Agricultural Research & Technology
Open Access Journal
ISSN: 2471-6774

Juniper Publishers
key to the Researchers

Research Article
Volume 19 Issue 4 - January 2019
DOI: 10.19080/ARTOAJ.2019.19.556102

Agri Res & Tech: Open Access J

Determination of Antioxidant Content and Activity in Eight Jordanian Fresh Green Leafy Vegetables

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Submission: January 12, 2019, **Published:** January 25, 2019

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Abstract

The consumption of vegetables that contain natural antioxidants is thought to be an efficient way for reducing the risk for oxidative stress diseases. Determination of antioxidant content and capacity allows the screening of vegetables that are probably involved in the prevention and/ or treatment of oxidative stress diseases. This study aimed at comparing eight fresh Jordanian green leafy vegetables (namely: grape leaves, lettuce, mint, parsley, Jew's mallow, watercress, celery, and garden rocket) for their antioxidant content (using two methods namely: Folin-Ciocaltaeu method and total flavonoid method) and capacity (using two methods namely: 2,2-diphenyl-picrylhydrazyl (DPPH) and cupric antioxidant reducing capacity (CUPRAC) assays). Three solvents were used for the vegetable extraction (ethanol, methanol, and water). Different solvents as well as different vegetables showed significantly ($P<0.05$) different antioxidant contents and capacities. Significant correlations ($P<0.001$**; $r>0.90$) were found between antioxidant content and capacity of the studied vegetables.

Keywords: Jordan; Antioxidants; CUPRAC; DPPH; Folin-Cioculteau; Green leafy vegetables; Total flavonoids

Introduction

Free-radicals are highly reactive molecules that lost one or more of the electrons of their outermost orbit; enabling them for hitting the electronically stable outermost orbit of a stable atom or an atom of a molecule for the purpose of steeling one of the electrons to be stable. Free radicals are formed continuously within the human body during cell respiration. Simultaneously, the body has many mechanisms to get rid of these highly reactive molecules. When the formation of free radicals exceeds the body capacity to get rid of them, oxidative damage occurs. Oxidative damage is involved in many chronic diseases such as cardiovascular diseases, type I diabetes, cataract, arthritis and certain types of cancer. Antioxidants are substances that, when present at low concentrations –compared to oxidized substrates- delay or prevent that substrate oxidation significantly [1]. The consumption of vegetables that contain natural antioxidants is thought to be an efficient way for reducing the risk for oxidative stress diseases [2]. Determination of antioxidant content and capacity, therefore, allows the screening of vegetables that are probably involved in the prevention and/ or treatment of oxidative stress diseases.

The purpose of this study is to screen eight Jordanian fresh green leafy vegetables that are consumed regularly (namely: grape leaves, lettuce, mint, parsley, Jew's mallow, watercress, celery, and garden rocket) for their antioxidant content and capacity. Additionally, this study aimed to find a correlation between the antioxidant content and capacity of the selected vegetables. This study is probably the first study that evaluated the antioxidant content and capacity of locally grown fresh Jordanian vegetables extracted by three solvents (i,e. ethanol, methanol, and water) in order to add a value to the scientific antioxidant database. In Jordan, there were many attempts to determine the antioxidant content and capacity of Jordanian plants [3-5]. There is –as well- a great interest in plants in Jordan in terms of classification [6], studying of nutritional value and methods of use [7], and functional properties [8,9].

Materials and Methods

Fresh vegetables were purchased from local market and prepared in the same day. Vegetables were prepared by washing with tap water and gentle drying by towel paper. Samples were then chopped finely by knife or food chopper (Ariete®, China). Representative samples (1-3g) were extracted conventionally by 10ml of one of three extraction solvents (methanol, ethanol, and water) at 50 °C, 50 °C, and 90 °C respectively for 2 hours with intermittent shaking. The extracts were centrifuged at 3000rpm for 10-15 minutes (HuMax®, Germany) and filtered (Wattman filter paper No.4), purged with liquid nitrogen [2], and stored at -20 °C (for not more than two months) until analyzed. Deionized water was used for the preparation of all standard solutions and to complete the reactions [2].

