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Supercritical fluid carbon dioxide extraction of *Nigella sativa* (black cumin) seeds using taguchi method and full factorial design

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Abstract
Optimum condition for *Nigella sativa* seeds oil and its bioactive compound, thymoquinone (TQ) using supercritical fluid carbon dioxide extraction (SCFE-CO_2), were investigated. The optimization process was performed with Taguchi method and full factorial design (FFD) under the following condition: pressure (150, 200 and 250 bar), temperatures (40, 50 and 60°C) and carbon dioxide (CO_2) flowrate (10, 15 and 20 g/min), in which solvent to feed (SF) ratio was set constant at 24. The highest yield of *Nigella sativa* seeds oil from SCFE-CO_2 process with FFD method was 12% at 250 bar, 60°C and 20 g/min. Whereas Taguchi method was performed at 250 bar, 50°C, 10 g/min with oil yield 11.9%. The highest thymoquinone content in *Nigella sativa* seeds oil from both experimental design was achieved through SCFE-CO_2 extraction condition at 150 bar, 60°C and 20 g/min with thymoquinone content 20.8 mg/ml. In addition, conventional methods such as high pressure soxhlet with liquid CO_2, n-hexane soxhlet and percolation with ethanol were performed with oil yield 5.8%, 19.1% and 12.4%; and thymoquinone content 8.8 mg/g oil, 6.3 mg/g oil and 5.0 mg/g oil, respectively. The analysis of variance (ANOVA) with 95% confidence interval, indicates effect of pressure on *Nigella sativa* seeds oil yield and thymoquinone content from SCFE-CO_2 process. The oil was then evaluated for its activity. The antibacterial activity of the oil from Taguchi Method, shows that all samples were unable to inhibit *Escherichia coli* O157 and *Salmonella typhimurium*. For other pathogenic bacterias, all samples show similar inhibition at concentration 10% oil for MRSA, 3% oil for *Stapylococcus aureus* and 3% oil for *Bacillus subtilis*.

Keywords: SCFE-CO_2, *Nigella sativa*, taguchi method, full factorial design, thymoquinone, antibacterial activity

Introduction
Nigella sativa (black cumin) seeds, a dicotyledon of *Ranunculaceae* family, have been used for thousands years as spice and food preservative [1-3]. Black cumin is an annual herbaceous plant widely grown in Mediterranean countries, Middle East, Eastern Europe and Western Asia. In Middle East, Northern Africa and India, it has been used traditionally for centuries to treat asthma, cough, bronchitis, headache, rheumatism, fever, influenza and eczema [3,4]. It has also been used as anti-histamine, anti-diabetes and anti-inflammatory. The oil and seed constituents, in particular thymoquinone (TQ), have shown medicinal properties in treatment and prevention of various diseases [5-7,10-12]. They have various potent activities such as anti-inflammatory [3]; anti-oxidant [1,2,4]; anti-cancer [6,7,10], etc. In addition, they also expressed anti-microbial properties toward different microbes [8,9].

Supercritical fluid extraction (SCFE) has many advantages compared to conventional extraction methods such as its environmentally compatible fluids, low temperature, shorter extraction time and ease separation of solute from supercritical fluid solvent by simple expansion. Moreover, this extraction does not leave any trace of organic solvent [13-17]. The most frequently employed supercritical solvent is carbon dioxide (CO_2). It has low toxicity, good safety, cheap, abundant in pure form (food grade) and low critical temperature and pressure [13,14,16,17].

Optimization process is critical as various parameters may potentially affect the SCFE process. The optimum condition can be achieved by investigating multiple factors in all possible conditions [18]. This condition was considered achieved when high yield of oil and high content of thymoquinone was obtained. The objective of this present research is comparing *Nigella sativa* seeds oil extracted by SCFE process using two different experimental designs, Taguchi's method and full factorial design. Other conventional extraction methods were also performed as comparison.

Materials and methods
Nigella sativa seeds were imported from Arab by "Intisari Herbal" supplier (Solo, Indonesia). Prior to the extraction process, all dried plant materials were grounded in milling machine (China) with stainless filter mess #10. Particle distribution of grounded raw material is shown at (Table 1). Moisture content was determined using H83 Halogen Moisture Analyzer (loss on drying 6.87%) and Volumetric KF Titrator V30 (water content 5.99%).

