Analysis and Antibacterial Activity of *Nigella sativa* Essential Oil Formulated in Microemulsion System

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Abstract: The Essential oil (EO) of *Nigella sativa* (black cumin) was extracted from the crude oil and the volatile constituents were characterized using gas chromatographic analysis. The EO was formulated in water-based microemulsion system and its antibacterial activity against six pathogenic bacteria was evaluated using the agar well diffusion method. This activity was compared with two other well known biologically active natural and synthetic antimicrobials namely eugenol and Ceftriaxone®. Results showed that *N. sativa* EO microemulsion was highly effective against *S. aureus, B. cereus* and *S. typhimurium* even at the lowest tested concentration of that EO in the microemulsion (100.0 μg/well). Interestingly, the EO microemulsion showed higher antibacterial activity than Ceftriaxone solution against *S. typhimurium* at 400.0 μg/well and almost comparable activity against *E. coli* at 500.0 μg/well. No activity was detected for the EO microemulsion against *L. monocytogenes* and *P. aeruginosa*. Eugenol which was also formulated in microemulsion was less effective than *N. sativa* EO microemulsion except against *P. aeruginosa*. The synthetic antibiotic (Ceftriaxone) was effective against most of the six tested bacterial strains. This work is the first report revealing the formulation of *N. sativa* EO in microemulsion system and investigating its antibacterial activity. The results may offer potential application of that water-based microemulsion in controlling the prevalence of some pathogenic bacteria.

Key words: antibacterial activity, *Nigella sativa*, essential oil, eugenol, microemulsion, Ceftriaxone

1 INTRODUCTION

Essential oils (EOs) are natural mixtures of volatile compounds secreted as by-products by many aromatic and medicinal plants. These oils act as insect attracting agents for pollination and also as a defense line against microbial attack. EOs were first used as perfuming and flavoring agents then their inherent antimicrobial activity was discovered. Different investigations on the application of EOs against pathogenic bacteria, food spoilage and film forming microorganisms have been reported[^1]-[^4]. The antimicrobial properties of EOs against pathogens were studied in different sanitizing applications including sanitization of wounds[^5] and surfaces[^6], disinfection of animal houses[^7] and sewage grey water[^8]. The promising results of these investigations along with many others warranted EOs for consideration as potential candidates for application as adjuvant for the synthetic antibiotics. EOs can enhance the therapeutic efficiency of these antibiotics through a synergistic mechanism[^9,10]. Combination of antibiotics with some EOs can also reduce the well known antibiotic resistance in multidrug resistant bacteria[^11].

Interestingly, it was found that formulation of EOs as nanoparticles in water-based delivery systems can enhance their antimicrobial activity compared with the neat formulated form of these oils[^12,13]. The mechanisms that clarify the role of nanoparticle in boosting the antimicrobial activity of EOs were reviewed in details[^14]. The above mentioned literature included antimicrobial evaluations of wide varieties of EOs isolated from different aromatic plants. However, we noticed some limitation of conclusive data regarding the antibacterial activity of *Nigella sativa* EO especially after formulation in microemulsion. The seeds of that herbaceous plant (family Ranunculaceae) are the part that bears the EO. The abrasive evaluation of the seeds and their solvent extracts were studied[^15-18]. Specific targeting on the antimicrobial evaluation of the pure EO fraction of *N. sativa* has also been approached[^19]. However that study did not address...
some important issues that usually correlated with the antimicrobial activity of \textit{N. sativa} EO. These include the chemical composition and the percentage of the major volatile constituents of that EO especially thymoquinone (TQ). On the other hand, some other antimicrobial evaluations considered and correlated the chemical composition of the EO to the reported activity\textsuperscript{30-32}. However, these investigations did not include some important pathogenic bacteria in their evaluations like \textit{Bacillus cereus}, \textit{Listeria monocytogenes}, and \textit{Salmonella typhimurium}. Most importantly, no studies were found, to our knowledge, evaluating the antibacterial activity of \textit{N. sativa} EO as nanoparticles formulated in water-based microemulsion delivery system.

From the above mentioned, the following investigation was dedicated to provide more information on the antibacterial activity of that EO microemulsion which may not be addressed in previous studies. This included extraction and analysis of the EO followed by formulation in water-based microemulsion system and finally evaluation of the overall antibacterial activity of the formulated EO against different pathogenic bacteria. Moreover, the antibacterial activity of the EO microemulsion was compared with that of a well know plant-based phenolic antimicrobial model namely eugenol which was formulated also in microemulsion. A synthetic antibiotic model (Ceftriaxone) was used as a reference to help in ranking the antimicrobial activity of \textit{N. sativa} EO microemulsion among the natural (eugenol) and the synthetic (Ceftriaxone) antimicrobial models.

