Research Article

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Effects of Supercritical Carbon Dioxide Extraction (SC-CO₂) on the content of tiliroside in the extracts from *Tilia* L. flowers

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Abstract: Tiliroside is one of the main flavonoid compounds responsible for a wide spectrum of biological activity of *Tilia* L. Up to now, several extraction techniques have been reported for extracting this compound from *Tilia* L. In this work for the first time supercritical carbon dioxide extraction was used for this purpose. Experiments were performed using supercritical carbon dioxide with 5% and 10% of ethanol as solvent, aiming a recovery content of tiliroside, temperature from 45 to 80°C, pressure from 100 to 220 bar and time from 20 to 60 minutes. The statistically generated optimum extraction conditions to obtain the highest content of tiliroside were established as: pressure of 200 bar, temperature 65°C and 45-50 minutes for 5% ethanol concentration and pressure of 220 bar, temperature 65°C and 15 minutes for 10% ethanol concentration.

A liquid chromatography-electrospray ionization-tandem mass spectrometry method (LC-ESI-MS/MS) was used to determine the content of tiliroside in the obtained extracts. In addition, the total phenolic (TPC) and flavonoid (TFC) content and antioxidant activity (DPPH[•] method) were determined.

Keywords: SC-CO₂ extraction; tiliroside; flavonoids; linden extract; LC-ESI-MS/MS.

1 Introduction

Tiliroside (3-O-β-D-(6''-p-coumaroyl)-glucopyranoside) is a glycosidic flavonoid ester, one of the main constituent of *Tilia* L. responsible for the biological properties of the plant, for example: anti-inflammatory [1], antimicrobial [2] and anticoagulant activities [3]. Furthermore, it shows antiproliferative and anti-cancer effects [4–7]. Due to its broad spectrum of activity, tiliroside is of interest to many scientists, therefore effective analytical methods are still being developed for its study.

Many techniques have been used for the extraction of polyphenols including traditional and modern extraction. The most popular classical polyphenols extraction techniques are Soxhlet extraction [8], maceration [8,9] and percolation [10]. These methods have been used for more than a century, but some disadvantages render its application quite unprofitable due to excessive consumption of energy, time, large amount of solvents and thermal decomposition of labile compounds [11,12]. Disadvantages associated with classical extraction techniques lead to the development of new and more efficient ones e.g. ultrasound assisted extraction (UAE), microwave assisted extraction (MAE), supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), pressurized hot water extraction (PHWE) [13,14] and supercritical carbon dioxide extraction (SC-CO₂) [14–16].

Supercritical carbon dioxide extraction is an environment-friendly technology. Carbon dioxide (SC-CO₂) is the most frequently used supercritical fluid solvent because of its practical advantages, it is inert, non-toxic, non-flammable, low cost at high purity and is able to solubilize lipophilic substances [17,18]. In addition, it allows extraction at low temperatures and at comparatively low pressures [19]. Carbon dioxide is mainly used to extract non-polar compounds. However, polar compounds, e.g. phenolic compounds, have reduced solubility in supercritical carbon dioxide. The use of a co-solvent or polar modifier has been enhanced by the solubility of the target compounds and increasing

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the selectivity of the extraction, allowing action at lower pressures [17,20]. Until now, methanol, ethanol, propanol and acetone have been used for this purpose [21,22]. The addition of small percentages (1–10%) of this polar co-solvents to carbon dioxide increase its extraction range to include more polar compounds [22,23].

Up to now, several extraction techniques have been reported for extracting phenolic compounds from *Tilia* L. [24–27], but to our knowledge, the supercritical carbon dioxide extraction has not been carried out. In this work for the first time the influence of conditions of supercritical carbon dioxide extraction for the tiliroside content in obtained linden extracts was analysed.

In this study the main subject was to evaluate the potential of *Tilia* L. flowers extracts obtained using SC-CO₂ solvent with the addition of ethanol, in terms of source of tiliroside. The variables temperature, pressure and time were also evaluated in order to assess the extraction yield.

2 Materials and Methods

2.1 Plant material

Tiliae Inflorescentia (*Tiliae flos*) was purchased from the herb company Kawon (Gostyń, Poland). According to the information provided by the manufacturer, linden flowers were harvested from *Tilia cordata* Mill and/or *Tilia platyphyllos* L. on June and July 2015. The plant material was dried in air and powdered in accordance with the requirements of the Polish Pharmacopoeia IX.

