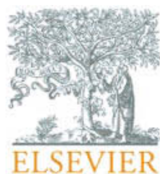

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Antioxidant and antipyretic properties of methanolic extract of *Amaranthus spinosus* leaves

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ABSTRACT

Objective: Methanolic extract of *Amaranthus spinosus (A. spinosus)* leaves was screened for antioxidant and antipyretic activities. **Methods:** Antioxidant activity was measured by 1,1-diphenyl-2-picryl-hydrazile (DPPH) free radical scavenging, superoxide anion radical scavenging, hydroxyl free radical scavenging, nitric oxide radical scavenging, 2,2′-azinobis-3-ethylbenzothiazole-6-sulfonic acid (ABTS) radical scavenging assays and total phenolic content was also determined. Antipyretic activity of methanolic extract of *A. spinosus* was measured by yeast induced pyrexia method at concentration of 200 and 400 mg/kg using paracetamol as standard drug. **Results:** Methanolic extract of *A. spinosus* showed potent antioxidant activity. The IC_{50} value was (87.50 ±3.52) μg/mL, (98.80±1.40) μg/mL, (106.25±0.20) μg/mL, (88.70±0.62) μg/mL and (147.50±2.61) μg/mL for DPPH, superoxide, hydroxyl, nitric oxide and ABTS radical scavenging activities. Methanolic extract of *A. spinosus* showed significant (P<0.01) antipyretic activity.

1. Introduction

Amaranthus spinosus (A. spinosus) Linn., (Amaranthaceae), commonly known as "Mulluharivesoppu" in Kannada, is an annual or perennial herb, native to tropical America and found throughout India as a weed in cultivated as well as fallow lands. In Indian traditional system of medicine (Ayurveda) the plant is used for analgesic, antipyretic, laxative, diuretic, digestible, antidiabetic, anti-snake venum, antileprotic, blood diseases, bronchitis, piles and anti-gonorrheal[1].

Some tribes in India apply *A. spinosus* to induce abortion. The juice of A. spinosus was used by tribal of Kerala, India to prevent swelling around stomach while the leaves are boiled without salt and consumed for 2-3 days to cure jaundice[2].

A. spinosus is also used as reported for its anti-inflammatory[3], antimalarial[4], immunomodulatory[5], anti-diabetic, anti-hyperlipidemic and spermatogenic activities[6]. The betalains in stem bark of *A. spinosus* were identified as amaranthin, isoamaranthine, hydroxycinnamates, rutin, quercetin and kaempferol glycosides[7,8]. It also contains amaranthoside, a lignan glycoside, amaricin, a coumaroyl adenosine along with stigmasterol glycoside. Betalains are well known for their antioxidant, anticancer, antiviral and antiparasitosis properties[9].

The whole plant of *A. spinosus* is used for the treatment of fever in traditional system of medicine. However, there is lack of scientific report regarding antipyretic activity of *A. spinosus*, thus this study aims to provide scientific validation for traditional uses and additionally antioxidant activity was also studied.

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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 B. V. Krishna, Department of Botany, Government first Grade College, Chickballapur, Karnataka (India). A voucher specimen (SKVCP 11) was deposited in college herbarium. The leaves are separated, shade dried and coarsely powdered. The coarse powder was subjected to extraction with methanol by soxhlet apparatus and extract was concentrated to dryness in vacuum. Methanolic extract was subjected to preliminary phytochemical screening[10].

2.2. DPPH radical scavenging activity

1,1-diphenyl-2-picryl-hydrazile (DPPH), a purple-coloured stable free radical, was reduced into a yellow-coloured diphenylpicryl hydrazine which is measured spectrophotometrically at 510 nm[11]. 200 μL different concentrations of methanolic extract of *A. spinosus*(MEAS) (50-250 μg/mL) were mixed with 50 μL DPPH (0.659 mM), then the solutions were incubated at 25 ℃ for 20 min and the absorbance was measured at 510 nm. A control reaction was carried out without the test sample. The experiments were performed in triplicate and % DPPH radical-scavenging was calculated by using the formula:

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

Where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract. IC_{50} value was also calculated. Ascorbic acid was used as standard.

