Antibacterial Effect of Black Pepper Petroleum Ether Extract against *Listeria monocytogenes* and *Salmonella typhimurium*

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The aim of the present study was to evaluate the antibacterial effect of black pepper petroleum ether extract (BPPE) against *Listeria monocytogenes* ATCC 19115 and *Salmonella typhimurium* ATCC 14028. The results showed that the BPPE had a strong antimicrobial activity against *L. monocytogenes* and *S. typhimurium*, and 2-methylene-4,8,8-trimethyl-4-vinyl-bicyclo[5.2.0]nonane (9.36%) and caryophyllene oxide (4.85%) were identified as the two primary components of BPPE. The ability of cells to break down hyperoxide was decreased, and the activities of POD and CAT were inhibited. The activities of key metabolic enzymes shed some light on the biochemical mechanism of aglycon cell growth inhibition, indicating that the energetic metabolism of *L. monocytogenes* and *S. typhimurium* was markedly influenced by the BPPE. The contents of key organic acids varied significantly, resulting in remarkable abnormalities in the energetic metabolism of *L. monocytogenes* and *S. typhimurium*. Thus, the consecution of energetic metabolism was destroyed by the BPPE, which contributed to metabolic dysfunction, the suppression of gene transcription, and cell death.

1. Introduction

With the development of food processing techniques, various chemical preservatives have been added to our food for daily sustenance [1]. However, food safety remains a very important public health issue, especially for the application of food preservatives in food processes. Therefore, a new methodology for reducing or eliminating food-borne pathogens has become a pressing issue [2].

Antibiotics, since their introduction over 60 years ago, quickly became the main strategy for controlling bacterial infections in clinical medicine. However, the increase in antibiotic use led to increased bacterial resistance, which is encouraging the search for new active compounds against pathogens. Researchers found that some natural products from plant sources (black pepper, ginger, garlic, onion, etc.) [3–5], animal sources (nucleoprotamine, antibacterial peptides, and chitosan) [6, 7], and microbial sources (kojic acid, ε-poly-L-lysine, and nisin) have significant effects on food-borne pathogens [8–11]. All of these natural products showed little influence on the health of the human body.

Since ancient times, black pepper has been commonly used as a spice in cooking. Moreover, this spice is highly valued as a folk medicine because of its antibacterial properties and other physiological benefits, particularly in treating pain, the flu, muscle aches, and rheumatism [12]. Recent studies have shown that black pepper extract can inhibit food spoilage and food pathogens [13–16]. The structure, preservatives, and antibacterial effect of the pepper extracts were reported. We previously explored the optimum...
extraction process as well as the inhibition and minimal inhibitory concentration (MIC) of the black pepper petroleum ether extract (BPPE) on L. monocytogenes and S. typhimurium. However, the antibacterial mechanisms contributing to the inhibition of these strains remain unclear.

Hence, the objective of this study was to investigate the inhibitory effect of the BPPE on Listeria monocytogenes and Salmonella typhimurium by determining the activities of key metabolic enzymes, the contents of organic acids involved in aerobic respiratory metabolism, and their possible lysis activity following antioxidant enzyme release in the broth medium.

2. Materials and Methods

2.1. Materials and Chemicals. The black pepper (Piper nigrum L., confirmed by Prof. Weimin Zhang, Hainan University) used in this study was purchased from Nanguo Supermarket (Haikou, China), grown in Wanning, Hainan Province, China, and picked in June 2017 (nine months after flowering). Black pepper was purchased from the Nanguo Supermarket (Haikou, China). The malate dehydrogenase (MDH) assay kit, succinate dehydrogenase (SDH) assay kit, and hydrogen peroxidase (POD) assay kit, and hydrogen peroxidase (CAT) assay kit were acquired from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). KH2PO4 and nicotinamide adenine dinucleotide (NADP+) were purchased from Amresco (Solon, OH, USA). Brain heart infusion (BHI) broth was purchased from the Guangdong HuanKai Microbial Tech Co., Ltd., and nutrient broth (NB) was acquired from the Beijing Solarbio Science & Technology Co., Ltd. All other chemicals were of analytical grade. BPPE was prepared according to our previous report (80% ethanol, 50°C, and 12 h) [17].

