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Supercritical Fluid Extraction from *Zataria multiflora Boiss* and Impregnation of Bioactive Compounds in PLA for the Development of Materials with Antibacterial Properties

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Highlights:

- The chemical composition of the *Z. multiflora* EO extract obtained by supercritical extraction was identified by GC-MS.
- DPPH assays demonstrated the antioxidant activity of *Z. multiflora* EO extract.
- *Z. multiflora* EO extract was impregnated in PLA films by supercritical impregnation.
- The operational parameters of the supercritical impregnation process were optimized using FFD.
- The impregnated samples were characterized by SEM, FTIR, DSC and XRD.
- Impregnated PLA films showed antibacterial activity against *E. coli* and *S. aureus*.

Abstract: In this research, the extraction with supercritical carbon dioxide (SC-CO_2) and the subsequent impregnation of the extracted bioactive compounds from *Zataria multiflora Boiss (Z. multiflora)* into polylactic acid (PLA) films was investigated. The effects of temperature (318 and 338 K), pressure (15 and 25 MPa) and cosolvent presence (0 and 3 mol%) on the extraction yield were studied. The SC-CO_2 assisted impregnation runs were carried out in a discontinuous mode at different pressure (15 and 25 MPa), temperature (318 and 328 K), and time (2 and 8 h) values, using 0.5 MPa min^{-1} as a constant value of depressurization rate. ANOVA results confirmed that pressure, temperature, and time influenced the extraction yield. Moreover, antioxidant activities of extracts of *Z. multiflora* were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays. In addition, the antibacterial activities of the extracts were screened against standard strains of *Staphylococcus aureus (S. aureus)* and *Escherichia coli (E. coli)*. The results of this investigation indicated that extracts obtained from the aerial parts of *Z. multiflora* possessed antioxidant and antibacterial properties. The impregnated samples presented strong antibacterial activity against the selected microorganisms.

Keywords: supercritical solvent impregnation; polylactic acid; *Zataria multiflora Boiss*; antibacterial active packaging

1. Introduction

Many medicinal plants (herbs) have antibacterial activity due to their high content of essential oils (EOs). Antibacterial properties of EOs from rosemary, oregano, clove, thyme, and *Zataria multiflora Boiss (Z. multiflora*) against bacteria and fungi have been reported previously [1]. Phenolic compounds, existing in essential oils, have antibacterial activity and are commonly categorized as Generally Recognized as Safe (GRAS) substances used to delay the growth of food spoilage microorganisms [2]. *Z. multiflora*, native to Iran with the Persian name of *Avishane Shirazi* appertaining to the family Labiatae, is traditionally used in food, and as natural medicine due to its antiseptic and antitussive properties. In addition, the antibacterial and antioxidant properties of *Z. multiflora* essential oil (EO) have been reported [3–5].

Over the past three decades, the technologies based on supercritical fluids (SCF) as clean and green methods, have attracted enormous interest in various industries, considering food, pharmaceutical, and biochemical fields [6]. Carbon dioxide (CO_2), due to its unique properties such as accessible critical point (Pc = 7.38 MPa and Tc = 304.1 K), diffusivity similar to the gas phase, and density like the liquid phase is the most used supercritical solvent. Moreover, CO_2 presents low viscosity, is non-toxic, non-flammable and it can be recycled with high degrees of purity [6–8]. SC-CO_2 is used in many food and pharmaceutical processes, such as extraction from solids (natural materials), supercritical fluid fractionation, highly selective separations and purification, supercritical reactions to increase the selectivity and improvement of reaction kinetics and enzymatic reactions, incorporation of active substances in food grade materials for functional foods, production of drug particles (micro/nano size) to increase the drug bio-activity and bio-availability, drug delivery, etc. [9–12].

Supercritical fluid impregnation is a modern technique with many applications at laboratory and industrial scales [13]. Hence, this method has received much attention from researchers and many authors around the world have published papers on its applications and advantages. In the supercritical impregnation process, SC-CO_2 acts as a solvent for the solute (e.g., active nutraceutical compounds, bioactive substances, drugs), as well as a polymer swelling agent to incorporate the dissolved solute into the solid matrix (e.g., polymer). Therefore, this method can be called supercritical solvent impregnation (SSI) process. The excellent mass transport properties of SC-CO_2 related to its high diffusivity and low surface tension are the main factors to select SC-CO_2 as impregnation medium. Comparing SSI to conventional impregnation methods, SSI is done in shorter times, there is no need for organic solvents and does not produce waste. In the SSI process, a drying step is not required, energy inputs to develop the process are lower than those of conventional processes and the excess of active substances can be recycled [13–15].

The SC-CO_2 assisted impregnation process consists of three steps; (i) dissolution step, in which the pure substances or active components dissolve in the supercritical fluid, (ii) sorption step, at this stage the swelling of the polymer take place by the sorption of SC-CO_2 and the dissolved active substances, and (iii) depressurization step; at this stage, by a fast decrease of pressure (i.e., decrease of solvent power) inside the high-pressure vessel, the active substances that have less affinity to the polymer could precipitate in higher extent on the polymeric matrix increasing the impregnation yield. However, a very fast decompression may damage the polymeric structure [16–20]. Today, the SC-CO_2 assisted impregnation has replaced conventional techniques for encapsulating active nutraceutical compounds into food grade substances to protect them against degradation (functional foods) or to incorporate active substances into polymeric matrices for active food packaging applications [21–23].

Today, the use of polymers from renewable sources with biodegradable properties has arisen as a way to decrease the negative effect on the environment of petroleum derived plastics [24]. Poly (lactic acid) (PLA) is an FDA-approved aliphatic polyester for application in foods, cosmetics, and pharmaceutical fields. Particularly in the food industry, the use of PLA has become relevant in packaging and active packaging development [25–27]. Active

food packaging is an innovative concept that involves the participation of natural active components with antioxidant and antibacterial properties in the packaging process to improve the shelf life and safety of food products using a high-tech method with lower processing costs than conventional methods [28–30]. To express the importance and various applications of the SSI technique, the researches and published papers on this subject in the last few years can be mentioned. Milovanovic et al. [26] used the impregnation with SC-CO_2 (batch and semi-continuous processes) to incorporate thymol (Thy) into poly (lactic acid) (PLA)/poly(ε-caprolactone) (PCL) films and analyzed the chemical, thermal and antibacterial properties of the films. Villegas et al. [31] applied the SSI process to incorporate cinnamaldehyde (Ci) as a natural antibacterial compound into PLA films for food packaging applications. Franco et al. [32] studied the adsorption of α-tocopherol (TOC) on monolayer and multilayer polyethylene terephthalate (PET)/polypropylene (PP) films via SSI at 17 MPa and 40 °C. They have shown that the impregnation using SC-CO_2 was a successful process for the preparation of active packaging films. Bastante et al. [33] examined the SSI process to incorporate antioxidants into multilayer PET/PP films and evaluated the main factors of the process, i.e., time, temperature, depressurization rate, the type of active material, and the modifier such as ethanol. Goñi et al. [34] used the SC-CO_2 assisted impregnation process to incorporate two insecticidal terpene ketones into LDPE/sepiolite nanocomposite films. Champeau et al. [16] reviewed the supercritical CO_2 assisted impregnation as a solvent free method to load drugs into drug-eluting implants. Performing SSI at low to medium temperatures for biomedical applications (temperature-sensitive) and obtaining a final solvent-free matrix were expressed as the advantages of it. The supercritical impregnation of Thy and Ci in bio nanocomposite films based on PLA and Cloisite 30B and the analysis of their antibacterial activity was studied by Villegas et al. [35]. Rojas et al. [20] wrote a review on the CO_2-assisted impregnation process in food applications and examined the effect of the operational variables (temperature, pressure, depressurization rate and time) on the incorporation of active substances. The authors showed that the effect of pressure and temperature on the active compound loading can be predicted mainly through the study of the phase behavior between the active compounds and SC-CO_2. Adenekan and Hutton–Prager [36] studied the impregnation of lkyl ketene dimer (AKD) in cellulose fibers using CO_2 and *n*-heptane at sub-and supercritical conditions. The optimized solubility of AKD dissolved in heptane and SC-CO_2 was determined between pressures of 10–20 MPa.

Based on our literature review, no study has been performed on the SC-CO_2–assisted impregnation of *Z. multiflora* EO extracts into PLA to produce antibacterial food packaging films. A Full factorial design (FFD) has been applied to optimize the operational parameters (time, pressure, and temperature) on the loading yield. The antibacterial activity of the films and the antioxidant properties of the *Z. multiflora* EO extract were investigated.