2 EXPERIMENTAL

2.1 Materials

Packages of \textit{N. sativa} crude oil encapsulated in hard gelatin capsules were purchased from a local pharmacy in Cairo, Egypt. Each capsule contains 450.0 mg of a 100.0% pure crude oil extracted from the seeds of \textit{N. sativa} by cold expression. No other additives are included in the capsules, as indicated by the manufacturing company (Pharco Pharmaceuticals, Cairo, Egypt).

The synthetic antibiotic Ceftriaxone\textsuperscript{®} sodium powder (containing 1000 mg Ceftriaxone) was also purchased from local pharmacy in Cairo, Egypt. The drug is produced by SmithKline Beecham Egypt under license from Sandoz. Eugenol 99.0% and Tween 20\textsuperscript{®} (polyoxyethylene sorbitane mono-laurate) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)

2.2 Extraction of the pure EO fraction

Sufficient numbers of capsules bearing \textit{N. sativa} whole crude oil (which is a natural mixture of fixed oil and EO), were broken and evacuated into a beaker. The pure EO fraction was extracted from the crude oil by the hydro-distillation method as previously indicated\textsuperscript{23}. In brief, the crude oil was mixed with distilled water (1:5 w/v) and distilled for three hours using Clevenger-type apparatus. The cup that receives the pure EO fraction in the side arm of the apparatus was wrapped with aluminum foil to protect the EO from light that can dimerize its main constituent thymoquinone into di-thymoquinone. At the end of the distillation process, the EO was collected, weighed and its content (yield percent) relative to the weight of total crude oil was calculated as mean of two extraction processes.

2.3 Gas chromatographic analysis

\textit{N. sativa} EO (20.0 \(\mu\)l) was diluted in 1.0 ml diethyl ether in a glass vial. Then 2.0 \(\mu\)l of this mixture were injected (at a split ratio 10:1) into a Perkin Elmer XL GC equipped with a FID. A fused silica capillary column (60 m \(\times\) 0.32 mm \(\times\) 0.25 \(\mu\)m) coated with DB-5 (5% phenyl, 95% methyl polysiloxane) was used to separate the different EO components. The oven temperature was programmed from 50\(^\circ\)C to 230\(^\circ\)C at a rate of 3\(^\circ\)C/min. The injector and detector temperatures were 230\(^\circ\)C and 250\(^\circ\)C respectively. Helium was used as carrier gas at a flow rate of 1.0 ml/min. The volatile oil constituents were quantified as percent of the total detected peak areas after flame ionization detection. All values were mean of two injections from two different extractions \(\pm\) SD. The available authentic samples were used to reveal the identity of \textit{N. sativa} EO constituents by matching their retention times with those of \textit{N. sativa} EO constituents after running on GC under the same conditions.

2.4 Gas chromatographic-mass spectroscopic analysis

GC-MS analysis was conducted using a Hewlett-Packard 5972 GC-MS system equipped with the same column and conditions used as in GC analysis. The ionization voltage was 70 eV and the ion source temperature was 170\(^\circ\)C. The scan range was from 29 to 300 AMU at 2.76 scans per second. Components identity that was previously revealed by running authentic samples in GC analysis was verified by automatic matching of their fragmentation patterns (m/z) with standard components stored in the electronic mass spectral library (NIST: National Institute of Standards and Technology) that was built-in the GC-MS soft ware.

2.5 Formulation of \textit{N. sativa} EO and eugenol in water-based microemulsions

Surfactant solution was prepared by dissolving the non-ionic surfactant (Tween 20\textsuperscript{®}) in de-ionized water at 5.0% (w/w) using magnetic bar. The clear solution was divided equally by weight among different glass vials wrapped with aluminum foil to protect from light. Different volumes of the hydrophobic \textit{N. sativa} EO and eugenol were titrated separately into each vial to end up with 10 concentrations for both EO and eugenol, ranging between 0.2% - 2.0%
with a concentration gradient of 0.2%. This percentage was equivalent to 2.0 mg - 20.0 mg EO or eugenol/ml microemulsion with concentration gradient 2.0 mg. All vials were vortexed for 1 minute to mix the ingredients and hasten the formation of microemulsion then left in dark at 27°C ± 1 to equilibrate. All formulations were also made in duplicates.

2.6 Formulation of Ceftriaxone in aqueous solution
Unlike the hydrophobic EO and eugenol, Ceftriaxone® hydrophilic powder was re-constituted directly in distilled water to give 1.0% aqueous solution (i.e. 10 mg/1 ml water). Then serial dilutions were made from that stock solution as required for utilization in the antibacterial assay. Thus there was no need to formulate Ceftriaxone in water-based microemulsion as it is a water-soluble antimicrobial.