Determination of antioxidant content

Chemicals were purchased from GCC® (UK), Fischer® (China), Labscan® (Thaihland), LabChem® (USA) and Sigma® (China). Standard curves were prepared to have r^2 value of 0.96-0.99. Samples were analyzed in duplicate with an accuracy of not less than 95% [10] and coefficient of variation not more than 15%. Absorbance values were measured using UV-visible spectrophotometer (SCO Tech, Model SPUV®) at the specified wavelength values against standard concentrations of certain antioxidants and blank solutions.

Folin-ciocultaeu method: Folin-Ciocultaeu method was used for the determination of antioxidant content according to Agbor et al. [11]. Sample concentration for antioxidants was measured against freshly prepared catechin standard at 750nm wavelength.

Total flavonoid method: Total flavonoids were analyzed by the method of Pękal and Pyrzynka [12]. The absorbance was measured at 400nm wavelength against different concentrations of rutin standard solutions.

Determination of antioxidant capacity

CUPRAC Assay: Sample antioxidant capacity was measured by CUPRAC assay according to Apak et.al. [2]. Absorbance was measured using a spectrophotometer at 450nm [2] against different concentrations of trolox standard solutions.

DPPH assay: The DPPH assay procedure was performed according to Molyneux [13]. The absorbance was measured at 517nm wavelength against ascorbic acid as a standard. The scavenging percentage was calculated according to the following equation:

$$Scavenging\ effect\ (\%) = \left(\left(A_0 - A_1\right) / A_0\right) * 100\%$$

Where: A_0: absorbance of the control; A_1: absorbance of the sample

Statistical Analysis

The statistical analysis of data was performed using the software package for social sciences (SPSS, version 23). To detect the differences between the 8 different vegetables as well as the extraction solvent, data were analyzed by factorial mixed (effect of type of plant and extracts types) analysis of variance (ANOVA) [14]. Significant differences were considered at $P<0.05$. Data are expressed in the tables as mean ± standard deviation. Pearson's correlation coefficient was calculated and considered significant at $P<0.05$.

Results and Discussion

Table 1: The antioxidant content of the ethanolic, methanolic, and water vegetable extracts determined by Folin-Ciocultaeu method (M catechin/100g) and total flavonoid method (M rutin/100g)[1,2]

Vegetable	Folin-Ciocultaeu Method			Total Flavonoid Method			
	Extract			Extract			
	Ethanol	Methanol	Water	Ethanol	Methanol	Water	P-value
Celery	0.9533±0.0265	0.6274±0.0773	0.9533±0.0265	0.4809±0.0524	0.3587±0.0088	0.1738±0.0092	<0.01** (Folin-Ciocultaeu method)
Garden rocket	3.5563±0.4517	1.3714±0.0519	3.4257±0.3939	0.8478±0.0075	0.3366±0.0038	0.1669±0.0021	<0.01** (Total Flavanoids Method)
Grape leaves	74.4792±1.8382	22.4282±0.3143	29.7666±1.3531	1.1103±0.0699	0.7320±0.0133	0.7168±0.0516	
Lettuce	1.1869±0.0227	0.9023±0.0218	1.4208±0.0288	0.0287±0.0043	0.0267±0.0007	0.02110±0.0021	
Mint	77.6903±9.6764	24.1357±0.3320	45.0805±1.3579	2.9404±0.0476	3.1849±0.01210	1.1894±0.0291	
Parsley	10.9093±1.0836	8.9465±0.7487	10.5018±0.7495	5.3971±0.4555	4.8651±0.3241	1.1349±0.0297	
Jew's Mallow	12.1150±0.1114	14.8060±0.3275	23.7710±0.9788	0.1090±0.0107	1.0704±0.0449	0.1512±0.0050	
Watercress	2.8772±0.2092	1.8392±0.1401	2.3765±0.0235	0.4103±0.0159	0.1824±0.0055	0.0042±0.0002	

[1]Values of the tables are average of duplicates ± SEM with c.v. of not more than 15%.

[2]P values are used to express significant differences between different vegetable extracts.

