Recovery of biologically active compounds from stinging nettle leaves part I: supercritical fluid extraction

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Article

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Abstract

Stinging nettle (Urtica dioica L.) is an annual plant from Urticaceae family growing wild all over the world. Throughout the history, this plant found its place as a both food and medicine. Due to the insufficient study, this work aimed to isolate the bioactive compounds from the stinging nettle leaves by supercritical fluid extraction. Extracts were analyzed and assessed for antioxidant and cytotoxic activities. Main fatty acids were α-linolenic, palmitic, and linoleic acids in all six samples. Beside fatty acids, chlorophylls and carotenoids were also found in all samples. Four empirical kinetic equations were effectively utilized for kinetic modeling of supercritical fluid extraction of stinging nettle leaves oil. As per proper statistical features, empirical models show good concurrence with experimental data. The numerical modeling of a process is gainful to foresee the process conduct and furthermore extend the methodology from laboratory to industrial scales. The principal component analysis was used to visualize the fatty acids profile and antioxidant capacity and cytotoxic activity of extract obtained from stinging nettle leaves. It was found that both composition and activity were strongly dependent on the parameters of the extraction process.

1. Introduction

Stinging nettle (Urtica dioica L.) is an annual plant from Urticaceae family growing wild all over the world. Throughout the history, this plant found its place as a both food and medicine. Its leaves have been used for flailing arthritic or paralytic limbs stimulating circulation and warmth the joints and extremities (urtication). Several studies reported different types of activity of this plant's extracts, such as antioxidant, anti-microbial, anti-inflammatory, anti-ulcer, and analgesic properties, as well as for treatment of various conditions and diseases, such as anemia, gout, eczema, urinary, bladder, and kidney problems. Moreover, this plant is also well-known as a dietary source of nutrients. It has been used for the preparation of different dishes such as omelets, soups, rice, salads, noodles, etc. Besides the application in the fresh form, extracts made from the leaves were also used for making the functional food products (bread). The wide diversity of usage this plant owns to its chemical composition. Different classes of compounds have been reported so far. Thus, numerous polyphenolic compounds, vitamins B, K, and C, terpenoids, fatty acids, minerals (especially iron), carotenoids, chlorophyll, essential amino acids, tannins, carbohydrates, sterols, polysaccharides, and isolectins.

Supercritical fluid extraction (SFE) is one of the green extraction techniques, which uses fluid in supercritical state as a solvent for the extraction. The most common solvent is carbon-dioxide because it is cheap, nonflammable, nontoxic, chemically inert, and has moderate critical parameters (pressure of 73.8 bar and temperature of 31.1°C). This extraction technique is very helpful for the extraction of thermally unstable compounds, for prevention of hydrolysis, and avoiding of occurrence of the solvent's residue in final extract. One of the advantages of this technique is easily modulated selectivity. It may be modulated by changing the pressure and temperature of the system. These two parameters significantly influence the property of the supercritical fluid and thus the solubility of different compounds in it.

The numerical modelling of the stinging nettle leaves oil process extraction could be especially useful since it gives understanding of transport mechanism for the extraction process. The kinetic models applied for assessment of the superficial fluid extraction (SFE) kinetics should incorporate mass-transfer based models, empirical models and models dependent on heat-transfer analog. The SFE mathematical modeling of a process is beneficial to predict the process behavior and also extend the procedures from laboratory to industrial scales. The reason of the current work was to contemplate the kinetic aspects of supercritical fluid extracted stinging nettle leaves oil by modeling the extraction curves.

There were several studies about the subcritical fluid extraction of the stinging nettle leaves. However, there is no report with the deeper analysis of the extracts regarding the chemical composition and biological activity. Therefore, aim of this study was to characterize extracts obtained under the different conditions (pressure and temperature). Moreover, principal component analysis (PCA) was used to display the fatty acids profile and antioxidant capacity and cytotoxic activity dataset to differentiate extracts obtained from stinging nettle leaves.

