Evaluation of \textit{in vitro} Antioxidant Potential of \textit{Amaranthus caudatus} L. Grown in Kashmir Region

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\textbf{ABSTRACT}

\textbf{Background:} Plants have a well-developed defensive machinery for minimizing the reactive oxygen species (ROS) associated damages in the form of enzymatic and non-enzymatic antioxidants. The \textit{in vitro} mechanism of antioxidant action of plant extracts may involve direct inhibition of the ROS generation or ROS scavenging. The antioxidant activity of the extracts may be due to active constituents alone or the combination of constituents. However, the amount of constituents is known to vary according to the change in environment. \textbf{Method:} In our study, antioxidant activity of \textit{Amaranthus caudatus} L. from two different sites (elevation sites) was investigated at three stages, (vegetative, pre flowering and post flowering) using ethanolic extract (EtOH). \textbf{Result:} The phenolic and flavonoid content increased at all stages from site 1 to site 2. The total reducing power, Ferrous reducing antioxidative power (FRAP), diphenyl picryl hydrazine (DPPH) radical scavenging assay, superoxide dismutase scavenging (SOD) assay and hydrogen peroxide ($\text{H}_2\text{O}_2$) scavenging activity increased from site 1 to site 2 at all the three stages. \textbf{Conclusion:} The results reveal that the altitude and the growth stage have a significant effect on antioxidative potential of \textit{Amaranthus}.

\textbf{Key words:} \textit{Amaranthus}, Ethanol extract, Phenolics, Hydrogen peroxide, Superoxide dismutase, Reactive oxygen species.

\textbf{INTRODUCTION}

Reactive oxygen species (ROS) are manufactured by living beings during metabolic activities of cell and are regulated by various machineries viz. enzymatic and non–enzymatic protective mechanisms. ROS are unstable which react with various biomolecules (DNA, lipids and proteins), modifying them structurally as well as functionally. The production of ROS is known to increase by various factors like environmental pollutants, hazardous chemicals, food adulterants, smoking and thus disturb the balance between the ROS production and ROS quenching which leads to oxidative stress.\textsuperscript{12} Due to the inadequacy of these antioxidative components (enzymatic and non-enzymatic components) in our body, we need to incorporate the plants or their products in our diets that possess good antioxidative activities so that the collective effect of oxidative stress is curtailed. Although there are choices of synthetic antioxidants, yet their use has been limited due their health related issues. Thus, various plant species or their products are increasingly investigated by researchers for antioxidant activity\textsuperscript{2} to protect cells from oxidative stress that accelerates age related diseases; Alzheimer’s disease, cancer and coronary heart disease.\textsuperscript{4,6} The protective nature of plants and their products is because of the diverse chemical constituents present in them such as enzymes, proteins, vitamins;\textsuperscript{7} carotenoids;\textsuperscript{8} flavonoids;\textsuperscript{9} and phenolic compounds.\textsuperscript{10} However, the medicinal properties are restricted to about two third of the total plant species globally\textsuperscript{11} and thus quest for a medicinal plant is increasing. \textit{Amaranthus caudatus}, belonging to family Amaranthaceae, is rare multifunctional crop grown in both temperate and tropical regions of world as vegetable, grains and coloring agent as well as for ornamental purposes.\textsuperscript{12} The plant grows in wide range of climatic conditions with minimum external inputs and shows tolerance towards heat, drought, diseases and pests.\textsuperscript{13} The plant is accredited with medicinal value as it possesses hepatoprotective;\textsuperscript{14} antihelminthic;\textsuperscript{15} antinociceptive and antipyretic\textsuperscript{16} as well as antidepressant activity\textsuperscript{17} and anti-hyperlipidemic activity.\textsuperscript{18,19} The presence of different compounds in \textit{Amaranthus caudatus} makes it a potential plant species for studying the antioxidative potential.\textsuperscript{20} The expression of compounds possessing antioxidant activity is influenced by the different environmental conditions. The concentration of active substances show positive correlation with increasing altitude, as it is associated with many ecological factors like temperature, humidity, duration of snow cover, length of vegetative period, soil composition and pH.\textsuperscript{21} Such ecological factors
control the production of secondary metabolites. Plants grown at high altitude may bring change in biochemical components to resist the stress conditions. \cite{22} Besides the altitudinal difference the bioactive components of plants show variation at different growth stages \cite{23} and thus the present study has been undertaken to check the in vitro antioxidative potential of *Amaranthus caudatus* grown in Kashmir region at two different sites at various stages of plant growth.

