Antioxidant Activities and Phenolic Compounds of Two Endemic Taxa of *Labiatae Sideritis*

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Sideritis species have very high antioxidant activity and and are used in a range of treatments by alternative medicine. This study examined the antioxidant activities and phenolic compositions of endemic 2 Sideritis species (Sideritis phrygia Bornm and ideritis bilgerana P.H. Davis), which were collected in the Konya region of Turkey. The oil content was extracted with petroleum ether using a soxhlet extractor. The defatted plant materials were extracted with methanol and then filtered and concentrated in vacuo at 45 °C. Finally, the extracts were lyophilized and stored in the dark at +4 °C until analyzed for antioxidant activity. Total phenol concentration of the extracts were estimated with Folin-Ciocalteu reagent using gallic acid as standard, free radical scavenging activities were determined based on DPPH(2,2-diphenyl-1-picrylhydrazyl) and ferric reducing antioxidant power (FRAP) was determined based on the method proposed by Oyaizu. Results were compared with standard BHT(Butylated hydroxytoluene) and BHA (Butylated hydroxyanisole). The phenolic composition of the samples was determined using HPLC. The results indicated that S. phrygia and S. bilgerana showed significant antioxidant effect and 6 phenolic compounds used in standard phenolic compounds. S. phrygia showed higher antioxidant capacity than S. bilgerana.

Keywords: Antioxidant activity, DPPH, reducing power, phenolic compound

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in biochemical reactions and physiological processes and cause oxidative stress leading to harmful oxidative reactions in organisms. This free radical reactions are the cause of certain human diseases including cardiovascular disease, cancer [1]. Antioxidants are of great importance in terms of preventing oxidative stress that may cause several degenerative diseases. The primary sources of antioxidants are plants; the preservative effect of plant spices and herbs suggests the presence of antioxidative and antimicrobial constituents in their tissues [2]. Dietary antioxidants are helpful in assisting the body to neutralize free radicals. Therefore, it is important to consume a diet high in antioxidants, such as fruits and vegetables, to reduce the harmful effects of oxidative stress. Fruits and vegetables are a rich source of phytochemicals, such as carotenoids, flavonoids and other phenolic compounds. Studies have indicated that these phytochemicals, especially polyphenols, have high free-radical scavenging activity, which helps to reduce the risk of chronic diseases [3]. Polyphenolic compounds are secondary metabolites which are of considerable physiological and morphological importance in plants. Researches showed that polyphenols have effective chemical structure for free radical scavenging activity, and they are more effective than some secondary metabolites. Generaly drugs such as antibiotics, antivirals, antiseptics and some bioactive substances based on polyphenols have been used. So phenolic compounds exhibit a wide range of physiological properties, such as anti-allergenic, antiatherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects. Therefore, plant materials containing phenolic compounds are increasingly of interest as they retard oxidative degradation of lipids [4-7].

The genus *Sideritis L. (Lamiaceae)* is represented by more than 150 species mainly found in the Mediterranean basin but also distributed from the Bahamas to western China and from Germany to Morocco. The genus *Sideritis* is represented in the Flora of Turkey by 46 species,of which 36 taxa are endemic to Turkey. Some *Sideritis* species are used widely in the treatment of diseases and their dried leaves are served as tea [8-12]. This study examined the antioxidant properties and phenolic compounds of *S. bilgerana* and *S. phrygia* endemic species that were collected in the Konya region of Turkey.

Experimental part

Plant material

The aerial parts of *Sideritis* species used in this study were collected in the flowering season from Konya and voucher specimens were deposited in the Biology Department Herbarium at Selcuk University (Konherbarium), Turkey.

Chemicals

The following chemicals were obtained for this study: Folin–Ciocalteu reagent, iron (III) chloride methanol (HPLC grade), trichloroacetic acid, BHA, BHT and petroleum ether (Merck; Darmstadt, Germany); Gallic acid (Acros Organics; NJ, USA); Anhydrous sodium carbonate (J.T. Baker; NJ, USA); DPPH and potassium ferricyanide (Sigma-Aldrich).