2. Materials And Methods

2.1 Plant material
Stinging nettle (Urtica dioica L.) leaves were collected in Vršac area (Southeastern Banat, Autonomous Province of Vojvodina, Republic of Serbia) during the period of April-May in 2015. Voucher specimens (Urtica dioica L., Vršac area, legator and determiner Saša Đurović, N° 2-1539) are deposited at the Herbarium BUNS, University of Novi Sad, Faculty of Science, Department of Biology and Ecology. Leaves were dried naturally in the shade on draft for one month. Dried plant material was grounded in the blender and kept in the paper bags before its usage.

2.2 Supercritical fluid extraction procedure

Supercritical fluid extraction (SFE) was performed using laboratory-scaled plant (HPEP, NOVA-Swiss, Effretikon, Switzerland) described elsewhere\(^\text{18}\). Extraction experiments were conducted under three different pressures (100, 200 and 300 bar), two different temperature levels (40 and 60°C), while CO\(_2\) rate flow was maintained constant (0.2 kg/h). Extractions were conducted for 5 h under the separator conditions of 15 bar and 23°C (Table 1S). After finishing the extraction, total extraction yield (\(Y\)) was measured and expressed as g per 100 g of SN leaves (g/100 g SN). Obtained extracts were transferred into the glass bottles, sealed and stored at 4°C in order to prevent any possible degradation of extract components until analysis.

2.3 Determination of total chlorophylls and carotenoids contents

SFE extracts were used for determination of chlorophylls and carotenoids contents. For this purpose, described spectrophotometric method was applied\(^\text{23,24}\), together with introduced modifications and equations\(^\text{25}\). Results were expressed as mg of chlorophyll A, chlorophyll B, total chlorophylls and total carotenoids contents per 100 g of extract (mg/100 g E), respectively.

2.4 Determination of fatty acids profile

Fatty acids profile of SFE extracts was established applying previously described method\(^\text{11–13}\) using GC-FID (Agilent 7890A) technique. Exact weight of extracts (0.50 g) was directly dissolved in \(n\)-hexane and 2M KOH solution in methanol was added (0.60 mL). Mixture was vigorously stirred for 20 seconds, heated in water bath (70°C), boiled for 1 min and stirred again (20 seconds). 1M HCl was added (1.20 mL) into the mixture which was stirred and left for layer separation. After layer separation, 3 mL of \(n\)-hexane was added, while 0.50 mL of hexane layer was transferred to the volumetric flask (5.00 mL) and filed with hexane. Such prepared solution was used for further analysis. Final results were expressed as a milligram of fatty acid per gram of extract (mg/g E).

2.5 Determination of biological activity of obtained extracts

2.5.1 Determination of antioxidant activity

Antioxidant activity of the SFE extracts was assessed by five previously described \textit{in vitro} spectrophotometric methods: DPPH radical scavenging activity\(^\text{26}\), lipid peroxidation assay\(^\text{27}\), metal ions chelation assay\(^\text{28}\), hydroxyl radical scavenging activity\(^\text{29}\), and ABTS radical scavenging assay\(^\text{30}\). Result for all tests were expressed as IC\(_{50}\) (\(\mu\)g/mL) which represents the concentration of the extract needed to neutralize 50% of reactive species (radicals).

2.5.2 Determination of cytotoxic activity

Cytotoxic activity of the SFE extracts was determined against the three different cell lines (RD (cell line derived from human rhabdomyosarcoma), Hep2c (cell line derived from human cervix carcinoma – HeLa derivative) and L2OB (cell line derived from murine fibroblast) by using the previously described MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay\(^\text{31}\). The results were expressed as IC\(_{50}\) (\(\mu\)g/mL) which was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control.

2.6. Extraction kinetics modeling

Four empirical models applied for stinging nettle leaves oil SFE kinetics fitting given in Table 1 are widely employed for this purpose and they are described in details in the literature, presented in Table 1.

2.7 Statistical analyses

All measurements were performed in triplicate, expressed by means \(\pm\) standard deviation (SD). The post hoc Tukey’s HSD test at \(p<0.05\) level was employed to investigate the differences in means between samples. The PCA analysis was applied to interrogate the intrinsic correlations between variables, and to classify the objects into groups. The data were investigated in the STATISTICA 10.0 software (StatSoft Inc., Tulsa, OK, USA).

