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ORIGINAL PAPER

# Functional Teas from the Leaves of *Arbutus unedo*: Phenolic Content, Antioxidant Activity, and Detection of Efficient Radical Scavengers

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**Abstract** The phenolic content/composition and antioxidant activity of hot/cold infusion and decoction from the leaves of *Arbutus unedo* were studied for the first time. 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS$^{\bullet+}$), crocin-bleaching, copper-reducing, and liposome accelerated oxidation assays were used for the evaluation of the activity *in vitro*. *In vivo*, the extracts were examined for their ability to protect *S. cerevisiae* cells from $H_2O_2$ induced oxidative stress. An on-line high-performance liquid chromatography-DPPH$^{\bullet}$ assay was applied to identify potent radical scavengers and comment on their contribution to the total activity. The addition of leaves to boiling water (decoction) was the most appropriate practice to apply since the highest phenol intake (220.2 mg gallic acid/cup served) was obtained. Additionally, its antioxidant activity was equal or superior to that of the other extracts. Flavonols (~51–61 mg/g dry extract) were the main phenols in all the extracts, with quercitrin accounting for ~20% of the total phenol amount. The on-line DPPH$^{\bullet}$ method verified the high potency of the decoction and indicated as the most active radical scavengers, two galloylquinic acid derivatives and myricitrin, accounting for ~28–45% and ~11–13% of the total scavenging, respectively. Present data may contribute to the future exploitation of *A. unedo* leaves by the food industry for health-promoting herbal tea preparations and dietary supplements.

**Keywords** *A. unedo* leaves · Decoction · Flavonoids · Antioxidant · On-line DPPH$^{\bullet}$ · *Saccharomyces cerevisiae*

## Introduction

*Arbutus unedo* (*Ericaceae* family), is an evergreen shrub widespread in the Mediterranean basin [1]. It is treasured for its fruits which are used to obtain alcoholic drinks, jams, jellies, and marmalades, whereas its flowers can serve as a source for the production of a high added value honey [1, 2]. The leaves are yet to be exploited despite the fact that a wide range of phenolic antioxidants has been identified, various extracts have been reported to present a range of biological properties *in vitro*, whereas their infusion has been used in the folklore medicine to counteract various diseases [2].

The published data on *A. unedo* leaf extracts may be promising for the food industry; even so, such information seems not adequate to promote the use of leaves as a dietary source of health promoting antioxidants. This is probably due to i) the examination in most cases of extracts prepared with solvents not suitable for human consumption [1, 2]; ii) the use of conditions (solid/solvent ratio, technique of preparation and duration), in the limited studies carried out on aqueous extracts [3–6], that do not resemble domestic ones; iii) the lack of quantitative data on individual constituents and identification of those ones that may contribute the most to the bioactivity of the extracts. These issues need to be addressed in order a plant material to pave its way in the field of dietary sources of bioactive constituents.

Considering the above, the infusion from the leaves of *A. unedo* prepared under domestic conditions was examined

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with regards to its phenolic content/composition and antioxidant activity. The decoction and cold infusion were also tested. The decoction, though recommended for harder plant parts (*e.g.*,roots), was selected since there are contradictory reports on its effect on the recovery of phenols from soft plant parts in comparison to the infusion [7–10]. The cold steeping, requiring a long time, has been shown to provide white tea extracts richer in flavanols with reduced caffeine levels [11, 12]. The preparations were characterized for their total or individual phenol content employing photometric, spectroscopic and chromatographic techniques. The antioxidant activity was tested *in vitro* using various assays (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, crocin-bleaching, copper-reducing, liposome accelerated oxidation), and *in vivo* toward the protection of *S. cerevisiae* cells from $H_2O_2$ induced oxidative stress. The yeast was used as a suitable model (proxy) to study various biological processes in humans [13, 14]. The radical scavenging activity of the extracts and the contribution of individual phenolic compounds/groups of phenolics to the total radical scavenging were estimated for the first time using an on-line HPLC-DPPH$^{\bullet}$ assay. The current study is expected to assist future commercialization of the *A. unedo* leaves for herbal tea preparations and dietary supplements that may increase consumer's health benefits.