2.7 Verification of microemulsion formation
Evidence of microemulsion formation for N. sativa and eugenol was based on the clear and transparent appearance of the formulations after the end of equilibration time which reached 4 weeks. The non-birefringent texture under polarized light microscope and the small hydrodynamic particle size (r) which did not exceed 100.0 nm was also taken as an evidence of microemulsion formation.

2.8 Particle size measurement
The particle size of microemulsions was measured using the dynamic light scattering instrument Zetasizer (Nano-ZS model ZEN3600, Nanoseries, Malvern Instruments, UK). Measurements were done at 27°C, with a fixed angle of 173°. The measurements are based on the Brownian motion of the hydrated particles, thus it provides information on the hydrodynamic diameter (nm) of the microemulsion particles. Sizes quoted are the z-average mean of the microemulsion hydrodynamic diameter (nm) obtained from 10 measurements (2 replicate ×5 measurements each). Before measurement, the samples were filtered through 0.20 μm single use syringe filter unit (Minisart®, Sarstien Stedium Biotech GmbH Germany) to remove impurities.

2.9 Antibacterial evaluation
2.9.1 Microorganisms and cultures
The tested microorganisms were provided from the culture collections of the Microbiological Department National Research Center (NRC) Dokki, Giza, Egypt. These include three strains of Gram-positive bacteria Staphylococcus aureus (ATCC 43300), Bacillus cereus (ATCC 11778), Listeria monocytogenes (ATCC 35152), and three strains of Gram-negative bacteria Salmonella typhimurium (ATCC 13311), Escherichia coli (ATCC 27325), Pseudomonas aeruginosa (ATCC 27853).

2.9.2 Antibacterial assay
The antimicrobials N. sativa EO, eugenol and Ceftriaxone formulated in their corresponding delivery systems (see formulation section) were evaluated against the six previously mentioned pathogenic bacteria. The evaluation was performed at five chosen concentrations of the three antimicrobials: 0.2%, 0.4%, 0.6%, 0.8% and 1.0%, unless otherwise stated. These percentages are equivalent to 2.0 mg antimicrobial/1.0 ml formula, 4.0 mg/ml, 6.0 mg/ml, 8.0 mg/ml and 10.0 mg/ml, respectively.

The agar well diffusion method was employed for the determination of antibacterial activities of the three formulated antimicrobials.

In details, 0.1 ml of the diluted inoculums (10⁷ CFU/ml) of test organism was spread on tryptone soy agar (TSA) plates. Wells of 5 mm diameter were punched into the agar medium and filled with 50 μl of each concentration of the three evaluated antimicrobial formulas. This volume delivered 10.0 μg, 200.0 μg, 300.0 μg, 400.0 μg and 500.0 μg of each antimicrobial (EO, eugenol and Ceftriaxone) per well. The plates were incubated for 18 h at 37°C. Antimicrobial activity was evaluated by measuring the zone of inhibition (mm) against the tested organisms. The evaluation was conducted twice and each time comprised three replicates for each concentration.

2.10 Statistical analysis
Statistical analysis was done through SPSS version 18.0. Quantitative data were represented in form of mean ± standard deviation (SD). Analysis of Variance (ANOVA) was used in the analysis of the results. The post hoc Duncan was used to make pair wise comparisons using a stepwise order of comparisons identical to the order used by the Student-Newman-Keuls test, but sets a protection level for the error rate for the collection of tests, rather than an error rate for individual tests. The level of significant was considered at p<0.05.

3 Results and Discussions
3.1 Analysis of N. sativa EO
The content of the pure N. sativa EO fraction after extraction from the whole crude oil using the hydro-distillation method was 2.2 ± 0.1 wt %. This percentage is considered to be much higher than other samples of N. sativa that yield 0.11-1.8 wt % EO relative to the crude oil. The genetic diversity of the seeds is among the most important factors that affect the quality and content of the EO. Table 1 showed the main constituents of the EO as revealed by the GC and GC-MS analysis. Thymoquinone (TQ) was the major constituent of the EO (52.6%) followed by p-cymene (25.8%), α-thujene (10.5%), β-pinene (3.0%) and α-pinene (2.8%). Other minor constituents were also
identified and illustrated in the table. This composition authenticated and confirmed the identity of *N. sativa* crude oil in the gelatin capsules because high p-cymene and TQ percentages in the EO are considered as markers for the *sativa* species. p-cymene and TQ can reach only 0.0-0.5 and 0.0-0.1, respectively in the EO of other *Nigella* species like for instance *Nigella orientalis*, *N. damascene* and *N. arvensis*.

