Optimization of essential oil extraction process from *Piper nigrum* L. by-products and investigation of its biological activities

Hanh T. Phan[1], Hang S. N. Vuong[1], Anh T. Ha[1], Anh V. T. Nguyen[1], Toan Q. Truong[2], Dong T. N. Le[2], Ly P. T. Trinh[1,2], & Biet V. Huynh[1,2*]

[1]Faculty of Biological Sciences, Nong Lam University, Ho Chi Minh City, Vietnam

[2]Research Institute for Biotechnology and Environment, Nong Lam University, Ho Chi Minh City, Vietnam

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***Corresponding author**

Huynh Van Biet

Email: hvbiet@hcmuaf.edu.vn

ABSTRACT

Black pepper (*Piper nigrum* L.) is an industrial crop commonly grown in Vietnam. Besides its economic value, its processing released large amounts of by-products into the environment including leaves, flatterned seeds, and seed-bearing branches. The objective of this study was to optimize the extraction of essential oil from the mixture of three black pepper by-products and evaluate its biological activities in order to exploit the potential value of the by-products. The essential oils were extracted by hydrodistillation in which the extraction conditions including extraction time, water to feed ratio, and ultrasonic pretreated time were optimized. The results showed that the highest essential oil yield was achieved after 4 h of extraction at a water to feed ratio of 10:1, and 10 min of ultrasonic pretreament. Isospathulenol, β-selinene, caryophyllene, α-pinene, and α-copaene were identified as the main components of the essential oil as a result of chemical composition analysis using gas chromatography mass spectrometry. The essential oils exhibited 2,2-Diphenyl-1-picrylhydrazyl radical scavenging capacity with an IC50 value of 4.205 mg/mL and antibacterial capacity against four strains of bacteria, including *Bacillus spizizenii, Bacillus cereus, Staphylococcus aureus* and *Salmonella enterica* with the diameters of inhibition zone of 11.37 mm, 4.12 mm, 7.75 mm, 5.37 mm, respectively.

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1. Introduction

Piper nigrum L. belongs to the family Piperaceae and is a perennial industrial crop with high economic value. Black pepper contains 0.4 - 7% essential oil with main valuable components such as α- and β-pinene, limonene, myrcene, 3-carene, linalool, α-phellandrene, sabinene, and β-caryophyllene (Myszka et al., 2018). Besides providing nutritional and flavoring values, pepper essential oil has biological activities such as antioxidant, antibacterial, anti-inflammatory, anti-cancer, analgesic, and stress relief properties, which make it be widely used in cosmetics and pharmaceuticals (Ashokkumar et al., 2021). According to statistics from the General Statistics Office of the Ministry of Planning and Investment in 2018, the pepper cultivation area of Vietnam was 149,900 ha, the pepper output reached 255,400 tons and it kept the leading position with pepper export output of over 200,000 tons/year.

These figures not only show a positive sign of economic development but also bring out a concern about increasing amount of pepper by-products because they have the potential to carry diseases to young plants if not treated strictly. Black pepper by-products include leaves, flat seeds, and seed-bearing branches containing high organic matter that might cause environmental pollution. If these by-products are effectively utilized and recycled, they will bring high economic efficiency and create jobs for workers. The study was carried out to optimize the essential oil extraction from black pepper by-products and to investigate its biological properties that provide useful information for further application from inexpensive and environmentally friendly raw materials.

2. Material and Methods

2.1. Plant materials, chemicals, and reagents

A mixture of three by-products including pepper leaves, flat seeds and seed-bearing branches were collected in Gia Nghia city, Dak Nong province in May 2022 and preserved according to the regulations of Vietnam Pharmacopoeia V (MOH, 2018).