## Materials and Methods

### Plant Material

Mature leaves from *A. unedo* were sampled from four random trees located in Kioumourtzi estate (Agios Andreas, Kavala, Eastern Macedonia, Greece) on October 2013. Sampling and plant material treatment was performed according to Papoti et al. [15]. The species was identified by Professor Stella Kokkini (Laboratory of Systematic Botany and Phytogeography, School of Biology, Aristotle University of Thessaloniki). Voucher specimens of the collected *Arbutus unedo* plants were deposited in TAU (Herbarium of Thessaloniki Aristotle University). The voucher number is Erkekoglou 20170121–01 (Fig. S1). Saffron red stigmas were donated by Saffron Cooperative of Kozani (Greece).

### Standards, Reagents, and Solvents

A detailed description can be found as supplementary material (Text S1). Quercitrin 98% was isolated with the aid of semi-preparative HPLC chromatography and characterized with spectroscopic techniques (Text S2, Fig. S2, Fig. S3, Table S1).

### Preparation of Leaf Extracts

Dry leaves were grounded in a laboratory mill (Arthur H. Thomas Co., Phil., PA, USA) to pass a 0.4 mm sieve before the extraction. Then hot infusion and decoction were prepared according to Papoti et al. [15] with some modifications. A detailed description is provided as supplementary material (Text S3).

### Phenol Content

Total polar phenol (TPP), total flavonol (TFLAVO) and total flavanol (TFLAVA) contents were determined as described by Nenadis et al. [16] using a leaf extract solution of 15 mg/10 mL. The results were expressed as gallic acid equivalents, GAE (mg GA/g of dry extract or dry leaf), quercetin equivalents, QUEE (mg QUE/g dry extract or leaf) and catechin equivalents, CATE (mg CAT/g dry extract or leaf) respectively. Each value is the mean of triplicate determinations ± standard deviation.

### HPLC-Mass Detection and HPLC-Diode Array-Fluorescence Detection

The HPLC systems and the conditions of analyses employed by Nenadis et al. [16] were used. An aliquot (10 μL) of a 15 mg/10 mL solution was injected. The concentration of those compounds (Fig. S4A, Text S4), tentatively assigned to gallic acid derivatives, was expressed as gallic acid equivalents ($\lambda$ = 270 nm), those that were fluorescent (flavanols) as catechin equivalents ($\lambda$exc = 280 nm/ $\lambda$em = 320 nm), myricitrin using the corresponding standard ($\lambda$ = 350 nm), quercetin glucosides as quercitrin equivalents ($\lambda$ = 350 nm), and kaempferol glucosides as astragalin equivalents ($\lambda$ = 350 nm). Each value is the mean of triplicate determinations ± standard deviation.

### *In Vitro* Antioxidant Activity Assays

The DPPH$^{\bullet}$, ABTS$^{\bullet+}$, crocin bleaching, copper reducing capacity and liposome oxidation assays were applied as described by Nenadis et al. [17]. Different aliquots of the leaf extract solution (15 mg/10 mL) were used in each case. The % radical scavenging activity (%RSA) values or corrected absorbance values (copper reducing) were converted to Trolox equivalents (μmol) using a calibration curve. A regression line (μmol vs μg dry extract or leaf) was then constructed and the slope was used as an index of the extract's antioxidant activity. The slope value given is the mean of triplicate determinations ± standard deviation. In liposomes, two levels of extracts were used (1500 or 3000 mg/L), whereas reference compounds (Trolox and quercetin) were added at 60 μM (final concentration).

### On-Line HPLC-DPPH$^{\bullet}$ Radical Scavenging Assay

On-line DPPH$^{\bullet}$ radical scavenging analysis was performed by a hyphenated HPLC-DPPH$^{\bullet}$ method using a single diode

array detector (Fig. S5, Supplementary material), a reaction coil of Teflon (7 m × 0.25 mm i.d.) and a DPPH$^{\bullet}$ solution $2 \times 10^{-5}$ M (pH 6.0) inserted to the eluents at a flow rate of 0.15 mL/min. Bleaching was recorded at 521 nm. The sum of the total negative area converted to nmol Trolox via a standard curve defined the total extract activity. Each value is the mean of triplicate determinations ± standard deviation. The contribution of each phenol or group of phenols to the total scavenging of the extract was calculated as % of the total negative area. Optimum parameters (coil length, the radical solution concentration, pH value) employed for the analysis were found with the aid of Taguchi experimental design/analysis using a set of phenolic compounds presenting different reaction kinetics with the radical. The selected conditions were finally adjusted to the available equipment aiming to maintain a straight baseline, low noise and low peak tailing factor (data not shown).

