DPPH AND NO RADICAL SCAVENGING ACTIVITY EFFECT USING EXTRACTION OF FOUR HALOPHYTE SPECIES IN KOREA

Man Kyu Huh
Division of Applied Bioengineering /Dong-eui University
S. KOREA
mkhuh@deu.ac.kr

Seung-Ju Park
Busanjin Girl's High School
S. Korea

Na-Kyung Ahn
Busanjin Girl's High School
S. Korea

Seo-Jin Yoo
Busanjin Girl's High School
S. Korea

Moon-Ju Kim
Busanjin Girl's High School
S. Korea

ABSTRACT

The 1- diphenyl 2-picrylhyorazyl (DPPH) assay and nitrite oxide (NO) were used to evaluate the radical scavenging activities of four halophyte species, *Phragmites australis, Limonium tetragonum, Suaeda japonica* and *Salicornia europaea*. DPPH scavenging activity was analyzed according to the method of Brand-Williams et al. Nitric oxide (NO) scavenging activity was measured by a Griess reagent. The plants in the coastal area were more antioxidant than those in inland plants. The DPPH results show the scavenging activity in the order of potency: *S. japonica* > *S. europaea* > *L. tetragonum* > *P. australis*. NO scavenging activity of *P. australis* leaf and stem extracts at inland area evaluated was 33.7% and that of root extracts was 38.0% on same concentration. NO scavenging activity of *P. australis* leaf and stem extracts at coastal area evaluated was 39.3% and that of root extracts was 48.1% on same concentration. NO scavenging activity of *S. japonica* leaf and stem extracts at coastal area evaluated was 47.5% and that of root extracts was 39.6%. NO scavenging activity of *S. europaea* leaf and stem extracts at coastal area evaluated was 47.6% and that of root extracts was 40.5%. A significant linear correlation was established between DPPH and corresponding NO radical activity of extracts of abalone tissues.

Keywords: 1, 1- diphenyl 2-picrylhyorazyl (DPPH), nitrite oxide (NO), *Limonium tetragonum, Phragmites australis, Salicornia europaea, Suaeda japonica*.

INTRODUCTION

There is an increasing interest in antioxidants, particularly in those intended to prevent the presumed deleterious effects of free radicals in the human body. Oxidation in living organisms is essential for the generation of energy during catabolism but these metabolic processes result in the continuous production of free radicals and reactive oxygen species (ROS) in vivo (Boora et al., 2014). The general methods of determination of antioxidant activity are summarized in many reviews, including (Huang et al., 2005; Frankel & Finley, 2008; Tirzitis & Bartosz, 2010).

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) is a well-known radical and a trap (scavenger) for other radicals. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form (Diphenylpicrylhydrazine; non radical) with the loss of this violet color (although there would be expected to be a residual pale yellow color from the picryl group still present) (Shekhar & Anju, 2014). DPPH free radical method is an antioxidant assay based on electron-transfer that produces a violet solution in ethanol (Huang et al., 2005). Therefore, rate reduction of a chemical reaction upon addition of DPPH is used as an indicator of the

radical nature of that reaction. DPPH is very popular for the study of natural antioxidants (Villano et al., 2007). The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometry (Huang et al., 2005), so it can be useful to assess various products at a time.

Nitric oxide (NO) and reactive nitrogen species (RNS) are free radicals that are derived from the interaction of NO with oxygen or reactive oxygen species (Tsai et al., 2007). Nitric oxide (NO) is an omnipresent intercellular messenger in all vertebrates, modulating blood flow, thrombosis, and neural activity. The biological production of NO is also important for nonspecific host defense, but NO itself is unlikely directly to kill intracellular pathogens and tumors (Pacher et al., 2007).

