



Antibacterial Activity of Black Pepper Essential Oil Nanoemulsion Formulated by Emulsion Phase Inversion Method

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Abstract

Black pepper essential oil has been proved to inhibit the growth of microorganisms in many recent studies. However, free essential oils are often lipophilic and difficult to use in food products. The nanoemulsion has some advantages such as good dispersion, long-term stability, and transparency. In our study, the Emulsion Phase Inversion method was utilized to formulate black pepper essential oil nanoemulsion. After 6 months, the nanoemulsion retained the droplet size about 18 nm and there was a rise in polydispersity index from 0.087 to 0.608. Besides, concentrations of important components (α -pinene, β -pinene, D-limonene, 3-carene, and β -caryophyllene) in the BPEO phase of nanoemulsion were similar to pure essential oil. This study also showed that *Escherichia coli* and *Salmonella entericawere* sensitive to black pepper essential oil nanoemulsion than free essential oil. Minimal Inhibitory Concentrations of nanoemulsion for *E. coli* and *S. enterica* (137 and 273 $\mu\text{g/mL}$, respectively) were higher than those of free essential oil (547 $\mu\text{g/mL}$). In addition, nanoemulsion inhibited these bacterial growth on pork samples. When utilizing nanoemulsion as a meat preservative, meat samples, which contained nanoemulsions, observed significantly lower aerobic microbial counts than control samples.



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Introduction


Essential oils (EO) derived from plant species have been demonstrated to contain lipophilic substances with proven bioactivities such as antioxidant,

antimicrobial, anti-inflammatory, anticarcinogenic, or antimutagenic activities and thus find a wide range of applications.^{1,2} Of which, the use as an antimicrobial agent in the food industry has been

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regarded as one notable application of essential oils extracted from spices and herbs, as reflected by the growing literature in this research direction.^{3,4} Such utilization of EO is realized mostly due to its antimicrobial activity against common food pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Bacillus cereus*.^{5,6,7}

Black pepper (*Piper nigrum* L.) is one of the popular food spices worldwide. Recently, black pepper essential oil (BPEO) has been gaining increasing scientific attention due to its abundance of compounds with useful bioactivities contained within.

BPEO contains at least twenty components, depending on material origins and extracted methods,^{8,9,10} most of which have been found to belong to monoterpene and sesquiterpene groups.¹¹ Major components have presented in BPEO included caryophyllene and limonene with concentrations ranging from 10% to 30%. Other less common compounds that accounted for more than 2% of BPEO mass are α -pinene, β -pinene, 3-carene, α -phellandrene, humulene, α -copaene, and sabinene.^{8,10,12,3,14,15,16}

It was also reported that *Staphylococcus aureus* was highly susceptible to BPEO, expressed by low MIC90% of 0.21 mg/ml.¹⁷ In another report, *Staphylococcus aureus* and *E.coli* were both sensitive to BPEO with MIC of 1,000 μ L/mL and 2,000 μ L/mL, respectively.¹⁸ *Pseudomonas orientalis* was another bacterial strain that was found to be inhibited by BPEO with inhibition zone and MIC of 20 mm and 7.6 mg/mL, respectively.¹⁹ Antibacterial activity of BPEO originating in ten different provinces of China was evaluated against *Aspergillus flavus*, *Candida fimbriata*, and *Candida albicans* and showed three of those BPEOs exhibited the lowest MIC for all tested fungi at 2 mg/mL.¹⁶

Therefore, BPEO has demonstrated potential for food preservation due to its inhibitory against many food pathogens. There have been more and more researchers that have fabricated essential oil nanoemulsions for enhancing essential oil bioactivity in the water-rich environment

such as food products. Because nanometre droplets could easily fuse with lipid bilayers of microbial membranes, essential oil activity loaded into nanoemulsion has increased.^{20,21,22}

In this study, Vietnamese black pepper essential oil was utilized to formulate nanoemulsion by the Emulsion Phase Inversion method. This has been a low-energy method with many advantages such as low cost for energy, protecting active compounds, easiness to do, and easiness to scale up.^{23,24} Then, BPEO nanoemulsion was evaluated antibacterial activity against food pathogens and the work was continued by using this system as a preservative for meat products.

Materials and Methods

Materials and Chemicals

Vietnamese BPEO was purchased from An Phong Dak Nong Investment and Import - Export Joint Stock Company (APEXDAKNONG) in Dak Nong Province, Vietnam. Tetrazolium Chloride (TTC) from Sigma-Aldrich was used as a dye for MIC assay. Dimethyl sulfoxide (DMSO) and polyoxyethylene sorbitan monooleate (Tween 80) were supplied by Biobasic, Canada. Amoxicillin antibiotic was also supplied by Sigma-Aldrich.

