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Comparison of Enzymic and Non-Enzymic Antioxidant Status in the Leaves of Five Selected Plants from Apiaceae Family

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ABSTRACT

The present study focused on comparing the enzymic and non-enzymic antioxidant status of the leaves of *Anethum sowa, Foeniculum vulgare, Trachyspermum ammi, Daucus carota* and *Coriandrum sativum*. The enzymic antioxidants analysed included superoxide dismutase, catalase, peroxidase, glutathione reductase, catechol oxidase and laccase, whereas the non-enzymic antioxidants included ascorbate, reduced glutathione, flavonoids, total phenols, tocopherol, carotenoids, lycopene and chlorophyll. Higher activities of superoxide dismutase, catalase and peroxidase, and higher levels of ascorbate, reduced glutathione, tocopherol, carotenoids and chlorophyll were observed in the leaves of *Foeniculum vulgare*. The leaves of *Coriandrum sativum* possessed more content of glutathione reductase, catechol oxidase, laccase, total phenols and lycopene. Flavonoids were found to be higher in the leaves of *Daucus carota.*

INTRODUCTION

Oxygen is an indispensable element for life. Oxidation process is crucial in living organisms for the production of energy in order to fuel the cellular processes. However, uncontrolled production of oxygen derived free radicals may result in the onset of various diseases like atherosclerosis, rheumatoid arthritis and cancer (Kaushik *et al*., 2015). Oxidative stress occurs due to an imbalance between the production and scavenging of reactive oxygen species (ROS) by the cells (Gilgun-Sherki *et al*., 2002). Reactive oxygen species (ROS) may include free radicals, such as superoxide anion ($O_2^{-\bullet}$) and hydroxyl ($HO^{\bullet}$) radicals, and non-free radical species, such as H_2O_2 and singlet oxygen ($^1O_2$). The excessive production of ROS induces lipid peroxidation as well as damages the lipids, proteins and nucleic acid bases. A variety of cellular responses occur via the generation of secondary reactive species, ultimately resulting in cell death by apoptosis or necrosis (Gutowski and Kowalczyk, 2013). Hence, to protect the cells against ROS, a highly sophisticated and complex antioxidant protection system has been evolved by the human system. These antioxidants function by either stabilizing or deactivating the free radicals. Antioxidants may include dietary antioxidants (vitamin C, vitamin E, carotenoids, glutathione and lipoic acid), antioxidant enzymes (superoxide dismutase, glutathione peroxidase and glutathione reductase) and numerous plant derived substances (Jacob, 1995). Thus, it is essential to consume plants that are rich in free radical scavenging molecules that can act as strong antioxidants (Supasuteekul *et al*., 2016).

In the present study, the enzymic and non-enzymic antioxidants in the leaves of *Anethum sowa, Foeniculum vulgare, Trachyspermum ammi, Daucus carota* and *Coriandrum sativum* belonging to Apiaceae/Umbelliferae family, were compared.

MATERIALS AND METHODS

All chemicals used were of analytical grade and were used without any further purification.

Plant material

Fresh leaves of *Daucus carota, Coriandrum sativum, Anethum sowa, Trachyspermum ammi* and *Foeniculum vulgare* were collected, washed thoroughly with tap water to remove the adhered dust particles, air dried and used for the following studies.

Enzymic Antioxidants

Superoxide dismutase (SOD)

Superoxide dismutase in the leaves was assayed according to the method of Kakkar *et al.* (1984). Fresh leaves (0.5g) were ground with 3.0ml of potassium phosphate buffer (50mM, pH6.4) followed by centrifugation at 2000xg for 10 minutes. The supernatant was used for the assay. The assay mixture contained 1.2ml of sodium pyrophosphate buffer (0.025M, pH8.3), 0.1ml of phenazine methosulphate (186µM), 0.3ml of nitro blue tetrazolium (300µM), 0.2ml of enzyme extract and water in a total volume of 2.8ml. The reaction was initiated by the addition of 0.2ml of NADH (700µM). The mixture was incubated at 30ºC for 90 seconds and arrested by the addition of 1ml of glacial acetic acid. The reaction mixture was shaken with 4ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in butanol layer was measured at 560nm in a spectrophotometer.