2. Material and Methods

2.1. Materials

The *Z. multiflora* (*Avishane shirazi*) used in this study has been provided from Shiraz, Iran. The samples were shadow-dried at room temperature to achieve a minimum of moisture content. PLA, was of commercial grade and purchased from Shiraz (Iran). Carbon dioxide (99.99% purity) was supplied by Aboghadareh Co. (Shiraz, Iran). Analytical-grade ethanol (99.9% HPLC grade) and methanol were provided by Merck (Darmstadt, Germany). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical, was procured from Sigma-Aldrich Chemie (Steinheim, German). The food-borne microbial strains, Gram-negative *Escherichia coli* (O157:H7) and Gram-positive *Staphylococcus aureus* (ATCC 25923), were supplied by the Laboratory of Biotechnology of the University of Kashan of Iran.

2.2. Method

2.2.1. Experimental Design

Several design of experiments (DOEs) techniques can be applied to evaluate and optimize experimental parameters. Accordingly, a full factorial design (FFD) has been applied to optimize the operational parameters of the impregnation of PLA with bioactive compounds through the SSI process. The effect of all the parameters and their interactions on the impregnation yield results were investigated in an FFD. In this experimental design, all the input parameters were set at two levels each one. These levels were termed as high and low or +1 and −1, respectively. If there are k parameters, each at 2 levels, a full factorial design will be of 2^k runs. Thus, a 2^3 factorial experimental design was developed to appraise the effect of three factors; pressure (X_1: 15 and 25 MPa), temperature (X_2: 318 and 338 K), and impregnation time (X_3: 2 and 8 h) on the impregnation yield. All the impregnation runs were performed in duplicate. The statistical impact of these three operational parameters on the impregnation yield of *Z. multiflora* EO in PLA (responses) was assessed by analysis of variance (ANOVA) using Design expert (version 12.0.3.0, Stat-Ease Inc., Minneapolis, MN, USA) software. Furthermore, the effects on the extraction yield of *Z. multiflora* EO of the operating temperature (318 and 338 K), pressure (15 and 25 MPa) and cosolvent use (0 and 3 mol%) were studied by FFD.

2.2.2. Supercritical Fluid Extraction Procedure

The extraction of bioactive compounds from the aerial parts of dried *Z. multiflora* was done in a SFE pilot plant illustrated in Figure 1. The capacity of the extraction vessel was 10 mL with an internal diameter of 0.01 m and a height of 0.12 m. The system was equipped with a high-pressure pump and 316 stainless steel fittings and pipes with high-pressure tolerance. After loading the dried plant (*Z. multiflora*) with glass beads, used to increase the contact surface between dried *Z. multiflora* and SC-CO_2 and therefore improve the mass transfer during extraction, CO_2 from a gas cylinder, that has been previously liquefied by a refrigeration unit, entered into the main extraction column after passing through the surge tank. The pump creates the pressure required to reach the supercritical condition and temperature was controlled by placing the extraction column inside an oven. The fixed time for the process was 120 min. The extracted EO (bioactive compounds) was carefully collected since it was a small amount and very sensitive. The extraction yield of the *Z. multiflora* EO was calculated using the Equation (1):

$$Yield\ (\%) = \frac{Amount\ of\ extracted\ oil\ (g)}{Amount\ of\ total\ sample\ (g)} \times 100 \quad (1)$$

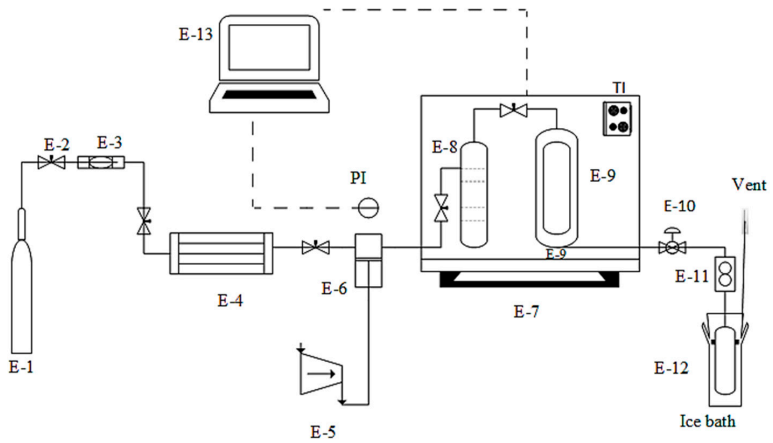

Figure 1. Schematic representation of the supercritical CO_2 extraction set-up. E-1: CO_2 cylinder; E-2: Needle valve; E-3: Filter; E-4: Refrigerator unit; E-5 Compressor; E-6: High pressure pump; E-7: Oven; E-8: Surge tank; E-9: Extraction cell; E-10: Back pressure; E-11: Micro metered valve; E-12: Sampler; E-13: Automation.

The details of the supercritical extraction apparatus and description of the method are given in our previous works [37–41].

2.2.3. Supercritical Solvent Impregnation Process

Figure 2 presents the setup used for the SSI process of *Z. multiflora* EO extract in PLA films. This setup was consisting of a CO_2 cylinder (E-1), needle valve (E-2), a molecular sieve filter (E-3), a refrigerator unit (E-4), a high-pressure pump (air driven liquid pump, type-M64, Shineeast Co., Shandong, China) (E-5), an air compressor (E-6), an oven (Memert) (E-7), a magnetic stirrer (100 rpm, Alfa, D-500 180)) (E-8), a heating coil, a stainless-steel impregnation cell (E-9), pressure gage (E-10), a back-pressure valve (Xi'an Shelok Instrument Technology Co., Xi'an, China) (E-11), micro metered valve (E-12), and an automation system (E-13).

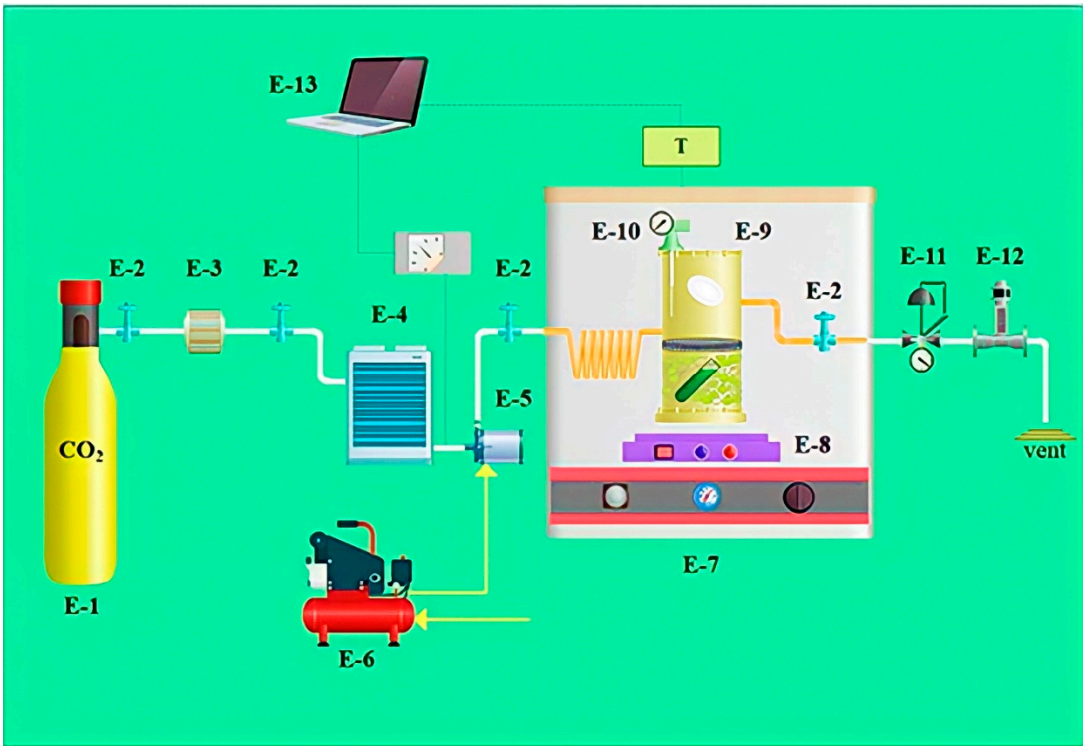

Figure 2. Schematic representation of the SC-CO_2-assisted impregnation apparatus. E-1: CO_2 cylinder; E-2: Needle valve; E-3: Filter; E-4: Refrigerator unit; E-5 High pressure pump; E-6: Compressor; E-7: Oven; E-8: Magnetic stirrer; E-9: Impregnation cell; E-10: Pressure gauge; E-11: Micro metered valve; E-12: Back pressure; E-13: Automation.