Table 1 shows the antioxidant content determined by Folin-Ciocultaeu and total flavonoid methods for the ethanolic, methanolic, and water vegetable extracts. Regardless of the extraction solvent; the antioxidant content (expressed as M catechin/100 g sample) was the highest ($P<0.05$) for mint>grape leaves>parsley>Jew's mallow>garden rocket. Nonetheless, there were no significant differences ($P>0.05$) among garden rocket, watercress, lettuce, and celery in the same context. In terms of the mixed effect of plant and extract types, different vegetables seem to have different ($P<0.01$**) results in terms of the extraction solvents. For instance, ethanol followed by ($P<0.05$) water had extracted higher amounts of antioxidants than methanol ($P<0.05$)

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from mint, grape leaves, parsley, garden rocket, watercress, and celery than methanol (P<0.05). From Jew's mallow only, water followed by (P<0.05) methanol had extracted higher (P<0.05) amounts of antioxidants than ethanol did. From lettuce only, water followed by (P<0.05) ethanol extracted higher amounts (p<0.05) of antioxidants than methanol did.

Regardless of the extraction solvent, parsley> mint>grape leaves>garden rocket>lettuce had the highest (P<0.05) antioxidant contents (Mrutin/100g). In terms of the extraction solvent, ethanol followed by methanol (P<0.05) had extracted more antioxidants (P<0.05) than water from parsley, grape leaves, garden rocket, celery, watercress, and lettuce. Methanol followed by ethanol (P<0.05) had extracted more antioxidants than water from mint. On the other hand, methanol followed by water (P<0.05) had extracted more antioxidants than ethanol from Jew's mallow.

Table 2 shows the antioxidant capacity determined by CUPRAC assay for the ethanolic, methanolic, and water vegetables extracts. Regardless of the extraction solvent, watercress> mint>grape leaves>lettuce>parsley>Jew's mallow>celery had the highest antioxidant capacity values. In terms of the extraction solvent, ethanol followed by methanol (P<0.05) had extracted antioxidants more powerfully (P<0.05) than water from parsley, garden rocket, and celery. Methanol followed by (P<0.05) water had extracted antioxidants more (P<0.05) powerfully than ethanol from Jew's mallow and watercress. On the other hand, water followed by methanol (P<0.05) had extracted antioxidants more powerfully (P<0.05) than ethanol from mint and grape leaves. Water followed by methanol (P<0.05) had extracted antioxidants more (P<0.05) powerfully than ethanol from mint and grape leaves. From lettuce, water followed by ethanol (P<0.05) had extracted antioxidants more (P<0.05) powerfully than methanol did.

Table 2: The antioxidant capacity (M trolox/100g) of the ethanolic, methanolic, and water vegetable extracts determined by CUPRAC assay[1,2].

Vegetable	Antioxidant Capacity (M trolox/100g) as Determined by Total CUPRAC Assay			
	Extract			
	Ethanol	Methanol	Water	P-value
Celery	1.7725±0.2361	1.1155±0.0391	1.0298±0.0165	0.002**
Garden rocket	3.8115±0.0786	3.7643±0.017	2.2329±0.1187	
Grape leaves	17.9871±0.3409	18.4574±0.1032	53.6694±1.3151	
Lettuce	0.6584±0.0000	0.54944±0.0253	2.4399±0.2667	
Mint	20.5841±0.4903	23.8290±1.5477	23.9893±2.8210	
Parsley	11.5835±0.0162	6.4861±0.1226	4.1143±0.2099	
Jew's mallow	9.8804±0.2549	15.8392±0.3314	15.0906±0.7481	
Watercress	2.2432±0.1172	3.4036±0.24078	2.3272±0.0648	

[1]Values of the tables are average of duplicates ± SEM with c.v. of not more than 15%.

[2]P values are used to express significant differences between different vegetable extracts.

Table 3: The antioxidant capacity of the ethanolic, methanolic, and water vegetable extracts determined by DPPH assay (expressed as % of DPPH radical scavenging and mg vitamin C/ml extract)[1,2].

