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Augmentation of antioxidative potential of *in vitro* propagated *Mentha piperita* L.

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Mentha piperita L., as an aromatic culinary herb and a source of variety of phytochemicals including effective antioxidants, is overexploited by food industry. It demands rapid conservation by means of *in vitro* propagation of improved clones. Here, we have made an attempt to evaluate and augment the antioxidative potential of *M. piperita* L. by additing a precursor to the tissue culture derived clones and compared it with the *in vivo* plants so that tissue culture derived plants can serve as an alternative source of drug. *M. piperita* L. were analyzed for total phenol, flavonoids, total antioxidant activity, free radical scavenging activity and lipid peroxidase activity. Total phenol content in *in vivo* plants was lesser than in *in vitro*. In case of total flavonoid content, it also varies through the season where tissue culture derived plants showed similar and continuous production of total flavonoids content. The percentage inhibition of the *in vitro* plant extract of precursor fed clone was higher than that of *in vivo* plant extract. Antioxidant capacity of ascorbic acid was used as a reference standard from which plant extracts with potential antioxidant activity were compared. After addition of precursor, the *in vitro* mint plant proved more efficient in inhibiting lipid peroxidation after one hour than the *in vivo* plant, which has high absorbance value indicating lipid peroxide formation.

Keywords: Antioxidant activity, Mint, Nutraceuticals, Peppermint, Phenylalanine

Mentha piperita L., (Fam. Lamiaceae), commonly called Peppermint, is the aromatic culinary herb widely used ancient time to flavour foods[1]. This herb is also a source of various phytochemicals, including polyphenols which are highly effective antioxidants and less toxic than the well-known synthetic antioxidants as BHA and BHT[2]. As all species of this genus contain high amounts of secondary metabolites, it is overexploited by the food and drug industries making the natural resource threatened. *In vitro* rapid propagation for production of improved clones is desirable for rapid conservation and commercial exploitation of this economically important plant[3]. Tissue culture techniques are used as alternative methods for propagation and conservation of germplasm of this medicinal plant in many countries. Micropropagation technique provides new possibilities for *in vitro* propagation and multiplication of plants and also recognized as an efficient tool for rapid clonal propagation[4]. In this context, application of plant tissue culture has gained major industrial importance in three main areas: (i) Inbreeding and genetics for conservation[5]; (ii) Model systems for plant biochemistry and pathology to produce disease free crops; and (iii) Production of secondary metabolites for exploitation by food, drug and pharmaceutical industries[6]. The growing demand for natural renewable products has brought researchers' attention to *in vitro* plant materials as potential factories for secondary phytochemical products, and has been driving research focus on secondary product expression *in vitro*. The deliberate stimulation of defined chemical products under highly controlled microenvironment regimes provides an excellent forum for in-depth investigation of biochemical and metabolic pathways[7].

In the present study, we have made an attempt to conserve the medicinally important culinary herb, *Mentha piperita* L. through *in vitro* propagation and also evaluated the augmented antioxidative potential of the tissue culture derived clones and compared the values with that of *in vivo* field grown plants.

Materials and Methods

Collection of plant material

Mentha piperita L. was collected from NBPGR, New Delhi bearing strain no IC:54537. *In vivo* plants were maintained in the medicinal garden of Lady Brabourne College. The antioxidant potential of the *in vivo* plants in four seasons, January (Juvenile stage), May (4 months of age), July (6 months of age), October (10 months of age) were taken into account and an average was calculated and the average annual productivity was compared with the *in vitro* regenerates of same age maintained in the laboratory.

Multiplication of shoot buds

Apical and axillary buds of 2-3 cm length from *in vivo* plants were taken as explants. Media used for

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in vitro culture was Murashige and Skoog (MS) modified basal medium supplemented with 3% (w/v) sucrose, 0.05 mg/L (w/v) ascorbic acid, 0.1 mg/L (w/v) glutamine, 0.25% (w/v) Gelrite® and different combinations of growth regulators[8]. Different growth regulators, NAA: α-naphthaleneacetic acid and BAP: 6-benzylaminopurine was added in different combinations and concentrations in MS basal media. The culture vessels containing media were autoclaved at 121ºC for 15 min. Explants were inoculated in sterile nutrient media contained in culture vessel under aseptic condition in a laminar air flow chamber. All the cultures were maintained in a culture room at 24±1°C in a light intensity of 48 µmol m^{-2} s^{-1} photosynthetic photon fluxes. Cultures were maintained by sub-culturing the material after every four weeks under aseptic condition.

