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Research Article

Determination of Total Phenolic, Tannin, Flavonoid Contents and Evaluation of Antioxidant Property of *Amaranthus tricolor* (L)

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ABSTRACT
Free radicals or reactive oxygen species are involved in various pharmacological conditions. As synthetic antioxidants possess numerous adverse health effects, the medicinal plants possessing antioxidant components can be used to prevent harmful effects of reactive oxygen species. In the present study leaves of *Amaranthus tricolor* Linn were used to prepare chloroform (CEAT), methanolic (MEAT) and aqueous (AEAT) extracts, analyze the presence of phytochemicals and evaluation of *in-vitro* antioxidant property. Quantitative determination of phenols, tannins and flavonoids in leaves *A.tricolor* was carried out using spectrophotometric methods. The antioxidant activity was performed by DPPH, p-NDA radical scavenging methods for different extracts of the plant. The plant species showed that methanolic extract (MEAT) on higher concentration possess better antioxidant potential when compared with reference standard ascorbic acid. The plant extracts exhibited strong antioxidant DPPH radical scavenging activity with the IC_{50} values 290, 657, 830 & 130µg/ml of MEAT, CEAT, AEAT & ASA respectively. In scavenging hydroxyl radical by p-NDA method the MEAT showed maximum activity, CEAT showed moderate and AEAT showed minimum activity. The strongest antioxidant activity of MEAT could be due to the presence of flavonoids and phenols.

Keywords: Amaranthus tricolor, phenols, flavonoids, DPPH, p-NDA.

INTRODUCTION

Phenolic compounds are one of the main secondary metabolites derived from pentose phosphate, shikimate and phenyl propanoid pathways in plants[1-3]. They are commonly found in non-edible and edible plants and posses' numerological biological effects[4]. They are essential for reproduction and growth of plants. Phenolic compounds posses' redox properties, which allows acting as hydrogen donors, reducing agents, metal chelators and singlet oxygen quenchers and hence they are antioxidants. Traditionally fruits, vegetables, tea and spices are used as antioxidants. Some of these are commercially exploited either as nutritional supplements or antioxidant additives.

The most common group of poly phenolics are flavonoids that are ubiquitously found in leaves, flowering tissues, woody parts such as stems and bark[4]. They posses free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action because of antioxidant activity[5,6]. Antioxidant is an important property which posses the ability of protecting organisms from damage caused by free radical-induced oxidative stress[7]. The antioxidant activity of phenolics is because of their redox property that allows them to act as hydrogen donors, reducing agents, metal chelators and single oxygen quenchers[8]. Flavonoids are also known for their free-radical scavenging and antioxidant activities

Biological metabolism involved in various processes produce reactive oxygen species or free radicals which are harmful to living cells. Excess accumulation of these radicals may cause asthma, cancer, liver diseases, cardiovascular diseases, muscular degeneration and inflammatory processess[9], resulting into oxidative stress. Oxidative stress is defined as imbalance between oxidants and antioxidants and causes damage in all types of biomolecules such as DNA, RNA, nucleic acid and protein[10]. Hence, the balance between reactive species or free radicals and antioxidants is believed to be a critical concept for maintaining a good biological system. Antioxidants act as free radical scavengers, reducing agents, quenchers of singlet of age-related diseases which could be due to the presence of various antioxidant compounds oxygen molecule, and activators for antioxidative enzyme to suppress the damage induced by free radicals in biological system. Many researchers found that consumption of plant products[11] reduces the mortality, especially, phenolics, which are the most reactive compounds. Antioxidants present in plant products help in the stimulation of cellular defense system and biological system against oxidative damage.

A.tricolor (Amaranthaceae) is an ornamental plant commonly known as ''Red amaranth'' or "Joseph's coat" cultivated throughout South-East Asia and many tropical countries. It is highly nutritious and hence extensively used as green leafy vegetable. *A. tricolor* L. is one of the traditional medicines used in many folk claims and the plant has been extensively used in ayurveda and sidda for

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Table 1: Preliminary phytochemical screening of MEAT, CEAT & AEAT.

Name of the Test	MEAT	CEAT	AEAT
Carbohydrates	+	+	+
Proteins	+	+	+
Amino acids	+	+	+
Steroids	+	+	+
Cardiac glycosides	+	+	+
Flavonoids	+	+	+
Alkaloids	+	+	+
Tannins & phenolic compounds	+	+	+

Table 2: Total phenolic, Non-tannin, Tannin & Flavonoid content Present in MEAT, CEAT & AEAT.