Food grade liquid carbon dioxide (purity 99.99%) was supplied in cylinder tube by PT. Inter Gas Mandiri (Cikarang, Indonesia); Analytical grade n-hexane was purchased from

Table 1. Particle distribution of grounded raw material.

Sieves		W_{before} (gram)	W_{after} (gram)	W_{after}-W_{before} (gram)	%
2	mm	345.3	345.3	0	0
1	mm	316.9	324.8	7.9	77.26
500	µm	279.4	280.2	0.8	7.824
250	µm	259.1	259.9	0.8	7.824
180	µm	242.0	242.4	0.4	3.912
125	µm	254.3	254.3	0	0
63	µm	242.0	242.0	0	0
45	µm	244.5	244.6	0.1	0.978
0	-	358.1	358.1	0	0

Table 2. Standard L_9 orthogonal arrays.

Exp. No.	Independent Variables		
	A	B	C
1	1	1	1
2	1	2	2
3	1	3	3
4	2	1	2
5	2	2	3
6	2	3	1
7	3	1	3
8	3	2	1
9	3	3	2

Table 3. Parameters and levels used in both experimental design.

LEVEL	FACTOR		
	Pressure (bar)	Temperature (°C)	CO_2 Flowrate (gr/min)
	A	B	C
1	150	40	10
2	200	50	15
3	250	60	20

Merck, Darmstadt, Germany; Technical grade (96%) ethanol was obtained from PT. Brataco, Bekasi, Indonesia and thymoquinone (purity 99.9%) was purchased from Sigma–Aldrich Co., St. Louis, Missouri, USA.

Design of experiment

Taguchi method

The Taguchi method utilizes orthogonal array design (OAD) to study a large number of parameters with a small number of experiments. It significantly reduces number of experimental configurations to be studied without affecting the quality of results [19] and maintain the experimental cost at minimum level [20]. Taguchi Method was employed a special set of OAD in each number of experimental conditions to systematically look for the favorable operating conditions [18]. In this study, the following parameters are considered in the SCFE-CO_2 process: pressure (A), temperature (B) and CO_2 flowrate (C), and each parameter consists with three levels (values). Thus, the L_9 orthogonal arrays improved by Taguchi method as shown in (**Table 2**), was chosen to determine the experimental design.

The quality of results were measured by the signal-to-noise (S/N) ratio with three different characteristic of target values: "larger is better", "smaller is better" or "nominal is better" [18,20]. Since purpose of this study was the highest oil yield and TQ content, target values of "larger is better" was chosen. In this study, interactions among variables were not considered and focus was placed on the main effects of three parameters.

Full factorial design (FFD)

FFD is the most basic structures experimental design. In FFD, responses are measured at all combinations of experimental factor levels [21]. Each experimental condition is called a "run" and each run represents variation of one variable. Each response will be measured for an observation. The entire set of runs is the "design". Since, we consider three parameters with three levels, the total run with full factorial design is 3^3 (27) number of trials. The variation levels for parameters is shown in (**Table 3**).

Supercritical carbon dioxide extraction

The supercritical carbon dioxide (SCFE-CO2) extraction was carried out using supercritical fluid extractor with CO_2 cycle system (KIST-Korea) as shown in (**Figure 1**). The extractor vessel with 2 x 1000 ml capacity was loaded with 100 g powdered material of *Nigella sativa*. Food grade liquid CO_2 was delivered to extraction vessel using high pressure pump (Thar, USA). Extraction pressure was varied from 150-250 bar and temperature 40-60°C. The pressure in the extraction vessel was controlled by back pressure regulator (Tescom, USA). Heat exchangers (Lab. Companion, USA) were provided in system to maintain temperature in the extractor and separator vessel. CO_2 flow rate was varied from 10 to 20 g/min for static extraction time of 60 minutes (fixed for all the extractions) and followed by dynamic extraction time (120, 160 and 240 min) that had been determined based on each CO_2 flowrate. Therefore, the solvent to feed (SF) ratio was constant at 24. Extract was collected every 20 min (cummulated) until dynamic extraction time finished. Yield of extract was determined as summation of extract from the experiments.