### *In Vivo* Antioxidant Activity Evaluation

The cellular-based antioxidant activity assay was performed according to Di Paola-Naranjo et al. [18] with modifications. The wild-type yeast strain *S. cerevisiae* BY4741, generously provided by Dr. Antonios Makris (Mediterranean Agronomical Institute of Maich, Crete, Greece), was used. Cells were exposed to $H_2O_2$ (5 mM, unless otherwise stated) with or without extracts (18 mg/L) and incubated for 1 h at 28 °C under aerated conditions (working volume/flask volume ratio of 1:5, 160 rpm). Two control plates were used, one with untreated cells (control A) and one with yeast cells treated with the extracts, without exposure to $H_2O_2$ (control B). The plates were incubated at 28 °C for 72 h. The number of colonies observed in the control A was set to 100%. *S. cerevisiae* activity and results were expressed as the survival percentage ($SP_{S.cerevisiae}$) with regard to the control. All assays were carried out in triplicate.

### Statistical Analysis

Statistically significant differences among the mean values were performed by one-way analysis of variance using the multiple Duncan test at $p < 0.05$. The analyses were carried out with SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). Pearson's correlations between phenolic content/composition and total antioxidant activity at $p < 0.05$ were carried with Minitab 16.1.1 (Minitab Inc., Coventry, UK).

## Results and Discussion

### Phenolic Content

The yield of extraction and the phenol content are presented in Table 1. The results are given in terms of dry extract and dry leaf, practices that are followed in the literature [6, 19], as well as in terms of polar phenol content per serving cup (200 mL) [20].

As it is observed, the more drastic the conditions of extraction the higher was the yield of the process and the TPP content of the extracts, despite the fact that these two parameters do not necessary correlate [6]. The values obtained for decoction were equal or up to 1.43-fold higher than those reported in other publications but prepared with different solid mass to the solvent volume ratio (1:125, 1:50, 1:10 *w*/*v*) and boiling duration (30–45 min) [3–6]. Such a difference, however, is difficult to be clearly attributed to the preparation protocol taking into account that both the yield of extraction and TPP content may vary even within samples of the same genotype [6].

Regarding the flavanol levels, the decoction did not differ significantly from the hot infusion in terms of mg/g dry extract. Nevertheless, when the yield of extraction was taken into account and results were expressed as mg/g dry leaf, the

**Table 1** Extraction yield, total polar phenol, total flavanol and total flavonol contents of herbal teas prepared from *A. unedo* leaves

| Herbal preparation | Extraction yield [% *w*/w] | TPP [GAE] | TFLAVA [CATE] | TFLAVO [QUEE] |
|---|---|---|---|---|
| Hot infusion | 44.9 | 173.8 ± 2.2a$^{*}$ | 147.2 ± 3.6a | 11.0 ± 0.4a |
| | | (78.0 ± 1.0a)$^{**}$ | (66.1 ± 1.6a) | (4.9 ± 0.2a) |
| | | [156.0 ± 2.0a]$^{***}$ | [132.1 ± 3.2a] | [9.8 ± 0.4a] |
| Cold infusion | 33.9 | 153.3 ± 6.6b | 110.3 ± 5.4b | 11.8 ± 0.2b |
| | | (52.0 ± 2.2b) | (37.4 ± 1.8b) | (4.0 ± 0.2b) |
| | | [104.0 ± 4.4b] | [74.8 ± 3.6b] | [8.0 ± 0.4b] |
| Decoction | 51.5 | 213.8 ± 3.2c | 153.4 ± 8.0a | 12.6 ± 0.3c |
| | | (110.1 ± 1.7c) | (79.0 ± 4.1c) | (6.5 ± 0.2c) |
| | | [220.2 ± 3.4c] | [158.0 ± 8.2c] | [13.0 ± 0.4c] |

The results for all tested samples are reported as the mean value ± standard deviation ($n = 3$)

Values within the same column bearing different lowercase letters are significantly different ($p < 0.05$)

$^{*}$ mg/ g dry extract; $^{**}$ mg/ g dry leaf; $^{***}$ mg/ serving cup (200 mL); GAE: gallic acid equivalents; CATE: catechin equivalents; QUEE: quercetin equivalents

