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Phenols and Antioxidant Activity *in Vitro* and *in Vivo* of Aqueous Extracts Obtained by Ultrasound-Assisted Extraction from Artichoke By-Products

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Artichoke by-products are rich in phenolic compounds although they represent a waste for the food industry. This paper examines the application of ultrasound-assisted extraction (UAE) for obtaining organic solvent-free extracts rich in nutraceuticals from artichoke scraps. Application of ultrasounds for 60 minutes on test samples, using water as a solvent, improved recovery of phenolic substances compared with untreated samples. Among the phenols detected by high performance liquid chromatography, 5-*O*-caffeoylquinic and 1,5-di-*O*-caffeoylquinic acids were identified. *In vivo* treatments of tobacco BY-2 cells with ultrasonic extracts consistently enhanced their antioxidant power, making the cells more resistant to heat stress. UAE applied to artichoke by-products, using water as a solvent, appears to be a powerful eco-friendly technique that can provide extracts rich in nutraceuticals and turn waste products into resources. The extracts could be advantageously utilized in the food industry to produce functional foods.

Keywords: Artichoke by-products, Ultrasound-assisted extraction, Phenols, Antioxidant activity, Tobacco BY-2 cells.

Artichokes play an important role in human nutrition. They are considered functional food because of their antioxidant properties and beneficial effects on liver diseases [1]. Apart from their bile expelling and hepatoprotective effects [2], artichokes also help lower blood cholesterol and enhance anticholestatic activity [3]. The beneficial properties of artichokes have been ascribed to compounds such as phenolic acids (gallic, caffeic, cumaric) and their glucosidic derivatives, and to simple and glycosylated flavonoids [4]. The phenolic content and antioxidant activity of artichokes vary based on the cultivar [5], and it has been demonstrated that bioactive compounds are contained both in the edible part (heart) and in the by-products [6], which account for about 60% of fresh weight (leaves, stems and outer bracts). For this reason, recovery of antioxidants from these by-products may represent an excellent solution for re-using the large amounts of vegetable discarded during processing, as has already been shown for other plant by-products [7,8].

The traditional methods used to extract phenolic compounds from plants are time-consuming and require relatively large amounts of organic solvents. Hence, nowadays there is greater demand for new time-saving and solvent free extraction techniques. Ultrasound-assisted extraction (UAE) could satisfy these requirements [9]. The technique has been used for various processes in the chemical and food industry for many years to produce emulsions, disrupt cells, and disperse aggregated materials [10]. Recently, it has been applied to enhance the efficiency of mechanical olive oil extraction [11]. Ultrasound treatment has also been used to extract food components such as aromas, antioxidants, pigments, polyphenols, triterpenic acids, and other organic and mineral components from a variety of matrices [12-17]. UAE also plays an important role as a potentially sustainable technique for industrial applications of polyphenol extraction [18]. The aims of our investigations were to: i) test the efficacy of UAE in obtaining organic solvent-free extracts rich in phenolic compounds from artichoke by-products, and ii) assess whether extracts obtained by UAE could enhance the antioxidant power of tobacco cells and protect them from severe stress.

Extraction with methanol was performed to measure the total phenol content (TPC) and antioxidant activity (AA) of the different parts of artichokes. Hearts were found to have the highest TPC values, which were 10% greater than those of leaves, 60% greater than those of the outer bracts, and about 100% greater than those of the stems (Table 1).

Table 1: TPC and AA of methanol extracts of different parts of artichoke (means ± SD).

Samples	TPC (mg kg^{-1} FW)	Antioxidant activity (μmoles TE kg^{-1} FW)	
		ABTS	DPPH
Hearts	1446 ± 15a	4977 ± 151a	5214 ± 301a
Leaves	1343 ± 25b	3197 ± 212b	4629 ± 278b
Outer bracts	907 ± 37c	2002 ± 144d	2077 ± 151d
Stems	774 ± 28d	2361 ± 195c	2495 ± 187c

In the columns, data followed by different letters indicate statistically significant differences at $P < 0.05$.