Parameter	Unit	MEAT	CEAT	AEAT
Total phenolic content	mg of GAE/gm of extract	19.4	16.7	13.8
Non tannin content	mg of GAE/gm of extract	12.2	10.8	9.3
Tannin content	mg of GAE/gm of extract	7.2	5.9	4.5
Flavonoid	mg of rutin/gm of extract	4.5	3.8	3.2

treating menorrhagia, diarrhea, dysentery, haemorrhagic colitis, bowel hemorrhages, cough and bronchitis. It is also used externally as an emollient poultice or a mouth wash to treat ulcerated conditions of the throat and mouth[12].

The ethno-botanical properties of the plant were reported as astringent[13], hepatoprotective[14], antinociceptive and anti-inflammatory activities[15], *in-vitro* antioxidant, anti-amylase, anti-arthritic and cytotoxic activity[16].The root decoction along with *Cucurbita moschata* is used to control haemorrhage following abortion[17]. The plant decoction is taken internally to strengthen the liver and to improve vision. Scientific study on the plant suggests that it may inhibit calcium retention[18]. It was also reported to posses' antibacterial activity against urinary tract pathogens of clinical origin[19]. The authors had undertaken the present work to explore the antioxidant property of leaves of *A. tricolor*.

In vitro antioxidant activity of plant extracts were carried out using DPPH and p-NDA methods. DPPH (2, 2-Diphenyl-1-picrylhydrazyl) is a rapid, simple and inexpensive method to measure antioxidant capacity of food. It involves the use of the free radical (DPPH), which is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity[20]. The DPPH assay method is based on the reduction of DPPH, a stable free radical[21]. The free radical DPPH with an odd electron gives a maximum absorption at 517 nm (purple colour). When Antioxidants react with DPPH, which is a stable free radical becomes paired off in the presence of a hydrogen donor (e.g., a free radical scavenging antioxidant) and is reduced to the DPPHH and as consequence the absorbance's decreased from the DPPH[22]. Radical to the DPPH-H form, results in decolorization (yellow colour) with respect to the number of electrons captured[23]. More the decolorization more is the reducing ability. This test has been the most accepted model for evaluating the free radical scavenging activity of any new drug[24]. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Diphenyl picryl hydrazine; non radical) with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present)[25].

MATERIALS AND METHODS

Plant material

The plant *Amaranthus tricolor* L was collected, identified and authenticated by BSI, Coimbatore. The healthy leaves were shade dried and powdered using electric blender to get a coarse powder.

Extraction

The powdered leaf material was extracted by successive solvent extraction using soxhlet apparatus. The solvents were selected according to the increasing order of polarity. Different solvents like chloroform (CEAT), methanol (MEAT) & water (AEAT) were used for extraction and the extracts were concentrated and preserved in a desiccator for further study.

Phytochemical screening

The phytochemical screening for the crude extracts of *Amaranthus tricolor* was carried out by standard protocols[26,27]. The presence of alkaloids, glycosides, saponins, carbohydrates, proteins, aminoacids, phenolic compounds, flavonoids, steroids, tannins was analyzed.

Determination of Total Phenolic Content

The total phenolic content was determined using Folin Ciocalteau reagent. A standard calibration curve was prepared and the absorbance against concentration of tannins at 725nm was estimated spectrophotometrically. Gallic acid was used as a standard and the total phenolic content was expressed as µg/ml gallic acid equivalents (GAE). Concentration of 0.01, 0.02, 0.03, 0.04 and 0.05 mg/ml of gallic acid were prepared in methanol. Concentration of 1mg/ml of plant extract was prepared in methanol and 0.5ml of each sample were introduced into test tubes and mixed with 0.5ml of a 1N dilute Folin-Ciocalteau reagent and 2.5ml of 20% sodium carbonate. The tubes were covered with parafilm and allowed to stand for 40 minutes at room temperature and absorbance was read at 725nm spectrophotometrically[28].

Determination of Tannin Content

Tannin content was determined using insoluble polyvinyl-polypyrrolidone (PVPP), which binds tannins[29]. Briefly 1ml of extract (1mg/ml) in which the total phenolics was determined, was mixed with 100mg of PVPP, vortexed, kept for 15min at 4^0C and then centrifuged for 10 min at 3000 rpm. In the clear supernatant non-tannin phenolics were determined the same way as that of total phenolics.