Temperature and pressure were measured with an accuracy of ±0.1 K and ±0.1 MPa, respectively. In all experiments, the mass ratio of PLA and *Z. multiflora* EO extract was maintained constant at 1:1. For each experiment, 3 mL of *Z. multiflora* EO extract was deposited at the lower part of the impregnation cell and PLA was incorporated at its upper side, a metal mesh separated both sides of the cell. After a determined impregnation time, the impregnated film was taken out from the cell, softly cleaned and stored in glass flasks at 277 K for their posterior characterization.

At the first step, CO_2 gas was filtered and liquefied using a micro filter and a refrigerator unit, respectively. Then, the system was pressurized to the working pressure introducing more liquefied CO_2 using a reciprocating pump. A pressure gauge and a pressure transducer were used to control the pressure of the system. In addition, the impregnation cell was placed inside an oven to achieve the required temperature. The completely mixing of the *Z. multiflora* EO with SC-CO_2 was guarantee by magnetic stirring at 100 rpm allowing the impregnation of PLA with a SC-CO_2 phase saturated with the

Z. multiflora EO extract. During this time, the swelling and impregnation process proceeded. Finally, the impregnation cell was depressurized at a controlled rate of 0.5 MPa/min by manually regulating a micrometric valve. During the sudden pressure drop in the system, the entire pipe and outlet valve were heated to avoid their freezing due to the CO_2 expansion (Joule–Thomson effect).

The amount of impregnated oil in the film of PLA was gravimetrically measured using a precision digital balance (±0.0001 g). The impregnation yield ($Y\%$) of the *Z. multiflora* EO extract was calculated conforming to Equation (2):

$$Y\% = \frac{m_f - m_i}{m_i} \times 100 \quad (2)$$

In this equation m_i and m_f are the mass of the PLA film before and after the impregnation process, respectively. It is noteworthy that in the initial tests, it was found that CO_2 was expelled quickly from the polymer surface due to the low thickness of the used PLA films, and the amount of bioactive substance on them could be obtained gravimetrically without interference.

2.2.4. Gas Chromatography-Mass Spectrometry (GC-MS)

The main components of the *Z. multiflora* EO extract obtained using SC-CO_2 at the optimal processing conditions were determined by GC-MS analysis. For these assays used an Agilent 7890A chromatograph coupled with an Agilent 5975C mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) and a HP-5MS capillary column (phenyl methyl siloxane, 30 × 0.25 mm; 0.25 µm film thickness) (Agilent Technologies, USA) were used. Ionization energy was set at 70 eV, scans were developed every 0.5 s and the mass range was between 35–400. The temperature profile inside the oven was between 60 and 240 °C with an increase rate of 3 °C/min. The temperature in the injector and detector were 240 and 250 °C, respectively. The gas carrier was Helium used at 0.9 mL/min with a split ratio of 1:50. The relative percentages of the determined components of the *Z. multiflora* EO extract were obtained from the integrations of the peak areas without using a correction factor. ChemStation software was used to process the mass spectra and chromatograms. Know retention times of patter alkanes were used to determine the retention rate of the components of the *Z. multiflora* EO extract under the same chromatographic conditions stablished by Van Den Dool and Kratz [42]. Finally, the components of the *Z. multiflora* EO extract were identified by cross-check their mass spectra with the Wiley library or with the published mass spectra.

2.2.5. Antibacterial Activity of Impregnated Films

In this work, *E. coli* and *S. aureus* selected as representative Gram-negative and Gram-positive bacterium, respectively, were used to evaluate the antibacterial effect of the impregnated samples. In this regard, the standard test method was used to estimate the stabilized antibacterial activity in dynamic contact conditions [31]

The mean initial concentration of microorganisms and serial dilutions in buffer were determined at 10^6 CFU/mL and from 10^1 to 10^8, respectively. On agar, which was already divided into 1/4, nano culture suspensions of microorganisms (10 µL) were plated. They were then incubated at 37 °C for 24 h and after this time, the colonies per quarter were counted. The results were evaluated as the survival of microorganisms or viability (CFU/mL), and their ability to multiply in the solid medium and finally the formation of a colony [43]. According to the following equation, the quantification in CFU/mL was calculated,

$$Viability\left(\frac{\text{CFU}}{\text{mL}}\right) = \frac{Number\ of\ colonies}{\text{mL}\ of\ seeded\ microorganisms} \cdot Dilution\ factor \quad (3)$$

2.2.6. Antioxidant Activity of *Z. multiflora* EO Extract

The antioxidant properties of the *Z. multiflora* EO extract was determined through the DPPH method [44]. Aliquots of 50 µL of different concentrations of *Z. multiflora* EO extract were mixed with 5 mL of a methanol solution with 0.004% of DPPH and left in dark at room temperature during 30 min. Then, the absorption of the different samples was read at 517 nm and the percentage of inhibition (%I) of the free radicals of DPPH was calculated as follows:

$$I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100 \quad (4)$$

A_{blank} correspond to the absorption of the control sample (containing all the reagents except the EO extract) and A_{sample} i the absorption of the EO extract sample. The EO extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotting inhibition percentage against EO extract concentration. All tests were done in triplicate.

2.3. Physical Characterization of Impregnated Samples

The crystalline structures of the neat PLA and PLA samples impregnated with *Z. multiflora* EO extract were study by XRD using a Philips X pert Pro MPD diffractometer (PANalytical, Almelo, The Netherlands). The XRD assays were developed using Cu-Kα radiation (λ = 0.154 nm) within 2θ range of 10–80° at room temperature. On another hand, the change of the thermal properties of the PLA films due to the impregnation of the *Z. multiflora* EO extract was evaluated by DSC assays (DSC 404 F3 Pegasus, Netzsch Co., Hanau, Germany). In these assays, samples of 5 mg were placed in hermetically sealed capsules and heated at a rate of 10 °C/min up to 300 °C under nitrogen purge. The chemical structure of the impregnated bioactive compounds and their intermolecular interactions with PLA were investigated by FTIR spectroscopy analyses in the range of 4000 to 500 cm^{-1} at room temperature. For these assays, KBr disks prepared by pressing 3 mg of each sample and 300 mg of spectral-grade potassium bromide (KBr) were used. The morphology of the impregnated samples was analyzed by FESEM on a VEGA 3 XMU system (TESCAN, Brno, Czech Republic). For this test, the film samples were sputter-coated with gold-palladium alloy using an SDC005 coater machine (BAL-TEC-SDC005, Pfäffikon, Switzerland) at 25 °C for 90 s.

3. Results and Discussion

3.1. Supercritical Fluid Extraction of EO from Z. multiflora

Figure 3 shows the effect of pressure, temperature, and the use of cosolvent over the extraction of *Z. multiflora* EO extract using a fixed *Z. multiflora* particle size (0.30 mm) and extraction time (150 min). As presented in Figure 3, pressure and cosolvent had a positive effect on the extraction yield while temperature had a negative effect on this parameter. In this case, as seen in Figure 3a, the yield of extraction was increased by increasing pressure from 15 to 25 MPa. This may be attributed to the increment of the density of CO_2 which consequently caused an increase in the solubility of the *Z. multiflora* EO extract in SC-CO_2 [45–47].

Figure 3b shows that the yield of extraction of the EO decreased as temperature increased. This result could be explained by the fact that the increase of temperature not only improves the diffusion coefficient of SC-CO_2, increases vapor pressure and volatility of the EO in SC-CO_2 but also leads to a decrease in the density of SC-CO_2, which consequently decreases the solvent power of SC-CO_2. Thus, in this work, the effect of reducing density on solubility prevailed over the effect of increasing the EO vapor pressure. The analysis of variance (ANOVA) is presented in Table 1. ANOVA was conducted considering R^2, adjusted R^2, predicted R^2 and *p*-values. In addition, ANOVA was applied to analyze the significance of the experimental model and its suitability. Based on ANOVA results (Table 2), the effect of temperature on the extraction yield was lower than the other parameters.

