Direct ultrasound-assisted extraction and characterization of phenolic compounds from fresh houseleek (*Sempervivum marmoreum L.*) leaves

Ivana T. Karabegović[1], Saša S. Stojičević[1], Dragan T. Veličković[2], Nada Č. Nikolić[1], Miodrag L. Lazić[1]

[1]*Faculty of Technology, University of Niš, Leskovac, Serbia*
[2]*College of Agriculture and Food Technology, Prokuplje, Serbia*

Abstract

The effects of ultrasound power and frequency on the yield of total extractive substances (*TES*), total phenolic content (*TPC*), total flavonoid content (*TFC*) and antioxidant activity (*AOA*) of fresh houseleek leaves extracts obtained by direct ultrasound-assisted extraction (DUAE) were studied. Preliminary extraction of plant material was performed using methanol, acetone and 2-propanol by Soxhlet extraction. It was found that maximum *TES* yield could be obtained by methanol extraction (2.91±0.02), followed by acetone and 2-propanol with a *TES* yield of 2.32±0.01 and 2.01±0.03 g per 100 g of fresh plant material, respectively. In the fresh houseleek leaves extracts obtained by DUAE and methanol as the chosen solvent, *TPC*, *TFC* and *AOA* were in the ranges of: 40.5–85.9 mg gallic acid/g dry extract, 12.7–19.3 mg rutin/g dry extract and 24.6–108.2µg/ml, respectively. The results showed that the increase in the ultrasound power and extraction time have positive and significant ($p < 0.05$) effects on the *TPC*, *TFC* and *AOA*, while the increase in the ultrasound frequency leads to a decrease in the *TPC*, *TFC* and *AOA* of the extracts. A chromatographic analysis of crude extract identified the following: kaempferol 3-*O*-(6''-*O*-malonylglucoside)-7-*O*-glucosyde, kaempferol 3-*O*-glucoside-7-*O*-rhamnoside, luteolin 5-*O*-(6''-*O*-malonylglucoside), kaempferol 3-*O*-(6''-*O*-acetylglucoside)-7-*O*-rhamnoside, genkwanin 5-*O*-glucoside, luteolin 5-*O*-(6''-*O*-malonylglucoside), kaempferol 3-*O*-(6''-*O*-malonylglucoside), kaempferol 3-*O*-rhamnoside, quercetin, genkwanin 4'-*O*-glucoside and hyperoside.

Keywords: ultrasound, direct ultrasound-assisted extraction, *Sempervivum marmoreum*, phenol, flavonoid, antioxidant, HPLC-DAD.

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Medicinal and aromatic plants represent a large source of biologically active compounds with numerous beneficial properties, including anti-inflammatory, anticancer, antiviral, antibacterial and cardio protective activities [1]. *Sempervivum marmoreum L.* (*Crassulaceae,* the common houseleek) is a species found in the Carpathian-Balkan area [2]. In traditional medicine houseleek leaves have commonly been used for the treatment of earaches, warts, skin burns and ulcers [3–6].

Previous studies on the genus *Sempervivum L.* were mainly related to *S. tectorum* species whose chemical composition and biological activities have already been described [7,8]. The presence of compounds with immunomodulatory properties, hepatoprotective and lipid level lowering effects for the *S. tectorum* extract were well documented [9–11]. In addition, strong and moderate antimicrobial activities against *Staphylococcus aureus*, *Bacillus cereus*, *Geotrichum sp*. and *Enterococcus faecalis* were reported [7]. In our previous study [12] three extraction techniques, including indirect ultrasound assisted extraction (UAE), classical solvent and Soxhlet extraction from fresh *S. marmoreum* leaves were investigated, and it was found that the obtained extracts possessed good antioxidant activity and exhibited antimicrobial activities against yeast such as *Aspergillus niger* and *Candida albicans*, while the range of *TPC* and *TFC* were 0.56–0.73 mg gallic acid and 0.51–0.62 mg rutin per g of fresh plant material, respectively. However, no reports of the identification and quantification of the main constituents in *S. marmoreum* leaves were found.

