Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 3, No. 5, p. 94-101, 2013
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Polyphenolic content and antioxidant activity of hydroalcohlic and alcoholic extract of *Thymus vulgaris*

Anoosh Eghdami*, Mojtaba Eizadi, Fatemeh Sadeghi

Department of biology, Saveh branch, Islamic Azad University, Saveh, Iran

Article published on May 16, 2013

Key words: Reducing power, Phenolic compound, DPPH, Antioxidants, *Thymus vulgaris*.

Abstract

Thymus vulgaris (thyme) (like some other wild Thymus species (wild thyme)) possesses a wide range of biological activities including expectorant, spasmolitic, sedative, antibacterial, antifungal, antioxidant. The objective of this paper was a screening of flavonoid content in Thymus vulgaris, the methanol extract of plant material was used for High-performance liquid chromatography (HPLC) analysis. Additionally, the antioxidant activities were also determined by ferric reducing antioxidant power (FRAP), 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity and reducing power methods. Hydroalcoholic extract had the higher flavonoid and total phenolic contents than alcoholic extract of Thymus vulgaris .A strong positive correlation of R2 = 0.92 between total phenolic content and antioxidant activity was observed in this investigation. This study indicated that Thymus vulgaris exhibited the high antioxidant activity, flavonoid and phenolic content and can be used potentially as a readily accessible source of natural antioxidant.Antioxidant activities of polyphenols have been suggested to exert beneficial pharmacological effects on some diseases.

***Corresponding Author:** Anoosh Eghdami ✉ eghdami_a@yahoo.com

Introduction

Thymus vulgaris (thyme) (like some other wild Thymus species (wild thyme) possesses a wide range of biological activities including expectorant, spasmolitic, sedative, antibacterial, antifungal, antioxidant .In general, investigations of biological activity in most of the studies were carried out on extracts, essential oils or pure compounds isolated from dried herbs of *Thymus* species. Nowadays, there is great world-wide interest in finding new and safe antioxidants from natural sources, to prevent oxidative deterioration of foods and to minimize oxidative damage of living cells. Within Lamiaceae species, examples of new antioxidants include phenolic diterpenes, phenolic carboxylic acids, biphenils, and flavonoids isolated from rosemary, sage, oregano and thyme .In many cases phenolic compounds have antioxidant activities more effective than ex-tocopherol and activity comparable to that of synthetic antioxidants, BHA and BHT (SvetIana *et al.,* 2001) .Plants are the oldest friends of mankind. They not only provide food and shelter but also serve humanity by preventing and curing different ailments. Herbs and spices have always been helpful to cure diseases. In modern animal feeding, they are forgotten because of use of antimicrobial growth promoters (AGP). But due to the prohibition of most of AGP, plant extracts have gained interest in animal feed strategies. Many plants also produce secondary metabolites such as phenolic compounds, essential oils and sarasaponins (Tipu *et al.,* 2006) .Phenolic compounds are secondary plant metabolites and are involved in a wide range of specialized physiological functions. They are very important for the normal growth, development and defense mechanisms of plants. These compounds are capable of inhibiting free radicals, and hence can retard the aging process. Aging causes pathogenesis of the skin. Theories involving free radicals and glycation processes are commonly used to explain the mechanism of skin aging. Aging proceeds by means of highly complicated biochemical processes in which the involvement of reactive oxygen species (ROS) and free radicals have been implicated. The overproduction of ROS and reactive nitrogen species (RNS) is a common underlying mechanism of aging, as they can damage various cellular components, including proteins, lipids and DNA. Ultraviolet (UV) light produces ROS in the skin, which accelerates aging bydamaging DNA, proteins, lipids and other cellular constituents (Nasapon *et al.,* 2010) .These compounds are capable of inhibiting free radicals, and hence can retard the aging process. Aging causes pathogenesis of the skin (Vioux-Chagnoleau *et al.,* 2006). Thymol and carvacrol constituted the main phenolic compound of Thyme oil. The major non phenolic compounds were linalool and p-cymene. Thyme oil with high thymol content strongly inhibited the bacterial growth. Also, thymol has the higher activity against fungi, followed by carvacrol and geraniol, but linalool, terpineol and thujone exhibited the least effect (Mensure *et al.,* 1998). Zheng & Wang found moderate levels of phenolics and antioxidant activity in thyme in comparison with other herbs (Table, 1). They also found a variation according to species, with common garden thyme ranking more highly in both respects than creeping or lemon thyme (Zheng *et al.,* 2001). Shan et al. identified the major phenolic compounds as gallic aicd, caffeic acid,rosmarinic acid, thymol, phenolic diterpenes and (unspecified) flavonoids. There have been a few trials involving human cells (Shan *et al.,* 2005). Youdim & Deans demonstrated a beneficial effect of supplementation with thyme essential oil or thymol on antioxidant status and fatty acid composition in the brains of ageing rats, both of which are believed to be important in maintaining brain function. In an animal study investigating its use as a burn treatment, a traditional herbal remedy of thyme oil served as a tissue-protective agent by decreasing the amount of nitrous oxide produced after the burn injury. It was also observed that more new tissue formed on the wounds of rats receiving the treatment than on the control animals (Yamamoto *et al.,* 2005). In two laboratory studies Braga et al. Investigated the effects of thymol on human neutrophils in relation to inflammation. It was shown that thymol was able to interfere with the production of reactive oxygen species released by neutrophils, as

