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Antioxidant activity of *Piper nigrum* L. essential oil extracted by supercritical CO_2 extraction and hydro-distillation

Hossein Bagheri*, Mohd Yazid Bin Abdul Manap, Zeinab Solati

Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, UPM, Serdang, 43400 Selangor, Malaysia

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ABSTRACT

The aim of this study was to optimize the antioxidant activity of *Piper nigrum* L. essential oil extracted using the supercritical carbon dioxide (SC-CO_2) technique. Response surface methodology was applied using a three-factor central composite design to evaluate the effects of three independent extraction variables: pressure of 15–30 MPa, temperature of 40–50 °C and dynamic extraction time of 40–80 min. The DPPH radical scavenging method was used to evaluate the antioxidant activity of the extracts. The results showed that the best antioxidant activity was achieved at 30 MPa, 40 °C and 40 min. The extracts were analyzed by GC-FID and GC–MS. The main components extracted using SC-CO_2 extraction in optimum conditions were β-caryophyllene (25.38 ± 0.62%), limonene (15.64 ± 0.15%), sabinene (13.63 ± 0.21%), 3-carene (9.34 ± 0.04%), β-pinene (7.27 ± 0.05%), and α-pinene (4.25 ± 0.06%). The essential oil obtained through this technique was compared with the essential oil obtained using hydro-distillation. For the essential oil obtained by hydro-distillation, the most abundant compounds were β-caryophyllene (18.64 ± 0.84%), limonene (14.95 ± 0.13%), sabinene (13.19 ± 0.17%), 3-carene (8.56 ± 0.11%), β-pinene (9.71 ± 0.12%), and α-pinene (7.96 ± 0.14%). Radical scavenging activity of the extracts obtained by SC-CO_2 and hydro-distillation showed an EC_{50} of 103.28 and 316.27 μg mL^{-1} respectively.

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1. Introduction

Piper nigrum L., also known as black pepper, is a perennial woody evergreen climber native to South India that can grow to a height of 50–60 cm. It is an aromatic plant very well known for its antioxidant, antimicrobial, carminative, and antiseptic properties [1]. It has been used traditionally for the relief of pain, atrophic arthritis, apathy, influenza, and febricity, and as a nerve tonic, antibacterial agent, stimulant, digestive, and antitoxin [2]. The essential oil of *P. nigrum* L. has been found to possess multiple applications in the food and pharmacological industries, perfumery, cosmetics and home remedies [3].

Essential oils are secondary metabolites produced by aromatic plants. They are also known as ethereal oils, and are characterized as volatile, natural, liquid, complex components with a strong aroma. The organoleptic properties and biological activities of essential oils are distinguished by their respective compositions [4,5]. They have been found to have antioxidant properties, which are attributed to the presence of terpenoid and phenolic compounds. The main compounds are made up of terpenes and terpenoids, and also aromatic and aliphatic components [6]. There is a growing interest in natural food antioxidants from plant origins due to the carcinogenic effects of synthetic antioxidants; this interest has led to the extraction of these biologically active components from a range of raw vegetable substances [6]. Biologically, antioxidants have been described as substances that can delay or inhibit the oxidation process when they are present in concentrations lower than the oxidation substrate. No definition could limit the antioxidant activity to a specific group of compounds or to any specific mechanism of action [7]. The quality of these substances is greatly influenced by how they are extracted. The essential oil of *P. nigrum* L. is traditionally extracted by hydro-distillation. However, this hot method of extraction can affect the essential oil's properties and may influence most of the minor constituents, which have many functional and antioxidative effects [8]. Furthermore, the essential oils can undergo hydrolysis and solubilization in water, which result in the alteration of the flavor and fragrance profile, and significantly decreasing the quality of the essential oil [5].

An alternative method has been suggested for extracting natural products, and more particularly volatile fractions, from plants by using supercritical carbon dioxide extraction (SC-CO_2). In comparison to traditional extraction methods (i.e., solvent extraction, steam distillation and hydro-distillation), the crucial benefit of the low temperatures used in this method is the conservation of

* Corresponding author. Tel.: +603 894 863 14.
E-mail addresses: hosseinbagheri_1983@yahoo.com, hosseinbagheri.1983@gmail.com (H. Bagheri).

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their biological properties [9,12]. The obtained extract has a high-quality base for the production of pharmaceutical drugs and additives in the perfumery, cosmetology, and food industries, and do not need any specific refining procedure [9]. SC-CO_2 extraction has been recommended for the extraction of antioxidant constituents from sage, rosemary leaves, and herbaceous matrixes [10]. It was found that the extract obtained using SC-CO_2 has significantly higher antioxidant activity compared to conventional methods [11]. The effects of different extraction parameters, including temperature, pressure and dynamic time on the antioxidant activity of *P. nigrum* L. essential oil, have not been studied before. These factors can be collectively studied to validate the optimal extraction conditions using response surface methodology (RSM). Accordingly, the main objective of this study was to establish the optimum SC-CO_2 extraction conditions for *P. nigrum* L. essential oil with a high antioxidant activity using RSM.

2. Materials and methods

2.1. Materials and reagents

Black pepper (*P. nigrum* L. var. Kuching) seeds were provided by the Malaysian Pepper Board, Sarawak, Malaysia. Carbon dioxide (purity 99.99%) was supplied in a diptube cylinder by the MOX Company (Petaling Jaya, Malaysia). The standard chemicals, including piperine, α-pinene, β-pinene, limonene, camphene, sabinene, thujene, and myrcene, were purchased from Sigma Chemical Co. (St Louis, USA). All chemicals and required solvents were of either analytical grade or GC grade, and were obtained from Merck (Darmstadt, Germany).

2.2. Sample preparation

P. nigrum L. seeds were ground with a suitable grinder (MX-335, Panasonic, Malaysia) for 2 min carefully and gently in order to prevent the increase in temperature. Each sort of increase of sample′s temperature results the reduction of quality and quantity of extractive essential oil. Afterwards, the powdered sample passed through 1–2 mm screens. The sample was prepared and weighted exactly before each experiment and was used immediately to avoid the loss of essential oil.

