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Study on the antioxidant and antimicrobial activities of *Allium ursinum* L. pressurised-liquid extract

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Allium ursinum L. is widely used as a spice as well as a traditional medicine. The aim of this work was to evaluate the antioxidant and antimicrobial activities (AMAs) of *A. ursinum* extract, obtained by pressurised-liquid extraction. Several reliable procedures such as 2,2-diphenyl-1-picrylhydrazyl, 2,2-azinobis-3ethyl benxothiazoline-6-sulphonic acid, ferric-reducing antioxidant power assay and oxygen radical absorbance capacity assays were carried out. Vegetable oil stability was evaluated by using Rancimat test. Moreover, AMA was performed on different microorganisms. On the basis of the results obtained, it is confirmed that the *A. ursinum* extract could be used as a natural ingredient in food and/or pharmaceutical industries.

Keywords: *A. ursinum*; polyphenols; antioxidant activity; antimicrobial activity; Rancimat test

1. Introduction

Lipid autoxidation is a free radical chain reaction, leading to increase in reactive radicals and hydroxides, which initiate further transmutations (Frankel 1985; Min & Boff 2002). The use of antioxidants for the control of oxidation process is desirable from technological and nutritional points of view. Recently, the research is aimed to isolate and characterise bioactive compounds from natural sources which possess antioxidant ability and are safe for human consumption. The species belonging to the *Allium* family have been used for a long time as a remedy for the prevention and treatment of certain diseases (Bagheri et al. 2011).

Allium ursinum L., which is known as 'wild garlic', 'ramsons', 'bear's garlic', is a wild relative of chives native to Europe. It is widely used as a spice as well as medicinal plant (Janeczko & Sobolewska 1995). In medieval medicine, the leaves of *A. ursinum* were used as a therapy for cardiovascular diseases (Richter 1999). Several biological activities of this plant, such as antioxidative (Wu et al. 2009), cytostatic (Saniewska & Zuradzka 2001) and antimicrobial (Ivanova et al. 2009; Sapunjieva et al. 2012), were reported.

The extraction process is the first crucial step in the preparation of plant formulations. Handa et al. (2008) described the pressurised-liquid extraction (PLE) technique as the conventional method used to recover natural products. PLE is a sustainable and green technique to extract bioactive compounds from food by-products of plant origin using aqueous ethanol or water as food-grade solvents (Wijngaard et al. 2012). This extraction technique is gaining popularity because it requires less time and a lower consumption of organic solvents than conventional techniques (Mendiola et al. 2007). In addition, PLE can be used to extract more polar

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compounds such as polyphenols recognised for many health benefits due to their antioxidant activity (AOA). However, their extracting efficiency depends mainly on temperature, type of solute and extraction medium (Wijngaard et al. 2012).

This work focuses on the characterisation of AOA and antimicrobial activities (AMAs) of an *A. ursinum* extract obtained for the first time by PLE technique in mild conditions in order to apply it in the food and/or the pharmaceutical industries.

2. Results and discussion

2.1 *Total polyphenol content*

The total polyphenol content (TPC; Table 1) of the investigated extract was 60.92 ± 0.32 mg gallic acid equivalent (GAE) 100 g^{-1} fresh weight (FW) of leaves. Sapunjieva et al. (2012) reported a higher concentration such as 145 mg GAE 100 g^{-1} FW using the 96% ethanol as solvent in heat-reflux, probably due to the different extraction technique and the raw material. In this respect, Pârvu, Toiu, et al. (2010) reported qualitative and quantitative differences in the polyphenolic composition of *Allium* species. Moreover, Schmitt et al. (2005) showed that the concentration of volatile precursors of *A. ursinum* depends on the harvesting time.