The extraction procedure is a crucial step in the isolation of valuable bioactive compounds from plant material. Development and improvement of extraction techniques with optimization of the operating conditions is a very important activity in every specific case. In order to overcome the drawbacks of classic extraction techniques (time and energy consuming, possible degradation of the target compound, high costs), non-conventional extraction methods (ultrasound, microwave and enzyme-assisted extraction, subcritical and supercritical fluid extraction) are increasingly being used. UAE is one of the “green” extraction techniques, which is recognized as an efficient technique that increases yield, while significantly reducing extraction

Correspondence: M.L. Lazić, Faculty of Technology, University of Niš, Bulevar oslobođenja 124, 1600 Leskovac, Serbia.
E-mail: lazic@tf.ni.ac.rs
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time and solvent consumption. The main advantages of sonication, *i.e*., the reduction of particle size, cell disruption, greater penetration of the solvent into the plant material and easier release of extractable compounds, as well as the enhancement of mass transfer, are attributed to the acoustic cavitation phenomena [13,14]. With respect to different equipment and scales, UAE could be performed with indirect (an ultrasound cleaning bath) or direct (a horn or probe system, ultrasound reactor) sonication [15], but both UAE techniques satisfy green extraction principles [16,17] and could be easily scaled up [18]. So far, UAE has been widely applied in extraction of various bioactive compounds from plants [19–23], while recent comparative studies of indirect and direct UAE indicate higher efficiency of direct sonication for different target compounds [24–27]. Great potential and advantage of direct UAE (DUAE) for extraction of pharmacologically useful compounds from different plant materials are due to a stronger cavitation effect caused by a much higher specific ultrasound energy than that of indirect UAE [26].

As a continuation of our previous work [12] where fresh *houseleek (Sempervivum marmoreum* L.) leaves were extracted by various extraction techniques (classic, indirect UAE and Soxhlet extraction), the aim of this study was to examine the effects of low/high ultrasound frequency and power during DUAE on the yield of total extractive substances (TES) and phytochemical constituents such as total phenolic content (*TPC*), total flavonoid content (*TFC*) and the antioxidant activity (*AOA*) of houseleek leaves. In addition, HPLC analyses of crude houseleek extracts were performed.

EXPERIMENTAL

Materials

Houseleek leaves were collected at Mt. Ozren (Central Serbia, 43°34'04.3"N 21°50'54.5"E), before the blooming period (end of July 2013). The plant material was packed in paper bags and kept in a dry and dark place until use (no more than five days). Methanol, acetone, 2-propanol, Folin–Ciocalteu (FCR), 2,2-diphenyl-1-picrilhydrazil radical (DPPH), gallic acid and rutin, were of analytical grade and purchased from the Sigma Chemical Company (USA). Aluminium chloride, potassium acetate and sodium carbonate were purchased from Merck-Alkaloid (FYR Macedonia).

Extraction methods

Soxhlet extraction

Fresh houseleek leaves (10 g) were extracted with solvents (100 mL) of different polarity (methanol, acetone and 2-propanol) using the Soxhlet apparatus until the complete exhaustion of the plant material (150 min). The extracts were evaporated by a rotary vacuum evaporator at 40 °C. The obtained yield represents the total extractive substance (*TES*) content in the plant material.

Direct ultrasound-assisted extraction (DUAE)

DUAE was carried out in the ultrasonic laboratory reactor URS 1000 (ELAC Nautik Communications GmbH, Kiel, Germany, internal diameter: 106 mm; height: 200 mm; total volume of the reactor: 1.7 L). An ultrasonic transducer (25 cm^2, frequency range: 40–2500 kHz; power range: up to 250 W) was an integral part of the reactor bottom. The ultrasonic power was monitored on a digital display, while the temperature in the extraction system was measured with a type-K thermocouple digital thermometer (Symmetry, Leskovac, Serbia) and maintained at 25±0.1 °C by thermostated water that circulated through the reactor jacket. Fresh and chopped houseleek leaves (15 g) were submitted to extraction by methanol (150 mL) for 2.5, 5, 10, 20, 40 and 60 min. One series of experiments was performed at the constant ultrasound power of 20 W and three different frequencies (42, 211 and 1038 KHz), while the second series was performed at the constant ultrasound frequency of 211 kHz and three different powers (20, 30 and 75 W). The levels of ultrasound frequencies and powers, as well as their combinations, were chosen during preliminary experiments. Liquid extracts were separated from the solid phase by vacuum filtration and evaporated to dryness on a rotary vacuum evaporator (40 °C).