Selection of control plants

Shoots collected from plants regenerated *in vitro* in a medium with best response were sub-cultured in MS basal medium for four weeks for elongation. Some untreated tissue culture derived shoots were considered as control and the rest were selected for precursor treatment. Each experiment was carried in 5 replicates.

Addition of precursors in media

One biosynthetic precursor responsible for rosmarinic acid synthesis; phenylalanine is added as additive during culture. Stock solutions were prepared in distilled water and added at different concentrations in same media[9,10]. In control media same volume of water was added instead of precursor. A wide range of concentrations were chosen (2, 4, 6, 8 and 10.0 mg/L for clones P_1-P_5, respectively) for phenylalanine in a factorial arrangement for addition in media. The control had no phenylalanine added. After collection of *in vitro* regenerated shoots from different culture media at two months interval, different parameters were observed.

Preparation of the plant extract

The fresh leaves (5 g) was crushed with 50 mL 80% methanol in a homogenizer (REMI) and filtered. The content was decanted through a filter paper and the residue was re-extracted again with 80% methanol. The extracts were lyophilized (Hahntec Lyophilizer) till free of solvents and crude extract was prepared.

Estimation of phenolics

The amount of total phenolics in extracts was determined with the Folin-Ciocalteau reagent[11]. Gallic acid was used as a standard and the total phenolics were expressed as mg/g gallic acid equivalents (GAE). About 1 mg/mL of plant extract was prepared in methanol and 0.5 mL of each sample and introduced into the test tubes. It was mixed with 2.5 mL of 10 fold dilute Folin-Ciocalteau reagent and 2 mL of 7.5% sodium carbonate. The reaction mixture was allowed to stand for 30 min at room temperature (25◦ C) and the absorbance was read at 760 nm in UV-Vis spectrophotometer. The concentration was calculated using gallic acid as the standard and the results were expressed as mg gallic acid equivalents/g of fresh weight (mg GAE/gFW).

Estimation of flavonoids

Total flavonoid content was measured by the aluminium chloride colorimetric assay[12]. In this method, quercetin was used as standard and flavonoid contents were measured as quercetin equivalent from the standard curve (y=a*x, a= 0.001339+/-0.3133) in mg/mL QE/g FW. About 1 mL of extract (500 µg/mL) was added to a 10 mL volumetric flask containing 4 mL of distilled water followed by 0.3 mL 5% $NaNO_2$. After 5 min, 0.3 mL 10% $AlCl_3$ was added. At 6th min, 2 mL of 1 M NaOH was added and the total volume was made up to 10 mL with water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm by UV-Vis spectrophotometer.

Total antioxidant activity

The total antioxidative activity was measured using the method described by Prieto *et al.*[13]. Plant extracts were dissolved in methanol to obtain a concentration of 500 µg/mL and 0.3 mL of extract was placed in a test tube; 3 mL of reagent solution (0.6M H_2SO_4+28 mM sodium phosphate+ 4 mM ammonium molybdate) was added. The reaction mixture was incubated at 95ºC for 90 min. The mixture was cooled to room temperature; the absorbance of each solution was measured using Systronics UV-Vis spectrophotometer at 695 nm against blank. The experiment was performed in triplicate. A calibration curve was constructed, using ascorbic acid (100-500 µg/mL) as standard and total antioxidant activity of extract expressed as ascorbic acid equivalents (mg/mL Asc AE/g FW).