Halophytes are salt-resistant or salt-tolerant plants that thrive and complete their life cycles in soils or waters containing high salt concentrations. Although ROS have positive roles in the stress-response pathway, for example in signaling (Kranner et al., 2010), an imbalance between ROS synthesis and scavenging may cause severe damage to protein structures, inhibit the activity of enzymes of important metabolic pathways and result in the oxidation of macromolecules including lipids and DNA (Boestflisch et al., 2014).

The purpose of the present study is to evaluate four halophyte species as sources of antioxidants for DPPH and NO radical to examine whether the extractions of leaves, stems, and roots are shown significant DPPH and OH activity or not.

METHODOLOGY

Sample extract

Phragmites australis, Limonium tetragonum, Suaeda japonica and *Salicornia europaea* were collected from Busan district in Korea. *Suaeda japonica* and *Salicornia europaea* were distributed only in the coastal area and not in the inland and intermediate areas. The plants were washed and divided into three parts: leaves, stems, and roots. Then tissues were shade dried and milled into coarse powder using a high-speed blender (HC-BL5000, Korea). Blanched samples were blended, and the juices were squeezed out, prior to analysis.

They were squeezed out with the muslin cloth and was put in 500 mL beaker. The samples were blended with 80% ethanol, and then an aliquot of the mixture (100 µL, 200 mg sample / ml 80% ethanol) was further mixed with 100 mM Tris-HCl buffer (400 µL, pH 7.4).The mixture was further stirred with a magnetic bar at 65°C for 12 hours. The sample was treated with ultrasound at room temperature for a given duration. The ultrasound extraction was carried out using an ultrasonic bath (5510, Branson, USA). The mixture was shaken vigorously for one hour at room temperature and left in the dark at room temperature for 20 min. Extracted sample was filtered. The sample was evaporated to remove solvent under reduced pressure and controlled temperature by using rotary vacuum evaporator (N-1001S-W, Eyela, Tokyo, Japan). To get dry powder, samples placed in a low temperature vacuum chamber.

DPPH free radical

The hydrogen atoms or electrons donation ability of the corresponding extracts and some pure compounds were measured by bleaching the violet colored ethanoic solution of DPPH (Cornish & Garbary, 2010). The effects of ethanoic extract on DPPH radicals were evaluated according to the method described by Brand-Williams et al. (1995) with slight modifications. 1 ml of 0.1 mM DPPH solution in ethanol was mixed with 1 ml of the ethanol extracts of

various concentrations (0.1, 0.5, and 1.0 mg/ml). DPPH was added to the solutions prepared with sample extracts and standard antioxidant substances and stirred. A solution of DPPH was prepared by dissolving 5 mg DPPH in 2 ml of ethanol, and the solution was kept in the dark at 4°C. A stock solution of the compounds was prepared at 1 mg/ml in DMSO. The stock solution was diluted to varying concentrations in 96-well microplates. Then, 5 µL of ethanol DPPH solution (final concentration 300 µM) was added to each well. The plate was shaken to ensure thorough mixing before being wrapped with aluminum foil and placed into the dark. The radical scavenging reaction was carried out at 37 ºC in dark for 30 min. The optical density (OD) of the solution was read using the Microplate Reader (VersaMax, Califonia, USA) at the wavelength 515 nm. Corresponding blank sample was prepared and L-Ascorbic acid (1.0 µg/ml) was used as reference standard (positive control). Inhibition of free radical scavenging activity was calculated using the following equation.

Inhibition (%) = 100 × (absorbance of the control - absorbance of the sample)/ absorbance of the control.

EC_{50} is defined as the concentration of inhibitor necessary for 50% inhibition of the enzyme reaction of a maximum scavenging capacity. To determine the EC_{50} value of the active component, the technique using 96-well microplates was employed (Zubia et al., 2009). Regression analysis by a dose response curve was plotted to determine the EC_{50} values.

