

Extraction of Essential Oil from *Nigella sativa* Using Supercritical Carbon Dioxide: Study of Antibacterial Activity

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Abstract: The antimicrobial activity of *N. sativa* essential oil obtained by supercritical fluid extraction by carbon dioxide was investigated against Gram Positive and Gram negative strains, isolated from clinical specimens. Best conditions for Black cumin oil extraction are obtained at 400 bar, 40°C and a solvent flow rate of 25 g min⁻¹. The seed extracts were prepared by supercritical fluid extraction method. Filter paper discs impregnated with varying concentrations of *N. sativa* extract were tested by the disk diffusion method. Methicillin Resistant *Staphylococcus Aureus* (MRSA) ATCC strain (700968), *E. coli* ATCC strain (25922), *E. coli* 0157 ATCC strain (12799), Extended-Spectrum Beta-Lactamase (ESBL) *Klebsiella pneumoniae* ATCC strain (700603), Carbapenam Resistant *acinetobacter Baumannii* (CRAB) clinical strain and *Vibrio cholerae* 01 Ogawa and 0139 Bengal clinical strains were investigated. The inhibition zones of the Mueller Hinton agar in different extract concentrate ion showed that at 25 mg 20 µL⁻¹, 50 mg 20 µL⁻¹ and 100 mg 20 µL⁻¹, the inhibition zones increased accordingly in *S. aureu*. However, *N. sativa* was found to be inactive against ESBL producers (*E. coli* and *K. pneumoniae*).

Key word: Supercritical fluid extraction, nigella sativa, antimicrobial activity, gram positive and gram negative bacteria, university putra Malaysia, Yemen

INTRODUCTION

The extraction of essential oil components using solvent at high pressure, or Super-Critical Fluids (SCF), has received much attention in the past several years, especially in food, pharmaceutical and cosmetic industries, because it presents an alternative to conventional processes such as organic solvent extraction and steam distillation^[5-8]. Supercritical fluid extraction allows a continuous modification of solvent power and selectivity by changing the solvent density^[4,12,14]. Black cumin is an economically important umbel lifer growing wild in the dry temperature regions of Jammu-Kashmir, Himachal Pradesh, Afghanistan, Baluchestan and Iran. The seeds, rich in essential oil, are consumed widely as condiment. In the indigenous system of medicines, seeds are regarded as stimulants and carminatives and found to be useful in diarrhoea and dyspepsia^[1]. Also, this plant is used for culinary purposes and for flavouring foods and beverages. It should be noted that the name of black cumin is sometimes given to entirely unrelated spice

Nigella sativa. The crude extracts of *N. Sativa* were reported to have a promising effect on multi-drug resistant *S. aureus* and *Candida albicans*^[10], *Shigella* spp., *Vibrio cholerae*^[15]. Production of β lactamases is the most common mechanism of resistance among the Gram-negative. The vast majority of strains expressing these enzymes belong to the family *Enterobacteriaceae* like *K. pneumonia* and some *E. coli* strains, β-lactam antibiotics are the most common treatment for bacterial infections. Extended-Spectrum β Lactamases (ESBL) have become a widespread serious problem and several aspects of them are worrying. The potential use of alternative antibiotics in drug-resistant bacteria from various plant extracts have been studied by many researches. To document the antibacterial effects of *N. sativa* both gram positive and gram negative bacteria were tested.

MATERIALS AND METHODS

***Nigella sativa* seeds:** The seeds were purchased from a local herbal shop in Yemen. Seeds were stored in dark

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at 4°C for 20 days. Immediately prior to the extraction process, the seeds were ground in a blender to produce a powder with an approximate size of 150 g.

