

# In Vitro $\alpha$ -Amylase Inhibition and Antioxidant Activities of Methanolic Extract of *Amaranthus Caudatus* Linn

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## Abstract

**Objectives:** The present study was aimed to investigate the  $\alpha$ -amylase inhibition and antioxidant activities of methanolic extract of *Amaranthus caudatus* Linn (MeAc).

**Methods:** Methanolic extract of *Amaranthus caudatus* was screened for  $\alpha$ -amylase inhibition activity by CNPG3 method (2-chloro-p-nitrophenyl- $\alpha$ -D-maltotrioxide) and antioxidant activity was evaluated by 1,1-diphenyl-2-picryl-hydrazole (DPPH) free radical scavenging, superoxide dismutase (SOD) scavenging, hydroxyl free radical scavenging, nitric oxide (NO) radical scavenging, and 2,2'-azinobis-3-ethylbenzothiazole-6-sulfonic acid (ABTS) radical scavenging assays. MeAc was also screened for non enzymatic hemoglycosylation.

**Results:** The methanolic extract of *Amaranthus caudatus* showed potent  $\alpha$ -amylase inhibition activity ( $IC_{50}$  19.233  $\mu$ g/ml). MeAc showed significant antioxidant activity in all the *in vitro* antioxidant models. Furthermore, the MeAc was found to be extremely effective in scavenging ABTS radical activity ( $IC_{50}$  48.75 $\pm$ 1.1  $\mu$ g/ml) when compared to DPPH ( $IC_{50}$  77.5 $\pm$ 0.4  $\mu$ g/ml), SOD ( $IC_{50}$  62.5 $\pm$ 2.1  $\mu$ g/ml), hydroxyl ( $IC_{50}$  88.50 $\pm$ 1.8  $\mu$ g/ml) and NO ( $IC_{50}$  67.5 $\pm$ 2.2  $\mu$ g/ml) scavenging activity.

**Conclusions:** The methanolic extract of *A. caudatus* showed potent  $\alpha$ -amylase inhibition and antioxidant activities.

**Keywords:** *Amaranthus caudatus*;  $\alpha$ -amylase inhibition; Antioxidant activity; Superoxide dismutase.

## Introduction

*Amaranthus caudatus* Linn (Amaranthaceae) is commonly known as "Peddathotakura" in Telugu. The amaranthus plants are spread throughout the world, growing under a wide range of climatic conditions and the species is well known to produce grains and leafy edible vegetables.<sup>1</sup> Traditionally, the plant parts of *A. caudatus* were used in the treatment of jaundice, amoebiasis, and kidney diseases,<sup>2,3</sup> but also as a blood purifier, diuretic, abortifacient, vermifuge as well as an astringent.<sup>4</sup>

*A. caudatus* has been reported for its anti-atherosclerotic,<sup>5</sup> anthelmintic, antinociceptive and antipyretic activities.<sup>6,7</sup> The seeds of the plant were reported to possess cholesterol lowering, and antioxidant as well as  $\alpha$ -amylase inhibition activities *in vitro*.<sup>8,9</sup> The Amaranth seed oil from Ecuadorian flora is used as a nutraceutical resource.<sup>10</sup> The plant is known to contain antimicrobial peptides,<sup>11</sup> agglutinins,<sup>12</sup> triterpenoid saponins, ionol derived glycosides,<sup>13</sup> vitamin E isomers,<sup>14</sup> and amaranthin.<sup>15</sup>

The seed of *A. caudatus* has been proved to possess antioxidant and  $\alpha$ -amylase inhibition properties. Given this background, the present study was undertaken to investigate the  $\alpha$ -amylase inhibition and antioxidant activities of methanolic whole plant extract of *A. caudatus*.

## Methods

The fresh plant of *A. caudatus* was collected from Chickballapur (Karnataka, India), and was authenticated by Prof. Venkatesh, BK, Department of Botany, Government First Grade College, Chickballapur, Karnataka (India). A voucher specimen (SKVCP 12) was deposited in the college herbarium. The whole plant was shade dried and coarsely powdered. The coarse powder was exhaustively extracted with methanol by soxhlet apparatus and the extract was concentrated by vacuum drying.

