RESEARCH COMMUNICATION

Antioxidant Properties of Rajgira (*Amaranthus paniculatus*) Leaves and Potential Synergy in Chemoprevention

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Abstract

In recent years there has been a substantial increase in the use of functional foods for disease control. Fruits and vegetables produce phytochemicals such as flavonoids and antioxidants which can lower oxidative stress and reduce the risk of chronic ailments like cancer. The aim of the present study was to investigate the antioxidant capacity and the possible protective effects of *Amaranthus paniculatus* leaves on the antioxidant defense system in Ehrlich's ascites carcinoma (EAC) -treated mice. Oral administration of the leaf extract at different doses caused a significant decrease in tumor volume, viable cell count and tumor weight and elevated the life span of EAC bearing mice. It also showed an improved antioxidant potential as evidenced by a significant increase in the cellular antioxidant defense system such as catalase, superoxide dismutase and reduced glutathione and also significantly reduced the levels of TBARS. The levels of RBC, hemoglobin and lymphocyte count were altered in EAC bearing mice and were reverted back to near normal levels after the treatment with the leaf extracts. Their adequate content of total phenolics and flavonoids, DPPH scavenging activity which further suggests that the extracts exert a significant protection against oxidative stress conditions.

Keywords: *Amaranthus paniculatus* - ethanol extract - Ehrlich's ascites carcinoma (EAC) - anticancer agent

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Introduction

Cancer is the leading cause of mortality worldwide, and the failure of conventional chemotherapy to effect a major reduction in mortality indicates that new approaches are critically needed. The involvement of free radicals with tumor suppressor genes and proto-oncogenes suggest their role in the development of different human cancers (Bohr et al., 2002). Hence a major portion of the current pharmacological research is devoted to anticancer drug design customized to fit new molecular targets. The identification of new antioxidants remains a highly active research area, because these agents may reduce the risk of various chronic diseases caused by ROS. Plants are some of the most attractive sources of new drugs and have been shown to produce promising results in the treatment of a number of disorders. Literature reveals that plants contain a large diversity of natural antioxidants that might serve as leads for the development of new drugs (Badami et al., 2003).

Plant derived natural products such as flavonoids, terpenes and alkaloids have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemo preventive effects. The growing interest in the substitution of synthetic food antioxidants by natural antioxidants and in health implications of antioxidants in nutraceuticals has hastened the research on vegetable sources and the screening of raw materials for identifying antioxidants. The antioxidants could attenuate this oxidative damage of a tissue indirectly by enhancing natural defenses of cell and/or directly by scavenging the free radical species (Xia et al., 2004) .One of the best approaches in search for anticancer agents from plant resources is the selection of plants based on ethno medical claims. Among the vast number of medicinal plants that are claimed to be anticancer *Amaranthus paniculatus* is the one in which systematic pharmacological studies have not been carried out to support the claim made. Hence, the present study plans to systematically evaluate to verify the claim.

Amaranthus paniculatus (Amaranthaceae) is commonly called as "Rajgira" in India and "Amaranth" in English. In traditional medicine it is a valuable plant cultivated by native Americans for several thousand years and now cultivated worldwide. The plant has widespread uses in folk and traditional medicines for respiratory infections, vision defects, tuberculosis, fleshy tumors, liver complaints and inflammations. In ayurveda, the decoction of leaves was used for chest afflictions, gastroenteritis and the seeds are applied to sores. *Amaranthus paniculatus* is recommended as good food with medicinal properties and its leaves are utilized for use for human as well as for animal food. It has been shown that amaranth leaves are an excellent source of protein, with its maximal accumulation

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in the blossoming phase. However, it has been rated considerably higher in minerals, such as calcium, iron, phosphorous and caretonoids than most medicinal plants (Bhatia & Jain, 2003). In Ayurvedic medicine Amaranth seeds and leaves have been used effectively as an astringent for stopping diarrhea, bloody stools and urine, and excessive menstruation. It is an excellent wash for skin problems such as acne and eczema to psoriasis and hives. It is used as a douche for vaginal discharges; as a mouthwash for sore mouths, gums, teeth and throat and as an enema for colon inflammation and rectal sores (Vietmeyer, 1983).