Supercritical Fluid Extraction (SFE): A Suprex MPS/225 system (Thar, Germany) at the Food science and biotechnology Laboratory UPM in the SFE mode was used for the extraction. In this study, extraction was performed by filling extraction vessel with 150.0 g of the completely crashed seeds. The plant was then extracted with SFE under 400 atm pressure and 40°C temperature for 15 min static followed by 20 min dynamic. A Duraflow manual variable restrictor (Suprex) was used in the SFE system to collect the extracted analytes. The SFE flow rate through the Duraflow restrictor was approximately 25 g min⁻¹ (compressed). The extracted analytes were collected in a volumetric flask. The final volume of the extract was adjusted to 35 mL at the end of the extraction. In order to have better collection efficiency, the 35 mL volumetric flask was placed in an ice bath during the dynamic extraction stage.

Antimicrobial Susceptibility Testing (AST) by NCCLS and concentrations of extracts: Extracts were diluted two-fold and used at a concentration starting from 100% to an end dilution of 6.25% where 25 mg mL⁻¹ in 10% Dimethyl Sulfoxide (DMSO) were prepared using vortex mixture. Whatman AA paper discs were injected with 20 µL of different *N. sativa* extracts using a micropipette and were dried in the biosafety cabinet for 30 min. Negative control disc were prepared using a 10% DMSO. All the bacteria were cultured on Nutrient Agar (NA) (Merck). Inoculum were prepared in 5 mL Mueller Hinton broth with 3-5 colonies of each bacterial strain. The inoculum were incubated at 35°C for 2-3 h to get an approximately close to 0.5 McFarland standard for susceptibility testing^[13]. Two methods were used- 1) Disc diffusion method: Sterile paper discs impregnated with each extract were placed aseptically over the bacterial cultures and incubated at 37°C for 24 h. 2) Well diffusion method: wells were made on inoculated agar and each well filled with 25 µL of extract. Each inoculated plate was incubated with a positive-control antibiotic disc for each of the bacteria.

Bacterial strains: The bacterial strain were used Methicillin Resistant *Staphylococcus Aureus* (MRSA) ATCC strain 700968, *E. coli* ATCC strain 25922, *E. coli* 0157 ATCC strain 12799, Extended-Spectrum Beta-Lactamase (ESBL) *Klebsiella pneumoniae* ATCC strain 700603, carbapenam Resistant *Acenitobacter Baumannii* (CRAB) clinical strain and

Vibrio cholerae 01 Ogawa and 0139 Bengal clinical strains. Purchased from (Biosyntech Malaysia) and kept at the Medical Microbiology Laboratory, University Putra Malaysia (UPM).

RESULTS

All the bacteria tested as shown in the Table 1, only Methicillin Resistant *Staphylococcus Aureus* (MRSA) showed high susceptibility towards the extract the inhibition zones increased accordingly in *S. aureus*. However, *N. sativa* was found to be inactive against ESBL producers (*E. coli* and *K. pneumoniae*). For the disc diffusion method, MRSA showed large zones of inhibition for the first three dilutions (100, 50 and 25% concentration) and showed resistance for the last two dilutions. For the well diffusion method, all wells produced large zones of inhibition and the inoculated plate itself produced little or almost no growth of MRSA (Fig. 1 a-d). *V. cholerae* plates were tested using