3. Results And Discussion
3.1 Total extraction yield and kinetics of extraction processes

Total extraction yield ($Y$) was determined for all six samples after 300 min (5 h), and after certain amount of time during the extraction process: after each 15 min in the first hour, and after 30 min until the end of the process (up to 300 min). All yields are given in Table 1S, while final yields are graphically presented in Fig. 1.

Results showed that the highest yield was in extract obtained under 300 bar and 60°C (sample 6) followed by sample 4 (200 bar, 60°C). The lowest yield was obtained under 100 bar and 60°C (sample 2). In order to analyze the behavior of $Y$ in the case of SFE extraction process, kinetics curves were constructed and presented in Fig. 1. In the case of lower temperature (Fig. 1A), under the all three pressure levels $Y$ was increasing constantly, where the highest $Y$ was achieved under the pressure of 300 bar. Same behavior was noticed in the case of higher temperature (Fig. 1B) as well. Such trend for $Y$ was rather expected due to positive influence of pressure on extraction yield, where with the increasing in pressure of supercritical CO$_2$, its density also increases causing the observed increasing in $Y$\cite{36,37}, which was observed from the data given in the Tables 1S and 2S.

Temperature has more complex influence on $Y$ of the process. Changes in temperature of the system cause the changes in two important parameters: density of the CO$_2$ and vapor pressure of the isolated compounds. With the increasing in temperature, vapor pressure also increases resulting in higher solubility. On the other hand, density decreases with the temperature increasing causing the lower solubility. Combination of these two effects and domination of the particular one determines what will happened with the $Y$.

Value of the pressure where the previously mentioned change in domination occur is known as crossover pressure\cite{37,38}.

For determination of this pressure value, PY curve was created (Fig. 2) where it might be seen that two curves intersect at 131.4 bar, which is in this case crossover pressure, i.e., pressure at which above-mentioned changes in domination of effects occurred. Curve in the Fig. 2 showed that above 131.4 bar $Y$ was higher at higher temperature. Therefore, influence of the temperature on vapor pressure was dominant effect in this region. However, below 131.4 bar higher $Y$ was measured at lower temperature indicating that temperature exhibited stronger influence on the density of CO$_2$.

There are only few articles regarding the supercritical fluid extraction of the stinging nettle\cite{15,20-22}. Hojnik et al. (2007) also investigated impact of the different experimental condition on the extraction yield. They reported maximal yield under the maximal pressure and temperature (300 bar and 60°C) but with the significantly lower yield of only 2.2%\cite{20}, while we got 5.2% (Table 2S). However, Akhan et al. (2013) reported quite higher yield, but the authors used modifier (ethanol) during the extraction process, which strongly influenced final result\cite{21}.

The SFE process in this study was conducted under the process conditions with a pressure of 100, 200 and 300 bar and temperature of 40°C and 60°C, while the CO$_2$ mass flow was 0.2 kg/h. In order to study the dynamics of the separation process, extraction time sequences were set at 0, 15, 30, 45, 60, 90, 120, 150, 180, 240 and 300 min, as the extraction yield reached a plateau after 5 h for each of the extraction procedures. Results obtained from experimental runs were fitted to the four commonly used mathematical equations for the SFE modelling.

Adjustable parameters obtained from Model I, $Y_\infty$ (total yield obtained for infinite time of extraction process) $a$, and $b$ (rate constant) were calculated and presented in Table 2, while Fig. 3 shows extraction curves with experimental and model data obtained for all applied models. The obtained $Y_\infty$ was in the range from 0.876 to 4.561% while $a$ and $b$ were negative, between $-0.055$ to $-0.010$ min$^{-1}$ and $-0.246$ to $-0.37$ min$^{-1}$, respectively. The highest $Y_\infty$ was obtained for sample 6, while the highest $a$ and $b$ values were obtained for sample 2.

The $Y_\infty$ in Model II was in the range from 0.856 to 4.991%, while $k$ was in the range from 11.386 to 30.048 min$^{-1}$. The highest $Y_\infty$ was noticed for sample 6, while the highest $k$ was obtained for sample 4.