**Table 2** Antioxidant activity of herbal teas prepared from *A. unedo* leaves

| Herbal preparation | DPPH$^{\bullet}$ μmol Trolox | ABTS$^{\bullet+}$ μmol Trolox | ROO$^{\bullet}$ μmol Trolox | Cu(II) μmol Trolox |
|---|---|---|---|---|
| Hot infusion | 27.2 ± 0.2a$^{*}$ | 189.0 ± 19.0a | 4.8 ± 0.1a.b | 22.1 ± 1.0a |
| | (12.2 ± 0.1a)$^{**}$ | (84.9 ± 8.6a) | (2.1 ± 0.1a,b) | (9.9 ± 0.6a) |
| Cold infusion | 24.9 ± 1.0b | 139.0 ± 19.0b | 4.4 ± 0.6a | 18.2 ± 0.3b |
| | (8.4 ± 0.3b) | (47.1 ± 6.5b) | (2.0 ± 0.2a) | (6.2 ± 0.1b) |
| Decoction | 28.6 ± 0.5c | 193.0 ± 17.0a,c | 5.6 ± 0.9b | 25.1 ± 0.3c |
| | (14.7 ± 0.3c) | (99.4 ± 8.9c) | (2.5 ± 0.4b) | (12.9 ± 0.2c) |

The results for all tested samples are reported as the mean value ± standard deviation ($n = 3$)

Values within the same column bearing different lowercase letters are significantly different ($p < 0.05$)

$^{*}$ per μg dry extract; $^{**}$ per μg dry leaf

decoction outweighed the hot infusion. The cold infusion was inferior to the other two extracts regardless of the result expression. Thus, soaking in water at room temperature for a long time, and particularly more than the 16 h applied by Yang

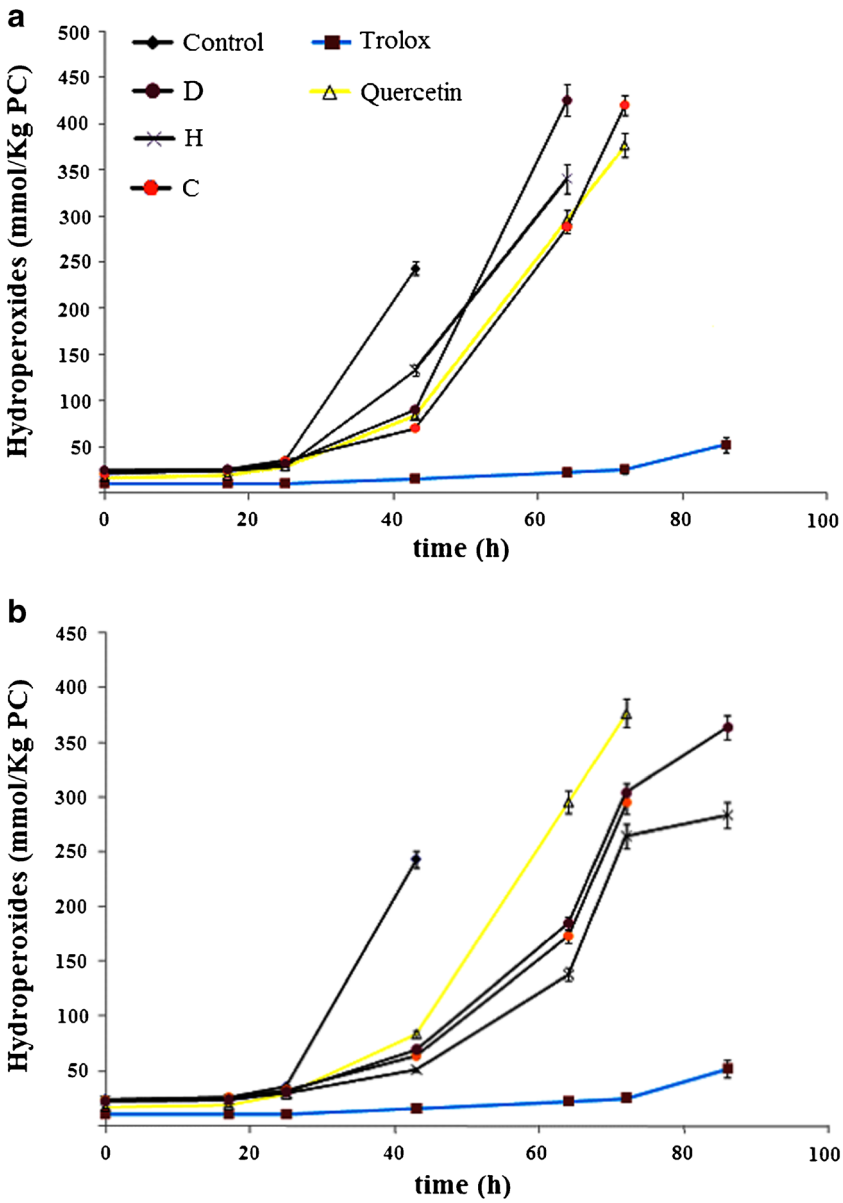


**Fig. 1** Liposome oxidation induced with cupric acetate (3 μM final concentration) at 37 °C in the absence or presence of *A. unedo* leaf herbal preparations (D: Decoction, H: Hot infusion, C: Cold infusion) **a** at 1500 mg/kg, **b** at 3000 mg/kg or Trolox and quercetin (60 μM final concentration). Values of hydroperoxides are means of three measurements ± standard deviation

