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Evaluation of the volatiles' chemical profile and antibacterial activity of *Lavandula stoechas* L. extracts obtained by supercritical carbon dioxide

Jelena Vladić [a, b], Igor Jerković [c], Sandra Svilović [c], Valentina Pavić [d], Kristian Pastor [b], Alexandre Paiva [a], Stela Jokić [e], Silvia Rebocho [a], Ana Rita Duarte [a, *]

[a] *Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516, Caparica, Portugal*
[b] *Faculty of Technology, University of Novi Sad, Bulevar cara Lazara 1, 21000, Novi Sad, Serbia*
[c] *Faculty of Chemistry and Technology, University of Split, Ruđera Boškovića 35, 21000, Split, Croatia*
[d] *Department of Biology, Josip Juraj Strossmayer University Osijek, Cara Hadrijana 8/a, 31000, Osijek, Croatia*
[e] *Faculty of Food Technology, Josip Juraj Strossmayer University Osijek, Franje Kuhača 18, 31000, Osijek, Croatia*

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ABSTRACT

The goal of this study was to establish a green methodology for obtaining safe, high-quality, and potent antibacterial extracts of *Lavandula stoechas* flowers. Supercritical carbon dioxide ($scCO_2$) at different conditions (pressure 100–300 bar, temperature 40 and 60 °C, and CO_2 flow 10–30) was applied. Moreover, the impact of the parameters on the extraction yield, chemical profile of the extracts, and the activity against Gram-positive and Gram-negative bacteria was investigated. $ScCO_2$ extraction kinetics was investigated by modelling the extraction curves using the models described by Brunner, Martinez, and their modifications. *In vitro* assays were applied to estimate the susceptibility of *Escherichia coli*, *Pseudomonas aeruginosa, Bacillus subtilis*, and *Staphylococcus aureus* to extracts. A pattern recognition approach was applied to explore the correlations between the employed $scCO_2$ parameters, detected volatile compounds, and extracts' antibacterial properties. The achieved yield ranged from 1.17 to 2.4% (w/w), with oxygenated monoterpenes fenchone (5.76–22.72%) and verbenone (8.04.-18.65%) as the most abundant ones. The most significant susceptibility of Gram-positive and Gram-negative bacteria was attributed to extracts obtained at 200 bar, with minimal inhibitory concentration in the range 2.71 ± 1.31–20.69 ± 0.91 $mgmL^{-1}$ and 3.39 ± 0.48–34.28 ± 5.94 $mgmL^{-1}$, respectively. The obtained extracts represent safe, viable, and promising alternatives for tackling antimicrobial resistance. Moreover, by varying process conditions, it is possible to adjust the chemical profile and the activity of the extracts according to the target purpose. Furthermore, the requirements of clean and sustainable technologies, such as environmental preservation, rational use of renewable resources, and provision of natural, safe, and high-quality extracts can be met.

1. Introduction

According to the World Health Organization (WHO), antimicrobial resistance (AMR) represents one of the 10 global public health threats (World Health Organization, 2021). Moreover, there is an estimate that drug resistance infections play a part in close to 5 mil-

* Corresponding author.
E-mail address: ard08968@fct.unl.pt (A.R. Duarte).

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lion fatalities annually (Antimicrobial Resistance Collaborators, 2022). Due to the increase in AMR, it is expected that the additional health expenditure costs will increase to 1.2 trillion USD by 2050. To tackle this problem as efficiently as possible, the Global Action Plan on Antimicrobial Resistance, 2015 (World Health Organization, 2021) was created which includes multisectoral collaboration, and the development and implementation of actions that aim to address and reduce the spread of AMR. One of the four priorities in addressing the AMR is the promotion of research and development of new antimicrobials. At the same time, the mitigation of AMR is necessary to achieve sustainable development goals (SDGs).

Natural resources represent a potential for solving this global problem because the preference of people to use natural resources can result in diminishing the uncontrolled use of synthetic antibiotics, which is one of the main causes of such the global and far-reaching AMR. Aromatic medicinal plants are rich in terpenes, which can be divided into oxygenated and hydrocarbon terpenes (Rout et al., 2022). Oxygenated terpenes are considered to be potent antimicrobial agents due to their specific chemical structure and the presence of an aromatic nucleus containing polar functional groups which enable their effective antimicrobial activity (Guimarães et al., 2019). Species from the *Lavandula* genus (*Lamiaceae*) are among the species rich in oxygenated terpenes.

Aromatic medicinal plants that belong to the *Lavandula* genus are highly valued in different areas including the production of pharmaceuticals, cosmetic, perfume, fragrance, repellent, and food products. According to the Brandessence Market Research, the global lavender oil market was valued at USD 34.2 million in 2020 and expected to reach USD 49.4 million by 2027 with the compound annual growth rate (CAGR) of 5.4% over the forecast period (Brandessence Market Research, 2021). Until now, 39 lavender species have been established and they are present across the world, however, *L. angustifolia, L. x intermedia*, and *L. latifolia*, which are the most cultivated species, are the only ones that possess commercial importance. The species that has oil of higher quality in lower content is *L. angustifolia*, while the other two species are characterised by a higher level of oil of lower quality and less commercial value (Wells et al., 2018). Namely, the higher presence of camphor in the oil of these two species contributes to the formation of a specific and unwanted odour, which contributes to the oil's poor quality (Lane et al., 2010). Other *Lavandula* species are not exploited sufficiently although they possess biologically significant components and are commonly used in folk medicine. One of the species of high ethnopharmacological importance is *L. stoechas*. This aromatic medicinal plant is widely present in the Mediterranean region and found across the world (Ez Zoubi et al., 2020). The biological potency, presence, and wide acceptance of *L. stoechas* point to a significant economic potential of this herbal species.

There is great variability in the chemical profile of the essential oil of *L. stoechas* originating from different geographical locations which can be caused by different physiological and environmental factors (Chizzola, 2013). A uniform chemical profile that would further secure the quality of the product can be achieved by cultivation under controlled conditions. This would have to be preceded by scientific studies in the areas of improvement and development of the extraction procedures that could provide an adequate utilisation of this natural resource and attainment of a high-quality product, in accordance with the green principles. Scientific validation can impact the higher use of this natural resource.

The majority of studies that investigated the *L. stoechas* species were focused on the chemical profile of the essential oil obtained by hydrodistillation (Labri et al., 2022; Küçük et al., 2019; Karaca et al., 2018), steam distillation (Asghari et al., 2016; Cherrat et al., 2014), and microwave-assisted extraction of the essential oil (Sadani and Shakeri, 2016). These procedures represent simple processes of obtaining oil, however, they do not provide an adequate, rational use of the material and can cause thermal degradation of the components. Also, it is not possible to tune the process conditions, hence it is not possible to adjust the selectivity towards certain components of interest and, consequently, the quality (Gavarić et al., 2021; Morsy, 2020).

Supercritical carbon dioxide ($scCO_2$) extraction represents an alternative to conventional procedures because it employs the use of an environmentally safe solvent with a solvation power that can be varied easily. Moreover, it provides a high selectivity and efficiency of the extraction with the attainment of safe extracts (Gavarić et al., 2021). The $scCO_2$ possesses mild critical conditions of temperature 31.1 °C and pressure 73.8 bar, it is easily removed from products by decompression, providing a safe product without the need for additional purification. Also, CO_2 is non-flammable, low-cost, available, inert, and chemically stable (Čižmek et al., 2021). Because of its positive characteristics, this solvent is increasingly implemented in various industries (Nikolai et al., 2019).

The studies in the area of $scCO_2$ extraction of *L. stoechas* are very scarce compared to other species of the Lavandula genus, such as *L. hybrida*, *L. luisieri*, *L. angustifolia*, *L. viridis* Giménez-Rota et al. (2019); Jerković et al. (2017); Kiran Babu et al., 2016; Babu et al. (2016); Kamali et al. (2015); Costa et al. (2012). Marongiu et al. (2010) explored the antimicrobial properties of *L. stoechas* extracts attained at 90 bar and 40 °C and determined that they do not exhibit antimicrobial inhibitory activity, while Topal et al. (2008) conducted $scCO_2$ extraction and investigated the antioxidative activity of extracts of several different herbal species among which was *L. stoechas* spp. However, the conditions that were used were not defined precisely (pressure ranging from 20 to 30 MPa and temperatures from 40 to 60 °C, with a constant CO_2 flow rate of 3 mL/min for 5 h). Akgün et al. (2000) conducted the $scCO_2$ extraction of the Turkish species L. *stoechas* subspecies *cariensis* Boiss and investigated the impact of the process conditions (80–140 bar pressure, 35–50 °C temperature, and $1.092–2.184 \times 10^{-3}$ kg/min CO_2 flow rate) on the extraction yield. In their following study, Akgün et al. (2001) conducted the supercritical fluid extraction with the process conditions in the ranges of 80–120 bar pressure, 35–55 °C temperature, 700–1300 rpm stirring rate, and 2–4 h extraction time, investigating the impact on the overall extraction yield. Moreover, by applying the conditions of 80 bar and 35 °C, a semicontinuous system was used and the impact of the flow rates on the extraction yield and four major components was explored. Therefore, the studies that comprehensively investigate the $scCO_2$ extraction of *L. stoechas* with the aim to establish a detailed impact of the extraction parameters on the quality, antibacterial activity, and yield of extracts had not been conducted.

Considering the bioactivity potential of this herbal species, its wide geographic presence, acceptance among consumers, and the necessity for the development of safe and clean processes, this work focused on the design of sustainable green process of obtaining lipophilic antibacterial extracts of *L. stoechas* by applying the $scCO_2$ extraction. The objective of this study was to investigate in detail

the impact of the process parameters temperature, pressure, and CO_2 flow on (i) the extraction yield of *L. stoechas* by modelling the extraction curves and (ii) the chemical composition of the volatiles (analyzed by gas chromatography-mass spectrometry (GC/MS)) by applying the pattern recognition approach for dimensionality reduction, principal component analysis (PCA), and (iii) antibacterial activity of extracts against Gram-positive and Gram-negative bacteria. This study represents the first scientific report with a systematic evaluation of the relation between the parameters of extraction and the yield, chemical profile, and antibacterial activity of the *L. stoechas* extracts. Developing the $scCO_2$ extraction methodology would provide the attainment of safe and potent extracts of *L. stoechas* that have the potential to be applied in different industrial productions.