2.2 *AOA of* A. ursinum *extract*

Recently, polyphenolic content and AOA have been successfully correlated by several studies (Tepe et al. 2006; Mihaylova et al. 2013). However, Štajner, Igić, et al. (2008) and Štajner, Popovic, et al. (2008) showed that the investigated *Allium* species exhibited antioxidant properties in all plant organs, the highest having been observed in the leaves. The AOA of *A. ursinum* pressurised-liquid extract was evaluated with different assays in order to better understand the mechanism of action of an antioxidant (Jimenez-Alvarez et al. 2008). The results of $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ assays were, respectively, 0.25 ± 0.01 and 2.65 ± 0.04 μM Trolox equivalents (TE) g^{-1} FW (Table 1). In comparison, Sapunjieva et al. (2012) reported different $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ values, respectively, 1.07 and 16.34 μM TE g^{-1} FW with 96% ethanol probably due to the different origin of the plant samples and the extraction technique. *A. ursinum* extract showed a ferric-reducing antioxidant power (FRAP) assay value of 2.02 ± 0.01 μM TE g^{-1} FW and a oxygen radical absorbance capacity (ORAC) value of 60.35 ± 0.98 μM TE g^{-1} FW as reported for lovage and chilli pepper (Číž et al. 2010). However, it is very hard to evaluate deeply the AOAs of an extract on the basis of *in vitro* assays only. Taking into account that *A. ursinum* was employed for a long time in the folk medicine without data concerning its toxicity, other *in vivo* assays to assess the possibility of technological application may be more useful.

2.3 *Vegetable oil stability using* A. ursinum *extract and AMA of the extract*

Edible fats and fat containing products undergo oxidation, both during production and storage, causing a sequence of unfavourable changes. The enrichment of lipids with plant polyphenols may improve their oxidative stability and positively influence human health (Kozłowska et al.

Table 1. Total polyphenol content (mg GAE 100 g^{-1} FW) and antioxidant activity (μM TE g^{-1} FW) of *A. ursinum* extract.

Sample/assay	TPC	$TEAC_{DPPH\cdot}$	$TEAC_{ABTS\cdot+}$	FRAP	ORAC
PLE	60.92 ± 0.32	0.25 ± 0.01	2.65 ± 0.04	2.02 ± 0.01	60.35 ± 0.98

Note: The data are expressed as mean ± SD ($n = 3$).

2012). In this respect, many authors studied the stability of vegetable oils using various plant extracts (Ivanova et al. 2009; Proestos et al. 2013) and essential oils (Quiroga et al. 2013). The results on the oxidative stability of corn, peanut, soybean and sunflower oils emulsified with *A. ursinum* PLE reported statistically significant differences (Table 2). The peanut oil emulsion exhibited the highest value of AOA index (AAI, 1.69), suggesting an improved oxidative stability (69.85%). On the contrary, the soybean oil emulsion had the lowest AAI value (1.40), indicating a worse oxidative stability (39.52%). In addition, *A. ursinum* PLE exhibited a better result than red chicory extract tested in a previous study (Lante et al. 2011).

The results of antimicrobial screening given in Table 3 did not show any significant activity of the *A. ursinum* PLE against the tested organisms (TOs). However, higher AMAs were obtained towards *Staphylococcus aureus* (12 and 10 mm inhibition zones) and towards *Aspergillus niger* (6 mm inhibition zones). Other studies showed that the antibacterial and antifungal effects of *Allium* species depend mostly on the pathogenic species and on the type of plant extract (Sobolewska et al. 2006; Ivanova et al. 2009; Pârvu, Pârvu, et al. 2010, Sapunjieva et al. 2012).

3. Experimental

3.1 *Plant material and extraction procedure*

For each experiment, about 100 g of vegetable was randomly sampled from 500 g of *A. ursinum* L. (leaves and flowers) harvested in 2012 in Veneto region (Italy) during the flowering period and stored in a freezer until use. The sample was cut into small pieces and extracted as described by Mihaylova et al. (2014) with 500 mL of ethanol/water solution (85:15, v/v) containing 0.1 M HCl (pH 3.5), previously deoxygenated by flushing with nitrogen (Rossetto et al. 2005). The volume of each sample was measured to calculate the extract concentration ($g\,L^{-1}$), the extraction rate was 0.1754. Then, PLE was split up in dark glass bottles without headspace and stored at -20°C.

3.2 *Determination of TPC*

The TPC was analysed using the Folin–Ciocalteu method of Kujala et al. (2000) with some modifications (Mihaylova et al. 2014). Results were expressed as mg GAE per g FW.

3.3 *DPPH radical-scavenging activity assay*

The DPPH radical-scavenging activity was determined following the method of Brand-Williams et al. (1995) slightly modified by Mihaylova et al. (2014). The results were expressed as TE antioxidant capacity – TEAC value ($\mu M\,TE\,g^{-1}$ FW).