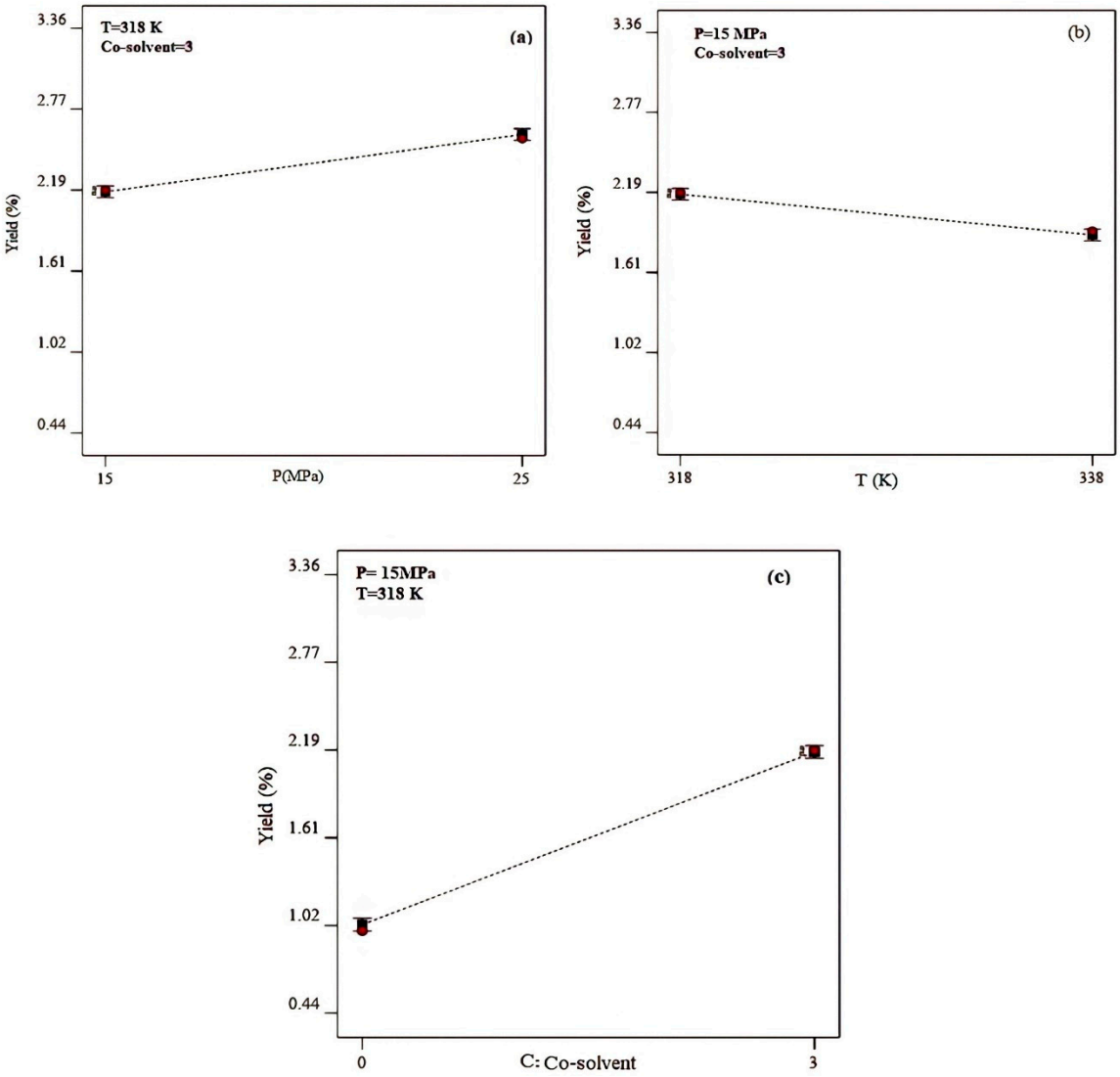

Figure 3. One factor plot to represent the effect of (**a**) pressure at a constant co-solvent concentration of 3% and temperature of 318 K (**b**) temperature at a constant pressure of 15 MPa and a co-solvent concentration of 3%, and (**c**) concentration of co-solvent at a constant temperature of 318 K and pressure of 15 MPa, on the yield of extraction.

Table 1. Analysis of variance (ANOVA) for the model fitted to the SC-CO_2 extraction process.

	Sum of Squares	df	Mean Square	F-Value	*p*-Value	Source
Model	3.15	3	1.5	555.12	<0.0001	significant
A-P	0.3409	1	0.3409	179.97	0.0002	significant
B-T	0.1768	1	0.1768	93.36	0.0006	significant
C-CO-Solvent	2.64	1	2.64	1392.03	<0.0001	significant
Residual	0.0076	4	0.0019			
Cor Total	3.16	7				

R^2	Adjusted R^2	Predicted R^2
0.9976	0.9958	0.9904

Table 2. Chemical composition of *Z. multiflora* EO extract by GC/MS.

No	Compounds	Result (%)	No.	Compounds	Result (%)
1	α-Thujene	1.01	14	Spathlenol	0.62
2	α-Pinene	3.37	15	Caryophyllene oxide	0.68
3	β-Pinene	0.74	16	Monoterpene hydrocarbons	23.91
4	β-Myrcene	1.32	17	Oxygenated monoterpenes	70.65
5	*p*-Cymene	0.67	18	Total monoterpenoids	94.56
6	Cis-Ocimene	6.43	19	Sesquiterpene hydrocarbons	2.83
7	γ-Terpinene	10.37	20	Oxygenated sesquiterpenes	0.62
8	Linalool	6.02	21	Total sesquiterpenoids	3.45
9	Carvacrol methyl ether	2.19	22	Total	98.02
10	Thymol	39.92			
11	Carvacrol	21.37			
12	Thymol acetate	0.47			
13	Trans-Caryophyllene	2.83			

Figure 3c shows the effect of the cosolvent addition on the extraction yield of *Z. multiflora* EO extract. Using ethanol as cosolvent at 3% enhanced the extraction yield of the EO given that it improves the polarity of the extraction fluid. Particularly, the addition of ethanol to the supercritical solvent seems to provide an enhanced extraction of pigment and polar compounds. This behavior has been previously reported [48].

In this work, parameters ranged as follows: [P = 15 and 25 MPa], [T = 318 and 338 K], and [co-solvent = 0 and 3%]. The optimized process conditions were determined as: P = 25 MPa, T = 318 K, and co-solvent = 3%. The extraction yield under the optimized process conditions was 2.56 ± 0.04 wt.%.

Miranda-Villa et al. studied the effect of pressure, temperature and depressurization rate in the SC-CO_2-assisted impregnation of R-(−)-carvone in PLA films, reporting the highest carvone impregnation (30 wt.%) at 40 °C, 9.8 MPa, and 0.6 MPa/min [49]. Torres et al. [50] reported the impregnation of thymol in PLA films using pressures ranging between 9 and 12 MPa, 40 °C and three different values of depressurization rate (0.1, 1.0, and 10 MPa/min), obtaining thymol loadings between 13.5 to 20.5 wt.%. Milovanovic et al. [51] reported the batch impregnation of thymol in PLA/PCL blended films using SC-CO_2 at 10 MPa and 40 °C. The maximized thymol loading with different operating times was 35.8 wt.%. Ivanovic et al. [52] investigated the impregnation of thymol EO in PCL and PCL-HA films by SSI. The process was done at temperatures of 35–40 °C and pressures of 13–17.

