Dear Prof. Dr Obradović,

I am sending to you the revised manuscript entitled "Direct ultrasound-assisted extraction and characterization of phenolic compounds from houseleek (*Sempervivum marmoreum* L.) fresh leaves". All the referees' recommendations are accepted, as you can see from the answers to the referee comments and some of them are very valuable for the final style of paper. Sentences which we added were red and blue colored in the final electronic version.

Sincerely,

Prof dr Miodrag Lazić

Reviewer A:

***Comment 1)*** *Although the authors reports new data, their paper cannot be recommended in its present form, i.e. a major revision is suggested. The main weakness of their work is the absence of comparasion of direct UAE with a control silence extraction (without ultrasonication) and the indirect UAE. Although the same research group has already published a paper dealing with indirect UAE from fresh houseleek leaves (ref. 12), the authors missed to emphasize the novelty of their present work, compared to the previously published one, especially with respect to the employed direct UAE. In my opinion, having in the mind the fields of interest of the Chemical Industry Journal, “the identification and quantification of the main constituents in S. marmoreum leaves” does not seem to be enough for qualifying this paper for publishing in it. However, the authors should have explained what they expected with the use of direct UAE instead of indirect UAE, which they had already employed, regarding the basic phenomena related to these two extraction techniques. Furthermore, the authors should focus on the effect of low/high frequency and power input on the extraction efficacy.*

**Answer:** We would like to thank the reviewer for all comments and constructive suggestions, which help to improve the quality of our manuscript.

Related to the reviewer's comments, comparison of DUAE with our previously published results, as well as the highlights of DUAE potential and advantage compared to the indirect UAE, were added to the Introduction and Results and discussion sections (the blue colored text and Table 2).

***Comment 2)*** *The best solvent among the tested ones was selected on the basis of their extraction efficacy using the Soxhlet extraction. Thus, the selection was not related to “the capacity of solvent to absorb and transmit the energy of the ultrasound”. In line with this, either the selection method or the discussion about the relation of polarity and UAE efficiency is inappropriate and superfluous. The solvent could be selected on the basis of the well-known effect of polarity (page 7, lines14-19), and methanol might be a solvent of choice even without testing by the Soxhlet extraction. Besides that, the authors stated that they used three solvents but they presented only two TES yields.*

**Answer:** It is true that it is well-known that polarity of solvent highly affectsthe extraction efficiency. However, at the beginning of our experiments, the solvents with different polarity and acoustic properties (methanol, acetone and 2-propanol) were chosen for the extraction by Soxhlet apparatus until the complete exhaustion of the plant material. Since the high content of water in the plant material (88.1 %) significantly affects the polarity of the extraction system, as well as the fact that the amount of water in water/organic solvent mixtures had higher impact on the extraction efficacy than the solvent itself, it was important to test more solvents of different characteristic, not just selected on the basis of the well-known results. But, based on the reviewer's comments and in order to explain the solvent selection criteria more precisely, we rewrote the first paragraph of Section 3.1 (as follows) and added the missing value for the TES yield.

Selection of the best solvent for the extraction of TES from the plant material is a most important step in any method of extraction. Since the high content of water in the fresh houseleek leaves (88.1 %) significantly affects the polarity of the extraction system, as well as the fact that the amount of water in water/organic solvent mixtures had higher impact on the extraction efficacy than the solvent itself [Dent et al., 2013, Food technology and biotechnology, 2013; 51:84-91.], it was important to test more solvents of different polarity and properties. In order to select the best solvent for the further experiments, the plant material was extracted by Soxhlet apparatus using three solvents of different characteristics, methanol, acetone and 2-propanol and 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.

***Comment 3)*** *While the authors used the Soxhlet extraction for selecting the best solvent, they did not conduct a control silence extraction (without sonication under the some other conditions) to evaluate directly the effect of direct ultrasonication on the efficacy of TES, TPC, TFC and AOA extraction from houseleek leaves*.

**Answer:** In our previous paper (ref. 12) we have already compared TES yield, TPC, TFC, AOA and antimicrobial activities of the extracts obtained from fresh houseleek leaves by the classical (silence), indirect ultrasound and Soxhlet extraction.