Vegetable	% DPPH scavenging			mg vitamin C/ml			
	Extract			Extract			
	Ethanol	Methanol	Water	Ethanol	Methanol	Water	P-value
Celery	4.823±0.455	15.264±2.060	17.546±0.056	59.915±5.649	194.946±26.300	161.8631±0.521	0.000** (% DPPH scavenging)
Garden rocket	11.362±1.566	14.441±0.211	18.745±0.754	92.598±12.764	93.712±1.425	111.856±4.275	0.000** (mg vitamin C/ml extract)
Grape leaves	35.128±1.583	57.981±0.506	88.426±0.982	213.543±9.624	439.470±3.595	506.874±5.567	
Lettuce	9.220±0.134	28.465±0.390	21.805±2.528	56.052±0.817	184.715±2.529	129.2051±14.329	
Mint	25.289±0.204	50.184±0.676	88.209±0.2111	206.104±1.666	384.090±4.800	428.4068±1.025	
Parsley	7.298±0.818	6.066±1.196	14.129±1.267	59.476±6.662	70.754±8.493	68.6224±6.151	
Jew's mallow	12.808±0.257	15.399±3.355	36.319±0.689	104.388±2.091	137.040±23.824	176.393±3.344	
Watercress	29.020±0.561	23.463±0.424	39.667±4.687	236.508±4.570	194.317±3.012	192.660±22.761	

[1]Values of the tables are average of duplicates ± SEM with c.v. of not more than 15%.

[2]P values are used to express significant differences between different vegetable extracts.

Table 3 shows the antioxidant capacity determined by DPPH assay and expressed as % DPPH scavenging and as mg vitamin C/ml extract. Regardless of the extraction solvent, the highest antioxidants capacities were for grape leaves> mint>watercress> Jew's mallow> garden rocket>parsley. In terms of the extraction solvent, water followed by methanol (P<0.05) had extracted

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antioxidants more powerfully (P<0.05) than ethanol from grape leaves, mint, Jew's mallow, garden rocket, and celery. Methanol followed by water had extracted antioxidants more powerfully (P<0.05) than ethanol from lettuce. On the other hand, water followed by ethanol (P<0.05) had extracted antioxidants more (P<0.05) powerfully than methanol from watercress and parsley.

In terms of DPPH scavenging expressed as mg vitamin C/ml solvent and regardless of the extraction solvent, grape leaves>mint> watercress>Jew's mallow>lettuce>parsley had the highest antioxidant capacity values. Within the same context and in terms of the extraction solvent, ethanol followed by methanol (P<0.05) extracted antioxidants more powerfully (P<0.05) than water from watercress. On the other hand, water followed by methanol (P<0.05) had extracted antioxidants more powerfully (P<0.05) than ethanol from grape leaves, mint, Jew's mallow, and garden rocket. Methanol followed by water (P<0.05) had extracted antioxidants more powerfully (P<0.05) than ethanol from celery, lettuce, and parsley.

With reference to correlations, antioxidant content (measured by Folin-Ciocultaeu method) of the studied vegetables correlated highly significantly with antioxidant capacity (measured by DPPH assay as percentage of scavenging (r=0.883, P=0.004**), as mg vitamin C/ml (r=0.857, P=0.007**), and as M trolox by CUPRAC assay (r=0.958, P=0.000**)). Additionally, there was a highly significant agreement expressed as a highly significant correlation between antioxidant capacity values measured by CUPRAC and DPPH assay (as DPPH scavenging percentage (r=0.860, P=0.005) and as mg vitamin C/ml (r=0.845, P=0.008)).

Table 4: Comparison between values of antioxidant content and capacity found in this research and those found in other reports.