well as inhibiting the release of elastase, which degrades tissue and is a marker of inflammatory diseases. A further non-cell-based assay showed thymol to have significant scavenging activity, which the authors attributed to its phenolic structure. Thymol also showed significant anti-thrombotic activity *in vitro* and *in vivo* in mice (Braga[a] *et al.,* 2006; Braga[b] *et al.,* 2006).

All investigated extracts contained various contents of flavonoids. The correlation between the antioxidant activity and the flavonoid content was poor, meaning that, B-sides flavonoids, other components in the extracts were also responsible for the antioxidant activity. These components could be phenol acids *or* phenolics from the essential oils (Kadifkova *et al.,* 2000). In the present work antioxidant activity of wild thyme extract was investigated.

Materials and methods

Plant collection

The plants were collected in July 2010 from Saveh, one of Markazay province cities of Iran. The area falls within the latitudes 35°.15' and longitudes 49°. 45' and the altitude of area is 1680m.

Chemicals

1-Diphenyl-2-picryl hydrazyl (DPPH) and quercetin were purchased from Sigma Chemical Co. (St., Louis, USA). Gallic acid, Folin Ciocalteu reagent, trifluoroacetic acid (TFA) and methanol and ethanol were purchased from Merck Co. (Germany).

Sample preparation

The samples were first ground to fine powder. Dried and ground fresh *Thymus vulgaris* (5 g) was extracted with 300 mL of alcohol(ethanol and methanol) and hydroalcoholic (50%) at by soucselet apparatus for 8 hours and then filtered and stored at 40C.The samples were then cooled down to room temperature and centrifuged at 4500 rpm for 15 min. The supernatant was recovered and used for the antioxidant assay and total phenolic analysis. We used HPLC analyzing for polyphenol types determination.

Determination of total phenolic content: The total phenolic content of the *Thymus vulgaris* extracts was determined using the Folin- Ciocalteu reagent (Wolfe *et al.,* 2003). The reaction mixture contained: 200 µl of diluted thyme extract, 800 µl of freshly prepared diluted Folin Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The final mixture was diluted to 7 ml with deionized water. Mixtures were kept in dark at ambient conditions for 2 h to complete the reaction. The absorbance at 765 nm was measured. Gallic acid was used as standard and the results were expressed as mg gallic acid (GAE)/g *Thymus vulgaris.*

Determination of total flavonoid content

Total flavonoid content was determined using aluminium chloride ($AlCl_3$) according to a known method (Ordon *et al.,* 2006), using quercetin as a standard. The plant extract (0.1 ml) was added to 0.3 ml distilled water followed by 5% $NaNO_2$ (0.03 ml). After 5 min at 25°C, $AlCl_3$ (0.03 ml, 10%) was added. After further 5 min, the reaction mixture was treated with 0.2 ml of 1 mM NaOH. Finally, the reaction mixture was diluted to 1 ml with water and the absorbance was measured at 510 nm. The results were expressed as mg quercetin (QE)/g Thymus vulgaris.