2.2. Bacterial Strains. Salmonella typhimurium (ATCC 14028) and Listeria monocytogenes (ATCC 19115) were purchased from Guangdong Microbiology Culture Center (Guangzhou, China). L. monocytogenes was grown and maintained on slants of brain-heart infusion agar (HuanKai Microbial Sci. & Tech. Co., Ltd., Guangdong, China). S. typhimurium and L. monocytogenes were cultured at 37°C for 24 h in the master slant culture tubes. The bacteria were washed twice with sterile NaCl water (0.9%, w/w) and then resuspended in sterilized saline water at a concentration of 1-2 × 10⁸ CFU/mL as measured by the Maxwell turbidimetry. One milliliter of S. typhimurium or L. monocytogenes was added into sterile Erlenmeyer flasks with 49 mL of fresh liquid culture bath. The BPPE was dissolved in ethanol (80%, v/v) and then added into the treated group to yield the minimum inhibitory concentration (MIC), and the MIC tests have been conducted previously [18]. The negative control group had the same volume of ethanol but without BPPE, and the positive control group had no ethanol and BPPE. All of the groups were agitated at 130 rpm in an environmental incubator shaker at 37°C.

2.3. Time-Kill Curve. The antimicrobial activity of BPPE was determined in triplicate using a plate colony-counting method [19]. In brief, Salmonella typhimurium and Listeria monocytogenes were cultured with shaking at 37°C for 24 h, washed twice with sterile NaCl water (0.9%, w/w), and resuspended. Subsequently, the BPPE was dissolved in ethanol and added to two test tubes containing Salmonella typhimurium and Listeria monocytogenes to make the concentration of the antibacterial 1.25 mg/mL and 0.625 mg/mL, respectively. Then, the antibacterial effects of the BPPE on the two bacteria at 0, 0.5, 1, 2, 4, 6, and 8 h were observed. Ten milliliters of the suspension were serial diluted by sterile NaCl (0.9%, w/w), coated, and cultured at 37 °C for 24 h. Finally, the bacterial population was counted.

2.4. Determination of the Antioxidant Enzyme Assay. Peroxidase reduces hydrogen peroxide and oxidizes a wide number of compounds including thiocyanate ions and fatty acids [20]. Peroxidase plays a role in the extracellular defense against stress, biosynthesis and degradation of lignin, intracellular removal of hydrogen peroxide, and oxidation of toxic reductants [21]. The effects of the BPPE on the activities of POD and CAT were determined at different concentrations. Concentrations of 1/4 MIC, 1/2 MIC, MIC, 2 MIC, and 4 MIC of the BPPE were added to the bacterial suspension (1.0 × 10⁹ cfu/mL), and the mixture was cultured at 37°C in an incubator shaker for 24 h. Then, 10 mL of the suspension was broken by an ultrasonic liquid processor (UP200S, Hielscher Ultrasonics GmbH, Teltow, Germany) in an ice bath for 6 min (550 W, working for 10 s with an interval of 5 s). The cell debris was removed, and the supernatants were collected by centrifugation at 5600 g for 12 min at 4°C. The activities of POD and CAT were explored by a POD assay kit and CAT assay kit, respectively. The content of the protein was determined by the Bradford method at 595 nm, and the standard curve was made using bovine serum albumin [22].

2.5. Dehydrogenase Activity Measurements and Total Protein Content. The bacteria strains grown in liquid nutrient medium were agitated at 146 g in an environmental incubator shaker at 37°C overnight, and the concentration was diluted to approximately 1-2 × 10⁷ CFU/mL with sterile nutrient broth. Then, the bacteria were cultured with shaking at 37°C for 24 h, washed twice with sterile phosphate buffered saline (PBS, pH = 7.4), and resuspended in 5 mL of sterile PBS. The bacterial suspensions were treated by an ultrasonic liquid processor in an ice bath for 6 min (550 W, working for 10 s with an interval of 5 s), and the supernatants were collected by centrifugation at 10,000 g for 15 min at 4°C, followed by storage in an ice bath [17].

