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EVALUATION OF PHENOLICS, THE NITRIC OXIDE RADICAL SCAVENGING ACTIVITY, AND FERRIC REDUCING/ANTIOXIDANT POWER OF *SALICORNIA EUROPAEA*

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ABSTRACT

We investigate here the antioxidant capacity in local halophytic species, *Salicornia europaea*, well known for their ethnopharmacological utilizations in traditional medicine. The objectives of this study were to determine antioxidant activities and reducing power activity of ethanol extracts of *Salicornia europaea* (Amaranthaceae) leaves, stems, and roots. The antioxidant activity of the *S. europaea* extracts was measured on the basis of the scavenging activity of the phenolic, nitric oxide radical ($NO^{\cdot}$)-scavenging activity, and ferric reducing/antioxidant power (FRAP). Total phenolic contents scavenging activity of leaves of *S. europaea* was evaluated at 8.0 mg/ml was 49.8%, that of stems was 36.1% at same concentration, and root was 24.7%. NO scavenging activity of leaves of *S. europaea* was evaluated at 8.0 mg/ml was 39.8% and that of stems was 38.9% at same concentration, and that of root was 29.9%. FRAP activity of leaves of *S. europaea* was evaluated at 8.0 mg/ml was 34.8% and that of stems was 29.6% at same concentration, and root was 22.8%.

KEYWORDS: Antioxidant capacity, Total phenolic contents, Nitric oxide radical (NO), ferric reducing antioxidant power (FRAP), *Salicornia europaea*.

INTRODUCTION

There is great number of methods for determination of antioxidant capacity of foods and beverages based on different principles: peroxyl radical scavenging (Oxygen Radical Absorbance capacity, ORAC); Total Radical-trapping Antioxidant Power (TRAP); ferric reducing antioxidant power (FRAP); Cupric Reducing Antioxidant Power (CUPRAC); hydroxyl radical scavenging (-OH assay); organic radical scavenging (2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid, ABTS); 2,2-Diphenyl-1picrylhydrazyl (DPPH); etc.[1,2]

Oxygen is a highly reactive atom that is capable of becoming part of potentially damaging molecules commonly called “free radicals”. Oxidative stress occurs when reactive oxygen/nitrogen species overwhelm the antioxidant defense system. Most aerobic organisms have developed antioxidant defense systems to offset harmful effects caused by free radicals. In particular, the plant kingdom is a good source to produce a wide range of natural antioxidants. Therefore, there is a growing interest day by day in the substances exhibiting antioxidant properties, which are supplied to humans and animals as food components or as specific preventative pharmaceuticals.[3] In nature there are a wide variety of naturally occurring antioxidants which are different in their composition, physical and chemical properties, mechanisms and site of action.[4] Their compounds in food play an important role as a health protecting factor. This free radical is stable at room temperature and is reduced in the presence of an antioxidant molecule.

Phenolics are secondary plant metabolites ranging from simple structures with one ring to complex polymers such as tannins and lignins. Plant phenolics, in particular phenolic acids, tannins and flavonoids are known to be potent antioxidants and occur in vegetables, fruits, nuts, seeds, roots and barks. In the case of phenolic compounds, the ability of the phenolics to act as antioxidants depends on the redox potential of their phenolic hydroxyl groups that allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. In addition, they have a metal chelation potential.[5]

Nitric oxide (NO) is an omnipresent intercellular messenger in all vertebrates, modulating blood flow, thrombosis and neural activity.[6] The biological production of NO is also important for nonspecific host defense, but NO itself is unlikely directly to kill intracellular pathogens and tumors.

The ferric reducing/antioxidant power (FRAP) assay is a developed, direct test of total antioxidant power.[7] The

FRAP assay is robust, sensitive, simple and speedy and facilitates experimental and clinical studies investigating the relationship among antioxidant status, dietary habits and risk of disease.

Salicornia europaea Fern. & Brack is a halophytic annual dicot which grows in various zones of intertidal salt marshes. The genus *Salicornia* is a plant belonging to the family Amaranthaceae. In the UK, it is one of several plants known as samphire (rock samphire).[8] *S. europaea* is edible, either cooked or raw. Due to its high salt content, it must be cooked without any salt added, in plenty of water.