Amaranthus paniculatus is the world's most nutritious plant. It is best for eye related problems. It protects hair and improves overall health. It is used for stomach problems especially constipation. The aqueous extract of *Amaranthus paniculatus* leaves was found to have radioprotective effect in whole body gamma radiation (Krishna & Kumar, 2005). It is also said to overcome the problems of psychological stress and its effect has been tested in stress – induced memory dysfunction and also improves learning after radiation stress (Bhatia & Jain, 2003). High content of nutritional critical lysine, methionine, carotenoids, Vitamin C and proteins have been indentified in *Amaranthus paniculatus* leaf extracts (Maharwal et al., 2003). However, so far there is no pharmacological validation for the anticancer activity in the leaves of this plant. Therefore, based on its traditional use and significance, the present study was undertaken to investigate the anticancer efficacy against EAC tumor model.

Materials and Methods

Plant Material and Extraction

The plant material *Amaranthus paniculatus* was collected from Tanjore District, Tamil nadu, India and authenticated by the Botanical survey of India, Coimbatore, Tamilnadu, India, where a voucher specimen has been preserved for future reference (BSI/SC/5/23/Tech-915). The leaves of *Amaranthus paniculatus* were shade dried, powdered, and extracted (150g) with ethanol and water in the ratio 1:4 in a Soxhlet extractor for 18-20h. The extracts were concentrated to dryness under reduced pressure and controlled temperature. The crude extracts obtained were a dark brown solid weighing 36g with the yield of the extracts as 18.01% respectively. The extracts were preserved in a refrigerator at 4°C until further use.

Chemicals

5-Fluorouracil was obtained from Ranbaxy Laboratories, Ltd., India. All other chemicals used were of analytical grade.

Phytochemical Screening & Total Phenolics & Flavonoids Content.

The leaves were subjected to preliminary phytochemical screening to identify the presence of various phytoconstituents present in the extract (Kokate et al., 1997). Antioxidant compounds generally contain phenolic group(s) and hence, the amounts of phenolic compounds in the extracts of the leaves were estimated by using Folin–Ciocalteau reagent (Chandler & Dodds, 1993). In a series of test tubes, 0.4 mL of the extract in methanol was taken, mixed with 2 mL of Folin–Ciocalteau reagent and 1.6 mL of sodium carbonate. After shaking, it was kept for 2 h and the absorbance was measured at 750 nm using a Shimadzu-UV-1800 spectrophotometer. Using gallic acid monohydrate, a standard curve was prepared. The linearity obtained was in the range of 1–10 µg/mL. Using the standard curve, the total phenolic compounds content was calculated and expressed as Gallic acid equivalent in mg/g of extracts.

Flavonoids were extracted and estimated by the method where (Chang et al., 2002) an aliquot of the extract was pipetted out and evaporated to dryness. 4.0ml of vanillin reagent was added and heated for 15 minutes in a boiling water bath. The standard was also treated in the same manner. The optical density was read at 340 nm. The values are expressed as mg flavonoids/g leaf.

DPPH (2, 2-Diphenyl-1-Picrylhydrazyl) -scavenging activity

The free radical scavenging activity of the extract was measured in terms of hydrogen donating or radical scavenging ability using the stable free radical DPPH (Blois, 2002). One milliliter solution of the extract in methanol was added to 0.5 ml of 0.15 mM DPPH solution in methanol. The contents were mixed vigorously and allowed to stand at 20 °C for 30 min. The absorbance was read at 517 nm. IC_{50} value (the concentration required to scavenge 50% DPPH free radicals) was calculated. The capability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = $[(A_0 - A1/A_0) - 100]$, where A_0 was the absorbance of the control reaction and A1 the absorbance in the presence of the sample.

Tumor cells

Ehrlich's ascites carcinoma cells were obtained from Amala Cancer Research Centre, Trissur, and Kerala, India. The EAC cells were maintained by intraperitoneal inoculation of $2x10^6$ cells/mouse. EAC cells aspirated from the peritoneal activity of mice were washed with saline and were given intraperitoneally to develop ascitic tumor.