Nitric oxide (-NO) scavenging assay

Nitrite oxide scavenging activity was measured by the method described by Kato et al., (1987). The reaction mixture contained 1 mM $NaNO_2$ 120 µℓ, 0.1 N HCl 840 µℓ, and various concentrations of samples, making final volume 1.2 ml. After reacting for 1 hour at 37 ℃, 1 ml of the reaction mixture was mixed with 3 ml of 2 % acetic acid and 400 µℓ of Griess reagent, and the mixture was reacted at room temperature for 15 minutes. Absorbance was measured at 550 nm using a spectrophotometer and the amount of remaining nitrite was also measured. Gallic acid was used as the positive control. The percentage inhibition of the extract and standard was calculated as DPPH formula.

Statistical Analyses

The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. All experiments were carried out in triplicate. The mean results were expressed as the mean±SD. One-way analysis of variance was applied to determine differences in means. $p \leq 0.05$ values or less were considered to indicate statistically significant difference. Correlation co-efficient (R) to determine the relationship between two or more variables among Radical Scavenging activity tests were calculated using the SPSS software (Release 21.0).

RESULTS

The DPPH scavenging activities of root extracts were higher than those of leaf and stem extracts. It was observed that inhibition percentage values go on increasing with enhancements in concentration of research plant extracts in the assay mixture. Table 1 was only shown the antioxidant activities for DPPH radical on 1.0 mg/ml. The results of 0.1 mg/ml and 0.5 mg/ml were omitted. DPPH scavenging activity of *P. australis* leaf and stem extracts at inland area evaluated was 35.2% and that of root extracts was 45.6% on same concentration. The plants in the coastal area were more antioxidant than those in inland plants. DPPH scavenging activity of leaf and stem extract of *P. australis* evaluated at coastal

area was 42.0% and that of root was 50.6% at same concentration. DPPH scavenging activity of leaf and stem extract of *L. tetragonum* evaluated at coastal area was 51.8% and that of root was 53.5% at same concentration. However, the all did not show a statistically significant difference ($p < 0.05$).

The high antioxidant activity for DPPH found on *S. japonica* and *S. europaea* extracts. The DPPH results show the scavenging activity in the order of potency: *S. japonica* > *S. europaea* > *L. tetragonum* > *P. australis.*

Nitrite oxide (NO) scavenging activity was measured by a Griess reagent. The results of the NO test of ethanol extracts of four halophyte species in comparison with the standard gallic acid) at 550 nm were shown in Table 2. The rates of antioxidant activities of the ethanol extracts for four halophyte species were dependent on concentrations. The plants in the coastal area were more antioxidant than those in inland plants. NO scavenging activity of *P. australis* leaf and stem extracts at inland area evaluated was 33.7% and that of root extracts was 38.0% on same concentration. NO scavenging activity of *P. australis* leaf and stem extracts at coastal area evaluated was 39.3% and that of root extracts was 48.1% on same concentration. NO scavenging activity of *L. tetragonum* leaf and stem extracts at coastal area evaluated was 41.4% and that of root extracts was 46.7%. NO scavenging activity of *S. japonica* leaf and stem extracts at coastal area evaluated was 47.5% and that of root extracts was 39.6%. NO scavenging activity of *S. europaea* leaf and stem extracts at coastal area evaluated was 47.6% and that of root extracts was 40.5%.

DPPH radical scavenging activity and NO as determined by the EC_{50} values of the different tissues (Tables 3 and 4). An EC_{50} value is the concentration of the sample required to scavenge 50% of the free radicals present in the system. EC_{50} value was inversely related to the antioxidant activity of crude extracts. The total DPPH contents activity of shell muscle (EC_{50} = 5.56 mg/ml for *P. australis* leaf and stem extracts at inland area and 4.98 mg/ml for *P. australis* root extracts was at the same levels as that of L-Ascorbic acid. The values of EC_{50} for *L. tetragonum* were 4.81 mg/ml for leaf and stem and 4.83 mg/ml for root. The NO activity of *S. japonica* (EC_{50} = 4.85 µg/ml for leaf and stem and 5.58 mg/ml for root) was at the same levels as that of positive control and EC_{50} of *S. europaea* was 4.85 mg/ml for leaf and stem, and that of root was 5.49 mg/ml.