Nanoemulsion Formulation by Emulsion Phase Inversion Method

The emulsion Phase Inversion process for formulating BPEO nanoemulsion was done following our previous reports.^{25,26} Briefly, 10 mL of BPEO and 20 mL of Tween 80 as a surfactant were mixed at 800 rpm stirring for 15 min. The volume of 70 mL of distilled water was titrated into the oil phase and surfactant mixture at a rate of 1 mL/min. After titration, the system was continuously stirred on a magnetic stirrer for an additional 30 min. Next, samples were stabilized for 24 hours at -15°C for 24 hours then completely thawed at room temperature. Then, BPEO nanoemulsion was kept at room temperature and utilized for testing antibacterial activity in the next experiments

Determination of Volatile Compounds by Gas Chromatography-Mass Spectrometry Method

GC-MS (Gas Chromatography-Mass Spectrometry) method was used for determining compounds in black pepper essential oil and nanoemulsions

by GC Agilent 6890N coupled with MS 5973 inert and HP5 – MS capillary column (30m x 0.25mm; 0.25µm film thickness). The input carrier gas (helium) pressure was 9.3 psi. The furnace temperature was programmed as follow: 50°C for 2 min, 50 – 80°C at 2°C. min⁻¹, 80 – 150°C at 5°C. min⁻¹, 150 – 200°C at 10°C. min⁻¹, 200 – 300°C at 20°C. min⁻¹ and maintaining at 300°C for 5 min. The nanoemulsion samples (250µL) were diluted with 250µL distilled water, then, 500µL n-hexane was added and vortexed before keeping for 2 days. The fraction in n-hexane (0.5µL) was injected for component analysis.²⁵

Determination of Droplet Size and Polydispersity Index

Mean droplet size and polydispersity index were determined by the Dynamic Light Scattering method with HORIBA SZ 100 Nanoparticle Analyzer. Before measuring, a 20-fold dilution of nanoemulsions was prepared. Additionally, the size distribution of the samples was also shown as a result of this device.²⁵

Determination of Minimal Inhibitory Concentration (Mic) and Minimal Bactericidal Concentration (Mbc) by Dilution Method

Antibacterial activity of BPEO and BPEO nanoemulsion was carried out over four bacteria including *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 14028, and *Staphylococcus aureus* ATCC 25823. Firstly, bacteria colonies were transferred from maintaining agar media to TSB (tryptone soy broth), followed by incubation at 37°C for 48 hours to increase cell mass. Afterward, bacteria suspension was diluted to 10⁸ CFU/mL by comparing with Mc. Farland 0.5 turbidity. Then, bacteria suspension was diluted to 10⁶ CFU/mL.

The lowest possible concentration of BPEO that causes no visual growth of bacteria after incubation was identified as minimal inhibitory concentration (MIC). The MIC parameter was determined by the dilution method.¹⁶ Firstly, nine glass tubes, which contained 2 mL of sterilized TSB media, were prepared. Then, 2mL of BPEO in 5% DMSO solution or BPEO nanoemulsion (EO concentration of 17,500µg/mL) was added into the first tube. Next, 2 mL solution in the first tube was transferred

to the second one for two-fold dilution. Two-fold dilution was continued for seven residue tubes. After dilution, essential oil's final concentrations in nine tubes are as follows: 8,750µg/mL, 4,375µg/mL, 2,188µg/mL, 1,094µg/mL, 547µg/mL, 273µg/mL, 137µg/mL, 68µg/mL and 34µg/mL. Finally, 0.2mL bacterial suspension (10⁸ CFU/mL) was added to the tubes. Additionally, a negative tube containing TSB only and a positive tube containing TSB and bacteria were also prepared.

Amoxicillin was also diluted by the same method to prepare nine different concentrations. All of the tubes were incubated at 37°C for 24 hours before being added with 400µL Tetrazolium Chloride (TTC) 0.4%. The lowest concentration of each drug (essential oils or antibiotic) that did not cause the tube turning into red was identified as MIC.

On the other hand, before adding TTC solution, 0.2 mL volume of each tube was used to spread on the TSA Petri dishes to count the colonies and determine as MBC (the least concentration without bacterial colony).

Antibacterial Activity of Bpeo Nanoemulsion on Meat Infected By Bacteria

After indicating bacterial inhibition of BPEO nanoemulsion by dilution method, our system was tested antibacterial activity on pork against *E.coli* and *S. enterica*. In this experiment, each sample of 20-gram lean pork was sterilized by rinsing with 70% v/v of ethanol solution, then cleaned with sterilized water and dried for 15 minutes at room temperature. After that, samples were dipped into BPEO nanoemulsion at different concentrations (10, 25, 50, 100%) or sterilized water (control sample). All meat samples were spread with 1mL of bacteria culture (10⁶CFU/mL) on the surface and kept at 5°C for 6 days. The bacteria population was determined on the 2nd, 4th, and 6th day to evaluate nanoemulsion antimicrobial activity.^{9,27}

Meat Preservation by Bpeo Nanoemulsion

Many studies were done for testing the food preserving ability of essential oil nanoemulsion on meat products [28] [29]. In this experiment, BPEO nanoemulsion was formed and evaluated its antimicrobial activity in fresh meats. All the

experiments were replicated 3 times before collecting and analysing data.