Determination of total phenolic content

The total phenolic content (*TPC*) was determined using the Folin–Ciocalteu (*FC*) reagent [28]. The measurements were carried out in triplicate and the calculations were done using a gallic acid calibration curve (R^2 = 0.9922). *TPC* was expressed as mg of gallic acid equivalents (GAE) per gram of dry extract.

Determination of total flavonoid content

The total flavonoid content (*TFC*) in the extracts was determined using the aluminium chloride method [29]. Measurements were carried out in triplicate and calculations were done using a rutin calibration curve (R^2 = = 0.9994). Total flavonoid content was expressed as mg of rutin equivalents (RE) per g of dry extract.

Determination of DPPH radical-scavenging activity

The scavenging activity of the houseleek leaves extract against the DPPH free radical was determined according to the method described elsewhere [30]. Capability to scavenge the DPPH free radical (*I* / %) was calculated by the equation:

$$I(\%) = 100\left(1 - \frac{A_s - A_b}{A_c}\right) \quad (1)$$

A_s – absorbance of the sample with DPPH, A_b – absorbance of the sample with added methanol, A_c – absorbance of DPPH solution with added methanol.

Efficient concentration values (EC_{50}) were calculated according to the experimental data [31] by using a nonlinear regression model and SigmaPlot 2000 Software (trial version).

HPLC-DAD identification

The HPLC analyses were performed on an Agilent 1100 Series HPLC system (Agilent Technologies, Germany) according to the Viet *et al.* method [32]. Column: Agilent Eclipse XDB-C18 4.6 mm ID×150 mm (5 µm) 80 Å. Elution profile: *A* = 0.15%, phosphoric acid in H_2O:MeOH volume ratio 77:23 (pH 2); B = MeOH. Isocratic: 0–3.6 min 100% A; gradient: 3.6 min 100% A; linear–24.0 min; 80.5% A isocratic–30 min linear–60 min; 51.8% A-linear–67.2 min; 100% B; Flow rate: 1 mL/min. The dosing volume of crude or hydrolised extract was 20 µL (5 mg/mL). Spectrophotometric detection in the UV region at 350 nm was used. The peak identity was checked by comparison of their relative retention indices with previous ones and that of reference compounds.

The flavonoid component in the sample extracts was identified by comparing houseleek extract retention time spectra with the retention times of standard spectra. Solutions of all the standards were prepared in methanol (1.0 mg/ml) and diluted to a series of concentrations ranging from 5 to 200 µg/ml. On the basis of the obtained peak areas and the concentration of the standard solution, calibration curves were constructed and concentrations of the components in the samples determined.

Statistical analyses

All measurements were performed in triplicate, and the results were expressed as the mean ± standard deviation. Tukey's test was used for multiple statistical comparisons (WINKS SDA software package, TexaSoft, 6th ed., Cedar Hill, Texas, 2007 Trial version) and values were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Solvent selection

Selection of the best solvent for extraction of TES from the plant material is the most important step in any method of extraction. Since the high water content in fresh houseleek leaves (88.1%) significantly affects the polarity of the extraction system, as well as due to the fact that the amount of water in water/organic solvent mixtures had a higher impact on the extraction efficacy than the solvent itself [33], it was important to test more solvents of different polarities and properties. In order to select the best solvent for further experiments, the plant material was extracted by the Soxhlet apparatus using three solvents of different characteristics, methanol, acetone and 2-propanol, and the obtained yields of TES were 2.91±0.02, 2.32±0.01 and 2.01±0.03 g per 100 g of fresh plant material, respectively.

The results published by other authors [33,34] also suggest that higher polarity solvents are a better choice for extraction of plant bioactive compounds than lower polarity solvents. Addition of water to organic solvents could increase the polarity of the solvent system, which according to the available reports [35] played an important role in UAE efficiency, while Barbero *et al*. [36] pointed out that addition of water to methanol does not increase the extraction yield of capsaicinoids from peppers obtained by USE.

Effect of ultrasound power and frequency on the total extractive substances yield

Changes in TES yield from fresh houseleek leaves during DUAE are shown in Table 1.

Considering that after approximately 20 min more than 90% of TES was extracted, and taking into account the technical and economic feasibility of the process, 20 min was chosen as the optimal time for DUAE of fresh houseleek leaves.