2.3. Nitric oxide scavenging activity

Nitric oxide radical scavenging activity was measured by using Griess′ reagent[12]. Sodium nitroprusside (10 mM, 50 μL) in phosphate buffer saline was incubated with the 50 μL of different concentrations of MEAS (50-250 μg/mL) at room temperature for 15 min. 125 μL of Griess reagent [sulphanilamide 1%, orthophosphoric acid 2% and N-(1-naphthyl) ethylenediamine 0.1%] was added and incubated for 10 min at room temperature and the absorbance was measured at 546 nm. From the absorbance, % scavenging activity was calculated using the above formula. The experiments were performed in triplicate and IC_{50} value was calculated. Ascorbic acid was used as standard.

2.4. Superoxide anion scavenging assay

The assay was carried using method described by Mc Cord and Fridovich[13]. Superoxide radical anions are generated in PMS-NADH systems by oxidation of nicotinamide adenine dinucleotide sodium salt (NADH) and assayed by the reduction of nitroblude tetrazolium to blue diformazan. Briefly, $O_2^{\bullet -}$ were generated by adding 62.5 μL of 468 μM NADH solution, 62.5 μL 150 μM nitroblude tetrazolium (NBT) solution and 62.5 μL of 60 μM phenazine methosulphate (PMS) and different concentration of MEAS (25-250 μg/mL) solution were added to a microwell plate and incubated at room temperature of 25 ℃ 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. IC_{50} value was calculated. Butylated hydroxyl anisole was used as standard.

2.5. ABTS scavenging assay

The assay was performed as described by Auddy *et al.*,[14]. $ABTS^+$ radical cations were produced by reacting ABTS and ammonium persulphate and incubating the mixture at room temperature in dark for 16 h. 20 μL of different concentration MEAS (25-250 μg/mL) in 10 mM phosphate buffer pH 7.4 and 230 μL of ABTS+ radical solution (0.238 mM) were added. The absorbance is measured immediately at 734 nm. Ascorbic acid (ASC) was used as standard.

2.6. Hydroxyl radical scavenging activity

This activity was measured by degradation of deoxy-D-ribose method as described by Kunchandy and Rao[15]. The reaction mixture contained ascorbic acid (0.1 mM), deoxyribose (3 mM), ferric chloride (0.1 mM), ethylene diamine tetra acetic acid (EDTA) (0.1 mM), hydrogen peroxide (2 mM) in phosphate buffer (20 mM, pH=7.4), 0.3 mL of various concentrations of the MEAS (25-250 μg/mL) were added to give a final volume of 3.0 mL. After incubation for 30 min at ambient temperature, 1.0 mL of trichloroacetic acid and thiobarbituric acid (TCA-TBA) reagent (Equal volumes of TCA-2.8% and TBA-0.5% in 4 mM NaOH) was added, followed by boiling the tubes in a water bath for 30 min. The tubes were then cooled and thiobarbituric acid reactive substances (TBARS) formed and were measured spectrophotometrically at 532 nm against an appropriate blank. Mannitol was used as standard.

2.7. Animals

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

2.8. Acute toxicity studies

Methanol extracts of *A. spinosus* was studied for acute oral toxicity as the revised OECD (Organization for Economic Cooperation and development) guidelines No. 423[16]. The extract was devoid of any toxicity in rats when given in dose

up to 2000 mg/kg by oral route. Hence, for further studies, 200- 400 mg/kg doses of extract were used.