High pressure soxhlet (HPS) extraction

About 50 g of powdered *Nigella sativa* seeds were submitted to high pressure soxhlet extraction (KIST-Korea) with CO_2 liquid as solvent. The operating condition for HPS extraction is at boiler temperature 35°C, chiller temperature -5°C (Lab. Companion, USA), pressure 40-50 bar, CO_2 cycle-rate 5 min and extraction time 7 hours. The remaining extract was considered as experiment yield.

Soxhlet n-hexane extraction

About 50 g of powdered *Nigella sativa* seeds were extracted with 600 ml n-hexane (ratio 12:1). Operating condition is at boiler temperature 100°C, chiller temperature -5°C (Lab. Companion, USA), pressure 1 bar and extraction time 6 hours. The extract was filtered and then evaporated to separate

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Table 4. Experiment results for SCFE-CO2 process of Nigella sativa with Taguchi Method.

Run	Pressure (bar)	Temperature (C)	CO_2 flowrate (gpm)	Yield Total (%)	Yield Oil (%)	TQ (mg/g oil)
1	150	40	10	9.5	8.9	11.9
2	150	50	15	9.1	8.6	15.1
3	150	60	20	6.1	4.8	20.8
4	200	40	15	10.3	9.5	10.5
5	200	50	20	11.4	10.4	10.3
6	200	60	10	12.5	10.6	11.0
7	250	40	20	11.6	10.7	9.1
8	250	50	10	13.1	11.9	9.5
9	250	60	15	12.8	12.0	10.5

Table 5. Antibacterial activity of Nigella sativa oil with disc diffusion assay method.

Run	Average of inhibition zone (cm) against pathogen bacteria				
	EC O157 (100% NS)	BS (3% NS)	ST (100% NS)	SA (3% NS)	MA (10% NS)
1	-	2.50	-	0.60	0.20
2	-	2.50	-	0.63	0.25
3	-	2.80	-	0.60	0.35
4	-	1.00	-	0.20	0.20
5	-	2.15	-	0.30	0.20
6	-	0.90	-	0.30	0.30
7	-	0.75	-	0.30	0.15
8	-	0.95	-	0.30	0.15
9	-	1.00	-	0.30	0.15

Note:
NS : *Nigella sativa oil*
EC O157 : *E. coli* O157
BS : *Bacillus subtilis*
ST : *Salmonella typhimurium*
SA : *Stapylococcus aureus*
MA : Methicillin- Resistance *Staphylococcus Aureus* (MRSA)
\- : not show inhibition

the organic solvent (n-hexane). The remaining extract was considered as experiment yield.

Solvent extraction (ethanol 96%) with percolator

About 200 g of powdered *Nigella sativa* seeds were extracted with 2700 ml Ethanol (96%, Technical Grade). Operating condition for percolation process is at pressure solvent pump (HKS, Korea) 10 bar, room temperature with solvent flowrate at 180 ml/min and the extraction time 7 hours. The extract was filtered and then evaporated to separate the organic solvent (ethanol). The remaining extract was considered as experiment yield.

Gas chromatography analysis

Thymoquinone analysis was performed using Perkin-Elmer Gas Chromatography Clarus 680 with Flame Ionized Detector (FID) detector. The entire analyses were carried out by Elite-5 column (30 m x ID 0.25 mm, DF 0.25 µm; T = 60–330ºC). Oven temperature was increased from 95ºC with ramp 20ºC/min to 280ºC and held isothermal for 5 min. The injector and detector temperature were maintained at 220ºC and 240ºC, respectively. Sample solution was prepared with concentration of 4000 ppm in 2-propanol (chromAR). The injection volume was 1.0 µl with a split ratio at 10:1. Flowrate carrier nitrogen (N_2) gas at 1.0 ml/min, Hidrogen (H_2) at 45.0 ml/min and compress air UHP (Ultra High Purity) at 450.0 ml/min.