The activities of malate dehydrogenase (MDH), succinate dehydrogenase (SDH), and kinase pyruvate (PK) were determined by the operation of an assay kit according to the manufacturer’s instructions [23, 24].
2.6. Pyruvic Acid Determination. The content of pyruvic acid was determined according to the method of Spoel and Dong [25]. At the designated time intervals, 5 mL of bacterial suspension was centrifuged at 6000 g for 15 min, and the supernatant was collected and stored at 4°C. The 2,4-dinitrophenol (1 mL), trichloroacetic acid (0.3 mL, 8.0%), and the supernatant (0.1 mL) were transferred to a test tube; the mixture was placed in a water bath at 37°C for 10 min; and then 10 mL of sodium hydroxide (0.4 mol/L) was added. The absorbance of the mixture was read at 520 nm, and the content of pyruvic acid was calculated using a pyruvic acid calibration curve.

2.7. Content of ATP. Adenosine 5'-triphosphate is the basic carrier of energy in organisms. The variation in the ATP content is directly related to the energy metabolism of organisms [26]. The content of ATP was determined by an ATP assay kit. Ten milliliters of culture suspension were washed and broken by ultrasonic treatment in the manner described above. Then, 30 µL of the supernatant was used to determine the content of ATP according to the instructions of the assay kit.

2.8. GC-MS Analysis. The chemical composition of BPCE was determined by gas chromatography-mass spectrometry (GCMS). GC-MS experiments were performed on an Agilent Technologies 7890A gas chromatograph (Santa Clara, CA) and an Agilent 7683B autoinjector coupled with a 240 Agilent Ion Trap mass spectrometer (MS/MS). The mass spectral scan rate was 2.86 scans/s. The GC was operated at a helium (ultrahigh purity) flow rate of 0.7 mL/min under a head pressure of 10 psi, and the injection volume was 1 µL. The MS was operated in the electron ionization (EI) mode using an ionization voltage of 70 eV and a source temperature of 230°C. The scan type used was the automated method development function (AMD), and the optimum MS/MS excitation amplitude was 1.20 V. Relative percentages of the primary components were calculated by integrating the registered peaks.

2.9. Statistical Analysis. The data of enzyme activity and bacteria count were statistically analyzed using IBM SPSS statistical software (version 21.0, SPSS Inc., Chicago, IL, USA). The differences between means were assessed by analysis of variance (ANOVA) with Duncan’s test using a significance level of $p < 0.05$.

3. Results and Discussion

3.1. Time-Kill Curve. The bacteriostatic activities of the BPPE against *Listeria monocytogenes* and *Salmonella typhimurium* are shown in Figure 1. The BPPE is particularly active against the Gram-positive bacteria *L. monocytogenes* ATCC 19115 and the Gram-negative bacteria *S. typhimurium* ATCC 14028. As shown in Figure 1, the BPPE exhibited good antimicrobial activity for *L. monocytogenes* and *S. typhimurium*. Almost 99.99% and 99.90% reductions in the populations were observed in *L. monocytogenes* and *S. typhimurium* after 8 h of the BPPE treatment.

The results showed that the BPPE at the MIC exerted strong bactericidal activity, as indicated by the significant reduction in microbial counts, which indicated that the BPPE has a perfect antibacterial activity against *L. monocytogenes* and *S. typhimurium*. Therefore, the BPPE can be regarded as a natural and efficient antiseptic of pathogens. Similar to our findings, the major components and essential oils of black and red pepper showed good antimicrobial activity for *Escherichia coli* O157:H7 and *Staphylococcus aureus* [27, 28].

3.2. The Antibacterial Effect on the Peroxidase Enzyme. The analysis of key enzymes provided further interesting information on the antimicrobial activity of the BPPE. As shown in Figure 2, the activities of POD and CAT were remarkably influenced by the BPPE. The activities of the enzymes decreased with increases of the BPPE concentration. In particular, POD of *L. monocytogenes* and *S. typhimurium* showed 80–85% activity inhibition in the presence of 4 MIC of the BPPE. The BPPE inhibits *L. monocytogenes* and *S. typhimurium* CAT activity by decreasing it to 70–50%, making cells more susceptible to oxidative injuries. The mechanisms that enabled microorganisms to survive after the release of POD and CAT include the degradation of *H₂O₂* and the inhibition of the ROS-mediated cell death. The BPPE inhibits the activities of POD and CAT, thereby making bacterial cells more susceptible to oxidative injury and reducing their capability to eliminate *H₂O₂* [29].