There were no report on antioxidant activity of phenolics, NO and FRAP on *Salicornia europaea* yet. The antioxidant potential was evaluated in relation to the scavenging of phenolics, Nitric oxide radical ($NO^{\cdot}$)-scavenging activity and ferric reducing/antioxidant power (FRAP). The purpose of the present study is to evaluate plant extracts as sources of natural antioxidants for phenolics, NO and FRAP and to examine whether the Halophyte (*S. europaea*) having significant anti-oxidant power activity.

MATERIALS AND METHODS

Sample extract

Whole plant of *S. europaea* in full bloom was collected in the August and September at Sacheon-ci in the South Korea. The plants of *S. europaea* divided into three parts: leaves, stems and roots. Each sample (100 g) of *S. europaea* was ground with pestles and liquid nitrogen at –70℃ and homogenized prior to beginning extraction experiments. The samples were blended with 50% ethanol and then an aliquot of the mixture (100 µL, 200 mg sample / ml 50% 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 2 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.

Assay of total phenolic contents

The concentration of phenolics in plant extracts was determined using Folin-Ciocalteau reagent with a slightly modified method of Ainsworth and Gillespie.[9] 1.0 mL of extract at various concentrations (0.1, 1.0, 2.0, 4.0 and 8.0 mg/ml) was used in the analysis. The reaction mixture was prepared by mixing 1.0 ml of ethanoic solution of extract (100 ug/mL), 5.0 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 5.0 ml 7.5% $NaHCO_3$. Blank was concomitantly prepared**,** containing 1.0 ml ethanol, 5.0 ml 10% Folin-Ciocalteu's reagent dissolved in water and 5.0 ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated in a thermostat at 45°C for 30 min. The absorbance was determined using double beam UV-VIS spectrophotometer (Shimadzu, UV-1800, Japan) at λmax = 765 nm. The same procedure was repeated for the standard solution of gallic acid and the linear equation of a contents of a standard curve was construed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

Assay of nitric oxide radical scavenging

Nitric oxide radical ($NO^{\cdot}$)-scavenging activity was measured by the Greiss reagent as described in a previous study.[10,11] The extracts were prepared from a 8.0 mg/mL ethanol crude extract. These were then serially diluted with distilled water to make concentrations from 0.1 mg/mL to 8.0 mg/mL. These were stored at 4°C for later use. Gallic acid solutions ranging from 0.1 to 8.0 mg/mL were used to prepare a standard curve. Griess reagent was prepared by mixing equal amounts of 1% sulphanilamide in 2.5% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid immediately before use. A volume of 0.5 mL of 10 mM sodium nitroprusside in phosphate buffered saline was mixed with 1 mL of the different concentrations of the ethanol extracts and incubated at 25°C for 180 mins. The extract was mixed with an equal volume of freshly prepared Griess reagent. Control samples without the extracts but with an equal volume of buffer were prepared in a similar manner as was done for the test samples. The color tubes contained ethanol extracts at the same concentrations with no sodium nitroprusside. A volume of 150μL of the reaction mixture was transferred to a96-well plate. The optical density (OD) of the solution was read using the UVmini-1240 Reader (Shimadzu, Kyoto, Japan) at the wavelength 546 nm. Corresponding blank sample was prepared and gallic acid was used as reference standard (positive control). The percentage inhibition of the extract and standard was calculated and recorded. The percentage nitrite radical scavenging activity of the ethanol extracts and gallic acid were calculated.

Ferric reducing antioxidant power (FRAP) Assay

FRAP assay was determined according to the method described by Nenadis et al.[12] This assay is based on the reducing power of antioxidants in which a potential antioxidant reduces the oxidized ferric ions to produce ferrous ions, which form a blue colored complex with tripyridyl triazine. The FRAP reagent was freshly prepared by mixing 2,4,6-tripyridyl triazine (10 mM, 1 mL) and ferric chloride (20 mM, 1mL) in 0.25 M acetate buffer (10 mL, pH 3.6). Plant extract sample (50 µL) was added to the FRAP reagent (3 mL) and the absorbance was measured at 593 nm after 8 min incubation at room temperature. In this assay the yellow colour of the test

solution changes to various shades of green and blue depending upon the reducing power of each compound. A calibration curve of ascorbic acid was established, the antioxidant capacity of the plant extracts was then expressed as mM ascorbic acid equivalent/g dry extract. Blank samples were prepared for both ethanol and deionized water extracted samples. All measurements were carried out in triplicates.