2.3. Extraction methods

2.3.1. Hydro-distillation

The extraction procedure was carried out according to the European Pharmacopoeia procedure (1983). Approximately 50 g of black-pepper powder was placed in a round-bottomed flask containing 1200 mL of distilled water, which was connected to a Clevenger′s distillation unit. The black-pepper powder was then hydro-distilled for 4.5 h. The obtained essential oil, which was collected in the side arm, was separated and dried over anhydrous sodium sulfate to eliminate traces of moisture. The extracted essential oil was placed in a glass amber bottle and refrigerated at 4 °C until further analysis.

2.3.2. Supercritical fluid extraction

Supercritical fluid extractions were performed using a laboratory-scale supercritical extraction unit equipped with two intelligent HPLC pumps (Model PU-1580, Jasco Corporation, Japan): a CO_2 pump, and a co-solvent pump. Liquid CO_2 contained in the cylinder was cooled using a cooling jacket attached to the intelligent HPLC pump. For all runs, the 50 mL stainless steel extractor (Model EV-3, Jasco Corporation, Japan) was charged with a mixture of 1:1 (v/v) milled *P. nigrum* L. seeds (5 g) and glass beads (1.00 mm in diameter), and the extraction vessel was placed in a column oven (model CO-1560, Jasco Corporation, Japan) at preferred temperature. The liquid CO_2 was pressurized to the desired pressure, then run through a long tubular preheating coil with a length of 4 m, where it reached the specified temperature prior to contacting the sample in the extractor vessel. The extractor was immersed in a temperature-controlled water bath. A back-pressure regulator (Model BP-1580-81, Jasco Corporation, Japan) was used to set the system pressure. Extractions were performed with a constant flow rate of 2 mL min^{-1}, and the extraction time was set by the timer. Three operating variables were used in the study: pressure, temperature, and dynamic extraction time. They were chosen according to the preliminary study. There were two stages of extraction: static and dynamic. The static stage was 30 min for all experiments, and the dynamic stage varied from 27–92 min. Extracts were collected during the dynamic stage in amber vials (5 mL) fitted with silicone septa. The essential oil was refrigerated at 4 °C prior to the analysis to prevent chemical reactions such as isomerization and oxidation.

2.4. DPPH radical scavenging assay

The capacity of the prepared extracts to scavenge the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was monitored according to the method described by Erkan et al.[13]. A freshly prepared DPPH solution exhibited a deep purple color with a maximum absorption at 515 nm. A volume of 1.5 mL DPPH (0.2 mM) in ethanol was added to 1.5 mL of varying concentrations of test samples. The reaction mixture was then kept at room temperature in a dark chamber for 30 min. The change in color from deep violet to light yellow was measured at 515 nm in a U-2000 spectrophotometer (Hitachi, Ltd, Tokyo, Japan). A blank experiment was also carried out by applying the same procedure to a solution without the test material. The decrease in absorbance was then converted to percentage antioxidant activity using the equation below:

$$\%\text{inhibition} = 100(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}$$

The antioxidant activity of test compounds or extracts was expressed as EC_{50}, defined as the concentration of test material required to cause a 50% decrease in initial DPPH concentration. The EC_{50} of each sample was expressed in μg mL^{-1} and calculated through the interpolation of linear regression analysis. The test was performed in triplicate for each extract.

2.5. Experimental design

In SC-CO_2 extraction, different factors affect the recovery of a target component. Due to the difficulties of conducting an experiment using high pressure and the need to decrease the number of tests, it was necessary to use an appropriate design of experiment (DOE). Generally, a DOE can decrease the costs and the time of operation and can be performed in different ways. RSM is the most popular design for an experimental approach, and a central composite design of the RSM is generally applied in optimization experiments [14]. In this study, 20 experimental treatments were assigned based on a three-factor CCD fitting with eight factorial points, six star points, six central points and $\alpha = \pm 1.682$ to assess the effects of the operating variables of SC-CO_2 process–pressure (15–30 MPa), temperature (40–50 °C), and dynamic extraction time (40–80 min) – on the antioxidant activity of *P. nigrum* L. essential oil.

Table 1 gives the matrix of the CCD and experimental data obtained for antioxidant activity. A six-time replication of the center point was used to calculate the repeatability of the method. The existence of six star points beyond the studied levels is to

Table 1
Matrix of the central composite design (CCD) and experimental data obtained for response variable studied.

Run	Block	Independent variable			Response variable
		Pressure (MPa)	Temperature (°C)	Time (min)	EC_{50} (μg mL^{-1})
1	3	10.25	45.00	60.00	401.73
2	3	22.50	45.00	27.34	181.74
3	3	22.50	53.16	60.00	268.31
4	3	22.50	36.83	60.00	173.45
5	3	34.74	45.00	60.00	105.67
6[a]	3	22.50	45.00	60.00	201.46
7	3	22.50	45.00	92.66	223.45
8[a]	3	22.50	45.00	60.00	212.82
9[a]	1	22.50	45.00	60.00	208.79
10	1	15.00	40.00	40.00	235.74
11	1	15.00	50.00	80.00	364.72
12[a]	1	22.50	45.00	60.00	204.25
13	1	30.00	50.00	40.00	143.58
14	1	30.00	40.00	80.00	108.56
15[a]	2	22.50	45.00	60.00	209.68
16	2	30.00	50.00	80.00	159.28
17[a]	2	22.50	45.00	60.00	205.81
18	2	30.00	40.00	40.00	103.28
19	2	15.00	40.00	80.00	281.29
20	2	15.00	50.00	40.00	298.67

[a] Center point.

realize the possible unexpected changes in the actual response that may happen outside the studied levels [15].