Regarding to the reviewer's comments and in order to evaluate the effect of direct ultrasonication on the efficacy of TES, TPC, TFC and AOA extraction from houseleek leaves, a more detailed explanation and comparison with previously published results were added to the **Results and discussion** section (blue colored text). Also, the Table 2 was expanded to include the results published in our previous paper.

In our previous experiment, the TES yields from fresh houseleek leaves were 2.3 and 2.4 g/100 g fresh plant material for the classical (silence) extraction and indirect UAE technique, respectively [12]. By comparison with the DUAE, it can be concluded that under the same operating conditions (plant material, plant material to solvent ratio, temperature, solvent) and independently on the ultrasonic power and frequency applied, the same yield of TES can be obtained for twice less time. These finding 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* (Smelcerovic et al., 2006), silymarin from *Silybum marianum* seeds (Saleh et al., 2015), ginseng saponins from ginseng roots and cultured ginseng cells (Wu et al., 2001) and oil from crushed dill seeds (Vinatoru et al., 1997).

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.

***Comment 4)*** *The experimental points in Figure 1 should be presented as mean values with standard deviation (with “bars”). The caption of Figure 1 is incomplete as the ultrasound frequency and power are not presented for sub-figures 1a and 1b, respectively. (extraction temperature and time are also desirable). This will make this figure self-standing completely.*

**Answer:** Since the TES yield obtained after three repeated experiments are very close, the error bars were smaller than the size of the symbols on the Figure 1. So, in order to avoid the reduction of the symbol size which could negatively affect the figure clarity, Figure 1 was replaced with the Table 1.

Table 1. Total extractive substances (TES) from fresh houseleek leaves during DUAEa, b

|  |  |  |
| --- | --- | --- |
| Power, (W) | Frequency (kHz) | Vreme (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 |
| 203075 |  | 1.19±0.02 | 1.59±0.07 | 2.06±0.02 | 2.43±0.01 | 2.47±0.01 | 2.57±0.08 |
| 211 | 1.23±0.01 | 1.65±0.02 | 2.12±0.01 | 2.48±0.06 | 2.53±0.03 | 2.59±0.07 |
|  | 1.34±0.04 | 1.71±0.01 | 2.32±0.01 | 2.56±0.03 | 2.73±0.04 | 2.79±0.09 |

a Data were expressed as the mean of three replicates±standard deviation. b Methanol, 1:10 g/mL, 25 oC and 20 minutes

***Comment 5)*** *Moreover, the authors should report the TES yields at various ultrasound powers and frequencies as a part of Table 1.*

**Answer:** The change in TES yield was monitored with time, so for each set of experimental conditions there were six experimental points. That is why we inserted a new table (Table 1) instead of adding the TES yield results to the existing Table 1.

***Comment 6)*** *The authors did not explain why the increase of ultrasound frequency did not affect the TES yield. In line with this the last paragraph of the sub-section 3.2 (page 9, lines 7-11) seems to be superfluous.*

**Answer:** The last paragraph of the sub-section 3.2 was rewritten as follows:

It has been reported previously by Wang and coworkers [38], that extraction yield did not increase significantly with the increase of ultrasound frequency. However, effects of ultrasound frequency largely depends on the nature of plant material of plant matrix. This is the reason that some author achieved best results with moderate ultrasound frequencies, while other gives priority to the lower ultrasound frequencies [39].

***Comment 7)*** *The authors should explain why the increase of power input from 20 W to 75 W resulted in a relatively small increase of TES (5.8%) and TFC (8%) but in a large increase of TPC (31%).*

**Answer:** The reviewer is right, with an increase of power input from 20 to 75 W, the TES yield increased only for about 6%. However, it can be calculated that under the highest applied ultrasound power (75 W) the TES yield achieves almost 96% of the maximum possible yield (obtained by Soxhlet extraction), so it is difficult to expect further yield improvement. On the other hand, different effects of the ultrasound power on TPC and TFC can be explained by the fact that UAE efficiency is strongly related to the type of extracted compounds (Carciochi et al., 2015, J Food Sci Technol. 52(7): 4396–4404). Also, it was published earlier that ultrasound could lead to degradation of some flavonoids, while flavonoid sensitivity depends on their chemical structure, number and type of substituents, as well position of hydroxyl group in flavonoid molecules. For example, higher number of hydroxyl groups in flavonoid promoted degradation of flavonoids, while sugar and methoxyl groups protected flavonoid from degradation (Biesaga M., 2011, J Chromatogr A 1218: 2505–2512).