**MATERIALS AND METHODS**

**Collection of plant material**
The seeds were collected from SKUAST-K (Sheri Kashmir University of Agricultural science and Technology-Kashmir) and were grown at two places viz. Nagbal (1581amsl, Site 1) and Wangath (2100 amsl, Site 2) (district Ganderbal). The leaves were collected at three different stages viz. vegetative stage (Stage I) i.e., after the 5th week of sowing, pre flowering (Stage II) i.e., after the 10th week of sowing and post flowering stage i.e., after the 17th week of sowing. Leaves were shade dried, powdered and stored in air tight bags until used.

**Extraction of plant material**
5g of dried powdered leaf material was extracted with 50ml of 80% ethanol using a shaker for 24 hrs at room temperature. The mixtures were filtered and the supernatant was kept in air tight tubes and was properly labelled and kept at 4°C until further use. The total phenolic content, total flavonoid content was estimated and antioxidant activity assays were performed using following methods.

**Total phenolic content determination**
Total phenolic content in leaf material was determined by using Folin–Ciocalteau reagent (FC) method \cite{25} and gallic acid was used as standard phenolic compound. The absorbance was measured at 650 nm using spectrophotometer (Shimadzu, Japan) against a blank reagent. The total soluble phenolic content in leaf extracts was expressed in mg/g of gallic acid equivalents.

**Total flavonoids determination**
The total flavonoids were determined by Aluminium chloride (AlCl₃) colorimetric method \cite{26} and absorbance was recorded at 415 nm. The total flavonoid content was estimated using rutin as a standard from which unknown concentration of the sample was estimated. The flavonoid content was expressed in mg/g of dry rutin equivalents.

**Total Reducing Power**
The total reducing power was assayed by the method of Yen and Duh, (1993) \cite{27} and the absorbance was measured at 700 nm. The increase in absorbance indicated an increase in reducing power.

**Ferrous Reducing Antioxidant Power Assay (FRAP)**
The antioxidant potential was assayed using the method of Benzie and Strain, (1996) \cite{28} with little modification. An aliquot of 10-50μl extracts was mixed with 2.9ml of FRAP reagent and kept in dark for 10 min. The bluish coloured product so formed was read at 593 nm. Here ferrous sulphate was used as standard. Results obtained were expressed in μMFe (II)/g.

**1,1-diphenyl-2-picryl hydrazine (DPPH) Percentage Inhibition Activity**
The antioxidative scavenging activity was assayed by using a stable free radical 1, 1-diphenyl-2-picryl hydrazine (DPPH) method. \cite{29} The reaction mixture was incubated in dark for 10 min and the absorbance was read at 517nm. The percentage inhibition activity was calculated by using formula.

\[
\text{Percentage inhibition} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100
\]

**Superoxide Radical Scavenging Activity**
Superoxide anions were measured using the method of Beauchamp and Fridovich, (1971). \cite{30} In this assay, PMS and riboflavin generate the superoxide radicals. These superoxide radicals are in turn assayed by the reduction of NBT with the formation of purple colour. The percentage inhibition of superoxide anion generation was estimated using formula.

\[
\text{Percentage inhibition} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100
\]

**Hydrogen peroxide scavenging activity**
Hydrogen peroxide scavenging activity was assayed using H₂O₂ reagent. \cite{31} The reaction mixture was incubated at room temperature for 10 min and the absorbance was measured at 230 nm against blank where no H₂O₂ was added.

\[
\text{Percentage inhibition} = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100
\]

**Statistical analysis**
The experimental data were expressed as mean ± standard deviation (SD) of three replicates. The results were analyzed by using analysis of variance (ANOVA) and the antioxidant potential of various assays was determined as IC₅₀ values by using Graph pad prism 5-software. Results were regarded as statistically significant at p ≤ 0.05.