2.6. Statistical and data analysis

RSM was employed to generate response surface models to (1) determine regression coefficients and statistical significance of model terms and (2) help in fitting the regression models to the experimental data, aiming at an overall optimum region for the response variable studied. The prediction of the optimum SFE condition was expressed in accordance with the following overall polynomial model:

$$Y_i = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} {X_1}^2 + \beta_{22} {X_2}^2 + \beta_{33} {X_3}^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where Y_i is the response variable predicted by the model; β_0 is an offset value; β_1, β_2, and β_3 are the regression coefficients for the main (linear) terms; β_{11}, β_{22},and β_{33} are quadratic effects; β_{12}, β_{13}, and β_{23} are interaction effects; and x_1, x_2, and x_3 are the independent variables.

Using the method of Weng et al. [16], the adequacy of the response surface models was investigated by model analysis, a lack-of fit test and coefficient of determination (R^2) analysis. Joglekar and May [17] recommended that R^2 of at least 0.80 indicates a good fit for the response model. The corresponding variables are considered more significant ($p < 0.05$) if the absolute t-value becomes larger and p-value becomes smaller [18]. The terms found to be statistically insignificant ($p > 0.05$) were dropped from the initial model, and the experimental data were refitted to only the significant ($p < 0.05$) factors to obtain the final reduced model. Minitab version 15 (Minitab Inc., PA, USA) was applied for the creation of the experimental design, data analysis, and optimization procedure.

2.7. Optimization and validation procedures

In the present work, both graphical and numerical optimization procedures were applied to determine the optimum level of SC-CO_2 extraction conditions leading to the maximum antioxidant activity of *P. nigrum* L. essential oil. The numerical optimization was performed via the response optimizer (Minitab v.15) to determine the exact optimum level of the independent variables (pressure, temperature, and dynamic extraction time) to obtain the desired response goals. The response optimizer allowed us to interactively alter the input variable settings to carry out sensitive analyses. For the graphical optimization procedure, the final reduced models were expressed as three-dimensional (3D) response surface plots for better visualization of the significant ($p < 0.05$) interaction effect of SC-CO_2 extraction variables on the antioxidant activity of *P. nigrum* L. essential oil. The 3D plots were generated by fixing one variable constant at the center point and changing the other two variables within the experimental range. For method validation, a comparison was made between the experimental data and predicted values using a t-test of two samples to verify the adequacy of the final reduced models [19].

2.8. Chemical compositions analysis: GC–FID and GC–MS

Gas chromatography–flame ionization detection (GC–FID) and gas chromatography–mass spectrometry (GC–MS) were used to carry out quantitative and qualitative analysis of the essential oil. GC analyses were performed on an Agillent 6890N GC (Wilmington,USA) equipped with a FID and a non-polar fused silica capillary column of HP-5 MS (0.25 mm × 30 m, film thickness 0.25 μm, Hewlett-Packard, California, USA).The carrier gas used for this system was helium (purity 99.999%) with a flow rate of 1.5 mL min^{-1}. The temperatures of the injector and detector were set at 250 and 300 °C, and the column inlet pressure was set at 207 KPa. The oven temperature was programmed at 40 °C for 5 min, then increased to 260 °C at a rate of 3 °C/min; it was then kept constant for a further 10 min. Sample preparation was performed by injection of a single dilution of the essential oil collected in hexane at a ratio of 1:10 (v/v). 1 μL of each diluted sample was injected, using split mode at a ratio of 1:20. The same procedure was conducted for the standard chemicals (α-pinene, β-pinene, limonene, camphene, sabinene, thujene, and myrcene), and the main components of the essential oil were identified by comparing the retention times with those of the respective standards. Component quantification was performed by the normalization method of the GC peak areas, calculated as mean values of three injections, without using correction factors.

The GC–MS analyses were carried out on a Shimadzu model QP5050A GC (Japan) coupled to a GC-17A Ver.3 system and equipped with a quadrupole mass spectrometer. The chromatographic column and the program parameters were the same as that used in GC. The temperatures of the injector, GC–MS interface and ion trap were 250, 320, and 230 °C respectively. For GC–MS detection, the spectrometer was adjusted in electron impact mode, with an ionization energy of 70 eV in the mass scanning range of 50–400 m/z. The compounds of essential oil were identified by comparison of their retention indices relative to a homologous series of n-alkanes (C9-C24) and mass spectra and those recorded in the NIST 05 MS Library Database (similarity index > 80%), those reported in the MS data found in the literature[20], and by co-injection of available reference compounds. The samples were analyzed in triplicate.

3. Results and discussions

3.1. Response surface analysis

In the current study, multiple regression analysis was performed using response surface analysis to provide a relationship

between three SC-CO_2 extraction variables and the antioxidant activity of *P. nigrum* L. essential oil (Table 2). The application of RSM provided an empirically significant ($p<0.05$) model to estimate the variation of the antioxidant activity as a function of the SC-CO_2 extraction conditions. Table 2 contains the estimated regression coefficients for the response variable, along with the corresponding R^2, R^2 (adj), F-value, and *p*-value of lack of fit.

The results showed that the second-order polynomial regression model with a high R^2 of 0.994 and an adjusted R^2 value of 0 0.989 was significantly ($p<0.05$) fitted for the response variable studied (Table 2). Therefore, it can be deduced that more than 99% of the variability of the antioxidant activity of *P. nigrum* L. essential oil could be explained by the RSM model as the nonlinear function of the SC-CO_2 extraction variables.

Additionally, no significant ($p>0.05$) lack of fit at the 95% confidence level was observed for the final reduced model. Consequently, the results confirmed that the final reduced model was suitably and adequately fitted to the experimental data (Table 2). According to Montgomery [15], the final reduced model and recommended optimum region is only a valid statistical empirical model in the studied independent variable ranges. Therefore, it cannot be extrapolated beyond these ranges.