2.2 Extraction methods

The extraction of plant material (about 10 g) was carried out using the multi-purpose pilot unit for supercritical fluid extraction produced by Waters (Milford, MA, USA). The flow rate of CO₂ was 10.33 ml/min and ethanol - 0.49 ml/min for 5% EtOH and the flow rate of CO₂ was 13.65 ml/min and ethanol - 0.97 ml/min for 10% EtOH. The following ranges were investigated; extraction pressure between 100 to 220 bar, temperature between 45°C and 80°C and extraction times of 20, 40 and 60 minutes. The extracts were marked with the symbol “Ex. 1-15/5%” for 5% ethanol concentration and “Ex. 1-15/10%” for 10% ethanol concentration. The extraction details are shown in Tables 1 and 2.

All extracts obtained were filtered, evaporated to dryness under vacuum and lyophilized in the Free Zone 1

Table 1: Conditions of supercritical carbon dioxide extraction for 5% ethanol concentration.

Sample name	Temperature [°C]	Time [min]	Pressure [bar]
Ex. 1/5%	45	20	160
Ex. 2/5%	75	20	160
Ex. 3/5%	45	60	160
Ex. 4/5%	75	60	160
Ex. 5/5%	45	40	100
Ex. 6/5%	75	40	100
Ex. 7/5%	45	40	220
Ex. 8/5%	75	40	220
Ex. 9/5%	60	20	100
Ex. 10/5%	60	60	100
Ex. 11/5%	60	20	220
Ex. 12/5%	60	60	220
Ex. 13/5%	60	40	160
Ex. 14/5%	60	40	160
Ex. 15/5%	60	40	160

Table 2: Conditions of supercritical carbon dioxide extraction for 10% ethanol concentration.

Sample name	Temperature [°C]	Time [min]	Pressure [bar]
Ex. 1/10%	60	20	160
Ex. 2/10%	80	20	160
Ex. 3/10%	60	60	160
Ex. 4/10%	80	60	160
Ex. 5/10%	60	40	100
Ex. 6/10%	80	40	100
Ex. 7/10%	60	40	220
Ex. 8/10%	80	40	220
Ex. 9/10%	70	20	100
Ex. 10/10%	70	60	100
Ex. 11/10%	70	20	220
Ex. 12/10%	70	60	220
Ex. 13/10%	70	40	160
Ex. 14/10%	70	40	160
Ex. 15/10%	70	40	160

apparatus (Labconco, Kansas City, KS, USA). The residue was weighed and re-dissolved in ethanol to obtain stock solutions at the suitable concentrations.

For preparing the samples for LC-MS analysis of flavonoid aglycones the SPE extraction was used. For this purpose a portion of solubilised extract was passed through octadecyl SPE microcolumns (500 mg, J.T. Baker Inc., Philipsburg, USA) previously conditioned with methanol (10 ml) and water (10 ml). After the application of sample, the individual column was washed with 5 ml of 100% methanol. All obtained solutions were evaporated to dryness under vacuum and re-dissolved in a suitable solvent to obtain samples for LC MS/MS analysis.

2.3 Total phenolic and flavonoid content (TPC and TFC)

All spectrophotometric measurements were determined using 96-well transparent microplates (Nunc, Nunc Roskilde, Denmark) and the Infinite Pro 200F microplate reader (Tecan Group Ltd., Männedorf, Switzerland).

The total phenolic content (TPC) of the extract was determined by the Folin–Ciocalteu method with some modification [28]. The absorbance was measured at 680 nm after 20 minute incubation. The total phenolic content was calculated from the calibration curve, and the results were expressed as μg of gallic acid equivalent per g of dry extract.

The analysis of total flavonoid content (TFC) was carried out using the modified Lamaison and Carnet method [29,30]. The absorbance was read at 430 nm after 30 minute incubation against a blank solution containing methanol instead of the test sample. The total flavonoid content was calculated from a calibration curve, and the results were expressed as μg quercetin per g of dry extract.

2.4 Antioxidant activity

The DPPH radical scavenging capacity of each extract (with concentrations 1 g of dry plant material per mL) was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]) assay with some modifications [28,31].