Lipid peroxidation

The antioxidant potential of *M. piperita* L. was measured by inhibition of lipid peroxidation of linoleic acid[14]. About 0.5 mL (1 mg/mL) of mint extract was taken and mixed with 2.0 mL of linoleic

acid emulsion (pH 7.0) and the final volume was made up to 3 mL by adding 0.5 mL of phosphate buffer (0.2M, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.28 g of linoleic acid, 0.28 g of Tween-20 as emulsifier and 50 mL of phosphate buffer. The mixture was then homogenized. The reaction mixture was incubated at room temperature. Aliquots (0.1%) was drawn after every 30 min up to 4 hours to access peroxidation of linoleic acid using thiocyanate method by sequentially adding ethanol (4.7 mL, 75%), ammonium thiocyanate (0.1 mL, 30%), sample solution (0.1 mL) and ferric chloride (0.1 mL, 0.02M in 3.5% HCl). After 30 min the mixture was diluted using 1 mL of extract and 4 mL of distilled water. The mixture was allowed to stand for 3 min and absorbance was recorded by double beam spectrophotometer at 500 nm. A negative control was run in an identical manner where extract is replaced by water. Ascorbic acid was used as a positive control.

Free radical scavenging activity

The antioxidant activity of the extracts was assessed by their ability to scavenge 2-2 diphenyl-1picryl hydrazyl stable radicals (DPPH)[15]. 1 mL of methanolic extract and 5 mL of freshly prepared 0.1 mM DPPH methanolic solution were thoroughly mixed and kept in the dark for 60 min. The absorbance of the reaction mixture at 517 nm was measured by spectrophotometer. The blank set was prepared by replacing the extract with methanol (1 mL). The percentage of free radical scavenging activity was calculated as follows:

$$\text{Free radical scavenging activity (\%)} = 100 \times (1 - A_{sample}/A_{blank}),$$

where A_{blank} is the absorbance of the control and A sample is the absorbance of the extract or ascorbic acid[16]. All the tests were run in triplicate and averaged.

Statistical analysis

Data were analyzed statistically to determine the least significant difference (LSD). Three replications of each set of experiment were taken for study and an average was calculated using Statistical software R.

Results and Discussion

In vitro culture

Apical buds were most suitable explant sources for multiplication of *Mentha*. The bud break was observed mostly between 8-11 days after culture in all cases. All the cultures were maintained up to fifteen weeks. For *Mentha* various combinations of different cytokinins on shoot bud multiplication were used with one auxin NAA (0.25 mg/L). The media containing 0.25 mg/L NAA and 2.5 mg/L BAP produced highest number of shoot buds (58.0 ± 0.82 shoot buds per explant). BAP showed the highest potentiality for shoot bud multiplication than the other two cytokinins tested. Root formation started after fifteen days of culture. Presence of auxin only gave a better response in root production. After complete regeneration they were transferred to the pre acclimation chamber (PAC). The plants were maintained there up to 3 wk. The upper half of the PAC was removed after 3 wk and kept in the outer environment. It was seen that plants survived with a good growth. The 8-wk old plants were taken for further tests.

The present investigation on multiplication of *Mentha piperita* L. revealed that the cytokinin BAP is more efficient in shoot bud multiplication than other cytokinins as reported by earlier authors in different plants[17-19] and in other plant species[20]. The requirement of such different levels of cytokinins may be correlated with the difference in its endogenous level in different populations and species studied, that may also be linked to its habitats. Chromosome analysis revealed chromosome number stability in the regenerates. This may indicate the efficacy of BAP and the culture conditions in maintaining the genome stability of the regenerates. As the principal importance of the medicinal plants is being highlighted as the source of natural antioxidant and functional food, the role of clonal propagation attracts much attention[21,22].

Polyphenols have received much attention because of their role in several degenerative and age related diseases. Like other micropropagated medicinal plants the levels of phytochemicals and the resultant pharmacological potency such as antioxidant activity in the micropropagated *Mentha piperita* are important. Although there may not be always a positive correlation between phytochemical content and bioactivity of regenerated plants, it may provide vital information in the critical assessment of the quality of micropropagated plants[23].

Total phenol content

The amount of total phenol varies in the four seasons. For field grown plants with an average of 5.82±0.03 mg GAE/g FW of methanolic extracts where tissue culture derived plants showed continuous and constant production of total phenolics

(6.24±0.03 mg GAE/g FW) which is 7% more than the field grown plants. After addition of precursor the production was increased significantly giving 1.7 time augmentation in total phenol content (Table 1).

Total flavonoids content

In case of total flavonoids content, it also varies through the season with an average production of 68.64±0.24 mg/mL QE/g FW where tissue culture derived plants showed similar and continuous production of total flavonoid content. After addition of precursors the production was augmented by 91.54% (Table 1).