Toxicity Study

An acute toxicity study relating to the determination of LD50 was performed (Litchfield & Wilcoxon, 1949). Different groups of mice were treated with graded doses of the leaves (100 mg, 200 mg, 500 mg and 750 mg/kg) orally. One group was maintained as control and was given distilled water. The animals were observed continuously for 2 h, and then intermittently and after 24 h for 14 days. The animals were observed for behavioral, neurological and autonomic profiles.

Transplantation of tumor and treatment schedule

Healthy Swiss albino mice weighing 20±2g were procured from Tamilnadu University of Veterinary and Animal Sciences (TANUVAS) were used for the

study. The animals were housed in large polypropylene cages in a temperature-controlled room and provided with standardized pelleted feed (TANUVAS) and clean drinking water ad libitum. All the procedures described were reviewed and approved by the University Animals Ethical Committee. The animals were divided into five groups. All the animals were injected with EAC cells (0.2 ml of $2x10^6$ cells/mouse) intraperitoneally except for the normal group for the development of ascites tumor (Kuttan et al., 1998). After 24 hrs of tumor inoculation the leaf extracts of *Amaranthus paniculatus* of variable doses ,100 mg/kg and 200 mg/kg were administered orally once daily for 14 days to groups 3 and 4. .After the last dose and 24 hrs fasting, six mice from each group were sacrificed. The antitumor efficacy of plant extracts was compared with the standard which served as the fifth group. (5-Fluorouracil, 20mg/kg/day i.p).

Tumor growth response

The anticancer effect of EEAP was assessed by change in tumor weight, tumor volume, viable and nonviable cell count, mean survival time (MST), and percentage increased life span (%ILS). Median survival time (MST) for each group was noted and antitumor activity of the test compounds were compared with that of control group by measuring ILS (Gupta, 2000).

MST = (Day of first death +Day of last death)/2
Increase in Life Span (%) = MST of treated group/MST of control group

The viability and nonviability of the cell were checked by trypan blue assay. The cells were stained with trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were viable and those that took the dye were nonviable. These viable and nonviable cells were counted.

Determination of Blood Components

At the end of the experimental period, the next day after an overnight fasting blood was collected from freely flowing tail vein and used for the estimation of hemoglobin (Hb) content, red blood cell (RBC) count, white blood cell (WBC) count and differential count of WBC by standard procedures.

Determination of Enzymic Antioxidants& TBARS level in liver

After the collection of blood samples, the mice were sacrificed. Then the liver was excised, rinsed in ice cold normal saline followed by ice-cold 10% KCl solution, blotted, dried and weighed. A 10% w/v homogenate was prepared in ice-cold KCl solution and centrifuged at 1500 rpm for 15 min at 4°C.The supernatant thus obtained were used for the estimation of thio-barbituric acid substances(TBARS)(Ohkawa et al., 1979) glutathione(GSH) (Ellman,1979) superoxide dismutase (SOD)(Kakkar et al.,1984) catalase(CAT)(Luck,1974) and total protein(TP)(Lowry et al.,1951).

Statistical analysis

All the parameters studied were subjected to statistical treatment using Sigma Stat statistical package (Version 3.1). The data were expressed as mean ±S.D (n=6) where 'n' represents the no of samples. One-way ANOVA, followed by post-hoc analysis using Fischer's LSD was adopted to all the parameters under study to test the level of statistical significance. The difference was considered significant if $p<0.05$.

Results

Phytochemical Screening & Total Phenolics & Flavonoids Content.

EEAP was positive for alkaloids, phenolics, flavonoids and tannins. In the present study the total phenolic compounds of the extracts were expressed as Gallic acid equivalent in mg/g and the flavonoids were expressed as quercetin equivalent in mg/g of plant material. The amount of total phenolic and flavonoid content was found to be 25.23mg/g and 11.60mg/g respectively. EEAP extract had the highest phenolic content followed by the flavonoid content.

DPPH free radical scavenging activity

The free radical scavenging activity of EEAP extracts was assessed by the stable free radical DPPH.The amount of sample needed to decrease the initial DPPH concentration by 50% is a parameter widely used to measure antioxidant activity. The EEAP leaf extracts had an IC_{50} of 68.184±0.51μg/ml. The scavenging effects of EEAP leaf extracts on the DPPH radical illustrates that the leaf extract significantly reduced DPPH radicals. In comparison, the positive control, Trolox® had an IC_{50} of 2.16±0.12μg/ml.