The absorbance was measured at 517 nm after 30 minute incubation. DPPH[•] solution was used as control. Reduction rate of DPPH[•] was calculated by the following equation.

$$\text{Inhibition (\%)} = [(A_c - A_s)/A_c] \times 100$$

where A_c is the absorbance of control and A_s is the absorbance of extract.

2.5 LC-ESI-MS/MS analysis

The content of tiliroside and flavonoid aglycones was determined by reversed-phase high-performance liquid chromatography and electrospray ionization mass spectrometry (LC-ESI-MS/MS). For this purpose, an Agilent 1200 Series HPLC system (Agilent Technologies, USA) equipped with a binary gradient solvent pump, a degasser, an autosampler and column oven connected to 3200 QTRAP Mass spectrometer (AB Sciex, USA) was used.

Tiliroside was separated at 25°C, on the Zorbax SB-C18 column (2.1 x 50 mm, 1.8- μm particle size; Agilent Technologies, USA) using 5 μl injection. The solvent used were water containing 0.1% HCOOH (A) and acetonitrile (B). The flow rate was 200 $\mu\text{l}/\text{min}$ and the gradient were as follows: 0–0.5 min 10% B; 1–1.5 min 50% B; 2.5–3.5 min 80% B; 4–5.5 min 7% B; 5–8 min 90% B.

The negative-ion mode ESI was used. The optimum values of the source parameters were: capillary temperature 500°C, curtain gas 30 psi, negative ionization mode source voltage –4500 V. Nitrogen was used as the curtain and collision gas. Data acquired was processed using Analyst 1.5 software from AB Sciex.

Multiple reaction monitoring (MRM) was used for quantitative analysis of tiliroside and flavonoid aglycones. The calibration curves obtained in the MRM mode were used for quantification of analytes. The identified compounds were quantified based on their peak areas and comparison with a calibration curve for the corresponding standards. The linearity range for the calibration curve was specified and the limit of detection (LOD) and quantification (LOQ) for tiliroside and flavonoid aglycones were determined.

A summary of the optimized parameters for the quantitative analysis and validation parameters of tiliroside determined by the LC-ESI-MS/MS method are shown in Table 3.

The content of flavonoid aglycones was determined by the LC-ESI-MS/MS method previously described by Pietrzak et al. [32]. Table 4 summarizes the optimized parameters for the quantification of analysed flavonoid aglycones.

Table 3: Summary of optimized parameters for quantitative LC-ESI-MS/MS analysis of tiliroside.

Compound	Retention time [min]	Q1 [m/z]	Q3 [m/z]	LOD [ng/mL]	LOQ [ng/mL]	R ²	Linearity range [ng/mL]
Tiliroside	6.17	302.7	124.9 284.8	0.5	2	0,9995	2-500

Table 4: Summary of optimized parameters for quantitative analysis of flavonoid aglycones.

Compound	Retention time [min]	Q1 [m/z]	Q3 [m/z]	LOD [ng/mL]	LOQ [ng/mL]	R ²	Linearity range [ng/mL]
Eriodictyol	7.07	286.7	134.9 150.9	0.01	0.015	0.9980	0.015–5
Quercetin	7.13	300.7	150.9 178.8	0.0015	0.003	0.9960	0.03–3.1
Apigenin	7.77	268.8	117 106.8	0.002	0.004	0.9955	0.01–6.3
Naringenin	7.81	270.8	150.9 116.8	0.025	0.03	0.9980	0.04–3.3
Kaempferol	7.94	284.7	116.8 93	0.025	0.03	0.9990	0.04–20
Isorhamnetin	8.09	314.7	299.7 150.9	0.015	0.03	0.9965	0.04–60
Sakuranetin	9.89	284.7	118.9 164.8	0.035	0.04	0.9955	0.07–7.2

2.6 Statistical analysis

The results were expressed as a mean $\pm$ standard deviation (SD) of three repeats. In addition, the Pearson correlation coefficient between the two components (i.e. total phenolic and flavonoid content) and antioxidant activity was determined.

Calculations and surface chart 3W were performed in STATISTICA 10.0 (StatSoft).

3 Results and discussion

Our study was designed to find the optimal conditions of supercritical carbon dioxide extraction for the maximum increase of content of tiliroside in the extracts from *Tilia* L. flowers.