et al. [11] in white tea leaves, was not proven an efficient practice for the recovery of flavanols from *A. unedo* leaves.

Similar to the above were the observations made for the levels of total flavonols. Therefore, the decoction was the richest extract and the cold infusion the poorest, regardless of the result expression.

Considering the dietary contribution in antioxidants by the extracts, it is clear that the consumption of decoction is more beneficial since the intake in phenols was found ~1.4-fold higher than that of the infusion and ~2-fold than that of cold infusion, respectively. Still, hot infusion from the *A. leaves* provides significant amount of phenols per serving cup (200 mL) compared to the levels reported for infusions from other plant materials, namely rosemary (8.5 mg GAE), sage (34.5 mg GAE), thyme, (58.7 mg GAE), marjoram (98.4 mg GAE), Cretan dittany (83.0 mg GAE), mint (88.3 mg GAE), chamomile (90.8 mg GAE), eucalyptus (103.3 mg GAE), which, however, were prepared using different solid to liquid ratio and time of infusion [20, 21].

## Antioxidant Activity

The extracts were then examined with a set of *in vitro* assays. More specifically tests were carried out to estimate the radical scavenging activity (DPPH$^{\bullet}$, ABTS$^{\bullet+}$, peroxyl) and their ability to reduce Cu (II) ions. Results were expressed in terms of Trolox equivalents on dry extract or dry leaf basis. The respective values are provided in Table 2.

On the basis of the antioxidant activity assays used, the decoction was the most potent. This was clearer when the results were expressed per μg of the dry leaf. Such an observation was in line with the higher total phenol content. The hot infusion followed in activity, whereas the cold one was the least active.

The prepared extracts were also examined towards their activity to retard the Cu (II) oxidation of lecithin liposomes, a proposed model of the cell membrane [17]. The course of oxidation is given in Fig.1a and b.

Despite the fact that polar extracts were added in a polar system in the presence of a transition metal no pro-oxidant activity was observed. When added at 1500 mg/kg, the oxidation was retarded to a similar extent to that by pure quercetin at 60 μM. The addition of a 2-fold higher level improved the protection in comparison to quercetin, but still, the effect was poorer to that of Trolox. Despite the differences in phenol content and radical scavenging activity of the extracts, in the liposomes, the induction periods were rather comparable.

In order to evaluate if the findings *in vitro* could be extrapolated to living systems, the protective effect of the extracts was examined in *S. cerevisiae* BY4741 cells exposed to $H_2O_2$ oxidative stress (Fig. 2a and b).

Considering that different yeast strains may not have the same sensitivity to $H_2O_2$ stress [22], the lethal dose of $H_2O_2$ was first determined (Fig. 2a) at 1 h. Early exponential phase