Table 3 shows the main, quadratic and interaction effects of pressure (X_1), temperature (X_2) and dynamic extraction time (X_3) on the response variable, and the significance of each term assessed using the *p*-value and *F*-ratio. The main effects of the SC-CO_2 extraction variables were retained in the final reduced model, while the quadratic effect of temperature and also the interaction effect of temperature and extraction time were dropped (Tables 2 and 3). Among all SC-CO_2 extraction variables, the extraction pressure and extraction time indicated the most and least significant ($p<0.05$) effects on EC_{50} of *P. nigrum* L. essential oil, respectively (Table 3). As shown in Tables 2 and 3, the interaction effects of pressure and temperature, as well as pressure and extraction time, significantly ($p<0.05$) affected the EC_{50} of the essential oil.

Table 2
Regression coefficients, R^2, adjusted R^2, probability values and lack of fit for the final reduced model.

Regression coefficient	EC_{50} (Y_1,μg mL^{-1})
b_0	208.497
b_1	−86.189
b_2	29.421
b_3	15.052
b_1^2	13.631
b_2^2	–
b_3^2	−5.533
b_{12}	−6.918
b_{13}	−11.328
b_{23}	–
R^2	0.994
$R^2(adj)$	0.989
Regression (*p-value*)	0.000[a]
Lack of fit (*F-value*)	3.09
Lack of fit (*p-value*)	0.191[b]

b_i, b_{ii} and b_{ji}: The estimated regression coefficient for the main effects, quadratic effects and interaction effects, respectively; 1: Pressure; 2: Temperature; 3: Dynamic extraction time.

[a] Significant ($p<0.05$).

[b] Not significant ($p>0.05$).

3.2. Antioxidant activity of the extract

Plant extracts possess particular biological actions, including antioxidant activity, that are frequently attributed to specific constituents or classes of components. As shown in Tables 2 and 3, the EC_{50} value (Y_1) was positively proportional to the main linear effects of temperature and extraction time, and the quadratic effect of pressure. However, it was negatively affected by the main linear effect of pressure and the quadratic effect of extraction time, as well as the interaction effects between pressure and extraction time and between pressure and temperature. It is also noteworthy that temperature indicated the highest significant positive effect on the variation of EC_{50} value (Y_1). Consequently, the significant parameters mentioned above were considered as the main factors fitted for EC_{50} in the final reduced model. As shown in the results (Table 2), the final reduced model indicated an acceptable R^2 (0.994) when fitted for EC_{50} value. Accordingly, the final reduced model could accurately explain 99% of the variation in the EC_{50} value as a function of the independent variables within the experimental range. By fitting the constant and coefficients into Eq. (1), an empirical model can be obtained.

$$Y_1 = 208.497 - 86.189\ X_1 + 29.421\ X_2 + 15.052\ X_3 + 13.631\ X_1^2 - 5.533\ X_3^2 - 6.918\ X_1X_2 - 11.328\ X_1X_3 \quad (2)$$

As shown in the regression equation above, the EC_{50} value of *P. nigrum* L. essential oil increased with the linear effects of all variables except pressure, and the quadratic effect of pressure. However, the single effect of pressure, and its interaction with extraction time and temperature, could decrease the EC_{50} value.

Table 1 exhibits the variations in the antioxidant activity of extracted essential oils obtained under different experimental conditions at various levels of pressure, temperature and extraction time. It is clear that lower EC_{50} values represent higher antioxidant power. All the extracts obtained by SC-CO_2 indicated antioxidant activity, and the extraction performed at 30 MPa, 40 °C, and 40 min, gave the lowest EC_{50} (highest antioxidant activity) value of 103.28 μg mL^{-1}. No earlier studies are reported in the literature regarding the DPPH assay of *P. nigrum* L. essential oil obtained by the SC-CO_2 extraction method. According to the study of Nahak and Sahu [21], the EC_{50} value of crude ethanolic extract of *P. nigrum* L. from the Soxhlet extraction method towards DPPH radicals was found to be 14.15 ± 0.02 μg mL^{-1}. In Su

Table 3
F-ratio and *p*-value of SC-CO_2 extraction variables in the final reduced model.

Variables		Main effects			Quadratic effects			Interaction effects		
		x_1	x_2	x_3	x_1^2	x_2^2	x_3^2	x_1x_2	x_1x_3	x_2x_3
EC_{50} (Y_1,μg mL^{-1})	*p-value*	0.000*	0.000*	0.000*	0.000*	–	0.034*	0.039*	0.003*	–
	F-*value*	1464.36	170.64	44.662	36.469	–	6.007	5.659	15.178	–

x_1, x_2 and x_3: Main effect of pressure, temperature and extraction time, respectively.
x_1^2, x_2^2 and x_3^2: Quadratic effect of pressure, temperature and extraction time, respectively.
$x_1 \times_2$, $x_1 \times_3$ and $x_2 \times_3$: Interaction between pressure and temperature, interaction between pressure and extraction time, and interaction between temperature and extraction time, respectively.

* Significant at $p<0.05$.

et al. [22], an 80% methanolic extract of black peppercorn at ambient temperature exhibited an EC_{50} of 1460 μg mL^{-1}, while the 50% acetone extract of black peppercorn possessed a lower EC_{50} value than the 80% methanolic extract. TheEC_{50} results of this study are meaningful, because they present the antioxidant activity of crude extracts achieved without additional purification of the components responsible for the antioxidant activity.

According to Table 1, the antioxidant activity of the extracted essential oil increased with incremental increases in pressure due to the increase in the solvating power of SC-CO_2 resulting from greater density. In general, supercritical fluids of higher densities possess superior solvating power; consequently, they permit higher specific extraction of bioactive components [5]. Hu et al. [23] explained that pressure improved the antioxidant activity of extracts of antioxidants from natural sources. Solati et al. [24] demonstrated an increment in antioxidant activity of *Nigella sativa* L. oil obtained by SC-CO_2 extraction with an increase in pressure.