Although the main disadvantage of supercritical carbon dioxide extraction is the expensive equipment and the analysis process, the possibility of using a lower temperature during extraction avoids thermal degradation of the labile compounds and makes this method attractive. In addition, the lack of light and oxygen protects the extracts from oxidation and loss of biological activity [17,33,34].

Due to the non-polar nature of supercritical carbon dioxide, addition of 5% and 10% ethanol have been

used as polar co-solvent to change polarity and increase solubility.

30 different *Tilia* L. extracts were investigated. The effect of a range of solvents, temperature, pressure and time on tiliroside content in the extracts was studied. The results were expressed as mean $\pm$ standard deviation (SD) for three repeats. The extraction yields were calculated as percentage of dry extracts obtained from 1 g of raw material.

Determination of tiliroside was performed by the LC-ESI-MS/MS method as a rapid, simple and reliable analytical tool.

The total phenolic content (TPC) and flavonoid content (TFC) in SC-CO₂ extracts and their antioxidant activity measured by DPPH• method were also determined.

The obtained results of efficiency of extraction, content of tiliroside, TPC and TFC and antioxidant activity are shown in Table 5 and 6.

Statistical analysis is an effective method of collecting and summarizing data. In chemical research it is used to summarize (descriptive statistics) experimental data and variance (standard deviation, standard error of the mean, confidence interval or range) and conduct hypothesis testing. Statistics can be very helpful in formulating experimental design and drawing appropriate inferences from the collected data, too [35].

Table 5: Efficiency of extraction, content of tiliroside, TPC, TFC and antioxidant activity of SC-CO₂ extracts with 5% of ethanol obtained from *Tilia L.* flowers.

Sample name	Efficiency of extraction [%] ^a	Content of tiliroside [$\mu\text{g g}^{-1}$ of dry extract]	TPC [$\mu\text{g GA g}^{-1}$ of dry extract]	TFC [$\mu\text{g Q g}^{-1}$ of dry extract]	Inhibition DPPH* [%] ^b
Ex. 1/5%	0.66	4.024 $\pm$ 0.172	187.47 $\pm$ 5.30	36.96 $\pm$ 1.70	27.2 $\pm$ 1.7
Ex. 2/5%	1.49	<LOQ ^c	92.53 $\pm$ 2.82	46.37 $\pm$ 1.16	30.7 $\pm$ 0.7
Ex. 3/5%	2.53	2.121 $\pm$ 0.195	87.70 $\pm$ 2.07	104.65 $\pm$ 3.10	37.8 $\pm$ 1.1
Ex. 4/5%	0.96	<LOQ ^c	113.72 $\pm$ 4.64	100.15 $\pm$ 2.35	30.0 $\pm$ 0.8
Ex. 5/5%	0.79	6.938 $\pm$ 0.898	91.84 $\pm$ 0.35	47.28 $\pm$ 2.16	28.3 $\pm$ 2.5
Ex. 6/5%	1.02	<LOQ ^c	131.31 $\pm$ 1.10	29.30 $\pm$ 0.37	48.9 $\pm$ 2.7
Ex. 7/5%	1.04	<LOQ ^c	155.92 $\pm$ 7.07	68.50 $\pm$ 2.71	39.3 $\pm$ 0.7
Ex. 8/5%	1.62	<LOQ ^c	200.23 $\pm$ 7.62	57.07 $\pm$ 1.66	63.5 $\pm$ 2.0
Ex. 9/5%	0.86	6.152 $\pm$ 0.066	285.03 $\pm$ 0.11	44.47 $\pm$ 1.97	37.8 $\pm$ 1.0
Ex. 10/5%	1.31	<LOQ ^c	158.33 $\pm$ 4.66	57.52 $\pm$ 2.40	56.5 $\pm$ 0.7
Ex. 11/5%	0.63	<LOQ ^c	83.56 $\pm$ 3.82	74.06 $\pm$ 3.63	37.5 $\pm$ 1.5
Ex. 12/5%	0.99	<LOQ ^c	101.35 $\pm$ 1.22	212.46 $\pm$ 2.66	29.9 $\pm$ 0.8
Ex. 13/5%	1.37	9.268 $\pm$ 0.145	204.19 $\pm$ 2.52	115.34 $\pm$ 5.09	37.7 $\pm$ 0.5
Ex. 14/5%	1.18	2.721 $\pm$ 0	155.89 $\pm$ 5.35	114.68 $\pm$ 2.23	42.9 $\pm$ 0.6
Ex. 15/5%	0.98	2.927 $\pm$ 0.248	199.76 $\pm$ 4.47	124.68 $\pm$ 4.92	39.6 $\pm$ 2.0
Average	1.16	4.879	156.09	82.23	-

