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Antioxidant Capacity of Fresh and Dry Leaf Extracts of Sixteen *Scutellaria* Species

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ABSTRACT

The antioxidant capacity of 16 *Scutellaria* species was examined using Trolox equivalent antioxidant capacity (TEAC) assay. Total polyphenol, antioxidant capacity estimation and flavonoid content measurements were conducted on fresh and air dried leaf extracts. The highest total polyphenol content was obtained in dry leaf extracts of *S. ocmulgee* at 732.41 67 mg/g of gallic acid equivalent. Dry leaf extracts of *S. ocmulgee* registered 2480.93 µmol/g of Trolox equivalent antioxidant capacity and the highest total flavonoid content with 200.63 µg/mL from fresh leaf extracts of *S. alpina*. Rosemary (*Rosmarinus officinalis*), a common herb with known high antioxidant potential, was used as a standard for comparison with the *Scutellaria* species.

INTRODUCTION

The genus *Scutellaria,* commonly known as skullcap (scullcap) belongs to the family Lamiaceae (Mint family). Of the 400 known *Scutellaria* species, over 90 of these plants have been recorded as growing in North America. In the current study, 20 *Scutellaria* spp. that grow in and adjacent to the state of Georgia were used (Joshee et al., 2002). *Scutellaria ocmulgee* and *Scutellaria montana*, two species that grow in Georgia, are relatively rare and protected by the state and federal government (Chafin, 2007).

Scutellaria species are known for their potential pharmaceutical/therapeutic properties and have been used in the traditional medicine of many countries for their anti-inflammatory, antioxidant, and anti-viral properties useful in treating human ailments (Huang et al., 2005b; Shang et al., 2010). *S. baicalensis*, native to China, and *S. barbata*, from Korea, have been extensively employed in traditional Chinese medicine (TCM) and in Japanese Kampo medicine (JKM) (Watanabe et al., 2002; Murch et al., 2004). Leaf extract from *S. ocmulgee* has demonstrated inhibitory properties against malignant gliomas (Parajuli et al., 2009, 2011).

Plants, such as basil *(Ocimum* spp.), mint (*Mentha* spp.), rosemary (*Rosmarinus officinalis*), lavender *(Lavandula* spp.*),* and Baikal skullcap (*Scutellaria baicalensis*), are known to contain relatively high levels of phenolics and have demonstrated antioxidant activity (Zheng and Wang, 2001; Shao et al., 2004; Waisundara, 2010; Atanassova and Georgieva, 2010). Leaves from peppermint (*M.* x *piperita*), rosemary, sage (*Salvia* spp., spearmint (*M. spicata*), and thyme (*Thymus* spp. growing in a greenhouse exhibit total polyphenol (TPP) and Trolox equivalent antioxidant capacity (TEAC) as plants grown under field conditions, plus anti-tumorigenic activity against colon cancer cells (Yi and Wetzstein, 2010; 2011).

Free radicals can cause disorders, such as atherosclerosis, central nervous system injury, and gastritis in the human body (Kumpulainen and Salonen, 1999; Pourmorad et al., 2006). By boosting the human immune system, plant-based antioxidants block free radicals produced through oxidation **(**Schuler, 1990), thus inhibiting chain reactions that could lead to degradation and death of cells (Pratt, 1992; Velioglu et al., 1998; Hu and Willett, 2002). The antioxidant activity delays and inhibits oxidation of cellular components and molecules (Nijveldt et al., 2001). Thus, the level of activity of various phytochemicals is important for the evaluation of their potential health benefit to humans.

The consumption of a diet rich in natural anti-oxidants has been associated with a reduced risk of human illnesses, such as cardiovascular problems, inflammation, neurodegenerative diseases, and some cancers (Kong et al., 2003; Beretta et al., 2009). A recent study suggests that presence of wogonin in *Scutellaria* extract plays a key role in the biochemical pathways leading to anti-cancer properties (Patel et al., 2013).

Of the several classes of plant secondary metabolites known as antioxidants, phenolics, the major antioxidants are classified into six groups: simple phenolics and phenylpropanoids, coumarins, lignans, quinones, tannins, and flavonoids. The flavonoids consist of five sub-groups: antho-cyanidins, flavones and flavonols, flavanones, catechins, and leucoanthocyanidins and proantho-cyanidins based on their chemical structures (Jedinak et al., 2004). More than 295 flavonoid compounds have been isolated from 35 *Scutellaria* species (Shang et al., 2010).