Toxicity Study

In acute toxicity, no gross behavioral changes and mortality was observed up to a dose level of 400 mg/kg body weight. The LD_{50} value of EEAP leaves was found to be >2g/kg body weight of mice indicating that it is very less toxic to the animal.

Tumor growth response

Treatment with EEAP leaves at the dose of 100 and 200 mg/kg body weight increased the life span (ILS) and nonviable cell count and reduced the tumor volume, tumor

Table 1. Effect of *Amaranthus Paniculatus* Leaves on Tumor Growth Response

Parameters	EAC control	Treated		
	II	III	IV	V
Volume	4.9±0.91	3.8±0.83	2.8±0.53	1.9±0.48**
Weight (g)	5.5±0.42	3.6±0.67	2.4±0.21	1.7±0.36**
MST (days)	23.2±1.32	33.8±2.50	40.3±2.20	48.4±1.50**
%ILS	00.00	43.24	75.32	109.51**
Viable Tumour cell count (cells/ml)	10.20±0.31	7.30±0.03	4.80±0.09	3.10±0.19**
Non Viable Tumour cell count (cells/ml)	0.40±0.03	0.70±0.05	0.90±0.03	1.40±0.05**

Values are expressed as mean ± S.D, n=6. III - leaf extract (100mg/kg) +EAC; IV- leaf extract (200mg/kg) +EAC; V- Standard 5-FU (20mg/kg) +EAC. *P<0.001, **P < 0.01

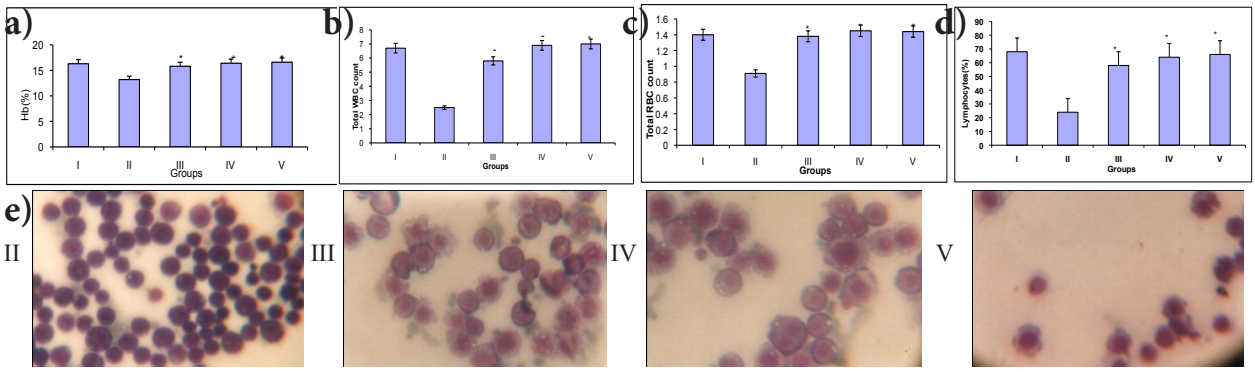

Figure 1. Effect of *Amaranthus Paniculatus* Leaf Extract on Hematological Parameters of EAC Treated Mice. a) Hemoglobin content (%) b) Total RBC count (x10^6 cells/ml) c) Total WBC count (x10^{6}cells/ml) d) Lymphocytes (%) e) II, III, IV and V. The data were expressed as mean ±S.D (n=6) where 'n' represents the no of samples. One-way ANOVA, followed by post-hoc analysis using Fischer's LSD was adopted to all the parameters under study to test the level of statistical significance. *P<0.001, **P < 0.01: between extract treated groups and EAC. e) EAC tumor smears of control & treated mice showing degenerative changes in form of membrane blebbing, vacuolated cytoplasm and reduction in staining intensity.