Preparation of methanol extracts

The samples were air-dried and then finely ground, were extracted using the method described elsewhere [13]. Briefly, a sample, weighing 100 g, was extracted in a soxhlet extractor with methanol at 60°C for 6 h. The extract was then filtered and concentrated in vacuum at 45°C. Finally, the extracts were lyophilized and stored in darkness at 4°C until tested.

Total Phenolic Assay

Total phenolic concentrations of the extracts were determined by the Folin-Ciocalteu colorimetric method [14]. Estimations were carried out in triplicate and calculated from a calibration curve obtained with gallic

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Species	(μg GAE / mL methanolic solution)
Sideritis phrygia Bornm	1,73±0,02
Sideritis bilgerana P.H. Davis	1,62±0,04

Table 1
TOTAL PHENOLIC CONTENT

acid. Total phenolic concentrations were expressed as gallic acid equivalents (μg GAE/mL methonolic solution). The phenol contents of the plant extracts were determined with the Folin-Ciocalteu method using 50 mL of each sample (0.4 mg/mL), 2.5 mL of 10 % dilution of Folin-Ciocalteu reagent and 2 mL of Na_2CO_3 (7.5 %, w/v). The resulting mixture was incubated at 45°C for 15 min. The absorbances of all samples were measured at 765 nm. Gallic acid was used as a standard for the calibration curve. The total phenolic concentrations were calculated and expressed as micrograms of gallic acid equivalent.

Free radical-scavenging method

The antioxidant activity of plant extracts was measured in terms of hydrogen-donating or radical scavenging ability, using the stable radical, (DPPH•). DPPH· is a stabile free radical and accepts a hydrogen radical to become a stable molecule. Free radical-scavenging activities were measured using a modified version of the method proposed by [15,16]. Methanolic solutions of samples (0.4-0.05 mg/ml) were placed in a cuvette and 4 mL of 6×10^{-5} mol/L methanolic solution of DPPH was added. After 0.5 h incubation period in darkness at room temperature, the absorbances were read against a blank at 515 nm. The same procedure was repeated with synthetic antioxidant, BHT and BHA, as positive control and a blank. Inhibition level (measured as percentage, I %) of the free radical DPPH• was calculated as follows:

$$I\ \% = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. The extract concentration required for 50% inhibition (IC_{50}) was calculated from the graph by plotting inhibition percentage against extract concentration. Tests were carried out in triplicate and BHT and BHA were used as positive controls.

Reducing power

The reducing power of the plant extracts was determined using the method proposed [17]. Different concentrations (0.4-0.04 mg/mL) of plant extracts in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, *p*H 6.6) and potassium ferricyanide ($K_3Fe(CN)_6$) (2.5 mL, 1 %). The mixture was incubated at 50°C for 20 min. Aliquots (2.5 mL) of trichloroacetic acid (10 %) were added to the mixture, which was then centrifuged for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1 %) and the absorbance at 700 nm was measured in a spectrophotometer. Increased absorbance of the reaction mixture indicates increased reducing power.

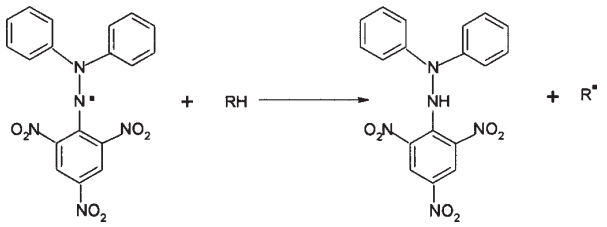
HPLC analysis of extracts

The phenolic compounds were extracted using the method described by [18]. Reversed phase (RP)-HPLC analysis used an SCL-10Avp system controller, and a Diode Array Detector with wavelengths set at 280, 320 and 360 nm. The flow rate was 1 mL/min, the injection volume was 10 mL, and the column temperature was set at 30°C. For gradient elution, mobile phase A contained 2% acetic acid in water; solvent B contained methanol. The following gradient was used: 0–3 min, from 100% A to 95% A, 5% B; 3–20 min, from 95% A, 5% B to 80% A, 20% B; 20–30 min, from 80% A, 20% B to 75% A, 25% B; 30–40 min, from 75% A, 25% B to 70% A, 30% B; 40–50 min 70% A, 30% B to 60% A, 40% B; 50–55 min, 60% A, 40% B to 50% A, 50% B; 55–65 min, 50% A, 50% B to 100% B. The data were integrated and analyzed using the Shimadzu Class-VP Chromatography Laboratory Automated Software system. The samples, standard solutions, and mobile phases were filtered with a 0.45 mm pore size membrane filter. The amount of phenolic compounds in the extracts was calculated as μg/g extract, using external calibration curves obtained for each phenolic standard.