Preliminary phytochemical screening of the methanolic extract of *A. caudatus* (MeAc) was subjected to various phytochemical tests for identification of secondary metabolites present in the extract, as described by Kokate.<sup>16</sup>

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The chemicals ABTS (2,2'-azino bis (3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt, DPPH (1,1- diphenyl-2-picrylhydrazyl), (TBA) thiobarbituric acid, Folin-Ciocalteu reagent, quercetin, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), potassium persulfate and Gallic acid were purchased from Sigma Chemical Co. Hemoglobin (Nice Chemicals Pvt. Ltd., Cochin), Glucose and  $\alpha$ -Tocopherol were procured from Merck, Mumbai. Ascorbic acid and gentamycin were obtained from Biokem Internationals Pvt. Ltd., Bangalore and Nicholas Piramal India Ltd., Pithampur.  $\alpha$ -amylase; Type VI-B from Porcine pancreas (A3176, Sigma, USA), 2-Chloro-4-Nitrophenol  $\alpha$ -D-Maltotrioxide [(CNP3) F120CH (77019), Chemadiagnostica, Italy], Acarbose (Glucobay, Bayer Pharma, India), Sodium dihydrogen orthophosphate dehydrate (RM1255, Himedia, India), Disodium hydrogen phosphate dehydrate (RM257, Himedia, India). All other reagents and solvents used were of analytical grade.

The total phenolic content of the methanol extract was determined using Folin-Ciocalteu reagent (FCR) according to the method of Slinkard and Singleton.<sup>17</sup> A 1 mg/ml solution of MeAc was prepared in ethanol: distilled water (1:1). Then 0.2 ml of MeAc was added to 0.5 ml of FCR and mixed thoroughly. After 10 min, 0.5 ml of sodium carbonate (2%) was added and allowed to stand for 30 min. The absorbance was read at 760 nm. The concentrations of total phenols in the extracts were calculated from the tannic acid graph. The total phenolic content was expressed as percentage.

The assay for  $\alpha$ -amylase inhibition was performed as described by Gella et al.<sup>18</sup> with slight modification. The  $\alpha$ -amylase enzyme solution was prepared by mixing 3.246 mg of  $\alpha$ -amylase enzyme in 100 ml of 40 mM phosphate buffer, pH 6.9. Positive control, Acarbose was obtained by dissolving 50 mg in 50 ml of phosphate buffer and diluted appropriately to get a concentration of 2.5  $\mu$ g/ml using phosphate buffer. *A. caudatus* was dissolved in buffer to give final concentrations of 10, 50 and 100  $\mu$ g/ml. The Acarbose and plant extract were separately mixed with 125  $\mu$ l of 2-Chloro-4-Nitrophenol  $\alpha$ -D-Maltotrioxide (CNP3) and incubated at 37°C for 8 min. The absorbance was measured at 405 nm. Similarly, a control reaction was carried out without the plant extract/Acarbose.

For determining the DPPH radical scavenging activity, the DPPH assay measured hydrogen atom donating activity and hence provided an evaluation of antioxidant activity due to free radical scavenging. DPPH, a purple-colored stable free radical, was reduced into the yellow-colored diphenylpicryl hydrazine; the color intensity of the reaction mixture was measured spectrophotometrically at 510 nm.<sup>19</sup> Briefly, 50  $\mu$ l of DPPH (0.659 mM) solution was added to 200  $\mu$ l of various concentration of MeAc and incubated at 25°C for 20 min. After incubation, the absorbance of the reaction mixture was read at 510 nm. Similarly, a control reaction was carried out without the MeAc. The DPPH radical-scavenging activity was expressed as percentage inhibition and calculated according to the following equation:

$$\text{Inhibition} = (A_0 - A_1)/A_0 \times 100 \%$$

Where  $A_0$  was the absorbance of the control (without extract) and  $A_1$  was the absorbance in the presence of the extract and  $IC_{50}$  value was calculated.

In terms of nitric oxide (NO) scavenging activity, the nitric oxide was liberated from sodium nitroprusside at physiological pH. This nitric oxide gets converted to nitrous acid and further forms nitrite ions ( $NO_2^-$ ), which may be quantified by the Griess reagent.<sup>20</sup> Sodium nitroprusside (10 mM, 50  $\mu$ l) in phosphate buffer saline was incubated with 50  $\mu$ l of various concentrations of MeAc at room temperature for 15 min. After incubation, 125  $\mu$ l of Griess reagent was added and incubated for 10 min at room temperature. The color developed was measured at 546 nm. The nitric oxide radicals scavenging activity was calculated according to the following equation:

$$\text{Inhibition} = (A_0 - A_1)/A_0 \times 100 \%$$

Where  $A_0$  was the absorbance of the control (without extract) and  $A_1$  was the absorbance in the presence of the extract and  $IC_{50}$  value was calculated.