Table 1. Total extractive substances (TES) from fresh houseleek leaves during DUAE; data were expressed as the mean of three replicates ± standard deviation; methanol, 1:10 g/mL, 25 °C and 20 min

Power, W	Frequency, kHz	Time, min					
		2.5	5	10	20	40	60
20	42	1.19±0.02	1.57±0.01	2.08±0.06	2.37±0.04	2.48±0.03	2.53±0.04
	211	1.19±0.02	1.60±0.03	2.07±0.02	2.43±0.01	2.47±0.02	2.57±0.01
	1038	1.17±0.01	1.56±0.01	2.06±0.02	2.35±0.01	2.44±0.01	2.51±0.02
20	211	1.19±0.02	1.59±0.07	2.06±0.02	2.43±0.01	2.47±0.01	2.57±0.08
30		1.23±0.01	1.65±0.02	2.12±0.01	2.48±0.06	2.53±0.03	2.59±0.07
75		1.34±0.04	1.71±0.01	2.32±0.01	2.56±0.03	2.73±0.04	2.79±0.09

By increasing the ultrasound power from 20 to 30 and 75 W at the constant frequency (211 kHz), the TES yield increased by 2.6 and 5.8%, respectively. Statistically significant differences ($p < 0.05$) between yields of TES obtained at different ultrasound powers were observed.

Increasing the ultrasound power increased the number of created and collapsed micro cavitation bubbles which are responsible for physical, mechanical, chemical and thermal effects of the ultrasound. Implosion of cavitation bubbles on the surface of plant material generates strong liquid microjets which could improve solvent penetration into the plant material and enhance the diffusion process, resulting in an increase in the TES yield [13,15]. The enhancement of extraction efficiency with an increase in the ultrasound power was also reported for UAE of fresh rosemary [37] and boldo leaves [38].

By increasing the ultrasound frequencies from 42 to 1038 kHz at the constant ultrasound power (20 W), the TES yield increased slightly (2.5%). It was determined that there were no statistically significant differences in the yields of TES obtained at frequencies of 42 and 211 kHz. It has been reported previously by Wang *et al.* [39] that extraction yield did not increase significantly with the increase in ultrasound frequency. However, effects of ultrasound frequency largely depend on the nature of the plant matrix. This is the reason that some authors achieved the best results at moderate ultrasound frequencies, while others give priority to lower ultrasound frequencies [40].

In our previous experiment, after 40 min at 25 °C, the TES yields from fresh houseleek leaves were 2.3 and 2.4 g/100 g of fresh plant material for the classical (silence) extraction and indirect UAE technique, respectively [12]. When compared to the DUAE, it can be concluded that under the same operating conditions (plant material, plant material to solvent ratio, temperature, solvent) and independently of the applied ultrasonic power and frequency, the same yield of TES can be obtained in half the time. These findings could be attributed to a higher ultrasound power per gram of the extraction suspension introduced to the extraction system during the DUAE. Similar results were also observed for the DUAE of bioactive compounds from *Hypericum perforatum* [24], silymarin from *Silybum marianum* seeds [25], ginseng saponins from ginseng roots and cultured ginseng cells [26], and oil from crushed dill seeds [27].

Effects of ultrasound power and frequency on the total content of phenolic and flavonoid compounds

The TPC and TFC of houseleek extracts obtained under different process conditions by DUAE are shown in Table 2.

Generally, increasing the ultrasound power at the constant frequency of 211 kHz positively affects the content of bioactive compounds in houseleek extracts. Namely, by increasing the ultrasound power from 20W to 30 W, the contents of total phenolic (11.5%) and flavonoid (5.0%) compounds were increased, while further increase in the ultrasound power up to 75 W, increases the contents of total phenolic (31.7%) and flavonoid (7.8%) compounds compared to the contents obtained at 20 W. Statistically significant differences in the TPC obtained by extraction at different ultrasound powers were observed at the 5% probability level ($p < 0.05$).

Similar results have been obtained for UAE of phenolic compounds from *Cassia auriculata* [41], curry [42], and fresh tulsi leaves [43].

This result is probably due to the improved mechanical effects of the ultrasound, such as the better solvent penetration into the plant material cells and easier release of target compounds into the solution. However, if the power continues to increase, a degeneration of polyphenolic compounds caused by extensive local temperature and pressure generation in the moment of cavitation bubble collapse can occur [15].