Results and discussions

Phenolic compounds (phenolic acids, flavonoids and proanthocyanidins etc.) form a major group of phytochemicals found in plants. The antioxidant properties of phenolics are mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers [19]. Polyphenols can be stabilized through intramolecular hydrogen bonding or by further oxidation. Total phenolic compound assay was measured by the Folin-Ciocalteu method. The amounts of total phenolics (μgGAE/mL) were determined as 1.73±0.02 μgGAE/mL for *S. phrygia* and 1.62±0.04 μgGAE/mL and *S. bilgerana*. (table.1). HPLC analyses produced similar results (table 4).

The radical scavenging method developed to determine the antioxidant activity of foods utilizes the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical. DPPH free radical gives a strong absorption maximum at 517 nm and is purple in color; the color turns from purple to yellow [20].



In the present experiment, methanolic extracts of two plants were evaluated for their free radical scavenging activity using the DPPH radical assay. Reduction of DPPH radicals can be observed by the decrease in absorbance at 517 nm. Two *Sideritis* extracts and synthetic antioxidant compounds reduced DPPH radicals significantly. Values of IC_{50}, connected to the percent decolorization of DPPH radicals, are shown in table 2 where IC refers to inhibitory concentration scavenging effect. The IC_{50} is the concentration of an antioxidant at which 50% inhibition of free radical activity is observed so a lower the IC_{50} number indicates higher antioxidant activity [21]. BHA and BHT are phenolic compounds known synthetic antioxidants. Naturally, their IC_{50} values are lower than those of *Sideritis* extracts. As a result, according to antioxidant powers, the antioxidant activity of BHA is higher than that of BHT and *Sideritis* extracts. *Sideritis* species have considerable antioxidant effect with *S. phrygia* showing greater

Species	IC_{50}
S. phrygia	0,0700±0,002
S. bilgerana	0,1230±0,0015
BHT	0,0543±0,002
BHA	0,0440±0,001