A significant linear correlation was established between DPPH and corresponding NO radical activity of extracts of abalone tissues (Fig. 1).

Table 1. The assay of DPPH by extraction of halophyte at tissues and different locations on 1.0 mg/ml

Species	Tissue	Location	Inhibition (%), repeats			Mean±S.D.
			1	2	3	
Phragmites australis	Leaf, stem	Inland	36.9	35.0	33.9	35.2±1.49
		Middle	39.6	38.7	39.6	39.3±0.55
		Coastal	41.5	41.5	44.8	42.6±1.88
	Root	Inland	40.1	42.6	43.5	42.0±1.76

		Middle	44.7	46.4	45.8	45.6±0.87
		Coastal	50.1	49.9	51.8	50.6±1.09
Limonium tetragonum	Leaf, stem	Inland	46.6	44.0	41.1	43.9±2.74
		Middle	48.5	47.4	43.0	46.3±2.89
		Coastal	50.1	54.6	50.8	51.8±2.42
	Root	Inland	46.0	44.3	41.0	43.7±2.56
		Middle	48.3	49.7	43.2	47.1±3.45
		Coastal	50.9	56.3	52.9	53.5±2.87
Suaeda japonica	Leaf, stem	Coastal	63.0	62.6	66.8	64.1±2.33
	Root	Coastal	53.8	51.0	53.9	52.9±1.65
Salicornia europaea	Leaf, stem	Coastal	62.0	58.6	59.6	60.0±1.86
	Root	Coastal	51.2	51.9	53.2	52.1±0.98

Suaeda japonica and *Salicornia europaea* were only occurred at low lands of river.

Table 2. The nitric acid (NO) by extraction of halophyte at tissues and different locations on 1.0 mg/ml

Species	Tissue	Location	Inhibition (%), repeats			Mean±S.D.
			1	2	3	
Phragmites australis	Leaf, stem	Inland	35.9	32.1	32.9	33.7±1.99
		Middle	36.7	35.1	38.7	36.8±1.77
		Coastal	38.5	38.4	41.0	39.3±1.48
	Root	Inland	38.2	37.6	38.2	38.0±0.32
		Middle	41.8	43.3	44.8	43.3±1.52
		Coastal	47.3	46.7	50.4	48.1±1.96
Limonium tetragonum	Leaf, stem	Inland	44.2	40.6	39.5	41.4±2.42
		Middle	45.2	44.9	42.0	44.1±1.77
		Coastal	47.3	48.8	44.5	46.9±2.18
	Root	Inland	43.3	40.2	39.1	40.8±2.16
		Middle	44.9	44.3	42.2	43.8±1.45
		Coastal	47.0	48.5	44.7	46.7±1.94
Suaeda	Leaf, stem	Coastal	48.2	46.6	47.6	47.5±0.82

japonica	Root	Coastal	38.9	38.8	41.0	39.6±1.22
Salicornia europaea	Leaf, stem	Coastal	47.5	49.1	46.1	47.6±1.50
	Root	Coastal	39.8	40.0	41.7	40.5±1.05

