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CuO nanoparticle mediated elicitation of polyphenols and antioxidant activity in chicory (*Cichorium intybus* L.)

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Abstract
The present study on effect of CuO nanoparticles on *Cichorium intybus* L. significantly enhanced the polyphenols content in treated plants at 10 and 20 DAT. Higher total phenol content i.e., 28.171±0.125 and 30.759±1.730 mg gallic acid equivalents g-1 extract, was observed 20 DAT in shoots and roots of treated plants, respectively. Higher flavonoid content i.e., 9.450±0.220 and 12.878±0.648 mg quercetin equivalents g-1 extract was also observed at 20 DAT in both shoots and roots of treated plants. The tannin content at 20 DAT in shoots and roots of treated plants also differed significantly w.r.t shoots and roots of control at 10 and 20 DAT, respectively. The shoots and roots of treated plants showed significantly ($p \leq 0.05$) higher total antioxidant activity at 10 DAT. The total antioxidant activity within shoots showed a significant ($p \leq 0.01$) positive correlation (0.938) with total antioxidant activity observed in roots of *Chicorium*. Phenol and flavonoid content within the roots showed a positive correlation with total antioxidant activity within the roots i.e., 0.241 and 0.384, respectively. Highest scavenging of DPPH radical was observed within shoots and roots of treated plants at 10 DAT, and it differed significantly ($p \leq 0.05$) w.r.t corresponding activity at 20 DAT. DPPH radical scavenging within shoots showed a significant positive correlation ($p \leq 0.01$) with corresponding activity in roots (0.939). DPPH radical scavenging activity within roots showed a negative correlation with total phenol, flavonoid and content within the roots (i.e., -0.453, -0.348 and -0.415, respectively) as observed by lower IC50 values of the extracts. Thus the results clearly show that CuO nanoparticles significantly enhanced polyphenols and corresponding antioxidant activity in *Cichorium*.

Keywords: Polyphenols, flavonoids, tannins, DPPH

Introduction
Chicory (*Cichorium intybus* L.) also known as Kasni is a traditional medicial plant and has been used as a therapeutic agent since ancient times. Its therapeutic properties were even recognized by Greeks and Romans. The herb is widely used in many traditional system of medicine including ayurvedic, unani system of medicine. Recently many known therapeutic effects of the plant have recently been confirmed by various researchers. The plant is a valuable source of several studies phytochemicals including coumarins, flavonoids, anthocyanins, fructans, and sesquiterpene etc., (Norbaek *et al.*, 2002; Innocenti *et al.*, 2005; Shah *et al.*, 2012; Montefusco *et al.*, 2015). Plant parts specially roots and leaves are frequently used as antipyretic, diuretic and laxative. The plant is known to possess antibacterial activity (Petrovic *et al.*, 2004) and have potential hypoglycemic and hypolipidemic properties. It has been used widely used as a traditional treatment for diabetes mellitus in India (Pushparaj *et al.*, 2007). It is also used as an appetizer as well as in the treatment of chronic hepatic failure, jaundice and skin diseases (Ghaderi *et al.*, 2012), apart from widely used for its anti-inflamatory and and anti-ulcerogenic properties.
Enhancing levels of phytochemicals in plants is a crucial step in the development of bioprocesses for their production within plants. Bioactives derived from the plants are important source valuable therapeutic drugs. Nanoparticle mediated elicitation of secondary metabolites within plants have been the subject of debate as at higher concentration they exhibit toxic effects within the plants. Thus the present investigation was planned with lower concentration of CuO nanoparticles and their effect on polyphenols as the secondary metabolites was investigated.

Recently nanoparticle mediated elicitation of secondary metabolites have been reported by many investigations, but the mechanism of enhanced production of these medicinally important compounds in plants is vastly complex and sometimes the interaction may be detrimental to the plant itself. Thus the present investigation was undertaken to evaluate the effect of CuO nanoparticles on polyphenols content and corresponding antioxidant activity in different plant parts of Chicory.