Table 2
IC_{50} VALUES OF *Sideritis* SPECIES AND SYNTHETIC ANTIOXIDANTS

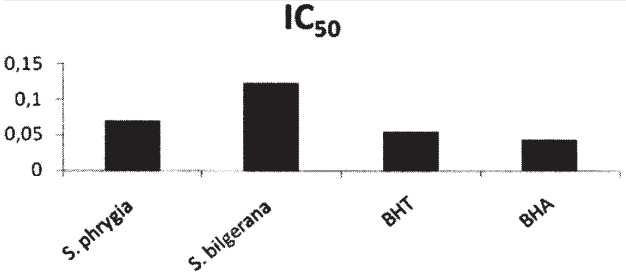


Fig.1 IC_{50} values of *Sideritis* species and synthetic antioxidant compounds

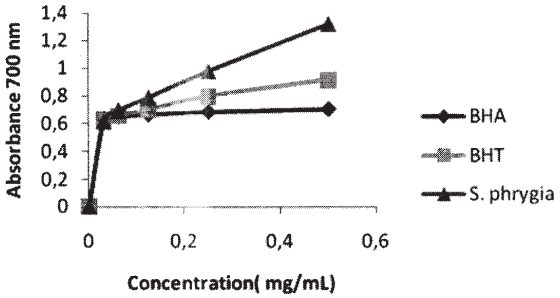


Fig.2 Reducing power of BHA, BHT and *S. phrygia*

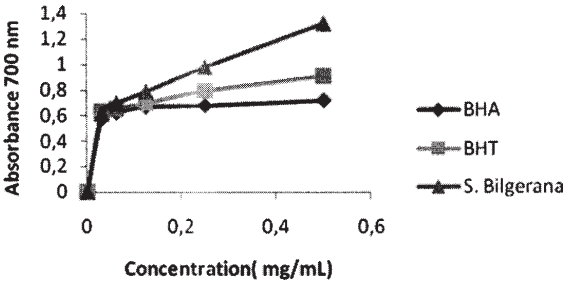


Fig.3 Reducing power of BHA, BHT and *S. bilgerana*

scavenging activity than *S. bilgerana* (fig.1). Parallel results were found using the Folin method.

After addition of the iron and hydrogen peroxide, they are going to react to generate hydroxyl radicals as shown in the following equations;

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$$

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + {}^{\bullet}OOH + H^+$$

In a Fenton reaction, Fe^{2+} reacts with H_2O_2, resulting in the production of hydroxyl radical, which is considered to be the most harmful radical to biomolecules. By many reductants, the oxidized form of iron ions can be converted to its reduced form Fe^{2+}, which can enhance the generation of hydroxyl radicals resulting in increased DNA damage [22]. The reducing power of the plant extracts was determined by the method proposed by [15] and then compared with BHA and BHTas shown in figures 2 and 3. According to FRAP values obtained via absorbance, BHA also showed a stronger antioxidant effect than the other extracts (table 3) as clearly seen in figures 2 and 3. FRAP values obtained for *S. phrygia* and *S. bilgerana* were very similar, at 7.61 μg/mL and 7.15 μg/mL (trolox equivalent) respectively. As BHA and BHT are synthetic antioxidant compunds, their FRAP values are much higher than those of lyophilized *Sideritis* extracts.

The identification of phenolics of *Sideritis* species by HPLC was achieved by comparing their retention times. A typical HPLC chromatogram of standard phenolic compounds is presented in (fig.5) and chromatograms of *S. phrygia* and *S. bilgerana* are shown in figures 6 and 7 respectively. Nineteen standard phenolics or phenolic acid compounds are used in the HPLC system of which 6 phenolic compounds were found in both *Sideritis* species. In total, 2205.5 μg/g *extract* phenolic compound was found

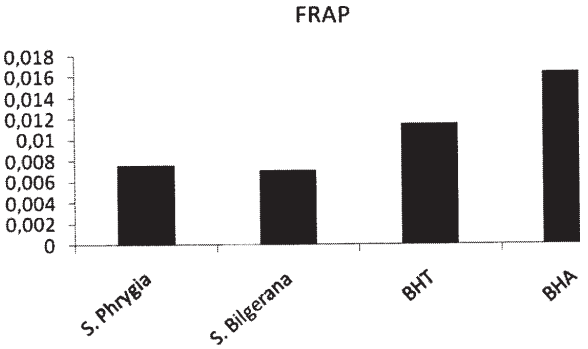


Fig.4 FRAP values of *Sideritis* species and synthetic antioxidants

in *S. Phrygia* compared with 1470.1 μg/g in *S. bilgerana*. (table 4). While apigenin is the most characteristic phenolic compound in both *Sideritis* species, apigenin glucoside is only found in *S. phrygia*. (687.1 g/g extract). Thirteen of the phenolic compounds used as standards were not found in both of the *Sideritis* species (table 4). Unnamed peaks visible in the chromatograms are probably different phenolic compounds that were not used as standard in this study. In conclusion, it is well known that many

Table 3
FRAP VALUES OF *Sideritis* SPECIES AND SYNTHETIC ANTIOXIDANTS

Species	FRAP (*Trolox equivalents*) (μg/mL)
S. Phrygia	7,61±0,01
S. bilgerana	7,15±0,02
BHT	11,5±0,02
BHA	16,3±0,03