Model III separated the SFE curve into two separated curves representing two fractions recovered during solubility-controlled and diffusion-controlled periods, respectively. The $Y_\infty$ in Model III was in the range of 2.009 to 9.643%, while the sum of $f_1$ and $f_2$ was equal to 1. The $f_2$ coefficient was larger than $f_1$, for samples 1, 2 and 4 (CO$_2$ density were lower: 0.629; 0.290 and 0.724 g/cm$^3$, respectively), which indicates that the SFE process was mostly controlled by diffusion, while the SFE process was mostly controlled by solubility for samples 3, 5 and 6 (CO$_2$ density were larger: 0.840; 0.910 and 0.830 g/cm$^3$, respectively).
The calculated parameters of the Model IV indicated that the constant extraction rate period ranged from 33.082 to 50.612 min (Table 2). The results suggested that the lowest $t_1$ was obtained at the higher pressures, according to the experimental results. The temperature exhibited the negative influence on $t_1$ observed by the experimental results (Table 2), which was in accordance with previous study. Following extraction step, i.e., falling extraction rate period was characterized by parameter $t_2$ which ranged from 5000 to 9900 min (Table 2). It could be observed that the falling extraction rate period did not terminate after 300 min, suggesting that experimentally applied total extraction time was not sufficient to completely exhaust a plant material. However, the falling extraction rate period is less important for the most of the industrial scale SFE processes, and the extraction would be stopped shortly after constant extraction rate period.

Sum of squared errors (SSE), coefficient of determination ($R^2$) and average absolute relative deviation (AARD) were calculated statistical parameters used for determination of fitting quality between experimental data and proposed models. Particularly high values of $R^2$ and low SSE and AARD suggested adequate fit in case of all applied models (Table 3). However, Model III and Model IV showed the best fit with the experimental data due to the highest $R^2$, the lowest SSE and AARD (mean for all experiments: 0.997, 0.08 and 0.09, respectively). Similar case was observed in recent study investigating SFE of wheat germ oil. According to statistical features, Model I provided the weakest fit compared to other models.

### 3.2 Determination of chlorophyll, carotenoids, and fatty acids

After the initial analysis of yield and kinetics of SFE, extracts were analyzed for content of chlorophyll, carotenoids, and fatty acids. Content of chlorophyll A, chlorophyll B, total chlorophyll, and total carotenoids contents were determined by using a spectrophotometric method. Results of the analysis are given in Table 4.

Presented results indicated that contents of both chlorophylls (A and B) as well as total chlorophylls increased with increasing in both pressure and temperature, although its content was slightly lower in sample 2 than in sample 1. Explanation for such results might be the degradation of chlorophylls which might occur at higher temperature. Chlorophylls are large molecules with high molecular masses. Therefore, they should be extracted more efficiently at higher pressures, which is actually case. Carotenoids who are planar molecules, and with lower molecular mass than chlorophylls, should be better extracted at lower pressure. Presented results in the Table 1 confirms that, where the carotenoids reached their maximal yield at 200 bar and 60°C (sample 4, Table 1S).

Presence of these pigments in stinging nettle leaves has been previously reported. Đurović et al. (2007) reported 208.96 mg, 95.68 mg, 304.63 mg, and 69.07 mg per 100 g of SN leaves for chlorophylls A and B, total chlorophylls, and total carotenoids content, respectively isolated by using the Soxhlet extraction technique. Other authors also reported presence of these pigments with certain diversity of the results which are connected with seasonality, maturation of the leaves, geographical origin, etc. Sovova et al. (2004) performed extraction of pigments and oleoresins by supercritical fluid in combination with ethanol as polar modifier, and confirmed presence of both chlorophylls (A and B) as well as carotenoids in stinging nettle leaves. Authors reported increasing in concentration of chlorophylls with amount of added ethanol. Same behavior was noticed with β-carotene, where concentration of this pigment also rose with the increasing of ethanol’s amount in the system.

Beside the pigments, extracts were analyzed for fatty acids (FAs), and the results are shown in Table 5, while their full names and abbreviations are given in Table 3S (Supplementary data). Fatty acids are divided into two major groups, i.e., saturated (SFA) and unsaturated (UFA) fatty acids. The latter is consisting of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids.