Statistical analysis

The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The results were expressed as the mean ±SD. 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). The percent inhibition was calculated as the decolourization percentage of the test sample using the following formula:

Inhibition % = (IA-As)/IA×100

Where IA is the absorbance of the 100% initial and As is the absorbance of the sample. IA and As were the values which were subtracted the average absorbance of the blank wells.

IC_{50} is defined as the concentration of inhibitor necessary for 50% inhibition of the enzyme reaction of a maximum scavenging capacity. To determine the IC_{50} value of the active component, the technique using 96-well microplates was employed.[13] Regression analysis by a dose response curve was plotted to determine the IC_{50} values.

RESULTS

The total phenolic contents of the extracts were determined by Folin-Ciocalteu method as gallic acid equivalents. Table 1 was shown the antioxidant activities for total phenolic contents radical of the *S. europaea.* Various concentrations of leaf extracts were higher than those of stem and root extracts. The rates of antioxidant activities of the ethanol extracts for *S. europaea* were dependent on concentrations. Total phenolic contents scavenging activity of leaves of *S. europaea* was evaluated at 0.1 mg/ml was 10.2%, that of stems was 7.4% at same concentration, and root was 4.5%. Total phenolic contents scavenging activity of leaves of *S. europaea* was evaluated at 8.0 mg/ml was 49.8%, that of stems was 36.1% at same concentration and root was 24.7%. The all values of total phenolic contents scavenging activity of leaves were higher than those of stems and roots. However, the all groups for stems and roots did not show a statistically significant difference ($p < 0.05$).

Nitric oxide radical (NO)-scavenging activity was measured by the Greiss reagent. NO scavenging activity of leaves of *S. europaea* was evaluated at 0.1 mg/ml was 9.5% and that of stems was 3.9% at same concentration, and that of root was 3.7% (Table 2). It is also observed that inhibition percentage values go on increasing with enhancements in concentration of research plant extracts in the assay mixture. NO scavenging activity of leaves of *S. europaea* was evaluated at 8.0 mg/ml was 39.8% and that of stems was 38.9% at same concentration, and that of root was 29.9%. The antioxidant activity for NO found on leaf and stem extracts. The all values of NO scavenging activity of leaves and stems were higher than those of roots. However, the all groups for stems and roots did not show a statistically significant difference ($p < 0.05$).

The results of FRAP assay of ethanol extracts of *S. europaea* in comparison with the standard (ascorbic acid) at 593 nm were shown in Table 3. The rates of reducing power activity of the ethanol extracts were also dependent on concentrations. The reducing power activity of leaves of *S. europaea* was evaluated at 0.1 mg/ml was 2.7% and that of stems was 1.9% at same concentration, and root was 1.9%. FRAP activity of leaves of *S. europaea* was evaluated at 8.0 mg/ml was 34.8% and that of stems was 29.6% at same concentration, and root was 22.8%.

Table 1: The assay of total phenolic contents by *Salicornia europaea* at different concentrations.

Concentration (mg/ml)	Leaf	Stem	Root
0.1	10.15±1.07	7.38±2.16	4.46±0.85
0.5	18.09±0.88	11.69±0.77	7.91±0.90
1.0	26.33±1.59	18.48±1.35	11.94±1.53
2.0	32.28±3.49	23.66±3.83	16.38±1.80
4.0	38.73±2.04	29.91±0.42	20.63±1.63
8.0	49.76±1.01	36.05±1.93	24.74±2.23
F-test	1.747		

Data represent the mean ± SD from three replicates.