3.2. Gas Chromatography Results

The chemical compositions of the *Z. multiflora* EO extract obtained by SC-CO_2 extraction at the optimal conditions is presented in Table 2. 15 compounds were identified that made up 98.02% of the *Z. multiflora* EO. As shown in Table 2, the main components were oxygenated monoterpenes compounds such as thymol (39.2 wt.%), carvacrol (21.37 wt.%) and γ-terpinene (10.37 wt.%). Other compounds with high well-known antibacterial activity such as *cis*-ocimene (6.43 wt.%), linalool (6.02 wt.%), α-pinene (3.37 wt.%), *trans*-caryophyllene (2.83 wt.%), and β-Myrcene (1.32 wt.%) were found in the extract. In this way, the *Z. multiflora* EO extract obtained by SC-CO_2 extraction was mainly composed of three compounds with well-known antibacterial properties: thymol, carvacrol and γ-terpinene [3–5]. This result agrees with the chemical composition of *Z. multiflora* EO extracts reported in other studies. Saei–Dehkordi et al. reported the chemical composition, antioxidant and antibacterial properties of the EO extracted from *Z. multiflora* collected from five different regions of Iran. Particularly, thymol and carvacrol were identified as the

main components of the EO of *Z. multiflora* collected from Hajiabad (47.46 and 9.64 wt.%), Farashband (46.61 and 17.26 wt.%), Yazd (64.87 and 22.39 wt.%), Najaabad (40.49 and 4.65 wt.%), and Pldokhtar (27.05 and 2.7 wt.%) [53]. Abbasi et al. extracted the EO of *Z. multiflora* collected from the Zanjan region in Iran by a hydro-distillation method and determined as its major constituents to carvacrol (36.62%), thymol (17.86 wt.%), and *p*-cymene (11.35 wt.%) [54]. Thymol and carvacrol have also been found as the main components of *Z. multiflora* EO extracts obtained by other methods of extraction. The steam-distilled method was used to extract the EO from *Z. multiflora*, the results indicated that the highest quantitative component was thymol [55–57]. Sadeghi et al. [58] reported the same trend for a *Z. multiflora* sample from Neyriz in Iran. In other cases, carvacrol has the highest amount in the oil composition [59–62].

3.3. Supercritical Solvent Impregnation of PLA Films

The results of impregnation yield of *Z. multiflora* EO extract into PLA films at different operating conditions, including the three independent variables which are pressure, temperature, and impregnation time are reported in Table 3. The SSI process allowed to obtain PLA films with impregnation yields ranging between 6.67 ± 0.76 and 23.76 ± 1.18 wt.%. The graph of the experimental results of *Z. multiflora* EO extract impregnation against the theoretical values predicted by the model, are shown in Figure 4. The experimental data and the values predicted by the model were confirmed together, which means that there was a good distribution of the data points near the straight line and can be evaluated by the coefficient of determination (R^2). The obtained values of R^2, Adjusted R^2, Predicted R^2 and Adeq Precision by FFD were 0.9982, 0.9969, 0.9929, and 70.4160, respectively. The predicted R^2 of 0.9929 was in reasonable agreement with the Adjusted R^2 of 0.9969; i.e., the difference was less than 0.2. Adeq Precision (AP) compares the range of predicted values at design points to the average prediction error and measures the signal-to-noise ratio (S/N). A ratio greater than 4 is desirable. The ratio of 70.416 indicates an adequate signal.

Table 3. Actual variables used in the FFD and impregnation yields of EO in PLA films.

Run	Pressure (P), X_1 (MPa)	Temperature (T), X_2 (K)	Impregnation Time, X_3 (min)	Actual Impregnation Yield (wt.%)	Predicted Impregnation Yield (wt.%)
1	15	338	8	17.34	17.08
2	25	338	2	10.21	10.40
3	15	338	2	6.67	6.52
4	25	338	8	20.76	20.96
5	15	318	2	8.98	9.35
6	25	318	2	13.65	13.22
7	15	318	8	19.89	19.91
8	25	318	8	23.76	23.78

The statistical analysis of variance (ANOVA), based on the FFD was applied to investigate the significance and determinate the effects of the independent variables on the response. The ANOVA results are reported in Table 4. Small *p*-values (less than 0.05) showed that pressure, temperature and impregnation time have significant effects on the EO impregnation yield.

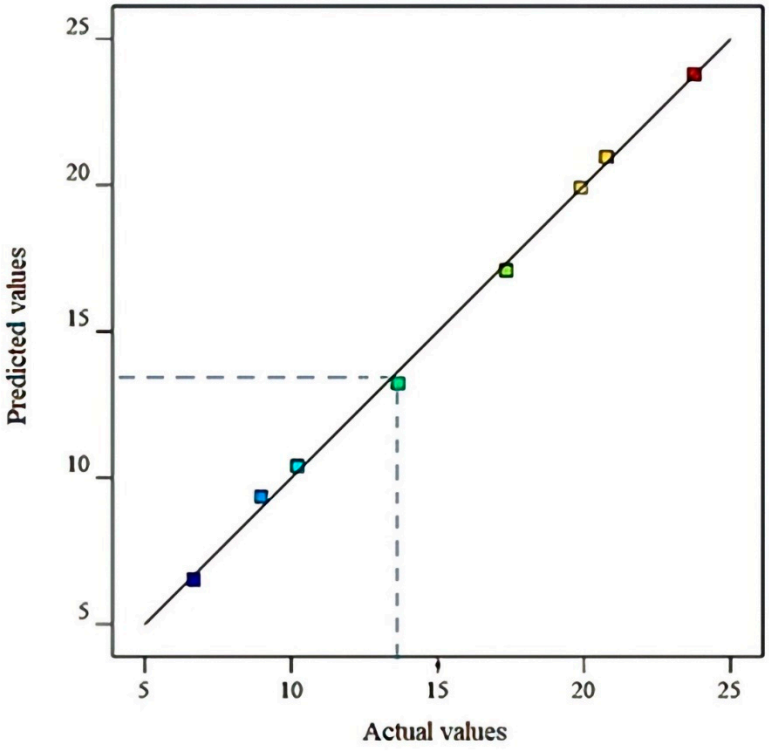

Figure 4. Diagnostic plots (predicted vs. actual) of model adequacy for the impregnation process.

Table 4. Analysis of variance (ANOVA) for the model fitted to the SC-CO_2 impregnation process.

Source	Sum of Squares	df (Degree of Freedom)	Mean Square	F-Value	*p*-Value
Model	269.02	3	89.67	746.27	<0.0001
A-P	30.03	1	30.03	249.92	<0.0001
B-T	15.96	1	15.96	132.83	0.0003
C-Time	223.03	1	223.03	1856.05	<0.0001
Residual	0.4806	4	0.1202		
Cor Total	269.50	7			

After full design, the coded equation was presented to provide the optimal parameters and indicate which parameters were significant as follows:

$$y = 15.16 + 1.94X_1 - 1.4X_2 + 5.28X_3 \quad (5)$$

where y, X_1, X_2 and X_3 are Impregnation yield, pressure, temperature and impregnated time, respectively.

3.4. Effect of Operational Conditions on the Impregnation of EO in PLA Films

In this study, pressure was one of the operational parameters in the SSI process. As shown in Figure 5a, with pressure increasing from 15 to 25 MPa at the constant temperature of 338 K, the EO impregnation yield increased from 6.67 to 10.21 wt.%. The increase in the impregnation yield as pressure increased, could be related to the increase of the SC-CO_2 density, which improves the solubility of the EO extract in the dense CO_2. Furthermore, the swelling and plasticizing effect of CO_2 on the polymer increased with pressure, which could provide more spaces for the diffusion of the supercritical mixture into the polymeric matrix improving the impregnation yield [63].