Major flavonoids in *Scutellaria* species are apigenin, luteolin, scutapins, and diterpenoids (scutalpin C), neo-clerodanes, chrysin, iridoids, isoscutellarein, wogonin, baicalin, and baicalein. These polyphenolic compounds are known to scavenge free radicals, inhibit hydrolytic and oxidative enzymes, and act in anti-inflammatory pathways (Frankel, 1995). In this study, the polyphenol content and antioxidant capacity of leaf extracts of *Scutellaria* species were analyzed and compared.

MATERIALS AND METHODS

Plant material. A total of 16 *Scutellaria* species, grown and maintained in a greenhouse at Fort Valley State University, Fort Valley, Georgia, were used in this study (Table 1). Rosemary (*Rosmarinus officinalis*) leaves collected locally were used fresh and dried as comparison standards for antioxidant capacity. For each species of the *Scutellaria* and for the rosemary, two samples (2 g each) of leaf tissue were randomly collected from multiple plants, cleaned, and weighed. One sample was extracted fresh while the second sample was dried at room temperature (25^{o}C) in the dark for seven days, weighed, and then extracted.

Constituent extraction. For extraction, the tissue samples were homogenized in a 50 mL capacity chilled mortar using a pestle kept at -20 ^{o}C (by submerging in liquid nitrogen for 2 h) to facilitate grinding and minimize any degradation of bioactive constituents. The homogenized leaf powder was transferred to a 125 mL Erlenmeyer flask containing 50 mL of HPLC grade 100% methanol (Burdick and Jackson, USA). The flasks were left overnight (18 h at 28.5 ^{o}C) in the dark on an orbital shaker at 200 RPM (Benchmark Mini Incu-Shaker, Edison, NJ, USA). The suspension was then transferred to 50 mL Falcon tubes (BD, Franklin Lakes, NJ, USA) to be centrifuged at 2057 x g (Eppendorf centrifuge 5810 R, Brentwood, NH) at 25 ^{o}C for 40 min. The supernatant was collected and the remaining pellet was extracted again for 1 h, with 25 mL of methanol.

Table1. Natural distribution of *Scutellaria* species used in the present study.

Distribution	Species
North America	*S. drummondii & S. elliptica, S. incana & S. integrifolia, S. montana & S. ocmulgee, S. ovata & S. suffrutescens*
Central & South America	*S .costaricana & S. racemosa*
Europe	*S. albida, S. alpine, & S. altissima*
East Asia	*S. baicalensis & S. barbata*
South Asia	*S. angulosa & S. scandens*

After the second extraction, the two extracts were combined and the pellets were discarded. The combined extract was filtered through a double layer of Whatman filter paper No. 2 (GE Healthcare Life Sciences, Piscataway, NJ, USA) and stored in air tight 50 mL Falcon tubes at 4 ^{o}C in the dark, until analysis of the extracts, following the procedures optimized by Vaidya (2013) for various *Scutellaria* species.

Extract analysis. Total polyphenol content was determined by the Folin-Ciocalteu reagent method (Lowry et al., 1951) as modified by Yi and Wetzstein (2010) for the herbs in the Lamiaceae family. Gallic acid (3, 4, 5-trihydroxybenzoic acid) (Sigma Life Science, USA) was used to develop standard reference points as outlined by Singleton and Rossi, 1965. A spectrophotometer (Nanodrop 2000c, Thermo Scientific, USA) was used to read the

solution absorbance at 765 nm. For each sample, five replicates were measured at 20 sec intervals. Total phenolic content was expressed as mg gallic acid equivalent/g dry or fresh extract (GAE mg/g).