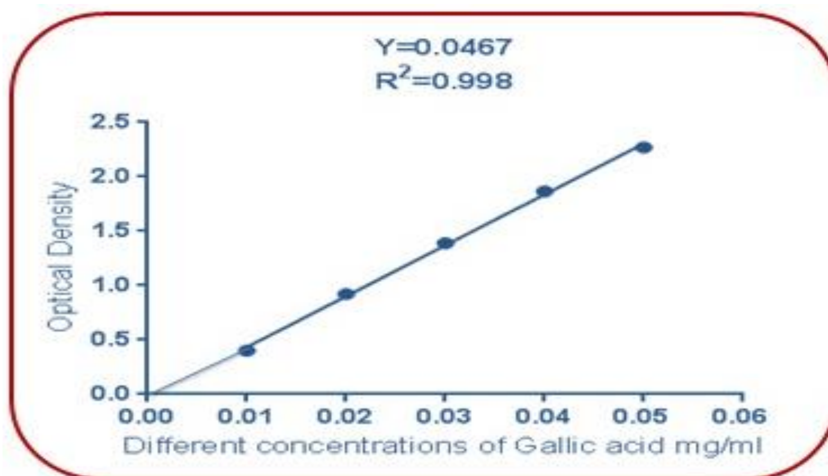

Figure 1: Standard curve of different concentrations (mg/ml) of Gallic acid and their respective optical densities at 725nm.

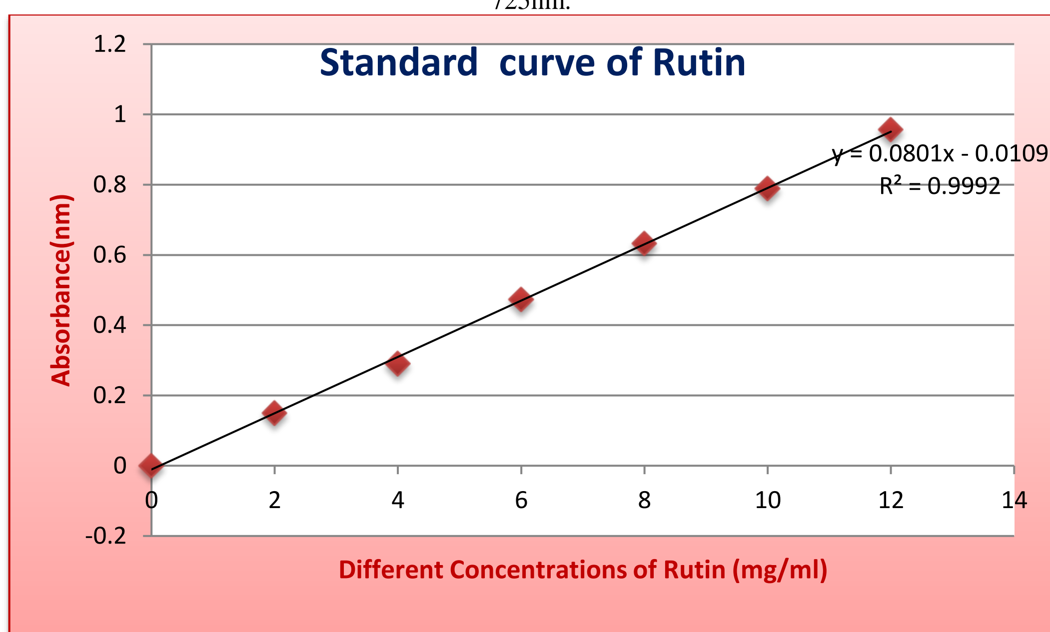

Figure 2: Standard curve of different concentrations (mg/ml) of Rutin and their respective optical density at 506nm.

Tannin content was calculated as a difference between total and non-tannin phenolic content.

Determination of Flavonoid Content

The aluminum chloride method was used for the determination of the total flavonoid content of the sample extracts[30]. Aliquots of extract solutions were taken and made up the volume 3ml with methanol. Then 0.1ml AlCl3 (10%), 0.1ml Na-K tartarate and 2.8 ml distilled water were added sequentially. The test solution was vigorously shaken. Absorbance at 415 nm was recorded after 30 minutes of incubation. Rutin was used as a standard compound in the range of 2-12 mg/ml concentration to construct a standard curve.