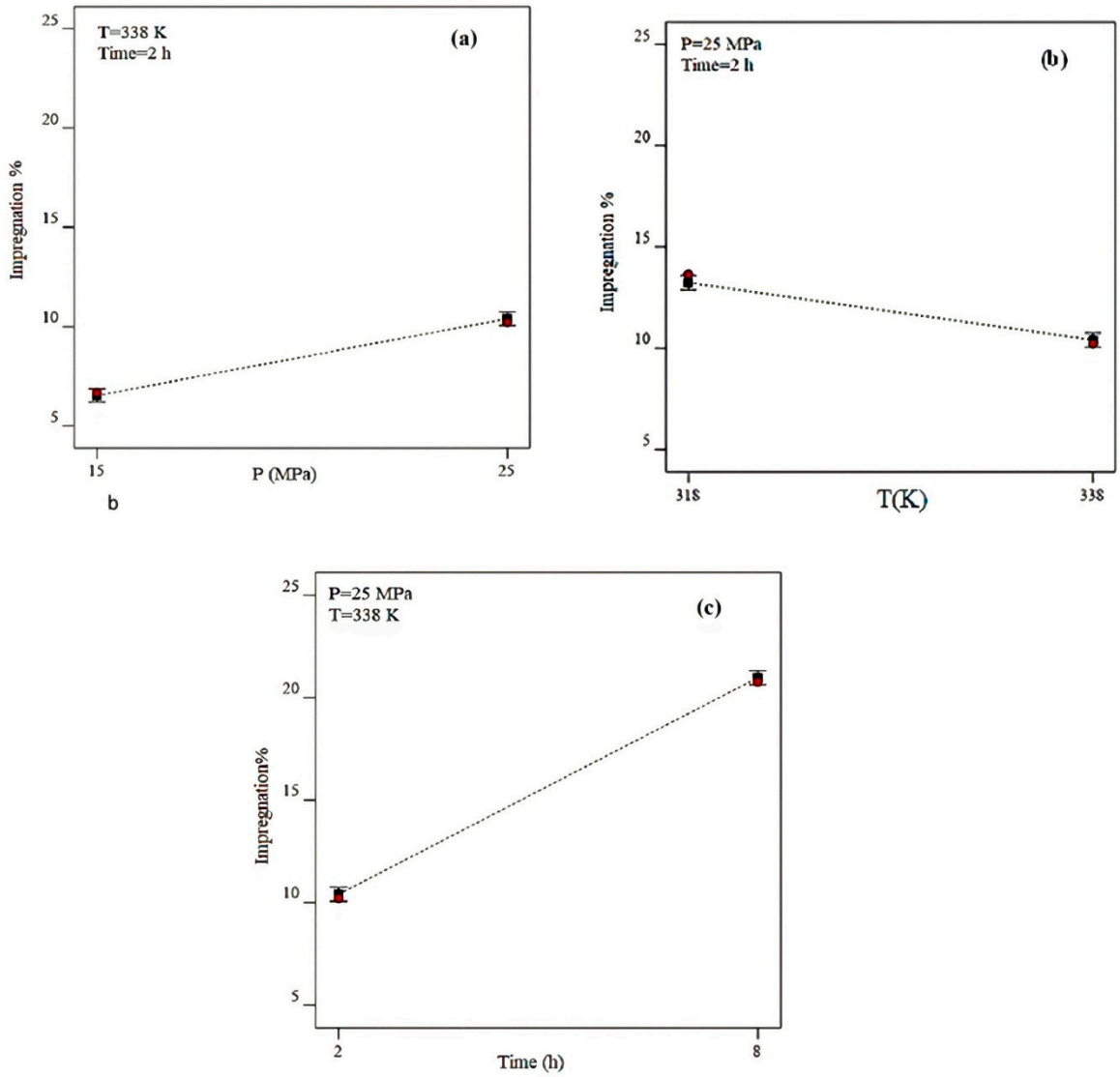

Figure 5. One factor plot to represent the effect of (**a**) pressure at a constant temperature of 338 K and impregnation time of 2 h (**b**) temperature at a constant pressure of 25 MPa and time of 2 h, and (**c**) time at a constant temperature of 338 K and pressure of 25 MPa, on the loading of EO in PLA films.

Some authors in literature reported an increase in impregnation yield with increasing pressure. Li and Han [64] reported the greatest impregnation of styrene in LDPE films when pressure was raised to 13 MPa (at 35 °C). Meanwhile, Torres et al. [65] indicated an increase in the impregnation of thymol when pressure was raised from 7 to 12 MPa at 40 °C. Shen et al. [18] reported the same behavior for the impregnation of vanillin in cellulose acetate fibers.

The effect of temperature on the impregnation yield of *Z. multiflora* EO extract in PLA films at constant pressure and time can be seen in Figure 5b. As shown in Figure 5b, increasing temperature from 318 to 338 K, at constant pressure (25 MPa) and impregnation time (2 h), decreased the EO impregnation yield from 13.65 to 10.21 wt.%, which indicates a negative effect of temperature on the impregnation of *Z. multiflora* EO in PLA. The same trend was obtained for 15 MPa and both impregnation times (Figure 6a,b). The effect of temperature over an active compound loading can be explained according to the interactions between the components of the system (EO extract, SC-CO_2 and PLA) and the changes in the physical properties of the polymers induced by temperature. Particularly, increasing temperature at constant pressure decreases the density of CO_2 which increases the diffusion coefficient of CO_2 in a polymer structure allowing to increase the

amount of CO_2 adsorbed in the polymer, negatively impacting on the interaction between the solute to be impregnated and the polymer [20]. This sorption phenomenon could be improved due to the increase of the movement of PLA chains as temperature increases. Moreover, the solubility of an active solute decreases as temperature increases at constant pressure due to the decrease in CO_2 density which establishes a lower gradient of concentration for the mass transfer process of the active compound from the dense CO_2-phase to the polymer. These facts explained the negative effect of increasing temperature from 35 to 55 °C on the caffein loading in PET/PP films using pressure values between 10 and 40 MPa [33]. Finally, the degree of impregnation of the substance in the polymeric matrix depends on the temperature tolerance of the bioactive and the polymers. The obtained results are consistent with the findings of other researchers [66–69].

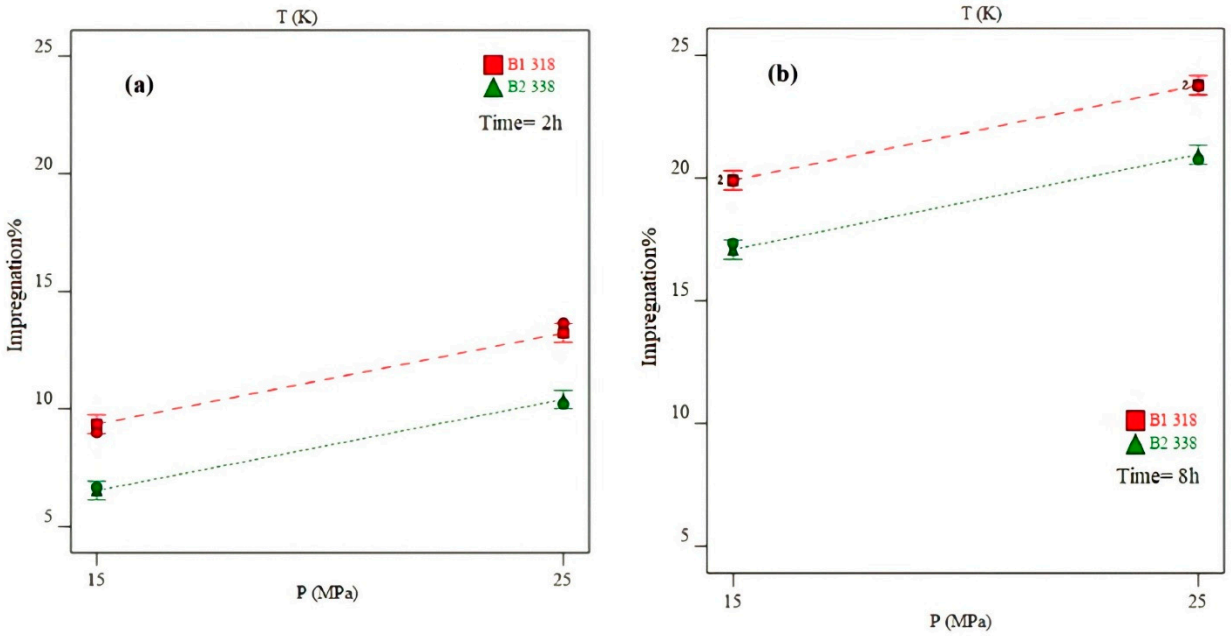

Figure 6. The plots to represent the effect of (**a**) the interaction of temperature and pressure with a time of 2 h, (**b**) the interaction of temperature and pressure with a time of 8 h, on the loading of EO in the PLA film.

The influence of increasing the impregnation time from 2 to 8 h, at constant temperature and pressure, on the impregnation yield of *Z. multiflora* EO extract is shown in Figure 5c. The necessary time to reach the equilibrium condition is one of the main factors in the SSI process appertaining to the type of the bioactive component, the physical properties of the polymer and the operational process conditions pressure and temperature. According to Table 4, with increasing the impregnation time from 2 h to 8 h, at a constant pressure of 25 MPa and temperature of 338 K, the loading of *Z. multiflora* EO extract in PLA increased from 10.40 to 20.96 wt.%. Particularly, a prolongation of the impregnation process leads to an increased swelling and flexibility of the polymer and thus increases the adsorption of SC-CO_2 into the polymer. Therefore, with more swelling of the polymer, the diffusion of bioactive molecules and the amount of transferred bioactive compounds from the SC-CO_2 to the polymer increases. In fact, the swelling and plasticization of the polymer and diffusion of the bioactive molecules into the polymeric structure are time-dependent phenomena during the SSI [70]. The positive effect of the impregnation time on the quantity of the loaded material in the polymeric matrix has been also reported by other researchers [67,71–73].