Table 3. Inhibitory effects {EC_{50} (mg/ml)} of DPPH by halophyte at tissues

Species	Tissue	Location	EC_{50} (mg/ml), repeats			Mean±S.D.
			1	2	3	
Phragmites australis	Leaf, stem	Inland	5.42	5.65	5.60	5.56±0.10
		Middle	5.18	5.33	5.12	5.21±0.09
		Coastal	5.02	5.08	4.68	4.93±0.17
	Root	Inland	5.14	4.99	4.79	4.98±0.14
		Middle	4.74	4.66	4.59	4.66±0.06
		Coastal	4.28	4.36	4.08	4.24±0.12
Limonium tetragonum	Leaf, stem	Inland	4.58	4.87	4.99	4.81±0.17
		Middle	4.42	4.57	4.83	4.61±0.17
		Coastal	4.28	3.95	4.17	4.13±0.14
	Root	Inland	4.63	4.84	5.01	4.83±0.15
		Middle	4.43	4.37	4.82	4.54±0.20
		Coastal	4.21	3.77	4.00	3.99±0.18
Suaeda japonica	Leaf, stem	Coastal	3.17	3.25	2.81	3.08±0.19
	Root	Coastal	3.96	4.26	3.91	4.04±0.15
Salicornia europaea	Leaf, stem	Coastal	3.26	3.62	3.42	3.44±0.15
	Root	Coastal	4.18	4.18	3.98	4.11±0.10

Suaeda japonica and *Salicornia europaea* were only occurred at low lands of river.

Table 4. Inhibitory effects {EC_{50} (mg/ml)} of NO by halophyte at tissues

Species	Tissue	Location	EC_{50} (mg/ml), repeats			Mean±S.D.
			1	2	3	
Phragmites australis	Leaf, stem	Inland	5.86	6.25	6.27	6.14±0.19
		Middle	5.80	5.97	5.74	5.84±0.10
		Coastal	5.63	5.67	5.52	5.61±0.06
	Root	Inland	5.66	5.74	5.78	5.73±0.05

		Middle	5.33	5.22	5.16	5.24±0.07
		Coastal	4.82	4.90	4.64	4.79±0.11
Limonium tetragonum	Leaf, stem	Inland	5.11	5.47	5.66	5.41±0.23
		Middle	5.01	5.07	5.43	5.17±0.18
		Coastal	4.82	4.71	5.19	4.91±0.21
	Root	Inland	5.19	5.51	5.70	5.47±0.21
		Middle	5.04	5.12	5.41	5.19±0.16
		Coastal	4.85	4.74	5.18	4.92±0.19
Suaeda japonica	Leaf, stem	Coastal	4.74	4.92	4.90	4.85±0.08
	Root	Coastal	5.59	5.63	5.52	5.58±0.05
Salicornia europaea	Leaf, stem	Coastal	4.81	4.69	5.04	4.85±0.15
	Root	Coastal	5.51	5.52	5.45	5.49±0.03