Firstly, minced beef was preserved by BPEO nanoemulsion. Briefly, 50-gram minced beef was mixed with BPEO nanoemulsion at some concentrations (0; 0.5; 1.0; 2.0; or 5.0 % w/w) and stored at 5°C for 6 days. The total aerobic microbial count (log CFU/g) was determined on the 2nd, 4th, and 6th day and data were analysed to identify the optimum concentration of BPEO nanoemulsion for minced beef preservation.

In the next experiment, lean pork and chicken were used for testing BPEO nanoemulsion bioactivity. Each meat sample was weighed at 100 g and cut into pieces (size at 4 x 5 x 1 cm). Then, all of the meat samples were cured with a seasoning mixture including 1.0g of salt; 1.0g of monosodium glutamate; 0.5g of sugar; 1.0g of fish sauce. BPEO nanoemulsion was added into meat samples at various mass percentages (0; 1; 2%). Then,

meats were kept at 5°C and evaluated total aerobic microbial count (log CFU/g) on the 2nd, 4th, and 6th day.

Statistical Analysis

All of the experiments were replicated at least three times and data were analysed by the Analysis of Variance method by Statgraphics Ver.3.0. The charts were made with Microsoft Excel.

Results And Discussion

Characteristics of Black Pepper Essential Oil Nanoemulsion

For using BPEO nanoemulsion as a food preservative, this system must be physicochemical steadiness and well-encapsulated bioactive components. After fabricating, BPEO nanoemulsion was kept at room temperature for six months. The nanoemulsion was then analysed droplet size distribution by DLS method and volatile components by GC – MS method.

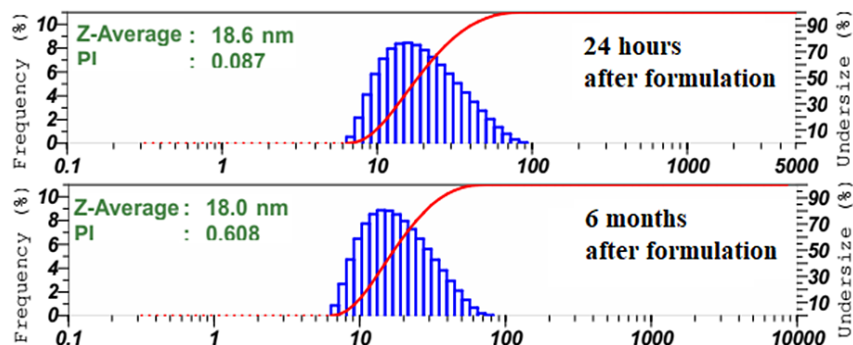


Fig. 1: Droplet size distribution of BPEO nanoemulsions

In Figure 1, the droplet size distribution of BPEO nanoemulsion after 24 hours and after six-month storage was compared. The data indicated that there was almost no difference in average droplet size after long-term storage. The similarity of the two charts (in shape and location) showed the physicochemical stabilization of BPEO nanoemulsion.

There was an increase in the polydispersity index (Pdl) from 0.087 to 0.608. This variation presented a decrease in system homogeneity. According to many authors, nanoemulsion is thermodynamically unstable so its average droplet size rises over time. However, nanoemulsion is kinetically stable and this

system could retain steadiness for a very long time if it has appropriate properties.^{23,24} In this case, our BPEO nanoemulsion had been high homogeneous with very small droplets (about 18 nm) and had retained steadiness for six months.

In recent studies, nanoemulsions fabricated by the EPI method also obtained highly homogeneous. D-limonene nanoemulsion formed by the EPI method determined the average droplet size of 47.5 nm and was stable for 12 days at 28°C with the rise in droplet size to 57.8 nm.³⁰ Vitamin E nanoemulsion with 40 nm of average droplet size was also successfully fabricated by the

EPI method.³¹ In another study, using the same method, clove and lemon grass oil nanoemulsion got the smallest droplet size of 76.73 nm and Pdl of 0.20. This system showed antimicrobial activity against *Fusarium oxysporum*.³²

For evaluating the protection for bioactive compounds, BPEO nanoemulsion after six months was determined volatile components by GC – MS method (Figure 2).