a-calculated as percentage of dry extract obtained from 1g of raw material; b-antioxidant activity, % inhibition of DPPH* for extract (1 g of dry plant material per 1 mL); c-<LOQ - below the quantitation limit

In order to obtain the maximum amount of tiliroside and other flavonoids in linden SC-CO₂ extracts the experimental traditional optimization (one at a time method) was carried out. In the present research the independent variables were: time of extraction, pressure and temperature (Table 1 and 2). The other effective variable was the amount of co-solvent (ethanol) obtained by screening experiment as 5% and 10% ethanol. Total yield of extract, amount of tiliroside, total phenolic content (TPC), total flavonoid content (TFC) and additionally antioxidant activity were response variable (dependent variable).

The obtained responses are shown in Tables 5 and 6. The average extraction yields obtained using different ethanol concentrations was comparable (1.16% and 1.19% for 5% and 10% of ethanol concentration, respectively). However, there was a significant increase of tiliroside content, TPC and TFC for extracts with 10% ethanol, especially for tiliroside where around a ten-fold increase was recorded.

The tiliroside content for 5% ethanol concentration ranges between 2.121 $\pm$ 0.195 $\mu\text{g per g}$ of dry extract and 9.268 $\pm$ 0.145 $\mu\text{g per g}$ of dry extract for extracts Ex. 3/5%

and Ex. 13/5%, respectively. So far, for 10% ethanol the lowest content of tiliroside was 3.742 $\pm$ 0.289 $\mu\text{g per g}$ of dry extract for Ex. 8/10% and the highest content of tiliroside was 309.033 $\pm$ 5.50 $\mu\text{g per g}$ of dry extract for Ex. 11/10%. So, the use of higher ethanol concentration during extraction slightly increases the extraction efficiency and strongly influences the content of tiliroside.

Based on the surface chart 3W model it is possible to determine optimal extraction conditions for using 5% and 10% ethanol concentration. Figures 1, 2 and 3 relate to 5% ethanol concentration. The analysis of all extracts (Figure 1) indicated that the extraction of tiliroside increased with the increase of temperature and extraction time. The use of higher pressure and temperature also increased the extraction of tiliroside (Figure 2). Figures 4, 5 and 6 relate to 10% ethanol concentration. The analysis of all data indicated that the extraction of tiliroside decreased with the increase of temperature and pressure.

The extraction time is a very important parameter affecting the quantity of compounds extracted. As we can see in Figures 1-6 higher addition of ethanol concentration shortened the extraction time.

Table 6: Efficiency of extraction, content of tiliroside, TPC, TFC and antioxidant activity of SC-CO₂ extracts with 10% of ethanol obtained from *Tilia L.* flowers.