Antioxidant activity DPPH (2, 2'-diphenyl-1-picrylhydrazyl)

The antioxidant activity of tea samples was measured by using the DPPH assay with some minor modifications. *Thymus vulgaris* extract (80 µL) diluted 15-fold with distilled water The antioxidant activity was tested by the DPPH (2,2'-diphenyl-1-picrylhydrazyl) free radical scavenging method. For each extract, then we also prepared a dilution 1 M of DPPH. The absorbance of a mixture of 1 ml of the extract and 1 ml of the DPPH solution was measured at 517 nm. The radical scavenging activity was calculated from the equation: Percentage of radical

scavenging activity = (Abs control - Abs sample)/Abs control X 100.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay is based on the ability of antioxidants to reduce Fe3+ to Fe2+ in the presence of 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), forming an intense blue Fe2+-TPTZ complex with an absorption maximum at 593 nm. This reaction is pH-dependent (optimum pH 3.6). The absorbance decrease is proportional to the antioxidant content (Benzie and Strain, 1996). 0.2 ml of the extract is added to 3.8 ml of FRAP reagent (10 parts of 300 Mm sodium acetate buffer at pH 3.6, 1 part of 10.0 mM TPTZ solution and 1 part of 20.0 mM FeCl3. 6H2O solution) and the reaction mixture is incubated at 37°C for 30 min and the increase in absorbance at 593 nm is measured. FeSO4 is used for calibration. The antioxidant capacity based on the ability to reduce ferric ions of sample is calculated from the linear calibration curve and expressed as mmol FeSO4 equivalents per gram of sample.BHT, BHA, ascorbic acid, quercetin, catechin or trolox (Benzie *et al.,* 1996) can be used as a positive control.

Reducing power

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants (Yen *et al.,* 1996). The reducing power can be determined by the method of Athukorala et al. (2006). 1.0 ml extract is mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of potassium ferricyanide (30 mM) and incubated at 50°C for 20 min. Thereafter, 2.5 ml of trichloroacetic acid (600 mM) is added to the reaction mixture, centrifuged for 10 min at 3000 rpm. The upper layer of solution (2.5 ml) is mixed with 2.5 ml of distilled water and 0.5 ml of FeCl3 (6mM) and absorbance is measured at 700 nm. Ascorbic acid can be used as positive control (Chen *et al.,* 1996).

HPLC analysis

Identification of individual phenolic compounds of the plant extracts was performed by HPLC.The analysis was performed with a flow rate of 0.75 mL/min. using 0.2% tri fluoro acetic acid (TFA) as solvent A and Methanol as solvent B, with a linear gradient from 5 to 80% methanol in 50 min(table). AIl solvent used were filtered on Acetate Plus (0.22) before analysis. The selected flavonoid standards required a greater concentration of Methanol (80%) and a longer HPLC run for their proper elution than phenolic acids. Each standard was first injected individually to determine the exact retention time. And chromatographic characteristics (λ.max, absorbance ratio) followed by the analysis of the standard mixture. The methanolic extract of *Thymus vulgaris* was filtered on qualitative circle, Whatman filter paper No. 1 (Whatman International Ltd., Maidstone U.K.) under vacuum and subsequently on Acetate Plus filters (0.22 µm) to remove the undesirable contaminants. The limitations conceming the UV detector are that the compound must absorbs ultraviolet light and any other contamination that also absorbs UV radiation May interfere in the analysis (Isabelle, 2000).

Results and discussion

All the analysis was performed in triplicate. Results were expressed as means ± standard deviation. Descriptive statistical analysis, Pearson correlation coefficients, one-way analysis of variance (ANOVA) were performed using SPSS .extract of *Thymus vulgaris* showed scavenging effects against DPPH radical. The hierarchy for antioxidant capacity with respect to their EC50 values was methanolic extract > aqueous extract (table 2).Correlation coefficient showed that total phenolic content was responsible for antiradical efficiency in Thymus vulgaris, extracs. The antioxidant and total phenolic content levels are also positively and significantly correlated our results strongly suggested Thymus vulgaris, extracts can be promising sources of potential antioxidants. Many different detection methods are available in HPLC analysis. In general ultraviolet (UV) detectors are

most popular and have been extensively used in the detection of phenols. The limitations concerning the UV detector are that the compound must absorbs ultraviolet light and any other contamination that also absorbs UV radiation May interfere in the analysis. On the other hand the analyte is not destroyed by this type of detection and can be recuperated after separation for further characterization. According to table standards curve (fig.1, 2) and retention time of standards (table, 3) ,methanolic extract of *Thymus vulgaris* have polyphenolic compounds(fig,2) . HPLC chromatogram of polyphenols was showed that quercetin is the most important polyphenols compound in the *Thymus vulgaris* extract.