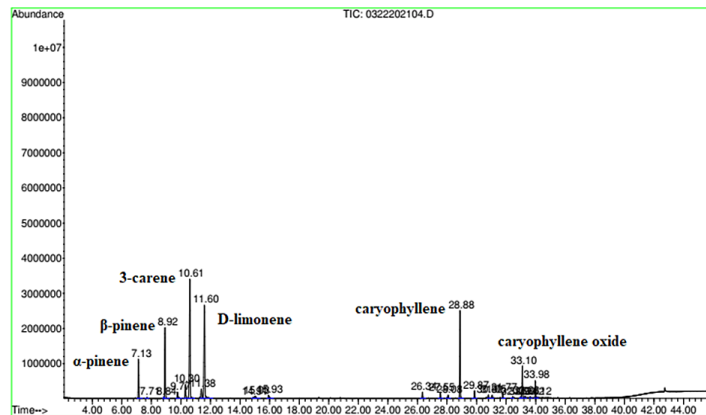


Fig. 2: GC – MS chromatogram of BPEO nanoemulsion volatile components after six-month storage

Figure 2 showed the existence of five main volatile compounds in BPEO nanoemulsion. These compounds, including α -pinene, β -pinene, D-limonene, 3-carene, and β -caryophyllene, obtained high concentrations of 5.88%; 12.44%; 20.37%; 24.93%, and 13.93%, respectively. Comparison with pure BPEO volatile components, which presented in our previous reports,^{25,26} concentrations of four compounds (α -pinene, β -pinene, D-limonene, 3-carene) almost remained. However, the concentration of β -caryophyllene was remarkably decreased from 21.94% of pure BPEO to 13.90% of BPEO in nanoemulsion after six months. The decrease in β -caryophyllene content might be due to the formation of caryophyllene derivatives such as caryophyllene oxide and caryophylladienol (at a retention time of 34.12 min in GC – MS spectrum). Caryophyllene oxide content increased from 1.05% (in pure BPEO) to 5.00% (in BPEO of nanoemulsion). While caryophylladienol, which was not identified in pure BPEO, presented in BPEO nanoemulsion after six months at low concentration (0.15%).²⁵

After six months, BPEO nanoemulsion maintained not only droplet size distribution, but also volatile components' content. Therefore, the BPEO nanoemulsion could use as a loading system for improving dispersion and bioactivity of lipophilic BPEO in practical application.

Antibacterial Activity of Bpeo and Bpeo Nanoemulsion by Dilution Method

The BPEO nanoemulsion in our research was determined antibacterial activity against some common food pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Salmonella enterica*). Free BPEO and amoxicillin were also used in this experiment as control samples, and the results were shown in Table 1.

All samples (BPEO, BPEO nanoemulsion, and amoxicillin) observed high antibacterial activity against *E. coli* and *S. enterica* than *S. aureus* and *P. aeruginosa*. MIC and MBC of BPEO nanoemulsion for *E. coli* were equal to amoxicillin (137 and 273 μ g/mL, respectively) and lower than free BPEO (547 and 1,094 μ g/mL, respectively). It was meant that BPEO nanoemulsion inhibited *E. coli* more effectively than free BPEO but presented similar bioactivity to amoxicillin.

In the case of *S. enterica*, BPEO nanoemulsion showed a better inhibiting effect than free BPEO, but less than amoxicillin. MBCs of nanoemulsion and free BPEO were equal (1,094 μ g/mL) and they were higher than MBC of amoxicillin (273 μ g/mL), Amoxicillin showed the best antibacterial activity against *S. enterica* in our experiment.

Table 1: MICs and MBCs of free BPEO, BPEO nanoemulsion, and amoxicillin against bacteria

Bacteria	BPEO nanoemulsion		BPEO		Amoxicillin	
	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)	MIC ($\mu\text{g/mL}$)	MBC ($\mu\text{g/mL}$)
<i>Escherichia coli</i>	137	273	547	1,094	137	273
<i>Salmonella enterica</i>	273	1,094	547	1,094	68	273
<i>Pseudomonas aeruginosa</i>	1,094	1,094	1,094	4,375	8,750	8,750
<i>Staphylococcus aureus</i>	2,187	4,375	2,187	8,750	8,750	8,750

Free BPEO and BPEO nanoemulsion showed better antibacterial activity against *P. aeruginosa* than amoxicillin with MICs of 1,094; 1,094; and 8,750 $\mu\text{g/mL}$, respectively. All three samples presented the worst antibacterial activity against *S. aureus* with very high MICs and MBCs.