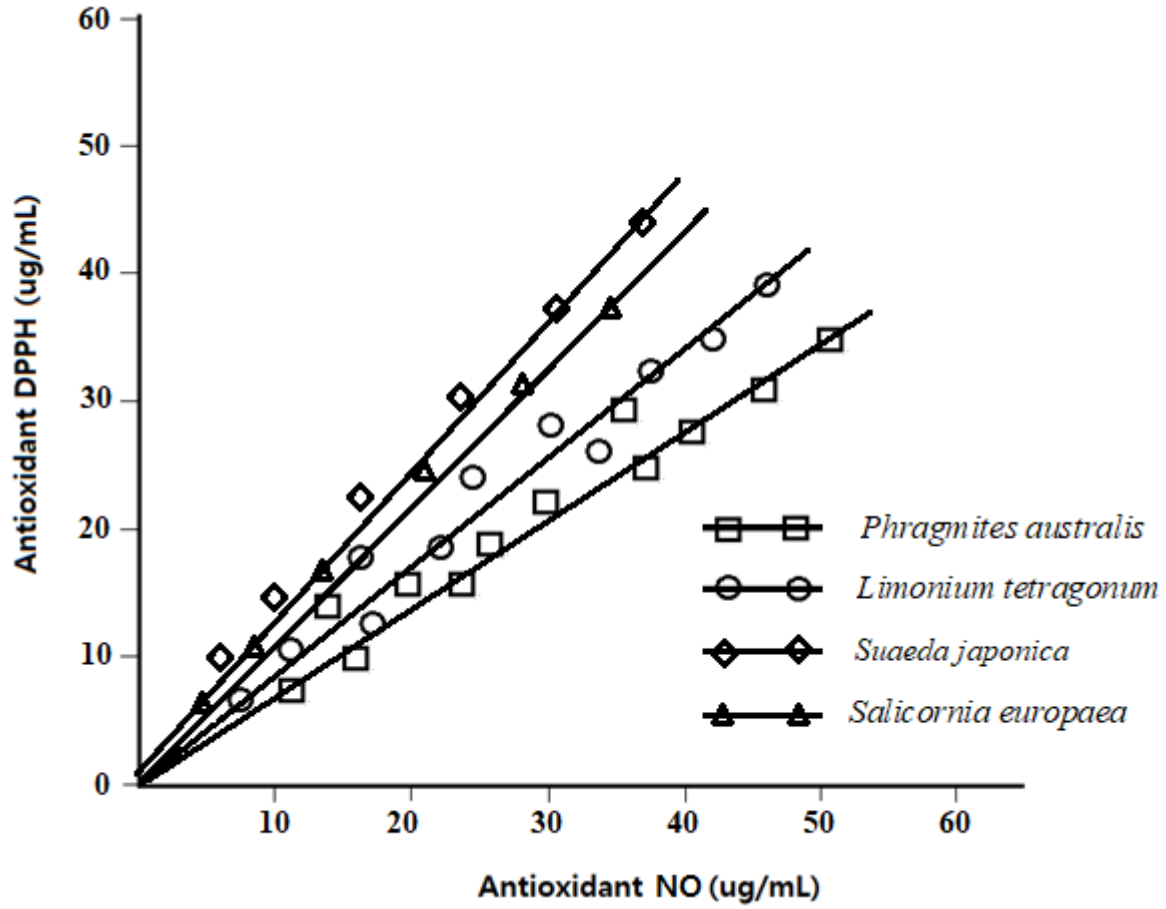

Fig. 1. Linear correlation between the amount of DPPH and NO for *Phragmites australis*, *Limonium tetragonum*, *Suaeda japonica* and *Salicornia europaea*.

DISCUSSION

For halophytes, functional traits, those plant attributes that significantly influence establishment and survival, include any mechanisms that contribute to their tolerance of high soil or water salinity as well as other abiotic stresses of their habitats, such as drought or flooding. Papers in this Special Issue address the ecophysiological mechanisms of salinity tolerance in halophytes (Flowers & muscolo, 2015).

The association of a raised antioxidant capacity with salt tolerance has been demonstrated in a number of salt-tolerant halophytes (Ben Amor et al., 2005; Cornish & Garbary, 2010; Alhdad et al., 2013). When salinity was applied over 24 h to plants of the annual halophyte *Lepidium latifolium*, an increase in total antioxidant capacity in addition to phenols, ascorbate and flavonoids was observed, demonstrating that manipulation of the antioxidant capacity is possible through salinity treatment (Boestflisch et al., 2014). This result was similar to our results. For other some species, the antioxidant capacity decreased salt exposure, suggesting that increased exposure time to salinity causes failure in these protective mechanisms, which in turn leads to a loss of vigour and eventually death of the organism (Kranner et al., 2010; Kranner & Seal, 2013). A variety of candidate salt tolerance genes been identified in *Arabidopsis thaliana*, among which genes encoding Na^+ and K^+ transporters, and genes involved in the general stress or anti-oxidant response, or in compatible solute metabolism. Some halophytic Amaranthaceae (Salicornioideae, Chenopodioideae and Suaedoideae) are not just highly salt tolerant, their growth rate is stimulated at a salinity range of 150-300 mM NaCl. Alternatively this may be described as depressed growth at low salinity (Rozema & Schat, 2013).

As mentioned earlier studies of halophyte species, our results appeared to be very similar to some halophyte species. Antioxidant capacity was related to the concentration of salinity (Tables 1 and 2).