For the evaluation of Superoxide anion scavenging assay, the superoxide anion radical scavenging activity of the extract was measured by Yen and Chen's method.<sup>21</sup> Phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system was used for the generation of superoxide anion. It was assayed by the reduction of nitro blue tetrazolium (NBT). Then 62.5  $\mu$ l of various concentrations of MeAc, 0.1 M phosphate buffer, 62.5  $\mu$ l of 46862.5  $\mu$ M NADH solution, 62.5  $\mu$ l of nitro blue tetrazolium (156  $\mu$ M) and 62.5  $\mu$ l of 60  $\mu$ M PMS were added to microwell plate and incubated at room temperature for 5 min. The absorbance of the mixture was measured at 560 nm against blank samples. The percentage inhibition was determined by comparing the results of control and test samples and  $IC_{50}$  values were calculated.

In determining the ABTS scavenging activity, the assay was performed as described by Auddy et al.<sup>22</sup> ABTS<sup>+</sup> radicals were produced by reacting ABTS and APS (Ammonium persulphate) and incubating the mixture at room temperature in the dark for 16 hrs. Then 20  $\mu$ l of various concentration of MeAc and 10 mM phosphate buffer pH 7.4 and 230  $\mu$ l of ABTS<sup>+</sup> radical solution (0.238 mM) were added. The absorbance was measured immediately at 734 nm. A control reaction was carried out without the extract.

Hydroxyl radical scavenging activity was measured by the formation of hydroxyl radicals ( $OH^\cdot$ ) from Fenton reagents, which was quantified using 2-deoxyribose oxidative degradation as described previously.<sup>23</sup> The principle of the assay is the quantification of the 2-deoxyribose degradation product malonaldehyde, by its condensation with thiobarbituric acid (TBA). The reaction mixture contained deoxyribose (2.8 mM);  $FeCl_3$  (100 mM);  $KH_2PO_4$ -KOH buffer (20 mM, pH 7.4); EDTA (100 mM);  $H_2O_2$  (1.0 mM); ascorbic acid (100 mM) and various concentrations of the MeAc in a final volume of 1 ml. Ferric chloride and EDTA (when added) were premixed just

before addition to the reaction mixture. The reaction mixture was incubated at 37°C for 60 min. After incubation at 37°C for 1 hr, 1.0 ml of 2.8% trichloroacetic acid (TCA) and 1.0 ml of 0.6% aqueous solution of TBA were added to 0.5 ml of MeAc; test tubes were heated at 95°C for 15 min to develop the color. After a cooling period, thiobarbituric acid reactive substances (TBARS) formation was measured spectrophotometrically at 532 nm against an appropriate blank. The hydroxyl radical-scavenging activity was determined by comparing absorbance of the control with that of the test compounds.

In terms of non-enzymatic hemoglycosylation, the antioxidant activity of the extract was investigated by estimating the degree of non-enzymatic hemoglobin glycosylation, measured colorimetrically. In order to find out the best condition for hemoglobin glycosylation, 60 mg/100ml of hemoglobin was incubated with 0.01 M phosphate buffer (pH 7.4) in the presence of 2g/100 ml glucose for 72 hrs. The assay was performed by adding 1 ml of glucose solution, 1 ml of hemoglobin solution and 1 ml of gentamycin (20 mg/100 ml) into 0.01 M phosphate buffer (pH 7.4) and MeAc 0.5 and 1 µg/ml. The mixture was incubated in the dark at room temperature for three days. The degree of glycosylation of hemoglobin in the presence of different concentrations of extract and their absence were measured colorimetrically at 520 nm.<sup>24</sup> The activity of MeAc was compared with positive control  $\alpha$ -Tocopherol.

## Results

In the Preliminary phytochemical screening; methanolic extract of *A. caudatus* showed the presence of flavonoids, saponins, glycosides, terpenoids, aminoacids, alkaloids, carbohydrates, phenolic compounds and proteins. While for the determination of total phenolic content, the phenolic compounds may contribute directly to antioxidant activity. The methanolic extract of *A. caudatus* was found to contain 48% total phenolic content.

In the study, the Alpha Amylase inhibition assay by CNPG3 revealed that the methanol extract of *A. caudatus* showed significant inhibition of  $\alpha$ -amylase enzyme activity. MeAc at 10, 50, 100µg/ml concentration showed 44.01±0.12, 65.56±0.18 and 74.98±0.11 percentage inhibition of  $\alpha$ -amylase activity respectively and IC<sub>50</sub> value was found to be 19.233 µg/ml.