Total flavonoid content was evaluated using the aluminum chloride ($AlCl_3$) colorimetric method (Chang et al., 2002). A standard solution (1 mg $AlCl_3$/mL) was prepared by dissolving HPLC grade quercetin dihydrate (Alfa Aesar, UK) in 80% ethanol. This solution was further diluted with 80% ethanol to provide concentrations of 10, 25, 50, 80, 100 and 125 µg/mL concentrations in distilled water. 0.5 mL of diluted solutions were mixed with 1.5 mL of 95% EtOH, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M potassium acetate (CH_3CO_2K) and 2.8 mL of distilled water in 50 mL beakers separately and incubated at 25 ºC for 30 min. Absorbance was read at 415 nm using spectrophotometer to generate the standard curve where regression equation is determined by y=128x + 4.5545. Test solutions included a mix of 0.5 mL of plant extracts with 1.5 mL of 95% EtOH, 0.1 mL of 10%, 0.1 mL of 1 M CH_3CO_2K and 2.8 mL distilled water. These solutions with fresh and dry extract samples were also incubated at 25 ºC for 30 min and measurements were taken at 415 nm. The samples were run in triplicate and blank was prepared without the addition of $AlCl_3$. The absorbance data was then converted to total flavonoid content using standard curve generated by quercetin dihydrate.

Antioxidant capacity measurement. Trolox equivalent antioxidant capacity (TEAC) assay of a sample was calculated based on the inhibition of radical cation absorption exerted by the standard TROLOX solution (6-hydroxy-2, 5, 7, 8-tetramethy-chroman-2-carboxylic acid) (Sigma-Aldrich, USA), a vitamin E analogue (Davies et al., 1988). A 7 mM ABTS solution [2, 2'-azinobis (3-ethylbenzothiazo-line-6-sulfonic acid) diammonium salt] (Sigma-Aldrich, USA) was mixed with 6.6 mg of potassium persulfate (Fisher Scientific, USA) to make final concentration of 2.45 mM. This ABTS radical solu-tion was incubated in the dark at 25 ºC for 16 h, and then diluted with ethanol (EtOH) to get an optical density (OD) A_{734} =0.70 ± 0.02 with spectrophoto-meter, Nanodrop 2000c using a disposable cuvette (Plasibrand, NJ, USA). Once the OD A_{734} =0.70 ± 0.02 was achieved, 2970 µL of ABTS was re-measured. To the same measured ABTS, 30 µL of a *Scutellaria* species extract was added and then remeasured after 6 min. The measurements from all extracts were plotted against Trolox standards for percent inhibition at 6 min. Calculation of anti-oxidant capacity was expressed as percent inhibition according to the equation:

$$\% \text{ Inhibition} = [(Abs_{Control} - Abs_{Sample})/Abs_{Control}] \times 100$$

Where $Abs_{Control}$ is the absorbance of the control reaction (containing all reagents except the test compound), Abs_{Sample} is the absorbance of the test compound, and % inhibition is the inhibition of ABTS absorbance by TROLOX.

Rosemary, a plant known to have a high antioxidant capacity (Yi and Wetzstein, 2011), was used as a standard to establish a comparative measure of the medicinal potential of the tested *Scutellaria* species on the basis of TPP content and TEAC assays.

Total polyphenol measurements. The TEAC assay (Re et al., 1999; Yi and Wetzstein, 2010) was used to measure the antioxidant capacity of the polyphenols, as compared with the standard Trolox. Measurements of TEAC utilized the ABTS decolori-zation assay where the change in color was directly proportional to the concentration of antioxidant present in the liquid (Huang et al., 2005a). The intensity of color in these assays were measured with a spectrophotometer to provide a numerical value of total polyphenol and antioxidant capacity of fresh and dry extracts. Redox properties of polyphenol prevent peroxide decomposition to free radicals (Li et al. 2009).

Statistical analysis. All data are presented as means ± SE for at least three replications for each sample. Statistical analysis was based on two way analysis of variance (ANOVA) with results at $P \leq 0.05$ level and means were separated using Tukey's post-hoc mean separation test.

RESULTS

Fresh and dry leaf extracts of the *Scutellaria* species and rosemary exhibited large variations in antioxidant capacity (Table 2). In fresh extracts, TPP results ranged from 52.11 to 281.93 mg/g gallic acid equivalent (GAE). In dry extracts, two species, *S. drummondii* and *S. incana*, failed to register any TPP

values. In the other species, TPP values ranged between 50.56 and 281.93 mg/g GAE in *S. albida* to 732.41 mg/g GAE in *S. ocmulgee*. Both *S. ocmulgee* and *S. montana*, exhibited a relatively high TPP content in both fresh and dry extracts. Total polyphenol content in the fresh leaf sample of *S. ocmulgee* was 281.93 mg/g GAE whereas dry leaf sample contained 732.41.

