Antibacterial Activities of Nepetalactones Against Public Health-Related Pathogens

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Abstract
The antimicrobial activities of (Z,E)- and (E,Z)-nepetalactones, 2 major compositional compounds from the essential oil of catnip (Nepeta cataria), were first discovered from fly larval development media studies with over 98% inhibition of bacterial growth. Further investigation demonstrated inhibition of the growth of various bacterial species of public health significance. Catnip oil showed antibacterial activity against 5 Gram-positive and 9 Gram-negative bacteria. The antimicrobial activity varied among the original essential oil from the plant and its major compositional compounds as a blended mixture or an individual compound. Growth inhibition was observed against 5 Neisseria species, with particularly strong inhibition against Neisseria sicca (with MICs ranging from 0.5 to 5 mg/mL) that provided comparable or increased levels of growth control produced by 2 antibiotics (Ceftiofur and Cephalothin). The development of plant-based antibacterial agents to prevent or delay the emergence of antimicrobial resistance in bacteria is discussed.

Keywords
Nepeta cataria, antibacterial, nepetalactones, public health pathogen, Neisseria spp

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Natural products, such as plant essential oils, have been used throughout history for therapeutic purposes. Catnip, Nepeta cataria, is a mint herbaceous plant originating from Eurasia, North Africa, but found in North America as well.1,2 Recently, strong antifeedant and spatial repellent activity of catnip oil were reported against various blood-sucking vectors, with a further inhibitory effect on fly larval growth from application of wax-based catnip oil.3,4 During our laboratory bioassays of oviposition deterrence studies, we observed the catnip oil treated media with strong inhibition of larval development, compared with the control diet. This phenomenon may indicate its potential suppression of bacterial growth which is required for stable fly larvae development via feeding bacterial growth byproducts. The present study aimed to investigate whether these natural products, in essential oil forms or individual compositional compounds, would further suppress the growth of other bacteria than those living inside the fly larval media. Studies were further carried out to evaluate whether they possess similar antimicrobial activities against some pathogens of human as well as animal health significance.

Results and Discussion
Strong larvicidal activity was obtained using catnip oil wherein over 98% mortality of 3rd instar larvae was observed when larval development media was incubated with 250 mg of catnip oil (Figure 1., $t = 2.10, P < 0.05$). The number of colony-forming units (CFU) in the larval development media treated with catnip oil showed a significant decrease in CFU compared with those from the control (Figure 1, $t = 2.07, P < 0.05$). A total of 61 bacterial isolates were recovered from the stable fly larval media. Among them, significant growth reduction ranging from 76% to 99% was observed in 7 bacterial isolates identified as Bacillus cytotoxicus, B. sphaericus, B. fortis, Pseudomonas xianmenensis, Alcaligenes faecalis, and 2 strains of Myroides odoratimimus (Table 1). The majority of these identified bacterial species

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are associated with soil and livestock animal manure.\textsuperscript{5} Fly larval development requires rich supplies of a bacterial community in their natural habitat, which in turn, depends upon the microbial-derived products for their food as nutrients.\textsuperscript{6} More studies are under way to investigate which of these 6 bacterial species may play a key role either individually or in consortia in stable fly larval growth.

The essential oil of catnip purchased commercially contained 3 major compounds, (Z,E)-nepetalactone, (E,Z)-nepetalactone, and β-caryophyllene. The relative ratio of the 3 compositional compounds is 49:43:8, respectively. However, the concentrations of the 2 nepetalactones in commercially available catnip oils may vary based on catnip plant materials from different geographic locations, extraction methods, and different parts of the catnip plants used (flowers, leaves, stems, and seeds), as well as seasonal changes during plant collection from the same area, as reported by Schultz et al.\textsuperscript{7} In addition to the strong antibacterial activity of catnip oil found against bacteria as shown in the stable fly larval media, its antibacterial activity was further evaluated against 5 Gram-positive and 9 Gram-negative bacteria of clinical origins (Table 2). It was noticed that the extent of growth inhibition varied with different doses tested in the bioassays. The strongest antibacterial activity was demonstrated against \textit{Neisseria subflava}, followed by moderate antibacterial activities against several other Gram-negative bacteria, including \textit{Citrobacter freundii}, \textit{Branhamella ovis}, \textit{Aeromonas caviae}, \textit{Escherichia coli} O157, and \textit{Serratia marcescens}. Growth inhibitions of several Gram-positive bacteria, including 3 \textit{Enterococcus} species and \textit{Staphylococcus aureus}, by catnip oil were also observed. Bacteria, such as \textit{Citrobacter} species, have been identified from fly larval development sites (horse manure) collected from the field; among them, only \textit{C. freundii} sustains stable fly development.\textsuperscript{6} Although \textit{Aeromonas} \textit{spp.} have been found from horse manure, they do not support stable fly development.