Material and Methods

Chemicals and Reagents

The compounds 1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade. CuO nano-particles used in the present investigation were prepared and provided by Dr. Karishma Joshi, Department of Biochemistry, Dolphin (P.G.) Institute of Biomedical and Natural Sciences, Dehra Dun 248007, Uttarakhand, India.

Samples

The plants of *Chicorium intybus* L. were purchased from Forest Research Institute, Dehradun and grown under controlled conditions. The plants were homogeneous in terms of growth stage and maturity. The plants were divided into two groups one group was treated with 1 ppm CuO nanoparticle solution and the remaining halves were left untreated (control). Samples were taken from roots and shoots of control and treated plants after 10 and 15 days after treatment (DAT), the roots and leaves of the plants from control (untreated) and treated were collected and washed nicely with distilled water separately and kept for drying in hot air oven at 45± 5ºC temperature for one week. After drying, the roots and leaves were grinded separately using pestle and mortar and stored in air tight containers for further use. The powdered dried shoots and roots were dissolved in 30 ml methanol kept for 24 hours. The extracts were filtered using Whatman No.1 filter paper. The residue was redissolved in methanol and filtered (thrice). The combined filtrate was evaporated to dryness using Rotatory flask evaporator (50ºC). The dried methanolic extracts were redissolved in methanol to prepare stock solutions (10 mg/ml). The methanolic extracts were stored at -20^{0}C until further used. Methanolic shoot and root extracts were used for quantification of polyphenols and antioxidant activity.

Determination of Polyphenols and Antioxidant activity

Total phenol content

The total phenolics content in the methanolic shoot and root extracts was estimated using Folin-Ciocalteu method as described by Swain and Hills (1959). 100 µl of the methanolic extract was used to estimate total phenol content. It was diluted 10 folds by adding 900 µl methanol. 0.5ml Folin-Ciocalteu reagent (FCR) was added to the diluted extract and mixed well. After 6 min, 1ml of saturated Na_2CO_3 was added. The total volume of the reaction mixture was made upto 7.5 ml using distilled water and kept to stand for some time. The absorbance of the resulting reaction mixture was measured at 725 nm against blank. The total phenol content was expressed as mg gallic acid equivalents (GAE) g^{-1} extract, using a calibration curve of gallic acid (200-1000 µg).

Determination of total flavonoid content

The total flavonoids contents within the extracts were estimated by Aluminium chloride colorimetric assay as described by Kim *et al.*, 2003. 100 µl of the methanolic extract was used to determine flavonoid content. It was diluted with 900 µl in a test tube. The volume was made to 4 ml using distilled water. To this 0.3 ml of 5% Na_2NO_3 was added and kept for 5 minutes. After few minute 0.3 ml of 10% $AlCl_3$ and 2 ml of 1M NaOH was added. The reaction mixture was allowed to stand for few minutes and the absorbance was measured at 510 nm against blank. The amount of flavonoids in the methanolic extracts was expressed as mg equivalents of quercetin equivalents (QE) g^{-1} extract, using a calibration curve of quercetin (20-100 µg).

Determination of total tannin content

Tannins content within the extracts were determined using Folin-Denis method (Polshettiwar *et al.*, 2007). To 100 µl of the extract, 900 µl of methanol was added. The volume was made upto 7.5 ml using distilled water. 0.5 ml of Folin-Denis reagent (FDR) was added to the reaction mixture along with 1 ml saturated sodium carbonate. The total volume was made upto 10 ml using distilled water. The reaction mixture was kept for 2-3 hours and respective absorbance was measured at 760 nm against blank. The amount of tannins was expressed as mg tannic acid equivalent g^{-1} extract, using a calibration curve of tannic acid (20-100 µg).

Determination of total antioxidant capacity (Phosphomolybdate assay)

The total antioxidant capacity of the methanolic extracts was estimated by Phosphomolybdate method (Prieto *et al.*, 1999) using ascorbic acid as a standard. An aliquot of 100 µl of sample solution was mixed with 400 µl methanol and 1ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) making total volume 1.5 ml. The reaction mixture was incubated in a water bath at 95ºC for 90 min. After cooling absorbance of the reaction mixture was measured at 695 nm against a blank. The total antioxidant capacity was expressed as mg ascorbic acid equivalents g^{-1} extract, using a calibration curve ascorbic acid (20-100 µg).

