 1999; Stintzing et al., 2004; Ashok Kumar et al., 2008; Hilou et al., 2006). It also contains amaranthoside, a lignan glycoside, amaricin, a coumaroyl adenosine along with stigmasterol glycoside, betaine such as glycinebetaine and trigonelline (Blunden et al., 1999; Azhar-ul-Haq et al., 2006). Betalains are well known for their antioxidant, anticancer, antiviral and antiparasitosis properties (Kapadia et al., 1995, 1996; Patkai et al., 1997).

The whole plant of *A. spinosus* was previously screened for anti-diabetic activity in streptozotocin-induced diabetic rats (Sangameswaran and Jayakar, 2008) and our study deals with the *in vitro* alpha-amylase inhibition and *in vivo* antioxidant potential of methanolic extract of leaves of *A. spinosus* in alloxan-induced diabetic rats.

2. Materials and methods

2.1. Collection of plant material and extraction

The fresh plant of *A. spinosus* was collected from Chickballapur, and was authenticated by Dr. Rajan, Department of Botany, Government Arts College, Ootcamund, Tamil Nadu. A voucher specimen (SKVCP 13) was deposited in the college herbarium. The leaves are separated, shade dried and coarsely powdered. The coarse powder was subjected to extraction with methanol by soxhlet apparatus and the extract was concentrated to dryness in vacuum.

2.2. Chemicals

CNPG3 was purchased from Chemadiagnostica, Italy, Acarbose was purchased from Glucobay, Bayer Pharma, India, sodium dihydrogen orthophosphate dehydrate, disodium hydrogen phosphate dihydrate (Himedia, India), alloxan was purchased from Sigma–Aldrich, St. Louis, USA. Glibenclamide was purchased from Sun Pharmaceuticals Ltd., India, Glucose assay kit was obtained from the diagnostic division of Dr. Reddy's Laboratories, Hyderabad. Trichloro acetic acid (Loba Chemie, Mumbai, India), alpha amylase, bovine serum albumin, thiobarbituric acid, 5,5′ dithio-bis-2-nitrobenzoic acid, reduced glutathione were procured from Sigma–Aldrich, St. Louis, USA. All the other chemicals used were of analytical grade.

2.3. Preliminary phytochemical screening

MEAS was screened for the presence of various phytoconstituents like steroids, alkaloids, glycosides, flavonoids, carbohydrates, amino acids, proteins and phenolic compounds (Kokate, 1986).

2.4. Alpha-amylase inhibition assay of MEAS by CNPG3

The α-amylase inhibitory activity for MEAS was determined based on the spectrophotometric assay using acarbose as the reference compound (Gella et al., 1997). The MEAS was dissolved in DMSO to give concentrations from 10, 50 and 100 μg/ml. The enzyme α-amylase solution (0.5 U/ml) was prepared by mixing 3.246 mg of α-amylase (EC 3.2.1.1) in 100 ml of 40 mM phosphate buffer pH 6.9. Add 60 μl of 40 mM phosphate buffer (pH 6.9)/acarbose/MEAS and 30 μl of α-amylase enzyme and are preincubated at 37 °C for 10 min and then 120 μl of CNPG3 was added, mixed and incubated at 37 °C for 8 min. The absorbance was measured at 405 nm and control reaction was carried out without the extract. Percentage inhibition was calculated by the expression:

$$\%\,\text{inhibition} = \frac{\text{Absorbance}_{\text{Control}} - \text{Absorbance}_{\text{Test}}}{\text{Absorbance}_{\text{Control}}} \times 100$$

2.5. Animals

Albino Wistar rats (150–250 g) of either sex were acclimatized to the experimental room at a temperature of 23 ± 2 °C, controlled humidity conditions (50–55%) and 12 h light and 12 h dark cycles. They were caged with a maximum of two animals in a polypropylene cage and were fed with standard food pellets (Kamadenu Enterprises, Bangalore) and water *ad libitum*.

2.6. Acute toxicity studies

Methanol extracts of *A. spinosus* were studied for acute oral toxicity as per revised OECD (Organization for Economic Cooperation and Development, 2001) guidelines No. 423 (2000). The extract was devoid of any toxicity in rats when given in doses up to 2000 mg/kg by an oral route. Hence, for further studies 200–400 mg/kg doses of the extract were used.