2. Material and methods

2.1. Material

Lavandula stoechas L. ssp. *stoechas* flowers were purchased in Celeiro, Lisbon, Portugal. *L. stoechas* is not an endangered species, and as a commercially registered product, the collection of the plant material complies with relevant institutional, national, and international guidelines and legislation. The mean particle size (0.31 ± 0.05 mm) of the material was determined using the vibration sieve sets (CISA, Cedaceria, Spain).

2.2. Supercritical carbon dioxide extraction

$ScCO_2$extraction was carried out in a lab-scale apparatus with the main specifications as follows: pneumatic pump (Williams P250V300), mass flow meter (Rheonik RHM 007), tubular extractor (316SS; 570 mm length, 24 mm I.D.; HiP), back-pressure regulator (Tescom Europe, model 26–1700, Selmsdorf, Germany), and separator (Swagelok 316L-HDF4-500).

The extractions were performed using 30 g of plant material under the following conditions: pressure 100, 200, 300 bar, temperature 40 and 60 °C, CO_2 flow 10, 20, 30 g/min, and extraction time 3 h. Moreover, the extraction kinetics was monitored during the extraction time intervals (0.5, 1, 1.5, 2, 2.5, and 3 h). The pressure and temperature in the separator were 50 bar and 40 °C, respectively. The extraction conditions were selected based on a previous study (Nađalin et al., 2014). The obtained extracts were placed in glass bottles and stored at 4 °C prior to further analysis. Each extraction was performed in triplicate. The extraction yield was determined gravimetrically and calculated using the following equation (Eq. 1):

$$\textbf{Extraction yield } (\%) = \frac{\textit{mass of obtained extract } (g)}{\textbf{mass of feed material } (g)} \times 100$$

To determine the initial content of the solute in the solid phase (x_0) to be used for modelling the extraction kinetic curves, the Soxhlet extraction was conducted. For the extraction, 10 g of the milled plant material and 150 mL of methylene chloride were used. The Soxhlet extraction was performed using the Soxhlet apparatus with a connected reflux condenser for 6 h. The extraction yield (x_0) of 2.3% (w/w) was determined after solvent evaporation.

2.3. Modelling the extraction kinetic curves

The extraction kinetic curves of *L. stoechas* extraction were fitted using two kinetic models, which are usually used for the supercritical fluid extraction, and their modified versions.

The first model used was proposed by Brunner (1984). It represents a specific case of Fick's law, and is expressed as (Eq. 2):

$$Y = x_0 \left(1 - e^{-k\,t}\right)$$

where Y_E is extraction yield (%), x_0 is the initial content of the solute in the solid phase obtained via Soxhlet (%), k is the constant rate (min^{-1}), and t is the extraction time (min).

Experimental data can also be tested using the so-called modified Brunner which is a model with two adjustable parameters. Bojanić et al. (2019) included a second adjustable parameter, Y_∞, and removed x_0 from the Brunner model. The Modified Brunner model is expressed as (Eq. 3):

$$Y = Y_\infty \left(1 - e^{-k\,t}\right)$$

where Y_∞ represents the yield obtained for infinite time of extraction process (%).

The next model presumes that the extract is a mixture of compounds (Marinho et al., 2019) and was developed considering the mass balance of the extraction bed while neglecting the accumulation and axial dispersion in the fluid phase (Piva et al., 2018; Martínez et al., 2003). The kinetic study herein presented considered the extract as one compound (pseudocomponent), therefore, the model used was presented as a single-component (Eq. 4):

$$m = \frac{m_t}{exp\left(B\,t_m\right)} \left\{ \frac{1 + exp\left(B\,t_m\right)}{1 + exp\left(B\,t_m - t\right)} - 1 \right\}$$

If the Martínez model is expressed in the form of yield, Eq. 4 becomes Eq. 5 (Jokić et al., 2015):

$$Y = \frac{x_0}{\exp(B\,t_m)} \left\{ \frac{1+\exp(B\,t_m)}{1+\exp(B\,t_m - t)} - 1 \right\}$$

where B and t_m are the adjustable parameters, m_t is the total mass of compounds (solute).

Parameter t_m represents the time in which the extraction rate reaches its maximum and parameter B is related to the extraction rate (Galvão et al., 2013; Martínez et al., 2003). In cases when m_t (x_0) is not determined experimentally, it also needs to be adjusted (Martínez et al., 2003).

The last tested model was the Martínez model modified by adding the adjustable parameter Y_∞ instead of x_0 (Eq. 6):

$$Y = \frac{Y_\infty}{\exp(B\,t_m)} \left\{ \frac{1+\exp(B\,t_m)}{1+\exp(B\,t_m - t)} - 1 \right\}$$

2.4. Gas chromatography-mass spectrometry (GC-MS) analysis

Agilent 8890 gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) connected to a mass spectrometer (series 5977E, Agilent Technologies, Palo Alto, CA, USA) was used for the analysis. The components of *L. stoechas* lipophilic extracts were separated on HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm, Agilent Technologies, Palo Alto, CA, USA) after the extracts were diluted in hexane (0.01 g/1 mL). The injector temperature was set at 250 °C with the injected sample of 3 μL in split mode of 1:50. Helium of 99.99% purity was used as a carrier gas in a constant flow regime of 1 mL/min. The following temperature program was set at 70 °C for 2 min and a temperature ramp of 3 °C/min to reach 200 °C, which was then maintained at constant temperature for 15 min. The separated components were analyzed with mass spectrometry (70 eV) with a scanning *m/z* range of 30–300. The injector and detector temperatures were 250 and 300 °C, respectively. Qualitative identifications of the compounds were performed using Wiley 9 (Wiley, New York, NY, USA) and NIST 17 (National Institute of Standards and Technology, Gaithersburg, MD, USA) mass spectral libraries as well as the literature data of retention indices calculated with C_9–C_{25}alkanes. The analysis of each sample was performed in three replicates and the results are expressed as mean data.

2.5. Data analysis

The concordance between the experimental data and the calculated values obtained by using the kinetic models was validated by the average absolute relative deviation (AARD) (Eq. 7). The values of AARD up to 5% were considered acceptable.

$$\mathrm{AARD} = \frac{1}{n} \cdot \sum_{i=1}^{n} \left| \frac{y_{exp} - y_{cal}}{y_{exp}} \right|$$

Moreover, n is the number of experimental points, y_{exp} is the yield value obtained in the experiment, y_{cal} is the yield value calculated by the kinetic model.

Multivariate method, the principal component analysis (PCA), was applied to reduce the data and observe the grouping, distribution, and qualification, and observing the connection between the monitored variables (Tharwat, 2016). Moreover, the extracts obtained under various conditions of the $scCO_2$ extraction process ((1) temperature 40 and 60 °C and pressure 100, 200, and 300 bar with a constant flow of 20 g/min; (2) temperature 40 and 60 °C and CO_2 flow 10, 20, and 30 g/min under constant pressure of 200 bar)) were imported into an open-source JASP software (https://jasp-stats.org/). After performing the PCA analysis, the bi-plots were obtained, visually representing the correlations between $scCO_2$ extracts obtained under varying conditions on one hand and volatile chemical information obtained by employing the GC/MS analysis on the other. The algorithm combined the original variables in the PCA bi-plots presented by the two-dimensional PC1 *vs* PC2 space of the initial dataset, which was thereby simplified and the dimensionality reduced.

2.6. Determination of antibacterial activity of $scCO_2$ extracts

The determination of minimal inhibitory concentrations (MIC) of obtained extracts was performed by modified broth microdilution method in Mueller Hinton Broth (Fluka, BioChemica, Germany) according to Clinical Laboratory and Standard Institute (CLSI) M7-A7 document (Weinstein, 2018) and described in our previous paper (Jokić et al., 2015). Briefly, four tested human pathogens reflecting gram-positive and gram-negative bacteria, *Bacillus subtilis, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*, isolated from various clinical specimens obtained from the Microbiology Service of the Public Health Institute of Osijek-Baranja County, Croatia were used. Midlogarithmic-phase bacterial cultures (5×10^5 CFU mL^{-1}) in Muller Hinton Broth (MHB) (Fluka, BioChemica, Germany) were added to two-fold serially diluted extracts (150–0.073 $mgmL^{-1}$). Each plate contained a growth control (bacterial inoculum without extracts) and negative background control (broth and ethanol). The antibacterial standard ciprofloxacin (Hospira, Hurley, Maidenhead, Berkshire, England, UK) was co-assayed under the same conditions in a concentration range 0.122–250 μgmL^{-1}. After incubation at 37 °C for 24 h, an additional 3-h incubation at 37 °C was performed with triphenyl tetrazolium chloride as a reducing agent and indicator for microbial growth. The MIC value was derived from triplicate analyses, normalized against the negative control, and expressed as milligrams per millilitre.

3. Results and discussion

3.1. Influence of extraction conditions on extraction yield

L. stoechas is a herbal species of high potential, creating the need to develop an efficient, safe, and clean process for the attainment of its lipophilic fraction. Furthermore, this study aimed to explain the correlation between process conditions and (i) extraction efficiency and (ii) chemical composition of the obtained products. The impact of pressure (100–300 bar) and temperature (40 and 60 °C) under a constant flow of CO_2 (20 g/min) on the yield of extraction of *L. stoechas* was investigated. The achieved yield was 1.17–2.16% (w/w). In addition, the impact of the flow of CO_2 (10–30 g/min) at constant pressure (200 bar) and temperature (40 and 60 °C) was investigated, with the achieved yield in the range from 1.51 to 2.4% (w/w).