Table 2. Levels of Lipid Peroxidation and Antioxidant Enzymes in Liver

Parameters	EAC control		Treated		
	I	II	III	IV	V
LPO (nmol MDA/mg protein)	0.95±0.70	1.41±0.50	1.02±0.20	0.97±0.80**	0.97±1.60**
GSH (mg/g)	2.33±0.21	1.29±0.32	2.62±0.15	2.39±0.32**	2.13±0.51**
SOD (U/mg protein)	4.24±0.05	2.12±0.07	3.19±0.06	4.52±0.06**	4.31±0.09**
CAT (U/mg protein)	25.4±0.21	11.9±0.92	25.9±0.32	26.9±0.42**	24.9± 0.52**

Values are expressed as mean ± S.D, n=6. Groups I-Normal; II- EAC control; III- EEAP, extract+EAC (100mg/kg); IV- EEAP extract+EAC (200mg/kg); V- Standard 5-FU +EAC (20mg/kg). *P<0.001: between normal and EAC; **P < 0.01: between extract treated groups and EAC

weight and viable tumor cell count significantly when compared to that of EAC control group (Table 1).

Levels of Blood Components

As shown in Fig-1, hemoglobin content and RBC count decreased significantly and the total WBC count increased significantly in EAC group as compared to normal group. Treatment with EEAP restored the hematological parameters to more or less normal values. The number of RBC count and hemoglobin content also increased, while the WBC and the differential count decreased as compared to that of EAC control.

Antioxidant Enzyme Response in Liver

The levels of TBARS were significantly increased in the EAC treated animals when compared to the normal group. The treatment with EEAP of leaf at 200 mg/kg body weight reversed these changes towards the normal levels (Table-2) and found to be significant. Significant decrease in the levels of SOD,GSH and CAT was observed in EAC control group which was reversed significantly towards normal in the EEAP treated group at 200 mg/kg dose level. Almost similar results were observed with 5-FU treatment

Discussion

Several studies have shown the possible benefits of antioxidants from plant sources in altering, reversing, or forestalling the negative effects of oxidative stress. Antioxidant containing foods or natural antioxidants may be used as effective substances for the prevention of diseases of higher age (Paredes-Lopez et al., 2010). Adequate amounts of phenolics, flavonoids and ascorbic acid found in the leaves of *A. paniculatus (Linn.)* denotes from the dietetic point of view that food intake with high flavonoid content is an important element in the prevention of diseases. Polyphenols, ascorbates and flavonoids are known to be responsible for antioxidant and free radical scavenging potentials. The total flavonoids and the phenolic contents proved the extracts to possess higher values of antioxidant phytochemicals which suggest that the leaves can be exploited as an important source of natural antioxidants with health protective potentials. Earlier studies have also shown strong antioxidant and free radical scavenging activities of *Amaranthus paniculatus* seeds (Bhatia & Jain, 2003).

Cancer chemoprevention refers to the use of agents to inhibit, reverse or retard tunorigenesis.Several experimental and epidemiological studies documented the chemo preventive activities of many herbal plants and their bioactive constituents. Antioxidant containing foods may exert the carcinogenic potential by modulating carcinogen detoxification, inhibiting lipid peroxidation, or by enhancing antioxidant defence mechanism (Davis & Kuttan, 2001).In the present study the chemopreventive potential of EEAP in EAC-bearing mice was monitored by observing the status of tumor volume, percentage of life span, levels of blood components and by determining the status of antioxidants in liver and lipid peroxidation.

The present study was carried out to evaluate the anticancer activity of EEAP in EAC-bearing mice. The present study showed that EEAP significantly increased

the life span than that of EAC control. The reliable criteria for judging the value of any anticancer drug are prolongation of life span and decrease of WBC count (Gupta et al., 2004). The results demonstrate the indirect inhibitory effect of the extract in EAC bearing group, which is probably mediated by the enhancement and deactivation of either macrophages or cytokine production. The result of the present study has shown the anticancer effect of the ethanolic extract of EEAP against EAC in swiss albino mice. Furthermore a significant enhancement of mean survival time and decrease in tumor volume suggest the delaying impact of EEAP in cell division. Cytological studies of Leishman-stained tumor cell smear also have revealed a decrease in the number of mitotic cells following EEAP treatment when compared with that of EAC control (Figure 1).