The antibacterial activity of the extracted essential oil was investigated on five strains of bacteria, including *Escherichia coli* (ATCC® 8739™), *Salmonella enterica* (ATCC®700623™), *Bacillus spizizenii* (ATCC® 6633™), *Bacillus cereus* (ATCC® 10876™), and *Staphylococus aureus* (ATCC® 6538™). These strains were provided by the Research Institute for Biotechnology and Environment, Nong Lam University, Ho Chi Minh City, Vietnam.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Merck. Other chemicals, including petroleum ether, Na2SO4, and dimethyl sulfoxide (DMSO) were obtained from Sinochem Group.

2.2. Optimization of essential oil extraction conditions from mixture of three by-products by response surface method (RSM - CCD)

2.2.1. Experimental design and statistical analysis

Optimization of extraction conditions for total essential oil content was carried out using a central composite design (CCD) of response surface methodology (RSM). Experimental design included five levels of the three independent variables including extraction time (X1), ultrasonic time (X2), water to feed ratio (X3). The design model consists of 16 experiments with 2 runs at the central point (000).

Table 1. Experimental design-matrix encoding the independent variables

Variable name	Unit	Code variable	Levels of evidence				
			- α	- 1	0	+ 1	+ α
Extraction time	h	X_1	0	2	4	6	8
Ultrasonic time	min	X_2	0	5	10	15	20
Water to feed ratio		X_3	6	8	10	12	14

α =2; The maximum and minimum points of each interval are equal to the levels +α and –α. The maximum or minimum levels are equal to the levels +1 and -1. The center points of each interval are equal to the levels 0.

The experimental data were analyzed using the statistical software JMP 10.

2.2.2. Determination of essential oil content

Essential oils in the mixture of three by-products were extracted by hydrodistillation using Clevenger apparatus system. The essential oil was isolated from the obtained mixture by extracting with 50 mL of petroleum ether (3 times) before dried on anhydrous sodium sulfate. Finally, the essential oil was obtained by removing water using a rotary vacuum evaporator.

2.3. Compositional analysis of essential oils from mixture of three by-products by GC-MS

The components of essential oils obtained from the by-product mixture were analyzed using gas chromatography-mass spectrometry (GC-MS). The essential oil was dissolved in n-hexane (1:50, v/v), mixed well and filtered through a PTFE syringe filter with a 0.22 µM pore size. The GC-MS was performed with an Agilent 6890 gas chromatography instrument coupled with an Agilent 5973 mass spectrometer. A capillary column (30 m x 0.25 mm i.d.) coated with 0.25 mM film of 5% phenyl methyl siloxane was used for separation. High purity helium was used as carrier gas with a flow-rate of 1.0 mL/min.The mass spectrometer was operated using electron-impact (EI) mode with the ionization energy of 70 eV and the scan rate was 0.34 s per scan. The quadrupole and ionization source temperature were 150 and 230°C. The experiment was performed once.

The components of essential oils were identified based on a comparison with data in NIST 14 (National Institute of Standards and Technologies, Mass Spectra Libraries).

2.4. Determination of biological activities of the essential oils from mixture of three by-products

2.4.1. DPPH radical scavenging assay

Antioxidant activity of the essential oils was estimated by DPPH assay (Kedare & Singh, 2011). Each 1.0 mL of sample was added to 1 mL of 0.2 mM DPPH. The mixture was incubated in darkness at room temperature for 30 min. The absorbance was measured at 517 nM. A control experiment was performed by adding 1.0 mL of water to 1 mL of the DPPH solution. Ascorbic acid was used as a reference.

The antioxidant activity was determined according to the following equation:

$$\text{The antioxidant activity (\%)} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

A correlation between sample concentration and antioxidant capacity was established, from which the IC50 value (the concentration of essential oil at which 50% of free radicals is captured) was determined to serve as a basis for comparing the antioxidant capacity between experiments. The lower the IC50 value, the higher the antioxidant activity.