At constant temperatures (40 and 60 °C) and constant flow (20 g/min), the increase in pressure caused an increase in yield due to the elevated density of CO_2 (Fig. 1). Namely, in the supercritical state, CO_2 is characterised by low viscosity and high diffusivity, allowing more efficient penetration into the material, and consequently leading to the improvement in mass transfer (Da Silva et al., 2016). Therefore, by increasing the pressure and density of $scCO_2$, its solvation power is improved, and a higher extraction yield is achieved. Nađalin et al. (2014) reported an increase in the yield of extraction of *L. officinalis* occurring with the increase in pressure from 100 to 200 bar under isothermal conditions (40 °C), while Akgün et al. (2000) increased the yield of the lipophilic fraction of *L. stoechas* subspecies *Cariensis Boiss* by increasing the pressure (from 80 to 140 bar).

Conversely, the impact of temperature on the yield was negative, thus with the increase in temperature at each applied pressure (constant CO_2 flow 20 g/min), a decrease in the extraction yield was observed. Namely, the elevation of the extraction temperature increases the vapour pressure of the components, and the increase in the yield of extraction can consequently occur. However, the increasing temperature lowers the density of CO_2 which, on the other hand, can cause a reduction in yield. Therefore, the impact of temperature on the extraction yield depends on the relation between the density of the fluid and vapour pressure of the components, that is, which parameter will have a greater effect (Confortin et al., 2019). In the case of the extraction of *L. stoechas*, the impact of the density of CO_2 was dominant, hence the extraction yield was reduced with elevated temperature and reduced density (Fig. 1). The lowest yield (1.17%, w/w) was achieved at conditions 100 bar and 60 °C, which corresponded to the lowest density of CO_2 (289.86 kg/m^3), while the highest yield was achieved at conditions 300 bar and 40 °C and the highest density of CO_2 (909.29 kg/m^3) (flow 20 g/min).

Furthermore, the impact of the flow (10–30 g/min) on the extraction yield was investigated, at a constant pressure of 200 bar and two different temperatures, 40 and 60 °C. At the lower temperature of 40 °C, the increase in the extraction yield with the increase of flow was observed (1.77–2.40%, w/w). The elevated flow of CO_2 enables a higher contact surface between the CO_2 and the material, increasing the concentration gradient. In addition, the resistance of the material is decreased causing external and internal mass transfer (Wei et al., 2021). Jerković et al. (2017) noted that the increased flow could significantly increase the extraction yield of *L. angustifolia* Mill.

However, the higher flow can also lead to a decrease in the yield of extraction due to the reduction in the time of contact between the solvent and the material, causing an inadequate release of components in the CO_2 (Wei et al., 2021). If the flow is too high, the solvent does not diffuse adequately through the pores of the material and the extraction rate is lowered. At the higher temperature of 60 °C, the highest yield (2.11%, w/w) was achieved with a flow of 10 g/min, while 20 g/min was the least adequate in terms of yield (1.51%).

Marongiu et al. (2010) stated a yield of 1.2–1.8% (w/w) of $scCO_2$ extraction at 90 bar and 40 °C, however, the study investigated three aromatic plants (lavender, thyme, and rosemary) and the exact yield of individual species was not defined. Akgün et al. (2001) determined that at 80 bar and 35 °C, the maximal yield was 96%, which matches the amount of the extract obtained by $scCO_2$ divided by the amount of extract obtained by solvent extraction which equalled 0.0539 g/g. According to literature data, the yield of *L. stoechas* obtained by hydrodistillation or steam distillation varies significantly: 0.3–1% (w/w) (Carrasco et al., 2015); 1.33% w/w (Gören et al., 2002); 0.71–1.97% (Kaya et al., 2012).

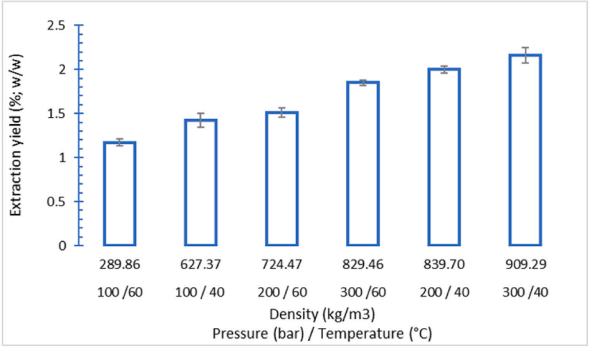

Fig. 1. Extraction yield of supercritical carbon dioxide extraction of *Lavandula stoechas* (pressure 100–300 bar, temperature 40 and 60 °C, CO_2 flow 20 g/min, extraction time 3h).

3.2. Modelling of supercritical carbon dioxide extraction

The kinetics of the $scCO_2$ extraction of *L. stoechas* was investigated by modelling the overall extraction curves using Brunner, modified Brunner, Martínez, and modified Martínez models.

As shown in Table 1, the AARD values for the Brunner model were, for all experiments but two, above 10%. This suggested that the kinetic of the studied experiments could not be fitted very well into the Brunner model. The modified Brunner model provided a slightly better fitting due to the higher number of adjustable parameters.

The Martínez model and its modification showed a better fitting with the experimental results than the Brunner model and its modification (Table 2). Calculated Y∞ values, with two exceptions, were similar to the experimental x_0. For the two experiments (200 bar/60 °C/20 g/min and 200 bar/60 °C/30 g/min), the maximum extraction value, with respect to the experimental data, could also be estimated to be lower than x_0, and the AARD value for the modified model vs. original was significantly lower. The amount of obtained extract was related to the solvent power i.e., experimental conditions (Kitzberger et al., 2009). The extraction yield obtained in the Soxhlet extraction could not be achieved in all $scCO_2$ experiments and for this reason, it would be more accurate to use models with an additional parameter, such as Y_∞.

The values of the calculated parameters were affected by experimental conditions and, almost the same trend could be observed for the change of parameters B and t_m for both models. Pressure had a positive effect on B values at both temperatures. An increase in the flow had a positive effect on B at both temperatures for the modified Martínez model. The only exception from the trend was observed for the Martínez model at 200 bar/60 °C/10 g/min. Change of parameter t_m mainly had the same trend as B. For both models, the deviation from the trend could be observed for the experiments carried out at 100 bar. The parameter t_m for all experiments was positive, the only negative value was obtained for 200 bar/40 °C/10 g/min. A negative value meant that the rate extraction had a maximum value at the initial instance (Sousa et al., 2005). Figs. 2 and 3 showed a good agreement between the experimental data and the approximation data using the Martínez modified model for the extraction yield.

3.3. Chemical composition

The chemical composition of volatiles of the obtained extracts was investigated with GC-MS analysis. The presence of 59 compounds in the supercritical extracts of *L. stoechas* was determined. Moreover, the components belonged to the groups of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, and others (Table 3). The extracts were characterized by high contents of oxygenated monoterpenes in the range from 41.78% (300 bar, 60 °C, and 20 g/min) to 63.99% (200 bar, 40 °C, and 30 g/min). Oxygenated sesquiterpenes were the following most present group of compounds with 11.17% (200 bar, 40 °C, and 10 g/min) to 21.90% (300 bar, 60 °C, and 20 g/min). Hydrocarbons were less present, namely, sesquiterpene hydrocarbons 2.39–5.02% and monoterpene hydrocarbons 0.13–0.95%. The group *Others* (Table 3), which encom-

Table 1
Calculated parameters of the Brunner and modified Brunner model for supercritical carbon dioxide extraction of *L. stoechas*.

Extraction conditions			Brunner model		Modified Brunner model		
Pressure (bar)	Temperature (°C)	Flow (g/min)	k (min^{-1})	AARD (%)	k (min^{-1})	Y∞ (%)	AARD (%)
100	40	20	0.0035	12.323	0.0021	4.229	9.634
100	60	20	0.0026	11.633	0.0035	2.226	12.089
200	40	20	0.0066	6.451	0.0044	3.695	4.782
200	60	20	0.0051	5.746	0.0091	1.952	2.975
300	40	20	0.0071	12.217	0.0032	4.966	6.496
300	60	20	0.0051	14.604	0.0021	5.832	12.272
200	40	10	0.0055	10.266	0.0029	4.584	10.092
200	60	10	0.0068	21.928	0.0029	5.259	17.019
200	40	30	0.0092	24.211	0.0030	6.147	14.158
200	60	30	0.0067	17.487	0.0046	3.628	15.085

Table 2
Calculated parameters of the Martínez and modified Martínez model for supercritical carbon dioxide extraction of *L. stoechas*.