Table 1. Total phenolic, flavonoids contents and antioxidant activities of hydroalcoholic and alcoholic extract of *Thymus vulgaris*

Variety	Phenolic content(mg GAL/g)	Flavonoids content((QE)/g)	Antioxidant activity By DPPH (Inhibition %)	Antioxidant (FRAP) Mmol/l	Reducing Power
Methanolic extract	5.82±0.42	4.303± 0.05	25.2±0.75	10.9 ±2.1	0.39±0.01
50% methanolic extract	23.34±1.3	13.12±1.03	66. 82±6.0	18.28±2.5	0.67±0.01
50% ethanolic extract	32.34±2.63	14.30±1.14	74.34 ±0.32	18.67±2.3	0.94±0.00

Each value in the table was obtained by calculating average of three experiments ą standard deviation

Table 3. Retention time of some poliphenols.

standards	Retention time
Vanilic acid	20,10
quercetin	37,80
Gallic acid	6,25
Coumaric acid	30.02
catechin	27.3
Cafeic acid	20.28

The reducing power of fresh *Thymus vulgaris* leave extracts, using solvents of different polarities was found to different range between 0.39-.094 (Table 1). Aqueous solvents (50%) gave higher reducing power values than the corresponding absolute solvents. It is clear that polar aqueous solvents dissolve more polar plant poly phenols with higher RP at all different extraction times. This conclusion is in agreement with the results of (Negi *et al.,* 2005) who reported that methanol extract of sea buckthorn seed had higher reducing power than extracts using low polarity chloroform. Based on this evidence, higher reducing powers of aqueous *Thymus vulgaris* extracts can be attributed to their containing more hydrophilic phenolics. The results of the free radical scavenging activity of tea extracts with different extraction solvents are shown in Table 2. The activities obtained for *Thymus vulgaris* extracts were found to vary from to95.4%. 50% aqueous solvent extracts exhibited higher and more significant inhibitory activity against DPPH radical in comparison with their corresponding absolute solvent ones for all extraction times and this trend was similar to that observed for polyphenol content and reducing power measurements. This can be attributed to higher concentration of polyphenol present in these extracts. The effect of the extracting solvent on DPPH scavenging activity has also been reported by previous studies (Negi *et al.,* 2005; Canadanovic-Brunet *et al.,* 2005; Yuan *et al.,* 2005).