Table 1. Bacteria Identification From Stable Fly Larval Media With Significant Growth Reduction (EGR) in Media Containing Catnip Oil.

| Isolates no. | Identification         | % EGR | ID match % |
|-------------|------------------------|-------|------------|
| 3           | \textit{Bacillus fortis} | 99.5  | 97.\textsuperscript{78} |
| 6, 8        | \textit{Myroides odoratinimus} | 99.2, 92.8 | 99.\textsuperscript{68, 84} |
| 28          | \textit{Aldaligenes fassalis} | 90.5  | 98.\textsuperscript{82} |
| 29          | \textit{Pseudomonas xiamenensis} | 99.3  | 97.\textsuperscript{38} |
| 43          | \textit{Basilis cyptotinus} | 99.1  | 98.\textsuperscript{32} |
| 62          | \textit{Basilis phaerinus} | 76.6  | 97.\textsuperscript{52} |

\textsuperscript{+} indicates the zone of growth inhibition. No inhibition was found from the control.

Table 2. Antibacterial Activity of Catnip Oil Against Selected Clinical Pathogens at 3 Dosages.

| Catnip oil | 0.1 mg | 1 mg | 10 mg |
|------------|--------|------|-------|
| Gram-positive |        |      |       |
| \textit{Enterococcus faecalis} V583 | - | + | + |
| \textit{E. faecalis} MMH594 | + | ++ | ++ |
| \textit{Micrococcus luteus} | - | + | ++ |
| \textit{E. faecium} VanR | - | ++ | ++ |
| \textit{Staphylococcus aureus} | - | ++ | ++ |
| Gram-negative |        |      |       |
| \textit{Branhamella ovis} | - | + | ++ |
| \textit{Aeromonas caviae} | - | + | ++ |
| \textit{Neisseria subflava} | ++++ | ++++ | ++++ |
| \textit{Salmonella enteriditis} | - | - | + |
| \textit{E. coli} O157 | - | + | ++ |
| \textit{Citrobacter freundii} | - | ++ | +++ |
| \textit{Pseudomonas aeruginosa} | - | + | ++ |
| \textit{Serratia marcescens} | - | ++ | ++ |
| \textit{E. coli} K12 | - | - | + |

\textsuperscript{+} indicates the zone of growth inhibition. No inhibition was found from the control.
 generally weak response against bacteria. Although antimicrobial agents to be able to work effectively due to their development of an enormous variety and quantity of small-molecule 20 times higher magnitude (Table 5).

At doses of $2^{-\text{MIC}}$ of catnip oil required to affect the bacterial growth were observed against few Neisseria species. Till date, there is no antibacterial of plant origin available with specific cellular targets, but resistant bacteria more efficiently and potentially delay the development of anti-microbial resistance.

Table 3. Growth Rates of Selected Pathogens Affected by Catnip Oil at 3 Dosages and B-Caryophyllene.

| Bacteria                  | Catnip oil 50,000µg/mL | Catnip oil 5,000µg/mL | Catnip oil 500µg/mL | Control |
|---------------------------|-------------------------|------------------------|----------------------|---------|
| Neisseria subflava        | 0.045 ± 0.005 a         | 0.147 ± 0.009 b        | 0.153 ± 0.029 b      | 0.805 ± 0.058 c |
| Neisseria gonorrhoeae     | 0.050 ± 0.001 a         | 0.113 ± 0.003 b        | 0.187 ± 0.033 b      | 0.299 ± 0.005 c |
| E. fæcalis V583           | 0.300 ± 0.007 a         | 0.379 ± 0.002 a        | 0.415 ± 0.019 b      | 0.921 ± 0.050 c |

Conclusion

Our findings on catnip oil and its major compositional compounds (nepetalactones) as alternative antibacterial agents may provide vital information to be used not only as a larvicide to combat stable fly larval development, but also by pharmaceutical industries for novel drug discovery, especially to combat the emerging threat of multi-drug resistance. The supplementary use of plant-based antibacterial substances and drugs may provide new opportunities to treat infections caused by multi-drug resistant bacteria more efficiently and potentially delay the development of anti-microbial resistance.