Table 2. Polyphenol content, antioxidant activity, and flavonoid content of *Scutellaria* species.

Scutellaria species	Total polyphenol		Inhibition	TEAC	Inhibition	TEAC	Flavonoid content	
	Fresh extract	Dried extract	Fresh extract		Dried extract		Fresh extract	Dried extract
	(mg/g GAE)		(%)	(µmol/g)	(%)	(µmol/g)	(µg/mL)	
S. albida	52.11^{h} ± 0.74	50.56^{i} ± 0.16	26.17 ± 0.64	654.34^{cde} ± 19.70	20.95 ± 1.23	523.68^{ef} ± 37.64	95.80^{efg} ± 1.27	74.02^{ef} ± 2.13
S. alpine	198.13^{cd} ± 0.18	112.79^{f} ± 0.26	50.10 ± 0.99	1252.55^{abc} ± 30.33	36.21 ± 1.46	905.26^{cde} ± 44.69	200.63^{a} ± 21.16	105.62^{bcde} ± 0.95
S. altissima	76.18^{gh} ± 0.32	60.58^{hi} ± 0.25	35.64 ± 4.34	891.08^{bcde} ± 132.8	28.25 ± 4.19	706.33^{def} ± 128.15	110.23^{defg} ± 4.86	92.63^{bcdef} ± 0.83
S. angulosa	215.80^{c} ± 0.29	264.35^{d} ± 0.63	54.35 ± 1.26	1358.65^{ab} ± 38.51	54.74 ± 0.86	1368.51^{bc} ± 26.40	167.87^{ab} ± 2.56	122.75^{bc} ± 7.47
S. baicalensis	91.83^{g} ± 0.13	186.65^{e} ± 0.07	44.98 ± 0.45	1124.37^{abcd} ± 13.71	55.12 ± 2.39	1377.93^{bc} ± 73.08	157.87^{abc} ± 6.67	135.55^{ab} ± 1.00
S. costaricana	205.25^{cd} ± 0.12	81.78^{h} ± 0.12	68.90 ± 2.19	1722.55^{a} ± 67.16	35.74 ± 1.14	893.41^{cde} ± 35.05	140.43^{bcd} ± 3.27	87.93^{cdef} ± 1.31
S. drummondii	70.34^{gh} ± 0.13	NA*	16.99 ± 1.16	424.74^{e} ± 35.66	9.38 ± 0.09	234.58^{f} ± 2.87	84.97^{fg} ± 2.78	21.29^{g} ± 0.30
S. elliptica	179.93 ± 0.15^{cde}	81.78^{h} ± 0.12	31.37 ± 1.90	784.20^{bcde} ± 58.10	17.52 ± 0.87	438.07^{ef} ± 26.65	116.90^{cdefg} ± 0.64	87.88^{cdef} ± 3.82
S. incana	78.54^{g} ± 0.05	NA*	22.92 ± 0.87	572.91^{de} ± 26.55	16.02 ± 0.54	400.49^{ef} ± 16.58	105.97^{defg} ± 6.36	84.63^{cdef} ± 2.82
S. integrifolia	118.31^{f} ± 0.29	51.15^{i} ± 0.16	26.00 ± 1.58	650.04^{cde} ± 48.36	26.88 ± 1.90	671.95^{def} ± 58.28	90.22^{efg} ± 18.15	95.53^{bcdef} ± 2.93
S. lateriflora	85.95^{g} ± 0.09	50.57^{i} ± 0.43	29.93 ± 0.97	748.20^{bcde} ± 29.66	26.48 ± 2.25	661.94^{def} ± 68.77	112.63^{defg} ± 9.82	78.70^{def} ± 4.35
S. montana	250.24^{b} ± 0.58	630.67^{b} ± 5.79	65.20 ± 12.96	1630.04^{a} ± 396.71	76.14 ± 16.16	1903.44^{ab} ± 494.85	129.93^{bcde} ± 13.11	119.30^{bcd} ± 11.23
S. ocmulgee	281.93^{a} ± 0.29	732.41^{a} ± 10.88	68.07 ± 2.36	1701.64^{a} ± 72.33	99.24 ± 0.28	2480.93^{a} ± 8.71	117.03^{cdefg} ± 1.39	170.80^{a} ± 5.17
S. ovata	70.78^{gh} ± 0.07	78.32^{h} ± 0.14	13.29 ± 0.36	332.35^{e} ± 10.98	18.19 ± 0.71	454.72^{ef} ± 21.64	73.93^{g} ± 0.80	61.77^{fg} ± 4.41
S. scandens	138.97^{f} ± 0.20	85.81^{gh} ± 0.27	48.22 ± 1.75	1205.53^{abc} ± 53.55	46.73 ± 2.44	1168.36^{cd} ± 74.61	130.95^{bdce} ± 7.91	104.00^{bcdef} ± 3.90
S. suffrutescens	259.50^{ab} ± 1.39	109.93^{fg} ± 0.11	60.80 ± 1.38	1519.98^{a} ± 42.39	50.27 ± 2.07	1256.79^{cd} ± 63.28	124.15^{bdcef} ± 3.66	102.12^{bcdef} ± 3.22
R. officinalis	175.51^{e} ± 0.43	325.28^{c} ± 1.17	53.77 ± 0.16	1344.11^{ab} ± 4.79	83.68 ± 2.59	2092.06^{a} ± 79.33	132.80^{bcde} ± 24.56	131.73^{ab} ± 17.83