Generally, free BPEO and BPEO nanoemulsion presented better antibacterial activity against Gram-negative bacteria (*E. coli*, *S. enterica*, and *P. aeruginosa*) than Gram-positive bacteria (*S. aureus*). This was corroborated by a previous study where the antibacterial activity of BPEO emulsion was evaluated against Gram-positive and Gram-negative bacteria, showing slightly higher activity against the latter than the former, possibly due to differences in the cell wall and membrane components.³³ The mechanism was further elaborated that lactic acid bacteria and *Brochothrix* spp. (Gram-positive) were more resistant to BPEO than *Pseudomonas* spp. and *Enterobacteriaceae* (Gram-negative). Accordingly, the high susceptibility of Gram-negative bacteria against lipophilic essential oil could be explained by a thinner peptidoglycan layer but higher content of lipid (lipoprotein and lipopolysaccharide) than Gram-positive. As a result, lipophilic essential oil could easily penetrate the Gram-negative bacteria cytoplasmic.²⁸

The bacteria inhibitory of BPEO have been reported in numerous studies. In a previous study, BPEO MICs for *S. aureus* and *E. coli* were indicated at 1,000 and 2,000 $\mu\text{g/mL}$, respectively.¹⁸ In another report, BPEO showed a high inhibiting effect on *Pseudomonas fluorescens* with the MIC of 5 $\mu\text{g/mL}$.⁹ Other authors pointed that there was a significant difference in inhibition zone diameter

between Indian BPEO and control sample against *Pseudomonas aeruginosa* (11.8 mm and 6.7 mm, respectively), suggesting potent antimicrobial activity of BPEO against this microorganism.³⁴

According to these studies, BPEO could inhibit bacteria but is more effective on Gram-negative bacteria than Gram-positive ones. Our work had given more proof for the sensitiveness of Gram-negative bacteria, especially *E. coli* and *S. enterica*, to BPEO nanoemulsion than free BPEO.

Inhibitory of Bpeo Nanoemulsion on Pork Infected by Bacteria

Depending on the high antibacterial activity of BPEO nanoemulsion against *E. coli* and *S. enterica* in our previous experiment, nanoemulsion was diluted by sterilized water to form dipping solutions with various concentrations (10, 25, 50, 100%). Then, meat samples were dipped into these solutions or sterilized water (control sample). Next, 1 mL of bacteria (*E. coli* or *S. enterica*) suspension (106 CFU/mL) was dropped and spread on the meat surface. Then, pork samples were put on plastic dishes, packed over by PE layer, and kept at 5°C. Bacterial colony counts were analysed on the 2nd, 4th, and 6th days. The data were given in Table 2.

As in Table 2, on the second day, all of the samples dipped into BPEO nanoemulsion solutions could retain *E. coli* population below 5.0 log CFU/g and it was significantly lower than that of the control sample. From the 2nd to the 4th day, the number of *E. coli* increased in all samples at different rates. On the fourth day, *E. coli* population of the control sample was the highest (5.5 log CFU/g) and these parameters were significantly lower in nanoemulsion

samples. After six days, *E. coli* number of treatment and control samples were not significantly different.

As in Table 2, BPEO nanoemulsion could also inhibit the growth of *S. enterica* on cold pork. The bacteria count of the control sample was

5.5 logCFU/g on the second day and rose to 5.7 logCFU/g on the sixth day. While the sample at the lowest nanoemulsion concentration (10%) obtained *S. enterica* count of 5.4 log CFU/g on the sixth day.

Table 2: Escherichia coli and Salmonella enterica population (log CFU/g) on bacteria-infected pork during cold storage

Nanoemulsion concentration (%)	<i>E. coli</i> count (log CFU/g)			<i>S. enterica</i> count (log CFU/g)		
	2 nd day	4 th day	6 th day	2 nd day	4 th day	6 th day
0	5.2 ^b ±0.16	5.5 ^c ±0.09	5.7 ^{ns} ±0.04	5.5 ^b ±0.20	5.7 ^b ±0.03	5.7 ^c ±0.02
10	4.7 ^a ±0.19	5.2 ^{ab} ±0.11	5.3±0.18	5.2 ^a ±0.17	5.4 ^{ab} ±0.08	5.4 ^{ab} ±0.18
25	4.8 ^a ±0.13	5.2 ^{ab} ±0.29	5.6±0.05	5.2 ^a ±0.14	5.2 ^a ±0.24	5.5 ^{bc} ±0.09
50	4.7 ^a ±0.11	5.4 ^{bc} ±0.04	5.5±0.10	5.0 ^a ±0.29	5.3 ^a ±0.20	5.2 ^a ±0.18
100	4.8 ^a ±0.22	5.0 ^a ±0.28	5.4±0.20	4.9 ^a ±0.14	5.1 ^a ±0.21	5.3 ^{ab} ±0.06
P	0.0190	0.0496	0.0764	0.0205	0.0488	0.0052

Note: Different superscript letters (a, b, c, ...) showed the significant difference of data in the same column at 0.05 level

In general, BPEO nanoemulsion could reduce the growth of both *E. coli* and *S. enterica* on pork. These results could give the hope to use this system for meat preservation.