### 3.2 Formulation of microemulsions

After the characterization of *N. sativa* EO, five concentrations of this oil and the pure eugenol were formulated separately in water-based microemulsion systems with the aid of surfactant \( \text{Twe} \text{e} \text{n} \text{20} \). Micro-emulsification technique is simple and economically feasible on small as well as large scale for solubilizing hydrophobic bioactive oils in water-based environment as nano-particles. This process is spontaneous and driven by the reduction of the system’s free energy due to interaction of the hydrophobic solubilizes (\( N. sativa \) EO and eugenol) with the surfactant molecules at certain energetically favorable orientation. One should consider that formulation of both EO and eugenol in water-based microemulsion is necessary due to the hydrophobic nature of these antimicrobials which prevent direct dissolution in water. On the other hand, the synthetic antibiotic Ceftriaxone was re-constituted directly in water due to its hydrophilic nature.

**Figure 1** showed that the particle size of the EO and eugenol in the microemulsion formulations was 8.7 ± 0.1 nm and 8.9 ± 0.1 nm, respectively with mono-modal size distribution. Examination of the different formulation under the polarized light microscope revealed no birefringence. Visual observation showed clear and transparent

<table>
<thead>
<tr>
<th>Component</th>
<th>Retention time (min)</th>
<th>Area % #</th>
<th>Mass fractions of each component (m/z)**</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )-thujene*</td>
<td>14.3</td>
<td>10.50 ± 0.7</td>
<td>93 (100.0%), 77, 91, 41, 39, 27, 79, 94, 136</td>
</tr>
<tr>
<td>( \alpha )-pinene*</td>
<td>14.6</td>
<td>2.8 ± 0.2</td>
<td>93 (100.0%), 91, 77, 41, 39, 27, 79, 53, 29</td>
</tr>
<tr>
<td>sabinene</td>
<td>16.3</td>
<td>0.5 ± 0.07</td>
<td>93 (100.0%), 41, 91, 77, 39, 27, 69, 94, 43</td>
</tr>
<tr>
<td>( \beta )-pinene*</td>
<td>16.6</td>
<td>3.0 ± 0.3</td>
<td>93 (100.0%), 41, 69, 91, 77, 39, 27, 92, 53</td>
</tr>
<tr>
<td>O-cymene</td>
<td>18.3</td>
<td>0.1 ± 0.02</td>
<td>119(100.0%), 91, 134, 117, 77, 65, 115, 39, 120, 51</td>
</tr>
<tr>
<td>p-cymene*</td>
<td>18.7</td>
<td>25.8 ± 0.7</td>
<td>119(100.0%), 134, 91,120, 117, 41, 77, 39, 65, 115</td>
</tr>
<tr>
<td>limonene*</td>
<td>19.0</td>
<td>1.1 ± 0.1</td>
<td>68 (100.0%), 93, 67, 94, 39, 92, 107, 53, 79, 136</td>
</tr>
<tr>
<td>linalool*</td>
<td>22.3</td>
<td>0.5 ± 0.05</td>
<td>71 (100.0%), 93, 55, 43, 41, 69, 80, 121, 67, 39</td>
</tr>
<tr>
<td>iso-3-thujanol</td>
<td>22.9</td>
<td>1.36 ± 0.2</td>
<td>43 (100.0%), 55, 41, 93, 95, 81, 67, 79, 121, 69</td>
</tr>
<tr>
<td>thymoquinone*</td>
<td>29.6</td>
<td>52.6 ± 1.9</td>
<td>164 (100.0%), 121, 136, 93, 149, 39, 40, 53, 77, 91</td>
</tr>
<tr>
<td>( \alpha )-longipinene</td>
<td>33.4</td>
<td>0.3 ± 0.03</td>
<td>119 (100.0%), 105, 133, 93, 107, 91, 161, 120, 204, 121</td>
</tr>
<tr>
<td>longifolene</td>
<td>35.8</td>
<td>1.5 ± 0.1</td>
<td>161 (100.0%), 94, 91, 93, 107, 105, 95, 79, 55, 109</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>98.96</td>
<td></td>
</tr>
</tbody>
</table>

#: after GC analysis via flame ionization detection

* Component identity was confirmed by matching retention times with authentic samples, beside the GC-MS identification.

** Mass fractions of each component were arranged in descending order according to the % abundance.

**Fig. 1** Particle size distribution of *Nigella* EO and eugenol microemulsions measured at their maximum concentrations (1.0% and 0.9%), respectively.
formulation with water-like flowability. These data confirmed the formation of typical water-based microemulsion systems. Both microemulsions were found to be physically stable when stored at 37°C for 24.0h before the evaluation of their antibacterial activity. They were also physically stable towards different freeze-thaw cycles and toward centrifugation at 5000 rpm for 30 min which confirmed their kinetic and thermodynamic stability as microemulsions.