Extraction conditions			Martínez model			Modified Martínez model			
Pressure (bar)	Temperature (°C)	Flow (g/min)	B (min^{-1})	t_m (min)	AARD (%)	B (min^{-1})	t_m (min)	Y∞ (%)	AARD (%)
100	40	20	0.0079	95.477	6.867	0.0079	78.067	2.660	7.175
100	60	20	0.0053	70.868	10.059	0.0057	88.559	2.752	9.864
200	40	20	0.0112	27.416	5.962	0.0127	23.923	2.551	5.363
200	60	20	0.0084	13.593	11.007	0.0187	10.313	1.669	2.738
300	40	20	0.0129	40.133	5.072	0.0169	54.265	2.512	4.538
300	60	20	0.0092	36.373	12.566	0.0123	108.176	3.027	10.083
200	40	10	0.0078	−52.709	9.963	0.0072	−35.533	3.065	10.191
200	60	10	0.0122	37.295	15.367	0.0124	47.104	2.845	15.004
200	40	30	0.0254	76.718	5.828	0.0376	75.195	2.490	6.998
200	60	30	0.0115	27.336	11.516	0.0347	65.378	1.968	1.538

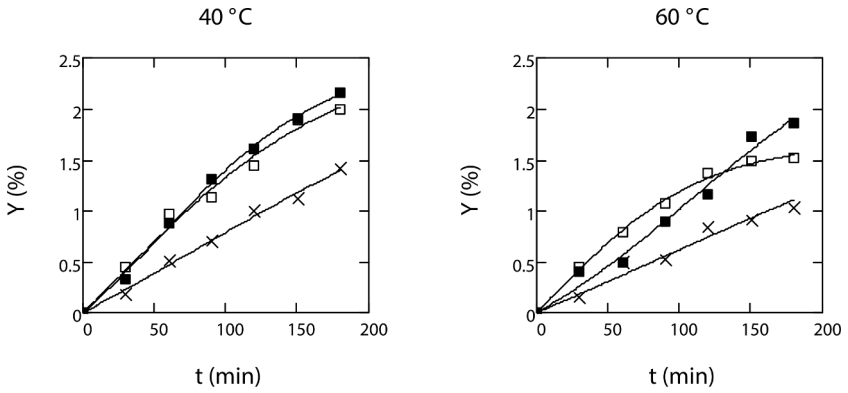

Fig. 2. Modified Martínez model (line) and experimental extraction data: effect of the pressure × – 100 bar; □ – 200 bar; ■ – 300 bar.

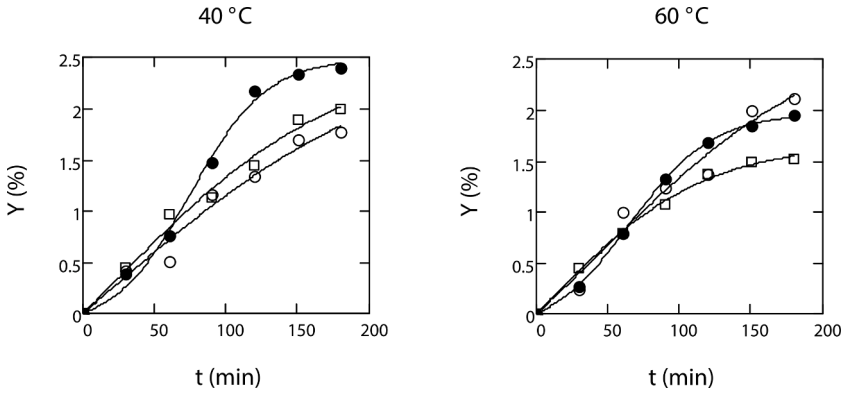

Fig. 3. Modified Martínez model (line) and experimental extraction data: effect of flow ○ 10 g/min; □ – 20 g/min; ● 30 g/min.

passed different compounds such as carboxylic acids and their esters, and aryl alkyl ketones, was present in the range of 4.56–12.05%.

Although it is highly variable depending on the location, external environmental conditions, ontogenetic, and morphogenetic factors, the chemical lipophilic profile of *L. stoechas* is commonly dominated by oxygenated monoterpenes (Ez Zoubi et al., 2020). Additionally, a significantly lower presence of hydrocarbons is characteristic of lavender species (Aprotosoaie et al., 2017) and represents a benefit if the extracts are used in food and cosmetic products. Namely, monoterpene hydrocarbons have the tendency to oxidize, hence they can be the cause of unpleasant odour and the decrease in quality induced by degradation (Aprotosoaie et al., 2017). Therefore, the increased presence of oxygenated terpenes matches the improvement of the sensory and pharmacological properties of the extracts (Aprotosoaie et al., 2017).

The chemical profile of the lipophilic fraction depended on the conditions of the $scCO_2$ extraction and their interactions. As previously explained, the increase in temperature can act in both directions – increase the presence of individual components due to the increase in the solubility of the components, or lower it due to the decrease in the density of CO_2 (Babu et al., 2016). Apart from the $scCO_2$ properties, the extraction of components is influenced by their properties such as molecular weight and boiling point.

At constant pressures of 100 and 300 bar with the increase in temperature, a drop in the presence of oxygenated monoterpenes was observed, which can be caused by the decreased CO_2 density (Pourmortazavi and Hajimirsadeghi, 2007). A reverse trend was recorded with sesquiterpenes, and at these pressures with the increase in temperature, the presence of sesquiterpenes rose. Due to the increase in temperature, the vapour pressure of volatile compounds increased leading to an increase in the solubility of sesquiterpenes. At 200 bar, the impact of temperature was reverse and significantly less prominent, causing a mild increase in the presence of oxygenated monoterpenes and a decrease in sesquiterpenes. The components with higher molecular weight mainly require more drastic conditions of extraction, higher pressure and temperature, while the low molecular weight constituents can be extracted easily under milder conditions. Also, with more drastic conditions, components with a high boiling point were coextracted such as oleyl alcohol and docosane, which were present in the highest percentage in the 300 bar/60 °C extract.

At 60 °C, an elevation of oxygenated monoterpenes was noticed with the increase in pressure from 100 to 200 bar, followed by a drop. At the same time, an opposite trend with oxygenated sesquiterpenes and group *Others* was recorded. This is in agreement with the conclusions reached by Glisic et al. (2010) which stated that the application of higher pressures led to a reduction in selectivity towards the monoterpenic compounds and an increase in the solubility of other components of higher molecular weight. Moreover, the changes in the presence of monoterpenes in the extracts following the increase in pressure and temperature could be caused by the dilution effect. Namely, sesquiterpenes have a higher molecular weight due to which a higher resistance to extraction can occur. This

Table 3
GC-MS analysis of *L. stoechas* extracts (%) obtained by supercritical carbon dioxide extraction (pressure 100–300 bar, temperature 40 and 60 °C, flow 10–30 g/min).

Compound	RI	100 bar		200 bar		300 bar		200 bar			
		40 °C	60 °C	40 °C	60 °C	40 °C	60 °C	40 °C		60 °C	
		20 g/min						10 g/min	30 g/min	10 g/min	30 g/min
Monoterpene hydrocarbons											
α-Pinene	945	0.13	0.30	0.07	0.05	0.13	0.07	0.09	0.22	0.09	0.39
Camphene	960	–	–	0.02	0.05	0.34	0.20	0.32	0.49	0.32	0.27
p-Cymene	1032	–	–	0.07	0.05	0.09	0.07	0.07	0.13	0.07	0.06
Limonene	1037	–	–	–	0.02	0.07	0.04	0.07	0.11	0.07	0.04
Oxygenated monoterpenes											
1.8-Cineole	1040	3.5	0.64	6.84	7.92	6.45	4.03	3.86	6.11	5.72	7.00
Lavender lactone	1048	0.29	0.15	0.29	0.28	0.22	–	0.18	0.18	0.22	0.21
trans-Linalool oxide	1079	1.17	0.58	1.28	1.20	0.87	0.69	0.81	0.80	1.05	0.96
α-Isophorone	1086	0.29	0.15	0.46	0.52	0.39	0.25	0.34	0.40	0.41	0.47
Fenchone	1094	18.45	5.76	11.42	12.44	12.12	7.08	17.48	22.72	19.6	11.19
Linalool	1104	0.51	0.55	0.49	0.31	0.20	0.18	0.38	0.42	0.45	0.35
Fenchol	1120	1.43	1.61	0.99	1.03	1.09	0.91	1.47	1.32	1.46	0.96
α-Campholene aldehyde	1133	0.13	0.06	–	–	0.02	0.04	0.11	0.09	0.11	0.08
trans-Pinocarveol	1146	0.29	0.30	0.24	0.17	0.22	0.18	0.2	0.24	0.28	0.29
trans-Verbenol	1149	0.18	0.21	–	0.11	0.11	0.07	0.16	0.16	0.17	0.14
Camphor	1152	10.4	6.49	6.87	6.79	6.67	5.19	10.44	10.92	10.57	7.57
Chrysanthenone	1156	0.33	1.01	0.40	0.39	0.39	0.73	0.29	0.29	0.3	0.43
Pinocarvone	1169	0.20	0.18	0.04	0.11	0.15	0.18	0.20	0.14	0.19	0.21
Borneol	1173	1.23	1.89	1.30	0.98	1.05	1.23	1.17	1.12	1.18	1.15
Eucarvone	1176	0.64	0.79	0.92	0.87	0.74	0.54	0.56	0.52	0.65	0.74
α-Cyclogeraniol	1180	1.06	1.89	1.47	1.42	1.12	0.98	0.88	0.99	1.14	1.27
Terpinen-4-ol	1183	0.37	0.61	0.22	0.17	0.2	0.04	0.32	0.29	0.32	0.35
p-Cymen-8-ol	1190	0.51	0.73	0.49	0.46	0.46	0.44	0.41	0.40	0.41	0.57
Verbenone	1194	8.09	13.10	18.65	18.37	16.60	13.47	8.04	9.33	9.92	12.54
Myrtenal	1200	0.51	0.55	–	0.06	0.24	0.18	0.34	0.11	0.28	0.33
cis-Chrysanthenone	1214	1.52	1.52	0.09	1.31	1.25	1.23	1.24	1.10	1.18	0.96
Fenchyl acetate	1225	0.59	0.73	0.71	0.83	1.20	0.44	0.52	0.81	0.90	0.92
2-Hydroxycineole	1229	0.88	1.31	0.11	0.28	0.31	1.63	0.59	0.20	0.22	0.25
Pulegone	1246	0.62	0.79	0.51	0.61	0.61	0.18	0.47	0.47	0.56	0.68
Carvone	1250	0.33	0.18	0.27	0.33	0.31	0.07	0.38	0.24	0.41	0.35
Linalyl acetate	1262	0.13	–	0.11	–	–	–	–	–	–	–
Isopulegyl acetate*	1284	1.03	1.68	0.88	0.88	0.87	0.76	0.88	0.96	1.08	1.25
Bornyl acetate	1290	2.2	2.99	1.69	1.58	1.31	0.84	2.03	2.28	2.39	2.36
Lavandulyl acetate	1295	0.84	1.61	1.28	1.27	1.01	0.22	0.81	1.27	1.14	1.42
Sesquiterpene hydrocarbons											
Cycloisosativene	1370	0.40	0.88	0.75	0.52	0.46	0.80	0.47	0.51	0.56	0.66
α-Selinene	1498	–	–	0.40	0.37	0.42	–	–	0.34	0.43	0.41
γ-Cadinene	1518	0.35	0.64	0.20	0.61	0.42	0.91	0.38	0.20	0.19	0.25
cis-Calamenene	1528	0.77	1.40	0.97	1.03	0.83	0.84	0.59	0.76	0.93	1.17
Cadalene	1679	1.76	2.10	0.82	0.87	0.92	2.36	1.13	0.58	0.62	1.42
Oxygenated sesquiterpenes											
Palustrol	1572	0.15	0.37	0.35	0.53	0.35	0.07	0.18	0.20	0.41	0.27
Caryophyllene oxide	1586	0.70	1.07	0.46	0.41	0.11	0.36	0.09	0.63	0.24	0.57
Globulol	1587	–	–	–	–	0.37	–	0.41	0.49	0.56	0.57
Viridiflorol	1595	2.51	4.27	3.85	4.18	4.09	4.94	1.87	2.42	2.73	2.85
Ledol	1608	0.97	1.34	3.37	3.44	3.5	4.43	1.78	1.95	2.43	2.57
Torreyol	1647	0.44	0.64	0.48	0.42	0.48	0.73	0.29	0.33	0.37	0.49
α-Cadinol	1660	7.98	10.21	5.22	3.53	4.37	7.63	4.72	5.75	5.31	9.05
Hexahydrofarnesyl acetone	1850	2.37	3.05	1.85	1.97	2.47	3.74	1.83	1.74	1.89	1.89
Others											
3-Methylbutanoic acid	< 900	0.02	0.03	0.02	0.02	0.04	0.04	0.02	0.02	0.06	0.06
2-Methylbutanoic acid	< 900	0.04	0.09	0.05	0.04	0.11	0.07	0.09	0.07	0.13	0.14
Hexanoic acid	980	–	0.34	–	–	0.09	–	0.20	0.02	0.09	0.12
Oct-1-en-3-ol	984	0.22	0.03	–	–	–	0.04	–	0.11	0.13	0.1
3-Acetyl-2,4-dimethylfuran	1096	0.31	0.27	0.77	0.74	0.66	0.44	0.34	0.40	0.43	0.47
Oct-1-en-3-yl acetate	1117	–	–	–	–	–	–	0.07	0.16	0.09	0.04
Methylacetophenone	1189	0.55	0.64	0.16	0.07	0.13	–	0.43	0.40	0.49	0.57
Estragole	1219	–	–	–	–	–	–	0.11	–	0.15	0.1