Table 2. Total phenolic, flavonoid compound content and antioxidant activity of houseleek extracts obtained by direct, indirect ultrasound assisted, classical and Soxhlet extraction; data were expressed as the mean of three replicates ± standard deviation

Extraction technique			Total phenolic content mg (GAE/g dry extract)	Total flavonoids mg RE/g dry extract	EC_{50} µg/ml DPPH	Reference
DUAE[b]	20 W	42 kHz	84.6±2.99	18.8±0.31	26.1±0.45	This work
		211 kHz	65.2±2.13	17.9±1.36	61.5±0.09	
		1038 kHz	40.5±0.72	12.7±0.37	108.2±2.34	
	211 kHz	20 W	65.2±2.13	17.9±1.36	104.0±2.78	
		30 W	72.7±1.39	18.8±0.31	76.2±0.44	
		75 W	85.9±3.19	19.3±0.14	24.6±0.45	
Classical (silent)[a]			32.6±0.53	18.9±0.39	56.94±0.20	[12]
Indirect UAE[a]			24.8±1.40	17.6±0.58	84.57±2.73	[12]
Soxhlet[b]			19.4±0.41	15.6±0.42	119.37±2.02	[12]

[a]Methanol, 1:10 g/mL, 25 °C and 20 min; [b]methanol, 150 min, boiling point

As can be seen from the Table 2, increasing ultrasound frequency from 20 to 1038 kHz at the constant ultrasound power (20 W) negatively affects TPC and TFC in the houseleek extracts. This reduction of bioactive compounds in the obtained extracts could be attributed to the fact that low frequency ultrasound (16––100 kHz) generates bigger cavitation bubbles, resulting in a more violent cavitation bubble collapse and higher localized temperatures and pressure which enhances the cavitational effect [44]. Further, as the ultrasound frequency increases the production of free radicals (hydroxyl and atomic hydrogen), which could induce the degradation of phenolic compounds, also increases [45]. The extraction of houseleek leaves was performed with methanol, a solvent which does not give rise to such a large proportion of radicals under cavitation [46], but the water present in the fresh houseleek leaves could.

Compared to our previous results [12], DUAE is more effective in TPC extraction than other extraction methods (indirect UAE, classical and Soxhlet extraction) which might be due to the strong cavitation effect causing an intensification of mass transfer [24]. On the other hand, direct application of the ultrasound did not significantly affect the TFC in houseleek extracts. Different effects of direct sonication on flavonoid compounds can be explained by the fact that UAE efficiency is strongly related to the type of extracted compounds [47]. Also, it was published earlier that direct ultrasound could lead to the degradation of some flavonoids, while flavonoid sensitivity depends on their chemical structure, number and type of substituents, as well the position of the hydroxyl group in the flavonoid molecules. Biesaga *et al.* [48] showed that a higher number of hydroxyl groups in flavonoid promoted the degradation of flavonoids, while sugar and methoxyl groups protected the flavonoid from degradation under sonication.

Antioxidant activity

The scavenging activity of the houseleek extracts obtained under different extraction conditions against stable DPPH radical are shown in Figure 1. Antioxidant activities of the extracts are compared using efficient concentration (EC_{50}) which could be defined as the concentration of the extract that causes reduction of DPPH concentration by 50%. For the interpretation of the results, higher EC_{50} values indicate lower antioxidant activity.

Increasing the ultrasound power, as well as decreasing the ultrasound frequency, significantly affects the *AOA* of the extracts. It could be calculated that the *AOA* of houseleek leaves extract obtained under 75 W was four times higher than that of the AOA extract obtained under 20 W. The positive effects of ultrasound power on the antioxidant activity of the extracts were previously published for curry [42] and fresh *Euphorbia tirucalli* tree leaves [49].

Significantly lower *AOA* was found for extracts obtained at the ultrasound frequency of 1038 kHz than that obtained at 42 kHz, probably because of radical reactions and degradation of bioactive compounds as these processes were reported as the major effects of sonication at higher ultrasound frequencies (>500 kHz) [50].

Effects of different ultrasound power and frequency levels on AOA were consistent with the previously described effects on the *TPC* and *TFC* in houseleek extracts. The good correlation between EC_{50} values and the TPC (R^2 = 0.999) and *TFC* (R^2 > 0.784) also confirms that those bioactive compounds are mainly responsible for the antioxidant activity of the extracts.