DPPH radical scavenging activity

DPPH radical scavenging activity of the methanolic extracts was measured using the method described by Shih *et al.*, (2010). This assay is based on the scavenging red colour by the antioxidants present within plant extracts, the red colour of DPPH solution turns yellow when scavenged (Braca *et al.*, 2001). The aliquot of the extract in varying concentration (ranging from 200-800 µg/ml) were mixed with 2 ml DPPH (0.1 mM in methanol). After incubating for 30 minutes at 37ºC the absorbance was measured at 517 nm. Antioxidants react with DPPH and reduce it to DPPH-H and as a consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds present in plant extracts in terms of hydrogen donating ability. The following formula has been used to determine the percentage of scavenging activity:

$$\%\ DPPH\ radical\ Scavenging = \left[1 - \left(\frac{Sample_{Abs}}{Control_{Abs}}\right)\right] \times 100$$

Statistical analysis

Analysis of variance (ANOVA) and Tukey HSD post hoc test were carried out on the experimental values obtained. Results are expressed as mean±SEm (n=3). A statistical difference at $p \leq 0.05$ was considered to be significant. Correlation analysis

(bivariate) was also carried out to determine the relationship between polyphenol content and antioxidant activity present in shoots and roots extracts of control and treated plants of *Chicorium intybus* L. under investigation. The software IBM SPSS Statistics 20 (IBM Corporation) and SigmaPlot for Windows 11.0 (Systat Software, Inc.) were used to perform statistical analysis and graphing respectively.

Results

Polyphenol content

Total Phenol Content

Total phenol content was expressed as mg gallic acid equivalents/g extract (Figure 1). The phenol content in shoots varied from 19.138±0.795 to 19.410±0.795 mg gallic acid equivalents/g extract in control plants from 10 to 20 days, respectively. In treated plants phenol content in shoots varied from 22.755±0.125 mg gallic acid equivalents/g extract, 10 DAT to 28.171±0.125 mg gallic acid equivalents/g extract, 20 DAT. In roots of control plants the phenol content varied from 27.974±1.673 mg gallic acid equivalents/g extract, 10 DAT to 26.455±1.577 mg gallic acid equivalents/g extract 20 DAT. In treated plants phenol content in roots varied from 29.810±1.291mg gallic acid equivalents/g extract, 10 DAT to 30.759±1.730 mg gallic acid equivalents/g extract, 20 DAT.

Total flavonoid Content

Total flavonoid content was expressed as mg quercetin equivalents/g extract (Figure 2). The flavonoid content in shoots varied from 9.450±0.220 to 9.484±0.254 mg quercetin equivalents/g extract in control plants from 10 to 20 days, respectively. In treated plants flavonoid content within shoots varied from 8.825±0.441 to 9.450±0.220 mg quercetin equivalents/g extract was observed from 10 to 20 DAT. In roots of control plants the flavonoid content varied from 7.143±0.220 mg quercetin equivalents/g extract, 10 DAT to 8.572±0.342 mg quercetin equivalents/g extract 20 DAT. In treated plants flavonoid content in roots varied from 12.410±0.872 mg quercetin equivalents/g extract, 10 DAT to 12.878±0.648 mg quercetin equivalents/g extract, 20 DAT.

Total Tannin Content

Tannin content was expressed as mg tannic acid equivalents/g extract (Figure 3). The tannin content in shoots varied from 2.981±0.281 to 6.219±0.286 mg tannic acid equivalents/g extract in control plants from 10 to 20 days, respectively. In treated plants tannin content in shoots varied from 5.295±0.162 mg tannic acid equivalents/g extract, 10 DAT to 9.257±0.224 mg tannic acid equivalents/g extract, 20 DAT. In roots of control plants the tannin content varied from 4.795±0.162 mg tannic acid equivalents/g extract, 10 DAT to 5.169±0.132 mg tannic acid equivalents/g extract 20 DAT. In treated plants tannin content in roots varied from 8.762±0.296 mg tannic acid equivalents/g extract, 10 DAT to 10.219±0.186 mg tannic acid equivalents/g extract, 20 DAT.