Anti-microbial analysis

Bacterial species used in this study were *Escherichia coli* O157, *Bacillus subtilis* ATCC 6633, *Salmonella typhimurium* ATCC 13311, *Stapylococcus aureus* ATCC 6538 and Methicillin Resistant *Staphylococcus Aureus* (MRSA). Antibacterial activity was determined by disc diffusion assay. *Nigella sativa* seeds oil was dissolved in dimethylsulphoxide (DMSO; Merck, Germany) to reach final concentration 3%, 5%, 10% and 100% (without dilution) and sterilized using filtration method with 0.22 µm membrane filters (Iwaki, Japan). The medium used were MHA (Mueller-Hinton Agar) supplied from Merck (Germany). The 6 mm diameter disc (Whatman, USA) were impregnated with 20 µl of the oil and placed on inoculated agar (10^6 CFU/ml of bacterial). Ciprofloxacin 0.2 mg/ml was used as positive reference standards to determine the sensitivity of tested microbial species. Control test was dissolved in 100% DMSO and showed no inhibition of microbial growth. The inoculated plates were incubated at 37ºC for 24 h for bacterial species growth. Antibacterial activity was evaluated by measuring zone of inhibition against those bacterial species. All inhibition assays and controls were carried out triplicate.

Statistical analysis

The data obtained from supercritical carbon dioxide extraction were subjected to analysis of variance (ANOVA) to determine significant difference among all extract yield. P-value less than 0.05 were considered significant. All statistical analysis were performed using MINITAB v.15 (Minitab Inc., USA) statistical software package.

Results and Discussion

(**Table 4** and **5**) show the yield and Thymoquinone (TQ) content of *Nigella sativa* seeds oil extracted by SCFE process using Taguchi Method and FFD, respectively.

Taguchi method

(**Figure 2a** and **2b**) show main effect plots for S/N ratio between oil yield and TQ content in *Nigella sativa* with different variables, including pressure (P, 150–250 bar), temperature (T, 40–60ºC) and CO_2 flowrate (10–20 g/min). Main effect plots were determined based on signal–to-noise (S/N) ratio which express the scatter around a target value. The larger the ratio means the smaller the scatter. Noise factors are viewed as the cause of variability in investigated responses. The S/N ratio measures level of performance and effects of noise factors on performance. According to Taguchi Method,