2.5 Antibacterial activity

Antibacterial activity of the essential oil was determined by Kirby-Bauer method (Bauer et al., 1966) at different ratios of essential oil to dimethyl sulfoxide (DMSO) of 1:1, 2:1, and 4:1. The antibacterial activity was tested on five strains of bacteria, including *Staphylococcus aureus* (ATCC® 6538™), *Escherichia coli* (ATCC® 8739™), *Salmonella enterica* (ATCC®700623™), *Bacillus spizizenii* (ATCC® 6633™) and *Bacillus cereus* (ATCC® 10876™). One hundred µL of the bacterial suspensions (10^8 CFU/mL) was spread over the Luria Bertani agar plate. Sterile filter paper discs impregnated with essential oils with one DMSO negative control and tetracycline positive control were placed on the surface of the agar plate in a triangular pattern and the samples were incubated at 37°C. Inhibition zones were measured after 24 h for *E. coli* and *S. enterica*, 48 - 72 h for *S. aureus*, *B. spizizenii*, and *B. cereus*. All experiments were conducted in triplicate.

2.6. Data analysis

All data are presented as mean ± standard deviation. The results were statistically processed using Microsoft Excel 2016 and Pearson's correlation coefficient (r) with the level of significance ($P \leq 0.05$).

3. Results and discussion

3.1. Effect of extraction conditions on the essential oil content from mixture of three by-products

The results of empirical experiments are shown in Table 2.

The relationship between the essential oil content and the independent variables is shown in the following function:

$$Y = (4.231 \times 10^{10}) - 5.59 \times 10^{10} X_2 X_3$$

The *P*-value of testing the model's incompatibility (lack of fit) of 0.094 indicated the above regression model was compatible with the experiments. The R-square value of 0.98 demonstrated high correlation between the experimental data and the predicted values. Essential oil contents obtained from the empirical experiments and predicted value on the model are shown in Figure 1.

The pretreatment of raw materials with ultrasonic strongly affected the essential oil yield. Ultrasound is able to disrupt the cell wall structure that might enhance essential oil extraction and shorten extraction time (Kumar et al., 2021).

Table 2. Experimental results of essential oil yield

No.	X_1 (h)	X_2 (min)	X_3	Y (%)
1	4	10	6	0.85
2	4	10	10	1.35
3	2	15	12	0.84
4	4	20	10	1.24
5	2	5	12	0.64
6	6	5	12	1.59
7	6	5	8	1.01
8	4	10	14	1.40
9	8	10	10	1.16
10	6	15	8	1.34
11	6	15	12	2.23
12	0	10	10	0
13	4	0	10	0.82
14	2	15	8	0.79
15	4	10	10	1.28
16	2	5	8	1.09

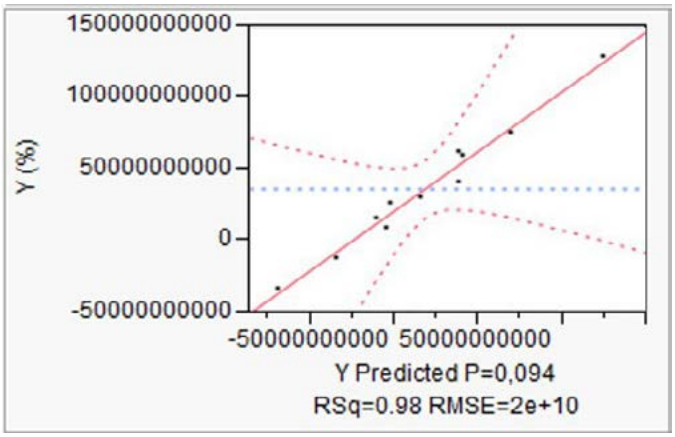

Figure 1. Essential oil contents obtained from the experimental data and the model.

In the extraction process, the steam penetrates the cell layers, breaking the essential oil bags and releasing the oil into the water. However, when the steam becomes saturated with essential oils, it doesn't give effectiveness to release more essential oils. Thus the water to feed ratio is an important factor. In addition, too much water reduces the economic efficiency of the distillation process by consuming heat and time. In this study the highest essential oil yield obtained from the by-

product mixture extract process of 3.9% was obtained by applying 10 min of pretreament with ultrasonic, followed by 4 h of extraction at a water to feed ratio of 10:1 (Figure 2).