It was interesting to note during formulation that eugenol took only ~ 30.0 minutes to equilibrate into a clear transparent microemulsion while N. sativa EO needed longer time that can reach 4.0 week to equilibrate especially at high concentration like 1.0 mg EO/ml microemulsion. The reason of that observation lies in the chemical structure of eugenol that contains hydrophilic and hydrophobic-like parts resembling the surfactant structure (Fig. 2). Due to that structure, eugenol can arrange itself at the interfacial film along with the original surfactant (Tween 20). That arrangement makes the original surfactant more efficient and facilitates the formation of microemulsion. That advantage is not available for N. sativa EO which is a complex mixture of volatile components of different chemical structures and polarities.

One of the liabilities of microemulsion formulation is the low pay load of the bioactive oil that can be incorporated in the water-surfactant solution to form microemulsion. Thus it was important to determine the maximum oil concentration that can be incorporated into the microemulsion before evaluating the antibacterial activity. For N. sativa EO that concentration was found to be 1.4 % (i.e. 14 mg EO/ml microemulsion) under the formulation conditions adopted in the current study. However at that concentration, crystals of TQ, which is the major constituent of the EO (52.6%), were precipitated out from the microemulsion. Therefore the upper limit of N. sativa EO used for assessing the antibacterial activity in the microemulsion was set at 1.0% (10 mg EO/ml microemulsion, or 500.0 μg/well). This concentration proved to be the safe margin for keeping the integrity of the EO constituents (including TQ) as being totally incorporated in the microemulsion. On the other hand concerning eugenol, the maximum pay load in the microemulsion was found to be 0.9% (9.0 mg eugenol/ml microemulsion) under the same formulation conditions. Trials to increase that load to reach 1.0% in order to match that of N. sativa EO was unsuccessful as it turned the transparent eugenol microemulsion into a cloudy dispersion of macroemulsion. The particle size of eugenol in that macroemulsion was out of the microemulsion size range (> 100 nm) as revealed by the visual cloudy appearance and the presence of large particles under the light microscopic.

3.3 Antimicrobial evaluation

Table 2 showed the antimicrobial activity of the three tested formulas namely, EO microemulsion, eugenol microemulsion and Ceftriaxone solution against some pathogenic bacteria. The table showed that the EO microemulsion was more antibacterial active than eugenol microemulsion at all concentrations against S. aureus, B. cereus and S. typhimurium, while eugenol was more effective against P. aeruginosa. The activity of eugenol microemulsion against P. aeruginosa reached its climax at 500.0 μg/well at which there was no significant difference between the antibacterial activity of eugenol microemulsion (40.0 mm) and that of Ceftriaxone (40.3 mm). Interestingly, there was no significant difference between the antimicrobial activity between N. sativa EO microemulsion (18.0 mm) and Ceftriaxone (19.3 mm) against S. typhimurium at 300.0 μg/well. In addition, the EO microemulsion showed even higher antibacterial activity than Ceftriaxone against S. typhimurium at 400.0 μg and 500.0 μg. The EO microemulsion also showed close antimicrobial activity (30.3 mm) to Ceftriaxone (31.3 mm) against E. coli at 500.0 μg.

From Table 2 it is evident that the EO microemulsion of N. sativa at high concentrations (400.0 μg/well and 500.00 μg/well) possesses antibacterial activity against four out of six of the tested pathogens. The EO microemulsion showed its highest antibacterial activity at the lowest concentration (100.0 μg EO/well) against S. aureus and B. cereus, respectively. E. coli was more resistant to EO microemulsion up to 300.0 μg/well, and then as the concentration of EO increased to 400.0 μg/well the antimicrobial activity also increased until reaching maximum (30.3 mm) at 500.0 μg/well. On the contrary, L. monocytogenes and P. aeruginosa were the most resistant bacterial strains toward EO microemulsion treatments at all concentrations. The highest antimicrobial activity for EO microemulsion at 500.0 μg/well was found against S. aureus (33.7 mm) followed by E. coli (30.3 mm), S. typhimurium (26.5 mm) and B. cereus (21.3 mm), respectively.

Table 2 also showed that eugenol microemulsion possessed high antibacterial activity against P. aeruginosa at all concentrations. Eugenol microemulsion was not effective at all concentrations against B. cereus and L. monocytogenes. However, it started to show concentration depen-
dent antibacterial activities above 300.0 μg/well against *S. aureus*, *E. coli* and *S. typhimurium*. The inhibition zone of *P. aeruginosa* was still significantly larger than that of these three strains at concentration above 300.0 μg/well.