(*continued on next page*)

Table 3 (*continued*)

Compound	RI	100 bar		200 bar		300 bar		200 bar			
		40 °C	60 °C	40 °C	60 °C	40 °C	60 °C	40 °C		60 °C	
		20 g/min						10 g/min	30 g/min	10 g/min	30 g/min
Phenylacetic acid	1266	0.62	–	–	–	–	0.25	0.14	–	–	0.23
Eugenol	1363	0.42	–	–	–	–	–	0.27	0.11	0.19	0.18
Hexadecanoic acid	1971	0.70	–	0.60	0.57	1.97	–	5.22	1.54	0.50	0.35
Oleyl alcohol	2061	2.18	4.66	3.96	3.31	3.41	7.48	2.30	2.93	1.72	1.58
Docosane	2200	2.00	2.83	2.27	2.03	2.62	4.18	3.12	1.05	1.25	0.9

can be overcome by applying higher pressures and temperatures. Therefore, with a more intensive extraction of sesquiterpenes, there could be a decrease in the percentage of monoterpenes in the extracts (Sonsuzer et al., 2004).

With the increase in flow from 10 to 30 g/min at 200 bar and 40 °C, the percentage of oxygenated monoterpenes increased from 54.94 to 63.99%, whereas the higher temperature (60 °C) combined with the increase in flow, promoted the presence of oxygenated sesquiterpenes (from 13.94 to 18.26%). Due to the higher flow, the diffusion of the solvent through the pores of the material was promoted and a higher concentration gradient was achieved. This decreased the resistance more efficiently resulting in a more efficient release of components with a higher molecular weight (Wrona et al., 2017). The higher temperature contributed to a higher solubility of the oxygenated sesquiterpens, while at the same time, a decreasing trend was observed for the oxygenated monoterpenes. However, considering that the percentage of oxygenated monoterpenes at 60 °C (10–30 g/min and 200 bar) was 55.28–62.65%, which was approximately close to the one achieved at a lower temperature, it is possible that the cause of the decreased presence of the oxygenated monoterpenes was the dilution effect due to the increase in extracted sesquiterpenes. Furthermore, the lowest flow was the most adequate for the sesquiterpene hydrocarbons at the temperature of 40 °C, while at the higher temperature, the highest abundance of hydrocarbons was in the extract obtained with the 20 g/min flow.

Among the oxygenated monoterpenes, the most dominant compounds were fenchone (5.76–22.72%) and verbenone (8.04–18.65%), while the most present sesquiterpenes that contain oxygen were α-cadinol (3.53–10.21%), viridiflorol (1.87–4.94), and ledol (0.97–4.43%). The dominant sesquiterpene hydrocarbon was cadalene in the range from 0.58 to 2.36%. Monoterpene hydrocarbons were present below 1%, with α-pinene and camphene as the principal ones.

The dominant biogenetic route in *L. stoechas* extracts was fenchone – fenchol – fenchyl acetate. The presence of fenchone as the principal component is common for the majority of *L. stoechas* species and, according to literature, the most common chemotype was fenchone/camphor type or 1,8-cineole/fenchone (Ez Zoubi et al., 2020). However, in the obtained extracts, the presence of camphor was 5.19–10.92% and 1,8-cineole 0.64–7.92%. Therefore, the obtained extracts could not be characterised according to usual chemotypes.

Aside from ketone verbenone which belongs to the biogenetic pathway α-pinene, other minor components that also represent products of this route were present, including alcohols *trans*-verbenol and *trans*-pinocarveol and corresponding ketones pinocarvone, and (*cis*-)chrysanthenone. In addition, among the components with the pinane skeleton, aldehyde myrtenal was present, while the bicyclic compounds with bornane skeletons, fenchole isomers, borneol (0.98–1.89%) and its acetate (bornyl acetate) (0.84–2.99) were determined.

As in the case of the group of oxygenated monoterpenes, where temperature had a more pronounced impact at pressures 100 and 300 bar than at 200 bar, the impact of temperature on the presence of certain individual monoterpene components was similar. At 100 and 300 bar with a decrease in the CO_2 density due to the temperature rise, the percentage of 1,8-cineole, fenchone, and camphor decreased. At 200 bar, temperature did not exhibit a significant effect on the presence of camphor, while the increased temperature and pressure 100 bar led to a decrease in the solubility of verbenene. However, at 300 bar, verbenene was dominant in the extract obtained at 40 °C.

Tertiary alcohol linalool was present in a lower percentage (0.18–0.55%) and favoured a lower pressure, while with the increase in pressure, its percentage dropped. A derivate of linalool, *trans*-linalool oxide (0.58–1.28%) was extracted more efficiently at a lower temperature. Additionally, the potential product of the conversion of linalool oxide, lavender lactone, was present in all extracts except the ones obtained at 300 bar/60 °C. The extracts contained esters in a low percentage, such as linalyl acetate, bornyl acetate, lavandulyl acetate, fenchyl acetate, and isopulegyl acetate. A trend of the decreasing percentage of esters with the increase in pressure at 60 °C temperature was recorded, which corresponded to the Babu et al. study (2016). Among the monoterpenes, other minor compounds, which had previously been reported in *Lavandula* essential oil and extracts, were present such as: *p*-cymen-8-ol, terpinene-4-ol eugenol, α-cyclogeraniol, α-campholene aldehyde, α-isophorone, hydroxyl-derivative of 1,8-cineole, and 2-hydroxycineole, which had previously been detected in lavender and lavender honey (Castro-Vázquez et al., 2014).

The selectivity towards individual sesquiterpenes mainly increased with the rise in temperature due to the increase in volatility. Therefore, the presence of viridiflorol, ledol and hexahydrofarnesyl acetone, and the less present γ-cadinene, increased with the rise in temperature at isobaric conditions. α-Cadinol also demonstrated growth with the rise in temperature at 100 and 300 bar. However, at pressure 200 bar, the impact of the CO_2 density was more dominant, so a decrease was observed. Also, the abundance of oxygenated sesquiterpenes, aromadendrane derivatives, viridiflorol, and ledol was favoured by the increase in pressure at a constant temperature, which corresponded to the increase in the density of CO_2. Due to increased pressure, the resistance of the material structures was potentially decreased and the extraction of components of higher molecular weight was facilitated.

Furthermore, the volatile profile of essential oil *L. stoechas* is highly variable and this is attributed to different environmental conditions, such as soil composition, temperature, climate, disease, phase of development, picking season, and ways of extraction. Tzakou et al. (2009) reported that fenchone and/or camphor represented 47.3–64.1% of the oils isolated from a species grown in Greece. It had previously been stated that in the essential oil of the species from Greece, aside from the predominant fenchone, a significant percentage of pinocarvyl acetate was recorded, which was slightly more dominant than camphor and 1,8-cineole (8.12%) (Kokkalou, 1988). In the aroma profile of a species originating from Corsica, fenchone and camphor were also dominant representing together more than 50% of the profile (Ristorcelli et al., 1998). Kaya et al. (2012) determined in *L. stoechas* essential oil a significant difference in the content of components: α-pinene (0.13–5.56%), fenchone (27.56–64.03%), camphor (16.73–50.94%), eucalyptol (1.80–20.29%), and myrtenyl acetate (0–8.65%) depending on the ontogenetic, morphogenetic, and diurnal factors. Karaca et al. (2018) reported camphor (46.7%), fenchone (28.9%), bornyl acetate (4.5%), and 1,8-cineole (3.5%) as the most dominant components. A similar division was determined by Küçük et al. (2019) with camphor (45.8%), α-fenchone (31.8%), and bornyl acetate (4.2%). According to Gören et al. (2002), pulegone had the highest percentage (40.4%), followed by menthol (18.1%) and menthone (12.6%).