Antioxidant Activity

Total Antioxidant Activity

Total antioxidant activity was expressed as mg ascorbic acid equivalents/g extract (Table 1). The total antioxidant activity in shoots varied from 57.811±7.811 to 45.127±1.010 mg ascorbic acid equivalents/g extract in control plants from 10 to 20 days, respectively. In treated plants total antioxidant activity in shoots varied from 72.443±0.679 mg ascorbic acid equivalents/g extract, 10 DAT to 47.364±1.188 mg ascorbic acid equivalents/g extract, 20 DAT. In roots of control plants the total antioxidant activity varied from 68.360±0.169 mg ascorbic acid equivalents/g extract, 10 DAT to 68.269±0.848 mg ascorbic acid equivalents/g extract 20 DAT. In treated plants total antioxidant activity in roots varied from 87.803±1.020 mg ascorbic acid equivalents/g extract, 10 DAT to 66.131±0.339 mg ascorbic acid equivalents/g extract, 20 DAT.

DPPH radical scavenging activity

DPPH radical scavenging activity was used as a measure of antioxidant activity in methanolic shoot and root extract of treated and untreated (control) plants. DPPH radical scavenging increased with increase in concentration of the extracts in both treated and untreated plants (Figure 4 A & B). The scavenging activity was expressed as IC_{50} values of the extracts, i.e., the inhibitory concentration which was able to scavenge 50% of DPPH radicals (Table 1). The IC_{50} values in shoots varied from 373.646±1.588 to 389.478±5.869 µg/ml in control plants from 10 to 20 days, respectively. In treated plants IC_{50} values in shoots varied from 263.657±2.131 µg/ml, 10 DAT to 387.929±13.678 µg/ml, 20 DAT. In roots of control plants the IC_{50} values varied from 375.724±19.357 µg/ml, 10 DAT to 461.542±5.303 µg/ml, 20 DAT. In treated plants IC_{50} values in roots varied from 262.396±4.940 µg/ml, 10 DAT to 421.982±6.891 µg/ml, 20 DAT.

Discussion

Polyphenol Content

Polyphenols within plants are the largest group of secondary metabolites with well known therapeutic properties including antioxidant properties against various reactive oxygen species (ROS) known to be associated with various diseases. Phenol, flavonoids and tannins are the major polyphenols responsible for many pharmacological effects shown by plant based formulations. The current investigation clearly shows significant ($p \leq 0.05$) elicitation effect of CuO nanoparticles on polyphenols content in shoots and roots of treated plants at 10 and 20 days after treatment (DAT). Both shoots and roots of *Chicorium* showed significantly ($p \leq 0.05$) higher phenol and flavonoid and tannin content at 20 DAT. The total phenol content in shoots and roots of treated plants at 20 DAT differed significantly ($p \leq 0.05$) w.r.t control and treated plants at 10 and 20 DAT, respectively. The flavonoid content in shoots and roots of treated plants at 20 DAT also differed significantly ($p \leq 0.05$) w.r.t control and treated plants at 10 and 20 DAT, respectively. Highest flavonoid content was observed at 10 and 20 DAT in both shoots and roots of treated plants. Higher tannin content was also observed in shoots and roots of treated plants at 20 DAT, which differed significantly ($p \leq 0.05$) w.r.t tannin content in shoots and roots of control at 10 and 20 DAT, respectively. The total phenol content in shoots showed a significant ($p \leq 0.01$) positive correlation with total phenol content in roots (0.757) and tannin content in shoots (0.830). Total phenol content in roots also showed a significant ($p \leq 0.01$) positive correlation with flavonoid and tannin content within the roots i.e., 0.770 and 0.871, respectively. A positive correlation (0.241) between total phenol content in roots and corresponding total antioxidant activity was also observed (Figure 5 A & B). Flavonoid content in roots showed a significant ($p \leq 0.01$) positive correlation (0.966) with tannin content within the roots. It also showed a positive correlation with corresponding total antioxidant activity in the roots (Figure 6 A & B). Tannin content within shoots and roots also showed a significant positive correlation (0.682). Flavonoid content in roots and shoots also showed a positive correlation (0.238). It also

showed a significant ($p \leq 0.01$) positive correlation with tannin content in roots (0.812).