Seasonal variation of phenolic content had been reported earlier. The total phenolic content of *Mentha piperita* varies in each season. In summer the plant growth was average. During monsoon a vigorous growth was observed with a slight decrease in total phenol content than in summer. During winter the growth rate was declined. Similar results were also observed in other plant species[24], where seasonal variation of total phenolic content was observed. The influence of environmental conditions on quantity of active constituents in various plants has been reported. It was observed that phenolic acid, flavonol and anthocyanin contents and antioxidant activity in fruit juice of *Fragaria ananassa* were considerably influenced by day and night growing temperature combinations[25]. It was observed by many researchers that the physiological and biochemical changes are synergistic effects of different biotic and abiotic parameters. In most of the plants exposure to high and low temperature than the optimum was regarded to be the main cause[26]. It is difficult to determine which environmental factor is mainly responsible for the variations. In laboratory experiments factors can be set and regulated exactly[27]. So by providing tissue culture approaches, a shoot based clonal line was tested here for experimental purpose and it was observed that a continuous stable production of total phenolics, total flavonoids and rosmarinic acid content was synthesized in shoot based clonal lines[28]. Tissue culture derived plant had a higher content of phenol as compared to the field grown plants. The reports mentioned by other authors[29] also are in accordance with our results. The results indicate that the phenolic compounds have a major contribution to the antioxidative capacity of the herbs.

The antioxidative potential of *in vitro* and *in vivo* regenerated plants was determined using the total antioxidant capacity, free radical scavenging activity and lipid peroxidation method.

Total antioxidant capacity

In the present study, the antioxidant activity in different plant parts (both field grown and micropropagated) were studied using different antioxidant assays i.e. flavonoids, TAC, free radical scavenging activity and lipid peroxidation and the data obtained by these antioxidant assays revealed that the methanolic extract of the micropropagated plants showed highest degree of antioxidant activity. Our results are in accordance with the results obtained in *Dendrobium nubile*-an important medicinal orchid used in herbal drugs.

Total antioxidant capacity of the methanolic extracts was determined from regression equation of calibration curve (y=ax, where a=0.001845) and expressed as mg/ml Asc AE/g FW (Table 2). The total antioxidant capacity of extracts of field grown *Mentha piperita* plants was found to be 497.03±0.08mg/mL Asc AE/g FW. 25.63% increase in TAC activity was observed after addition of precursor. Antioxidant capacity of Ascorbic acid has

Table 1—Determination of total phenol and total flavonoid

Plant sample	Total phenol (mg GAE/g FW)*	Total flavonoid content (mg/ml QE/g FW)*
Field grown plants	5.82±0.03^{a}	68.64±0.24^{a}
Control	6.24±0.83^{b}	71.03±0.48^{b}
P1	6.76±0.87^{b}	94.28±0.66^{c}
P2	7.68±0.66^{c}	78.39±0.98^{d}
P3	4.96±0.08^{d}	101.56±1.29^{e}
P4	9.63±0.76^{e}	118.26±0.32^{f}
P5	8.60±0.34^{f}	136.10±0.43^{g}
LSD	1.03	1.44

[*Data expressed as Mean ± S.E. from 3 replicates; $P \leq 0.0001$. Means followed by different superscript letters in the same column present significant difference. $P \leq 0.05$]

Table 2 — Determination of total Antioxidant and free radical scavenging activity

Plant sample	Total antioxidant capacity (mg/mL Asc AE/g FW)	Free radical scavenging activity (%) (500 µg/mL)	Free radical scavenging activity (%) (1mg/mL)
Field grown plants	497.03±0.08^{a}	5.82±0.09^{a}	10.10±0.19^{a}
Control	531.29±0.67^{b}	6.50±0.66^{b}	11.59±0.94^{b}
P1	573.11±0.33^{c}	6.50±0.33^{b}	11.28±0.82^{b}
P2	595.80±0.19^{d}	7.53±0.67^{c}	13.92±0.13^{c}
P3	608.86±0.48^{e}	7.53±0.48^{c}	13.27±0.22^{c}
P4	617.73±0.96^{f}	8.92±0.98^{d}	15.24±0.17^{d}
P5	624.40±0.24^{g}	9.70±0.18^{d}	17.36±0.18^{e}
Ascorbic acid		10.9±0.12^{e}	22.60±0.48^{f}
LSD	1.008	0.86	0.76