Catalase (CAT)

Catalase activity in the leaves of the selected plants was determined by adopting the method of Luck (1974). Fresh leaves (0.5g) were homogenized in 2.5ml of phosphate buffer (0.067M, pH 7.0) at 4ºC and the homogenate was centrifuged at 2000xg for 10 minutes. The supernatant was used for the assay. A volume of 2.9ml of H_2O_2 (2mM) in phosphate buffer (0.067M, pH 7.0) was pipetted into a quartz cuvette. The enzyme extract (0.1ml) was rapidly added and mixed thoroughly. The time required for the decrease in absorbance by 0.05 units was recorded. The H_2O_2-phosphate buffer served as the control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240nm by 0.05 units.

Peroxidase (POD)

The peroxidase activity in the plant samples was assayed following the method of Reddy *et al*. (1995). Fresh leaves (0.5g) of the plants were homogenized in 2.5ml of 0.1M phosphate buffer (pH 6.5), centrifuged and the supernatant was used for the assay. The assay mixture containing 3.0ml of pyrogallol solution (0.05M in 0.1M phosphate buffer, pH 6.5) and 0.1ml of enzyme extract was taken in a cuvette. The spectrophotometer was adjusted to read zero at 430nm followed by the addition of 0.5ml of H_2O_2 (1% in 0.1M phosphate buffer, pH 6.5) and mixed.

The change in absorbance was recorded every 30 seconds up to 3 minutes. One unit of peroxidase activity is defined as the change in absorbance per minute at 430nm.

Glutathione reductase (GR)

Glutathione reductase activity was determined by the method proposed by David and Richard (1983). Fresh leaves were crushed and extracted into 2.5ml of phosphate buffer (0.12M, pH 7.2). The debris was removed by centrifugation at 5000xg for 10 minutes and the supernatant was used for the assay. The reaction mixture had a final volume of 3ml, containing 0.1ml each of EDTA (15mM), sodium azide (10mM), oxidized glutathione (6.3mM), enzyme source and water. The reaction mixture was incubated for 3 minutes, after which 0.1ml of NADPH (9.6mM) was added. The absorbance at 340nm was recorded at an interval of 15 seconds for 3 minutes. For each series of measurement, controls that contained water instead of oxidized glutathione were used. One unit of glutathione reductase is expressed as the µmoles of NADPH oxidized per minute.

Polyphenol oxidases (PPO)

The activity of polyphenol oxidases, comprising of catechol oxidase and laccase, can be simultaneously assayed by the spectrophotometric method proposed by Esterbauer *et al*. (1977). Fresh leaves (0.5g) were homogenized in 2ml of reaction medium containing Tris HCl (50mM, pH 7.2), sorbitol (0.4M) and NaCl (10mM). The homogenate was centrifuged at 2000xg for 10 minutes at 4°C and the supernatant was used for the assay. The assay mixture contained 2.5ml of phosphate buffer (0.1M, pH 6.5) and 0.3ml of catechol solution (0.01M). The spectrophotometer was set at 495nm. The enzyme extract (0.2ml) was added to the assay mixture and the change in absorbance was recorded every 30 seconds up to 5 minutes. One unit of catechol oxidase (or laccase) is defined as the amount of enzyme that transforms 1µmole of dihydrophenol to 1µmole of quinine per minute under the assay conditions.