Polyphenols are important constituents of plant origin and major antioxidants of our diet. As antioxidants, they protect cell constituents against oxidative damage by reactive oxygen species (ROS) and, therefore, limit the risk of various degenerative diseases associated to oxidative stress (Scalbert *et al.*, 2005). Numerous investigations have shown that dietary polyphenols exhibit many biologically significant functions. They are important therapeutic agents which prevent the development of cancers, cardiovascular diseases, neurodegenerative diseases, diabetes, and osteoporosis etc. Most of these biological actions can be attributed to their ability to act as intrinsic antioxidants. Oxidative burst has been consistently reported in plants exposed to toxic levels of nanoparticles (Thwala *et al.*, 2013; Hossain *et al.*, 2015). But non toxic concentrations as in current investigation may be the reason for changes in polyphenols content in *Chicorium*. Further investigations needs to be done to precisely understand the possible mechanism of increase in polyphenols within the plant.

Antioxidant Activity

The total antioxidant activity as measured by Phosphomolybdate method is a sensitive method to determine total antioxidant potential of polyphenols. Significantly higher ($p \leq 0.05$) total antioxidant activity was observed in shoots and roots of treated plants at 10 DAT. Total antioxidant activity in shoots showed a significant ($p \leq 0.01$) positive correlation (0.938) with total antioxidant activity observed in roots of *Chicorium.* Total antioxidant activity in roots also showed a positive correlation with phenol and flavonoid content within the roots i.e., 0.241 and 0.384, respectively.

DPPH free radical scavenging assay is routinely used in different laboratories for evaluating antioxidant activity in plant extracts. The DPPH radical scavenging activity within shoots and roots of treated plants showed highest scavenging at 10 DAT, and it differed significantly ($p \leq 0.05$) w.r.t corresponding activity at 20 DAT. DPPH radical scavenging within shoots showed a significant positive correlation ($p \leq 0.01$) with corresponding activity in roots (0.939). It also showed a negative correlation with total phenol content within the shoots (-0.013), clearly showing that even at lower concentration the shoots extracts were able to scavenge DPPH free radicals, as observed by lower IC_{50} values of the extracts. Similarly within roots DPPH radical scavenging activity showed a negative correlation with total phenol, flavonoid and content within the roots (i.e., -0.453, -0.348 and -0.415, respectively). clearly showing that even at lower concentration the roots extracts were able to scavenge DPPH free radicals, as observed by lower IC_{50} values of the extracts.

Recent experimental evidences have shown that plant growth, development and physiology are significantly affected by nanoparticles (Marslin *et al.*, 2017). But, the effect of nanoparticles on plant secondary metabolism is still obscure. The induction of reactive oxygen species (ROS) following interactions with nanoparticles has been observed in various studies. Thus the results clearly shows that the variation in total antioxidant activity and DPPH radical scavenging activity within roots and shoots of treated plants are due to changes in the intrinsic levels of polyphenols (phenol, flavonoid and tannins) which may be induced against ROS generated due to CuO nanoparticles within the plants.

Conclusion

The findings of the present investigation clearly showed that treated plants showed significantly higher phenol, flavonoid and tannin content at different days of sampling. The shoots and roots of the treated plants even showed higher antioxidant activity. Thus the present study may serve as a basis for understanding the ability of Cuo nanoparticles to elicit polyphenols and corresponding increase in antioxidant activity within *Chicorium*.

Conflict of interest

We the authors declare that we have no conflict of interest.

Acknowledgements

The infrastructure provided by Department of Agriculture, Dolphin (P.G.) Institute of Biomedical and Natural Sciences, Dehra Dun, Uttarakhand and CuO nanoparticles provided by Dr. Karishma Joshi (Department of Biochemistry, Dolphin (P.G.) Institute of Biomedical and Natural Sciences, Dehra Dun, Uttarakhand, India) is duly acknowledged.