Phenolic compounds	*S. bilgerana* (µg/g extract)	*S. phrygia* (µg/g extract)
Gallic acid	*	*
Catechin	61.1±0,1	112.3±0,3
Caffeic acid	217.1±0,1	48.8±0,1
Epicatechin	*	*
p-coumaric acid	*	*
Ferulic acid	26,7±0,2	69.2±0,2
Viteksin	*	*
Rutin	*	*
Naringin	*	*
Hesperidin	*	*
Apigenin-glucoside	*	687.1±0,1
Rosmarinic acid	*	*
Eriodictyol	*	*
Quercetin	80.2±0,3	*
Naringenin	*	*
Luteolin	254.4±0,2	41.1±0,2
Apigenin	830.6±0,3	1247,0±0,1
Carvacrol	*	*
Acecetin	*	*
	1470,1	**2205,5**

*: could not be detected

Table 4
PHENOLIC COMPOUNDS IN *Sideritis* SPECIES (95% CONFIDENCE INTERVALS FOR PHENOLIC COMPOUNDS)

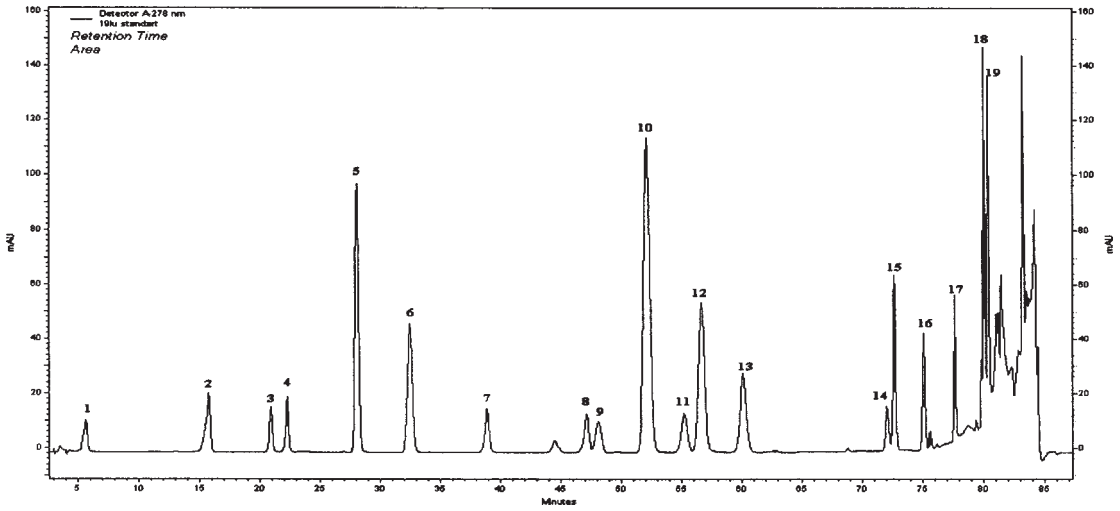


Fig.5 Chromatogram of standard phenolic compounds;1: gallic acid, 2: catechin, 3: caffeic acid, 4: epicatechin, 5: p-coumaric acid, 6: ferulic acid, 7: viteksin, 8: rutin, 9: naringin, 10: hesperidin, 11: apigenin-glucoside, 12: rosmarinic acid, 13: eriodictyol, 14: quercetin, 15: naringenin 16: luteolin,17: apigenin, 18: carvacrol, 19: acecetin