Considering these, our future effort will be to evaluate the effects of ultrasound power and frequencies on the extraction of specific compounds from houseleek extracts.

***Comment 8)*** *The authors are expected to compare the performances of direct and indirect UAEs in the extraction of TES, TPC, TFC and AOA from houseleek leaves. A short overview of the results of so far conducted comparative studies on direct and indirect UAE is desirable.*

**Answer:** A brief overview of the previously published comparative studies on direct and indirect UAE was added in Introduction section (blue colored text), while for the purpose of the comparison with our previous results Table 2 was expanded to include these results and some blue colored text was added to the **Results and discussion** section.

***Comment 9)*** *The temperature increase during DUAE experimental runs should be reported.*

**Answer:** During the DUAEthe temperature was measured and maintained at the constant level. We add these information in the section 2.2.2. as the following (red colored) sentences.

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 oC by thermostated water that circulated through the reactor jacket.

***Comment 10)*** *A more careful check of the manuscript is needed in order to correct the present errors, such as:*

*Page 3, line 11: „Geotrichum“ should be „Geotrichum“*

*Page 3, line 13: „indirect ultrasound“ should be replaced by „indirect ultrasound-assisted“. („Ultrasonic extraction“ and „ultrasound-assisted extraction“ are more appropriate than „ultrasound extraction“.)*

*Page 3, line 24 „grether“ should be „greater“*

*Page 4, line 6, „ultrasound assisted extraction“ should be*

*„UAE“(the same on page 7, lines 4-5;title of Table 1; page 15, lines10 and 12),*

*Page 4, line 25: „Direct ultrasound extraction (DUE)“ should be „Direct ultrasound-assisted extraction (DUAE)“ or „Direct UAE“; in fact,the authors should use DUAE instead of DUE all through the manuscript,*

*Page 7, line 19: „USE“ should be „UAE“, etc.*

**Answer:** In the revised version all these errors were corrected.

***Comment 11)*** *English should be checked!!! See, for instance (page 11, line 16),*

*„The four time lower AOA exhibited extract obtained by ultrasound frequency of 1038 Hz than 42 Hz, ...“*

**Answer:** The paper is checked again and the revised manuscript is improved in English grammar and writing.

Reviewer B:

***Comment 1)*** *The manuscript “Direct ultrasound extraction and characterization of phenolic compounds from houseleek (Sempervivum marmoreum L.) fresh leaves” by Stojicevic et al., presents extraction studies of houseleek leaves by ultrasound focusing on the effects of ultrasound power and frequency as well as chemical characterization of one of the extracts obtained under optimal extraction conditions. Presentation is clear, organized in a logical manner. However, experimental section lacks some important details about the extraction process as well as number of experiments so that some of the presented results and drawn conclusions cannot be fully evaluated. Also, the extraction kinetics is qualitatively described while for a critical analysis, mathematical modeling should be applied. Finally, the obtained chemical composition of the extract is not discussed. The manuscript should be corrected for many grammatical errors. Some of them are corrected in the attached pdf file but the authors should continue corrections throughout the text.*

**Answer:** We greatly appreciate all your comments and suggestions which are very helpful to improve the quality of our manuscript. Thank you.

***Comment 2)*** *2.2 Extraction methods*

*Experiments should be described in detail. In specific, authors should state the number of experiments and replicates, solvent volumes, sampling times, and volumes, experiment duration etc.*

**Answer:** We add these information in the text as the following (red colored) sentences.

Extraction 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 cm2, 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 oC by thermostated water that circulated through the reactor jacket. Fresh and chopped houseleek leaves (15 g) was extracted by methanol (150 mL) for 2.5, 5, 10, 20, 40 and 60 min. One series of experiments was performed at constant ultrasound power of 20 W and three different frequencies (42, 210 and 1.038 KHz), while the second series was performed at constant ultrasound frequency of 211 Hz and three different powers (20, 30 and 75 W). The levels of ultrasound frequencies and powers, as well as their combinations were chosen in a preliminary experiments. Liquid extracts were separated from the solid phase by vacuum filtration and dried under vacuum at 40 °C to constant weight.