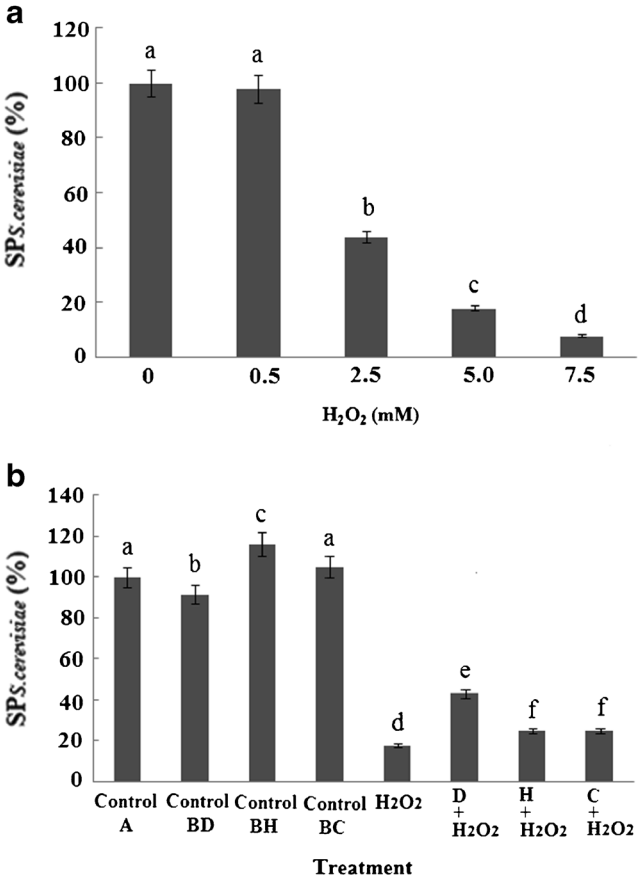


**Fig. 2** Viability of exponentially growing *S. cerevisiae* cells under treatment with different $H_2O_2$ concentrations for 1 h **a** Survival rates of cells treated with *A. unedo* leaf extracts (D: Decoction, H: Hot infusion, C: Cold infusion) and untreated with extracts and/or $H_2O_2$ (**b**). Survival percentage (SP%) with respect to untreated cells. Control A: untreated cells; Control BD: treated cells with decoction without $H_2O_2$; Control BH: treated cells with hot infusion without $H_2O_2$; Control BC: treated cells with cold infusion without $H_2O_2$. Data represent the mean values ± standard deviation of at least three independent experiments. Columns with different lowercase letters differ statistically at $p < 0.05$

cultures used were very sensitive to $H_2O_2$ concentrations greater than 2.5 mM resulting in less than 50% reduction in the survival of the cells under the conditions used in this study. The high sensitivity is due to the generation of the toxic and highly reactive hydroxyl radical, against which the organisms have no defense [23]. Phenols can act in a protective way through radical scavenging and/or through inducing the expression of antioxidant enzymes [23–25]. Thus, in subsequent experiments, an $H_2O_2$ of 5 mM (18% of the untreated cell viability) was chosen to test the extract's antioxidant activity. The lower concentration needed for improving the survival of yeast cells as compared with control was also determined by cell exposure to increased concentrations of extracts (data not shown). The minimum effective, non-cytotoxic final concentration of the extracts was 18 mg/L and as shown (Fig. 2b), all were able to increase the resistant of yeast cells to the oxidant. The decoction was significantly more efficient (a 2.5-fold

increase of survival rate) than the two infusions (~1.5-fold). Taking into account that the hydroxyl radical attacks every organic molecule at the site of formation, the contribution of other kind of co-extracted constituents, besides the various phenolic compounds, in the *in vivo* activity cannot be excluded. Such a study has to be carefully designed in the near future.

## HPLC Analysis and on-Line HPLC-DPPH• Radical Scavenging

The quantitative data for the corresponding peaks and the three groups of phenolics in all the tested extracts are given in Table 3 including the % contribution from the on-line DPPH• scavenging assay. A scavenging profile is illustrated for the decoction in Fig. S4B.

The range in TPP was 107.30 to 139.34 mg/g dry extract. The ranking was in accordance with the one obtained with Folin-Ciocalteu method. Thus, the use of drastic conditions to recover efficiently the phenols from the leaves was verified. In all extracts, flavonols were the major group of phenolics (51.04–60.88 mg/g dry extract), followed by flavanols (32.37–38.70 mg/g dry extract). The positive effect of drastic conditions was more pronounced for gallic acid derivatives. As a consequence, their concentration, which

**Table 3** Elution time, levels of phenolic compounds and % contribution on the radical scavenging efficiency of herbal teas prepared from *A. unedo* leaves based on the *on-line* HPLC-DPPH• technique