These results are in agreement with those reported for some Italian artichoke cultivars [19,20]. As expected, hearts also showed higher AA than scraps. Amongst the scraps, the highest AA value was found in leaves, which is an interesting finding for the purpose of recovering extracts rich in phenols, given that leaves account for a substantial part of artichoke by-products.

Ultrasonic treatment of artichoke scraps was conducted for 15, 30, 45 and 60 minutes. Figure 1 shows the mean TPC of ultrasonic extracts (UEs) and their corresponding control samples. TPC increased with time in both extracts, but the increase was greater in the ultrasonic samples. In particular, at the end of the experiment (60 min) TPC increased by almost 80% in the ultrasonic samples versus 26% in the control samples. However, TPC of UEs was

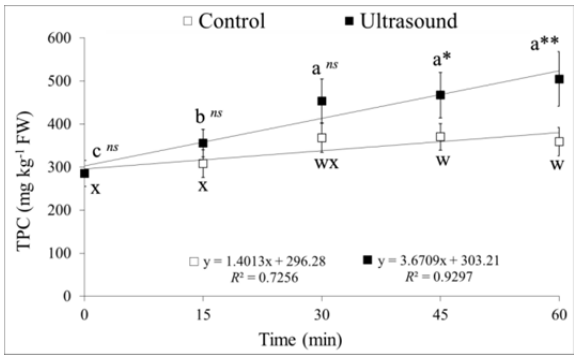

Figure 1: Total polyphenol content (TPC) of ultrasonic extracts and corresponding controls during treatment (means ± SD). Time of extraction: different letters (Ultrasound, a, b, c; Control, w, x) indicate statistical significance by ANOVA ($P < 0.05$). Technology: *ns*, * and ** indicate not significant or significant at $P < 0.05$ and $P < 0.01$, respectively.

significantly higher than controls already after 45 minutes. The increased yield in polyphenols may be ascribed to the thermal and mechanical effects of ultrasounds [21,22].

As expected, the AA increased both in ultrasound and control extracts with time (Figure 2) and showed a trend similar to that of TPC. This result confirms that phenolic compounds contribute heavily to the antioxidant properties of plant matrices [23,24]. UEs exhibited higher AA levels than did the corresponding controls, and the difference increased with time. In particular, at the end of the treatment, ABTS showed a value that was about five-fold higher than DPPH. This finding suggests that the ABTS assay reflects the antioxidant content of artichoke aqueous extracts better than the DPPH assay, as has already been indicated for other plant matrices [25].

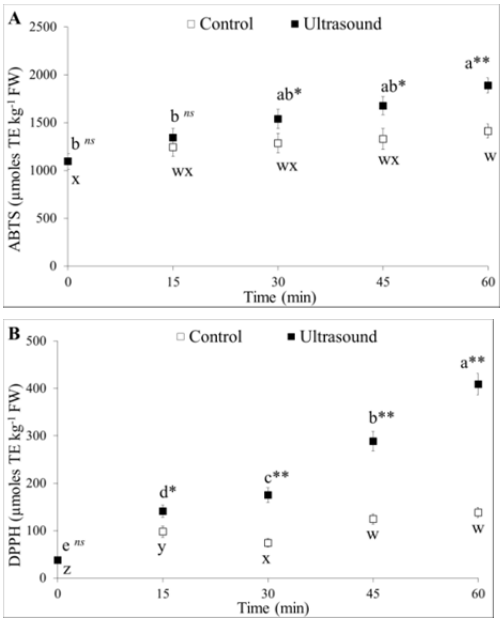

Figure 2: Antioxidant activity of ultrasonic extracts and corresponding controls during treatment assessed by ABTS (A) and DPPH (B) assays (means ± SD). Time of extraction: different letters (Ultrasound, a, b, c, d, e; Control, w, x, y, z) indicate statistical significance by ANOVA ($P < 0.05$). Technology: *ns*, * and ** indicate not significant or significant at $P < 0.05$ and $P < 0.01$, respectively.