Effect of Bpeo Nanoemulsion Concentration on Minced Beef Microorganism Qualification

Depending on the tendency for utilization of natural food preservatives, BPEO nanoemulsions

were blended in minced beef at different concentrations (from 0.5 to 5.0 % w/w). Next, these treated samples and control samples (without BPEO nanoemulsion) were put in plastic dishes, packed over with PE layer, and kept at 5°C. The total aerobic microbial count and pH of samples were determined on the 2nd, 4th, and 6th days.

Table 3: Total aerobic microbial count (log CFU/g) and pH index of minced beef

Nanoemulsion concentration (%)	Storage day					
	2		4		6	
	Microbial count (log CFU/g)	pH	Microbial count (log CFU/g)	pH	Microbial count (log CFU/g)	pH
0	6.2 ^a ±0.05	5.1 ^e ±0.02	7.8 ^e ±0.00	5.1 ^e ±0.03	9.3 ^e ±0.02	4.9 ^e ±0.03
0.5	5.8 ^d ±0.07	5.2 ^d ±0.04	7.3 ^d ±0.03	5.2 ^d ±0.03	9.0 ^d ±0.03	5.0 ^d ±0.01
1.0	5.0 ^c ±0.03	5.4 ^c ±0.01	6.7 ^c ±0.01	5.3 ^c ±0.03	8.1 ^c ±0.01	5.1 ^c ±0.02
2.0	4.8 ^b ±0.03	5.5 ^b ±0.02	6.4 ^b ±0.02	5.4 ^b ±0.01	7.3 ^b ±0.02	5.3 ^b ±0.01
5.0	3.6 ^a ±0.03	5.6 ^a ±0.02	4.8 ^a ±0.08	5.5 ^a ±0.02	6.2 ^a ±0.08	5.4 ^a ±0.02
P	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000

Note: Different superscript letters (a,b,c,...) showed the significant difference of data in the same column at 0.05 level

In this experiment, the aerobic microbial number and the change of pH index significantly decreased with the increase in nanoemulsion concentration. It meant that BPEO nanoemulsion could function as a preservative for fresh beef against microorganisms. The microorganism population of untreated beef rose quickly to 9.3 log CFU/g on the sixth day while beef at 2% and 5% nanoemulsion just reached 7.3 and 6.2 log CFU/g, respectively. The pH index of beef at 5% nanoemulsion was 5.4 after 6 days and this index was just 0.1 units lower than material, fresh beef (Table 3). BPEO nanoemulsion at concentrations ranging from 1% to 5% was observed bacteriostatic effect of microbial flora on minced beef.

BPEO had been also utilized in some recent studies as a preservative. For example, Malaysian BPEO was used as an ingredient in a salad made by fresh-cut lettuce, and 10mL of *Pseudomonas*

fluorescent suspension (10^4 CFU/mL) was added. The results showed that bacterial inhibition of BPEO was 30% higher than the control sample.⁹ In another report, a product of orange juice (50mL) mixed with 10% (v/v) BPEO solution in ethanol (100 μ L) was formed. After storage at 4°C for 28 days, the total aerobic microbial count in orange juice with BPEO was lower than in the control sample, 2×10^3 CFU/mL and 6.5×10^3 CFU/mL, respectively.²⁷

Effect of Bpeo Nanoemulsion Concentration on Microorganism Qualification of Seasoning Cured Meat

In this experiment, pork and chicken were cured with seasoning and BPEO nanoemulsion and kept at 5°C for six days. The samples were determined total aerobic microbial count during cold storage to evaluate the microbial inhibiting effect of BPEO nanoemulsion on meat. The data were shown in Table 4.

Table 4: Total aerobic microbial count (log CFU/g) on seasoning-cured pork and chicken

Nanoemulsion concentration (%)	Total aerobic microbial count in pork samples (log CFU/g)			Total aerobic microbial count chicken samples (log CFU/g)		
	2 nd day	4 th day	6 th day	2 nd day	4 th day	6 th day
0	5.10c \pm 0.09	5.15b \pm 0.06	5.17b \pm 0.05	5.09c \pm 0.64	5.21c \pm 0.13	5.22c \pm 0.03
1	4.72b \pm 0.19	4.78a \pm 0.12	4.94a \pm 0.02	4.73b \pm 0.21	4.80b \pm 0.04	4.99b \pm 0.08
2	4.37a \pm 0.04	4.62a \pm 0.12	4.77a \pm 0.17	4.33a \pm 0.04	4.54a \pm 0.05	4.82a \pm 0.07
P	0.0120	0.0020	0.0080	0.0010	0.0020	0.0008

Note: Different superscript letters (a, b, c, ...) showed significant differences in data in the same column at 0.05 level

The results revealed that all of the meat samples with BPEO nanoemulsion were a significantly lower microbial number than the control samples. The control samples obtained above 5.0 log CFU/g on the second day, while the others retained microbial count below that level even on the sixth day. When rising the BPEO nanoemulsion concentration from 1% to 2%, the total microbial counts just decreased on the second day for pork samples and the efficacy of microbial count decrease was observed through six days for chicken samples. Generally, BPEO nanoemulsion used as a natural preservative could inhibit microbial growth in meat samples (both pork and chicken).