Means ± standard error; those means followed by the same letter within a column are not significantly different, P ≤ 0.05; NA*= no sample.

Flavonoid content. Total flavonoid content of leaf extracts ranged between 73.93 µg/mL for *S. ovate* to 200.63 µg/mL for *S. alpine* in fresh leaf extracts. In dry leaf extracts, flavonoid content ranged from 21.29 µg/mL in *S. drummondii* to 170.80 µg/mL in *S. ocmulgee*. *S. angulosa*, which grows at 2000 m above sea level in the Central Himalayas, had a high flavonoid content in both fresh and dry leaf extracts with 167.87 µg/mL for dry and 122.75 µg/mL for fresh leaf extracts. Similarly, *S. baicalensis* also exhibited high flavonoid content of 157.87 µg/mL in fresh leaf and 135.55 µg/mL in dry leaf extract.

Antioxidant capacity measurement. The percent inhibition exhibited against Trolox by *Scutellaria* leaf extracts ranged from 9.38% to 99.24%. In case of *S. ocmulgee* fresh and dry leaf extracts, average inhibition values were 68.07% and 99.24%, respectively, and for *S. montana* 65.20% for fresh and 76.14% for dry leaf extract, respectively. These values were among the highest of all the samples tested in the current study. On the contrary, *S. drummondii*, registered the lowest values for both fresh and dry leaf extracts, 16.99% and 9.38%, respectively.

The TEAC values obtained ranged from 234.88 µmol/g (dried leaf extract of *S. drummondii*) to 2,480.93 µmol/g (dried leaf extract of *S. ocmulgee*). Next to *S. ocmulgee*, *S. montana* along with *R. officinalis* had the highest measured TEAC values in the dried extract. In the fresh tissue extracts, *S. ocmulgee* with 1701.64 µmol/g and *S. montana* with 1630.04 µmol/g extracts were the *Scutellaria* species with the highest TEAC values.

Of the three test assays, the TPP concentration in fresh tissue and TEAC value for antioxidant capacity in fresh tissue exhibited a strong correlation (Table 3). Significant correlation was also observed for TPP fresh, TPP dry, and TEAC fresh with the flavonoid levels in dry tissue.

Table 3. Pearson's correlation coefficients for antioxidant capacity of *Scutellaria* species.*

	Species*	TPP Fresh	TPP Dry	TEAC Fresh	TEAC Dry	FLAV Fresh	FLAV Dry
Species	1.00000						
TPP Fresh	0.24459 0.3441	1.00000					
TPP Dry	0.16498 0.5568	0.65721 0.0078	1.00000				
TEAC Fresh	0.04772 0.8557	0.86689 <.0001	0.62131 0.0134	1.00000			
TEAC Dry	0.10240 0.6957	-0.10473 0.6891	-0.18025 0.5203	-0.22532 0.3846	1.00000		
FLAV Fresh	-0.35783 0.1585	0.49601 0.0429	0.13553 0.6301	0.63366 0.0063	-0.26573 0.3026	1.00000	
FLAV Dry	-0.00190 0.9942	0.64458 0.0052	0.79487 0.0004	0.73609 0.0008	-0.02040 0.9381	0.53054 0.0285	1.00000