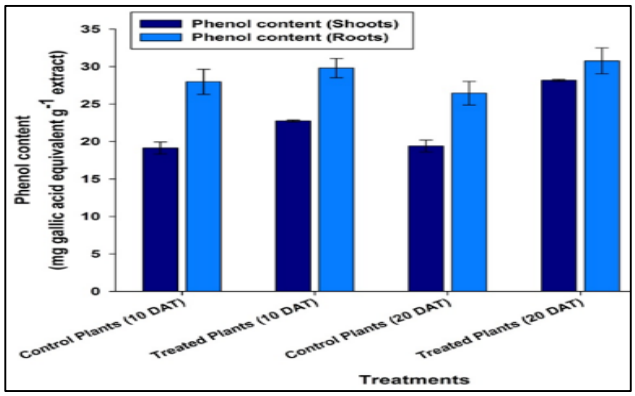

Fig 1: Total Phenol content in (Shoots and Roots) Control and Treated plants of *Chicorium intybus* L. Data shown below are mean value ± standard error (n=3).

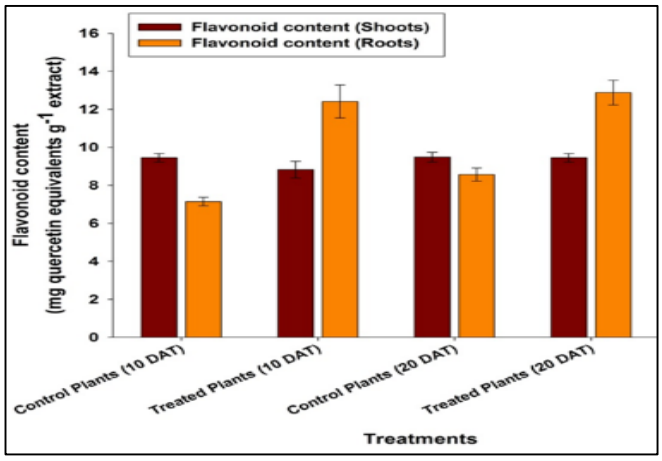

Fig 2: Flavonoid content in (Shoots and Roots) Control and Treated plants of *Chicorium intybus* L. Data shown below are mean value ± standard error (n=3).

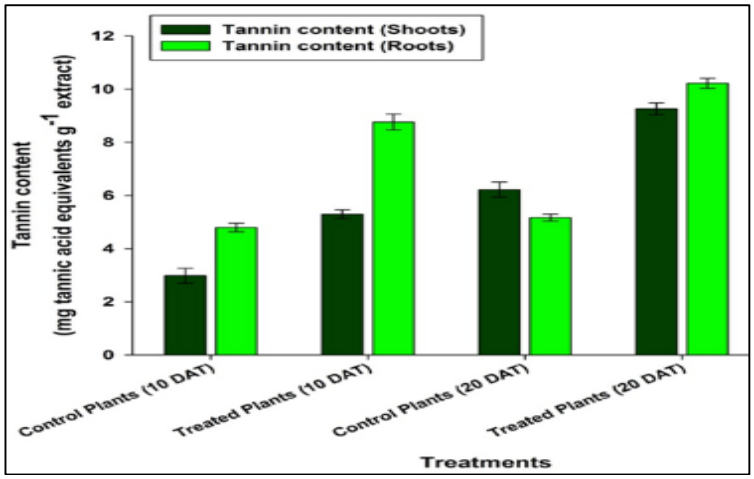

Fig 3: Tannin content in (Shoots and Roots) Control and Treated plants of *Chicorium intybus* L. Data shown below are mean value ± standard error (n=3)