Presented results clearly showed the impact of the main SFE parameters, i.e., pressure and temperature, on the occurrence and yield of the fatty acids in obtained extract. The total yield of the FAs was in the sample 3 (200 bar and 40°C), while the lowest was in the sample 2 (100 bar and 60°C). Pressure influenced positively on the total yield of FAs at the both temperature levels, where the yield increased from 100 to 200 bar, and that decreased at 300 bar. Results for total yield of SFA showed the same tendency. Generally, higher pressure and higher temperature is mostly more suitable for the extraction of SFAs, with the exception of the samples 1 and 2 where lower temperature gave higher yield of SFA. When it comes to the UFAs, MUFA, and PUFAs, results were completely different. Pressure of 200 bar was suitable for the isolation of the SFAs, where the similar results were obtained at the both temperature levels. On the other hand, UFAs were better extracted at lowest levels of both parameters (sample 1). Same trend might be noticed for MUFA.
and PUFAs, where both classes achieved maximal yield in the sample 1 (100 bar and 40 °C). It might be concluded that lower pressure and temperature are more suitable for the extraction of UFAs, which was also the case for MUFAs and PUFAs. This is supported by the SFA/UFA ratio. The highest ratio was obtained for sample 4 (1.51), while the lowest was noticed in sample 1 and 2 (0.42).

Presence/absence of individual FAs in the samples, together with their yields would provide valuable insight into a structure-solubility relationship. Previously published studies showed that length of carbon chain and number of double bonds in the structure would significantly influence miscibility of the molecule in the supercritical CO2. It was reported that miscibility decreases with the increasing of the chain length. On the other hand, double bonds decrease melting point and molecular weight, thus increasing the miscibility.

Results showed that C6:0 was not found in samples 4 and 6. This indicated that temperature negatively influenced on the miscibility of this acid because it was found in samples 3 and 5. same influence was observed for C13:0. The C10:0 and C11:0 acids were found in sample 1, which indicated that both pressure and temperature had negative impact on their miscibility. C15:0 acid was not found in sample 5, C21:0 was not found in sample 2, while C23:0 was not found in sample 3. All other analyzed SFAs were found in all samples. Investigation of the parameters’ influence on yield of individual FAs yield showed following: under isothermal conditions, at 40°C, yield decreased with the pressure up to C18:0 acid. After it, yield initially increased (at 200 bar) and then decreased (at 300 bar). At 60°C, yield decreased initially and then increased for all SFAs. However, different behavior of SFAs was noticed under isobaric conditions. At 100 bar, yield decreased with the temperature. Exceptions were C12:0, C20:0, and C23:0 acids whose yield increased with the temperature. At 200 bar, yield increased with the temperature in some cases, with the exception of C15:0, C17:0, C18:0, C20:0, C22:0 C23:0, and C24:0. In this case, there were much more acids which achieved the maximal yield at higher temperature comparing to the samples obtained at 100 bar. At the highest level of pressure (300 bar) only four acids (C6:0, C13:0, C14:0, and C23:0) showed decreasing in their yield with the temperature.

In the case of MUFAs, trans-C18:1 acid was found in sample 2, C24:1 acid was detected in sample 3, C15:1 acid was found in all samples except in sample 4, while C20:1 was not detected in sample 3 and 6. Following acids, C14:1, C17:1, and cis-C18:1, were detected in all 6 samples. Comparing the results under the isothermal conditions it might be noticed that at 40°C yield of C14:1, C17:1, and cis-C18:1 increased with the pressure, while at 60°C acids reached their maximal yield at 200 or 300 bar, depending on acid. In the case of isobaric conditions, at 100 bar yields decreased with the temperature, with the exception of trans-C18:1 acid. Same behavior might be noticed at 200 bar, but exceptions in this case were C16:1 and C20:1. At 300 bar almost all yields increased with the temperature except for C16:1 and C17:1 whose yield decreased.