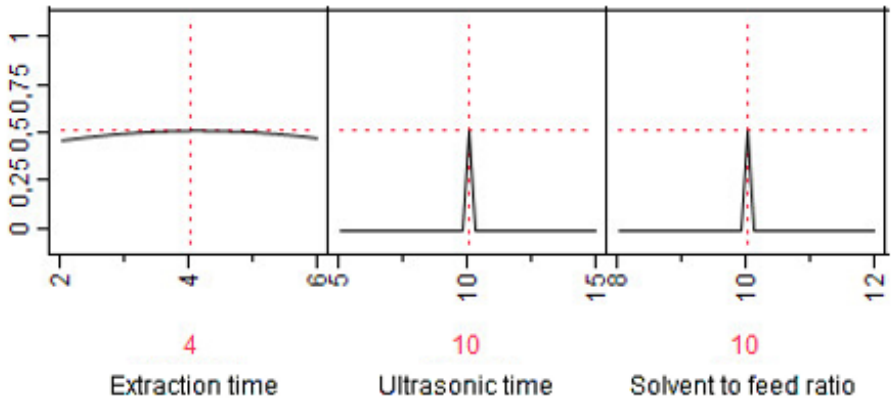

Figure 2. Optimized conditions for the highest response level.

3.2. Chemical compositions of essential oils

There were 16 main compounds found in the essential oil extracted from a mixture of three by-products pepper and the majority belongs to the monoterpenes and sesquiterpenes (Table 2 and Figure 3). These compounds are responsible for the aroma and spicy taste and important biological properties of essential oils (Salzer & Furia, 1977).

The main component of the essential oil is isospathulenol (20.87%) calculated based on the ratio of the peak area of the substance to the total peak area and does not accurately reflect the content present in the essential oil, which has antioxidant activity by decreasing MDA, which is a toxic product due to the degradation of cell membranes due to lipid peroxidation, causing cell rupture and inhibiting inflammatory parameters such as leukocyte migration and

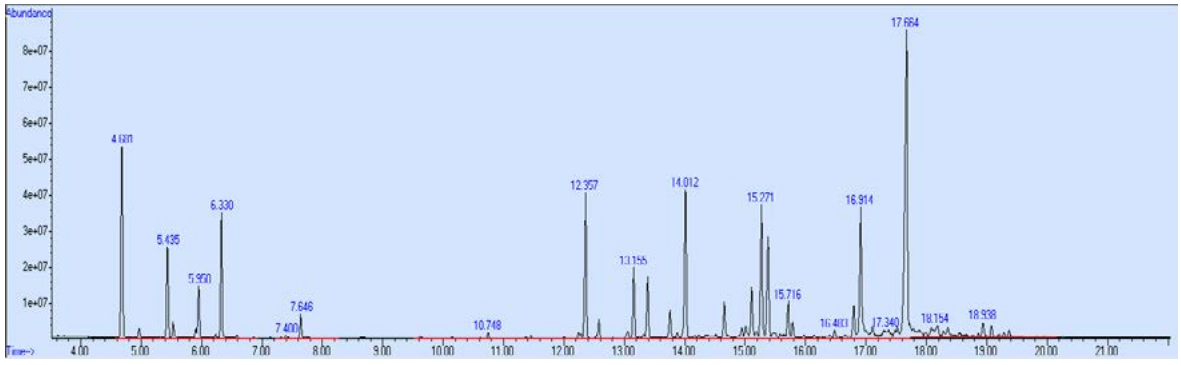

Figure 3. Gas chromatography-mass spectrometry chromatograms of the essential oil.