**RESULTS AND DISCUSSION**
The antioxidant potential of *Amaranthus caudatus* L. was checked by the in vitro antioxidant assays viz. total reducing power, DPPH radical scavenging activity, superoxide radical scavenging activity and ferrous reducing antioxidant power. Besides, the total phenolic and total flavonoid content were also estimated in the plant extracts.

**Total Phenolic Content**
*Amaranthus caudatus* L. plants were grown at two different sites and the total phenolic content was monitored in the leaves collected at various stages of growth. In this method, phenols get oxidized by a molybdenum tungstate reagent (FC reagent) which gives blue coloured complex. The phenolic content at site 1 and 2 was 1.250 ± 0.066 mg per gram and 3.340 ± 0.05 mg/g GAE, stage I; 1.193 ± 0.09 and 5.37 ± 0.34 mg/g GAE, stage II and 1.41 ± 0.07 and 7.58 ± 0.43 mg/g GAE, stage III (Figure 1A). The total phenolic content in *Amaranthus caudatus* increased from vegetative to post-flowering stages at both sites. However, the phenolic content was significantly higher at site 2 with 1.67, 3.5 and 4.4 fold increase at vegetative, pre-flowering and post-flowering stages respectively. The phenolics present in the plants contribute to their antioxidant activity due to their typical structure that induces the property of electron donating capacity, the chain breaking function of free radicals and termination of Fenton reaction. \cite{32} It is clearly illustrated that plants grown at low altitude (Site 2) exhibit low phenolic content than those grown at high altitude (Site 2) at all the three stages. It was also spotted that ethanolic extracts showed maximum activity than other extracts used during this study. The results were supported by Enujjihaga et al. (2014) \cite{33} who examined the phenolics in ethanolic extracts from leaves of *Amaranthus caudatus*. After comparing the three stages, it was revealed that stage III showed highest phenolic content at both the sites followed by the stage I and the least phenolic content was observed in stage II. As per the earlier reports, the phenolic content increased with the maturity of plant and was present in less quantity in newly opened leaves. The low phenolic content in stage II at both the sites indicates that besides altitudinal variations, this might be due to seasonal variation that renders profound effect on antioxidant activity.
activity. As per Shahid and Bhanger, (2006) during hotter seasons the phenolic content decreases as was also observed in our study during the stage II.

**Total Flavonoid Content**

Determination of total flavonoids using Aluminium Chloride (AlCl₃) method is based upon the formation of stable complex between AlCl₃ and hydroxyl group or ketonic group of flavones and flavonoids present within extract. Here rutin was used as a standard compound. The flavonoid content at site 1 and site 2 was 0.95 ± 0.031 and 1.63 ± 0.17 mg/g RE, stage I 0.47 ± 0.026 and 0.83± 0.019 mg/g RE, stage II and 0.929 ± .007 and 1.140 ±.200 mg/g RE respectively (Figure 1B). The total flavonoid content varied significantly with the developmental stages (I, II and III) at both sites. Sites I when compared to their respective stages at site 2, attaining a decline of 0.715, 0.772 and 0.212 respectively at three stages. The flowering content increased at post flowering stage by 2.27% at stage 1 and 30.09% present at stage 2 when compared to vegetative stage. The pre-flowering stage showed sharp decline when compared to other two developmental stages. The flavonoid content increased significantly from vegetative to post flowering stage. Flavonoids, the polyphenolic compounds, ubiquitous in nature have awakened interest because of beneficial effects on humans and possess antiviral, antiallergic, anti-inflammatory, antitumor, anti-neoplastic, antiviral and vasodilatory and antioxidiant activities. The antioxidant and free radical scavenging activity of these compounds can be attributed to their molecular structure as the position of hydroxyl groups and other properties. The present study in *Amaranthus caudatus* L. revealed that the total flavonoid content increased with the increase in altitude at all the three stages, however, site 2 exhibited higher flavonoid content compared to site 1. In general, highest flavonoid content was observed at stage I in both the cases followed by stage III and lowest at stage II. The flavonoid content in our study was far better than the values reported in same plant by Akubugwo et al. (2008) that ranged between 0.38-0.83 mg/100g but were less than 28.19-42.84 mg/g and 69.67mg/g. The high phenolic and flavonoid content present in plants and their products result in their antioxidant activities.