The extracts were subjected to HPLC analysis to identify the phenolic compounds. The analysis was conducted only on extracts obtained at the beginning of the treatment (C0, control) and after 60 minutes (with and without ultrasounds, U60 and C60). Four compounds were tentatively identified by comparing the retention

Table 2: Phenolic composition of artichoke by-product extracts detected by HPLC at 280 nm (mg kg^{-1} FW; means ± SD).

Compounds	C0	C60	U60
5-*O*-Caffeoylquinic acid	48.5 ± 3.2^c	67.8 ± 3.5^b	74.2 ± 4.1^a
1,5-di-*O*-Caffeoylquinic acid	19.3 ± 0.8^b	22.0 ± 1.2^a	23.2 ± 1.9^a
Apigenin 7-*O*-glucoside	0.2 ± 0.01^c	0.4 ± 0.02^b	0.5 ± 0.04^a
Luteolin	1.9 ± 0.1^c	4.3 ± 0.3^b	5.7 ± 0.4^a
Unidentified phenols*	129.6 ± 10.0^c	156.0 ± 13.5^b	222.8 ± 20.1^a
Total	175.0 ± 15.0^c	218.1 ± 20.0^b	295.7 ± 26.8^a

C0, control at beginning; C60, control after 60 min; U60, ultrasound after 60 min. *Quantification was performed using 1,5-di-*O*-caffeoylquinic acid calibration curve.
In the rows, data followed by different letters indicate statistically significant differences at $P < 0.05$.

times with those of reference standards, and analyzing their UV spectra and the data available in the literature [26,27]. In particular, two caffeoylquinic acids (5-*O*-caffeoylquinic acid and 1,5-di-*O*-caffeoylquinic acid) and two flavonoids (apigenin 7-*O*-glucoside and luteolin) were detected. 5-*O*-Caffeoylquinic and 1,5-di-*O*-caffeoylquinic acids exhibited the highest levels (Table 2), which is noteworthy since these caffeoylquinic acids have an antioxidant activity [28] and are well absorbed after ingestion [29]. After 60 minutes, the extracts exhibited a higher content of total phenols than did the C0 (25% for C60 and 70% for U60, respectively), and at the end of the experiment the ultrasound treatment led to a significant increase compared with the untreated samples (about 35%). The results highlighted that the ultrasound treatment significantly increased the amount of phenolic compounds but did not influence their relative composition, indicating that this technique does not exert a selective extraction of phenols.

In order to test the ability of living cells to absorb artichoke by-product extracts, *in vivo* experiments were conducted on tobacco BY-2 cells. Figure 3A shows that tobacco BY-2 cells treated with 2 mL of artichoke ultrasonic extract collected after 60 minutes (U60) significantly increased their endogenous phenolic content. Moreover, cells treated with ultrasonic extract (U60) and their relative control (C60) showed a greater total antioxidant content than the untreated (NT) cells. However, the increase in antioxidants in the U60 cells was higher than that observed in the C60 cells (Figure 3B).

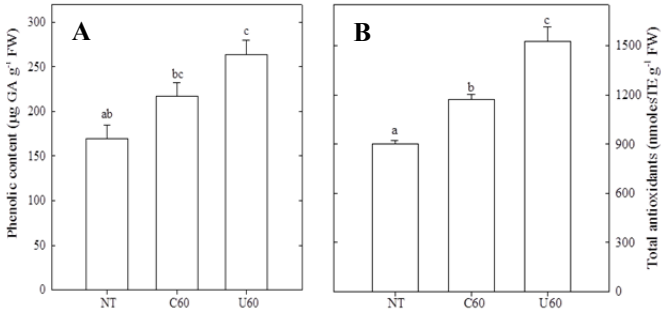

Figure 3: Phenolic content (A) and total antioxidants (B) of untreated tobacco BY-2 cells (NT) and cells treated for 24 hours with ultrasonic artichoke extract (U60) and control artichoke extract (C60), collected after 60 min. Values are means ± SE. Different letters indicate statistical significance by ANOVA ($P < 0.05$).