Presence of PUFAs was also noticed in all samples. Principal PUFA in all 6 samples was α-C18:3 acid, which was also the principal FA in all samples in the same time. The γ-C18:3 acid was not found in samples 3 and 6. C20:3(n-3) acid was found in samples 2 and 6, C22:2 was detected in sample 3, while C20:5 was found in sample 2. Most of detected PUFAs achieved their maximal yield in sample 1 (100 bar and 40°C). The exceptions were C20:3 (n-3), C20:3 (n-6), and C22:2 acids, whose maximal yields were achieved in the samples 6, 5, and 3, respectively. It might be easily noticed that both C20:3 acids achieved their maximal yield at 300 bar, at different temperature. Obviously that positions of double bonds have significant impact on miscibility of these isomers at different density of supercritical CO2.

Under the isothermal conditions, at 40°C, yields of the most PUFAs decreased initially, but the increased, with the exception of C18:2 and C 20:2 acids. On the contrary, at 60°C yields of C18:2 and γ-C18:3 acids decreased with the pressure, while other acids showed different behavior where yield increased or decreased up to 200 bar. Generally, it might be noticed that low pressure is the most suitable for extraction of UFAs (both MUFAs and PUFAs), while higher pressure is to used when SFAs are the primary target. Increasing in temperature negatively influenced the yield of both classes of UFAs. However, for SFA this was the case only at 100 bar, but under higher pressure yields of SFAs increased with the temperature.

Đurović et al. (2018) previously investigated influence of the SFE extraction parameters on the fatty acid profile in extract obtained from commercially available stinging nettle leaves 12. Authors reported very similar trends for yields of SFAs, MUFAs, and PUFAs. However, comparing the quantitative data, it might be noticed that wild-growing stinging nettle synthetizes higher amounts of fatty acids than commercially breading one.

The PCA of the antioxidant and cytotoxic activity of extract obtained from exhausted leaves data explained that the first two principal components explained 69.17% of the total variance (these components covered 46.59 and 26.98%, respectively, while the calculated
eigenvalues were 18.17 and 8.81, respectively) in thirty nine variables factor space (fatty acids profile). Based on the PCA analysis, the content of C14:1 (which contributed 5.2% of total variance, based on correlations), α-C18:3n-3 (5.4%), UFA (5.4%) and PUFA (5.4%) exhibited positive influence according to the first principal component calculation (Fig. 4). The positive contribution for the second principal component was observed for C20:3n-6 (6.6%, of total variance, based on correlations) and C20:5 (9.0%), while the influences of C16:0 (7.3%), C18:0 (7.1%), SFA (6.6%) and total content (11.2%) were negative.

3.3 Biological activity of SFE extracts
Biological activity of the SFE extracts was assessed by using different in vitro assays and methods. Thus, five spectrophotometric methods were employed to assess the antioxidant activity: DPPH radical scavenging activity (DPPH), lipid peroxidation assay (ILP), metal ions chelation assay (MCA), hydroxyl radical scavenging activity (HRSA), and ABTS radical scavenging assay (ABTS) (Table 6). Additionally, the MTT test was used for estimation of the cytotoxic activity (Table 7) against three different cancer cell lines.

Results given in Table 6 showed that sample 6 was the most potent in the case of all used assays. It scavenged DPPH radicals better than BHT (butylated hydroxyanisole) and was comparable to vitamin C and gallic acid. Sample 6 proved to be better antioxidant agent against hydroxyl radicals (HRSA assay) comparing with all three used standards (vitamin C, gallic acid, and BHT). In the case of ABTS assay, sample 6 scavenged these radicals more efficient than gallic acid and BHT. Anyway, all analyzed samples showed competitive activity against all radicals, which indicate that FSE extracts could be the promising sources of natural antioxidant compounds used for food preservation and improvement of human health.

Cytotoxic activity is given in Table 7. Values of IC\textsubscript{50} < 30 µg/mL for plant extracts is considered to be the criterion for the anticancer agent according to the American National Cancer Institute \textsuperscript{45}. Herein, extracts 3–6 fulfilled this criterion against all three cell lines, as well as sample 2 against Hep2c cell line. Sample 1 showed significant activity close to this criterion, while activity of sample 2 against RD and L2OB cell lines were slightly higher than 30 µg/mL. Although activities of all tested samples were much higher than activity of the cis-DDP (cis-diamminedichloroplatinum) \textsuperscript{46}, it should be born in mind that this well-known cytostatic is very toxic and nonselective, while SFE extracts are clean products obtained from the nature without any solvent traces.