At a constant pressure, the antioxidant activity decreased with an increase in temperature because of the reduction in the density of the solvent. It should be noted that in high temperatures some thermolabile compounds could have decomposed and therefore the antioxidant activity decreased [5]. In SC-CO_2 extraction, an increase in temperature results in the decrease in density of the supercritical fluid, but an increase in the volatility of the solute. A reduction in the density of SC-CO_2 would diminish its extraction potential. Conversely, an increase in solute vapor pressure due to increased temperature is expected to enhance solubility and extraction, which results in the enhancement of the percentage of desired components with antioxidant activity [25]. In general, the effect of temperature on the EC_{50} of the extracted essential oil was controlled by the combination of the two competing factors of fluid density and solute volatility. Consequently, the solubility of solute that has no significant vapor pressure decreases with an incremental increase in temperature at a constant pressure due to the reduction in CO_2 density [26]. In the present study, the influence of lower SC-CO_2 density dominated the influence of further solute vapor pressure, as shown by the higher EC_{50} value (lower antioxidant activity), which in turn demonstrates that lower antioxidant components are extracted under these conditions.

The antioxidant activity of extracted essential oil is shown in Fig. 1a as a function of pressure and temperature at an extraction time of 60 min. The effect of pressure and extraction time on antioxidant activity at 45 °C is illustrated in Fig. 1b. Pressure had the most significant effect on antioxidant activity, as mentioned earlier, while the antioxidant activity of extracted essential oil moderately decreased with an increase in extraction time. This result may be explained by the fact that a portion of the extracted antioxidant compounds could have been dragged by CO_2 from the collecting container. It also seems possible that this result is due to the co-extraction of the inactive components. Another possible explanation for this might be the degradation of antioxidant compounds with the increase in extraction time.

As shown in Fig. 1a and b, and concluded from Tables 2 and 3, the variation of antioxidant activity was explained by a nonlinear function of SC-CO_2 extraction variables. The presence of a relatively complex EC_{50} curve (Fig. 1a and b) demonstrated that the interaction effects of independent variables on antioxidant activity of *P. nigrum* L. essential oil depended on the ranges of all variables.

3.3. Optimization procedure

In the present work, the optimum *P. nigrum* L. essential oil would have the highest antioxidant activity (lowest EC_{50}). The multiple optimization procedure was performed to measure an optimum set level of independent variables to obtain the desired response goals [9]. Overall, the optimum SC-CO_2 extraction parameters were found through running the graphical and numerical response optimizations. For the graphical optimization procedure, 3D response surface plotting was strongly suggested; the optimal condition was found by superimposing all plots [27]. A numerical optimization was also performed using the response optimizer to determine the exact optimum level of SC-CO_2 extraction variables. The numerical optimization result indicated that the most desirable *P. nigrum* L. essential oil with optimum antioxidant activity was predicted to be achieved by SC-CO_2 extraction at a pressure of 30 MPa, temperature of 40 °C, and dynamic extraction time of 40 min. Under the optimal extraction conditions, the corresponding predicted response value for EC_{50} was found to be 105.1544 μg mL^{-1}, based on the final reduced model.

3.4. Verification of the final reduced models

The validity of the final reduced model was checked by comparing the experimental values with those predicted by the final reduced model. A two-sample *t*-test was applied to do this comparison. Table 4 indicates the experimental and predicted values in accordance with the final reduced response surface model. No significant ($p > 0.05$) difference was observed between the experimental and predicted values (Table 4). The results also exhibited a close correspondence between the experimental and predicted EC_{50} of essential oil, confirming the adequacy of the corresponding response surface model for predicting the variation of the antioxidant activity of *P. nigrum* L. essential oil as a function of the studied SC-CO_2 extraction conditions.

The desirable SC-CO_2 extraction conditions were prepared according to the recommended optimum levels, then assessed in terms of the antioxidant activity of *P. nigrum* L. essential oil. The corresponding experimental value for EC_{50} at the optimum SC-CO_2 extraction conditions was 113.12 ± 0.65 μg mL^{-1}. It was found that the response values obtained from the experimental data were

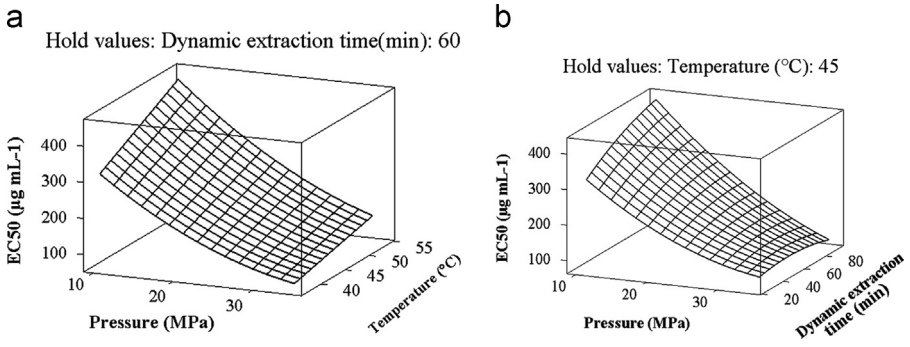

Fig. 1. Response surface plots indicating (a) interaction effect of pressure (MPa) and temperature (°C) and (b) the interaction effect of pressure (MPa) and dynamic extraction time (min) on the EC_{50} (μg mL^{-1}) of essential oil.

Table 4
Comparison between experimental and predicted values based on the final reduced models (validation procedure).

Run	EC_{50} (μg mL^{-1})[a]		
	Y_0	Y_i	Y_0-Y_i
1	401.73	392.776	8.9541
2	181.74	176.345	5.3949
3	268.31	263.725	4.5847
4	173.45	167.635	5.8150
5	105.67	111.284	−5.6138
6	201.46	215.680	−14.2202
7	223.45	225.505	−2.0546
8	212.82	215.680	−2.8602
9	208.79	205.541	3.2486
10	235.74	237.110	−1.3697
11	364.72	362.546	2.1738
12	204.25	205.541	−1.2914
13	143.58	146.230	−2.6501
14	108.56	108.671	−0.1111
15	209.68	204.270	5.4102
16	159.28	152.407	6.8728
17	205.81	204.270	1.5402
18	103.28	99.951	3.3293
19	281.29	288.597	−7.3068
20	298.67	308.516	−9.8458

Y_0: Experimental value; Y_i: Predicted value; Y_0–Y_i: Residue value.