3.5. Characterization of the PLA-Z. multiflora System

3.5.1. Fourier Transform Infrared (FTIR) Spectroscopy

Fourier transform infrared spectroscopy (FTIR) analyses were carried out for *Z. multiflora* EO extract that are presented in Figure 7. In additional, the FTIR analyses were done to determine the bioactive compounds-polymer interactions. In this regard, FTIR spectra of the neat PLA and a PLA sample impregnated with the *Z. multiflora* EO are also illustrated in Figure 7.

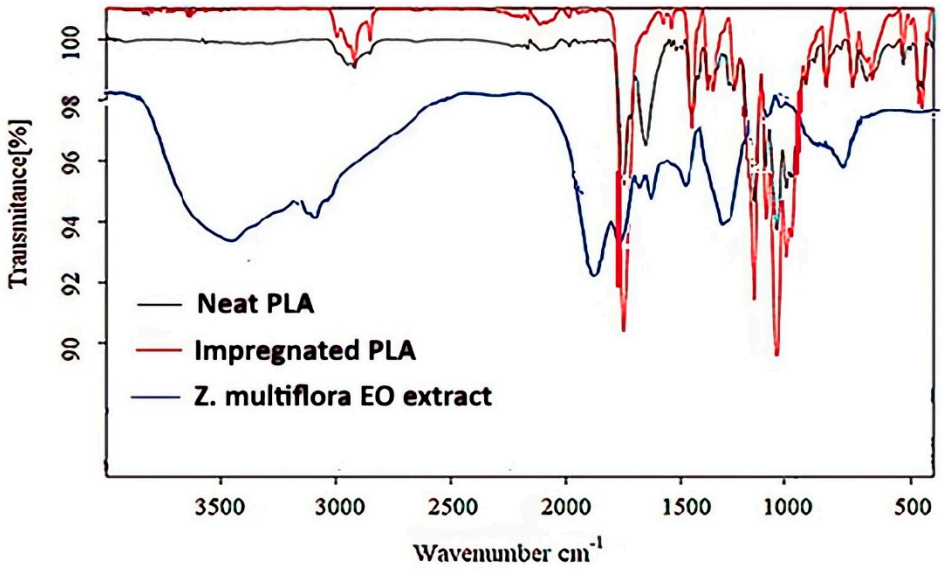

Figure 7. FTIR spectra of neat PLA, impregnated PLA, and *Z. multiflora* EO extract at the optimum conditions.

Neat PLA films presented peaks at 2994 and 2944 cm^{-1} related to the characteristic symmetric and asymmetric stretching of C-H vibrations in PLA. FTIR peaks at 1747, 1450, 1381 and 1360 cm^{-1} have been ascribed to the stretching of C=O group, bending of CH_3 and bond of C-H symmetric and asymmetric vibrations of CH_2, respectively. Peaks at 1180 and 1127 cm^{-1} are attributed to stretching vibrations of -C-O-C- and the peak at 1079 cm^{-1} corresponded to stretching vibrations of -C-O-C- and -C-O- bonds. Eventually, the peak at 867 cm^{-1} is assigned to amorphous PLA and the peak at 754 cm^{-1} is assigned to the crystalline phase of PLA [74–76].

On the other hand, the incorporation of the *Z. multiflora* EO extract in the films of PLA was confirmed through the apparition of a new band at 815 cm^{-1} associated to the ring vibration of thymol, the major constituent of the *Z. multiflora* EO extract [76,77].

3.5.2. Thermal Properties

DSC analyses were performed to study the thermal properties of the different samples. The DSC thermograms for the neat PLA film and PLA films impregnated with *Z. multiflora* EO extract are reported in Figure 8. By analyzing the PLA film after processing with SC-CO_2, it was found that the process had no effect on the glass transition temperature (T_g) of the polymer, but a reduction in crystallinity and melting temperature (T_m) from 168.3 to 167.8 °C was obtained due to the reported plasticizing effect of some of the phenolic compounds presented in the EO extract on PLA, such as thymol. These results are in agreement with those reported by Torres et al. [50].

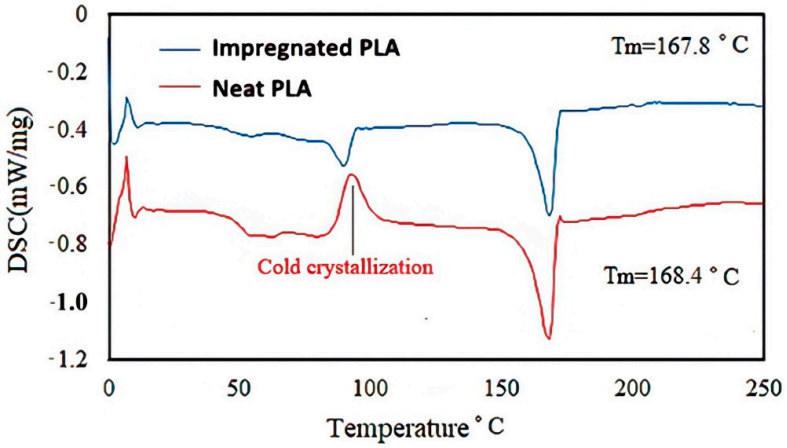

Figure 8. DSC analyses results of the neat PLA and PLA impregnated at the optimum conditions.

3.5.3. X-ray Diffraction

The XRD spectra of the neat PLA film and the impregnated PLA film at the optimum conditions are presented in Figure 9a,b, respectively. The XRD pattern of the neat PLA film was crystalline with the characteristic peaks at 9.603°, 17.647°, and 29.278°, in agreement with the report of Mihai et al. [78]. The comparisons between neat PLA and impregnated PLA indicated that the bioactive components of the *Z. multiflora* EO extract were dispersed in PLA in an amorphous state, which agrees with DSC findings. In addition, the differences between the samples illustrated that the impregnated PLA film had a lower crystallinity degree than the neat PLA film. This Phenomenon could be attached to two reasons; (i) the reported plasticizing effect of SC-CO_2 on PLA and (ii) the reported plasticizing effect of some of the components of the *Z. multiflora* EO extract on the PLA structure [79].

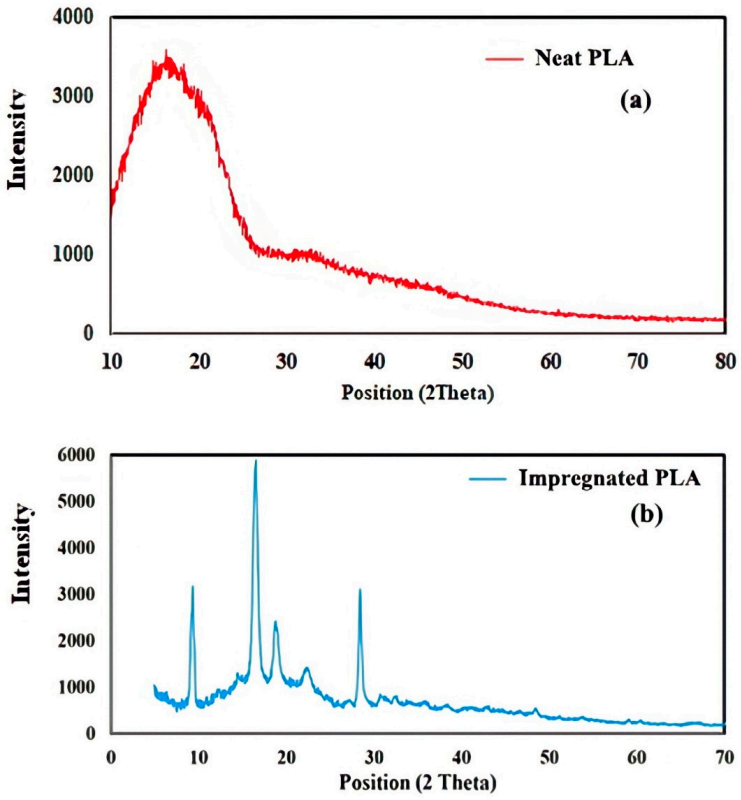

Figure 9. XRD patterns of (**a**) neat PLA and (**b**) PLA impregnated at the optimum conditions.

3.5.4. Film Surface Morphology (SEM)

Scanning electronic microscopy (SEM) was used to analyze the morphology of PLA before and after the impregnation with *Z. multiflora* EO extract. Figure 10a,b show the surface images of the neat, and impregnated sample, respectively. It was possible to observe a homogeneous surface morphology on the impregnated sample. However, the initial average thickness of the PLA film was increasing after the impregnation process. This phenomenon may be due to the swelling of PLA after the supercritical process, which leads to a more amorphous polymeric film [80].