In order to verify whether artichoke extracts were able to protect BY-2 cells from severe heat stress, NT, C60 and U60 cells were subjected to heat shock (HS) at 55°C for 10 minutes and cell death was analyzed after 24 hours. Figure 4 shows that HS caused the death of about 40% NT cells, as previously reported [30]. Interestingly, rescue of cell viability in U60 treated cells was 25% higher than in C60 treated cells. This finding indicates that the increase in cellular antioxidants due to treatment with U60 confers BY-2 cells with a good defence against severe abiotic stress (HS).

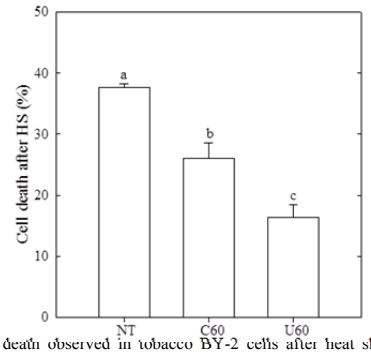

Figure 4: Cell death observed in tobacco BY-2 cells after heat shock (HS) at 55°C. NT, untreated cells; U60 and C60, cells treated for 24 hours respectively with ultrasonic artichoke extract and control artichoke extract, collected after 60 min. Values are means ± SE. Different letters indicate statistical significance by ANOVA ($P < 0.05$).

The results obtained in this study showed that application of ultrasounds for 60 minutes yielded aqueous extracts that were richest in TPC and with the highest AA. When compared with controls, these extracts were better able to increase the total antioxidants present in cells and protect the cells from death after severe heat stress. In conclusion, UAE applied to artichoke by-products using water as a solvent appears to be a powerful technique able to provide extracts rich in phenols with antioxidant activity. They could be utilized in the food industry in order to produce functional food at a low environmental impact, thus turning a waste product into a resource.

Experimental

Chemicals and reagents: 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), methanol, gallic acid and trypan blue were obtained from Sigma-Aldrich Co. (St. Louis, USA), Folin–Ciocalteu reagent from Carlo Erba Reagent (Milano, Italy), potassium persulfate from AppliChem GmbH (Darmstandt, Germany) and sodium carbonate from J. T. Baker (Deventer, Holland).

Plant material: On April 2013, artichokes of the Catanese cultivar were harvested from a field located in Mola di Bari (Apulia region, southern Italy). Fifteen artichokes were picked when they reached commercial maturity and subjected to extraction of the phenolic fraction, using methanol as a solvent. About 100 kg of scraps (outer bracts, leaves and stems) were taken from the same artichoke field, and subjected to extraction of phenols using UAE technology and water as a solvent.

Methanol extraction from different parts of artichokes: Hearts, leaves, outer bracts and stems of 5 artichokes were separately cut into pieces of about 1 cm and then minced using a Waring Blender (Waring Commercial, Torrington, USA). One g of each part was weighed in a flask with a screw cap, macerated in 10 mL of methanol for 60 min, stirred at room temperature in the dark and shielded from the air. The extracts were then separated from the solid residues using a Büchner funnel with a filter paper disk, centrifuged at 9.000 *g* for 10 min at room temperature and filtered on 0.22 μm regenerate cellulose (RC) membranes before analysis. The experiment was carried out in triplicates.

Ultrasound-assisted extraction (UAE) from artichoke by-products: Artichoke by-products were shredded in a Bio-Shredder R130B/E (Negri Garden Equipment, Mantova, Italy). Eight kg aliquots of shredded scraps and 24 L of water were put into an ultrasonic pilot plant (Weal, Milano, Italy) consisting of a 35-L reactor, Sonic Digital LC 1000 SD 25-P ultrasonic generator (25 kHz frequency, 1200 W power output), Sonopush Mono SPM 1200 titanium transducer, centrifugal electrical-pump with open impeller and electrical control panel. Effective power was about 50 W/L water. In order to monitor phenol extraction, 30 mL of extracts were taken during treatment at 0, 15, 30, 45 and 60 min (via a ball cock). The extracts were then centrifuged at 9.000 *g* for 10 min and filtered on 0.22 μm RC membranes. A trial without ultrasound treatment was performed as a control. Experiments were carried out in triplicates.