**Total Reducing Power**

In this assay potassium ferricyanide \([\text{K}_3\text{Fe} (\text{CN})_6]\) reacts with ferric chloride \((\text{FeCl}_3)\) in presence of extract to give potassium ferrocyanate \([\text{K}_3\text{Fe} (\text{CN})_6]\) and ferrous chloride \((\text{FeCl}_2)\). This complex shows maximum absorbance at 700 nm and oxidation state changes from \(\text{Fe}^{3+}\) (ferric cyanate complex) to \(\text{Fe}^{2+}\) (ferrous form). Increase in OD indicates increase in antioxidative activity. Thus reducing power indicates the electron donating capacity and antioxidative activity and hence colour changes from yellow to green or prussian blue. The total reducing power at site 1 and 2 were 0.91 ± 0.007 nm and 1.56 ± 0.004 nm, stage I; 0.35 ± 0.03 nm and 1.32 ± 0.012 nm stage II and 1.17 ± 0.005 nm and 1.38 ± 0.009 nm, stage III (Figure 2A). There was an increment from site 1 to site 2 by 0.71 fold, 2.82 fold and 0.18 fold at vegetative, pre-flowering and post flowering stages. However, comparing different developmental stage, the pre-flowering stage showed decline of 0.61 fold at site1 and 0.15 fold at site 2 when compared to vegetative stage. Furthermore, the total reducing power at post flowering stage increased by 0.28 fold at site 1 and decreased by 0.11 fold from vegetative to post flowering stage. The reducing power is correlated to the antioxidant activity as is apparent in the in- vitro system where they donated electrons. It was also observed that with the increase in the altitude the total reducing power increased i.e., total reducing power was high at site 2 compared to site 1. After comparing three stages, it was also observed that total reducing power was higher in case of stage I. However, the total reducing power decreased at stage II and then exhibiting an increase at stage III. This again indicated that besides the altitudinal variation, seasonal effect is clearly playing an important role as the less activity of total reducing power was observed in stage II. However, the inherent resource allocation pattern of the plant can also be a decisive factor in the overall physiological and biochemical development of the plant.

**Ferrous Reducing Antioxidative Power (FRAP)**

The ferrous reducing antioxidant power assay is used to observe the ability of extract to reduce ferric ions. In FRAP assay complex between 2, 4, 6-tri-pyridyl-s-triazine (TPTZ) and Ferric chloride (FeCl₃) is formed which is then reduced to ferrous (Fe II-TPTZ) complex which is strongly absorbed at 593nm. The reaction is pH dependant and reaction proceeds only in acidic medium (pH 3.6). In stage I the FRAP activity was 36.49 ± 1.29 μMFe (II)/g, 65 ± 0.51, stage I; 34.96 ± 0.86 μMFe (II)/g and 38.2 ± .20 μMFe (II)/g, stage II and 42.06 ± 0.89 μMFe (II)/g and 43.99 ± 1.95 μMFe (II)/g stage III (Figure 2B). The FRAP activity increased significantly from Site 1 to site 2 at all the three stages of development i.e. the FRAP activity increased by 78.39% , 9.27% and 4.59% at vegetative, pre-flowering and post flowering stage respectively. However, the FRAP activity decreased from vegetative to pre-flowering stage by 4.19% and 41.20% and it increased from pre-flowering to post flowering stage by 20.29% and 15.14% from site 1 and 2 respectively. The ferrous
reducing antioxidant power (FRAP) assay method offers the quantitative estimation of Fe II-TPTZ that are formed as products. In this assay ferrocene 2,4,6-tripryidyl-s-triazine are utilized and changed into its ferrous form (intense blue colour) by the plant extracts possessing antioxidant capability. The results clearly depict that plants grown at site 2 had better activity and similar to earlier assays vegetative stage was better than pre-flowering stage indicating that activity is also affected by agroclimatic zonation and seasonal variation.