Topal et al. (2008) performed $scCO_2$ extraction of *L. stoechas* spp. and determined that the dominant components in the supercritical extract were fenchone (37.29%), camphor (48.04%), and 1,8-cineole (4.84%). Sesquiterpenes were not detected although they were present in the oil obtained by steam distillation. The precise conditions were not mentioned, only the range in which the extractions were conducted: pressure ranging from 200 to 300 bar and temperatures from 40 to 60 °C, with a constant CO_2 flow rate of 3 mL/min for 5 h. Marongiu et al. (2010) in the extract obtained at 90 bar and 40 °C, identified camphor (31.7%), fenchone (20.7%), 1,8-cineole (10.9%), bornyl acetate (5.0%), and myrtenyl acetate (2.1%) as the most dominant components. In the supercritical extract of *L. stoechas* L. *ssp. cariensis Boiss* obtained at conditions 80 bar, 35 °C, 4 h, principal compounds were camphor 55.8%, fenchone 33%, and α-pinene 3.5% (Akgün et al., 2001).

Verbenone was detected in three *L. stoechas* samples collected from different locations in Spain where the principal compounds were fenchone, eucalyptol, camphor, α-pinene, camphene, and limonene (Carrasco et al., 2015). Also, in the essential oil *L. stoechas* from Algeria, the presence of verbenone 0.6% was found (Dob et al., 2006), while Marongiu et al. (2010) reported 0.7% of verbenone in the supercritical extract of *L. stoechas* from Sardinia. A significant presence of verbenone 2.67%, as well as *trans*-verbenol up to 2% was recorded in the Algerian L. *stoechas* where the major essential oil components were fenchone (11.27–37.48%), camphor (1.94–21.8%), 1,8-cineole (0.16–8.71%), and viridiflorol (2.89–7.38%) (Benabdelkader et al., 2011). Furthermore, the presence of verbenone (0.84–13.97%) in the chemical profile was reported in the supercritical extract of *L. viridis* L'Hér, while the most dominant compound was camphor (1.61–22.48) (Costa et al., 2012).

For the application in aromatherapy, fragrance, and perfume industries where it is important for the oil to possess a herbal-rosy scent, the parameters that provide a low presence of camphor and 1,8-cineole are desirable. On the other hand, these two components are of high medicinal importance. Their anticancer and antimicrobial activities were established (Yuceturk et al., 2021; Cai et al., 2021). For 1,8-cineole, it was determined that due to its antioxidative and anti-inflammatory effects, this compound can be used in the treatment of respiratory, cardiovascular, digestive, and Alzheimer's disease (Pokajewicz et al., 2022; Tardugno et al., 2019). Therefore, the extracts rich in these components can be applied in health-related products, or in food products as a preservation agent. In addition, other components identified in the extracts also exhibit a broad spectrum of biological activity. Verbenone's *in vivo* antidiabetic action was reported together with the conclusion that it could reduce the diabetes-associated complications such as cardiovascular disease, by enhancing lipids' metabolism (Tijjani et al., 2022). Fenchone exhibited an antidiarrheal effect (Souza de Souza Pessoa et al., 2020), as well as a significant analgesic activity without inducing motor incoordination (Him et al., 2008). Furthermore, borneol showed promising results as an effective adjuvant for improving drug delivery to the central nervous system (Zhang et al., 2017). Yang et al. (2014) suggested the anti-inflammation potentials of bornyl acetate in patients with osteoarthritis. Peana et al. (2002) reported that due to the dominant presence of linalool and linalyl acetate, the essential oil exhibited an anti-inflammatory effect, while linalyl acetate could be efficient in the prevention of hypertension-related ischemic injury (Hsieh et al., 2018). Viridiflorol exerted anti-mycobacterial, anti-inflammatory, and antioxidant activities (Trevizan et al., 2016), whereas the supercritical extract and oil of *Ledum palustre* L. with palustrol and ledol as their main components, were reported to exert anti-inflammatory activity (Baananou et al., 2015). Therefore, the obtained extracts represent highly valuable products with aromatic properties. Apart from their potential to be used in human and animal-related medicinal products, these extracts can be used in cosmetic, food, and pesticide industries. Moreover, by adjusting the parameters of extraction, the composition of extracts can be influenced and adjusted accordingly.

3.4. Pattern recognition of extraction parameters using PCA

The PCA bi-plots represented in the two-dimensional PC1 *vs* PC2 space, indicating both scores as the extract samples obtained under various $scCO_2$ extraction conditions, and loadings as the volatile compounds obtained after the GC-MS analysis, were shown in Figs. 4 and 5.

Fig. 4(a–e) presents PCA bi-plots of extracted samples obtained when applying different extraction temperatures (40 and 60 °C) and pressures (100, 200, and 300 bar) while maintaining a constant CO_2 flow of 20 g/min, thus explaining 95.76% (71.68 and 24.08), 68.88% (42.54 and 26.34), 82.84% (57.55 and 25.29), 76.98% (42.62 and 34.36), and 65.90% (36.74 and 29.16) of the overall variance of the input data, respectively. This suggested that most of the variability in the datasets could be explained by the first principal component.

Fig. 4a shows that most of the monoterpene hydrocarbons (camphene, limonene, and *p*-cymene) in the extracts obtained at 300 bar correlated positively with PC1, regardless of the applied temperature. This means that these compounds were most effec-

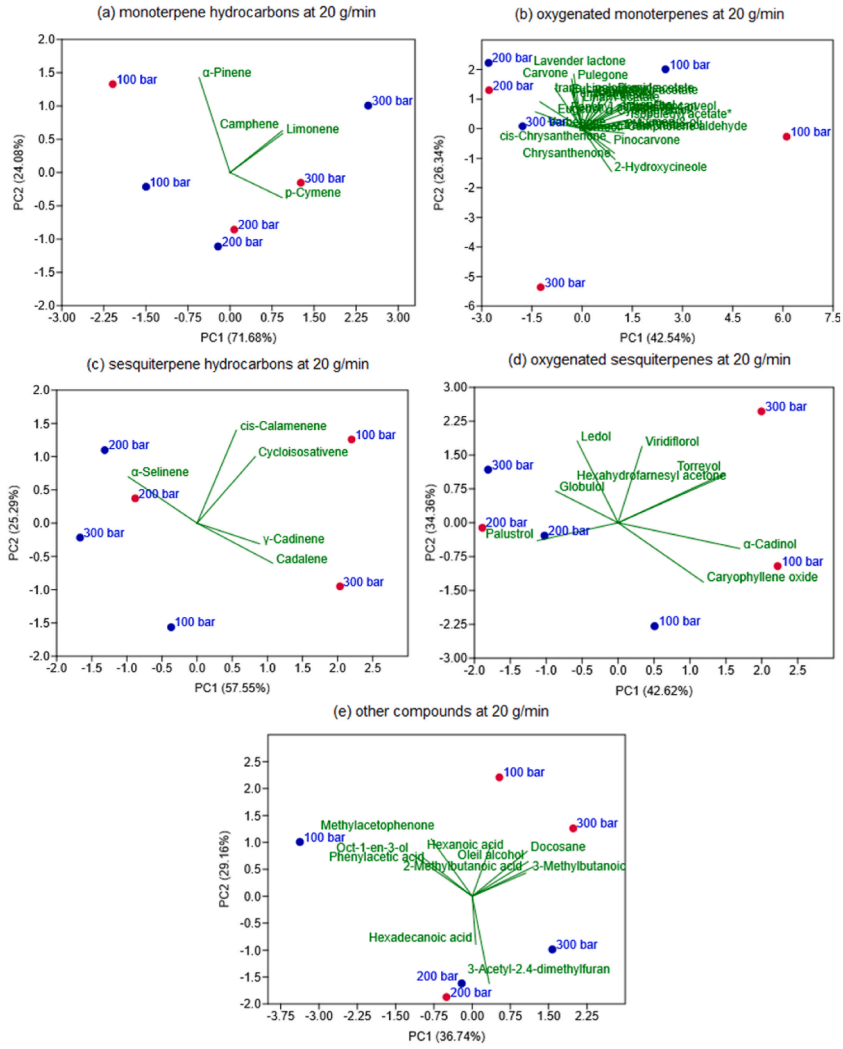

Fig. 4. (a–e). PC1 *vs* PC2 space bi-plots obtained under various conditions of the $scCO_2$ extraction process (temperature 40°C-blue dots and 60°C-red dots; pressures 100, 200, and 300 bar; CO_2 flow 20 g/min). The represented compounds are in accordance with the ones shown in Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

tively extracted under high pressures. As shown in Fig. 4b, most of the oxygenated monoterpenes and samples obtained under 100 bar with both temperatures applied, correlated positively with PC1, which suggested that this chemical class was more effectively extracted when using milder pressures. A PCA graph in this case also indicates that a significant amount of the components belonging to this chemical class could be extracted under 200 bar, showing no meaningful difference when applying lower (40 °C) or higher temperatures (60 °C), nor the pressure of 300 bar at 40 °C. Sesquiterpene hydrocarbons (Fig. 4c), *cis*-calamenene and cycloisosativene were most efficiently extracted using a pressure of 100 bar, and γ-cadinene and cadalene at pressure of 300 bar (60 °C). On the other hand, the pressure of 200 bar at both temperatures applied was the most effective for obtaining α-selinene. As Fig. 4d suggests, the higher pressure of 300 bar contributed to the extraction of most oxygenated sesquiterpenes in the obtained extracts (globulol, ledol, viridiflorol, hexahydrofarnesyl acetone, and torreyol), while palustrol was most efficiently obtained under 200 bar, at 40 and 60 °C. The lowest pressure (100 bar) was shown as the most suitable for obtaining caryophyllene oxide and α-cadinol. Regarding the chemical compounds defined as *Others* (Fig. 4e), hexadecanoic acid and 3-acetyl-2,4-dimethylfuran were most efficiently extracted under 200 bar both at 40 and 60 °C; docosane, oleyl alcohol, 2- and 3-methylbutanoic acid under 300 bar at 60 °C; hexanoic acid under 100 bar at 60 °C; and methylacetophenone, oct-1-en-3-ol and phenylacetic acid at 100 bar and 40 °C.