From the table it is also clear that Ceftriaxone solution had high antibacterial active against all bacterial strains at all concentrations. However, it was more effective against *S. aureus* and *P. aeruginosa* compared with *L. monocytogenes* and *S. typhimurium* at the lowest and highest tested concentrations.

It is impotent to note that the control sample of the surfactant and water at 5.0 % did not show any antibacterial activities.

These results in general indicated a selective and high antibacterial activity of *N. sativa* EO. This activity is thought to be due to the high content of TQ which constitutes 52.6 % of the EO composition (Table 1). This compound is a phyto-quinone present only in the EO of *N. sativa*. One of the most important antimicrobial characteristics of TQ is its ability to modify the resistance of bacteria to antibiotics by acting as efflux pump inhibitor38. Efflux pumps are proteins in the cell membrane of the bacteria that extrude the antimicrobial agents outside the cell. Thus multi-drug resistant bacterial strains can arise which threaten the lives of patients infected with these strains. TQ was efficient in inhibiting several multi-drug resistant pathogenic bacteria39. It can kill antibiotic resistant *S. aureus* at 6.0 μg/ml40. TQ also has anti-biofilm properties against some Gram-positive and Gram-negative bacteria41.

Beside literature evidences that points out to TQ as the major antibacterial active principle of *N. sativa* EO one should bear in mind the potential synergistic effect of TQ with other component(s) that constitute the EO. For instance, p-cymene which constitutes 25.8 % of the EO composition may act as a potential synergistic agent. This terpenic hydrocarbon was previously found to act synergistically with carvacrol against *B. cereus* in rice42 and against *Escherichia coli* O157:H7 in un-pasteurised apple Juice43. p-Cymene also showed a high affinity for microbial membranes however it does not affect the membrane permeability but it may decrease its enthalpy and melting temperature44. In addition, this compound can perturb the microbial membranes, causing them to expand thus affecting the membrane potential of the intact cells45. Synergism can also be rationalized in some cases by the protection of the major antimicrobial principle of a given EO from degradation by the microbial enzymes or by con-

### Table 2 Effect of the three tested antibacterial formulas against some pathogenic bacteria.

<table>
<thead>
<tr>
<th>Concentration*</th>
<th>Antibacterial formulas</th>
<th>S. aureus</th>
<th>E. coli</th>
<th>B. cereus</th>
<th>L. monocytogenes</th>
<th>P. aeruginosa</th>
<th>S. typhimurium</th>
</tr>
</thead>
<tbody>
<tr>
<td>100.0 μg/well</td>
<td>EO microemulsion</td>
<td>15.7</td>
<td>0.6</td>
<td>0.0*</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Eugenol microemulsion</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0*</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Ceftriaxone solution</td>
<td>39.67</td>
<td>0.6</td>
<td>24.0</td>
<td>1.0</td>
<td>27.0</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P&lt; 0.0001</td>
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<tr>
<td>200.0 μg/well</td>
<td>EO microemulsion</td>
<td>19.7</td>
<td>2.1</td>
<td>0.0*</td>
<td>0.0</td>
<td>16.3</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>Eugenol microemulsion</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0*</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td></td>
<td>Ceftriaxone solution</td>
<td>41.7</td>
<td>0.6</td>
<td>25.7</td>
<td>1.2</td>
<td>28.3</td>
<td>1.5</td>
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<td>P&lt; 0.0001</td>
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<tr>
<td>300.0 μg/well</td>
<td>EO microemulsion</td>
<td>25.3</td>
<td>2.3</td>
<td>0.0*</td>
<td>0.0</td>
<td>18.0</td>
<td>1.0</td>
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<td></td>
<td>Eugenol microemulsion</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0*</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td></td>
<td>Ceftriaxone solution</td>
<td>43.3</td>
<td>0.6</td>
<td>27.3</td>
<td>1.6</td>
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<tr>
<td>400.0 μg/well</td>
<td>EO microemulsion</td>
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<td>1.0</td>
<td>25.3</td>
<td>1.5</td>
<td>19.6</td>
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<td>Ceftriaxone solution</td>
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<td>P&lt; 0.0001</td>
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<tr>
<td>500.0 μg/well</td>
<td>EO microemulsion</td>
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<td>1.5</td>
<td>30.3</td>
<td>1.5</td>
<td>21.3</td>
<td>1.5</td>
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<td>Eugenol microemulsion</td>
<td>12.7</td>
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<td>Ceftriaxone solution</td>
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<td>34.7</td>
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<td></td>
<td>P&lt; 0.0001</td>
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</table>

* The content of the antimicrobial agent (μg) which present in 50.0 μl of each antimicrobial formula /well
a : Means with the same letter are not significantly different.