2.7. Induction of diabetes

The animals were fasted for 12 h prior to the induction of diabetes as described by Joy and Kuttan (1999) with a slight modification. ALX freshly prepared in 0.5% tween 80 was administered intraperitoneally (i.p.) at a single dose of 140 mg/kg. Development of diabetes was confirmed by measuring blood glucose concentration 5 days after the administration of ALX. Rats with blood glucose levels of above 200 mg/dl were considered to be diabetic and used for the studies. Tween 80, MEAS (200 and 400 mg/kg) and glibenclamide (10 mg/kg) were administered for diabetic rats for 15 days and the blood sugar level was measured on 0, 1, 10 and 15th day, respectively.

On the 15th day of the study, blood samples were collected for biochemical estimations. Later animals were sacrificed and the liver was removed and homogenized for *in vivo* antioxidant activity study.

2.8. In vivo antioxidant activities

Lipid peroxidation was estimated by thiobarbituric acid (TBA) reaction with malondialdehyde (MDA) a product formed due to the peroxidation of membrane lipids (Gelvan and Saltman, 1990). Glutathione (GSH) was determined according to the method of Moran et al. (1979), catalase (CAT) activity was determined according to the method of Claiborne (1985) and total thiols (TT) were determined according to the method of Moran et al. (1979).

2.9. Statistical analysis

Results were expressed as the mean ± SEM, for statistical analysis of the data group, means were compared by one-way analysis of variance (ANOVA) followed by Tukey's post-test for multiple comparisons. $p < 0.001$ was considered to be statistically significant.

3. Results

3.1. Preliminary phytochemical analysis

Preliminary phytochemical analysis showed the presence of carbohydrates, steroids, alkaloids, phenolic compounds, flavonoids, saponins and amino acids in methanol extract of *A. spinosus*.

3.2. Alpha-amylase inhibition assay of MEAS by CNPG3

Table 1 results revealed that MEAS showed a significant inhibition of α-amylase enzyme. MEAS at concentrations 10, 50, 100 µg/ml showed 38.02 ± 0.1%, 55.9 ± 0.11% and 63.14 ± 0.1% of α-amylase enzyme inhibition and IC_{50} value was 46.02 µg/ml. The acarbose used as a reference standard at concentrations of 0.1, 0.5 and 1.0 µg/ml showed 31.52 ± 0.1%, 72.49 ± 0.14% and 82.92 ± 0.09% inhibition of α-amylase activity and IC_{50} value was 0.312 µg/ml.

Table 1 Alpha-amylase inhibition assay of methanolic extract of *A. spinosus* (MEAS).

Sample	Concentration (µg/ml)	% inhibition	IC_{50} (µg/ml)
Acarbose	0.1	31.53 ± 0.14	0.312
	0.5	72.49 ± 0.09	
	1.0	82.92 ± 0.12	
MEAS	10	38.02 ± 0.1	46.02
	50	55.9 ± 0.11	
	100	63.14 ± 0.1	

The data are expressed in mean ± S.E.M. $n = 3$ in each group 0.

3.3. Effect of MEAS on blood sugar

MEAS (200 and 400 mg/kg) and glibenclamide (10 mg/kg) showed significant ($p < 0.001$) fall in blood glucose levels 56.6%, 57.6% and 66.8% respectively when compared with diabetic control group (Table 2).

3.4. In vivo antioxidant activities

Table 3 shows the effect of administration of MEAS on MDA, GSH, CAT and TH in liver tissue of different groups of rats. There was a significant ($p < 0.001$) elevation in tissue MDA in diabetic rats as compared to normal rats. Treatment with MEAS for 15 days resulted in a significant ($p < 0.001$)

Table 2 Effect of methanolic extract of *A. spinosus* (MEAS) on blood glucose level in diabetic rats.

Groups	Treatment	Blood glucose level (mg/dl)			
		0 day	1st day	10th day	15th day
I	Control	86.75 ± 2.18	87.44 ± 2.34	62.66 ± 5.6	84.98 ± 3.8
II	Diabetic control (DC)	316.84 ± 47.13	354.74 ± 39.98	378.94 ± 7.05	342.83 ± 40.06
III	DC + MEAS 200 mg/kg	259.7 ± 27.9	221.1 ± 41.9[a]	108.4 ± 7[c]	89.84 ± 9.01[c]
IV	DC + MEAS 400 mg/kg	248.6 ± 31.4	138.3 ± 4.7[c]	95.7 ± 4[c]	81.26 ± 6.86[c]
V	DC + glibenclamide (10 mg/kg)	271.7 ± 33.1	185.2 ± 14.9[b]	86.6 ± 5.6[c]	72.16 ± 7.48[c]

The data are expressed in mean ± SEM. $n = 6$ in each group.
[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$ compared with corresponding value of diabetic control animals.

Table 3 Effect of methanolic extract of *A. spinosus* (MEAS) on MDA, GSH, CAT and TT levels in the liver homogenates of ALX-induced diabetic rats.