Free Radical Scavenging Activity (DPPH Method)

The antioxidant activity of various extracts of *A. tricolor* and ascorbic acid were assessed on the basis of radical scavenging effect on the DPPH (2, 2-Diphenyl-1-picrylhydrazyl) stable free radical. In this method different concentrations of the crude extracts of *Amaranthus tricolor* 100 to 500µg/ml concentrations of extracts were prepared with methanol. 1ml of each prepared concentration was mixed with 3 ml of DPPH (0.1 mM) solution in methanol. The test tubes were incubated for one hour at room temperature in dark and the absorbance is measured at 517nm in UV-Visible Spectrophotometer[31,32]. Ascorbic acid is used as a standard and the same concentrations were prepared as to test solution. The differences in absorbance between the test and the standard was calculated and expressed as

% scavenging of DPPH radical Scavenging effect (%) = (Ac-As)/Ac x100

Where, Ac is absorbance of control, As is absorbance of sample or standard.

p-Nitroso dimethyl aniline radical scavenging method (p-NDA)

Hydroxyl radical scavenging is measured by the inhibition of p-NDA bleaching. Hydroxyl radicals generated through Fenton reaction can bleach p-NDA specifically. Scavenging activity was measured by the extent of inhibition of bleaching in the presence and absence of the

Table 3: % inhibition of DPPH radical by MEAT, CEAT, AEAT & ASA.

Extract	Quantity in micrograms(μg/ml)					IC_{50}
	100	200	300	400	500	
MEAT	32.58±0.85	42.37±0.22	51.55±1.27	57.34±1.06	63.92±0.54	290
CEAT	11.93±0.75	17.65±0.47	26.28±0.74	33.50±0.70	39.75±0.47	657
AEAT	5.70±0.47	9.75±0.53	15.72±0.63	19.61±0.36	23.37±0.35	830
ASA	48.88±0.36	59.83±0.65	72.93±0.95	81.86±0.55	85.96±0.25	130

Values are mean ± SD of triplicates
AT: Amaranthus tricolor (L); MEAT: Methanolic Extract of AT; CEAT: Chloroform Extract of AT; AEAT: Aqueous Extract of AT; ASA: Ascorbic Acid

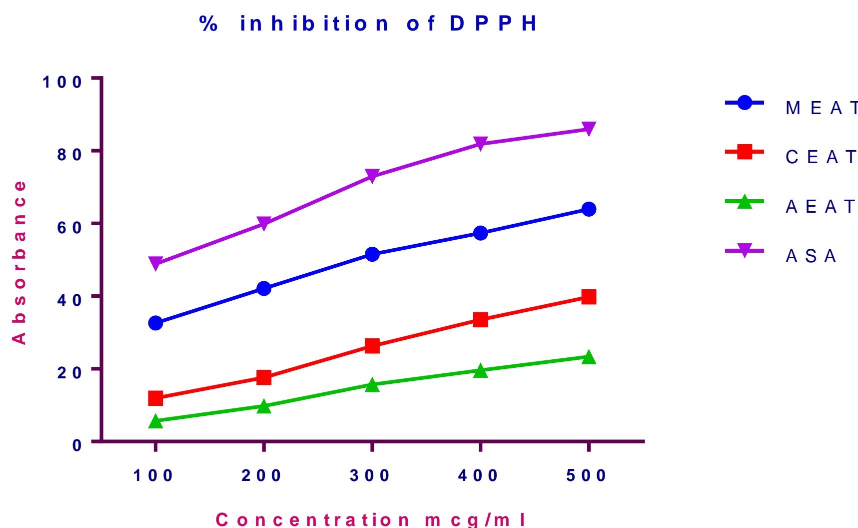

Figure 3: Graphical representation of % inhibition of DPPH radical by MEAT, CEAT, AEAT & ASA.

extract solutions[33]. In this method to the different concentrations of the crude extracts of *Amaranthus tricolor* 500 to 1000µg, respectively dissolved in distilled DMSO (or) any solvent, alcohol add ferric chloride (0.1mM, 0.5ml), EDTA (0.1mM, 0.5ml), ascorbic acid(0.1mM, 0.5ml), hydrogen peroxide(2mM, 0.5ml) and p-nitroso dimethyl aniline (0.01mM, 0.5ml) in phosphate buffer (P^H 7.4, 20Mm) to make a total volume of 3ml.The absorbance was measured at 700nm. Increased absorbance of the reaction mixture indicated increased antioxidant activity. Ascorbic acid was used as a reference standard. The percentage of absorbance by this method can be calculated by using the following formula.

p-NDA radical scavenging activity(%) = (Ac-As)/Ac x100

Where, Ac is absorbance of control, As is absorbance of sample or standard.