Variable	Vegetable	Value Found in this Research	Value Found in Other Reports	Reference
Antioxidant Content: Total Polyphenol (Folin-Cioculteau Method)	Celery	0.39126 M catechin/100g. This value corresponds to 22.8309g catechin equivalent/100g.	20.55g catechin equivalent/100g	[15]
	Celery	0.39126 M catechin/100g. This value corresponds to 11.36ppm in fresh celery.	18.43ppm catechin in fresh celery.	[36]
	Garden Rocket	2.7845 M catechin/100g fresh garden rocket; a value which corresponds to 80.8219mg catechin/g fresh garden rocket.	1.2mg total poly phenols/g fresh weight.	[28]
	Grape Leaves	42.2247 M catechin/100g fresh grape leaves; a value which corresponds to 1.226mg catechin/g fresh grape leaves.	19.8 and 22.8mg catechin/g fresh grape leaves by extraction with 80% ethanol and 80% acetone respectively.	[30]
	Lettuce	1.1700 M catechin/100g fresh lettuce; a value which corresponds to 3.39mg catechin/100g fresh lettuce.	4.85 mg gallic acid equivalent/100g fresh weight.	[19]
	Mint	48.9688 M catechin/100g fresh mint; a value which corresponds to 101.52mg/g fresh leaves.	25.62mg catechin equivalent/g fresh leaves.	[24]
	Parsley	10.1102 M catechin/100g corresponds to 2.098 g catechin equivalent/100g.	3.698g catechin equivalent/100g.	[15]
	Jew's mallow	16.8973 M catechin/100g fresh sample. Assuming 82.4% moisture content [42,43], our value corresponds to 27.867mg catechin/g dry Jews mallow.	16.54 mg gallic acid/g dry weight.	[23]
	Watercress	2.3643 M catechin/100g fresh watercress. Assuming 95.11% moisture content [44]; our value corresponds to 140.6274mg catechin equivalent/g dry water cress.	217.14 mg gallic acid equivalent/g dry water cress.	[33]
Antioxidant Content: Total Flavonoid Content	Celery	0.3378 M rutin/100g fresh celery. This value corresponds to 29.3601mg rutin/100g fresh celery	4.51mg total flavonoids/100g edible celery	[25]
	Garden Rocket	0.4602 M rutin/100 g fresh watercress. Assuming 95.11% moisture content [44]; our value corresponds to 575.7403mg rutin/g dry weight.	0.82µg rutin/g dry weight.	[29]
	Grape Leaves	0.8530 M rutin/100 sample; a value which corresponds to 8.362g rutin/L extraction solution.	56.38 and 100.08 mg rutin/L grape leaves extracts of May and September respectively.	[31]
	Lettuce	0.0255 M rutin/100g fresh lettuce; a value which corresponds to 1.55mg rutin/g fresh sample.	4.24µg rutin/g fresh lettuce.	[32]

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	Mint	2.438 M rutin/100g. This value corresponds to 148.844mg rutin/g fresh sample.	The amount of mint flavonoids is 60.48 mg/100g fresh leaves.	[25]
	Mint	2.438 M rutin/100g. This value corresponds to 0.02438mg rutin/g fresh sample.	0.01mg rutin/g fresh leaves.	[39]
	Parsley	3.7390 M rutin/100g; a value which corresponds to 23.1937mg rutin/100g.	4.32mg rutin/100g fresh parsley.	[40]
	Jew's mallow	0.4435 M rutin/100g fresh Jews mallow; a value which corresponds to 0.154mg rutin/g dry sample.	0.33mg rutin/g dry Jew's mallow stems.	[38]
	Watercress	0.1990 M rutin/100g fresh watercress. Assuming 95.11% moisture content [44]; our value corresponds to 248.962µg rutin/g dry weight.	126.57µg rutin/g dry weight.	[29]
Antioxidant Capacity: CUPRAC Assay	Celery	1.3059 M trolox/100g; a value which corresponds to 1.747µmol TE/ml.	9 and 11µmol TE/ml and 0.25 and 3.5µmol TE/ml broth of fresh leaves and stalk respectively.	[21]
	Garden Rocket	3.2696 M trolox/100 g fresh garden rocket; a value which corresponds to 18.16µmol TE/g fresh garden rocket.	8.18 and 32.08µmol TE/g fresh garden rocket by DPPH and ORAC1 assays for antioxidant capacity.	[29]
	Grape Leaves	30.0380 M trolox/100g fresh sample; a value which corresponds to 3.0038 mmol trolox/g fresh sample.	0.12mmol trolox/g fresh sample.	[30]
	Lettuce	our value for lettuce corresponds to 675.507µmol TE/100g.	491µmol TE/100g fresh lettuce (ORAC method).	[17]
	Mint	22.8008 M trolox/100g fresh leaves. Assuming 69.6% moisture content [44], our value corresponds to 7500.2632µmol/100 g dry weight.	386µmol trolox/100g dry weight.	[27]
	Parsley	Our value corresponds to 18.508g trolox/100g fresh parsley.	987.51 mg trolox/100g fresh parsley.	[39]
	Parsley	Assuming 25.3% dry matter. Our value corresponds to 1623.715µmol TE/100g dry matter.	340.68 µmol TE/100 g dry matter (average of 2 methods; DPPH and ABTS2).	[26]
	Jew's mallow	13.3043 M trolox/100 g fresh sample; a value which corresponds to 75.593µmol trolox/g dry weight.	antioxidant capacity by ABTS method to be 139.55µmol trolox/ g dry weight.	[23]
	Watercress	2.658 M trolox/100g fresh garden rocket; a value which corresponds to 14.767µmol TE/g fresh garden rocket.	7.76 and 32.92µmol TE/g fresh watercress by DPPH and ORAC assays for antioxidant capacity.	[29]
Antioxidant Capacity: DPPH Scavenging Percentage	Celery	Our values ranged between ~5 and ~17%.	DPPH% scavenging capacity value of the essential oils of celery herb and seeds to be 56.68 and 74.35 respectively.	[34]
	Garden Rocket	Our values ranged between ~11 and ~17.8%.	20-60% inhibition of alcohol and hydro alcohol extracts of garden rocket (range is due to the extract concentration; ranged between 10-320µg/ml).	[20]
	Grape Leaves	Ranged between ~35% and ~88%. Referring to our raw data records, our samples were purchased in August.	61.69% and 70.32% for May and September vine leaves.	[31]
	Lettuce	Our values ranged between ~9 and ~28%.	80.9 in 12 US lettuce varieties.	[35]
	Mint	Our values ranged from ~25% to ~88%.	18 and 35 for mint stem and leaves respectively (diethylether extracts).	[16]
	Parsley	Our values ranged between ~6 and ~14%.	20-30% of DPPH radical scavenging capacity in field and aeroponic grown 500µg/ml parsley extracted dimethylsulfoxide extracts.	[23]