Total phenol content (TPC): The TPC of methanolic and aqueous (ultrasonic and control) extracts was determined spectrophotometrically using the Folin-Ciocalteu (FC) reagent according to the procedure reported by Gambacorta *et al.* [31]. Results were expressed as gallic acid equivalents (mg of gallic acid kg^{-1} of fresh weight).

Free radical-scavenging activity by ABTS and DPPH: Antioxidant activity was assessed by ABTS and DPPH assays performed according to Li *et al*. [32]. Trolox standard solutions were prepared at a concentration ranging from 20 to 1000 μM. Results were expressed as μmoles of Trolox Equivalent kg^{-1} of fresh weight.

Phenolic profile of artichoke by-product extracts: The extracts were preliminarily freeze-dried, rinsed with 3 mL of 70% ethanol and filtered on 0.22-μm RC membranes. Analysis was performed by high performance liquid chromatography (HPLC) using a Waters 600 E apparatus (Waters, PA, USA) monitored with a photodiode array detector at 280, 310, and 350 nm [27]. Quantification was performed by an external standard method using 6 point (10 to 1000 ppm) standard calibration curves of 5-*O*-caffeoylquinic acid, 1,5-di-*O*-caffeoylquinic acid, luteolin and apigenin 7-*O*-glucoside, with R^2 values ranging between 0.9995 and 0.9999. Results were expressed as mg kg^{-1} of fresh weight.

Analysis of tobacco BY-2 cells treated with artichoke by-product extracts: *Nicotiana tabacum* L. cv. Bright Yellow 2 (BY-2) cell suspensions were routinely propagated and cultured according to Nagata *et al*. [33]. On the third day of culture the BY-2 cells were treated for 24 h with either 2 mL artichoke ultrasonic extract or the relative control collected after 60 min extraction (U60 and C60, respectively). A sample of untreated (NT) cells was used as reference. The BY-2 cells were then subjected to heat shock at 55°C for 24 h [30], filtered, ground in liquid nitrogen and homogenized at 4°C with acidified methanol (80% methanol-7% acetic acid). Homogenates were centrifuged at 20,000 *g* for 15 min. Supernatants were used to evaluate total antioxidant power (ABTS method) and phenolic content, as described above. Cell death was measured by Trypan Blue staining, as described in a previous study [34].

Statistical analysis: All experiments and determinations were performed in triplicate and results were expressed as means ± SD (standard deviation) or ± SE (standard error). Statistical analysis was carried out using IBM SPSS software v 19. Significant differences were determined using one-way ANOVA with contrast analysis and Student's *t*-test.

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References

[1] Ceccarelli N, Curadi M, Picciarelli P, Martelloni L, Sbrana C, Giovannetti M. (**2010**) Globe artichoke as a functional food. *Mediterranean Journal of Nutrition and Metabolism*, ***3***, 197-201.

[2] Saenz Rodriguez T, Garcia Gimenez D, De La Puerta Vazquez R. (**2002**) Choleretic activity and biliary elimination of lipids and bile acids induced by an artichoke leaf extract in rats. *Phytomedicine*, ***9***, 687-693.

[3] Gebhardt R. (**2001**) Anticholestatic activity of flavonoids from artichoke and their metabolites. *Medical Science Monitor*, ***7***, 316-320.

[4] Schütz K, Kammerer D, Carle R, Schieber A. (**2004**) Identification and quantification of caffeoylquinic acids and flavonoids from artichoke (*Cynara scolymus* L.) heads, juice, and pomace by HPLC-DAD-ESI/MS^n. *Journal of Agricultural and Food Chemistry*, ***52***, 4090-4096.