[*Data expressed as Mean ± S.E. from 3 replicates; $P \leq 0.0001$. Means followed by different superscript letters in the same column present significant difference. $P \leq 0.05$]

been used as a reference standard from which plant extracts with potential antioxidant activity are compared[30]. The high antioxidant activity was exhibited in *in vitro* regenerated plants compared with the *in vivo* plants. The result of this study demonstrates that the phenolic acid levels increased in *in vitro* regenerated plants and directly influenced their antioxidant potential[31]. Our results are similar to the results obtained from the extracts of *Phyllanthus niruri*, where the *in vitro* plants had higher TAC value than the samples obtained from the market[32]. There is an interesting correlation in the samples between the antioxidant capacity determined by the DPPH method and the total phenolic content. Antioxidant activity will probably depend mainly on the content of the total phenolic compounds in the herbs. Our results are in accordance because the higher the amount of total phenolics, the higher the total antioxidant capacity and the percentage inhibition of the free radical.

The DPPH radical scavenging activities

The DPPH radical scavenging activities of the extracts increased by increasing the concentration of the sample extract. This assay has been largely used as a quick, reliable and reproducible parameter to search for the *in vitro* antioxidant activity of pure compounds as well as plant extracts. This manifested in the rapid discoloration of the purple DPPH to light yellow suggesting the radical scavenging activity of the methanol extract of *Mentha* was due to its proton donating ability. The percentage inhibition of the *in vivo* plant extract is 5.82±0.09, while that of the precursor feeded clone is 9.70± 0.98, nearly 1.66 times higher than the *in vivo* plants. Ascorbic acid, the well-known antioxidant has a percentage inhibition of 10.90±0.12.The results indicate that the *in vitro* cultured plants contain a high concentration of antioxidant compounds (Table 2). The present results suggests extracts are apparently good free radical scavenger and probably have the ability to inhibit auto oxidation of lipids and could thus be beneficial in the treatment of various diseases where lipid peroxidation is an important mechanism for pathogenesis[33].

Lipid peroxidation

Polyunsaturated fatty acids, such as linoleic acids are easily oxidized in the presence of oxygen of the air. During Linoleic acid oxidation, peroxides are formed. Primary antioxidants react with lipid peroxy radicals to convert them into stable products. Secondary antioxidants, such as oxygen scavengers, reduce the rate of chain initiation[34]. The FTC method measures the amount of peroxidases in the beginning of lipid peroxidation where ferric ion was formed upon reaction of peroxide with ferrous chloride. The ferric ion later unites with ammonium thiocyanate producing ferric thiocyanide, a red coloured substance[35]. The absorbance data of linoleic acid peroxidation after adding plant sample with positive control ascorbic acid is shown in Table 2. Thus, a high absorbance value was an indication of high peroxide formation during incubation. The results indicate that after addition of precursor (Phenylalanine 8 mg/mL) the *in vitro Mentha piperita* plant sample becoming more efficient in inhibiting lipid peroxidation after one hour than filed grown plants, which has high absorbance value indicating lipid peroxide formation. The results are comparable to the standard antioxidants used. Similar values were established[36,37] for the aromatic plants of Labiatae family. After the completion of the second hour all the samples have shown a lower absorbance value indicating inhibition of lipid peroxidation. The positive control, ascorbic acid have also shown similar results. Our results shows that all the peppermint plants, both field grown and regenerated plants with precursors can inhibit the formation of lipid peroxides. After addition of precursors (phenylalanine 8 mg/mL), the *in vitro* regenerated mint plant becomes more efficient in inhibiting the lipid peroxidation after one hour than the field grown plants which has a high absorbance value indicating formation of lipid peroxides (Table 3). The phenolic compounds present in *Mentha piperita* may donate hydrogen and can terminate the free radical reaction chain by changing into stable compounds. Leaves of Lamiaceae, have higher antioxidant activity compared to the plants belonging to other families. Peppermint also has a hepatoprotective effect through antiperoxidation. The hydrophyllic fractions of the Lamiaceae plants in which bitterness and lipophilic odorants were reduced may be useful as foodstuffs for liver protection[38].