Materials and Methods

Essential Oils and Their Ingredient Compounds

The catnip essential oil was purchased from Liberty Natural Products, Inc. (Oregon City, Oregon, USA). Its chemical composition was determined by gas chromatography-mass spectrometric (GC-MS) analysis via confirmed characteristic fragments and retention times from their synthetic standards. One mg of oil was dissolved in 2 ml n-hexane, and 0.5 µl of the aforementioned solution (approx. 100 ng) was injected in the injection port of a coupled HP 7890 GC interfaced to a HP 5975 Mass Selective Detector (Agilent Technologies, Inc., Santa Clara, CA, USA). The GC was equipped with a FFAP and DB-5 column (30 m × 0.25 µm ID, Agilent) with the mode of split-less injection (250 °C). The oven temperature program started at 50 °C for 3 minutes, increased to 170 °C at 5 °C/min, and then increased to 240 °C at 15 °C/min. Helium was used as the carrier gas (3 mL/min). Mass spectra were

Table 4. Antibacterial Activities of Catnip Oil and Its Compositional Compounds at 50,000 µg/mL Against 5 Bacteria in Neisseria.

| Bacteria                | Catnip oil | EZ-Nepetalactone | ZE-Nepetalactone | Caryophyllene |
|-------------------------|------------|------------------|------------------|---------------|
| N. subflava             | 12.7 ± 0.7b| 13.0 ± 1.5a      | 8.7 ± 0.6b       | 9.0 ± 1.0 b   |
| N. perflava             | 18.3 ± 0.8a| 10.0 ± 1.1a      | 10.3 ± 0.9ab     | 6.0 ± 0.0 b   |
| N. sicca                | 13.3 ± 0.6b| 8.0 ± 1.2ab      | 7.3 ± 1.3 b      | 6.0 ± 0.0 b   |
| N. gonorrhoeae          | 11.3 ± 0.3b| 6.7 ± 0.6b       | 6.0 ± 0.0 b      | 7.0 ± 3.5 b   |
| N. catarrhais           | 17.7 ± 0.8a| 12.7 ± 0.6a      | 11.3 ± 0.3 ab    | 6.0 ± 0.0 b   |

Catnip oil and its ingredient chemicals added to disc diffusion assays; the zone of inhibition measured along the diameter including disc (Means ± SE, diameter of the disc, 6 mm). At 50 000 µg/mL, different letters followed by means indicated significant differences among catnip oil, nepetalactones and β-caryophyllene.

In contrast to mammalian immune systems, plants have to develop an enormous variety and quantity of small-molecule antimicrobial agents to be able to work effectively due to their generally weak response against bacteria. Although significant antibacterial activity of catnip oil has been demonstrated, especially against Neisseria species, its effectiveness is still relatively weaker in contrast to antibiotic drugs. Plants produce over 1 00 000 small-molecule compounds exhibiting antimicrobial activity, of which many are considered to contribute to the success of plant defense mechanisms in combating pathogen infection. Antimicrobial efficacy against 25 different genera of bacteria from 6 plant essential oils have been reported by Dorman and Deans. Zaika proposed that Gram-positive species, its effectiveness is still relatively weaker in contrast to antibiotic drugs. Plants produce extremely strong antibacterial activity of catnip oil has been demonstrated, compared with antibiotic drugs, especially against Gram-positive species. Till date, there is no antibacterial of plant origin available with specific cellular targets, but several antibiotics have been successfully produced by modifying chemical structures of active plant-based natural antibiotics, such as penicillin. As suggested by Tiwari et al., the development of novel plant-based antibiotics can reduce the selective pressure on bacterial pathogens to develop antimicrobial resistance by capitalizing upon the advantages of using various chemical strategies in plants against bacterial infection.
Table 5. Comparisons of Antibacterial Activity of Selected Antibiotics and Catnip Oil Against *Neisseria*.

| Bacteria     | Cefotiofur | Cephalexin | Catnip oil |
|--------------|------------|------------|------------|
| *N. subflava*| 26         | 35         | 13         |
| *N. perflava*| 40         | 10         | 18         |
| *N. sicca*   | 6          | 13         | 13         |
| *N. gonorrheae* | 30       | 38         | 18         |
| *N. catarrhali* | 30       | 13         | 17         |

1 µl/mL antibiotics and 10 mg catnip oil added to disc diffusion assays; the zone of inhibition measured along the diameter including disc (diameter of the disc, 6 mm).