Conclusion

In conclusion, our nanoemulsion formulated by the EPI method could load BPEO and it had been stable for at least 6 months. The MIC and MBC values in the dilution assay revealed that BPEO nanoemulsion observed higher antibacterial activity against food pathogens than free BPEO. *E. coli* and *S. enterica* were more sensitive than other bacteria (*P. aeruginosa* and *S. aureus*). BPEO nanoemulsion could also inhibit the growth of bacteria on meat infected by *E. coli* and *S. enterica*. Last but not least, BPEO nanoemulsion could be an effective preservative for meat samples in our study because this system could reduce total aerobic microbial growth in these samples at 5°C.

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Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Properzi A., Angelini P., Bertuzzi G. and Venanzoni R. Some Biological Activities of Essential Oils. *Medicinal & Aromatic Plants*. 2013; 2(5):136.
2. Dhifi W., Bellili S., Jazi S., Bahloul N. and Mnif W. Essential Oils' Chemical Characterization and Investigation of Some Biological Activities: A Critical Review. *Medicines*. 2016; 3(4):25-41.
3. Tongnuanchan P., Benjakul S. and Prodpran T. Comparative studies on properties and antioxidative activity of fish skin gelatin films incorporated with essential oils from various sources. *Int Aquat Res*. 2014;6:62-74.
4. Perricone M., Arace E., Corbo M. R., Sinigaglia M. and Bevilacqua A. Bioactivity of essential oils: a review on their interaction with food components. *Frontiers in Microbiology*. 2015;6:76.
5. El Moussaoui M., Talbaoui A., Gmouh S., Aberchane M., Benjou A., Bakri Y. and Kamdem D. P. Chemical composition and bactericidal evaluation of essential oil of *Tetraclinis articulata* burl wood from Morocco. *J Indian Acad Wood Sci*. 2010; 7(1-2):14-18.
6. Akarca G. Composition and antibacterial effect on foodborne pathogens of *Hibiscus urrattensis* L. calyces essential oil. *Industrial Crops & Products*. 2019;137: 285-289.
7. Sayout A., Ouarhach A., Dilagui I., Sora N., and Romane A. Antibacterial activity and chemical composition of essential oil from *avandula tenuisecta* Coss.ex Ball. an endemic species from Morocco. *European Journal of Integrative Medicine*. 2020;33:101017.
8. Jelen H. H. and Gracka A. Analysis of black pepper volatiles by solid phase microextraction-gas chromatography: A comparison of terpenes profiles with hydrodistillation. *Journal of Chromatography A*. 2015;1418:200-209.
9. Myszka K., Schmidt M. T., Majcher M., Juzwa W. and Czaczyk K. β -Caryophyllene-rich pepper essential oils suppress spoilage activity of *Pseudomonas fluorescens* KM06 in fresh-cut lettuce. *LWT - Food Science and Technology*. 2017;83:118-126.
10. Bastos L. P. H., Vicente J., Santos C. H. C., Carvalho M. G. and Garcia-Rojas E. G. Encapsulation of black pepper (*Piper nigrum* L.) essential oil with gelatin and sodium alginate by complex coacervation. *Food Hydrocolloids*. 2020;102:105605.
11. Kumoro A. C., Hasan M. and Singh H. Extraction Of Sarawak Black Pepper Essential Oil Using Supercritical Carbon dioxide. *Arabian Journal For Science And Engineering*. 2010;35(2B):7-16.
12. Jirovetz L., Buchbauer G., Ngassoum M. B. and Geissler M. Aroma compound analysis of *Piper nigrum* and *Piper guineense* essential oils from Cameroon using solid-phase microextraction-gas chromatography, solid-phase microextraction-gas chromatography-mass spectrometry and olfactometry. *Journal of Chromatography A*. 2002;976:265-275.
13. Aziz S., Naher S., Abukawsar M. D. and Roy S. K. Comparative Studies on Physicochemical Properties and GC-MS Analysis of Essential Oil of the Two Varieties of the Black Pepper (*Piper nigrum* Linn.). *International Journal of Pharmaceutical and Phytopharmacological Research*. 2012;2(2):67-70.
14. Wang Y., Li R., Jiang Z. T., Tan J., Tang S. H., Li T. T., Liang L. L., He H. J., Liu Y. M., Li J. T. and Zhang X. C. Green and solvent-free simultaneous ultrasonic-microwave assisted extraction of essential oil from white and black peppers. *Industrial Crops & Products*. 2018;114:164-172.