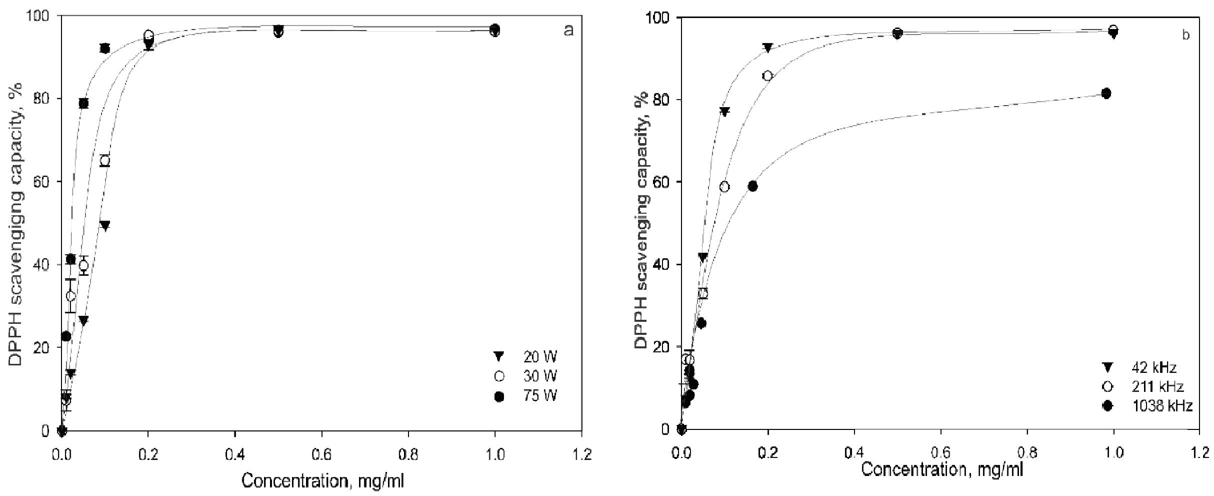

Figure 1. Antioxidant activity of the Sempervivum marmoreum L. extracts obtained at: a) the constant frequency of 211 kHz and different ultrasound powers; b) the constant ultrasound power of 20 W and different frequencies (methanol, 25 °C, 20 min. 1:10 g/mL). Error bars are not shown when they are smaller than the symbols.

Finally, as can be seen from Table 2, the extract of fresh houseleek leaves with the highest *TPC*, *TFC* and *AOA* was obtained at the ultrasound power of 75 W and frequency of 211 kHz. So, taking into account the results in the present study and in order to maximize the extraction of antioxidant phenolic compounds from fresh houseleek leaves, the optimal DUAE conditions were suggested as follows: extraction time of 20 min, methanol as solvent, ultrasound power of 75 W and frequency of 211 kHz. The extract obtained under the proposed optimal conditions was further subjected for preliminary characterization.

HPLC/DAD analysis of extracts

The HPLC chromatogram of crude extract obtained under proposed optimal DUAE conditions is presented in Figure 2.

Most of the peaks in the chromatogram were not identified due to the lack of referent compounds, but seven flavonoids were identified including: genkwanin 4'-*O*-glucoside, genkwanin 5-*O*-glucoside, kaempferol 3-*O*-rhamnoside, kaempferol 3-*O*-glucoside-7-*O*-rhamnoside and quercetin (Table 3).

The typical HPLC/DAD chromatogram of fresh houseleek leaves extract (75 W, 211 kHz obtained under optimum conditions) shows 23 peaks, and the most abundant components in the extract are: kaempferol 3-*O*-(6''-*O*-malonylglucoside)-7-*O*-glucosyde, kaempferol 3-*O*-glucoside-7-*O*-rhamnoside, luteolin 5-*O*-(6''-*O*-malonylglucoside), kaempferol 3-*O*-(6''-*O*-acetylglucoside)-7-*O*-rhamnoside, genkwanin 5-*O*-glucoside, luteolin 5-*O*-(6''-*O*-malonylglucoside), kaempferol 3-*O*-(6''-*O*-malonylglucoside), kaempferol 3-*O*-rhamnoside, quercetin, genkwanin 4-*O*-glucoside and hyperoside (according to retention time). Glyconic forms of the compound belongs to: glucosides (21.88%), ramnosides (24.89%) and sophorrosides (1.74%) while 22.38% are free flavonoids. The HPLC-DAD–MS/MS analysis of the *Sempervivum tectorum* leaves juice shows that solely flavonol glycosides are detectable in *Sempervivum* leaves juice. Kaempferol glycosides prevail, while quercetin glycosides are less characteristic. For unambiguous identification retention times, UV and mass spectra of kaempferol and quercetin were compared to those of a reference compound. However, flavonoid variation of the houseleek was studied only at the aglycone level and detailed data on glycosylation pattern of *Sempervivum* flavonols cannot be found in other sources [51].