Liner correlation of antioxidant DPPH activity were strong positively correlated with NO antioxidants. The results of the leaves with stem and roots under investigation and their mutual comparison have not shown significant differences, and in this way, the roots are a contribution to a further selection of the suitable tissues, which could become a part of nutrition participating in strengthening antioxidant effects of human organism.

CONCLUSIONS

The DPPH results show the scavenging activity in the order of potency: *S. japonica* > *S. europaea* > *L. tetragonum* > *P. australis.* The plants in the coastal area were more antioxidant than those in inland plants.

REFERENCES

Alhdad et al. (2013) The effect of combined salinity and waterlogging on the halophyte *Suaeda maritima*: the role of antioxidants. *Environmental and Experimental Botany, 87*, 120-5.

Ben Amor et al. (2005) Physiological and antioxidant responses of the perennial halophyte *Crithmum maritimum* to salinity. *Plant Science, 168*, 889-99.

Boestfleisch et al. (2014) Manipulating the antioxidant capacity of halophytes to increase their cultural and economic value through saline cultivation. *AoB Plants*, doi:

10.1093/aobpla/plu046.

Boora, F., Chirisa, E., & Mukanganyama, S. (2014) Evaluation of nitrite radical scavenging properties of selected Zimbabwean plant extracts and their phytoconstituents. *Journal of Food Processing*, article ID 918018.

Brand-Williams, W., Cuvelier, M.E., & Berset, C. (1995) Use of free radical method to evaluate antioxidant activity. *Food Science & Technology, 28,* 25-30.

Cornish, M.L., & Garbary, D.J. (2010) Antioxidants from macroalgae: Potential applications in human health and nutrition. *Algae, 25,* 155-71.

Flowers, T.J., & Muscolo, A. (2015) Introduction to the special issue: halophytes in a changing world. *AoB Plants, 7,* https://doi.org/10.1093/aobpla/plv020.

Frankel, E.N., & Finley, J.W. (2008) How to standardize the multiplicity of methods to evaluate natural antioxidants. *Journal of Agricultural and Food Chemistry, 56,* 4901-8.

Huang, D.J., Ou, B.X., & Prior, R.L. (2005) The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry, 53,* 1841-56.

Kato et al. (1987) Inhibitory of nitrosamine formation by nondialyzable melanoidins. *Agricultural and Biological Chemistry, 51,* 1333-8.

Kranner, I., & Seal, C.E. (2013) Salt stress, signaling and redox control in seeds. *Functional Plant Biology, 40,* 848-59.

Kranner et al. (2010) What is stress? Concepts, definitions and applications in seed science. *New Phytologist, 188,* 655-73.

Pacher, P., Beckman, J.S. & Liaudet, L. (2007) Nitric oxide and peroxynitrite in health and disease. *Physiological Reviews, 87,* 315-424.

RozemaJ., & Schat, H. (2013) Salt tolerance of halophytes, research questions reviewed in the perspective of saline agriculture. *Environmental and Experimental Botany, 92,* 83-95.

Shekhar, T.C., & Anju, G. (2014) Antioxidant activity by DPPH radical scavenging method of *Ageratum conyzoides* Linn. leaves. *American Journal of Ethnomedicine, 1,* 244-9.

Tirzitis, G., & Bartosz, G. (2010) Determination of antiradical and antioxidant activity: basic principles and new insights. *Acta Biochimica Polonica, 57,* 139-42.

Tsai et al. (2007) Evaluation of NO-suppressing activity of several Mediterranean culinary spices. *Food and Chemical Toxicology, 45,* 440-7.

Villano et al. (2007) Radical scavenging ability of phenolic compounds towards DPPH free radical. *Talanta, 71,* 230-5.

Zubia et al. (2009) Antioxidant and cytotoxic activities of some red algae (Rhodophyta) from Brittany coasts (France). *Botanica Marina, 52,* 268-77.