The highest tested concentration of eugenol in the microemulsion was 0.9% (450 μg/well) due to formulation restriction (as indicated previously in the results and discussion section).
Analysis and Antibacterial Activity of Nigella sativa Essential Oil Formulated in Microemulsion System

...tribution in altering the multi-drug resistance mechanism (30). To our knowledge, there is no data so far revealing the potential synergistic activity between p-cymene and TQ which could be a subject for a future research work.

Despite this evidence-based antibacterial activity of N. sativa EO microemulsion, it was challenging to find it not effective against L. monocytogenes and P. aeruginosa at all concentrations (Table 2). The relatively simple structure of the cell wall of L. monocytogenes as Gram-positive bacteria is supposed to make it vulnerable to a wide range of antibacterial agents. However the antimicrobial resistance of L. monocytogenes is not an unusual biological event. This phenomena was previously reported in different studies from samples taken from dairy farms (31), seafood products (32), and from ready-to-eat meat products (33). The resistance of L. monocytogenes originates from some antimicrobial resistance genes that limit the number of the effective anti-listerial agents, as revealed from the last three references. Details of the resistance mechanism of L. monocytogenes were reviewed elsewhere (34).

P. aeruginosa was found to be another resistant bacterium to N. sativa EO microemulsion. The complex cell envelope of this organism resembles that of other Gram-negative bacteria which consist of polysaccharide, protein, lipopolysaccharide, and lipid components (34). It is well known that P. aeruginosa possessed a great adaptability and metabolic versatility. It has adaptive resistance toward some antibiotics (35, 36) and biocides (37). Adaptive resistance refers to reversible refactoriness to the bactericidal action of an antibiotic following first exposure. This phenomenon has been well documented in P. aeruginosa following exposure to an aminoglycoside (38). Thus the potential reasons for resistance to antimicrobials are a combination between the inherent multidrug efflux pumps with chromosomally-encoded antibiotic resistance genes (39) and the barrier function of the outer complex cell membrane. Another acquired resistance factor may also develop by mutation in chromosomally-encoded antibiotic resistance genes (30). A detailed contribution explaining the mechanism of antibiotic resistance in P. aeruginosa is also available elsewhere (42).

Interestingly, with the presence of all the above mentioned resistance mechanisms against antibacterial agents, eugenol microemulsion was found to be highly effective against P. aeruginosa than N. sativa EO at all concentrations (Table 2). The activity of eugenol microemulsion against that pathogen was almost the same as that of Ceftriaxone at the highest concentration (500.0 μg/well). Another study also reported high antibacterial activity of clove bud ethanol extract, as a rich source of eugenol, against P. aeruginosa (42). However, the incidence of antimicrobial sensitivity for that organism varied depending on the isolate strain and source of isolation (human, animal or environment). This may explain why two strains of P. aeruginosa exhibited intrinsic tolerance to different plant-based volatiles including pure eugenol (53).

The disrupting effect of eugenol on the cytoplasmic membrane of the bacterial cell (47) may justify its observed effectiveness against P. aeruginosa in our study. Another reason may include a certain affinity between the surfactant (Tween 20) that associate with the active phenolic group of eugenol in the microemulsion, and the cell membrane of P. aeruginosa. That affinity can induce better adsorption of the surfactant/eugenol complex to the surface of the bacterial cell which in turn will increase its local concentration at that point. However, a conclusive justification of the enhanced antibacterial activity of eugenol microemulsion against P. aeruginosa in our study is certainly challenging. That is particularly due to the lack of any antibacterial activity of eugenol microemulsion against L. monocytogenes and B. cereus at all concentrations (Table 2).

In contrast to this result, eugenol microemulsion was found in other studies effective against L. monocytogenes at concentrations close to that used in the current study (48, 49). This contradiction may originate from using different strain or isolate of L. monocytogenes that lack some of the resistance genes. It may also be due to using different surfactants in the formulation of microemulsions or using different antibacterial evaluation techniques.