Table 3. Compounds detected in Sempervivum marmoreum leaves methanolic extract obtained under optimal conditions

Peak No.	Compound	TR, min	%
1	Unidentified	19.777	0.52
2	Kaempferol 3-*O*-(6”-*O*-malonylglucoside)-7-*O*-glucoside	21.216	2.48
3	Kaempferol 3-*O*-glucoside-7-*O*--rhamnoside	25.405	5.66
4	Kaempferol 3-*O*-sophoroside	28.557	1.74
5	Luteolin 5-*O*-glucoside	29.171	1.38
6	Unidentified	29.831	0.58
7	Kaempferol 3-*O*-glucoside-7-*O*--rhamnoside	31.109	0.86
8	Luteolin 5-*O*-(6''-*O*-malonylglucoside)	38.808	3.61
9	Kaempferol 3-*O*-(6”-*O*-acetylglucoside)-7-*O*-rhamnoside	39.501	2.99
10	Genkwanin 5-*O*-glucoside	41.300	9.50
11	Kaempferol 3-*O*-(6”-*O*-malonylglucoside)	43.155	0.72
12	Unidentified	43.761	2.74
13	Unidentified	44.209	4.40
14	Kaempferol 3-*O*-rhamnoside	45.465	15.38
15	Quercetin	48.133	22.38
16	Unidentified	51.445	13.13
17	Genkwanin 4'-*O*-glucoside	54.098	4.19
18	Unidentified	68.361	1.13

Similar phenolic profiles were detected for *S. tectorum* species with the presence of quercetin and kaem-

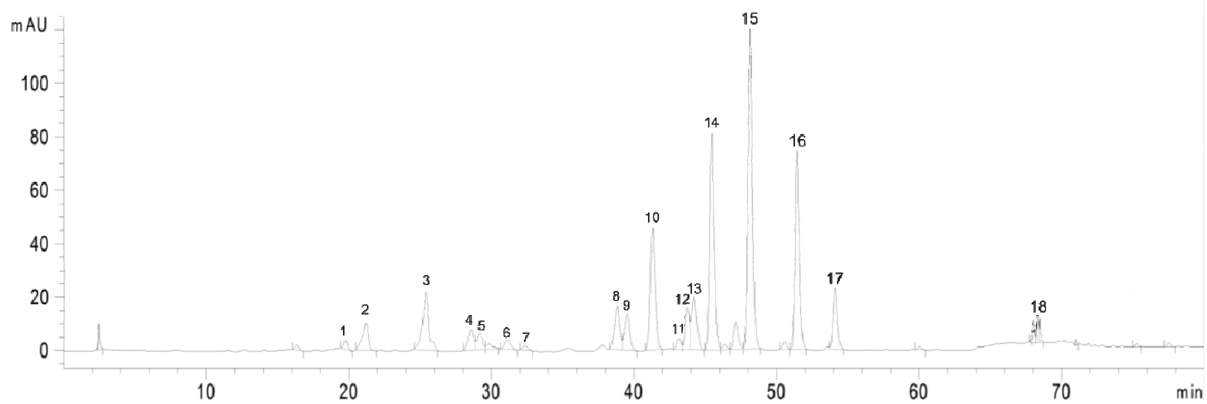

Figure 2. HPLC-DAD profiles of extract obtained by methanol (DUE, 75 W, 211 kHz, 20 min).

pferol glycosides as the most abundant components [52] and scutellarein-7-rutinoside found in *S. rhutenicum* [53]. The analysis of houseleek flower extracts shows the presence of carboxylic acids derivate as 3,4--dihydroxycinnamic acid and 1,4-dihydrocaffeic acid [54]. The flavonoid aglycone composition analysis of some *Sempervivum* species after acidic hydrolysis showed that kaempferol was the principal flavonoid of all the species [55].

CONCLUSION

The results emphasize that higher ultrasound power and lower frequency of DUAE were better for the extraction of a bioactive compound from the plant material. In addition, the extract of fresh houseleek leaves with the highest content of TPC, TFC and AOA was obtained at the ultrasound power of 75 W and the frequency of 211 kHz. Hence, the extraction time of 20 min, methanol as the solvent, ultrasound power of 75 W and the frequency of 211 kHz were suggested as the optimal conditions for the DUAE of fresh houseleek leaves.