However, Acarbose was used as reference standard, and showed 31.52±0.1, 72.49±0.14 and 82.92±0.09 percentage inhibition of  $\alpha$ -amylase activity at concentrations of 0.1, 0.5 and 1.0 µg/ml; the calculated IC<sub>50</sub> value was found to be 0.312 µg/ml. (Table 1)

**Table 1:** Inhibitory activity of methanol extract of *Amaranthus caudatus* Linn against  $\alpha$ -amylase(n=3)

Sample	Concentration (µg/ml)	% Inhibition	IC <sub>50</sub> (µg/ml)
Acarbose	0.1	31.53±0.14	0.312
	0.5	72.49±0.09	
	1.0	82.92±0.12	
MEAC	10	44.01±0.12	19.233
	50	65.56±0.18	
	100	74.98±0.09	

DPPH, which is a stable free radical with purple color, the intensity was measured at 510 nm spectrophotometrically to determine DPPH radical scavenging activity. The MeAc reduced DPPH in to 1,1-diphenyl-2-picryl hydrazine, a colorless compound. For Superoxide radical scavenging activity, the superoxide radicals generated from dissolved oxygen by PMS=NADH coupling can be measured by their ability to reduce nitroblue tetrazolium. The decrease in absorbance at 560 nm with the MeAc is shown in the Table 2, the IC<sub>50</sub> value MeAc was 62.5±2.1 µg/ml.

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interact with oxygen to produce nitrite ions. The MeAc had significantly scavenged the generated nitric oxide radicals with an IC<sub>50</sub> value of 67.5±2.2 µg/ml, (Table 2). The ABTS radical scavenging activity of MeAc was measured spectrophotometrically at 734 nm. Upon Interaction with various concentrations of the extract, the absorbance of the ABTS<sup>+</sup> radical cation was decreased dose dependently and the IC<sub>50</sub> value of MeAc was 48.75±1.1 µg/ml, (Table 2).The Hydroxyl radical scavenging activity assay shows the abilities of the extract to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe<sup>3+</sup>-EDTA-ascorbic acid and H<sub>2</sub>O<sub>2</sub> reaction mixture. The IC<sub>50</sub> value of MeAc in this assay was 88.50±1.8 µg/ml. (Table 2)

The effect of MeAc on hemoglycosylation was demonstrated by the degree of glycosylation of hemoglobin in the presence of methanol extract of *A. caudatus* and their absence was measured by means of a colorimetric method. MeAc exhibited 36.19% and 56.07% inhibition of hemoglycosylation at 0.5 and 1 mg/ml concentrations and the positive control standard  $\alpha$ -Tocopherol showed 61.53% and 86.6% inhibition at 0.5 and 1 µg/ml concentrations respectively. (Table 3)

**Table 2:** Antioxidant activities of methanol extract of *Amaranthus caudatus* Linn (IC<sub>50</sub> values) (n=3)

Plant extract	DPPH Scavenging (mg/ml)	SOD Scavenging (mg/ml)	ABTS Scavenging (mg/ml)	Nitric oxide scavenging activity	Hydroxyl Scavenging (mg/ml)
MEAC	77.5±0.4	62.5±2.1	48.75±1.1	67.5±2.2	88.50±1.8

**Table 3:** Effect of methanol extract of *Amaranthus caudatus* on hemoglycosylation

S.No.	Sample	Concentration (mg/ml)	% Scavenging
1	Methanolic extract of <i>A. caudatus</i>	0.5	36.19
		1	56.07
2	$\alpha$ -Tocopherol	0.5	61.53
		1	86.6

## Discussion

In living systems, free radicals are constantly generated and they can cause extensive damage to tissue and biomolecules leading to various disease conditions, especially degenerative diseases, and extensive lysis.<sup>25</sup> Many synthetic drugs protect against oxidative damage but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplements and traditional medicines.<sup>26,27</sup> Recently, many natural antioxidants have been isolated from different plants materials.<sup>28,29</sup> Phenolic constituents are very important in plants because of their scavenging ability towards their hydroxyl groups.<sup>30</sup> A number of studies have focused on the biological activities of phenolic compounds, which are potential antioxidants and free radical scavengers. It has been suggested that up to 1 g of poly phenolic compounds ingested daily have inhibitory effects on mutagenesis and carcinogenesis in humans. This activity is believed to be mainly due to their redox properties,<sup>31</sup> which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposition of peroxides.