Table 2. Oxidative stability of *A. ursinum* extract oil emulsions measured by Rancimat® test.

Sample	IT (h)	AAI
Corn oil	7.71 ± 0.30a	1.00
Corn oil + extract	11.37 ± 0.21a	1.47
Peanut oil	7.43 ± 0.20b	1.00
Peanut oil + extract	12.62 ± 0.27b	1.69
Soybean oil	5.39 ± 0.32c	1.00
Soybean oil + extract	7.52 ± 0.15c	1.40
Sunflower oil	3.50 ± 0.49d	1.00
Sunflower oil + extract	5.23 ± 0.74d	1.50

Note: a, b, c, d: $P < 0.05$.

Table 3. AMA of *A. ursinum* extract.

Test organism/sample	Inhibition zone (mm)		Concentration of TO, CFU mL^{-1} in the media
	Control	Sample	
Escherichia coli ATCC 25922	–	Bacteria growth	5.0×10^{10}
Klebsiella	–; 1	1; 1	9.6×10^{10}
Salmonella enterica subsp. *enterica* ATCC BAA-2162	–	–	1.0×10^{11}
Pseudomonas aeruginosa ATCC 9027	–	–	1.27×10^{12}
S. aureus ATCC 25093	–	12; 10	5.0×10^{11}
Listeria monocytogenes	–	–	2.5×10^{6}
Bacillus subtilis	–	–	1.0×10^{5}
Saccharomyces cerevisiae	–	–	8.0×10^{4}
A. niger	–	6; 6	1.0×10^{5}
Rhizopus	–	–	1.0×10^{5}

Note: (–) No activity detected. The data are results of two parallel determinations. CFU, colony-forming units.

3.4 *ABTS radical cation decolourisation assay*

The scavenging activity of the extract against radical cation ($ABTS^{\cdot+}$) was estimated according to Re et al. (1999). The results were expressed as TEAC ($\mu M\,TE\,g^{-1}$ FW).

3.5 *FRAP assay*

The FRAP assay was carried out according to the procedure of Benzie and Strani (1996) with some modifications (Mihaylova et al. 2014). The results were expressed as $\mu M\,TE\,g^{-1}$ FW.

3.6 *ORAC assay*

The ORAC assay measures the antioxidant-scavenging function against peroxyl radical induced by (2,2′-azobis(2-amidinopropane) dihydrochloride at 37°C. The loss of fluorescence of fluorescein indicates the extent of damage due to its reaction with the peroxyl radical (Ou et al. 2001). The ORAC values were expressed as $\mu M\,TE\,g^{-1}$ FW.

3.7 *Determination of the oxidative stability*

Oxidative stability is expressed in hours and determined according to AOCS official method Cd 12b (AOCS 1992), using a Rancimat apparatus (Metrohm, model 743, Herisau, Swirzerland). The determination was made at 110°C with a $20\,L\,h^{-1}$ air flow, using 3 g, respectively, of corn, peanut, sunflower and soybean oils with *A. ursinum* PLE dissolved in a water/ethanol emulsions and 60 mL of distilled water in flasks containing electrodes. The volatile decomposition products were detected as reported by Lante et al. (2011).

The induction time (IT) is the time (h) at the break point of the two extrapolated straight parts of the curve obtained using the Rancimat® apparatus. The AAI was calculated by the following equation:

$$\mathrm{AAI} = \frac{\text{IT of oil emulsions with extract}}{\text{IT of oil emulsions without extract}}.$$

3.8 *Determination of AMA*

The AMA of the examined extracts was analysed by the agar-well diffusion method as described by Mihaylova et al. (2014). All the plates were examined for any zones of growth inhibition, and the diameters of the zones were measured in mm.

3.9 *Statistical analysis*

All measurements (except AMA – in duplicate) were carried out in triplicates. The results were expressed as mean ± SD and statistically analysed using MS-Excel software.

Rancimat data were subjected to analysis of variance. The significance of differences was determined using Tukey's multiple-range test ($P < 0.05$) using the CoHort software package (CoHort Software, Monterey, CA, USA).

4. Conclusions

On the basis of the results obtained, *A. ursinum* PLE exerted significant AOA and improved vegetable oil stability. Further studies will focus on optimising the extraction technique and the polyphenol concentration, in order to apply *A. ursinum* extract as a non-toxic ingredient in food and/or pharmaceutical industries.

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