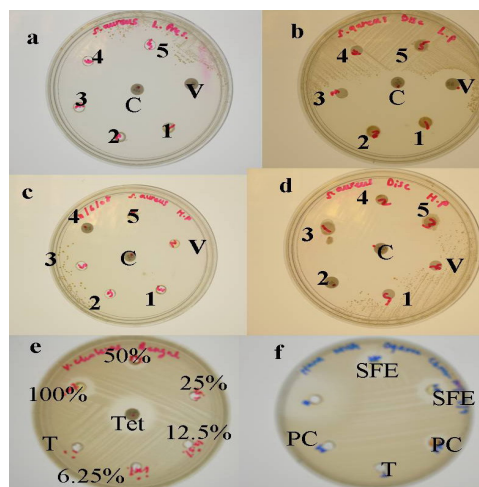


Fig. 1: Susceptibility of MRSA towards extracts- Concentrations 1- 100, 2- 50, 3- 25, 4- 12.5 and 5- 6.25%; V- Vancomycin, C- Cefoxitin: (a): Well diffusion of SCF extract; (b): Disc diffusion of oil extract; (c): Well diffusion of SFE extract; (d): Disc diffusion of SFE extract. (e and f): Susceptibility of *V. cholerae* towards extracts: (e): Well diffusion with different concentrations of extract on *V.cholerae* 0139 (Bengal)-100, 50, 25, 12.5 and 6.25% concentration, Tet-tetracycline, T- Tryptic soy broth (as solvent negative control); Inhibition zone diameter at 100% extract concentration is 13mM; at 50% concentration is 9 mM (f): Well diffusion of 100% (PC pure control of DMSO) extracts on *V. cholerae* 01 (Ogawa), T- Tryptic soy broth

Table 1: Commercial antibiotic discs were included as controls

Bacteria	Antibiotic control	SFE extract
MRSA	Cefoxitin/ Vancomycin	Sensitive
<i>E. coli</i> 25922	Gentamycin	Resistant
<i>E. coli</i> 0157:H7	Gentamycin	Resistant
<i>E. coli</i> 12799	Gentamycin	Resistant
ESBL <i>K. pneumoniae</i>	Cefotaxime/ Ceftazidime	Resistant
CRAB	Imipenam/ Carbapenam	Resistant
<i>V. cholerae</i> Bengal	Tetracycline	Small inhibition zone

only the well diffusion method, where *V. cholerae* 0139 Bengal was tested with different extract concentrations (100, 50, 25, 12.5 and 6.25%) producing small but clear zones of inhibition at 100 and 50% extract. For *V. cholerae* Ogawa strain, the extracts used were SFE extract and methanol extracts, producing also small but clear inhibition zones (Fig. 1e and f).

DISCUSSION

Supercritical extraction with carbon dioxide is a feasible technique for the isolation of active substances from *N. sativa*. The supercritical extract is characterized by higher concentrations of the main extracted aromatic compounds thymoquinone. The SFE extract showed stronger antimicrobial activities against the tested bacteria *S. aureus* strains. The SFE extract had a very strong antimicrobial activity. This confirms the study of Agarwal *et al.*^[2] who reported that the oil inhibited one strain of *S. aureus* even up to 1:100 dilutions, the least concentration tested. However, the zones of inhibition observed in our study were larger which may be due to difference of the strains tested. The oil was found to be more effective on Gram positive than Gram negative bacteria, which is in conformity of a number of earlier studies compounds derived from plants often show considerable activity against Gram positive bacteria but not against Gram negative species. Gram-negative bacteria have an effective permeability barrier, comprised of the outer membrane, which restricts the penetration of amphipathic compounds and multidrug resistance pumps that extrude toxins across this barrier. It is possible that the apparent ineffectiveness of plant antimicrobials is largely due to the permeability barrier. Results of the study indicate that black seed oil possesses significant antimicrobial activity against Methicillin resistant *Staphylococcus aureus* (MRSA) and experiments evaluating the antibacterial properties of black seed oil in suitable foods are underway in our laboratory. Super-Critical Fluids (SCFs) are increasingly replacing organic solvents, e.g., n-hexane, dichloromethane, chloroform and so on, that is

conventionally used in industrial extraction, purification and recrystallization operations because of regulatory and environmental pressures on hydrocarbon and ozone-depleting emissions. In natural product extraction and isolation, Supercritical Fluid Extraction (SFE), especially that employing SFE, has become the method of choice. Sophisticated modern technologies allow precise regulation of changes in temperature and pressure and thus manipulation of solvating property of the SCF, which helps the extraction of natural products of a wide range of polarities. According to these advantages of supercritical fluid extraction we recommended to use as clean tool for essential oil extraction.

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