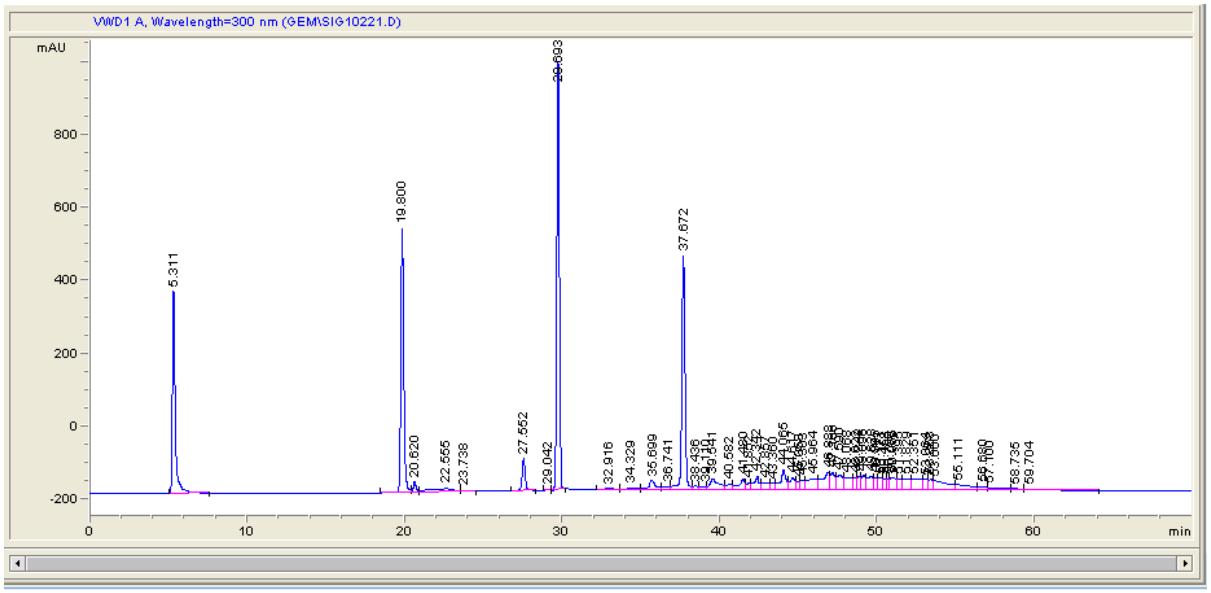

Fig. 1. HPLC chromatogram some of polypehnol standard (gallic acid, vanillic acid, coumaric acid, benzoic acid respectively).

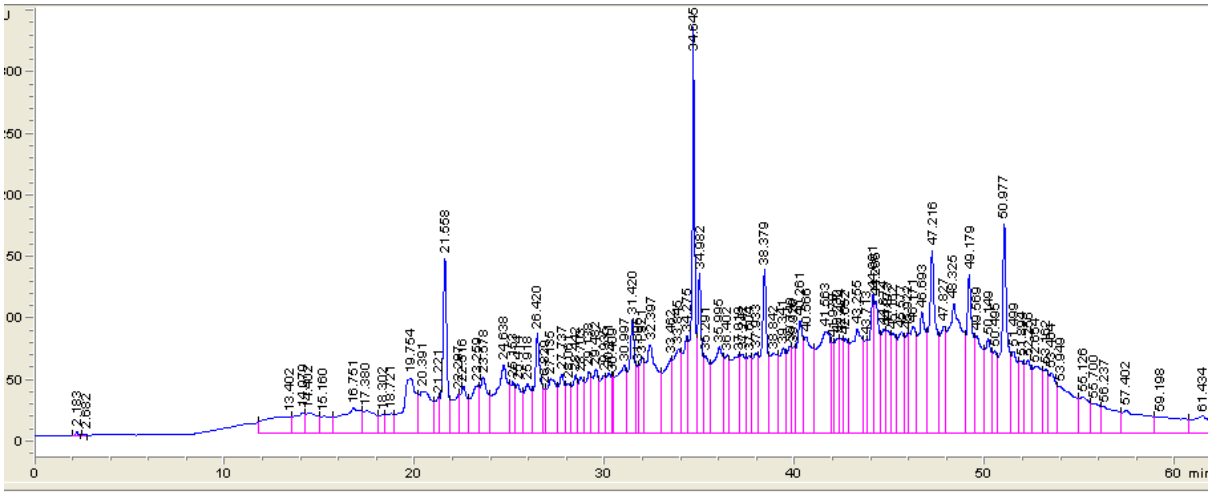

Fig. 2. HPLC chromatogram of *Thymus vulgaris*.

Conclusion

The results from this study indicate that the leaves and stem extracts of thyme possess antioxidant properties and could serve as free radical inhibitors or scavenger or, acting possibly as primary antioxidants. The results of the current study showed that the methanol extract of thyme had the highest scavenging activity. The amounts of phenol compounds of thyme correlate to their scavenging effect. These results demonstrate that the antioxidant activities observed can be ascribed both to mechanisms exerted by phenolic compounds and also to synergistic effect of different phyto compounds. This study reveals that tested plant materials have moderate to significant antioxidant activity and free radical scavenging activity. The result of the present study suggests this plant can be used as a source of antioxidants for pharmacological preparations which is very well evidenced by the present work.

References

Benzie IFF, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anals Biochemical **239,** 70–76.