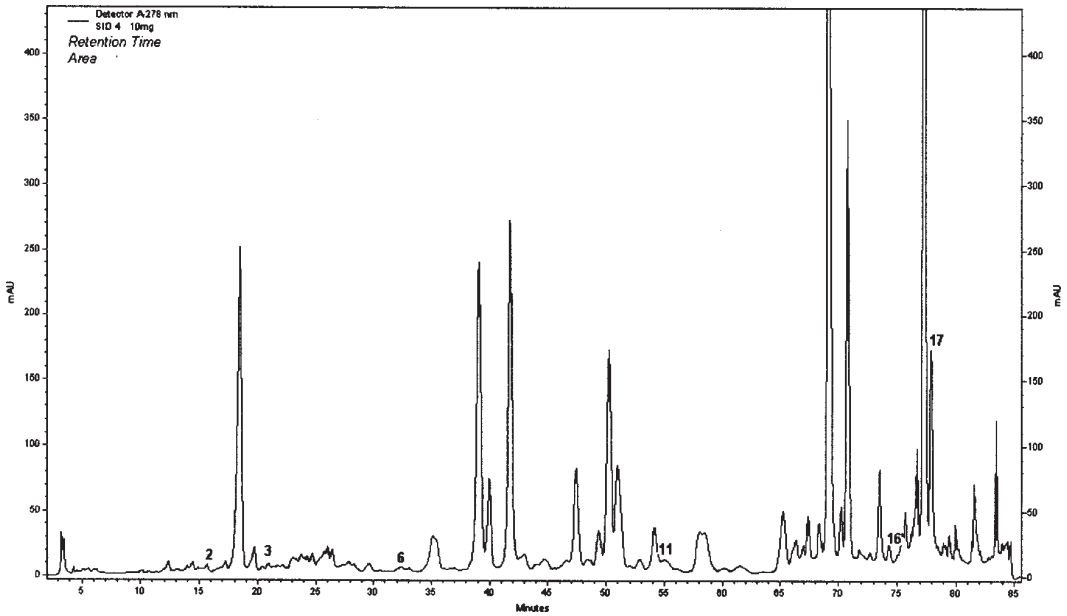


Fig.6 Chromatogram of *Sideritis bilgerana*

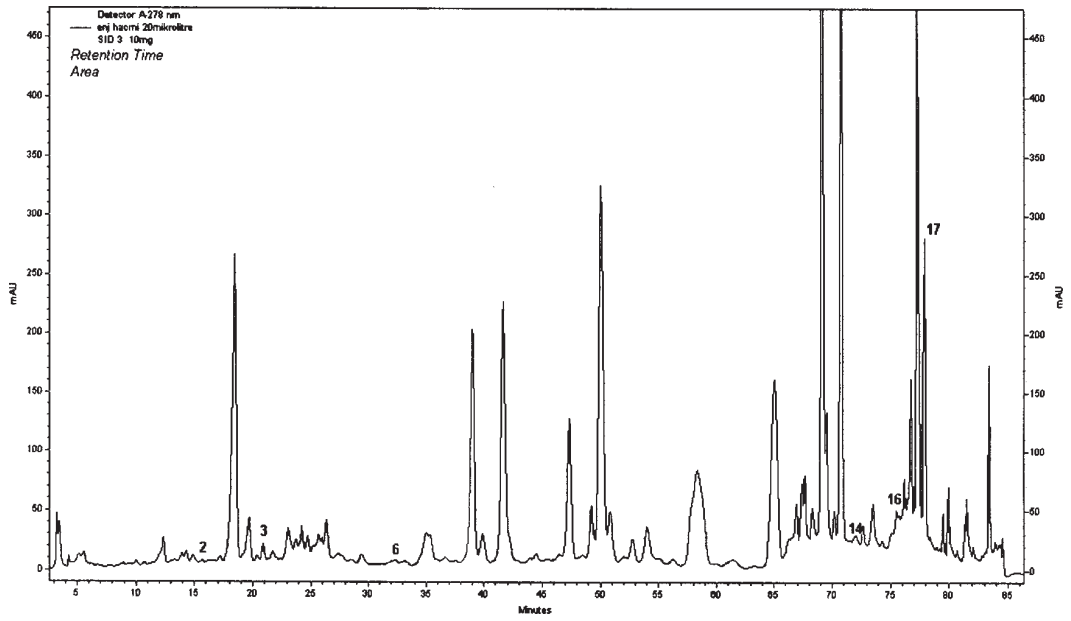


Fig.7 Chromatogram of *Sideritis phrygia*

medicinal plants are also excellent sources of phenolic and polyphenolic compounds, many of which have potent antioxidant activities that are often exploited in food products and in various medicinal treatments [23]. *Sideritis* species are a group of plants known in Turkey as "mountain tea". Therefore, some of the species are used as tea, flavoring agents and for medicinal purposes [24]. The results of this study support the use of *Sideritis phrygia* Bornm and *Sideritis bilgerana* P.H. Davis in traditional remedies and confirm that both species have significant antioxidative activity.

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