[5] Bonasia A, Conversa G, Lazzizera C, Gambacorta G, Elia A. (**2010**) Morphological and qualitative characterisation of globe artichoke head from new seed-propagated cultivars. *Journal of the Science of Food and Agriculture*, ***90***, 2689–2693.

[6] Llorach R, Espín JC, Tomás-Barnerán FA, Ferreres F. (**2002**) Artichoke (*Cynara scolymus* L.) byproducts as a potential source of health-promoting antioxidant phenolics. *Journal of Agricultural and Food Chemistry*, ***50***, 3458-3464.

[7] Larrosa M, Llorach R, Espín JC, Tomás-Barberán FA. (**2002**) Increase of antioxidant activity of tomato juice upon functionalisation with vegetable byproduct extracts. *Lebensmittel-Wissenschaft and Technologie*, ***35***, 532-542.

[8] Albishi T, Jhon JA, Al-Khalifa AS, Shahidi F. (**2013**) Phenolic content and antioxidant activities of selected potato varieties and their processing by-products. *Journal of Functional Foods*, ***5***, 590-600.

[9] Vinatoru M. (**2001**) An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrasonics Sonochemistry*, ***8***, 303-313.

[10] Chemat F, Zill-e-Huma, Khan MK. (**2011**) Applications of ultrasound in food technology: Processing, preservation and extraction. *Ultrasonics Sonochemistry*, ***18***, 813-835.

[11] Clodoveo ML, Durante V, La Notte D, Punzi R, Gambacorta G. (**2013**) Ultrasound-assisted extraction of virgin olive oil to improve the process efficiency. *European Journal of Lipid Science and Technology*, ***115***, 1062-1069.

[12] Caldeira I, Pereira R, Climaco MC, Belchior AP, Bruno de Sousa R. (**2004**) Improved method for extraction of aroma compounds in aged brandies and aqueous alcoholic wood extracts using ultrasound. *Analytica Chimica Acta*, ***513***, 125-134.

[13] Virot M, Tomao V, Le Bourvellec C, Renard CM, Chemat F. (**2010**) Towards the industrial production of antioxidants from food processing by-products with ultrasound-assisted extraction. *Ultrasonics Sonochemistry*, ***17***, 1066-1074.

[14] Chen F, Sun Y, Zhao G, Liao X, Hu X, Wu J, Wang Z. (**2007**) Optimization of ultrasound-assisted extraction of anthocyanins in red raspberries and identification of anthocyanins in extract using high-performance liquid chromatography-mass spectrometry. *Ultrasonics Sonochemistry*, ***14***, 767–778.

[15] Coletta A, Trani A, Faccia M, Punzi R, Dipalmo T, Crupi P, Antonacci D, Gambacorta G. (**2013**) Influence of viticultural practices and winemaking technologies on phenolic composition and sensory characteristics of Negroamaro red wines. *International Journal of Food Science and Technology*, ***48***, 2215-2227.

[16] Alexandru L, Cravotto G, Giordana L, Binello A, Chemat F. (**2013**) Ultrasound-assisted extraction of clove buds using batch -and flow- reactors: A comparative study on a pilot scale. *Innovative Food Science and Emerging Technologies*, ***20***, 167-172.

[17] Yang Y-C, Wei M-C, Chiu H-F, Huang, T-C. (**2013**) Development and validation of a modified ultrasound-assisted extraction method and HPLC method for the quantitative determination of two triterpenic acids in *Hedyotis diffusa*. *Natural Product Communications*, ***8***, 1683-1686.

[18] Khan MK, Abert-Vian M, Fabiano-Tixier AS, Dangles O, Chemat F. (**2010**) Ultrasound assisted extraction of polyphenols (flavanone glycosides) from orange (*Citrus sinensis* L.) peel. *Food Chemistry*, ***119***, 851-858.