Sample name	Efficiency of extraction [%] ^a	Content of tiliroside [$\mu\text{g g}^{-1}$ of dry extract]	TPC [$\mu\text{g GA g}^{-1}$ of dry extract]	TFC [$\mu\text{g Q g}^{-1}$ of dry extract]	Inhibition DPPH* [%] ^b
Ex. 1/10%	0.96	<LOQ ^c	179.53 $\pm$ 5.52	123.77 $\pm$ 4.69	54.6 $\pm$ 1.3
Ex. 2/10%	1.00	<LOQ ^c	252.94 $\pm$ 0.44	107.95 $\pm$ 5.21	60.4 $\pm$ 0.2
Ex. 3/10%	1.34	<LOQ ^c	241.51 $\pm$ 7.71	145.76 $\pm$ 5.13	65.5 $\pm$ 2.5
Ex. 4/10%	1.13	<LOQ ^c	254.68 $\pm$ 3.84	166.93 $\pm$ 1.74	76.1 $\pm$ 0.6
Ex. 5/10%	0.81	<LOQ ^c	113.70 $\pm$ 4.01	123.48 $\pm$ 3.86	54.8 $\pm$ 1.3
Ex. 6/10%	0.87	23.326 $\pm$ 3.017	211.04 $\pm$ 3.04	82.40 $\pm$ 3.77	60.9 $\pm$ 1.0
Ex. 7/10%	1.32	73.036 $\pm$ 12.974	260.09 $\pm$ 5.63	145.90 $\pm$ 5.34	74.6 $\pm$ 1.9
Ex. 8/10%	1.42	3.742 $\pm$ 0.289	287.30 $\pm$ 6.27	159.48 $\pm$ 7.09	73.9 $\pm$ 1.0
Ex. 9/10%	0.96	50.785 $\pm$ 0.444	184.00 $\pm$ 5.62	86.61 $\pm$ 4.46	73.7 $\pm$ 1.2
Ex. 10/10%	1.29	10.902 $\pm$ 1.078	242.05 $\pm$ 8.81	112.71 $\pm$ 2.02	73.6 $\pm$ 0.4
Ex. 11/10%	1.26	309.033 $\pm$ 0	273.87 $\pm$ 8.57	148.03 $\pm$ 5.96	75.8 $\pm$ 1.1
Ex. 12/10%	1.49	18.578 $\pm$ 0.952	245.22 $\pm$ 7.74	154.45 $\pm$ 6.93	72.6 $\pm$ 1.0
Ex. 13/10%	1.22	8.485 $\pm$ 1.297	303.99 $\pm$ 5.50	105.82 $\pm$ 2.66	75.8 $\pm$ 0.6
Ex. 14/10%	1.47	25.798 $\pm$ 1.152	267.37 $\pm$ 8.25	153.00 $\pm$ 0.017	74.7 $\pm$ 0.4
Ex. 15/10%	1.35	8.752 $\pm$ 0.741	215.80 $\pm$ 10.17	101.53 $\pm$ 5.02	72.6 $\pm$ 0.4
Average	1.19	53.244	235.53	133.01	-

a-calculated as percentage of dry extract obtained from 1g of raw material; b- antioxidant activity, % inhibition of DPPH* for extract (1 g of dry plant material per 1 mL); c-<LOQ - below the quantitation limit

Extraction pressures investigated were from 100 to 220 bar and it was found that the content of tiliroside increased with higher extraction pressure. This is due to compounds with higher molecular mass (for example tiliroside as a glycosidic kaempferol ester) requiring a higher pressure for extraction.

In the case of the extraction temperature used, it was noted that higher temperatures increase the extraction of tiliroside. However, there is the risk that higher temperatures could cause thermal decomposition of labile compounds.

The overall optimum set of conditions found by maximizing all the dependent variables were as follows; for 5% ethanol concentration temperature 65°C, time 45–50 minutes and pressure 200 bar, and for 10% ethanol concentrations temperature 65°C, time 15 minutes and pressure 200–220 bar.

Table 5 and 6 show that the highest amount of TPC was 285.03 $\pm$ 0.11 μg per g of dry extract was obtained for Ex. 9/5% and 303.99 $\pm$ 5.50 μg per g of dry extract for Ex. 13/10%. The lowest polyphenol amount was observed for Ex. 11/5% (83.56 $\pm$ 3.82 μg per g of dry extract) and for Ex. 5/10% (113.7 $\pm$ 4.01 μg per g of dry extract).

Figure 1: Surface chart 3W of the content of tiliroside vs. temperature and pressure of extraction (for 5% ethanol concentration) (Smoothing of smallest squares weighted by distance).

The highest result of TFC was obtained for Ex. 12/5% (212.46 $\pm$ 2.66 μg per g of dry extract). In turn, the lowest flavonoid content (47.28 $\pm$ 2.16 μg per g of dry extract) was obtained for Ex. 5/5%. For 10% ethanol concentration, the total of flavonoid content varied between 82.40 $\pm$ 3.77 μg

Figure 2: Surface chart 3W of the content of tiliroside vs. time and pressure of extraction (for 5% ethanol concentration) (Smoothing of smallest squares weighted by distance).

Figure 4: Surface chart 3W of the content of tiliroside vs. temperature and time of extraction (for 10% ethanol concentration) (Smoothing of smallest squares weighted by distance).