Table 3. Chemical compositions of mixture pepper by-products essential oil

Number	Chemical composition	Retention time (min)	% Area
1	-Pinene	4.683	7.70
2	-Pinene	5.438	4.80
3	3-Carene	5.952	2.18
4	D-Limonene	6.329	4.72
5	Terpinolene	7.398	0.12
6	-Myrcene	7.644	0.99
7	.delta.-Elemene	12.359	6.02
8	-Copaene	13.153	6.67
9	Caryophyllene	14.011	10.16
10	β-Selinene	15.274	14.25
11	Cadinene	15.714	3.08
12	Germacrene B	16.485	0.86
13	Unknown	16.914	8.84
14	Isospathulenol	17.663	20.87
15	Alloaromadendrene	18.154	3.87
16	-Bisabolene	18.937	3.04

protein extravasation (Nascimento et al., 2018). Other major compounds included β-selinene (14.25%) and caryophyllene (10.16%). These were demonstrated to show pharmacological activities, such as immunoinhibitory, anti-inflammatory, anti-cancer, apoptosis inducer, and highly repellent to insect (Mohammed et al., 2016).

3.3. Antioxidant activity of the essential oil

The essential oil was extracted at optimized conditions and diluted at 5 concentrations of 4 mg/mL, 13 mg/mL, 22 mg/mL, 31 mg/mL, and 40 mg/mL. In the DPPH assay, the purple color

Figure 4. The 2,2-Diphenyl-1-picrylhydrazyl scavenging capacity of the essential oil with increasing concentrations.

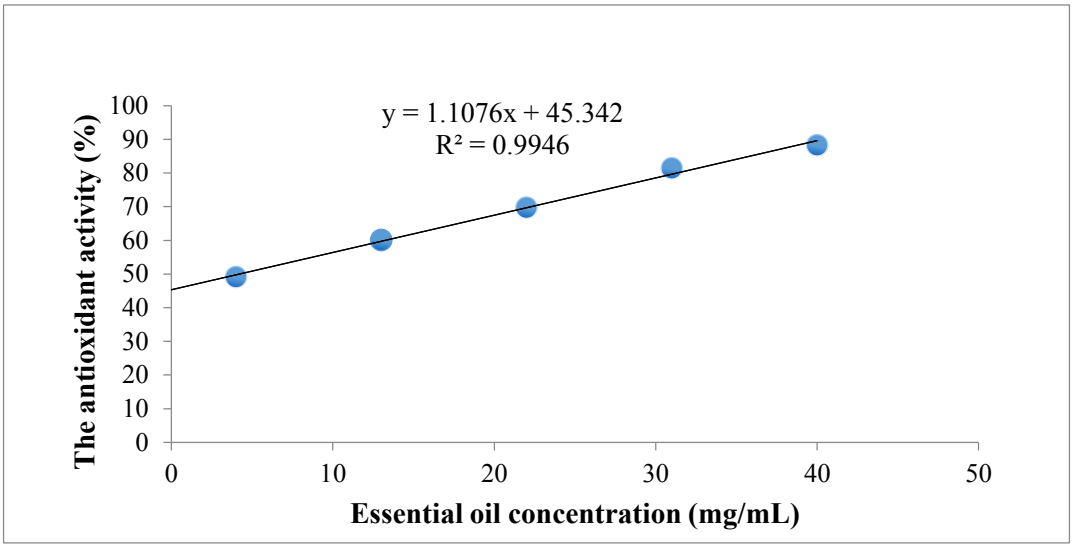

Figure 5. The 2,2-Diphenyl-1-picrylhydrazyl scavenging activity of the essential oil.

intensity gradually decreased with increasing essential oil concentrations (Figure 4). This proves that in the optimal extract, there are groups of substances with antioxidant activity combined with free radicals of DPPH leading to this phenomenon. As shown in Figure 5, IC50 value of the essential oil from the mixture of three black pepper by-products was approximately 4.205 mg/mL, which was much higher than the IC50 of ascorbic acid (0.024 mg/mL). As a result, the essential oils have lower antioxidant capacity. Its antioxidant properties are attributed to main components such as isospathulenol (Nascimento et al., 2018) and D-limonene (Yu et al., 2017).