3.3. Influence of the BPPE for EMP. Pyruvate kinase (PK) could promote the production of pyruvate from glycolysis and is helpful with regard to energy metabolism. Additionally, pyruvic acid is the raw material to produce acetyl-CoA, which is the initial reactant in the Krebs cycle, one of the most important metabolic pathways for the generation of energy and metabolites in living organisms [30]. Pyruvic acid, an important intermediate metabolite, is associated with many metabolic pathways in microorganism. For example, pyruvic acid connects EMP, TCA, and HMP. If pyruvic acid is accumulated, normal physiological metabolism would be inhibited, especially the TCA pathway of bacteria [31].

The activity of the tested bacteria’s dehydrogenase enzyme was markedly influenced by the presence of the BPPE. In detail, the activity of PK for the *L. monocytogenes* has been significantly decreased by the addition of the BPPE for 6 h before the test. Additionally, the activity of PK for *L. monocytogenes* was chaotic compared with that of PK for the control group. The content of pyruvic acid can be controlled by the activity of PK. The activity of PK for *S. typhimurium* was remarkably inhibited by the presence of the BPPE, which resulted in the content of pyruvic acid decreasing. Thus, the tricarboxylic acid cycle was inhibited when there is BPPE in the culture.

The content of pyruvic acid in the culture solution is shown in Figure 3. Throughout the entire incubation process...
of *L. monocytogenes*, the pyruvic acid contents in the control groups slightly decreased within 0–6 h, and then they increased from 0.1193 g/L to the final concentration of 0.1602 g/L (Figure 4). However, the concentration of pyruvic acid significantly increased during the incubation process in the culture, and the final concentration value was 0.1795 g/L.

**Figure 1:** The time-kill curves of *L. monocytogenes* (L) and *S. typhimurium* (S) in the presence of the BPPE. The differences were analyzed by one-way ANOVA. The asterisks (**) indicate a significant difference compared with the control at *p* < 0.05.

**Figure 2:** The effect of the BPPE on the activities of POD and CAT for *L. monocytogenes* (L) and *S. typhimurium* (S). The letters (a, b, c, d, e, f, and g) indicate a significant difference at *p* < 0.05.
The value of the treated group for *S. typhimurium* increased within 6–24 h, and the value was above that of the control group during the incubation process. After 24 h, the concentration of pyruvic acid in the treated group was 0.0931 g/L, whereas those in the control group and alcohol group were 0.0747 g/L and 0.0763 g/L, respectively.

3.4. Dehydrogenase of TCA. MDH and SDH are the catalyzing enzymes in the Krebs cycle, which is conducive to providing ATP. The SDH could catalyze succinic acid to fumaric acid, and the MDH catalyzes malic acid to oxaloacetate [32]. Thus, the succinic acid reaction and malic acid reaction were the important points for producing energy by the Krebs cycle. As is shown in Figure 3, there were significant differences in the activities of the test enzymes between the BPPE and blank groups.

As is shown in the picture, the activity of SDH in the BPPE was higher than that of the control group; however, the MDH was inhibited for *L. monocytogenes* by the BPPE. The inhibition of MDH indicates a decrease in its capability to utilize malic acid to restore NAD⁰⁺, which is essential for the reaction of glyceraldehyde 3-phosphate dehydrogenase during glycolysis.

In particular, SDH showed a 100–300% activity increase in the presence of 1.25 mg/mL and 0.625 mg/mL BPPE for *L. monocytogenes* and *S. typhimurium*. The promotion of the key enzyme in the aerobic energetic metabolism of the bacteria, which exists in the mitochondrial membrane, indicates an increase in its capability to utilize succinic acid to restore FADH₂, an essential reaction for biological oxidation. The BPPE also influences the activity of another fundamental enzyme for energy metabolism, malate dehydrogenase (MDH). This enzyme is also the key regulation
point of the Krebs cycle, one of the most important metabolic pathways for the generation of energy and metabolites in living organisms. In particular, MDH showed remarkable inhibition in the presence of the BPPE for \textit{L. monocytogenes}, indicating that malic acid was accumulated, the content of oxaloacetate was decreased, and NAD$^+$ accepting H$^+$ to form NADH was blocked. However, the activity of MDH increased at 12 h before, and then the activity of MDH was inhibited by the presence of the BPPE. These results were consistent with the findings of Hu et al. [33].