[a] No significant ($p > 0.05$) difference between experimental (Y_0) and predicted value (Y_i).

close to the predicted ones. From the validation process and the fact that no significant ($p > 0.05$) difference was found between the experimental and predicted values, the sufficiency of the final reduced model fitted by RSM was verified.

3.5. Comparative study of SC-CO_2 and conventional hydro-distillation extraction

Hydro-distillation is the easiest, oldest and most frequently employed method to isolate *P. nigrum* L. essential oil from the plant material. In the current study, the essential oil of SC-CO_2 extraction with the highest antioxidant activity (lowest EC_{50} value) was compared with conventional hydro-distillation extraction, inasmuch as the parameters studied here are correlated with the quality of the essential oil.

3.5.1. Chemical compositions

The water-distilled *P. nigrum* L. essential oil was yellowish in color, with a fresh and peppery aroma. This essential oil was slightly darker in color compared to the essential oil obtained by SC-CO_2. GC and GC–MS analysis of *P. nigrum* L. essential oil revealed its major compounds, which are principally responsible for its characteristics of odor and taste. Chemical components of *P. nigrum* L. essential oil obtained by SC-CO_2 extraction under pressure 30 MPa, temperature 40 °C, and extraction time 40 min, and that obtained by hydro-distillation, are presented in Table 5.

Fig. 2 shows the gas chromatogram of *P. nigrum* L. essential oil obtained by both techniques. The essential oil of *P. nigrum* L. extracted through SC-CO_2 extraction and hydro-distillation possessed similar groups of compounds in different relative abundances. The essential oil extracted by hydro-distillation was identified as having 43 compounds, representing about 99.87% of the oil, whilst 39 components representing about 99.84% of the oil were detected in the SC-CO_2-extracted essential oil.

Table 6 presents the relative abundance of some important components along with the classification index described by Salzer and Furia [28]. The monoterpene hydrocarbon fraction in SC-CO_2 extraction was 56.31%, compared to that in hydro-distillation (61.07%). On the other hand, the sesquiterpene hydrocarbon fraction was relatively higher in SC-CO_2 extraction (40.65%) than in hydro-distillation (34.86%). In addition, in SC-CO_2 extraction the oxygenated monoterpene (1.67%) and the sesquiterpene (1.21%) hydrocarbons decreased in comparison with their quantities in hydro-distillation. However, the principal compositions for both SC-CO_2 extraction and hydro-distillation were β-caryophyllene, limonene, sabinene, β-pinene, 3-carene, α-pinene, and copaene. The recovery of β-caryophyllene as the most abundant component by SC-CO_2 extraction (25.38%) was better than that of hydro-distillation (18.64%). As stated by Salzer and Furia [28], the classification index discriminates the quality of the essential oil, which is a function of the ratio of monoterpene hydrocarbons (i.e., limonene, sabinene, β-pinene, 3-carene, and phellandrene) to sesquiterpene hydrocarbons (β-caryophyllene). A lower classification-index value represents a higher quality of essential oil [28]. SC-CO_2 yielded oil with a lower classification index value (1.87%), whilst the classification index of hydro-distillation was found to be 2.57%. Consequently, the essential oil obtained by SC-CO_2 extractionwas of higher quality compared to hydro-distilled essential oil.

3.5.2. Antioxidant activity

SC-CO_2 extraction was able to obtain an essential oil richer in antioxidant activity (103.28 ± 0.05) than hydro-distillation (316.27 ± 0.12). This may be due to the thermal degradation, hydrolysis, and solubilization of some components in water, which can change their antioxidant capacity. Furthermore, it could be due to the way that the water used in hydro-distillation makes several antioxidants unstable or degrades them by enzymatic action in the wet plant material. SC-CO_2 provides the best capability to alter solvent conditions through controlling simple factors (pressure and temperature), and could offer the best selectivity [5]. In fact, in hydro-distillation, samples are usually extracted in boiling water over a long period of time, which could lead to thermal decomposition of thermolabile target compounds from *P. nigrum* L., as indicated by lower antioxidant activity [29]. The temperature in SC-CO_2 extraction is considerably lower than that in hydro-distillation, which could result in lower degradation of antioxidant compounds.

GC–MS analysis revealed that *P. nigrum* L. constituents obtained in considerable percentages through SC-CO_2 extraction and hydro-distillation include β-caryophyllene, limonene, sabinene, β-pinene, 3-carene, α-pinene, and copaene. Consequently, they may contribute to the antioxidative potential of *P. nigrum* L. essential oil. On the other hand, it is complex to ascribe the antioxidant capacity of whole *P. nigrum* L. essential oil to only some principal constituents, due to the fact that an essential oil is a complex mixture of varying chemical compositions. As a general rule, the antioxidative potential of the entire essential oil is higher than that of the individual components. This suggests the presence the possible synergistic effects among various compositions of essential oils [30]. As stated by Singh et al. [30], the antioxidant activity of *P. nigrum* L. essential oil may be attributable to α-pinene, β-pinene, camphene, and camphor, which exist in the whole oil in substantial percentages. According to Kapoor et al. [31], β-caryophyllene, limonene, and β-pinene were the principal components, which may contribute to the antioxidant capacity of *P. nigrum* L. essential oil. Nevertheless, the compounds accountable for the antioxidative potential of *P. nigrum* L. essential oil are currently uncertain.

Table 5
Chemical components (% of total peak area) of *P. nigrum* L. essential oil obtained by hydro-distillation and SC-CO_2 extraction methods.