Previously reported study on stinging nettle leaves extracts showed slightly higher cytotoxic activity of water extracts than the activity reported here \textsuperscript{4}. However, antioxidant activity of SFE extracts proved to be better for scavenging the DPPH radicals. It was previously reported that water extracts showed higher reducing capability than α-tocopherol and better inhibitor for superoxide generation than BHT. The metal chelating capability was reported to be higher than both BHT and α-tocopherol \textsuperscript{2}. These results are similar with the ones reported in this study. It indicates that, despite the different nature of applied solvent for the extraction process, there are numerous of compounds in this plant which make it remarkable and very useful in medicine and food industries.

The PCA of the antioxidant and cytotoxic activity of extract obtained from leaves data explained that the first two principal components explained 99.65% of the total variance in eight variables factor space (antioxidant and cytotoxic activity of extract). According to the PCA analysis, all eight variables influenced similarly to the PC1 coordinate, showing the negative impact. (Fig. 5). The negative impact to the second principal component was noticed for DPPH (19.3%, of total variance, based on correlations), Hep2c (9.37%) and RD (7.52%). The positive contribution for the second principal component was obtained for ABTS assay (58.1%).

4. Conclusion
Stinging nettle is one of the remarkable plants which is well-known for its beneficial activity throughout the history of mankind. Despite such significance, this plant is still not studied enough. This study aimed to change this fact, and investigated the supercritical fluid extraction of the stinging nettle leaves, influence of the extraction's parameters (pressure and temperature) on chemical composition and biological activity of the extracts, and extraction kinetics. Results showed that leaves are quite rich in both chlorophylls (A and B), carotenoids and fatty acids. Significant biological activity of the extracts indicated the possible application in food and pharmaceutical industries as a food additive or dietary supplement.

Declarations
Acknowledgement
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Tables

Table 1. Commonly used empirical models applied for fitting of stinging nettle leaves SFE

<table>
<thead>
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<th>Model No.</th>
<th>Model</th>
<th>Reference</th>
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<tr>
<td>Model I</td>
<td>( Y = Y_\infty (1 - e^{-k \cdot t}) )</td>
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<td>Model II</td>
<td>( Y = \frac{a \cdot Y_\infty}{b + t} )</td>
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<td>Model III</td>
<td>( Y = Y_\infty \left[ 1 - \left( \frac{f_1 e^{-k_1 \cdot t} + f_2 e^{-k_2 \cdot t}}{f_1 + f_2} \right) \right] )</td>
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<tr>
<td>Model IV</td>
<td>( Y = \frac{CL-B \cdot G}{t} ), for ( t \leq t_1 = \frac{G}{k} ) ( ) ( Y = Y_\infty (1 - G) e^{\frac{t}{k}} ), for ( t \geq t_1 )</td>
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Table 2. Calculated adjustable parameters of Models I - IV applied for SFE modelling

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<thead>
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<th>Model III</th>
<th>Model IV</th>
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Table 3. Statistical parameters used for determination of fitting quality (SSE, AARD and \( R^2 \)) between experimental results and applied models (I - IV)

<table>
<thead>
<tr>
<th>Run</th>
<th>( Y_\infty )</th>
<th>Model I</th>
<th>Model II</th>
<th>Model III</th>
<th>Model IV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SSE</td>
<td>AARD</td>
<td>( R^2 )</td>
<td>SSE</td>
</tr>
<tr>
<td>1</td>
<td>0.119</td>
<td>0.087</td>
<td>0.921</td>
<td>0.055</td>
<td>0.056</td>
</tr>
<tr>
<td>2</td>
<td>0.110</td>
<td>0.084</td>
<td>0.818</td>
<td>0.081</td>
<td>0.066</td>
</tr>
<tr>
<td>3</td>
<td>0.598</td>
<td>0.211</td>
<td>0.922</td>
<td>0.241</td>
<td>0.127</td>
</tr>
<tr>
<td>4</td>
<td>1.622</td>
<td>0.235</td>
<td>0.870</td>
<td>1.083</td>
<td>0.287</td>
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<tr>
<td>5</td>
<td>0.602</td>
<td>0.199</td>
<td>0.946</td>
<td>0.159</td>
<td>0.095</td>
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<tr>
<td>6</td>
<td>2.097</td>
<td>0.376</td>
<td>0.897</td>
<td>0.910</td>
<td>0.232</td>
</tr>
<tr>
<td>Average</td>
<td>0.866</td>
<td>0.218</td>
<td>0.896</td>
<td>0.422</td>
<td>0.143</td>
</tr>
</tbody>
</table>