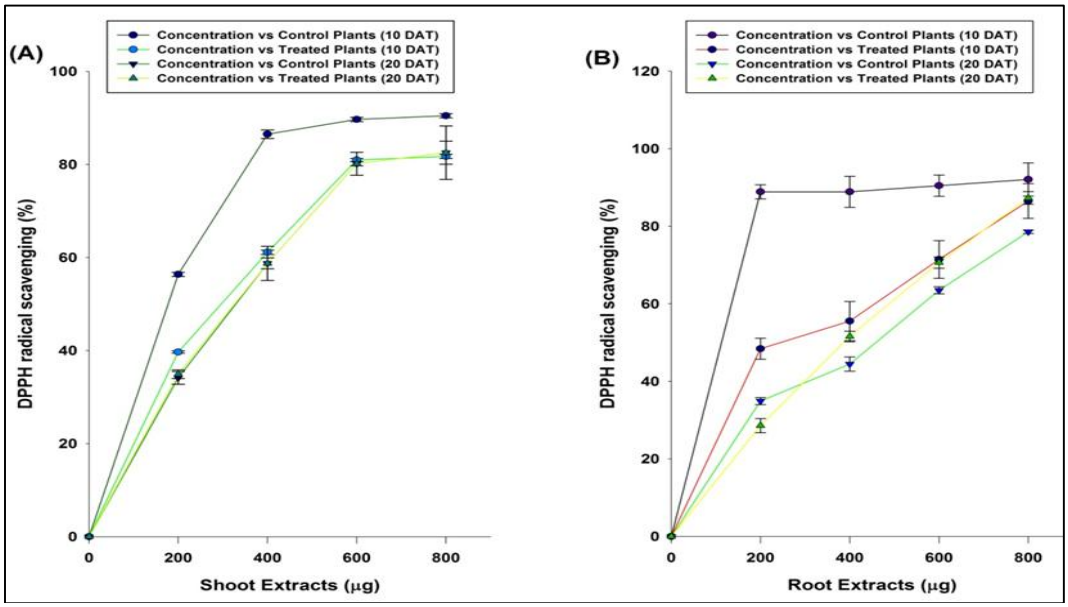

Fig 4: DPPH radical scavenging in (A) Shoots and (B) Roots of Control and Treated plants of *Chicorium intybus* L. Data shown below are mean value ± standard error (n=3).

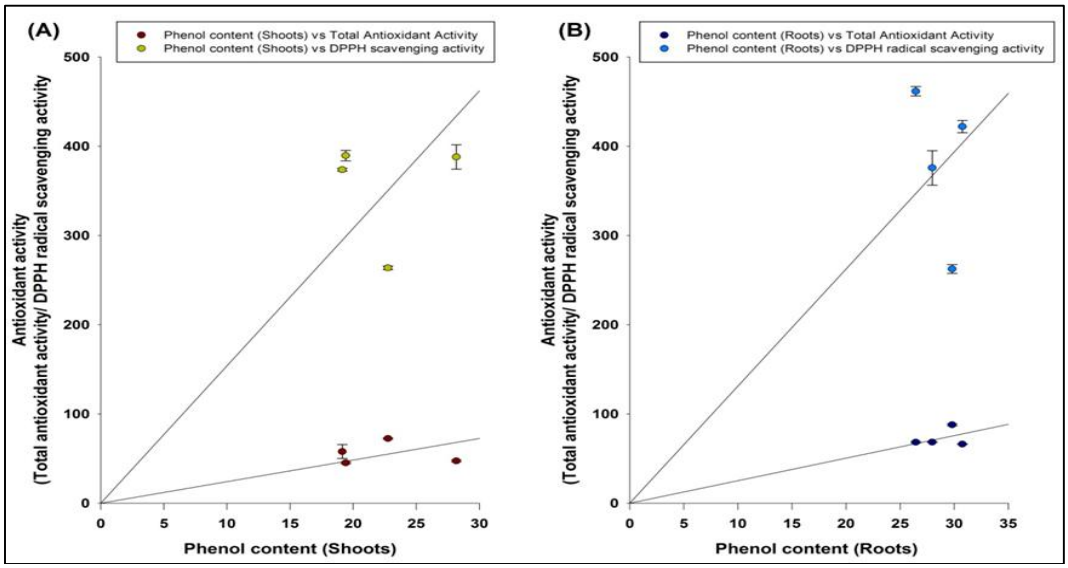

Fig 5: Correlation analysis (A): Between phenol content and antioxidant activity in shoots of control and treated plants of *Chicorium intybus* L. (B): Between flavonoid content and antioxidant activity in shoots of control and treated plants of *Chicorium intybus* L.