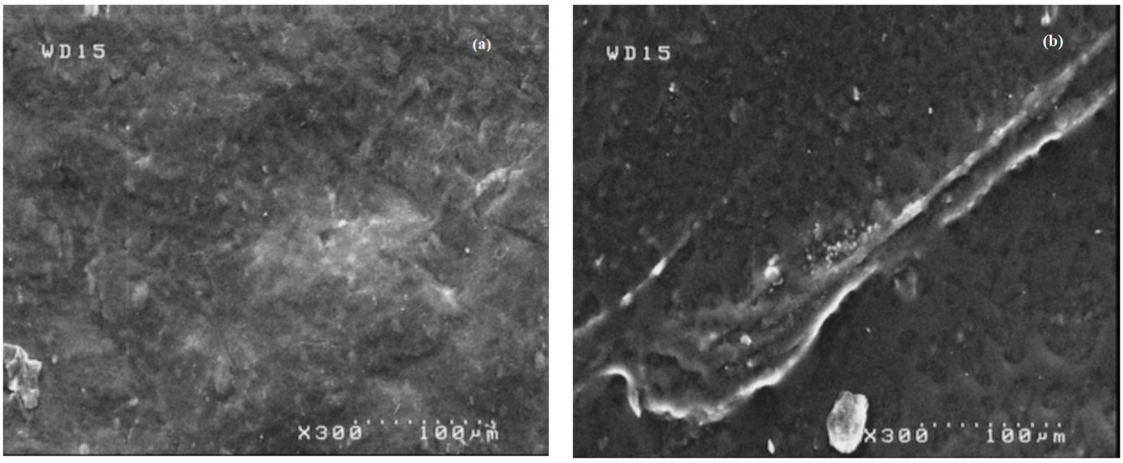

Figure 10. The surface images of (**a**) neat PLA, and (**b**) PLA impregnated at the optimum conditions.

3.6. Antibacterial Activity of Impregnated Films and Antioxidant Capacity of the Z. multiflora EO Extract

Table 5 shows the antibacterial effect of the PLA films impregnated with *Z. multiflora* EO extract against *E. coli* and *S. aureus*. The impregnation conditions for the obtention of the PLA films loaded with *Z. multiflora* EO extract were listed in Table 3. These results show viability for both bacteria strains in the control sample (media), neat PLA (bacteria + phosphate buffer + blank PLA), and some impregnated samples. As indicated in Table 5, the viability values for both bacteria strains were very similar for the control PLA and the neat PLA with values ranging between 7.755–7.771 log CFU/mL for *E. coli* and between 7.361–7.398 log CFU/mL for *S. aureus*. The GC Mass Analysis (Table 1) showed that the concentrations of thymol, carvacrol, γ-terpinene, and linalool in the *Z. multiflora* EO extract obtained by SFE at the optimal conditions were 39.92, 21.37, 10.37, and 6.02 wt.%, respectively. These four compounds have been identified as the main components of *Z. multiflora* EO in many researches [53,56–58,81] and the main responsible of the great antibacterial activity of the *Z. multiflora* EO [81]. Saei–Dehkordi et al. reported the antibacterial effect of *Z. multiflora* EO extracts obtained from five different places of Iran. EO extracts obtained from *Z. multiflora* from Najafabad had the highest antibacterial activity due to its higher content of thymol and carvacrol (69.52 wt.%) [53]. High antibacterial properties of other natural extracts have also been attributed to their high content of thymol and carvacrol [82]. In this context, the higher inhibition of the growth of *E. coli* and *S. aureus* (Table 5) using the impregnated samples for the runs 1, 4, 6, 7, and 8, where viability was not detected (N.D) for both bacteria, than the inhibitions obtained using the impregnated samples for the runs 2, 3 and 5, where viabilities ranged between 6.954 and 7.079 log CFU/mL, could be explained by the values of impregnation yields of *Z. multiflora* EO extract obtained for the first impregnation runs group (13.65 to 20.76 wt.%), which are higher than the impregnation yields obtained for the second group (6.67 to 10.21 wt.%). Thus, the initial content of *Z. multiflora* EO extract in the samples 1, 4, 6, 7 and 8 allowed to maintain a Minimal Inhibitory Concentration (MIC) along the tested time in contrast with the samples 2, 3 and 5. The reported minimum inhibitory concentrations (MIC) of thymol,

carvacrol, and linalool have been reported at 250, 125, 256 µg/mL against both *E. coli* and *S. aureus* [83].

Table 5. Antibacterial activity of *Z. multiflora* EO extract against *E. coli* and *S. aureus*.

	Viability (Log CFU/mL)	
Samples	*E. coli*	*S. aureus*
Control	7.755 ± 7.114 [b]	7.361 ± 6.633 [b]
PLA	7.771 ± 7.079 [b]	7.398 ± 6.716 [b]
Run 1	N.D [a]	N.D [a]
Run 2	6.954 ± 6.114 [b]	N.D [a]
Run 3	7.079 ± 6.23 [b]	6.491 ± 5.716 [b]
Run 4	N.D [a]	N.D [a]
Run 5	7.041 ± 6.204 [b]	6.415 ± 5.633 [b]
Run 6	N.D [a]	N.D [a]
Run 7	N.D [a]	N.D [a]
Run 8	N.D [a]	N.D [a]

[a] N.D: Not Detected; [b] mean ±standard deviation.

The antioxidant potential of an active substance is defined by its reducing power, radical scavenging ability and singlet oxygen quenching ability. The antioxidant capacity of the *Z. multiflora* EO extract obtained at the optimal extraction conditions was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. The IC_{50}, concentration to scavenge 50% of DPPH radicals, for the EO sample extracted from *Z. multiflora* using SC-CO_2 was 19.9 ± 0.7 µg/mL. This result agrees with the values reported by other authors for *Z. multiflora* extract [53]. The findings suggested that *Z. multiflora* can provide a good source of antioxidants to be used for food and medicinal purposes.

4. Conclusions

This work aimed to study the supercritical extraction of EO from *Z. multiflora* and its incorporation in PLA films by supercritical impregnation. Thymol, carvacrol and γ-terpinene were determined as the main constituents of the *Z. multiflora* EO extract using GC Mass spectroscopy. The optimized conditions for the EO extraction process were determined as: P = 25 MPa, T = 318 K, and co-solvent = 3%. The extraction yield under this condition was 2.560 ± 0.04 wt.%. The antioxidant potential of the extracted EO was determined by its reducing power and radical scavenging ability through a DPPH assay. The impregnation yield of *Z. multiflora* EO extract in PLA films ranged between 6.67 ± 0.86 and 23.76 ± 1.18 wt.%. The presence of the components of the *Z. multiflora* EO extract in PLA was confirmed by FTIR analysis. The full factorial method (FFD) was used to study the effect of pressure, temperature and time over the impregnation yield of *Z. multiflora* EO extract in PLA. In addition, the impregnated sample with the highest impregnation yield was analyzed via FTIR, DSC, SEM, and XRD tests. Impregnated PLA samples presented a decrease in the melting temperature which confirmed the incorporation of the *Z. multiflora* EO extract. The molecular dispersion of the constituents of the *Z. multiflora* EO extract impregnated in PLA by SII was confirmed by the DSC results. Furthermore, the antibacterial properties of this sample against two bacteria, Gram (+) and Gram (−) were studied. The results for antibacterial activity of impregnated PLA films with different contents of *Z. multiflora* EO extract against *E. coli* and *S. aureus* indicated no viability for both bacteria. The obtained results showed that supercritical extraction (SFE) and impregnation (SSI) are feasible techniques for the development of antibacterial food packaging materials.

Author Contributions: Conceptualization, S.A.S. and A.R.; methodology, S.A.S., N.S.A., A.R., N.E., A.B. and M.J.G.; validation, S.A.S., A.R., N.S.A. and N.E.; writing—original draft preparation, N.S.A., S.A.S., A.R., N.E. and A.B.; writing—review and editing, S.A.S., A.R., N.E. and M.J.G.; visualization, S.A.S. and N.S.A.; supervision, S.A.S., N.S.A. and M.J.G.; project administration, S.A.S.; funding acquisition, A.R. and M.J.G. All authors have read and agreed to the published version of the manuscript.

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