Figure 3: Surface chart 3W of the content of tiliroside vs. temperature and time of extraction (for 5% ethanol concentration) (Smoothing of smallest squares weighted by distance).

Figure 5: Surface chart 3W of the content of tiliroside vs. temperature and pressure of extraction (for 10% ethanol concentration) (Smoothing of smallest squares weighted by distance).

per g of dry extract (Ex. 6/10%) to 166.93 ± 1.74 µg per g of dry extract (Ex. 4/10%).

In our study, it was clear that the content of tiliroside, total phenolic and flavonoid increase with a higher concentration of ethanol. Tables 5 and 6 show the average content of TPC and TFC obtained from the linden extracts depending on the ethanol concentration. A higher ethanol concentration increased the phenolic and flavonoid content due to a similar polar solvent dissolving a similar polar solute. The addition of 10% ethanol concentration significantly improved the elution conditions of polyphenols and flavonoid compounds from the plant matrix. The study by Bitencourt et al. [36] indicated that

10% ethanol concentration as co-solvent has increased considerable of phenolic acids. Ethanol is capable of hydrogen-bonding and dipole–dipole interactions with phenols, so it is a good carbon dioxide co-solvent for extraction of the phenolic components.

The results obtained show that the content of tiliroside in the sample Ex. 4/10% with the highest concentration of flavonoids was below the level of quantification.

However the sample Ex. 11/10% extracted at higher pressure contains the highest amount of tiliroside and high concentration of TPC and TFC. So, in order to check and compare the flavonoid composition for samples Ex. 4/10% (sample with the highest concentration of flavonoids) and

Table 7: Flavonoid aglycones content for extracts with higher amount of tiliroside (Ex. 11/10%) and flavonoids (Ex. 4/10%) obtained from *Tilia* L. Values are presented in mean $\pm$ SD, n=3.

Sample name	Flavonoid aglycones ($\mu\text{g per g dry extract}$)					
	Eriodictyol	Quercetin	Apigenin	Naringenin	Kaempferol	Isorhamnetin
Ex. 4/10%	17.698 ± 0.616	8.364 ± 0.264	4.151 ± 0.239	72.444 ± 1.131	27.022 ± 1.006	10.338 ± 0.415
Ex. 11/10%	28.447 ± 0.560	32.330 ± 1.345	7.892 ± 0.202	74.485 ± 0.224	42.314 ± 1.569	11.569 ± 0.159

Figure 6: Surface chart 3W of the content of tiliroside vs. time and pressure of extraction (for 10% ethanol concentration) (Smoothing of smallest squares weighted by distance).

Ex. 11/10% (sample with the highest concentration of tiliroside) the LC MS/MS analysis of flavonoid aglycones was performed (Table 7).

As a result, the largest difference in the content of flavonoid aglycons for the analysed samples was obtained. The content of flavonol derivatives quercetin and kaempferol in Ex. 11/10% was about four and double Ex. 4/10%, respectively. However, the amount of other aglycones in the both samples was comparable. Since quercetrin, kaempferol and tiliroside belong to flavonol group of flavonoids, it can be concluded that the high pressure strongly and selectively affects not only the extraction of tiliroside but also other flavonol derivatives. Based on the above results, it can be assumed that the increase of the pressure has the highest influence on the content of flavonols in supercritical linden extracts.

Table 8 shows correlation coefficients between the data of TPC, TFC and extraction conditions (temperature, time and extraction pressure). The total flavonoid content (TFC) significantly depended on the extraction

time (Pearson correlation coefficients were 0.5415 for 5% ethanol concentration and 0.4269 for 10% ethanol concentration) and the extraction pressure (Pearson correlation coefficients were 0.4502 for 5% ethanol concentration and 0.6752 for 10% ethanol concentration). The total phenolic content (TPC), only for extracts with the addition of 10% ethanol concentration, was influenced by the temperature of extraction (Pearson correlation coefficients was 0.4121) and by the pressure of extraction (Pearson correlation coefficients was 0.5729).

Scavenging of DPPH[•] free radical is fast and is a common technique employed to evaluate antioxidative activity [25].

In this study the highest antioxidant activity (76.3% $\pm$ 0.4 inhibition of DPPH[•]) was observed for sample Ex. 4/10%, with the highest amount of phenolic compounds.