Myelosuppression is a frequent and major complication of cancer chemotherapy (Maseki et al., 1981). Present study indicates that EEAP has significantly enhanced the hemoglobin level and RBC count when compared to that of the EAC control. These observations assume great significance as anemia is a common complication in cancer and the situation aggravates further during chemotherapy since a majority of antineoplastic agents exert suppressive effects on erythropoiesis and there by limiting the use of drugs.Ascitic fluid is essential for tumor growth, since its constitutes a direct nutritional source for tumor cells(Prasad & Giri,1994). Reduction in viable cell count and increased non-viable cell count towards normal in tumor host suggest antitumor effect against EAC cell in mice. These results could indicate either a direct cytotoxic effect of EEAP on tumor cells or an indirect local effect which may involve macrophage activation and vascular permeability inhibition.

DPPH scavenging activity has become routine in establishing the antioxidant activity of herbal extracts and phytochemicals.The antioxidants react with DPPH, a purple colored stable free radical and convert it into a colorless α-α-diphenyl-β-picryl hydrazine (Naik et al., 2003). In several studies it was concluded that the plant flavonoids which show antioxidant activity *in vitro* also function as antioxidants *in vivo* (Geetha & Vasudevan, 2004). From the present study it is evident that the antitumor effect of EEAP may be due to the antioxidant and the free radical quenching property of the phytoconstituents of EEAP.

It was observed that tumor cells produced more peroxides when they proliferate actively after inoculation of tumor and also known to affect many functions of the vital organs, which indicated the intensification of oxygen free radical production. The elevation of lipid per oxidation is also known to be associated with cancer. LPO results in the formation of several toxic byproducts such as 4-hydroxy nonenal and malondialdehyde which form adducts with DNA and induce mutagenicity, carcinogenicity and apoptosis (Navvaro et al., 2001).The reduction in amount of TBARS or MDA equivalents in the Amaranthus administered mice suggests that it may either scavenge or reduce the free radicals generation which clearly explains that how the constituents of Amaranthus may deplete the augmentation of LPO.It has been reported that *A. paniculatus (Linn.)* contains natural sources of carotenoids, Vitamin C, folate, folic acid, high level of nutritional critical lysine and methionine, protein content and promising oil composition with regard to polyunsaturated fatty acid (Maharwal et al., 2003). Antioxidant free radical scavenging compounds such as beta-carotene and Vitamin C can protect DNA from oxidizing radical reactions. Beta-carotene has an excellent antioxidant property, a potent free radical quencher, singlet oxygen scavenger and lipid antioxidant .Vitamin C is considered to be the most important antioxidant in extracellular fluids(Bhatia, 1998) and has many cellular activities of an antioxidant nature to protect membranes against peroxidation. Hence it can be stressed that oxidative stress may be ameliorated by phytoantioxidants present in the leaf extract of Amaranthus.Cells with enzymatic mechanism also play an important role in the elimination of free radicals. SOD, CAT and glutathione peroxides are involved in the clearance of superoxide and hydrogen peroxide. Decrease in SOD, GSH and CAT activities described in tumors is regarded as markers of malignant transformation (Kavitha & Manoharan,2006). Therefore the significant elevation of SOD ,CAT and GSH and significant reduction in LPO by the extract treatment confirms the potent free radical quenching property and chemopreventive activity of EEAP.

The preliminary phytochemical studies indicated the presence of flavonoids, saponins, and tannins in the *Amaranthus paniculatus* leaf extract. Many such compounds are known to possess potent antitumor properties (Kintzios et al., 2006). Flavonoids have been shown to possess antimutagenic and antimalignant effects (Blois, 2002). The potent cytotoxicity and antitumor properties of the *Amaranthus paniculatus* may be due to the presence of any of these phytoconstituents especially flavonoids.The results from present study are consistent with the earlier reports which supports its antioxidant property and scavenging mechanism of free radicals (Bhatia & Jain, 2003; Maharwal et al., 2003).

In conclusion our present study suggests that the extracts of *Amaranthus paniculatus* leaves possess chemopreventive potential and can therefore be exploited for antitumor agents.

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