No	Component	Formula	RI[a]	HD (%)	SFE (%)	Identification
1	Thujene	$C_{10}H_{16}$	897	1.38 ± 0.06	1.11 ± 0.06	MS, RI,RT
2	α-Pinene	$C_{10}H_{16}$	943	7.96 ± 0.14	4.25 ± 0.06	MS, RI, RT
3	Camphene	$C_{10}H_{16}$	948	0.28 ± 0.04	0.25 ± 0.02	MS, RI, RT
4	Sabinene	$C_{10}H_{16}$	967	13.19 ± 0.17	13.63 ± 0.21	MS, RI,RT
5	β-Pinene	$C_{10}H_{16}$	969	9.71 ± 0.12	7.27 ± 0.05	MS, RI, RT
6	α-Phellandrene	$C_{10}H_{16}$	971	1.60 ± 0.04	1.80 ± 0.08	MS, RI
7	Terpinolene	$C_{10}H_{16}$	973	0.16 ± 0.03	0.13 ± 0.02	MS, RI
8	Myrcene	$C_{10}H_{16}$	1018	1.15 ± 0.02	1.98 ± 0.03	MS, RI, RT
9	Limonene	$C_{10}H_{16}$	1025	14.95 ± 0.13	15.64 ± 0.15	MS, RI,RT
10	β-Ocimene	$C_{10}H_{16}$	1032	0.46 ± 0.02	0.13 ± 0.04	MS, RI
11	γ-Terpinene	$C_{10}H_{16}$	1035	0.26 ± 0.04	0.16 ± 0.01	MS, RI
12	p-Cymene	$C_{10}H_{14}$	1042	0.89 ± 0.13	0.62 ± 0.08	MS, RI
13	3-Carene	$C_{10}H_{16}$	1044	8.56 ± 0.11	9.34 ± 0.04	MS, RI
14	trans-Sabinene hydrate	$C_{10}H_{18}O$	1047	0.16 ± 0.04	0.06 ± 0.00	MS, RI
15	cis-Sabinene hydrate	$C_{10}H_{18}O$	1049	0.28 ± 0.05	–	MS, RI
16	Isothujol	$C_{10}H_{18}O$	1079	0.19 ± 0.04	0.10 ± 0.01	MS, RI
17	Linalool	$C_{10}H_{18}O$	1082	0.61 ± 0.04	1.01 ± 0.06	MS, RI
18	Terpinen-4-ol	$C_{10}H_{18}O$	1137	0.59 ± 0.02	0.12 ± 0.02	MS, RI
19	α-Terpineol	$C_{10}H_{18}O$	1186	0.68 ± 0.16	0.38 ± 0.04	MS, RI
20	α-Cubebene	$C_{15}H_{24}$	1319	0.09 ± 0.01	0.05 ± 0.00	MS, RI
21	Carvone	$C_{10}H_{14}O$	1339	0.31 ± 0.04	–	MS, RI
22	β-Cubebene	$C_{15}H_{24}$	1344	0.23 ± 0.07	0.24 ± 0.05	MS, RI
23	δ-Elemene	$C_{15}H_{24}$	1377	2.00 ± 0.12	1.18 ± 0.06	MS, RI
24	α-Gurjenene	$C_{15}H_{24}$	1419	0.37 ± 0.05	0.52 ± 0.09	MS, RI
25	δ-Cadinene	$C_{15}H_{24}$	1435	0.23 ± 0.04	0.24 ± 0.02	MS, RI
26	Copaene	$C_{15}H_{24}$	1441	3.72 ± 0.17	3.84 ± 0.17	MS, RI
27	β-Elemene	$C_{15}H_{24}$	1458	0.85 ± 0.09	1.05 ± 0.04	MS, RI
28	β-Farnesene	$C_{15}H_{24}$	1461	0.18 ± 0.03	0.10 ± 0.03	MS, RI
29	γ- Elemene	$C_{15}H_{24}$	1465	0.23 ± 0.06	0.25 ± 0.03	MS, RI
30	β-Caryophyllene	$C_{15}H_{24}$	1484	18.64 ± 0.84	25.38 ± 0.62	MS, RI
31	γ-Muurolene	$C_{15}H_{24}$	1493	0.64 ± 0.04	0.56 ± 0.06	MS, RI
32	α-Selinene	$C_{15}H_{24}$	1498	0.82 ± 0.12	1.18 ± 0.04	MS, RI
33	β-Bisabolene	$C_{15}H_{24}$	1500	1.77 ± 0.15	1.47 ± 0.15	MS, RI
34	Germacrene-D	$C_{15}H_{24}$	1515	0.18 ± 0.02	0.15 ± 0.03	MS, RI
35	α-Caryophyllene	$C_{15}H_{24}$	1537	1.24 ± 0.13	1.65 ± 0.16	MS, RI
36	β-Selinene	$C_{15}H_{24}$	1559	0.71 ± 0.13	0.43 ± 0.11	MS, RI
37	L-calamenene	$C_{15}H_{22}$	1564	0.33 ± 0.04	0.29 ± 0.06	MS, RI
38	β-Cadinene	$C_{15}H_{24}$	1577	1.13 ± 0.08	1.16 ± 0.07	MS, RI
39	γ-Gurjunene	$C_{15}H_{24}$	1580	1.5 ± 0.13	0.91 ± 0.16	MS, RI
40	Spathulenol	$C_{15}H_{24}O$	1583	0.21 ± 0.06	0.09 ± 0.02	MS, RI
41	Caryophyllene oxide	$C_{15}H_{24}O$	1591	1.22 ± 0.11	1.12 ± 0.14	MS, RI
42	trans-Nerolidol	$C_{15}H_{26}O$	1594	0.10 ± 0.02	–	MS, RI
43	Cubenol	$C_{15}H_{26}O$	1597	0.11 ± 0.03	–	MS, RI

[a] Retention indices relative to C_9–C_{24} n-alkanes on the HP-5MS column. RT, Identification based on retention time. RI, Identification based on retention index. MS, Identification based on comparison of mass spectra. Values given are the mean of three determinations ± standard deviation.