Table 4. Chlorophyll A, chlorophyll B, total chlorophyll, and total carotenoids contents in SFE extracts of SN leaves

<table>
<thead>
<tr>
<th>Sample</th>
<th>CL-A (mg/100 g E)</th>
<th>CL-B (mg/100 g E)</th>
<th>CL (mg/100 g E)</th>
<th>Cx (mg/100 g E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.94 ± 0.52</td>
<td>16.06 ± 0.23</td>
<td>33.00 ± 0.33</td>
<td>166.88 ± 12.52</td>
</tr>
<tr>
<td>2</td>
<td>16.93 ± 0.36</td>
<td>11.89 ± 0.11</td>
<td>28.82 ± 0.52</td>
<td>169.93 ± 10.25</td>
</tr>
<tr>
<td>3</td>
<td>5007.84 ± 99.25</td>
<td>585.73 ± 68.52</td>
<td>3593.57 ± 102.52</td>
<td>556.40 ± 12.26</td>
</tr>
<tr>
<td>4</td>
<td>3396.41 ± 102.35</td>
<td>792.09 ± 84.23</td>
<td>4188.50 ± 135.23</td>
<td>722.62 ± 15.56</td>
</tr>
<tr>
<td>5</td>
<td>4221.36 ± 121.32</td>
<td>1046.66 ± 90.22</td>
<td>5268.02 ± 202.23</td>
<td>465.19 ± 10.11</td>
</tr>
<tr>
<td>6</td>
<td>5696.65 ± 152.36</td>
<td>1668.46 ± 101.23</td>
<td>7365.11 ± 225.23</td>
<td>587.38 ± 15.56</td>
</tr>
</tbody>
</table>

CL-A: chlorophyll A; CL-B: chlorophyll B; CL: total chlorophyll; Cx: total carotenoids.
Values are presented as means of three determinations ± standard deviation. Values in the same row with the different superscript lowercase letters are statistically different (\( p < 0.05 \)).

Table 5. Fatty acid profile of SFE extracts
Table 6. Antioxidant activity of SFE extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC_{50} (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPPH</td>
</tr>
<tr>
<td>1</td>
<td>22.55 ± 0.88&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>20.45 ± 0.65&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>17.86 ± 0.28&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>13.59 ± 0.12&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>9.96 ± 0.63&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>6.19 ± 0.44&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are presented as means of three determinations ± standard deviation. Values in the same row with the different superscript lowercase letters are statistically different (p < 0.05).
Table 7. Cytotoxic activity of SFE extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell line / IC&lt;sub&gt;50&lt;/sub&gt; (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hep2c</td>
</tr>
<tr>
<td>1</td>
<td>34.02 ± 0.56&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>29.15 ± 0.54&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>26.65 ± 0.77&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>20.36 ± 0.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>16.63 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>10.98 ± 0.96&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are presented as means of three determinations ± standard deviation. Values in the same column with the different superscript lowercase letters are statistically different (p < 0.05).

**Figures**

Figure 1

Kinetics curves for SFE extraction: a) pressures of 100, 200 and 300 bar, and b) temperatures of 40 °C and 60 °C.
Figure 2
PY curve for determination of crossover pressure.
Figure 3

Extraction curves with experimental and model data obtained for all applied models for SFE extracts of the stinging nettle leaves.
Figure 4

PCA ordination of variables based on component correlations.
Figure 5
PCA ordination of variables based on component correlations.

Supplementary Files
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- Supplementarydata.docx