| Peak No | Elution time | mg/g dry extract% | | | Contribution in the scavenging of DPPH• | | |
|---|---|---|---|---|---|---|---|
| | (min) | H | C | D | H | C | D |
| 1[C] | 11.5 | 21.58 ± 1.54 | 21.56 ± 1.02 | 22.26 ± 0.02 | 0 | 0 | 0 |
| 2[C] | 13.1 | 2.33 ± 0.01 | 2.29 ± 0.08 | 2.42 ± 0.01 | 2.7 ± 0.1 | 3.9 ± 0.2 | 2.7 ± 0.1 |
| 3[G] | 15.1 | 1.10 ± 0.04 | 1.11 ± 0.01 | 1.16 ± 0.01 | 0 | 0 | 0 |
| 4[C] | 15.6 | 3.88 ± 0.32 | 2.37 ± 0.08 | 4.21 ± 0.01 | | | |
| 5[G] | 16.1 | 4.98 ± 0.15 | 4.91 ± 0.05 | 5.57 ± 0.02 | 28.6 ± 0.6 | 45.5 ± 0.1 | 31.3 ± 0.2 |
| 6[G] | 16.6 | 1.90 ± 0.02 | 4.16 ± 0.07 | 4.66 ± 0.01 | | | |
| 7[G] | 27.0 | 1.27 ± 0.05 | 1.52 ± 0.02 | 1.84 ± 0.01 | 5.4 ± 0.4 | 2.6 ± 0.1 | 2.8 ± 0.1 |
| 8[C] | 33.0 | 8.93 ± 0.67 | 6.15 ± 0.29 | 9.81 ± 0.23 | 1.3 ± 0.1 | 1.0 ± 0.1 | 0.8 ± 0.1 |
| 9[G] | 33.6 | 4.77 ± 0.44 | 4.58 ± 0.17 | 5.09 ± 0.13 | 8.1 ± 0.2 | 9.8 ± 0.6 | 7.3 ± 0.3 |
| 10[G] | 39.2 | 2.21 ± 0.08 | 1.24 ± 0.03 | 3.71 ± 0.08 | 3.3 ± 0.2 | 2.0 ± 0.3 | 3.0 ± 0.2 |
| 11[G] | 41.4 | 1.00 ± 0.15 | 1.24 ± 0.07 | 2.00 ± 0.14 | 2.3 ± 0.1 | 1.6 ± 0.2 | 4.7 ± 0.1 |
| 12[G] | 42.4 | 1.21 ± 0.05 | <LOD | 1.43 ± 0.01 | 1.2 ± 0.1 | - | 1.5 ± 0.1 |
| 13[G] | 43.6 | 4.75 ± 0.30 | 3.25 ± 0.09 | 6.76 ± 0.18 | 7.8 ± 0.2 | 6.9 ± 0.2 | 7.3 ± 0.2 |
| 14[G] | 44.8 | 2.07 ± 0.11 | <LOD | 3.58 ± 0.14 | 4.6 ± 0.2 | - | 5.4 ± 0.1 |
| 15[G] | 46.4 | 2.43 ± 0.14 | 1.92 ± 0.03 | 2.96 ± 0.16 | 1.5 ± 0.1 | 1.0 ± 0.1 | 1.9 ± 0.2 |
| 16[Q] | 48.5 | 2.11 ± 0.08 | 1.93 ± 0.04 | 2.62 ± 0.02 | 2.9 ± 0.2 | 1.0 ± 0.2 | 2.7 ± 0.1 |
| 17[Q] | 49.5 | 4.41 ± 0.17 | 4.09 ± 0.05 | 4.55 ± 0.02 | 3.6 ± 0.2 | 3.8 ± 0.2 | 3.4 ± 0.1 |
| 18[M] | 50.7 | 9.98 ± 0.40 | 8.53 ± 0.18 | 10.00 ± 0.12 | 12.8 ± 0.1 | 12.1 ± 0.2 | 11.4 ± 0.1 |
| 19[Q] | 51.9 | 5.74 ± 0.08 | 4.71 ± 0.02 | 5.87 ± 0.19 | 1.7 ± 0.1 | 1.3 ± 0.1 | 1.6 ± 0.1 |
| 20[Q] | 52.7 | 1.97 ± 0.05 | 1.85 ± 0.05 | 2.07 ± 0.03 | 0.9 ± 0.1 | 0.8 ± 0.1 | 1.0 ± 0.1 |
| 21[Q] | 53.6 | 4.47 ± 0.19 | 3.89 ± 0.16 | 4.78 ± 0.05 | 0 | 0 | 0 |
| 22[Q] | 53.8 | 23.78 ± 1.06 | 22.37 ± 0.94 | 26.36 ± 0.27 | 4.3 ± 0.1 | 4.0 ± 0.1 | 4.5 ± 0.2 |
| 23[A] | 55.5 | 2.31 ± 0.10 | 1.87 ± 0.05 | 2.48 ± 0.03 | 0 | 0 | 0 |
| 24[A] | 56.0 | 2.01 ± 0.09 | 1.80 ± 0.03 | 2.15 ± 0.01 | 0 | 0 | 0 |
| TPP | | 121.19 ± 6.21b | 107.30 ± 3.50c | 139.34 ± 1.92a | | | |
| TFLAVO | | 56.78 ± 2.22b | 51.04 ± 1.52c | 60.88 ± 0.74a | 26.2 ± 0.8 | 23.0 ± 0.9 | 24.6 ± 0.7 |
| TFLAVA | | 36.72 ± 2.54a,b | 32.37 ± 1.47c | 38.70 ± 0.27a | 4.0 ± 0.2 | 4.9 ± 0.3 | 3.5 ± 0.2 |
| Total GA | | 27.69 ± 1.45c | 23.89 ± 0.51d | 39.76 ± 0.91a | 62.8 ± 1.9 | 69.4 ± 1.2 | 65.2 ± 1.3 |