**DPPH Antioxidative Scavenging Activity**

1,1-diphenyl-2- picryl hydrazine DPPH is a stable nitrogen containing free radical at room temperature which gives deep purple colour when dissolved in ethanol/methanol. The method is based on measurement of the percentage inhibition of DPPH by the plant extracts and upon reduction it changes colour from deep purple to yellow due to formation of diamagnetic molecule. The degree of discoloration is indicative of the antioxidative activity of tested samples with the decrease in absorbance with increase in the concentration of extracts. This *in vitro* method is widely used for the estimation of primary antioxidant activity. The DPPH inhibition was at site 1 and site 2 was 69.21 ± 0.80; and 84.23 ± 0.70 (stage I); 51.21 ± 0.93 and 69.31 ± 0.53 percent at site 1 and 2 respectively. Finally, at stage III the inhibition was 61.35 ± 1.3 percent and 69.46 ± 0.41 (stage III) (Figure 3A). In DPPH assay, the percentage inhibition activity in vegetative, pre-flowering and post flowering stage increased from site 1 to site 2 by 21%, 35.3% and 13.2% respectively. However, the vegetative stage, at site 1 showed increment of 11.36% and 17.54% at site 2 in comparison to post flowering stage. However, comparing the vegetative stage with pre-flowering stage there was decline in activity was 26.01% at site 1 and 17.71% site 2. It was observed that scavenging activity was higher at site 2 than site 1 and the percentage inhibition showed the following trend Stage I > Stage III > Stage II. This outcome is also supported by Nyonje, (2014) who also reported that Stage II showed least percentage inhibition. The values for DPPH scavenging percentage in our study was similar to Enujiugha (2014) who reported 74-90 % inhibition in case of ethanolic extract. It is clear from our results that altitudinal variation (Site 1-site2) has an impact on antioxidant activity. Besides the seasonal and temperature variation might have profound effect upon antioxidant activity of plant extracts. These conclusions are supported by Iqbal and Bhanger, (2006).41

**Superoxide Radical (SOD) scavenging activity**

In superoxide dismutase (SOD), superoxide anions are generated through PMS-Riboflavin coupling system followed by reduction with nitro blue tetrazolium (NBT). The decrease in absorbance with the increase in concentration of extract indicates consumption of the superoxide anions in the reaction mixture. Superoxide dismutase scavenging activity at site 1 and site 2 was 64.59 ± 0.80 percent and 73.14 ± 0.31 stage I; 53.60 ± 0.92 and 61.35 ± 3.43 percent and 70.55 ± 1.5 at site 1 and 73.08 ± 0.22 percent stage III respectively (Figure 3B). The SOD percentage inhibition activity increased from site 1 to site 2 by 13.24%, 20.68% and 3.59% at stages I-III. The SOD activity decreased at pre-flowering stage by 17% at site 1 and 11.56% at site2 but it increased at post flowering stage by 9.22% and 0.08% at site 1 and site 2 in comparison to vegetative stage. Superoxide anions result in the formation of more reactive species like hydrogen peroxide, hydroxyl radical, and singlet oxygen, which in turn damage lipids, proteins, and DNA. Therefore, studying the scavenging activity of plant extracts on superoxide radical is one of the most important ways of clarifying the mechanism of antioxidant activity. The results regarding the superoxide radical scavenging activity by various extracts of *Amaranthus caudatus* revealed that site 1 exhibited less inhibition of superoxide anions compared to site 2. It was also observed that maximum percentage inhibition was attained at stage I and lowest at stage II. The trend observed was similar to the earlier two assays specifying that SOD inhibition activity is also affected by agroclimatic zonation and seasonal variation.