RESULTS AND DISCUSSION

The present study revealed the presence of carbohydrates, proteins, aminoacids, steroids, cardiac glycosides, alkaloids, tannins and flavonoids. The results of preliminary phytochemical screening was reported in table:1. The presence of various phytoconstituents in plant parts received attention because of their biological activities. The presence of tannins and flavonoids in the plants exhibited various biological activities like antibacterial, antifungal, antioxidant and anthelmintic. The total phenolic content in MEAT (19.4) is maximum, CEAT (16.7) moderate and AEAT (13.8) is minimum. The tannin content is 7.2, 5.9, 4.5 mg of GAE/gm of extract in MEAT, CEAT and AEAT respectively. The total phenolic content and tannin content were estimated through the standard calibration curve of gallic acid (Fig:1).

Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenols. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties. The flavonoid content in MEAT is 4.5, CEAT is 3.8 and AEAT is 3.2 mg of rutin/gm of extract. The flavonoid content was estimated through the standard calibration curve of rutin (Fig:2).The results of total phenolics, tannin and flavonoid contents were represented in table:2.

The plant showed better antioxidant potential when compare to standard ascorbic acid by DPPH scavenging assay method. The leaf extracts strongly scavenge in dose dependent manner (Fig:3). The IC_{50} values of MEAT, CEAT, AEAT & ASA was found to be 290, 657, 830 & 130µg/ml respectively (table:3). In scavenging hydroxyl radical by p-NDA method the MEAT showed maximum activity, CEAT showed moderate activity and AEAT showed minimum activity (Fig:4). The results were reported in table:4.

Statistical analysis

Data were expressed as mean and standard deviation (SD).

Table 4: % inhibition by p-NDA radical by MEAT, CEAT & AEAT.

Extract	Quantity in micrograms (µg/ml)					IC_{50}
	100	200	300	400	500	
MEAT	6.83±0.35	12.83±0.35	20.33±0.47	24.7±0.45	29.13±0.30	>1000
CEAT	4.33±0.37	5.96±0.20	8.56±0.35	12.4±0.45	17.36±0.50	>1000
AEAT	2.43±0.35	4.13±0.321	6.16±0.25	8.53±0.35	12.30±0.45	>1000
ASA	24.9±0.65	39.0±0.62	46.26±0.50	59.0±0.70	64.16±0.35	>1000

Values are mean ± SD of triplicates

AT: Amaranthus tricolor (L); MEAT: Methanolic Extract of AT; CEAT: Chloroform Extract of AT; AEAT: Aqueous Extract of AT; ASA: Ascorbic Acid

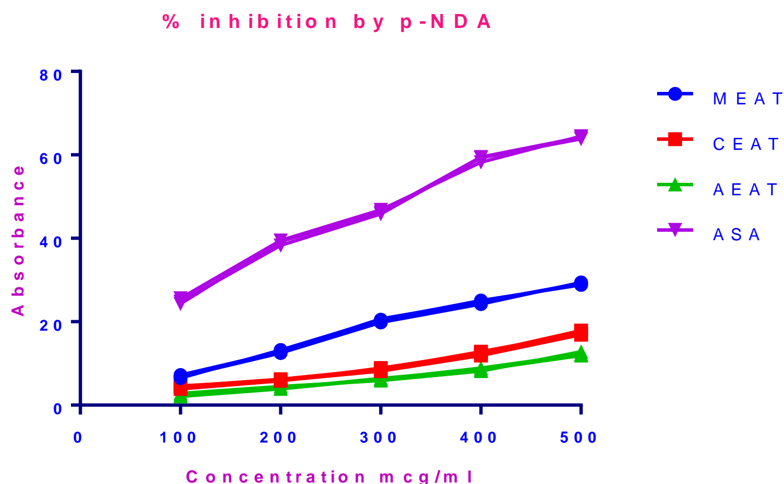

Figure 4: Graphical representation of % inhibition of p NDA radical by MEAT, CEAT & AEAT.

Statistical analysis of parametric data for IC_{50} was carried out using graph prism pad software.

CONCLUSION

The present study supports the use of *A.tricolor* as green leafy vegetable, which may be due to its antioxidant property. The presence of various phytochemicals was responsible for high antioxidant activity. However, further studies are required to confirm the same. The plant merits further investigation to isolate its active constituents and to establish the activity in animal models.

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