Treatment	MDA (*n* moles/g of tissue)	GSH (*n* moles/mg of protein)	CAT (U/mg of protein)	TT (μmoles/mg of protein)
Normal control	11.22 ± 0.65[b]	48.21 ± 2.05[b]	89.38 ± 2.53[b]	3.92 ± 0.094[b]
Diabetes control (DC)	60.76 ± 3.3[#]	16.26 ± 1.98[#]	24.28 ± 2.94[#]	0.6 ± 0.088[#]
DC + MEAS (200 mg/kg)	40.26 ± 2.12[c]	25.28 ± 1.26[a]	37.09 ± 2.03[b]	2.1 ± 0.19[c]
DC + MEAS (400 mg/kg)	27.89 ± 2.46[c]	33.47 ± 2.18[c]	53.94 ± 2.16[c]	2.6 ± 0.12[c]
DC + GLB (10 mg/kg)	19.75 ± 1.84[b]	39.85 ± 1.74[b]	67.18 ± 2.45[b]	3.1 ± 0.27[b]

The data are expressed in mean ± SEM. $n = 6$ in each group.
[a] $p < 0.05$.
[b] $p < 0.001$.
[#] $p < 0.001$ compared with corresponding value of diabetic control animals.

decrease in liver tissue MDA. GSH content in diabetic control rats was significantly ($p < 0.001$) depleted in liver tissue when compared with normal rats. MEAS treatment at both doses levels significantly ($p < 0.001$) restored GSH, CAT and TT levels as compared with diabetic control.

4. Discussion

Drugs that inhibit carbohydrate hydrolyzing enzymes have been demonstrated to decrease postprandial hyperglycemia and improve impaired glucose metabolism without promoting the insulin secretion of NIDDM patients. The results of *in vitro* studies showed that MEAS inhibits α-amylase activity. Natural health products of vegetable origin were clearly indicated as a promising avenue for the prevention of chronic diseases (Punitha and Manoharan, 2006).

Oxidative stress, altered lipid levels, and disturbances in glucose metabolism are important risk factors for diabetes, cardiovascular, oncologic and many other diseases. Diet undoubtedly plays a key role as a chemopreventive agent against various diseases and optimizing the diet in both quality and quantity has a preventive function. Fruit and vegetables are an invaluable source of many biologically active substances, including antioxidants. For this reason a diet rich in fruit and vegetable has a positive effect on reducing the incidence of these serious lifestyle diseases (Van Der Schouw et al., 2005).

The diabetogenic agent alloxan is a hydrophilic and chemically unstable pyrimidine derivative, which is toxic to pancreatic β-cells because it can generate toxic-free oxygen radicals during redox cycling in the presence of reducing agents such as glutathione and cysteine (Szkudelski, 2001). The increase in oxygen-free radicals in diabetes could be due to an increase in blood glucose levels, which generates free radicals due to autooxidation (Yadav et al., 2000). In the present work, involvement of free radicals in progression of disease and protective effects of *A. spinosus* has been examined. Administration of MEAS for 15 days showed significant effect on blood sugar level, and antioxidant levels in ALX-induced diabetic rats.

Elevation of LPO is attributed to the enhanced production of the reactive oxygen species. In the present study, we observed a MDA formation, the index of lipid peroxidation, was significantly increased in the liver of ALX-treated animals. MEAS supplementation potentially reduced MDA level, suggesting that MEAS might have antioxidant principles to produce such a response.

GSH protects the cellular system against the toxic effects of lipid peroxidation. A marked depletion in the GSH content of a liver was observed in diabetic control rats (Table 3). Furthermore, MEAS treatment showed a significant restoration in GSH content of diabetic rats.

The present data indicates that ALX-induced diabetes disrupts the actions of antioxidant enzymes. The decreased activities of these enzymes may be due to the production of a reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) that reduces the activity of these enzymes (Kaleem et al., 2005; Vincent et al., 2004). In the present study, MEAS potentiated the *in vivo* antioxidant activities.

The findings of the present study shows positive effects of *A. spinosus* on rats with ALX-induced disturbances in the glucose level and the antioxidant status. Thus, MEAS is beneficial in the control of diabetes and oxidative stress by activation of enzymatic and non-enzymatic antioxidants. These beneficial effects of *A. spinosus* are especially promising in the light of preventing lifestyle diseases of the cardiovascular system (Despres et al., 2000).

In conclusion, the result of the present study indicates that *A. spinosus* may have active principle(s) which were responsible for the potent anti-diabetic agent. However, more efforts are still needed for the isolation, characterization and biological evaluation of the active principle(s) of the *A. spinosus* extract.

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