15. Amalraj A., Haponiuk J. T., Thomas S. and Gopi S. Preparation, characterization and antimicrobial activity of polyvinyl alcohol/gum arabic/chitosan composite films incorporated with black pepper essential oil and ginger essential oil. *Journal of Biological Macromolecules*. 2020;151:366–375.
16. Li Y., Zhang C., Pan S., Chen L., Liu M. and Yang K. Analysis of chemical components and biological activities of essential oils from black and white pepper (*Piper nigrum* L.) in five provinces of southern China. *LWT - Food Science and Technology*. 2020;117:108644.
17. Andrade B. F. M. T., Barbosa L. N., Da Silva Probst I. and Júnior A. F. Antimicrobial activity of essential oils. *Journal of Essential Oil Research*. 2014;26(1):34-40.
18. Rakmai J., Cheirsilp B., Mejuto J. C. and Torrado-Agrasar A. Physico-chemical characterization and evaluation of bio-efficacies of black pepper essential oil encapsulated in hydroxypropyl-beta cyclodextrin. *Food Hydrocolloids*. 2017;65:157-164.
19. Leja K., Majcher M., Juzwa W., Czaczyk K. and Komosa M. Comparative Evaluation of *Piper nigrum*, *Rosmarinus officinalis*, *Cymbopogon citratus* and *Juniperus*. *Foods*. 2020;9:141.
20. Shams N. and Sahari M. A. Nanoemulsions: Preparation, Structure, Functional Properties and their Antimicrobial Effects. *Applied Food Biotechnology*. 2016;3(3):138-149.
21. Pathania R., Khan H., Kaushik R. and Khan M. A. Essential Oil Nanoemulsions and their Antimicrobial and Food Applications. *Current Research in Nutrition and Food Science*. 2018;6(3):626-643.
22. McClements D. J., Das A. K., Dhar P., Nanda P. K. and Chatterjee N. Nanoemulsion-Based Technologies for Delivering Natural Plant-Based Antimicrobials in Foods. *Frontiers in Sustainable Food Systems*. 2021;5:643208.
23. McClements D. J. Emulsions: Principles, practices, and techniques – Third Edition, New York: Taylor & Francis Group, LLC, 2016.
24. Gupta A., Eral H. B., Hattona T. A. and Doyle P. S. Nanoemulsions: formation, properties, and applications. *Soft Matter*. 2016;12:2826.
25. Hien L. T. M. and Dao D. T. A. Black pepper essential oil nanoemulsions formulation using EPI and PIT methods. *Journal of food processing and preservation*. 2021;45:15216.
26. Hien L. T. M., Khoa T. D. and Dao D. T. A. Characterization of black pepper essential oil nanoemulsion fabricated by emulsion phase inversion method. *J Food Process Preserv*. 2021;0:e16207.
27. Kapoor I. P. S., Singh B., Singh S. and Singh G. Essential oil and oleoresins of black pepper as natural food preservatives for orange juice. *Journal of Food Processing and Preservation*. 2012.
28. Zhang J., Wang Y., Pan D. D., Cao J. X. and Shao X. F. Effect of black pepper essential oil on the quality of fresh pork during storage. *Meat Science*. 2016;117:130–136.
29. Noori S., Zeynali F., and Almasi H. Antimicrobial and antioxidant efficiency of nanoemulsion-based edible coating containing ginger (*Zingiber officinale*) essential oil and its effect on safety and quality attributes of chicken breast fillets. *Food Control*. 2018;84:312-320.
30. Li Y., Zhang Z., Yuan Q., Liang H. and Vriesekoop F. Process optimization and stability of D-limonene nanoemulsions prepared by catastrophic phase inversion method. *Journal of Food Engineering*. 2013;119:419–424.
31. Mayer S., Weiss J. and McClements D. J. Vitamin E-enriched nanoemulsions formed by emulsion phase inversion: Factors influencing droplet size and stability. *Journal of Colloid and Interface Science*. 2013;402:122 - 130.
32. Sharma A., Sharma N. K., Srivastava A., Kataria A., Dubey S., Sharma S. and Kundu B. Clove and lemongrass oil-based non-ionic nanoemulsion for suppressing the growth of plant pathogenic *Fusarium oxysporum* f.sp. *lycopersici*. *Industrial Crops & Products*. 2018;123:353–362.
33. Jimenez M., Domínguez J. A., Pascual-Pineda L. A., Azuara E. and Beristain C. I. Elaboration and characterization of O/W cinnamon (*Cinnamomum zeylanicum*) and black pepper (*Piper nigrum*) emulsions. *Food Hydrocolloids*. 2018;77:902-910.
34. Swathy J., Mishra P., Thomas J., Mukherjee A. and Chandrasekaran N. Antimicrobial potency of high-energy emulsified black pepper oil nanoemulsion against aquaculture pathogen. *Aquaculture*. 2018;491:210–220.