$$\text{Catechol oxidase} = 0.272 \times (\Delta A/\text{minute})$$

$$\text{Laccase} = 0.242 \times (\Delta A/\text{minute})$$

Non-Enzymic Antioxidants

Ascorbic acid

The amount of ascorbic acid present in the fresh leaves was estimated using the method of Roe and Keuther (1943). Fresh leaves (1g) were taken and homogenized with 4% TCA (Trichloroacetic acid) to extract the ascorbic acid and the final volume was made up to 10ml with 4% TCA. The supernatant obtained after centrifugation at 2000 rpm for 10 minutes was treated with a pinch of activated charcoal, shaken well and kept for 10 minutes. Centrifugation was repeated once again to remove the charcoal residue. Aliquots (0.5ml and 1.0ml) of the supernatant were taken for the assay. The working standard solution containing 20-100µg of ascorbic acid was also taken. The assay volume was made up to 2.0ml with 4% TCA in all the test tubes. A volume of 0.5ml of 2,4-dinitrophenyl hydrazine reagent (DNPH, 2% in 9N sulphuric acid) was then added, followed by the addition of two drops of 10% thiourea solution. The assay mixture was then incubated at 37°C for 3 hours resulting in the formation of osazone crystals. The crystals were dissolved in 2.5ml of 85% H_2SO_4, in cold conditions, to avoid an appreciable rise in temperature. To the blank, only DNPH reagent and thiourea were added after the addition of H_2SO_4. The tubes were cooled on ice and the absorbance was read at 540nm. The concentration of ascorbic acid in the samples were determined using a standard graph constructed on an electronic calculator set to the linear regression mode and expressed as mg ascorbate/g leaf sample.

Reduced glutathione (GSH)

The method proposed by Moron *et al.* (1979) was used for the estimation of reduced glutathione. A 2.0g portion of the leaves was homogenized with 5% TCA and centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was used for the assay. To 0.1ml of the supernatant, 1.0ml of phosphate buffer (0.2M, pH 8.0) and 2.0ml of freshly prepared 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, 0.6mM in phosphate buffer) solution were added. The intensity of yellow color formed was measured at 412nm after 10 minutes. The blank was prepared by adding the reagent without DTNB. A standard curve was prepared using concentrations ranging from 2-10nmoles. The values are expressed as nmoles GSH/g tissue.

Flavonoids

Flavonoids were estimated using the method described by Cameron *et al*. (1943). The plant sample (0.2g) was initially extracted with methanol: water mixture (9:1) followed by extraction with the same mixture in the ratio 1:1. The extracts were shaken well, allowed to stand overnight, pooled, clarified using n-hexane and the volume was measured. This was concentrated and then used for the assay. An aliquot of the extract was pipetted out and evaporated to dryness. Different volumes of standard catechin (0.2 to 1.0ml) were taken and made up to 1.0ml with distilled water. About 4.0ml of vanillin reagent (1% in 70% sulphuric acid) was added and the tubes were heated for 15 minutes in a boiling water bath and allowed to cool. The optical density of the solution was read at 340nm. The standard curve was constructed and the concentration of flavonoids was calculated. The values are expressed as mg flavonoids/g tissue.

Total phenol

Total phenols in the plant sample were assayed by the method proposed by Mallick and Singh (1980). The homogenate was prepared by homogenizing 0.5g of the leaves in 10X volumes of 80% ethanol. The homogenate was centrifuged at 10,000xg for 20 minutes. The residue was re-extracted with 80% ethanol. The supernatants were pooled and evaporated to dryness. The residue was then dissolved in a known volume of distilled water. The diluted extract was taken and the volume was made up to 3.0ml with distilled water. Then 0.5ml of Folin-Ciocalteau reagent (1:1 ratio in distilled water) and 2.0ml of 20% sodium carbonate solution were added. After mixing the tubes thoroughly, the blue solution obtained was warmed for 1 minute, cooled and the absorbance was measured at 650nm against a reagent blank. Standard curve was prepared using known concentrations of catechol solution (0.2-1.0ml) corresponding to 2.0-10µg. Total phenol content in the sample was calculated using the standard curve and the values are expressed as mg phenols/g tissue.