2.9. Antipyretic activity

The antipyretic activity of methanolic extracts of MEAS was evaluated using Brewer's yeast-induced pyrexia in rats as described by Loux *et al*.,[17]. Fever was induced by administration of 20 mL/kg 20 % aqueous suspension of Brewer's yeast (Saccharomyces cerevisiae) in normal saline subcutaneously. MEAS (200 and 400 mg/kg), paracetamol (150 mg/kg) and distilled water were administered orally. Rectal temperatures of all the rats were recorded at 19 h, immediately before extract, vehicle or paracetamol administration, and again at 1 h interval up to 24 h by thermal probe Eliab themistor thermometer.

2.10. Statistical analysis

Data were recorded as Mean±S.E.M. The statistical significance of differences between groups was determined by analysis of variance (ANOVA), followed by *Dunnett's* test. Differences of $P<0.05$ were considered statistically significant.

3. Results

3.1. Preliminary phytochemical screening

Preliminary phytochemical screening of methanol extracts of all the three plants reveals the presence of steroids, flavonoids, glycosides, carbohydrates, terpenoids and amino acids respectively.

3.2. DPPH radical scavenging activity

DPPH is stable free radical with purple colour, the intensity of which is measured as 510 nm spectrophotometrically. The MEAS reduce DPPH to 1,1-diphenyl-2-picryl hydrazine a colourless compound. IC50 value of MEAS was (87.50 ±3.52) μg/mL.

3.3. Superoxide radical scavenging

The results showed that MEAS showed potent scavenging property against superoxide radicals generated from dissolved oxygen to by PMS=NADH coupling can be measured by their ability to reduce NBT. The IC_{50} values of MEAS and butylated hydroxyl anisole on superoxide scavenging activity were (98.80±1.40) μg/mL and (1.46±0.17) μg/mL respectively.

3.4. Hydroxyl radical scavenging activity

This assay shows the abilities of the MEAS to inhibit hydroxyl radical-mediated deoxyribose degradation in a Fe^{3+}-EDTA-ascorbic acid and H_2O_2 reaction mixture. IC_{50} value of MEAS and mannitol were (106.25±0.20) μg/mL and (2950.00±0.20) μg/mL.

3.5. Nitric oxide radical scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interact with oxygen to produce nitrite ions. MEAS scavenge nitric oxide radicals and IC_{50} value of MEAS and butylated hydroxyl anisole were (88.70±0.62) μg/mL and (1.46±0.17) μg/mL respectively.

3.6. ABTS radical scavenging activity

Methanolic extract of A.spinosus was found to scavenge ABTS radical and IC50 values of MEAS and ascorbic acid were (147.50±2.61) μg/mL and (1.64±0.05) μg/mL.

3.7. Acute toxicity studies

The preliminary acute oral toxicity test of MEAS at the highest dose of (2000 mg/kg) didn't show any mortality or behavioural changes in the animals.

3.8. Antipyretic activity

Table 1 revealed that MEAS at 400 mg/kg showed significant ($P<0.01$) antipyretic activity 20 h after administration of Brewer's yeast till the end of the experiment, while for the 200 mg/kg dosage, significant ($P<0.05$) reduction in temperature was shown 20 h after the administration of Brewer's yeast, which extended up to 23 h.

Table 1

Effect of methanolic extract of *Amaranthus spinosus* (MEAS) on yeast induced pyrexia in rats.

Treatment	Dose (mg/kg)	Rectal temperature after yeast injection (℃)						
		0 h	19 h	20 h	21 h	22 h	23 h	24 h
Control	-	37.39±0.03	39.16±0.02	39.20±0.15	39.68±0.15	39.20±0.15	39.05±0.18	38.58±0.21
Paracetamol	150	36.93±0.41	38.60±0.56	37.33±0.21**	37.33±0.31**	37.52±0.17**	37.41±0.20**	37.26±0.16**
MEAS	200	37.30±0.11	39.00±0.27	38.25±0.37	38.11±0.30*	38.45±0.18*	38.32±0.33*	38.04±0.22
	400	37.28±0.09	39.26±0.39	38.11±0.31*	37.75±0.27**	37.65±0.20**	37.43±0.20**	37.30±0.18**

Values are in mean ±SEM; (n=6) *$P<0.05$, ** $P<0.01$ vs control.