***Comment 3)*** *2.6. HPLC-DAD identification*

*L16: “Prepared stock solution standards in the methanol concentration of 1.0 mg/mL and a series of dilute solutions. ”The sentence is not grammatically correct. Please revise.*

**Answer:** We rewrote a sentence as follows:

Solutions of all standards were prepared in methanol (1.0 mg mL-1) and diluted to a series of concentrations ranging from 5 to 200 µg/mL.

***Comment 4)*** *3.1. Solvents selection Please modify as “Solvent selection”*

*Please specify all 3 yields obtained by different solvents used.*

**Answer:** The section title was modified and a third value was added (red colored).

In order to select the best solvent for extraction, the plant material was extracted by Soxhlet extraction using three solvents of different acoustic properties, methanol, acetone and 2-propanol and 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.

***Comment 5)*** *3.2. Effect of ultrasound power and frequency on the total extractive substances yield*

*Error bars should be inserted in the Figure 1, as well as in the other figures, in order to evaluate differences in the experimental data obtained in different experimental series.*

**Answer:** Since the TES yield obtained after three repeated experiments are very close, the error bars were smaller than the size of the symbols on the Figure 1. So, in order to avoid the reduction of the symbol size which could negatively affect the figure clarity, the results from Figure 1 was replaced with the new Table 1.

Considering that the error bars were visible for the lower concentration on the Figure 2, we added red colored text below the figure as a part of figure legends.

Error bars not shown when smaller than symbols.

Since the values for the correlation coefficients were given in the text, the Figure 3 has been omitted from the manuscript.

***Comment 6)*** *L5: “Curves have the same shape as those of the UAE of TES from garden (Salvia officinalis L.) and 5 glutinous (Salvia glutinosa L.) sage [33], satsuma mandarin peel [34] and from aerial parts of 6 Echinacea [35].”*

*In order to compare different kinetics results, kinetics models should be applied. Only then, discussion about the mechanism and potential stages of extraction could be made. A simple diffusion model should be applied followed by more complex models developed for extraction processes from different plant materials. This whole section should be revised accordingly*.

**Answer:** Detailed kinetics study of houseleek fresh leaves by direct ultrasound extraction were done by using four two-parameter kinetic models (unsteady diffusion through plant material, the film theory, the empirical equations of Ponomaryov and Weibull), but these results were part of other manuscript which was submitted in other journal. Accordingly, it is better to delete this sentence from the text.

***Comment 7)*** *Table 1 presents EC50 data, while these data are not discussed in that section. Furthermore, these data are presented again in Figure 3 and discussed there. Thus they could be omitted from the Table 1.*

**Answer:** The percentage of DPPH radical neutralization was plotted against extract concentration (new Figure 1), and from this graph EC50 was calculated by using a linear regression analysis. Hence, the EC50 values were calculated and could not be easily visible from the Figure, the authors still believed that both, values in Table and the Figure is necessary part of the manuscript. But, regarding to the reviewer's comments, in the section 3.4. where we discussed the EC50 values Table 2 was cited in the text.

***Comment 8)*** *3.4. Antioxidant activity, L8:*

*“Antioxidant activities of the extracts are compared using efficient concentration (EC50) which could be defined as the concentration of extract that causes reduction of DPPH concentration for 50%. For the interpretation of results, the higher EC50 values indicate lower antioxidant activity.” This whole section should be moved before the Figure 3, which shows EC50 data.*

**Answer:** OK, we accepted suggestion.

***Comment 9)*** *3.5. HPLC/DAD analysis of extracts*

*Discussion of the obtained results should be extended stressing the importance of the findings and potential relevance to the other results presented in the present study.*

**Answer:** We added a few sentences in this section, all of them are red colored.

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].

Similar phenolic profiles were detected for *Sempervivum tectorum* species with the presence of quercetin and kaempferol glycosides as the most abundant components [52] and scutellarein-7-rutinoside found in *Sempervivum 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].