Braga[a] PC, Dal Sasso M, Culici M, Bianchi T, Bordoni L, Marabini L. 2006. Anti-inflammatory activity of thymol: Inhibitory effect on the release of human neutrophil elastase. Pharmacology **77(3),** 130-136.

Braga[b] PC, Dal Sasso M, Culici M, Galastri L, Marceca MT, Guffanti EE . 2006. Antioxidant potential of thymol determined by chemiluminescence inhibition in human neutrophils and cell-free systems. Pharmacology **76(2),** 61-68.

Canadanovic-Brunet JM, Djilas SM, Cetkovic GS. 2005. Free-radical scavenging activity of wormwood (Artemisia absinthium) extracts. Journal of the Science of Food and Agriculture **85,** 265-272.

Chen ZY, Chan PT. 1996. Antioxidative activity of green tea catechins in canola oil. Chemistry and Physics of Lipids **82,** 163–172.

Isabelle D. Recovery ,separation and characterization of phenolic compounds and flavonoids from Maple produces. Oepartment of Food Science and Agricultural Chemistry. A tbesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment ofthe requirement of the degree of Master of Science. 2000.

Kadifkova P, Gj. Stefkov, S. Kulevanova. 2000. Proceedings of the First Conference on Medicinal and Aromatic Plants of Southeast European COlli1lries, Arangelovac, FR Yugoslavia **11,** 527-531.

Mensure Q, Sezen T. 1998. Drug Yield and Essential Oil of *Thymus vulgaris* L. as in Influenced by Ecological and Ontogenetical Variation. Turkish Journal of Agriculture and Forestry **22,** 537-542

Nasapon P , Ampai P, Maltree S, Chaiyavat C, Pimporn L. 2010. Phenolic content and in vitro inhibitory effects on oxidation and protein glycation of some thahi medicinal plants. Pakistan Journal of Pharmaceutical Sciences **23(4),** 403-408

Negi PS, Chauhan AS, Sadia GA, Rohinishree YS, Ramteke RS. 2005. Antioxidant and antibacterial activities of various seabuckthorn (Hippophae rhamnoides L.) seed extracts. Food Chemistry **92,** 119-124.

Ordon Ez AAL, Gomez JD, Vattuone MA, Isla MI. 2006. Antioxidant activities of *Sechium edule* (Jacq) Swart extracts. Food Chemistry **97**, 452–458.

Shan B, Cai YZ, Sun M, Corke H . 2005. Antioxidant Capacity of 26 Spice Extracts and Characterization of Their Phenolic Constituents. Journal of Agricultural and Food Chemistry **53(20),** 7749-7759.

SvetIana K, Tatjana KP. 2001. Antioxidant activity of essential oils of different wild Thymus vulgaris. Bulletin of the Chemists and Technologists of Macedonia **20(1),** 61-66.

Tipu MA, Akhtar MA, Anjum M, Raja MJ. 2006. New dime OF Mention dicinal plants as animal feed. Pakistan Veterinary Journal. J **26(3),** 144-148.

Vioux-Chagnoleau C, Lejeune F, Sok J, Pierrard C, 2006. Marionnet C and Bernerd F .Reconstructed human skin: from photodamage to sunscreen photoprotection and anti-aging molecules. Journal of Dermatological Science **2(1),** 1-12.

Wolfe K, Wu X, Liu RH. 2003. Antioxidant activity of apple peels. Journal of Agriculture and Food Chemistry **51,** 609–614.

Yamamoto J, Yamada K, Naemura A, Yamashita T, Arai R. 2005. Testing various herbs for antithrombotic effect. Nutrition **21(5),** 580-587.

Yen GH, Chen HY. 1996. Antioxidant activity of various tea extracts in relation to their antimutagenicity. Journal of Agricultural and Food Chemistry **43,** 27–32.

Yuan YV, Bone DE, Carrington MF. 2005. Antioxidant activity of dulse (Palmira palmata) extract evaluated in vitro. Food Chemistry **91,** 485-494.

Zheng W, Wang SY. 2001. Antioxidant Activity and Phenolic Compounds in Selected Herbs. Journal of Agricultural and Food Chemistry **49(11),** 5165-5170.