3.4. Antibacterial activity of the essential oil

The essential oil was extracted at optimized conditions prior to evaluating its antibacterial activity against five strains of bacteria, including *Escherichia coli, Salmonella enterica, Bacillus spizizenii, Bacillus cereus*, and *Staphylococcus aureus*. The bacterial optical density at 600 nM was measured and the suspension turbidity was adjusted according to McFarland turbidity standards by adding distilled water to 10 times the dilutions of the bacterial suspension so that OD600 of 0.5 was achieved, which was equivalent to a colony density of 108 CFU/mL. The agar plates were incubated from 24 to 72 h depending on the bacterial species at room temperature and the results are shown in Figure 6. The essential oil extracted from mixture of three by-products pepper showed good activities against *Salmonella enterica* (D = 5.37 mm), *Bacillus spizizenii* (D = 11.37 mm), *Bacillus cereus* (D = 4.12 mm), and *Staphylococcus aureus* (D = 7.75 mm) but didn't affect the growth of *Escherichia coli*. However, diameters of inhibition zone were smaller than those of the reported data due to different raw material source and extraction conditions such as, techniques, time, temperature, and also bacterial proliferation conditions.

Diameters of inhibition zones were significantly affected by essential oil concentrations (Table 4). Larger inhibition zones were obtained at higher essential oil