3.5. ATP. The ATP levels of \textit{L. monocytogenes} and \textit{S. typhimurium} are shown in Figure 5. In the control group, the ATP level was increased in first 6 h and 3 h for \textit{L. monocytogenes} and \textit{S. typhimurium}, respectively, which may be due to growth and reproduction. After 24 h, the concentration of ATP in the treated group was significantly lower than that in the control group, which could be due to that the ATP was rapidly degraded when the cells died. This result indicated that the BPPE could change the respiratory metabolism of the two bacteria. The biological function of ATP synthase is dependent on the membrane potential [34]. Therefore, we could further investigate the effects of the BPPE on the cytoplasmic membrane potential of \textit{L. monocytogenes} and \textit{S. typhimurium} [7], in which ATP production had been inhibited.

3.6. GC-MS Analysis of the Chemical Composition of BPPE. The GC-MS spectrum of BPPE is presented in Figure 6, in which 125 chemical constituents were identified. And the 30
primary substances are presented in Table 1. The results revealed that 2-methylene-4,8,8-trimethyl-4-vinyl-bicyclo [5.2.0] nonane (9.36%) and caryophyllene oxide (4.85%) were the two primary components of BPPE. In addition, BPPE was rich in decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha., 4a.alpha., 7a.beta., 7b.beta., 7b.alpha.)]-1H-cycloprop[e]azulen-7-ol (4.30%), piperonal (2.18%), 4,4-dimethyl- tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol (3.54%), n-hexadecanoic acid (3.70%), 6-octadecenoic acid (3.06%), (Z,Z)-9,12-octadecadienoic acid (2.46%), 3-octadecenoic acid (2.21%), trans-2-octadecenoic acid (2.67%), and longipinocarvone (2.29%). The volatile oil (12.00% of 2-methylene-4,8,8-trimethyl-4-vinyl bicyclo[5.2.0]nonane) obtained from Fusarium tricinctum showed excellent antimicrobial activity against eight bacteria and two fungi. Piperonal is a naturally occurring aromatic aldehyde, a secondary metabolite produced by higher plants (especially species in pepper genus), and well known as a volatile compound frequently used in perfumes, cosmetics, and flavoring agents. Piperonal showed significant inhibition effect on the bacterial growth [35]. The antimicrobial properties of caryophyllene oxide have also been confirmed in many reports [36–38]. In addition, those compounds such as decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1a.alpha., 4a.alpha., 7a.beta., 7a.beta., 7b.alpha.)]-1H-cycloprop[e]azulen-7-ol [39], 4,4-dimethyl- tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol [40], and 6-octadecenoic acid [41] have potential antimicrobial activities.

In addition, natural products and naturally derived compounds from black pepper may have applications in controlling pathogens in foods. The challenge is to isolate, purify, stabilize, and incorporate natural antimicrobials into foods without adversely affecting sensory, nutritional, and safety characteristics [42]. And the components analysis of GC-MS and the MIC could ensure the food security of BPPE, whereas the impact of BPPE on organoleptic properties of food requires further studies.

4. Conclusion

This study describes the antimicrobial effect of organic compounds in black pepper on the energy metabolism of
L. monocytogenes and S. typhimurium. The BPPE is particularly active against L. monocytogenes and S. typhimurium. First, the BPPE could rapidly destroy the permeability of the cell wall and membrane, and this phenomenon resulted in the substantial rapid loss of cell contents and freedom from the separation of macromolecular substances from intracellular substances. The POD and CAT were markedly influenced by the BPPE with the increase of its concentration. The active ingredients of the BPPE entered the cells and interacted with key enzymes in the glycolytic pathway, eventually hindering and disordering cell metabolism. Furthermore, the accumulation of pyruvic acid indicated that the ability to expend reducing sugars decreased and the ability of cells to produce energy was reduced. The activity of MDH was remarkably influenced by the BPPE, and this proved that the additive could influence cellular respiration by disrupting the TCA pathway, which could result in abnormal physiological metabolism in the cell. The reduction of ATP proved that the BPPE could destroy bacterial respiratory metabolism and lead to free access to intracellular material, which could result in cell death. The result of GC-MS revealed that 2-methylene-4,8,8-trimethyl-4-vinyl-bicyclo[5.2.0]nonane (9.36%) and caryophyllene oxide (4.85%) were the two primary potential antimicrobial components of BPPE. Overall, the current investigation would facilitate the development of antibacterial agents targeting energy metabolism. This experimental result provides an approach for developing convenient and efficient antimicrobial agents in the food and pharmaceutical industries, which is of great significance to food security.