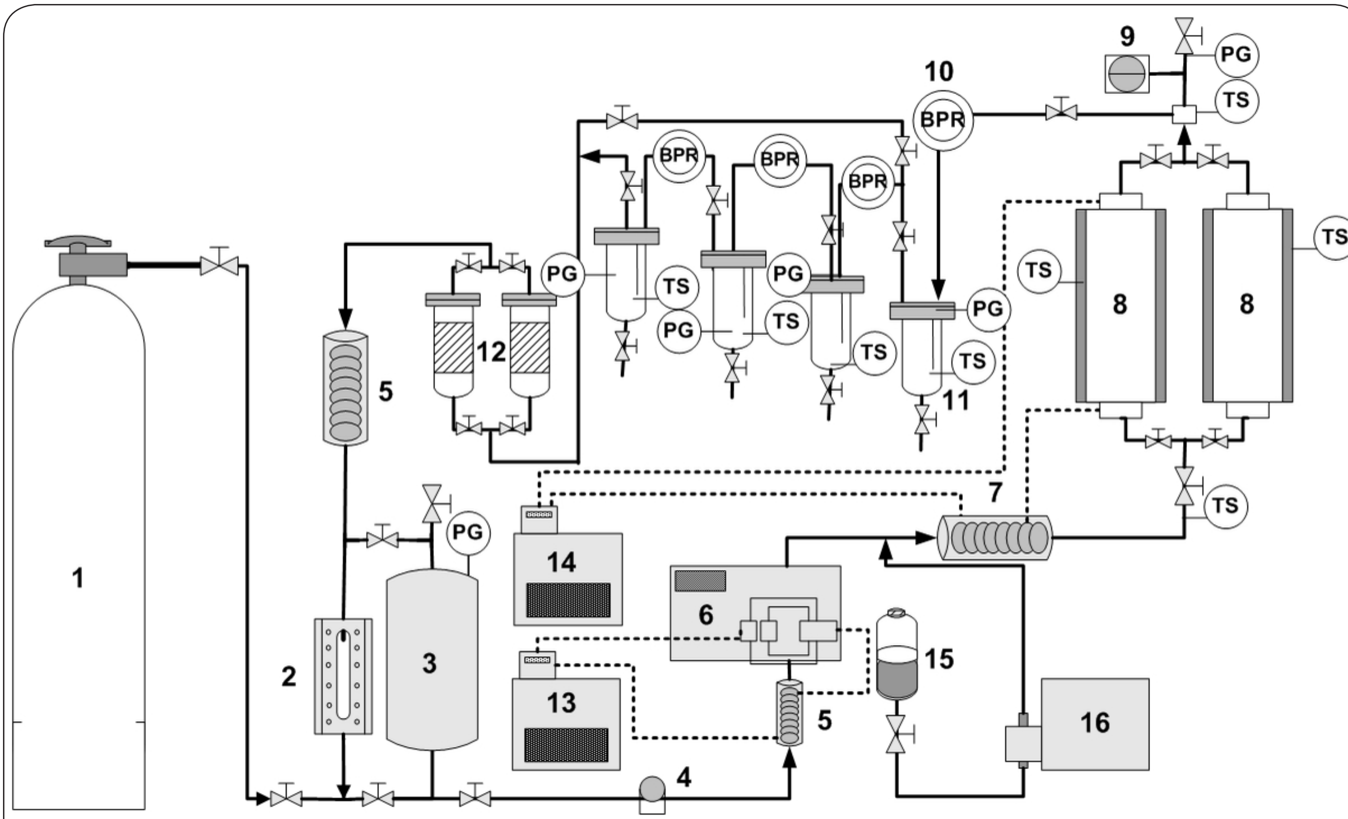

Figure 1. Schematic diagram of supercritical CO_2 extraction system.

Note :

1. CO_2 cylinder	9. Rupture
2. Level Cell	10. B.P. Regulator
3. CO_2 Feed Tank	11. Separator
4. Filter	12. Absorber
5. Condenser	13. Circulator (chiller)
6. CO_2 pump	14. Circulator (heater)
7. CO_2 Pre-Heater	15. Co-solvent feed Container
8. Extractor	16. Co-solvent pump

the optimum conditions for *Nigella sativa* seeds oil yield were at P = 250 bar, T = 50°C and CO_2 flowrate = 10 g/min (**Figure 2a**). Whereas the optimum conditions for thymoquinone content were obtained at P = 150 bar and T = 60°C with CO_2 flowrate 20 g/min.

The antibacterial activity of *Nigella sativa* seeds oil were tested with disc diffusion assay method on several strains of bacteria such as *Escherichia coli* O157, *Bacillus subtilis*, *Salmonella typhimurium*, *Stapylococcus aureus* and Methicillin-Resistant *Staphylococcus Aureus* (MRSA) (**Table 5**). Based on the results of antibacterial activity test, all samples did not inhibit *E. coli* O157 and *Salmonella typhimurium* even without oil dilution treatment. For other pathogenic bacterial, all samples show similar inhibition (insignificant difference) at 10% oil for MRSA, 3% oil for *Stapylococcus aureus* and 3% oil for *Bacillus subtilis*. All samples show a better inhibition against Gram-positive bacteria compared to Gram-negative bacteria. The higher resistance of Gram-negative bacteria to external agents is due to the presence of lipopolysaccharides in their outer membranes, which make them inherently resistant to antibiotics, detergents and hydrophilic dyes.