C, G, Q, M, A: indicate that the particular compound was quantified as catechin, gallic acid, quercitrin, myricitrin and astragalin equivalents

H: Hot infusion, C: Cold infusion, D: Decoction

The results are reported as the mean value ± standard deviation ($n = 3$); LOD: Limit of detection

Values within the same row bearing different lowercase letters for total polar phenols (TPP), flavonols (TFLAVO), flavanols (TFLAVA) and gallic acid (GA) derivatives are significantly different at $p < 0.05$

was lower than that of flavanols in infusions, became equal in the decoction.

The examination of the herbal preparations with the on-line HPLC-DPPH$^{\bullet}$ assay was made taking into account the recent observations of McDermott et al. [26]. Evaluation of the total DPPH$^{\bullet}$ expressed as nmol Trolox showed that the decoction ($10.3 \pm 0.02^{A}$ nmol Trolox, $n = 3$) was slightly better than the hot infusion ($9.98 \pm 0.18^{B}$ nmol Trolox, $n = 3$). The cold one was listed third ($7.77 \pm 0.17^{C}$ nmol Trolox, $n = 3$). The respective findings were similar to those obtained using the off-line protocol of DPPH$^{\bullet}$ scavenging when the results were expressed in terms of the same extract basis. Nevertheless, it should be stressed that contrary to the off-line examination, under the on-line evaluation, antagonism/synergism among phenols cannot take place due to the chromatographic separation.

Based on the % values obtained, gallic acid derivatives contributed the most (>60%) to the radical scavenging activity of the extract despite being 1.5–2.1-fold lower in concentration than flavonols. Half or more (45% in cold infusion) of this percentage is attributed to two galloylquinic acid derivatives. Less was the contribution of flavonols (23.0–24.6%) and almost negligible was that of catechin and related compounds (3.5–4.9%). A 1.1 ~ 7% of the scavenging efficiency was attributed to constituents (denoted * and *, Fig. S4) that could not be assigned to any of these three groups (data not shown). Within flavonols, most significant was the contribution of myricitrin with a pyrogallol moiety (11.4–12.8%). Quercitrin accounting for ~19–21% of TPP contributed only 4–4.5%. The low activity for quercitrin is in agreement with Exarchou et al. [27] who related such a finding to its slow kinetics with DPPH$^{\bullet}$. Even so, a low activity is expected considering that glycosylation of flavonols at C-3 position decrease the antioxidant efficiency. This was verified by examination on the same molar basis of quercetin (2.57 Trolox equivalents) and rutin (0.65 Trolox equivalents) (see details in Text S5).

Bearing in mind that in the other assays or the off-line DPPH$^{\bullet}$ evaluation such an insight was not feasible, correlation analysis between phenolic content/composition and the antioxidant activity values was sought. The correlation was limited and presented variability, indicating the complexity of the interaction of mixtures of phenols with each implicated radical (see Table S2).

## Conclusion

In summary, regardless of the culinary approach adopted, teas from the *A. unedo* leaves rich in phenols with expected antioxidant activity *in vivo* can be obtained. To increase the possible health benefits for the consumer, the decoction is the best practice. The most active radical scavengers (on-line DPPH$^{\bullet}$) in the teas were two galloylquinic acid derivatives and myricitrin, accounting for only 13.9–16.4% (*w*/w) of TPP content but being responsible for 41.4–57.6% of the total radical scavenging. Given that the fruits of the respective species are widely used for food applications, the leaves that are available throughout the year, if exploited for functional tea preparation or dietary supplements, can add to the income of people in rural areas where the particular species is thriving.

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**Compliance with Ethical Standards**

**Conflict of Interest** The authors declare that they have no conflict of Interest.

**Human and Animal Rights and Informed Consent** This article does not contain any studies with human or animal subjects

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