The correlation coefficients between the data of DPPH[•] inhibition and TPC and TFC (Table 9) suggest that TPC and TFC affect the antioxidant activity of *Tilia* L. flowers extracts obtained with the addition of 10% ethanol concentration. The research confirms that the use of higher ethanol concentration as co-solvent increases extraction of the phenolic compounds from plant material which has a significant impact on the antioxidant activity of the obtained plant extracts.

According to the literature reports, different extraction methods influence the content of phenolic compounds and flavonoids, among them tiliroside from *Tilia* L. [36–39]. Oniszcuk and Podgórski [26] have shown the higher content of tiliroside (1067.54 $\mu\text{g per g dry weight}$) after ASE extraction with 80% methanol. Nowak reported that content of tiliroside in *Tilia* L. inflorescences was 49.2–55.8 $\mu\text{g per g of dry weight}$ of obtained extracts after classical extraction with methanol and SPE [24]. In turn, depending on the part of the plant total content of phenolic ranges between from 16.494 to 17.548 mg GAE per g of dry sample, total content flavonoid varied between from 0.014 to 0.066 mg Q per g of dry sample. The scavenging activities expressed as EC₅₀ was 0.106–0.231 mg per ml of extract [39].

Table 8: Pearson correlation coefficients $R(X, Y)$ and coefficient of determination (R^2) showing relationship between TPC, TFC, % inhibition of DPPH*, temperature, time and pressure for *Tilia* L. extract (*Tiliae Inflorescentia*).

Parameter	R (X,Y)	R ²	R (X,Y)	R ²
	for 5% ethanol concentration		for 10% ethanol concentration	
TPC & temperature	0.0246	0.00	0.4121	0.17
TFC & temperature	-0.0426	0.00	-0.0976	0.01
% inhibition of DPPH* & temperature	0.1251	0.01	0.2804	0.08
TPC & time	-0.3082	0.10	0.1789	0.03
TFC & time	0.5415	0.29	0.4269	0.18
% inhibition of DPPH* & time	-0.3703	0.14	0.2978	0.09
TPC & pressure	-0.2049	0.04	0.5729	0.33
TFC & pressure	0.4502	0.20	0.6752	0.46
% inhibition of DPPH* & pressure	-0.3703	0.14	0.4344	0.19

Table 9: Pearson correlation coefficients $R(X, Y)$ and coefficient of determination (R^2) showing relationship between TPC, TFC and % inhibition of DPPH* for *Tilia* L. extracts (*Tilia Inflorescentia*).

Parameter	R (X,Y)	R ²
for 5% ethanol concentration		
TPC & % inhibition of DPPH*	0.1704	0.03
TFC & % inhibition of DPPH*	-0.1402	0.02
for 10% ethanol concentration		
TPC & % inhibition of DPPH*	0.7210	0.52
TFC & % inhibition of DPPH*	0.4272	0.18

Jabeur et al. [40] assessed antioxidant potential of *Tilia platyphyllos* L. hydroethanolic extract. Total flavonoid was 50.4 mg Q per g of dry extract and 62.0 mg GA per g of dry extract. The value of EC_{50} was 105 μ g per ml.

The results in our study revealed that the supercritical linden extracts were not a rich source of polyphenols.

4 Conclusion

In our research for the first time, the influence of conditions supercritical carbon dioxide extraction for content of polyphenols, mainly tiliroside, in linden extracts were analysed. The optimum extraction conditions to obtain the highest content of tiliroside were determined statistically.

All tested parameters of extraction had a significant effect on the yield of supercritical carbon dioxide extraction. Generally, higher content of tiliroside, total flavonoid and phenolic content was achieved for pressure

of 220 bar, temperature 65°C, time 15 minutes and addition of 10% ethanol concentration. Moreover, a increased of extraction pressure and average temperature improved the extraction efficiency in a shorter time. It should also be noted that the high concentration of ethanol has a significant influence on the content of active phenolic compounds and the biological activity of the extracts.

Despite many advantages, supercritical carbon dioxide extraction is not an appropriate method for obtaining tiliroside and other phenolic compounds from the linden extracts.

Conflict of Interest: Authors state no conflict of interest.

Ethical approval: The conducted research is not related to either human or animal use.

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