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	Jew's mallow	21.5090%. Assuming 82.4% moisture content [42], this value corresponds to 129.5723%.	52.29% in the dry weight.	[24]
	Watercress	Our values ranged between ~23 and ~39%	60-80% DPPH radical scavenging capacity of the aqueous and methanolic extracts of watercress stem and leaves.	[37]

The antioxidant content and capacity values of the studied vegetables have been published previously. Table 4 shows a comparison between values of antioxidant content and capacity found in this research and those found in other reports. The previously published values were sometimes comparable with the values found in this research [15-23], sometimes much higher [24-29] or much lower [23,30-39]. The differences found are probably attributed to the differences in the extraction solvent type, treatment, and concentration extraction methods, experimental methodology, plant growing conditions as Jordan has variation in agricultural environment because it meets the Mediterranean, Irano-turanian, and Saharo-Arabian region [40], parts of the analyzed plant, and the experimental standardization conditions. The expression of antioxidant capacity (measured by DPP radical scavenging assay) as mg of vitamin C is another method for expression that will add a value to the scientific antioxidant capacity database.

Many scientists agree that there is no single best method for measuring antioxidant content and capacity [2]. Nonetheless, in either method for determining antioxidant content; grape leaves, mint, and parsley showed the highest 3 levels of antioxidant contents. Furthermore, grape leaves, mint, and watercress showed the highest levels of antioxidant capacities. With reference to correlations; similar to the results found by Apak et.al. [2], the highly significant correlation between the antioxidant content (measured by Folin-Ciocultaeu method) of the studied vegetables with antioxidant capacity (measured by DPPH as well as CUPRAC) assays is not surprising since the antioxidants measured by Folin-Ciocaltaeu method contributed well to the antioxidant capacity. Similar to the results of Kaur & Mondal [41], there was no correlation between the antioxidant content measured by total flavonoid content and antioxidant capacity owing that the antioxidant capacity is not solely due to the total flavonoids content. The highly significant agreement expressed as a highly significant correlation between antioxidant capacity values measured by CUPRAC and DPPH assays is not surprising as both assays measure the same reaction kinetics (ET-assays).

Conclusion

Significant differences ($P<0.05$) were found among vegetables extracts in terms of antioxidant contents and capacities. Also, we find highly significant ($P<0.001$**) correlations between antioxidant content and capacity values. This study is limited by the types of extracts used and the extraction methods as well as the treatment of the vegetables (as the vegetables were fresh). However, this study is probably the first study which analyzed and compared three extracts types of the eight fresh green leafy vegetables and will start a database for the antioxidant content and capacity of Jordanian vegetables.

Acknowledgement

The authors would like to thank the Deanship of Academic Research at The University of Petra for financially supporting this research [Grant number 5/4/2015].

Conflict of Interest

The authors declare that there is no conflict of interest among them.

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