The results emphasize the possibility of successful application of DUAE in the extraction of bioactive compounds from fresh plant material. A great increase in the yield of TES was observed when DUAE was applied at a constant frequency and variable power. An increase in the frequency of ultrasonic waves at a constant power does not significantly affect the yield of total extractive substances.

Power and frequency of the ultrasound wave have a greater influence on antioxidant activity, and the total phenolic and flavonoid content in the extracts of houseleek. The good correlation between the total of phenolic and flavonoid content and antioxidant activities of houseleek extracts obtained at different frequencies and power of ultrasonic waves suggests that phenols are mainly responsible for the antioxidant activity of the extracts.

The most abundant components in the extract identified by HPLC-DAD are: kaempferol 3-*O*-(6''-*O*-malonylglucoside)-7-*O*-glucosyde, kaempferol 3-*O*-glucoside-7--*O*-rhamnoside, luteolin 5-*O*-(6''-*O*-malonylglucoside), kaempferol 3-*O*-(6''-*O*-acetylglucoside)-7-*O*-rhamnoside, genkwanin 5-*O*-glucoside, luteolin 5-*O*-(6''-*O*-malonylglucoside), kaempferol 3-*O*-(6''-*O*-malonylglucoside), kaempferol 3-*O*-rhamnoside, quercetin, genkwanin 4-*O*-glucoside and hyperoside.

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IZVOD

DIREKTNA ULTRAZVUČNA EKSTRAKCIJA FENOLNIH JEDINJENJA IZ SVEŽIH LISTOVA ČUVARKUĆE *Sempervivum marmoreum* L.

Ivana T. Karabegović[1], Saša S. Stojičević[1], Dragan T. Veličković[2], Nada Č. Nikolić[1], Miodrag L. Lazić[1]

[1]*Tehnološki fakultet u Leskovcu, Univerzitet u Nišu, Bulevar oslobodjenja 124, Leskovac, Srbija*
[2]*Visoka Poljoprivredno–prehrambena škola, Ćirila i Metodija 1, Prokuplje, Srbija*

(Naučni rad)

Cilj ove studije bilo je ispitivanje uticaja snage i frekvencije ultrazvuka i vremena ekstrakcije na prinos ukupnih ekstraktivnih materija (*TES*), ukupan sadržaj fenola (*TPC*) ukupnog sadržaja flavonoida (*TFC*) i antioksidativne aktivnosti (*AOA*) ekstrakata dobijenog iz svežih listova čuvarkuće. Preliminarna ekstrakcija polifenolnih jedinjenja urađena je primenom metanola, acetona i 2-propanola. Maksimalan prinos ukupnih ektraktivnih supstanci dobijen je ekstrakcijom po Soksletu (Soxhlet) sa metanolom kao rastvaračem. Rezultati su pokazali da povećanje ultrazvučne snage ima pozitivan i značajan ($p < 0,05$) uticaj na *TPC*, *TFC* i *AOA*. Povećanje frekvencije ultrazvuka od 42 do 1038 kHz dovodi do smanjenja *TPC*, *TFC* i *AOA*. Rezultati pokazuju da sonikacija na 75 W i 211 kHz ima uglavnom pozitivan efekat na *TPC*, *TFC* i *AOA* kod ekstrakata dobijenih iz svežih listova čuvarkuće. Najzastupljenije komponente ekstrakata identifikovanih pomoću HPLC-DAD su: kemferol 3-O-(6''-*O*-malonilglukozid)-7-*O*-glukozid, kemferol 3-*O*--glukozid-7-*O*-ramnozid, luteolin 5-*O*-(6''-*O*-malonilglukozid), kemferol 3-*O*-(6''-*O*--acetilglukozid)-7-*O*-ramnozid, genkvanin 5-*O*-glukozid, luteolin 5-*O*-(6''-*O*-malonilglukozide), kemferol 3-*O*-(6''-*O*-malonilglukozid), kemferol 3-*O*-ramnozid, kvercetin, genkvanin 4'-*O*-glukozid i hiperozid.

Ključne reči: Ultrazvuk • *Sempervivum marmoreum* • Fenol • Flavonoid • Antioksidant • HPLC