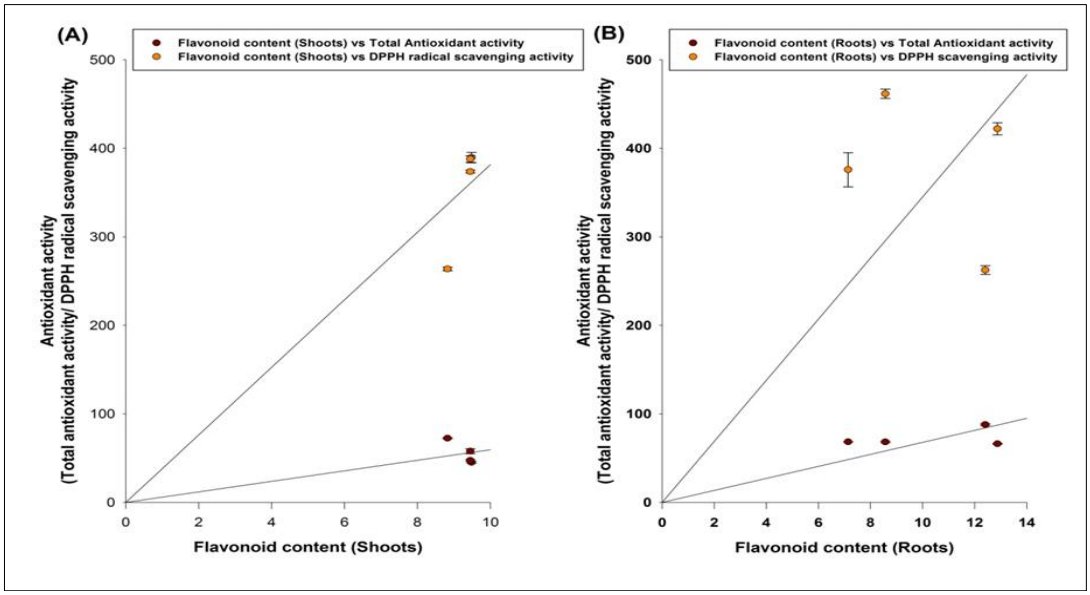

Fig 6: Correlation analysis (A): Between phenol content and antioxidant activity in roots of control and treated plants of *Chicorium intybus* L. (B): Between flavonoid content and antioxidant activity in roots of control and treated plants of *Chicorium intybus* L.

Table 1: Total antioxidant activity (mg ascorbic acid equivalent/g extract) and DPPH radical scavenging (IC_{50}) in shoots and roots of control and treated plants of *Chicorium intybus* L. Data shown below are mean value ± standard error (n=3).

Treatments	Total antioxidant capacity in Shoots (mg ascorbic acid equivalent g^{-1} extract)	Total antioxidant capacity in Roots (mg ascorbic acid equivalent g^{-1} extract)	DPPH radical scavenging in Shoots (IC_{50})	DPPH radical scavenging in Roots (IC_{50})
Control Plants (10 DAT)	57.811 ± 7.811^{b}	68.360 ± 0.169^{b}	373.646 ± 1.588^{b}	375.724 ± 19.357^{b}
Treated Plants (10 DAT)	72.443 ± 0.679^{a}	87.803 ± 1.020^{a}	263.657 ± 2.131^{a}	262.396 ± 4.940^{a}
Control Plants (20 DAT)	45.127 ± 1.010^{b}	68.269 ± 0.848^{b}	389.478 ± 5.869^{b}	461.542 ± 5.303^{c}
Treated Plants (20 DAT)	47.364 ± 1.188^{b}	66.131 ± 0.339^{c}	387.929 ± 13.678^{b}	421.982 ± 6.891^{bc}

***Note:** The values with same superscript are not significantly different at ($P\leq0.05$) according to Tukey HSD post hoc analysis

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