*Includes the *R. officinalis* sample and significance level

DISCUSSION

Plants are endowed with complex antioxidant activity that protects them against oxidative damage. This study demonstrates that among *Scutellaria* species the antioxidant capacity can vary considerably. Differences in total polyphenol content, Trolox equivalent antioxidant capacity, and total flavonoid content in the fresh and dried leaf extracts affected antioxidant levels. The relatively high levels of TPP, TEAC, and flavonoid content detected in *S. ocmulgee* and *S. montana* emphasize the need for conservation of the lesser known, rare, and threatened species of *Scutellaria*, such as *S. ocmulgee* and *S. montana* (Chafin, 2007).

S. ocmulgee, which registered one of the highest antioxidant values, is the most effective *Scutellaria* species against glioma cells (Parajuli et al., 2009, 2011, Dandawate et al., 2012). *S. lateriflora* has anxiolytic properties (Awad et al., 2003) and thus has been used as sedative and for treating nervous disorders (Islam et al., 2011). Specific flavonoids isolated from the leaf extracts of *Scutellaria* species are known to have specific medicinal properties (Shang et al., 2010).

Other species, such as lemon balm (*Melissa officinalis*, oregano (*Origanum vulgare*), marjoram

(*Origanum majorana*) and rosemary, are used in Traditional Chinese Medicine (TCM) and Ayurveda medicine practices because of noted beneficial effects on human health (Lutomski, 2001; Capecka et al., 2005). The polyphenolic compounds produced by these and other plants exhibit high levels of antioxidant activity (Rohman et al., 2010). Similarly, extensive work with *S. baicalensis* and *S. lateriflora* in relation to their chemical constituents and anti-oxidant properties, have been published (Li, et al., 2012; Shao et al., 2004). Previous studies conducted by our group using *S. ocmulgee* leaf extract have shown specific anti-tumor activity against gliomas through inhibition of Akt, GSK-3 αβ, and NF-kB phosphorylation in rat tumors (Parajuli et al., 2009, 2011).

Hence, a positive correlation between higher amounts of TPP, TEAC, and flavonoid content can be associated with potential medicinal property of a plant extract. The current study with *Scutellaria* supports the concept that determining the quantitative and qualitative elucidation of polyphenol levels in plant extracts will most likely enable connection of plants to various medicinal properties. Our study with several *Scutellaria* species demonstrated a positive correlation between TPP and TEAC values. Extracts with the highest antioxidant activity showed the highest flavonoid content, indicating an important relationship found among these variables.

Qualitative and quantitative analyses of major individual phenolics in plant extracts could be helpful in explaining the relationship between phenolic content and antioxidant capacity within a species. In a variety of medicinal plants, Djeridane et al., (2006) and Katalinic et al., (2006) have demonstrated a linear correlation between the content of total phenolic compounds and antioxidant capacity. Others, (Capecka et al., 2005; Wong et al., 2006), however, have indicated a poor linear correlation between antioxidant activity and phenolic content.

Observed differences among the presence of polyphenolics and antioxidant activity in our study with *Scutellaria* appeared to be due to the use of fresh or dry tissue. In fresh tissue extracts, TPP content positively correlated with TEAC activity, but in dry tissue extracts, TPP did not correlate with TEAC of the *Scutellaria* species. Although some variability was apparent among species as fresh extract had a higher value of TPP in *S. alpine* and *S. costaricana*, whereas, higher TPP values were measured in dry leaf extract from *S. montana* and *S. ocmulgee*.

These differences between fresh and dry tissues could due to differences in the quantity and type of flavonoid groups within the various *Scutellaria* species and/or the thermostability of flavonoid groups during leaf drying and extract preparation. Understanding the relationship among antioxidant activity, flavonoids, plant species, and extraction may help clarify differences in biological activity. A relationship between high antioxidant activity and therapeutic properties has been established for some plant materials (Kong et al., 2003; Beretta et al., 2009). Understanding the anti-oxidative mechanisms within plants and the effects of post-harvest environments and extraction methodologies could lead to the development and improvement of medicinal plant materials for human health.

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