[19] Romani A, Pinelli P, Cantini C, Cimato C, Heimler D. (**2006**) Characterization of Violetto di Toscana, a typical Italian variety of artichoke (*Cynara scolymus* L.). *Food Chemistry*, ***95***, 221-225.

[20] Fratianni F, Tucci M, De Palma M, Pepe R, Nazzaro F. (**2007**) Polyphenolic composition in different parts of some cultivars of globe artichoke (*Cynara cardunculus* L. var. *scolymus* (L.) Fiori). *Food Chemistry*, ***104***, 1282–1286.

[21] Kramer JF. (**1984**) Ultrasound: Evaluation of its mechanical and thermal effects. *Archives of Physical Medicine and Rehabilitation*, ***65***, 223-227.

[22] Suslick KS, Mdleleni MM, Ries JT. (**1997**) Chemistry induced by hydrodynamic cavitation. *Journal of the American Chemical Society*, ***119***, 9303-9304.

[23] Gambacorta G, Faccia M, Previtali MA, Pati S, La Notte E, Baiano A. (**2010**) Effects of olive maturation and stoning on quality indices and antioxidant content of extra virgin (cv. Coratina) during storage. *Journal of Food Science*, ***75***, C229-C235.

[24] Deng G-F, Lin X, Xu X-R, Gao L-L, Xie J-F, Li H-B. (**2013**) Antioxidant capacities and total phenolic contents of 56 vegetables. *Journal of Functional Foods*, ***5***, 260-266.

[25] Floegel A, Kim D-O, Chung S-J, Koo SI, Chun OK. (**2011**) Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. *Journal of Food Composition and Analysis*, ***24***, 1043-1048.

[26] Sànchez-Rabaneda F, Jàuregui O, Lamuela-Raventòs RM, Bastida J, Viladomat F, Codina C. (**2003**) Identification of phenolic compounds in artichoke waste by high-performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, ***1008***, 57-72.

[27] Negro D, Montesano V, Grieco S, Crupi P, Sarli G, De Lisi A, Sonnante G. (**2012**) Polyphenol compounds in artichoke plant tissues and varieties. *Journal of Food Science*, ***77***, 244-252.

[28] Heilmann J, Merfort I, Weiss M. (**1995**) Radical scavenger activity of different 3,4-dihydroxyflavonols and 1,5-dicaffeoylquinic acid studied by inhibition of chemiluminescence. *Planta Medica*, ***61***, 435-438.

[29] Azzini E, Bugianesi R, Romano F, Di Venere D, Miccadei S, Durazzo A, Foddai MS, Catasta G, Linsalata V, Maiani G. (**2007**) Absorption and metabolism of bioactive molecules after oral consumption of cooked edible heads of *Cynara scolymus* L. (cultivar Violetto di Provenza) in human subjects: a pilot study. *British Journal of Nutrition*, ***97***, 963-969.

[30] Locato V, Gadaleta C, De Gara L, de Pinto MC. (**2008**) Production of reactive species and modulation of antioxidant network in response to heat shock: a critical balance for cell fate. *Plant Cell and Environment*, ***31***, 1606-1619.

[31] Gambacorta G, Faccia M, Trani A, Lamacchia C, Gomes T. (**2012**) Phenolic composition and antioxidant activity of Southern Italian monovarietal virgin olive oils. *European Journal of Lipid Science and Technology*, ***114***, 958-967.

[32] Li H, Wang X, Li Y, Li P, Wang H. (**2009**) Polyphenolic compounds and antioxidant properties of selected China wines. *Food Chemistry*, ***112***, 454-460.

[33] Nagata T, Nemoto Y, Hasezawa S. (**1992**) Tobacco BY-2 cell line as the "HeLa" cell biology of higher plants. *International Review of Cytology*, ***132***, 1-30.

[34] de Pinto MC, Francis D, De Gara L. (**1999**) The redox state of the ascorbate-dehydroascorbate pair as specific sensor of cell division in tobacco BY-2 cells. *Protoplasma*, ***209***, 90-97.