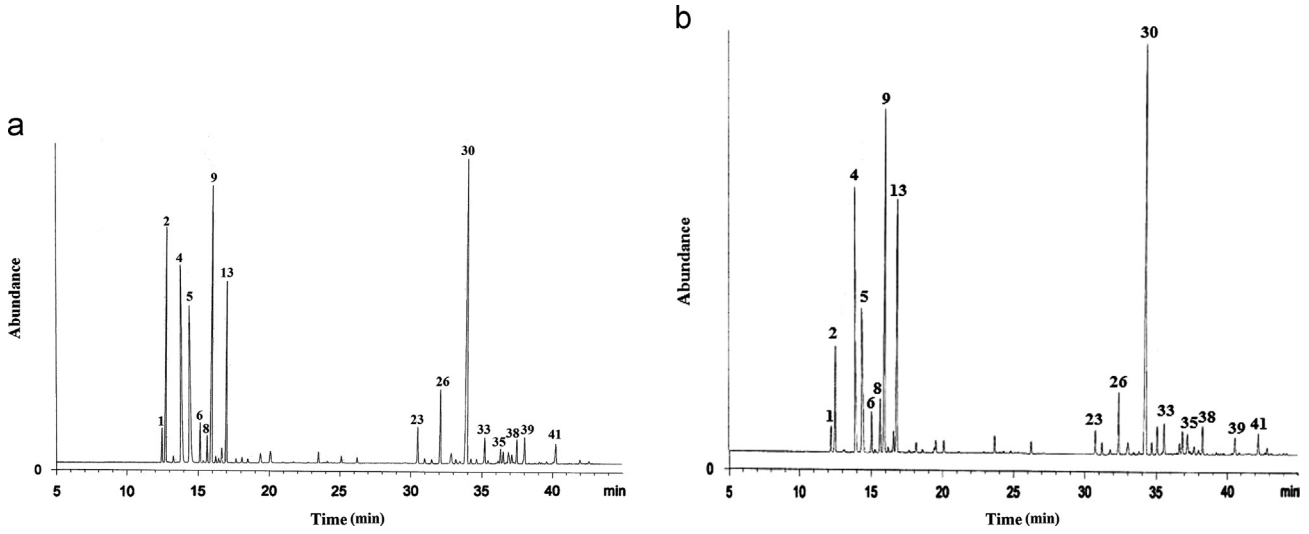

Fig. 2. GC–MS chromatogram of *P. nigrum* L. essential oil, (a) obtained by hydro-distillation extraction method, and (b) SC-CO_2 extraction for run 18 (temperature = 40 °C, pressure = 30 MPa and dynamic extraction time = 40 min) using a non polar HP-5MS column (for peak identification see Table 5).

Table 6
Proportional abundance (%) of specific major components of *Piper nigrum* L. essential oil.

Method	α-pinene	Sabinene +β-pinene, *X*	Limonene +3-carene +Phellandrene, *Y*	β-caryophyllene, *Z*	Classification index (*X*+*Y*)/*Z*	MT	ST	MTO	STO
SFE	4.25	20.9	26.78	25.38	1.87	56.31	40.65	1.67	1.21
HD	7.96	22.9	25.11	18.64	2.57	61.07	34.86	2.3	1.64

MT, ST, MTO and STO represent monoterpene hydrocarbon, sesquiterpene hydrocarbon, oxygenated monoterpene and oxygenated sesquiterpene respectively.

3.5.3. *Extraction yields*

The extraction yields were calculated as the weight of collected oil divided by the weight of dried material fed into the extractor. The yields obtained using varying extraction methods were significantly ($p<0.05$) different. Hydro-distillation using water as a solvent produced a higher yield ($2.88\pm0.07\%$), whilst SC-CO_2 extraction (30 MPa, 50 °C, and 80 min) resulted in a lower yield ($2.16\pm0.02\%$). The hydro-distilled *P. nigrum* L. essential oil can be defined as 100% recovery of essential oil from the seeds. According to the study of Ferreira et al. [32], the yield of *P. nigrum* L. essential oil extracted via SC-CO_2 in optimum conditions was significantly less (2.05%) than the yield obtained through steam distillation (3.46%). This may be explained by the fact that water is a polar solvent with different extracting properties to those of CO_2 as a non-polar solvent. It seems possible that this result is due to a minor escaping fraction of the extracted volatile components along with CO_2 from the container. Another possible explanation for this could be that in hydro-distillation, the time of exposure to water as a solvent is relatively long, and consequently the essential oil within glands could have been extracted.

4. Conclusions

The present study showed that the SC-CO_2 extraction conditions significantly ($p<0.05$) influenced the antioxidant activity of the essential oil. Pressure and extraction time showed the most and least significant ($p<0.05$) impact, respectively, on the antioxidant activity. The antioxidant activity of oil as measured by the DPPH method was significantly ($p<0.05$) higher for oil obtained via SC-CO_2 extraction than conventional hydro-distillation. The experimental results verify that SC-CO_2 extraction prevents the degradation of thermolabile constituents. Moreover, the SC-CO_2 method was more selective; hence, it produced an essential oil with superior antioxidant activity. Thus it could be the best option for obtaining essential oils high in antioxidant compounds. The chemical compositions of both essential oils demonstrated that they were mainly composed of monoterpene and sesquiterpene hydrocarbons, with small amounts of oxygenated terpenes. Due to the extensive use of black-pepper essential oil in therapeutic applications, the quality of the essential oil is of great concern. The present study revealed that essential oil extracted via SC-CO_2 is of higher quality than that obtained through hydro-distillation. In fact, the black-pepper essential oil from SC-CO_2 extracted could be more similar to the actual plant chemical composition, and consequently contain a fuller variety of components from the source *P. nigrum* L. than a hydro-distilled essential oil.

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