Full factorial design (FFD)

FFD requires more experimental points, however it can provide more information on the effect of various combinations. (**Table 6**) show various combination factors including pressure (P, 150-250 bar), temperature (T, 40-60 bar) and CO_2 flowrate (10-20 g/min). The optimum conditions to obtain high oil yield was at 250 bar and 60°C. All three level of CO_2 flowrate at this condition yield similar results 12% of *Nigella sativa* seeds oil. Therefore, 20 g/min was considered as optimum level for CO_2 flowrate This result is different compared to Taguchi method at 250 bar, 50°C and 10 g/min with oil yield 11.9%. However, the difference between these two optimum condition is

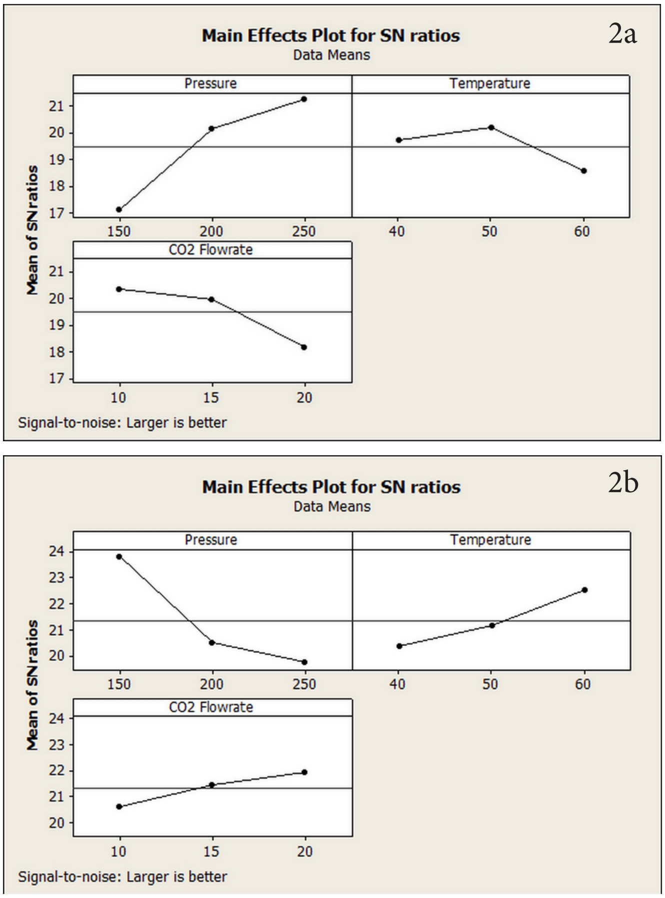

Figure 2. Main effects plot of Taguchi Method for : **a**) oil yield; **b**) thymoquinone content.

Table 6. Experiment results for SCFE-CO2 process of N. sativa with Full Factorial Design.

Run	Pressure (bar)	Temperature (C)	CO_2 flowrate (gpm)	Yield Total (%)	Yield Oil (%)	TQ (mg/g oil)
1	150	40	10	9.5	8.9	11.9
2	150	40	15	9.2	8.8	9.4
3	150	40	20	8.6	8.2	9.4
4	150	50	10	9.7	8.7	8.3
5	150	50	15	9.1	8.6	15.1
6	150	50	20	7.3	6.7	11.2
7	150	60	10	5	4.2	13.3
8	150	60	15	5.8	4.5	15.6
9	150	60	20	6.1	4.8	20.8
10	200	40	10	9.4	9.1	6.9
11	200	40	15	10.3	9.5	10.5
12	200	40	20	10.7	10.1	8.1
13	200	50	10	9.8	8.9	8.4
14	200	50	15	11.4	10.6	7.6
15	200	50	20	11.4	10.4	10.3
16	200	60	10	12.5	10.6	11.0
17	200	60	15	12.4	11.1	7.4
18	200	60	20	11.8	10.4	8.2
19	250	40	10	9.6	9.1	7.1
20	250	40	15	10.7	10.0	5.0
21	250	40	20	11.6	10.7	9.1
22	250	50	10	13.1	11.9	9.5
23	250	50	15	10.9	10.1	6.7
24	250	50	20	11	10.2	7.9
25	250	60	10	13.2	12.0	7.7
26	250	60	15	12.8	12.0	10.5
27	250	60	20	14.0	12.0	8.0
High Pressure Soxhlet (HPS) Extraction				5.8	-	8.8
Soxhlet n-Hexane Extraction				19.1	-	6.3
Perculator (Ethanol 96%)				12.4	-	5.0

insignificant (0.1%). For TQ content, both experimental designs gave similar results at 20.8 mg/g oil. Therefore, taguchi method was able to simplify the experimental procedure without affecting the quality of results.