**Hydrogen Peroxide (H₂O₂) radical scavenging activity**

H₂O₂ is converted into H₂O and O₂ in the body which might produce free radicals like hydroxyl radical that ultimately causes lipid peroxidation and DNA damage. In this assay the ability of different extracts is tested to scavenge this hydrogen peroxide. An stage I the hydrogen peroxide percentage inhibition at site 1 and site 2 was 81.29 ± 1.56 and 88.99 ± 0.035 percent stage I; 78.72±4.45 and 89.03 ± 1.08 percent stage II and 80.38 ± 1.29 and 91.14% ± 0.95 at site 1 and 2 respectively (Figure 3C). The percentage inhibition increased at vegetative, pre-flowering and post flowering stages from 9%, 13%, 13.4% from site 1 to site 2 respectively. However, comparing the vegetative stage with pre-flowering stage there was decline in activity was 26.01% at site 1 and 17.71% site 2. It was observed that scavenging activity was higher at site 2 than site 1 and the percentage inhibition showed the following trend Stage I > Stage III > Stage II. This outcome is also supported by Nyonje, (2014) who also reported that Stage II showed least percentage inhibition. The values for DPPH scavenging percentage in our study was similar to Enujiugha (2014) who reported 74-90 % inhibition in case of ethanolic extract. It is clear from our results that altitudinal variation (Site 1-site2) has an impact on antioxidant activity. Besides the seasonal and temperature variation might have profound effect upon antioxidant activity of plant extracts. These conclusions are supported by Iqbal and Bhanger, (2006).41
Figure 3: DPPH (A); SOD (B) and \( \text{H}_2\text{O}_2 \) percentage inhibition (C) in ethanolic leaf extract of *Amaranthus caudatus* L. grown at two different sites.

The IC\textsubscript{50} values for SOD, \( \text{H}_2\text{O}_2 \), and DPPH were calculated (Table 1). The table clearly depicts that the less concentration of leaf extracts belonging to site 2 is needed to get IC\textsubscript{50} values.

### Table 1: IC\textsubscript{50} values of ethanolic extracts of leaf material at different altitudinal sites in three different stages.

<table>
<thead>
<tr>
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<th>Site 1</th>
<th>Site 2</th>
<th>Site 1</th>
<th>Site 2</th>
<th>Site 1</th>
<th>Site 2</th>
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</thead>
<tbody>
<tr>
<td>DPPH</td>
<td>3.51</td>
<td>1.501</td>
<td>2.07</td>
<td>1.42</td>
<td>3.01</td>
<td>2.15</td>
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<tr>
<td>SOD</td>
<td>4.832</td>
<td>2.92</td>
<td>3.91</td>
<td>3.00</td>
<td>2.37</td>
<td>1.56</td>
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<tr>
<td>( \text{H}_2\text{O}_2 )</td>
<td>3.92</td>
<td>1.97</td>
<td>3.15</td>
<td>2.57</td>
<td>0.89</td>
<td>0.23</td>
</tr>
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</table>

The sites, the lowest activity was observed at stage II, which means the seasonal variation plays an important role. Further the results revealed that because of natural free radical quenching activity of *Amaranthus caudatus*, it can be utilized as a promising plant product for ROS associated diseases. The large scale cultivation of this crop especially at high altitudes is having a promising potential because in these areas there are climatic constrains for the cultivation of the conventional crops.

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### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with any party.

### ABBREVIATIONS

ROS: reactive oxygen species; EtOH: ethanolic extract; FRAP: Ferrous reducing antioxidative power; DPPH: diphenyl picryl hydrazine; SOD: superoxide dismutase; \( \text{H}_2\text{O}_2 \): hydrogen peroxide; TPTZ: 2, 4, 6-tri-pyridyl-s-triazine; FeCl\textsubscript{3}: Ferric chloride; Pre F: preflowering; Post F: post flowering; VEG: vegetative stage

### REFERENCES

Ramzan, et al.: Effect of altitudinal difference on antioxidant activity of Amaranthus Caudatus L.


PICTORIAL ABSTRACT


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