Tocopherol

The levels of tocopherol in the plant samples were estimated spectrophotometrically by the method reported by Rosenberg *et al.* (1992). Fresh leaves (2.5g) were homogenized in a small volume of 0.1N sulphuric acid and the volume was finally made up to 50ml by adding 0.1N sulphuric acid slowly, without shaking and the contents were allowed to stand overnight. The

contents of the flask were shaken vigorously on the next day and filtered through Whatman No.1 filter paper. Aliquots of the filtrate were used for the estimation of tocopherol. A volume of 1.5ml each of the plant extract, standard and water (test, standard and blank) was pipetted out into three centrifuge tubes. To all the tubes, 1.5ml each of ethanol and xylene was added, mixed well and centrifuged. After centrifugation, the xylene layer was transferred into another tube, taking care not to include any ethanol or protein. To 1ml of xylene layer, 1ml of 2,2'-dipyridyl reagent (1.2g/L in n-propanol) was added and mixed. This reaction mixture was taken in the spectrophotometric cuvettes and the extinctions of the test and the standard were read against the blank at 460nm. Then, in turn, beginning with the blank, 0.33ml of ferric chloride solution (1.2g/L in ethanol) was added, mixed well and after exactly 15 minutes, the test and the standard were read against the blank at 520 nm. The results are expressed as µg tocopherol/g of sample.

$$\text{Tocopherol}(\mu g) = \frac{A_{520} - A_{460}}{A_{520}\text{ of standard}} \text{ x } 0.29 \text{ x } 15$$

Total carotenoids and lycopene

The estimation of total carotenoids and lycopene was done by the method described by Fish *et al*. (2002). Fresh leaves (0.6g) were homogenized with 5ml of 0.05% BHT in acetone, 5ml of 95% ethanol and 10 ml of hexane. The mixture was then centrifuged and the supernatant was used for the assay. To the supernatant, 3.0ml of water was added and kept on ice for 5 minutes and then shaken well. The tubes were incubated at room temperature for 5 minutes. The upper hexane layer was carefully transferred to another tube. The absorbance of this layer was read at 450nm for carotenoid estimation and at 503nm for lycopene estimation.

$$\text{Carotenoids (mg)} = \frac{A_{450} \text{ x } 4 \text{ x Volume of sample x } 100}{\text{Weight of sample}}$$

$$\text{Lycopene (mg)} = \frac{3.12 \text{ x } A_{503} \text{ x Volume of sample x } 100}{\text{Weight of the sample x } 1000}$$

Chlorophyll

The chlorophyll content in the leaves was estimated by the method outlined by Witham *et al*. (1971). The leaves were homogenized with 80% pre-chilled acetone and centrifuged at 5000 rpm for 5 minutes. The supernatant was used for the assay. The extraction procedure was repeated

until the residue was colourless. The absorbance of the green colored supernatant was read at 645nm and 663nm against the solvent blank (80% acetone).

$$\text{Chlorophyll} = 20.2\ A_{645} + 8.02\ A_{663} \frac{\text{Volume of sample}}{1000 \text{x} \text{Weight of sample}}$$

RESULTS AND DISCUSSION

Enzymic Antioxidants

The activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione reductase (GR), catechol oxidase (CO) and laccase (Lac) were determined in the fresh leaves of *Anethum sowa, Foeniculum vulgare, Trachyspermum ammi, Daucus carota* and *Coriandrum sativum*.

SOD is a metalloenzyme which converts $O_2^{-\bullet}$ to H_2O_2. It may be classified as copper and zinc (Cu/Zn SOD), manganese (MnSOD) or iron (FeSOD) containing SOD based on the type of metal ion in its active site. Catalases are tetrameric heme containing enzymes, which catalyzes the conversion of H_2O_2 into O_2 and H_2O. Plant catalases may be of three types, of which class 1 and class 2 catalases are involved in removal of H_2O_2 produced during photorespiration and fatty acid degradation. Glutathione peroxidases are involved in the reduction of H_2O_2 and cytotoxic hydroperoxides to alcohols. In addition, they detoxify the products of lipid peroxidation. Glutathione reductase catalyzes the final step of the ascorbate-glutathione pathway (Ahmad *et al*., 2010). Polyphenol oxidases (PPOs) are copper containing enzymes which include catechol oxidases and laccases. Catechol oxidases catalyze the oxidation of o-diphenols to their corresponding o-quinones. They are also involved in the hydroxylation of monophenols to o-diphenols. Any wound or pathogen attack in plants will induce the activity of polyphenol oxidases (Sullivan, 2015). In the damaged tissues, PPOs produce quinines. These highly reactive quinines either cross-link or alkylate the proteins, resulting in brown pigmentation. This browning reaction is considered to be a defensive role of PPOs in plants (Lokhandwala and Bora, 2014).