Table 2: The nitric oxide radical scavenging (NO) by *Salicornia europaea* at different concentrations.			
Concentration (mg/ml)	**Leaf**	**Stem**	**Root**
0.1	9.52±3.82	3.93±1.56	3.70±1.94
0.5	14.71±3.68	11.67±1.39	7.41±2.51
1.0	21.62±1.92	18.97±2.75	11.41±1.80
2.0	29.65±1.86	27.64±2.09	16.33±2.88
4.0	34.42±2.79	31.42±1.27	24.72±5.44
8.0	39.83±3.04	38.86±3.58	29.86±2.88
F-test	0.171		

Data represent the mean ± SD from three replicates.

Table 3: The ferric reducing antioxidant power (FRAP) by *Salicornia europaea* at different concentrations.			
Concentration (mg/ml)	**Leaf**	**Stem**	**Root**
0.1	2.72±1.24	1.92±0.04	1.94±0.42
0.5	6.98±2.97	5.49±0.78	3.88±1.55
1.0	15.27±1.88	12.17±1.02	6.90±1.14
2.0	23.10±2.10	18.44±1.31	12.35±1.66
4.0	28.09±1.40	22.73±2.67	16.81±1.86
8.0	34.79±2.69	29.55±0.80	22.57±1.51
F-test	0.220		

Data represent the mean ± SD from three replicates.

Figure 1 was shown the comparative data of total phenolic contents, NO radical scavenging activity and reducing power as determined by the IC_{50} values of the different vegetable tissues. An IC_{50} value is the concentration of the sample required to scavenge 50% of the free radicals present in the system. IC_{50} value is inversely related to the antioxidant activity of crude extracts. The total phenolic contents activity of leaves (IC_{50} = 125.6 µg/ml) was at the same levels as that of gallic acid and IC_{50} of stem was 136.8 µg/ml, and that of root was 168.4 µg/ml. The NO activity of leaves (IC_{50} = 112.4 µg/ml) was at the same levels as that of gallic acid and IC_{50} of stem was 114.7 µg/ml and that of root was 205.5 μg/ml. The FRAP activity of leaves (IC_{50} = 168.9 µg/ml) was at the same levels as that of L-ascorbic acid and IC_{50} of stem was 188.1 µg/ml and that of root was 256.3 µg/ml.

A significant linear correlation (Correlation co-efficient r = 0.912, 95% confidence interval 0.116 - 0.138. Co-efficient of determination (r^2) = 0.832, $p < 0.01$) was established between total phenolics (as measured mg gallic acid/g dry material) and corresponding ferric reducing ability (as measured mg L-ascorbic acid/g dry material) of extracts of *S. europaea* parts (Fig. 2). A significant linear correlation (Correlation co-efficient r = 0.884) was established between total phenolics and NO ability of extracts of *S. europaea* parts. A significant linear correlation (Correlation co-efficient r = 0.866) was established between NO and corresponding ferric reducing ability of extracts of *S. europaea* parts.

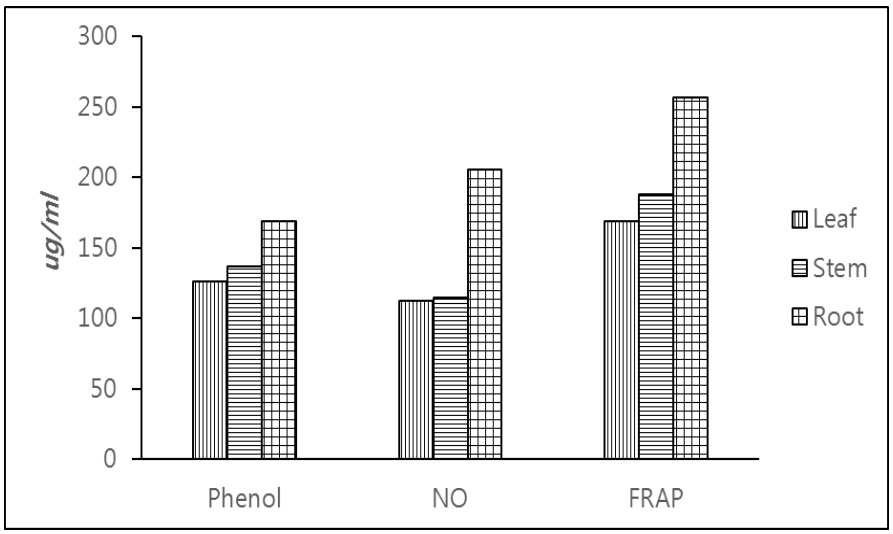

Figure 1: Inhibitory effects {IC_{50} (mg/ml)} on total phenolic contents, NO, and FRAP by *Salicornia europaea* on 1.0 M.