The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability.<sup>32</sup> Free radicals are known to be a major factor in biological damages and DPPH has been used to evaluate the free radical scavenging activity of natural antioxidants.<sup>33</sup> DPPH; which is a radical itself with a purple color, changes in to a stable compound with a yellow color by reacting with an antioxidant and the extent of the reaction depends on the hydrogen donating ability of the antioxidant.<sup>34</sup> The reduction capability of the DPPH radical is determined by the decrease in its absorbance at 510 nm, induced by antioxidants. In order to evaluate the antioxidant potency through free radical scavenging by the test sample, the change of optical density of DPPH radicals was monitored. Table 1 shows the decrease in absorbance of DPPH radical due to the scavenging ability of MeAc.

Superoxide anion is also very harmful to cellular components.<sup>35</sup> Robak and Glyglewski reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions.<sup>36</sup> The MeAc possesses free radical scavenging activity of superoxide radicals which regulate metabolites capable of signaling and communicating important information to the cellular genetic machinery. As shown in Table 1, the MeAc showed strong superoxide radical activity.

It is well known that nitric oxide plays an important role in various inflammatory processes. Sustained levels of production

of this radical are directly toxic to tissues and contribute to the vascular collapse associated with septic shock, whereas chronic expression of nitric oxide radical is associated with various carcinoma and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis.<sup>37</sup> The toxicity of NO increases greatly when it reacts with superoxide radical forming the highly reactive peroxynitrite anion (ONOO<sup>-</sup>). The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide. The present study proved that the extract studied has more potent nitric oxide scavenging activity.

Proton radical scavenging is an important attribute of the antioxidants. ABTS<sup>+</sup>, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals.<sup>38</sup> The MeAc was effectively scavenged the ABTS<sup>+</sup> radicals, (Table 1). In the present study, the MeAc significantly scavenged both DPPH and ABTS radicals. Among ABTS<sup>+</sup> radicals and DPPH radicals, the MeAc showed significantly higher scavenging of ABTS<sup>+</sup> radicals when compare to DPPH radicals. This further showed the capability of the extracts to scavenge different free radicals in different systems, indicating that they may be useful therapeutic agents for treating radical related pathological damage.

Recently, an international expert committee has proposed a revision of diagnostic criteria for diabetes, recommending that HbA<sub>1c</sub> may be a better means of diagnosing diabetes than measures of glucose and hence it should be adopted as a diagnostic criterion for diabetes.<sup>39</sup> Glycation of hemoglobin has been implicated in nephropathy and retinopathy associated with diabetes mellitus. Monitoring the HbA<sub>1c</sub> in diabetic patients may improve treatment. In the normal 120 day life span of the red blood cell, glucose molecules join hemoglobin, forming glycolated hemoglobin. In individuals with poorly controlled diabetes, increases in the quantities of the glycated hemoglobin are noted. In the present study, the MeAc showed strong inhibition of hemoglycosylation.

The antidiabetic properties of plants can be evaluated by several methods; *in vitro*  $\alpha$ -amylase inhibitory activity by 2-chloro-4-nitro phenyl  $\alpha$ -maltotrioxide (CNPG3) assay is one of such models used to study the antidiabetic effect of a test drug *in-vitro*. Alpha-amylase is an enzyme found in the salivary, intestinal mucosal and pancreatic secretions, functioning in the breakdown of the  $\alpha$ -1-4-glycosidic bonds in starch. Thus, this enzyme increases the bioavailability of glucose in the blood. For a substance to be antidiabetic, it should be able to reduce the amount of glucose in the blood or increase the efficacy of insulin. It has been reported that the inhibition of  $\alpha$ -amylase reduces the bioavailability of glucose, the determination of 40 drugs that inhibit carbohydrate hydrolyzing enzymes have been proved to decrease postprandial hyperglycemia and improve impaired glucose metabolism without promoting insulin secretion in Non-insulin dependent diabetes mellitus (NIDDM) patients. Our *in-vitro* studies demonstrate an appreciable  $\alpha$ -amylase inhibitory activity with the methanol

extract of *A. caudatus*. The study suggests that the polyphenolic compounds involved in radical scavenging activities may also be involved in the  $\alpha$ -amylase inhibition.

## Conclusion

This study indicates that the whole plant of *A. caudatus* possess antioxidant properties and could serve as free radical inhibitors or scavengers. The plant also inhibits the activity of  $\alpha$ -amylase at low concentrations. Further studies need to be conducted to isolate carbohydrate hydrolyzing enzyme inhibitors.

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