Fig. 5a-e presents PCA bi-plots of extracted samples obtained when applying different extraction temperatures (40 and 60 °C) and CO_2 flows (10, 20, and 30 g/min) while maintaining a constant pressure of 200 bar, thus explaining 92.05% (69.89 and 22.16), 72.76% (52.43 and 20.33), 76.27% (49.20 and 27.07), 85.36% (59.69 and 25.67), and 81.01% (54.55 and 26.46) of the overall variance of the input data, respectively. This again suggested that most of the variability in the datasets could be explained by the first principal component.

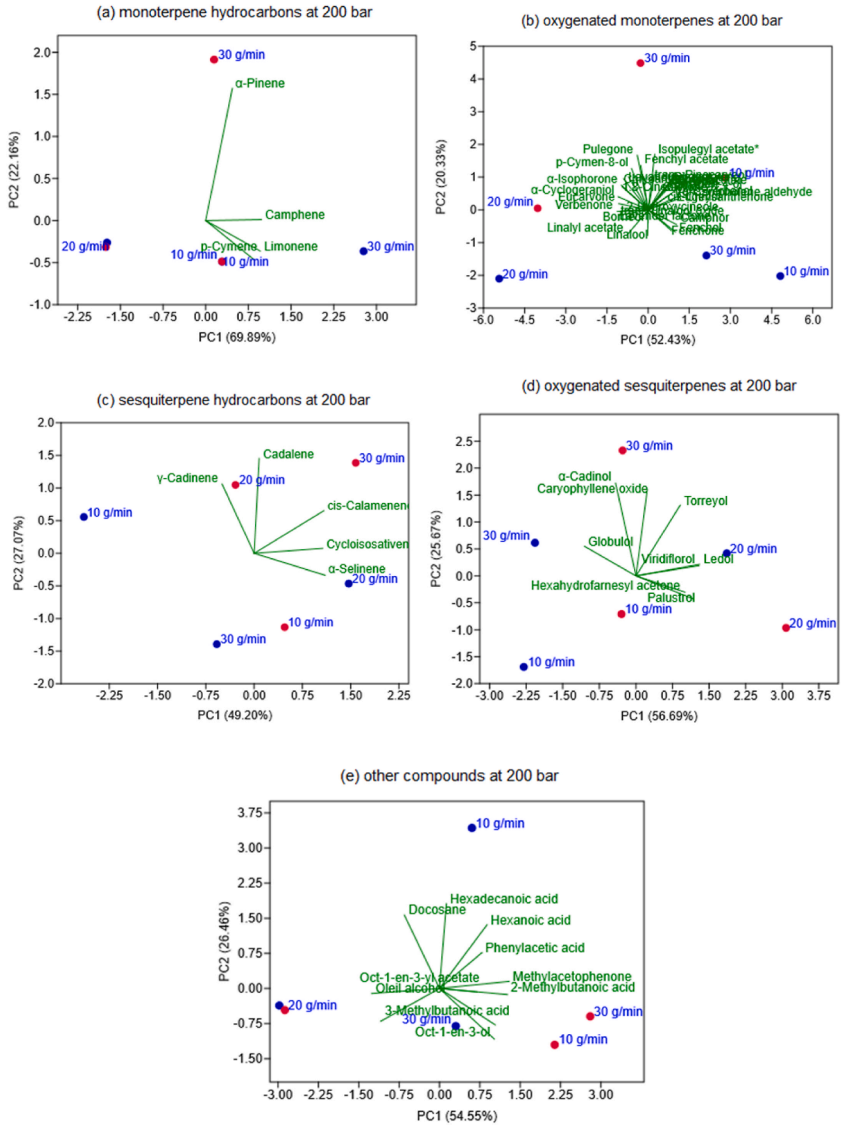

Fig. 5. (a–e). PC1 *vs* PC2 space bi-plots obtained under various conditions of the $scCO_2$ extraction process (temperature 40°C-blue dots and 60°C-red dots; CO_2 flow 10, 20, and 30 g/min; pressure 200 bar). The represented compounds are in accordance with the ones shown in Table 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The PCA bi-plots presented in Fig. 5a demonstrate that all monoterpene hydrocarbons positively correlated with PC1, as well as the samples obtained using the CO_2 flows of 10 g/min and 30 g/min at both applied temperatures (40 and 60 °C), and were, thus, most effectively extracted under these conditions. The oxygenated monoterpenes were quite equally distributed among all samples obtained under all $scCO_2$ extraction conditions investigated in this study (Fig. 5b). Fig. 5c demonstrates that sesquiterpene hydrocarbons: α-selinene, cycloisosativene and *cis*-calamenene could be most efficiently obtained using a flow of 20 g/min at 40 °C and 30 g/min at 60 °C, while γ-cadinene and cadalene with 20 g/min at 60 °C. According to Fig. 5d, the lowest CO_2 flow of 10 g/min was the least effective for extracting oxygenated sesquiterpenes, while the applied flows of (i) 20 g/min was suitable for the extraction of palustrol and hexahydrofarnesyl acetone at 60 °C, and ledol and viridiflorol at 40 °C; and (ii) 30 g/min for the extraction of torreyol, caryophyllene oxide, and α-cadinol at 60 °C, and globulol at 40 °C. The majority of compounds belonging to the group *Others* (Fig. 5e) were extracted more effectively with flows of 10 g/min and 30 g/min (at both 40 and 60 °C). Furthermore, this figure also implies that only oleyl alcohol and oct-1-en-3-yl acetate were abundant in the samples obtained when using the CO_2 flow of 20 g/min, with no differences between the applied temperatures of 40 and 60 °C.

3.5. Antibacterial activity

The antibacterial activity of the supercritical extracts was investigated against Gram-negative and Gram-positive bacteria. The MIC values are represented in Table 4. The inhibitory activity towards Gram-negative bacteria (*E. coli* and *P. aeruginosa*) was from

Table 4
Antibacterial activity of supercritical extracts expressed as MIC/mgmL^{-1}.

Label	Pressure/Temperature/Flow CO_2	Gram-negative bacteria		Gram-positive bacteria	
		Escherichia coli (EC)	*Pseudomonas aeruginosa* (PA)	*Bacillus subtilis* (BS)	*Staphylococcus aureus* (SA)
A	100 bar/40 °C/20 g/L	20.69 ± 0.91	20.69 ± 0.91	20.69 ± 0.91	10.35 ± 0.45
B	100 bar/60 °C/20 g/L	10.86 ± 1.22	10.86 ± 1.22	10.86 ± 1.22	10.86 ± 1.22
C	**200 bar/40°C/20 g/L**	**3.39 ± 0.48**	**6.77 ± 0.95**	**3.39 ± 0.48**	**3.39 ± 0.48**
D	**200 bar/60°C/20 g/L**	**5.05 ± 1.50**	**5.05 ± 1.50**	**10.09 ± 1.06**	**10.09 ± 1.06**
E	300 bar/40 °C/20 g/L	13.10 ± 0.25	13.10 ± 0.25	13.10 ± 0.25	13.10 ± 0.25
F	300 bar/60 °C/20 g/L	17.14 ± 2.97	34.28 ± 5.94	17.14 ± 2.97	17.14 ± 2.97
G	**200 bar/40°C/10 g/L**	**5.43 ± 1.25**	**5.43 ± 1.25**	**2.71 ± 1.31**	**5.43 ± 1.25**
H	200 bar/40 °C/30 g/L	12.85 ± 2.14	12.85 ± 2.14	6.43 ± 1.54	12.85 ± 2.14
I	**200 bar/60°C/10 g/L**	**4.46 ± 0.36**	**8.92 ± 0.84**	**4.46 ± 0.36**	**8.92 ± 0.84**
J	200 bar/60 °C/30 g/L	11.64 ± 1.02	23.28 ± 4.97	11.64 ± 1.02	11.64 ± 1.02
	Ciprofloxacin[a]	3.13	7.89	1.56	3.13

[a] MIC/μgmL^{-1}.

3.39 ± 0.48 to 34.28 ± 5.94 mgmL^{-1}, while the Gram-positive bacteria (*B. subtilis* and *S. aureus*) were more susceptible to the extracts with the MIC from 2.71 ± 1.31 to 20.69 ± 0.91 mgmL^{-1}. The reason behind the lower activity against Gram-negative bacteria could be the specific structure of the outer membrane which limits the passage of hydrophobic components through the lipopolysaccharide, reducing their activity (Santos et al., 2018).

Relying on the data of assessed antibacterial activities against the species *E. coli* (EC), *P. aeruginosa* (PA), *B. subtilis* (BS), and *S. aureus* (SA) (Table 4), a PC1 *vs* PC2 space bi-plot was created using principal component analysis (Fig. 6). The obtained bi-plot explained 90.83% of the overall variance of the examined data, thereby showing correlations between the obtained $scCO_2$ extracts and assessed antibacterial activities. The variability was noted in the antibacterial activity of the extracts obtained under different extraction conditions.