Phenylalanine is the precursor of phenyl propanoid pathway leading to the formation of phenolic acids, flavonoids and other phenolic compounds. Most natural phenolic compound in plants is derived from Transcinnamic acid formed by deamination of L-phenylalanine by Phenylalanine ammonia lyase (PAL). Phenylalanine has been used to increase the metabolite production *in vitro* in several different plant cultures[39]. These metabolites might increase by the stimulation of an enzyme Phenylalanine ammonia

Table 3 — Determination of Lipid peroxidation activity

Sample	Time (h)			
	1	2	3	4
Asc Acid(+ve control)	$0.156\pm0.06_a{}^a$	$0.80\pm0.12_b{}^a$	$0.120\pm0.12_c{}^a$	$0.159\pm0.13_d{}^a$
Field grown plants	$0.265\pm0.08_a{}^a$	$0.121\pm0.19_b{}^a$	$0.165\pm0.11_b{}^a$	$0.187\pm0.36_b{}^a$
Control	$0.251\pm0.07_a{}^a$	$0.126\pm0.03_b{}^a$	$0.140\pm0.09_c{}^a$	$0.166\pm0.18_d{}^a$
P1	$0.226\pm0.12_a{}^a$	$0.141\pm0.23_b{}^a$	$0.162\pm0.06_b{}^a$	$0.225\pm0.12_c{}^a$
P2	$0.212\pm0.23_a{}^a$	$0.201\pm0.06_b{}^a$	$0.190\pm0.28_b{}^a$	$0.239\pm0.16_b{}^a$
P3	$0.202\pm0.16_a{}^a$	$0.212\pm0.18_a{}^a$	$0.145\pm0.16_b{}^a$	$0.248\pm0.07_c{}^a$
P4	$0.229\pm0.14_a{}^a$	$0.094\pm0.08_a{}^a$	$0.059\pm0.10_b{}^b$	$0.107\pm0.08_c{}^b$
P5	$0.230\pm0.06_a{}^a$	$0.208\pm0.03_a{}^b$	$0.255\pm0.20_b{}^c$	$0.301\pm0.10_c{}^c$
Water (-ve control)	$0.430\pm0.22_a{}^b$	$0.490\pm0.17_b{}^c$	$0.494\pm0.11_b{}^d$	$0.503\pm0.12_b{}^d$
LSD	0.12	0.072	0.084	0.10

[*Data expressed as Mean ± S.E. from 3 replicates; $P \leq 0.0001$. Means followed by different superscript letters in the same column present significant difference. $P \leq 0.05$]

lyase by the addition of precursors[40]. Phenylalanine ammonia lyase (PAL) is the first and committed step in the phenyl propanoid pathway[41]. Phenylalanine used to increase the metabolite production in *in vitro* clones here might be mediated by stimulation of phenyl propanoid pathway.

The accumulation of monoterpenoids in the tissue culture of different *Mentha* species and hybrids has been reported by several workers[42]. Some studies have emphasized the need for high levels of differentiation to achieve the production of monoterpenoid by cell cultures. Maximum accumulation of terpenoids has been found in the late exponential phase of culture cycle and is higher in cell suspensions than in callus cultures[43]. Terpenoid production was enhanced in cell culture and callus culture of *Mentha piperita* but as no reports were found on terpenoid production from shoot culture it could be explored further.

Conclusion

In this study, we have proposed a simple and rapid *in vitro* propagation protocol. Both the *in vivo* and tissue culture derived plants have been shown to possess significant amount of polyphenolic components, expressed as gallic acid equivalents. The content of rosmarinic acid, the phenolic compound in *Mentha piperita*, was higher in the *in vitro* regenerated plants. The results of antioxidant evaluation based on two models, DPPH and TAC used in this study revealed that the methanolic extract of both field grown and tissue cultured *M. piperita* possess interesting antioxidant activity, and hence, excellent free radical scavenging capacity. Both the field grown and regenerated plants were able to inhibit the oxidation of lipids. It was observed that the antioxidative properties of the field grown plants were well maintained in the tissue culture as well as successfully augmented suggesting *in vitro* regeneration as an alternative for sustainable use of this medicinally important plant used as a food preservative apart from pharmaceutical and natural therapies for treatment of oxidative stress.

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Conflict of interest

The authors declare no conflicts of interest.

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