Generally, higher pressure at isothermal conditions result an increase in solvent density, subsequently the solvent power and solubility of compounds. As the density increased, the distance between the molecules decreased; the interaction between the compounds and CO_2 increased; which leads to increase compounds solubility in CO_2. Therefore, increase in pressure will also accelerate mass transfer analytes and solvent in supercritical extractor vessel system and increase the extraction yield of *Nigella sativa* seeds oil. Solubility of solutes in SCFE-CO_2 is affected by solvent density and volatility of solutes which depend on temperature in opposing ways. Higher temperatures increase the volatility of solutes and improve their solubility and extraction. On the other hand, at a given pressure, density of supercritical CO_2 decreases with increase in temperature, reduction in solvation power of CO_2 and thus reduction in solubility and extraction efficiency [23-25].

The significance of each independent variable determined by analysis of variance (ANOVA) are presented in (Table 7a and 7b). With 95% confidence interval, only pressure affect the oil yield, while two others parameters (temperature and CO_2 flowrate) have no significant effects ($P>0.05$). The ANOVA results are in accordance with S/N ratio that identify pressure as the main parameter.

Other extraction methods such as high pressure soxhlet with liquid CO_2, n-hexane soxhlet extraction and percolation with ethanol were performed with yield 5.8%, 19.1% and 12.4%, respectively (Table 6). Although, yield from n-hexane soxhlet extraction and percolation process higher than optimum results of SCFE-CO_2 but the extract from both methods still contain organic solvent. Moreover, the final extract from these three different methods has lower content of thymoquinone compared to the SCFE-CO_2 process, with concentration of 8.8 mg/g oil, 6.3 mg/g oil and 5.0 mg/g oil, respectively. Therefore, SCFE-CO_2 seems to be a better alternative for *Nigella sativa* seeds since it offers the usage of non-toxic, non-explosive, environmental friendly, cost effective, time saving and selectivity-adjustable solvent in the extraction process.

Table 7. Analysis of variance for Full Factorial Design: a) oil yield; b) thymoquinone.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pressure	2	73.916	73.916	36.958	16.18	0.000
Temperature	2	1.147	1.147	0.574	0.25	0.780
CO_2 flowrate	2	0.227	0.227	0.114	0.05	0.952
Residual Error	20	45.692	45.692	2.285	-	-
Total	26	120.983	-	-	-	-
Source	DF	Seq SS	Adj SS	Adj MS	F	P
Pressure	2	121.460	121.460	60.730	9.95	0.001
Temperature	2	36.816	36.816	18.408	3.02	0.072
CO2 flowrate	2	4.442	4.442	2.221	0.36	0.699
Residual Error	20	122.069	122.069	6.103	-	-
Total	26	284.787	-	-	-	-

Conclusion

The highest yield of SCFE-CO_2 of *Nigella sativa* seeds oil using FFD was 12%, with optimum conditions at 250 bar and 60°C with CO_2 flowrate 20 g/min. While Taguchi method optimum condition was at 250 bar, 50°C and 10 g/min with oil yield 11.9%. The difference between these two methods is insignificant (± 0.1%). For highest TQ content, both methods gave similar optimum results at 150 bar, 60°C and 20 g/min with the value of TQ 20.8 mg/ml. Hence, Taguchi method was able to simplify the experimental procedure without affecting the quality of results. The analysis of variance (ANOVA) from Taguchi method and full factorial design, indicate that pressure affect oil yield and TQ. The antibacterial activity *Nigella sativa* seeds oil from L_9 Taguchi Method, shows inhibition *i.e,* 10% for MRSA, 3% for *Stapylococcus aureus* and 3% for *Bacillus subtilis* but was unable to inhibit *Escherichia coli* O157 and *Salmonella typhimurium* even without oil dilution treatment.

Competing interests

The authors declared no conflict of interest with respect to the authorship and/or publication. All authors disclosed receipt of the following financial supports from PT Dexa Medica to conduct this study.

Authors' contributions

Each author provides substantial contributions to the concept and design of the work, conducting experimental procedures and validations, data acquisition, compilation, analysis, interpretation and draft manuscript preparation.

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