Table 1: The chemical components of BPPE.

| No. | Name of the compound                                                                 | RT (min) | Area% |
|-----|--------------------------------------------------------------------------------------|----------|-------|
| 1   | 2-Methylene-4,8,8-trimethyl-4-vinyl-bicyclo[5.2.0]nonane                             | 11.623   | 9.36  |
| 2   | 4-Ethenyl-alpha.,alpha.,alpha.,4-trimethyl-3-(1-methylethenyl)-, [1R-(Lalphal.,3alpha.,4beta.)]-cyclohexanemethanol | 12.996   | 1.86  |
| 3   | Decahydro-4a-methyl-1-methylene-7-(1-methylethenyl)-, [4aR-(4aalpha.,7alpha.,8alpha.beta.)]-naphthalene | 13.971   | 1.23  |
| 4   | Decahydro-4a-methyl-1-methylene-7-(1-methylhydridene)-, (4aR-trans)-naphthalene       | 14.052   | 0.99  |
| 5   | Caryophyllene oxide                                                                  | 19.011   | 4.85  |
| 6   | Longipinocarvone                                                                     | 20.059   | 2.29  |
| 7   | 1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octan-6-ol                                        | 22.485   | 0.80  |
| 8   | Piperonal                                                                            | 23.192   | 2.18  |
| 9   | Decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aalpha.,4alpha.,7beta.,7beta.,7balpha.)]-1H-cyclopentylazulen-7-ol | 23.576   | 4.30  |
| 10  | 4,4-Dimethyl-tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol                            | 24.261   | 3.54  |
| 11  | cis-Bicyclo[4.4.0]decan-1-ol-3-one                                                   | 24.871   | 0.85  |
| 12  | Alloaromadendrene oxide-(1)                                                          | 25.476   | 1.99  |
| 13  | Ethyl 9cis.,11trans-octadecadienoate                                                 | 27.855   | 1.21  |
| 14  | Behenic alcohol                                                                      | 28.605   | 1.02  |
| 15  | Phytole                                                                              | 29.019   | 1.33  |
| 16  | Dibutylyl phthalate                                                                  | 30.277   | 1.16  |
| 17  | Decahydro-1,1,7-trimethyl-4-methylene-, [1ar-(1aalpha.,4alpha.,7beta.,7beta.,7balpha.)]-1H-cyclopentylazulen-7-ol | 30.592   | 1.08  |
| 18  | Bendiocarb                                                                           | 31.856   | 0.79  |
| 19  | n-Hexadecanoic acid                                                                  | 33.032   | 3.70  |
| 20  | 1-(1-(2-Thienyl)cyclohexyl)pyrrolidine                                               | 33.943   | 1.85  |
| 21  | (8R,Z)-8-Methyl-6-((R)-2-methylpentylidene)                                          | 34.535   | 1.40  |
| 22  | octahydroindolizine                                                                  | 34.656   | 1.68  |
| 23  | 2-Ethyl-5-undecyl.-delta.1-pyrrole                                                  | 36.081   | 3.06  |
| 24  | 6-Octadecenoic acid                                                                  | 36.743   | 2.46  |
| 25  | (Z,Z)-9,12-Octadeicenoic acid                                                        | 39.283   | 1.47  |
| 26  | Pyrrolidine 4-hexadecenoic acid                                                      | 39.777   | 1.26  |
| 27  | cis-13-Octadecenoic acid                                                             | 40.183   | 1.37  |
| 28  | 3-Octadecenoic acid                                                                  | 41.818   | 2.21  |
| 29  | trans-2-Octadecenoic acid                                                            | 42.222   | 2.67  |
| 30  | 3-[9-Tridecenyl]-4,5,6,7-tetrahydrobenz[d]isoxazole-5-ol-4-one                       | 42.673   | 1.53  |
Data Availability
The data used to support the findings of this study are included within the article.

Disclosure
The authors alone are responsible for the content and writing of the paper.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Authors’ Contributions
Wenxue Chen and Haiming Chen conceived and designed the experiments; Hui Tang performed the experiments; Yueying Hu, Qiuping Zhong, and Weijun Chen analyzed the data; Wenxue Chen and Ningxin Jiang wrote the original and revised manuscript.

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