The most potent antibacterial activity was exhibited by the extracts attained at 200 bar labelled as C, D, G, and I (Table 4 and Fig. 6). Moreover, the combination of parameters of 200 bar, 40 °C and 20 g/L, yielded the extract (C) with the lowest MIC value of 3.39 mgmL^{-1} against *E. coli* and *S. aureus*, while extract (G) obtained at same temperature and pressure with a lower CO_2 flow (10 g/L), exhibited the strongest activity (2.71 mgmL^{-1}) towards *B. subtilis*. The most significant susceptibility of *P. aeruginosa* was determined towards the extract D (200 bar/60 °C/20 g/L) (MIC 5.05 ± 1.50 mgmL^{-1}). However, this extract had nearly a 3-fold weaker activity towards Gram-positive bacteria compared to the extract obtained at a lower temperature (200 bar, 40 °C and 20 g/L). The CO_2 flow also had a significant impact on the activity, hence the extracts obtained at 200 bar and flows 10 and 20 g/min had a more prominent antibacterial activity compared to the highest flow of 30 g/min, at both applied temperatures.

Extracts L. *stoechas* were characterized by a high content of oxygenated terpenes which have a prominent antimicrobial activity. According to Guimarães et al. (2019), the phenolic and non-phenolic alcohols exhibit the strongest inhibitory effects, followed by aldehydes and ketones (Griffin et al., 1999). Because of their terpene structure, they have a lipophilic character which is, in combination with hydrophilic properties of functional groups, considered responsible for the antimicrobial activity (Chizzola, 2013). Hydrocarbons demonstrate weak antimicrobial activity but can impact the membrane by modifying its structural characteristics, such as swelling which can inhibit respiratory enzymes cells and affect the cell's functional properties. For instance, it was demonstrated that *p*-cymene causes the swelling of the membrane and in that way improves the incorporation of carvacrol into the membrane, promoting its antimicrobial activity (Burt, 2004). The correlation between the presence of dominant individual compounds and the exhibited activity of the extracts was not determined, hence, the activity cannot be attributed to individual dominant constituents (Supplemen-

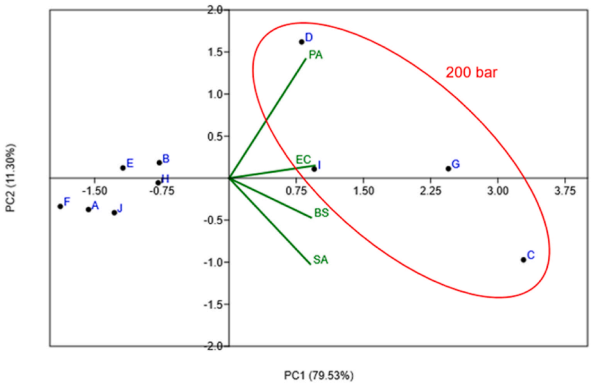

Fig. 6. PC1 *vs* PC2 space bi-plots of all studied extracts showing relationships with investigated antibacterial activities. (*Escherichia coli* (EC); *Pseudomonas aeruginosa* (PA); *Bacillus subtilis* (BS); *Staphylococcus aureus* (SA). Labels of extracts correspond to the ones shown in Table 4.

tary data 1 and 2). Potentially, the activity of extracts could be the result of the synergistic activity of the constituents, the additive activity, where minor components increase the activity of the major components, or the antagonistic activity (Farhanghi et al., 2022). Although the mechanism of activity was not clarified entirely, it had been previously determined that one of the possible mechanisms of activity of the lipophilic components is the passage through the cell membrane, leading to the leak of potassium and the modification of the cell's morphology due to the degradation of the electron respiratory chain (Bouyahya et al., 2017).

The weakest activity was demonstrated by the extracts obtained at 100 bar/40 °C/20 g/L and 300 bar/60 °C/20 g/L. At the lowest pressure applied (100 bar), the extract obtained at 60 °C exhibited a better activity against all bacteria than the extract obtained under the same conditions, but at lower temperature (40 °C). The extract attained at 60 °C had a higher presence of oxygenated sesquiterpenes among which some were confirmed antimicrobials, such as caryophyllene oxide (Schmidt et al., 2010) and hexahydrofarnesyl acetone (Balogun et al., 2017). At 300 bar, the extracts obtained at the lower temperature were richer in oxygenated monoterpenes and significantly more effective than the ones attained at the higher temperature and lower content of oxygenated monoterpenes. In the extract 300 bar/40 °C, the presence of certain oxygenated monoterpenes was more dominant, including verbenone, camphor, and 1,8-cineole which possess established antimicrobial properties (Santoyo et al., 2005).

Due to the significant variability of the chemical profile, the difference in used strains, and the methods, literature reports different activities of the extracts and essential oil of *L. stoechas* against the bacteria. According to Marongiu et al. (2010), the lavender supercritical extracts did not inhibit any of the tested pathogens, among which were *E. coli* and *S. aureus*, while our study determined a significant inhibitory effect of the supercritical extracts. Insawang et al. (2019) investigated the activity of five hydrodistilled essential oils of *L. stoechas* cultivars from different countries and regions towards different bacteria. Out of the five investigated oils, only one exhibited activity towards *E. coli* with a MIC of 25.00 $mgmL^{-1}$, and two demonstrated inhibitory activity against *S. aureus* and *P. aeruginosa*, at 6.25–50 and 12.50–50 $mgmL^{-1}$, respectively. Baali et al. (2019) also observed a more prominent activity of *L. stoechas* essential oil obtained by hydrodistillation against the Gram-positive *S. aureus* 0.78 ± 0.01 $mgmL^{-1}$ and *B. subtilis* 0.10 ± 0.01 $mgmL^{-1}$ than the Gram-negative *E. coli* 1.56 ± 0.01 and *P. aeruginosa* 6.25 ± 0.09 $mgmL^{-1}$. Sarac and Ugur (2009) established the antibacterial activity of *L. stoechas* hydrodistilled essential oil cultivated in Turkey against several Gram-positive and Gram-negative bacteria, including *S. aureus*, *E. coli*, and *B. subtilis*. Moreover, *P. aeruginosa* was resistant to the essential oil of *L. stoechas* ssp. *stoechas* (Sarac and Ugur, 2009). Another study also reported the resistance of *P. aeruginosa* against *L. stoechas* essential oil obtained with the microwave-assisted process, while the oil's activity against *E. coli* and *S. aureus* was determined, with *S. aureus* being more sensitive (Sadani and Shakeri, 2016). In addition, oil obtained by steam distillation showed antimicrobial activity towards *B. subtilis*, *E. coli,* and *S. aureus* (Cherrat et al., 2014).

Compared to the control, ciprofloxacin, the extracts had a significantly lower activity towards bacteria. However, the activity of the obtained extracts was in the range of natural antibacterial products. Considering the increase in resistance of pathogens to conventional medications and the rising tendency towards the use of natural components and products, there is a constant need for the development of new antimicrobial agents. The supercritical L. *stoechas* extracts exhibited an important inhibitory activity towards *E. coli*, *S. aureus*, and *P. aeruginosa* which represent three of the six leading pathogens with AMR associated with fatalities (Antimicrobial Resistance Collaborators, 2022). Therefore, the obtained extracts which represent safe and viable products can be applied as antimicrobial agents and an alternative or addition to synthetic antimicrobial products. In addition, the investigated bacteria are foodborne pathogens, thus *L. stoechas* supercritical extracts can contribute to food quality and safety, consequently decreasing the foodborne diseases.

4. Conclusions

The impact of pressure, temperature, and the CO_2 flow on the extraction yield, chemical profile, and the antibacterial activity of the extracts was determined. The kinetics of the extraction was investigated and modelled by using two models and their modifications. Moreover, the modified Martínez model was determined to be the most adequate for the description of the experimental data. Oxygenated monoterpenes were the most dominant group of organic compounds with fenchone (5.76–22.72%) and verbenone (8.04–18.65%) as the principal constituents, while sesquiterpenes were the following most present group with the predominant α-cadinol (3.53–10.21%), viridiflorol (1.87–4.94), and ledol (0.97–4.43%). Furthermore, by applying adequate parameters, pressure, temperature, and flow, a selective extraction of the components of interest can be achieved and the content of extracts can be influenced and adjusted accordingly. Moreover, the application of the principal component analysis enabled a facilitated observation of the correlation between the extraction conditions and the volatile components, as well as the antibacterial activities of the obtained extracts. *In vitro* assays demonstrated the significant difference in the antibacterial activity depending on the extraction conditions which implies the importance of the production process optimization. The most potent extracts were attained at 200 bar and demonstrated an inhibitory activity in the range 2.71 ± 1.31–12.85 ± 2.14 $mgmL^{-1}$ and 3.39 ± 0.48–23.28 ± 4.97 $mgmL^{-1}$ towards Gram-positive and Gram-negative pathogens, respectively. Therefore, the supercritical *L. stoechas* extracts represent a promising resource in the fight against the AMR and the achievement of the SDGs.

CRediT author statement

Jelena Vladić;: Conceptualization, Investigation, Resources, Writing-Original draft preparation, Writing - Review & Editing, Funding acquisition, Project administration; Igor Jerković;: Conceptualization, Software, Investigation, Writing - Review & Editing, Investigation, Data curation; Sandra Svilović;: Methodology, Software, Writing- Original draft preparation; Validation; Valentina Pavić;: Investigation, Writing- Reviewing and Editing, Validation; Kristian Pastor: Formal analysis, Writing- Original draft preparation, Software, Visualization; Alexandre Paiva: Writing- Reviewing and Editing, Funding acquisition; Stela Jokić;: Investigation, Writing-

Original draft preparation; Silvia Rebocho: Methodology, Investigation; Writing-Original draft preparation; Ana Rita Duarte: Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scp.2023.101126.

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