The results of the enzymic antioxidants assays are represented in Table 1. The results revealed high activity of SOD and CAT in the leaves of *Foeniculum vulgare* and the least activity in the

leaves of *Anethum sowa*. A higher activity of SOD is implicated to combat oxidative stress and hence it plays a crucial role in the survival of plants (Ahmed *et al*., 2008). An increase in CAT activity occurs as an adaptive trait, by reducing the toxic levels of H_2O_2, in order to overcome the damage to tissue metabolism (Vital *et al*., 2008).

Table 1: Enzymic antioxidant activities in the leaves of five selected plants of *Apiaceae* family

Enzymic Antioxidants (Units/g)	*Anethum sowa*	*Foeniculum vulgare*	*Trachyspermum ammi*	*Daucus carota*	*Coriandrum sativum*
SOD	118.89±5.6	729.9±2.12	187.05±9.04	241.61±2.13	396.41±4.57
CAT	0.11±0.01	0.41±0.02	0.19±0.01	0.15±0.03	0.13±0.04
POD	0.32±0.03	0.34±0.01	0.31±0.03	0.20±0.01	0.30±0.02
GR	0.31±0.04	0.63±0.02	0.76±0.01	0.61±0.01	1.20±0.11
PPOs					
CO	0.26±0.04	0.48±0.06	0.34±0.01	0.45±0.04	0.80±0.01
Lac	0.23±0.02	0.37±0.01	0.30±0.01	0.28±0.01	0.71±0.01

Values are Mean ± SD of triplicates

The activity of POD was found to be comparable in the leaves of all the five plants. *Coriandrum sativum* leaves exhibited more GR activity followed by the leaves of *Trachyspermum ammi*, *Foeniculum vulgare*, *Daucus carota* and *Anethum sowa*. A higher activity of GR helps in increasing the ratio of $NADP^+$/NADPH. This ensures the availability of $NADP^+$ to accept electrons from the photosynthetic electron transport chain resulting in minimal formation of $O_2^{-\bullet}$ (Sudhakar *et al*., 2001). *Coriandrum sativum* leaves further exhibited high CO and Lac activities, whereas the leaves of *Anethum sowa* exhibited the least activity.

Non-Enzymic Antioxidants

The levels of non-enzymic antioxidants namely ascorbate, reduced glutathione, flavonoids, total phenols, tocopherol, carotenoids, lycopene and chlorophyll in the leaves of the five selected plants are shown in Table 2.

Ascorbic acid is a water-soluble free radical scavenger. Flavonoids are benzo-γ-pyran derivatives and are found to be potent antioxidants. Flavonoids complex with copper or iron, to prevent ROS generation. Carotenoids are lipid soluble plant components that efficiently scavenge the peroxyl radicals that are generated during lipid peroxidation. Lycopene is the most potent naturally occurring antioxidant. It is engaged in quenching of singlet oxygen as it possesses more number of conjugated double bonds. Tocopherol protects polyunsaturated fatty acids from oxidation and hence stabilizes the membrane. Tocopherol functions by scavenging and quenching the lipid peroxyl radicals and modulating signal transduction. The tocopheroxyl radical generated in turn is recycled back to the corresponding α-tocopherol by reacting with ascorbate resulting in the termination of lipid peroxidation chain reactions (Kruk *et al*., 2005; Nimse and Pal, 2015). Glutathione is involved in scavenging H_2O_2 and reacts with singlet oxygen, superoxide radicals and hydroxyl radicals (Millar *et al*., 2003). Polyphenols serve as hydrogen or electron donors and can stabilize and delocalize the unpaired electron as a chain-breaking function. They also have the potential to chelate transition metal ions (Rice-Evans *et al*., 1997).