4. Discussion

In living systems, free radicals are constantly generated and they can cause extensive damage to tissue and biomolecules leading to various diseases, especially degenerative diseases and extensive lysis. Many synthetic drugs protect tissues against oxidative damage but they have adverse side effects. An alternative solutions to the problem is to consume natural antioxidants from food supplements and traditional medicines[18,19]. Recently, many natural antioxidants have been isolated from different plants materials[20]. Phenolic constituents are very important in plants because of their scavenging ability to their hydroxyl groups. 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 human. This activity is believed to be mainly due to their redox properties[21], which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposition peroxides.

4.1. DPPH radical scavenging activity

Free radicals are known to be major factor in biological damages and have been used to evaluate the free radical scavenging activity natural antioxidants[22], DPPH is a radical itself which is purple in colour. It changes into a stable yellow compound by reacting with an antioxidant, and the extent of the reaction depends on the hydrogen donating ability of the antioxidant. The reduction capability of the DPPH radical is determined by the decrease in its absorbance of 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.

4.2. Superoxide scavenging activity

Superoxide anion is also very harmful to cellular components, Robak and Glyglewski reported that flavonoids are effective antioxidants mainly because they scavenge superoxide anions[23]. The MEAS posses free radical scavenging activity and are capable of singling and communicating important information to the cellular genetic machinery. It was shown in this study, the superoxide radical scavenging activities of the MeAc had strong superoxide radical activity.

4.3. Hydroxyl radical scavenging activity

Hydroxyl radicals are the major active oxygen species causing lipid peroxidation and enormous biological damage[24]. They were produced in this study by incubating ferric-EDTA with ascorbic acid at pH 7.4 and reacted with 2-deoxy-2-ribose to generate a malondialdehyde (MDA) like product. This compound forms a pink chromogen upon heating with TBA at low pH (32). When MEAS was added to the reaction mixture, it removed the hydroxyl radicals from the sugar and prevented the reaction. IC_{50} value indicates that the plant extract is better hydroxyl radical scavenger than the standard mannitol.

4.4. Nitric oxide scavenging activity

It is well known that nitric oxide has 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[25]. The toxicity of NO increases greatly when it reacts with superoxide radical forming the highly reactive peroxynitrite anion (ONOO.). The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. MEAS 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.

4.5. ABTS radical scavenging activity

Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals[26]. The MEAS was effective scavenger of ABTS radical.

4.6. Antipyretic activity

Pyrexia or fever is considered as a secondary impact of infection, tissue damage, inflammation, graft rejection, malignancy or other diseased state. It is the body's natural defence to create an environment where infectious agent or damaged tissue can't survive. Most of the antipyretic drugs inhibit COX-2 expression to reduce the elevated body temperature by inhibiting PGE2 biosynthesis[27]. Moreover, these synthetic agents irreversibly inhibit COX-2 with high selectivity but are toxic to the hepatic cells, glomeruli, cortex of brain and heart muscles, whereas natural COX-2 inhibitors have lower selectivity with fewer side effects[27]. Search for herbal remedies with potent antipyretic activity received momentum recently as the available antipyretics, such as paracetamol, nimusulide etc, have toxic effect to the various organs of the body. The results showed that methanol extract of three plants of amaranthus possesses a significant antipyretic effect in maintaining reducing yeast-induced elevated body temperature in rats and their effects were comparable to that of the standard antipyretic drug paracetamol.

Preliminary phytochemical study indicated the presence of alkaloids, steroids, glycosides, flavonoids, phenolic

compounds, terpenoids, proteins and carbohydrates which may be responsible for the antipyretic activity.

Our investigation suggests that methanolic extract of leaves of *A. spinosus* possess antioxidant and antipyretic properties. This study provides the scientific evidence for the traditional claim.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgment

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