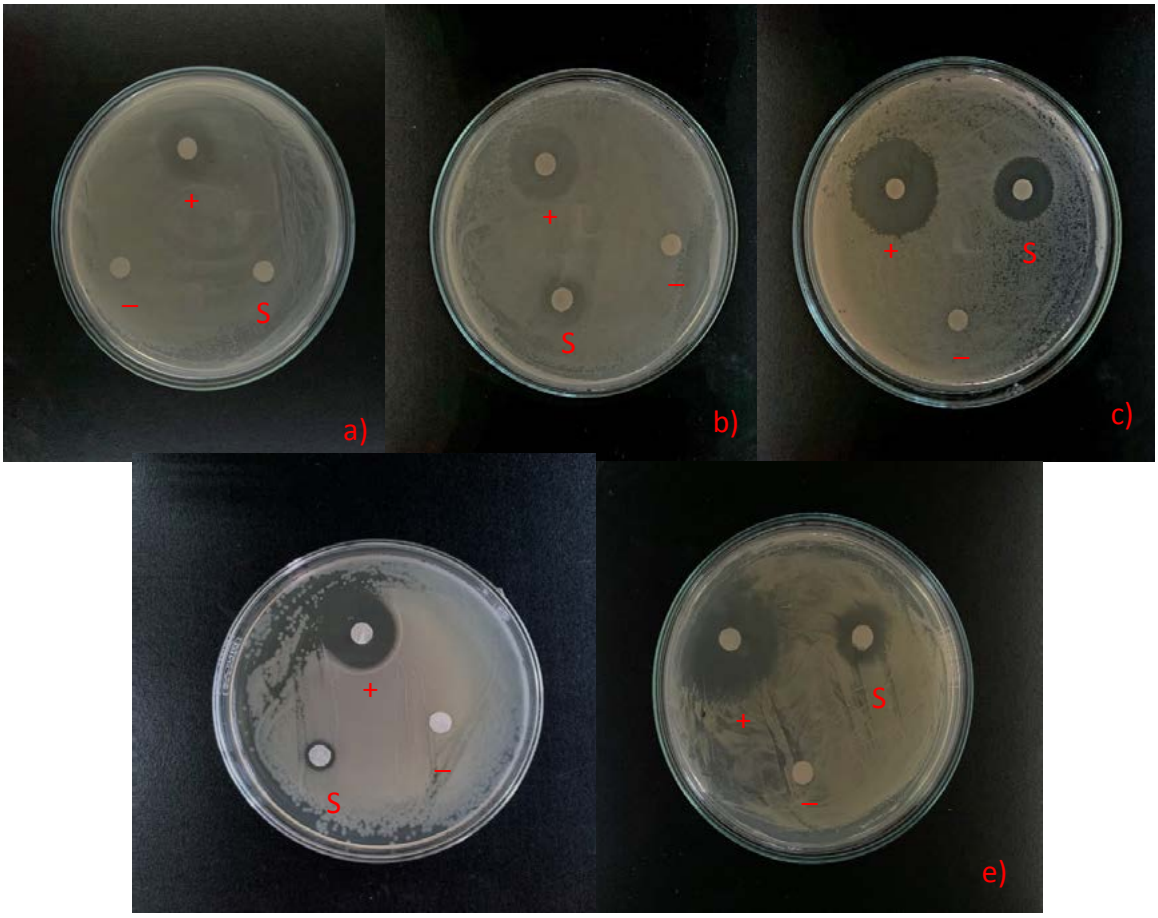

Figure 6. The antibacterial ability of the essential oil against (a) *Escherichia coli*; (b) *Salmonella enterica*; (c) *Bacillus spizizenii*; (d) *Bacillus cereus*; (e) *Staphylococus aureus*; (S) Ratio of essential oil to dimethyl sulfoxide (DMSO) was 4:1; (+) Positive control is Tetracylin; (-) Negative control is DMSO.

concentrations. *B. spizizennii* was the most susceptible strain to the essential oil, followed by S. aureus and S. enterica. The *E. coli* was the most resistant strain to the essential oil. Gram-positive bacteria have a cell membrane structure without lipopolysaccharide molecules. The hydrophilic components of essential oils can directly combine with the phospholipid bilayer of the cell membrane, disrupting the metabolism on both sides of the membrane bacterial cells.

Antibacterial capacity was commonly attributed to the terpenoids representing in the essential oil. Terpenoids are able to inhibit two crucial processes which are essential to microbial survival including oxygen uptake and oxidative phosphorylation. Aerobic microbes require oxygen in order to yield energy for their growth. Previously, it was proven that low oxygen levels caused limited respiration rates in bacteria. In addition, oxidative phosphorylation is a crucial biochemical process responsible for cellular respiration that takes place in the cytoplasmic membrane. Thus, terpenoids interfere cellular respiration, which later causes uncoupling of oxidative phosphorylation in the microbe (Mahizan et al., 2019).

Table 4. Diameter of the inhibition zones (mm)

Species	The ratio of essential oil to dimethyl sulfoxide		
	1:1	2:1	4:1
Escherichia coli	0	0.25 ± 0.10	0.31 ± 0.01
Salmonella enterica	3.00 ± 0.12	3.62 ± 0,14	5.37 ± 0.13
Bacillus spizizenii	2.87 ± 0.14	3.50 ± 0.26	11.37 ± 0.17
Bacillus cereus	1.12 ± 0.11	2.00 ± 0.16	4.12 ± 0.21
Staphylococus aureus	2.25 ± 0.13	2.62 ± 0.23	7.75 ± 0.24

4. Conclusions

The optimized conditions for extracting essential oil from three type by-products of black pepper were found to be 4 h of extraction time, water to feed ratio of 10:1 (v/w), and an ultrasonic time of 10 min. The main chemical compositions of the essential oil included isospathulenol, β-selinene, caryophyllene, α-pinene, and α-copaene. The essential oil obtained from the optimization process showed an antioxidant capacity with an IC50 of 4.205 mg/mL and antibacterial activity against four bacterial strains including *Bacillus spizizenii*, *Bacillus cereus*, *Staphylococcus aureus*, and *Salmonella enterica* with the diameter of inhibition zone of 11.37 mm, 4.12 mm, 7.75 mm, 5.37 mm, respectively. The results provide an understanding of the exploitation, development, and application of black pepper by-products in various areas such as foods, healthcare, and pharmaceuticals.

Conflict of interest

The authors have no conflicts of interest to declare.

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