Table 2: Non-enzymic antioxidant levels in the leaves of five selected plants of *Apiaceae* family

Non-Enzymic Antioxidants	*Anethum sowa*	*Foeniculum vulgare*	*Trachyspermum ammi*	*Daucus carota*	*Coriandrum sativum*
Ascorbate (mg/g)	160.20±0.51	282.60±0.11	156.72±0.32	216.12±0.15	56.87±0.38
Reduced Glutathione (nmoles/g)	215.82±2.12	653.41±5.41	144.85±3.62	260.19±1.69	262.81±1.13
Flavonoids(mg/g)	91.08±1.02	169.76±3.61	131.79±4.12	341.40±8.23	172.18±2.6
Total Phenols (mg/g)	132.33±6.13	273.52±6.12	154.89±2.31	112.14±2.69	351.06±3.19
Tocopherol (µg/g)	5.91±0.06	8.54±0.06	6.27±0.04	4.68±0.06	5.71±0.02
Carotenoid (mg/g)	732.67±5.44	791.11±3.14	384.44±8.32	582.16±2.69	751.86±4.68
Lycopene (mg/g)	37.20±0.49	51.40±0.25	16.47±0.65	32.68±0.13	61.52±0.16
Chlorophyll (mg/g)	0.48±0.01	1.69±0.13	0.78±0.03	0.31±0.03	1.45±0.02

Values are Mean ±SD of triplicates.

The leaves of *Foeniculum vulgare* possessed the highest ascorbate, reduced glutathione, tocopherol, carotenoids and chlorophyll contents. The level of flavonoids was found to be high in *Daucus carota* leaves and the least level was observed in *Anethum sowa* leaves. *Coriandrum*

sativum leaves possessed more content of total phenols and lycopene. The non-enzymic antioxidants play a key role in protecting macromolecules against the attack of free radicals. Vascular plants contain many phenolic compounds that serve as crucial plant products (Kumar *et al*., 2013).

A significant level of total phenols, flavonoids and flavonols was obtained for the aqueous and methanolic extracts of seeds of *Foeniculum vulgare* and *Trachyspermum ammi* (Chatterjee *et al*., 2012). The methanolic extract of seeds of *Foeniculum vulgare* L. and flowers of *Matricaria chamomilla* L. possessed highest total phenolic content compared to ethanol, diethyl ether and hexane extracts (Roby *et al*., 2013).

Starlin and Gopalakrishnan (2013) assessed the activities of enzymatic and non-enzymatic antioxidants in the whole plant of *Tylophora pauciflora*. Kumar *et al*. (2013) reported a higher level of enzymic antioxidants, ascorbic acid and non-protein thiol contents in the leaves of *Bacopa monnieri* L. when compared to stems. Non-enzymatic antioxidants like proline, ascorbic acid and carotenoids are involved in protecting macromolecules against attack by free radicals. The ethyl acetate extract of roots of *Coriandrum sativum* exhibited highest total phenolics content. A positive correlation obtained for total phenolic contents and FRAP and DPPH scavenging activities (Tang *et al*., 2013).

Khan *et al* (2013) quantified the phenolic content of methanolic extract of different parts of *Morus alba* and inferred a strong antioxidative potential of the extract due to high level of polyphenolic compounds. Thus, the level of antioxidants plays a key role in rendering free radical scavenging property to the plants. Polyphenols and flavonoids are found to protect the plants from various environmental stresses. The rhizome of *Nardostachys jatamansi* possessed considerable amounts of polyphenols, flavonoids and sesquiterpenes and hence rendered strong antioxidant and free radical scavenging activities (Razack *et al*., 2015).

CONCLUSION

In the present study, high contents of enzymic and non-enzymic antioxidants were found to be present in the fresh leaves of *Anethum sowa, Foeniculum vulgare, Trachyspermum ammi, Daucus carota* and *Coriandrum sativum*. This observation implies that the consumption of all

these leaves can help in combating diseases and disorders resulting from oxidative damage. Higher levels of antioxidants were recorded in *Foeniculum vulgare* leaves than the other four, suggesting a higher potential of these leaves in rendering antioxidant protection.

Conflict of interest

No conflict of interest is declared by the authors.

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