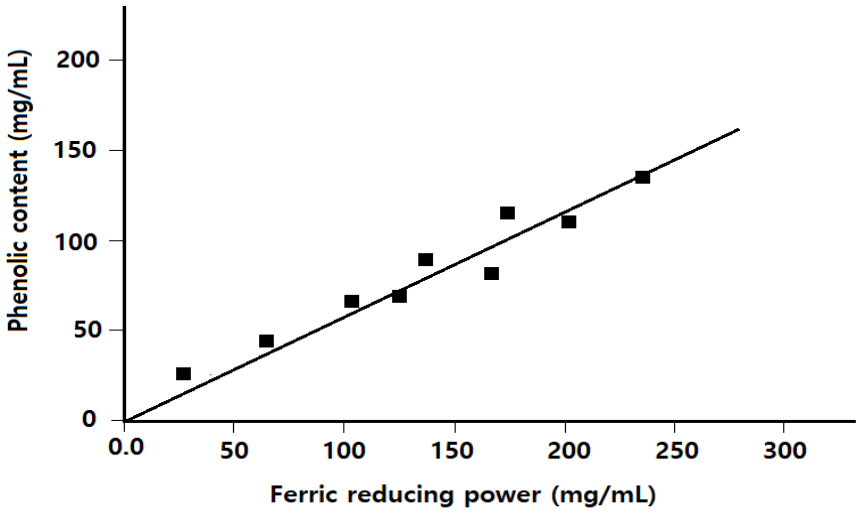

Figure 2: Linear correlation between the amount of total phenolic and ferric reducing power. Correlation coefficient r = 0.912, 95% confidence interval 0.116 – 0.138.

DISCUSSION

Halophytes are distributed from coastal regions to inland deserts and they have been used for medicinal and nutritional purposes. *S. europaea* has ability to grow in saline regions and arid to semiarid regions. The young leaves of this plant are lightly cooked and eaten as herbs. The ethyl acetate fraction exhibited broad-range antibacterial activities against gram-positive bacteria, and the butanol fraction exhibited growth inhibitory effect against only *Staphylococcus epidermidis*.[14] Extremophile species like halophytes represent physiological plasticity which provides these plants a competitive advantage over other species in saline habitats.[15] These results are not shown high antioxidant activities and in good agreement with previous findings reported in halophytic species.[15,16] However, the strong antioxidant activity was not shown in *S. europaea*. The amounts of total polyphenols varied significantly between plant parts and ranged from 24.7% to 49.8% (Table 1). The NO radical had been used widely in the model systems to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanins, or crude extracts of plants. In this study, NO values of *S. europaea* were moderate (Table 2). The extract of *S. europaea* was 34.8% inhibitory effects on the activation of FRAP (Table 3). The low antioxidant capacity for *S. europaea* could be attributed to the solvent (ethanol). Among the several parameters that influence antioxidant capacities in plant analysis, solvent nature is the most controversial one.[17] For example, when five solvent kinds (hexane, ethanol, acetone, methanol, and water) with different polarity were used to evaluate the antioxidant potential of Halophyte, *Limoniastrum monopetalum* leaves and revealed a wide range of leaf polyphenols contents as function of the used solvent, they were closely dependent on the solvent polarity.[16] The extraction with pure methanol showed the highest leaf polyphenol content, followed by acetone extract and ethanol was third. Several studies showed that solvent natures, notably polarity, have significantly different extraction capacities for phenolic compounds in plants.[18,20]

CONCLUSION

Phenolic concentrations, nitric oxide radical ($NO^{\cdot}$)-scavenging activity, and ferric reducing/antioxidant power (FRAP) in *S. europaea* were not significantly higher than those of other halophyte plants.

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