The bacterial isolates were obtained from 2 sources including stable fly larval development media and faucets’ handles of 3 public restrooms at the East Union of the University of Nebraska. Bacterial isolates were streaked onto TSA (Tryptic Soy Agar) plates for catnip oil treatment. TSA plates with 5.67 mL/dimethyl sulfoxide (DMSO), TSA plates with 5.57 mL/DMSO and 100 mg/catnip oil were further incubated at 37 °C for 24 hours. Antimicrobial activity of the isolate was measured according to the Poisoned Food Method used for antifungal activity of plant extracts and the antimicrobial effect was estimated by the following formula: Growth reduction (%) = \((growth size of bacterial isolate in control – growth size in catnip oil treated media) / growth size of bacterial isolate in control \times 100\). Single colonies with significantly reduced growth sizes were isolated on TSA and stored at 4 °C for further DNA analysis.

Antimicrobial Activity of Essential Oils on Bacterial Isolates

The bacterial isolates were obtained from 2 sources including stable fly larval development media and faucets’ handles of 3 public restrooms at the East Union of the University of Nebraska. Bacterial isolates were streaked onto TSA (Tryptic Soy Agar) plates for catnip oil treatment. TSA plates with 5.67 mL/dimethyl sulfoxide (DMSO), TSA plates with 5.57 mL/DMSO and 100 mg/catnip oil were further incubated at 37 °C for 24 hours. Antimicrobial activity of the isolate was measured according to the Poisoned Food Method used for antifungal activity of plant extracts and the antimicrobial effect was estimated by the following formula: Growth reduction (%) = \((growth size of bacterial isolate in control – growth size in catnip oil treated media) / growth size of bacterial isolate in control \times 100\). Single colonies with significantly reduced growth sizes were isolated on TSA and stored at 4 °C for further DNA analysis.

For the identification of bacteria, the genomic DNA was extracted from pure cultures using a Nucleospin Tissue kit and the 16S RNA (16S ribosomal RNA) gene was amplified following the method described by Romero et al. Sequences were determined using the same ebacterial universal primers and were analyzed for similarity to known sequences in the GenBank databases (www.ncbi.nlm.nih.gov/blast) by using the BLAST (basic Local Alignment Search Tool) program.

All tests for antibacterial effectiveness with catnip oil and its compositional compounds against pathogens were conducted in the Veterinary Medical Entomology Laboratory at Kansas State University and in the laboratories at the Departments of Entomology, Agronomy and Horticulture of University of Nebraska. Fresh cells of test bacterial strains were cultured on TSA (Difco ™ 2nd Edition). A bacterial suspension of OD600 0.5 (optical density at 600 nm using MacFarland standard kit) was prepared in phosphate buffered saline (PBS, MP Biomedicals) and 100 µl was spread plated on TSA. Discs (diameter of 6 mm) were prepared using sterile white filter paper and a paper punch machine. Test compounds of catnip oil, β-caryophyllene, and \((E,Z)-\) and \((Z,E)-\)-nepetalactones were dissolved in DMSO and added to the sterile discs at different dilutions. The plates were incubated at 30 °C for 48 hours and the zone of inhibition was measured. The values are presented as mean of triplicate ±SD.
Data Analysis

Student T-test and one-way factorial analysis of variance (ANOVA) was used to analyze differences in growth or inhibition of bacteria isolate, larval development and CFU counts among essential oil-added media, DMSO/ethanol-added media, and the control. Means were compared using the least square difference (LSD) test. Values of $P < 0.05$ were considered significant. Analyses were performed using SAS, version 9.1 (SAS Institute Inc.).

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Declaration of Conflicting Interests

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