Generally speaking, the surfactant used in microemulsion formulation can play a prominent roll on the antibacterial activities of the tested antimicrobial oils. Positively charged surfactant (cationic) can easily be adhering to the negatively charged bacterial cell wall via electrostatic attraction, making the antibacterial agent more effective. Such interaction is absent in the case of nonionic surfactants, as the case in the current study. However the role of nonionic surfactant is also thought to be crucial in contributing to the observed antibacterial activity. These surfactants tend to form micelles in water which incorporate (host) the antibacterial oils and form water-based microemulsions. The particle size of the antimicrobial (EO and eugenol) in the microemulsions formulated in the current study was ~8.00 nm. That nano-size can potentially increase the passive cellular absorption mechanisms of the antibacterial, therefore reducing mass transfer resistances and increasing antimicrobial activity. That was supported by previous studies which showed that nonionic surfactant-based microemulsions improved the antibacterial properties of eugenol (50-52). However in the current study we cannot confirm or deny that statement concerning N. sativa EO until that oil was tested in its neat versus its microemulsified states.

One may also consider the potential diminish of the antimicrobial activity of microemulsions due to the physical interactions between the antimicrobial oil and the nonionic surfactant that used in the formulation. For instance, some nonionic surfactants especially from the Tween family can...
bind to the polar bioactive group of some essential oil constituents by forming hydrogen bonds. That can lead to deactivation and partial loss of EO antibacterial activity. It worth also noting that, the water soluble fraction of the EOs and their constituents (via hydrogen bonds) in equilibrium with the amount incorporated in the surfactant micelles can also contribute to the antibacterial activity of emulsions. That is mainly because bacteria normally lives and proliferates in water. Therefore the extent of partition of the antibacterial EO in water along with its colloidal nano-size in microemulsion and its interactions with the surfactant can determine the over all antibacterial properties of the antimicrobial microemulsions.

On the other hand, the role of the surfactant is ignored in discussing the antibacterial activity of Ceftriaxone (the synthetic antibiotic) due to its hydrophilic nature. That property enabled direct re-constitution of Ceftriaxone in water without the need of formulation in surfactant-aided colloidal system as microemulsions.

4 CONCLUSIONS

This study is the first report that revealed the antibacterial activity of N. sativa EO microemulsion against different pathogenic bacteria. This activity resembled that of the synthetic antibiotic Ceftriaxone against S. typhimurium at 300.0 µg/well and surpassed Ceftriaxone at higher concentrations. In addition the EO microemulsion showed antibacterial activity comparable to that of Ceftriaxone against E. coli at 500.0 µg/well. The EO microemulsion was more effective than eugenol microemulsion against all tested pathogens except for P. aeruginosa. The revealed chemical composition of N. sativa EO can be imitated using synthetic ingredients to satisfy mass production if natural resources of that EO are not sufficient. In such case, more selective control over the chemical composition can be granted to secure reproducible antimicrobial activity and regular supply. A complementary work is planed by our team to investigate the combination of N. sativa EO microemulsion with other synthetic antibiotics in the same formula for potential enhanced antimicrobial activity. In addition, the validity of that EO microemulsion as a preserve in real food system is also a subject of future investigation.

References


10) Pereira, V.; Dias, C.; Vasconcelos, M.; Rosa, E.; Saavedra, M. Antibacterial activity and synergistic effects between Eucalyptus globulus leaf residues (essential oils and extracts) and antibiotics against several isolates of respiratory tract infections (Pseudomonas aeruginosa). Indust. Crops Prod. 52, 1-7 (2014).


Conflict of Interest

The authors declare that there is no any potential source of conflict of interest that editors may consider relevant to manuscript.
Studies have examined the antimicrobial activity of *N. sativa* against various bacterial strains. For instance, the essential oil of *N. sativa* has been found to exhibit antimicrobial activity against *Aspergillus flavus* in an *Industr. Crops Prod.* study (2014). Similarly, Salvia-Tujillo et al. (2007) assessed the antimicrobial activity of *N. sativa* seed extracts, demonstrating its potential as a natural antimicrobial agent.

Moreover, studies have explored the microbiological properties of *N. sativa* L. seeds and seedlings. For example, Salem et al. (2010) studied the microbiological properties of *N. sativa* L. seeds and seedlings grown in Egypt, highlighting their potential for use in food preservation and other applications.

The antibiotic activity of *N. sativa* L. seed extracts has also been evaluated in several studies. Islam et al. (2013) evaluated the antibiotic activity of *N. sativa* L. seed extracts against various bacterial strains and found it to be effective against *Escherichia coli* and other pathogens.

In conclusion, the *N. sativa* L. seed, its essential oil, and its components have shown promising antimicrobial and antibiotic properties against various bacterial strains. Further research is needed to fully understand the mechanisms behind these activities and to explore their potential applications in medicine and food preservation.


52) Kamel, G.; Ezz eldeen, N.; El-Mishad, M.; Ezzat, R. Susceptibility pattern of Pseudomonas aeruginosa against antimicrobial agents and some plant extracts with focus on its prevalence in different sources. Global Veterinaria. 6, 72-78 (2011).


