In the present research, we compared the efficacy of human use against disease vectors (Klun et al. 2003) to develop a more effective and safe repellent for repellent, was developed as a part of broader objective carboxamide (SS220), a newly recognized arthropod cine-South, Fort McPherson, GA 30330.

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mendation by the United States Department of Agriculture for its use. A proprietary product does not constitute an endorsement or a recom-

THERE ARE SEVERAL ALTERNATIVE mosquito repellents conducted (Bernier et al. 2005) and on the expected signiﬁcantly depending on how bioassays are con-

ents is not always certain because the apparent efﬁcacy of the presumed active ingredient differs signiﬁcantly depending on how bioassays are con-

However, in human volunteer bioassays, neither E,Z and Z,E-nepetalactone nor racemic nepetalactone deterred mosquito biting as effectively as SS220 or deet. All compounds differed signiﬁcantly from the control. We conclude that catnip oil and nepetalactone isomers are signiﬁcantly less effective than deet or SS220 in deterring the biting of Ae. aegypti.

KEY WORDS nepetalactone, repellents, yellowfever mosquito, deet

There are several alternative mosquito repellents containing penny royal oil, citronella, eucalyptus oil, soybean oil, or peppermint oil as putative active ingredi-

ents (Barnard and Xue 2004). Catnip oil was recently added to the list of alternative repellents and was formulated by a number of companies (Peterson et al. 2001). However, the effectiveness of all commercial products containing natural product ingredi-

ents is not always certain because the apparent efﬁcacy of the presumed active ingredient differs signiﬁcantly depending on how bioassays are con-

amples and are probably established originally from a colony at the United States Department of Agriculture Laboratory in Gainesville, FL (Rutledge et al. 1978). Insects were reared (Gerberg et al. 1994) by feeding larvae ground Tetramin Tropical Fish-Food Flakes (Tetra Sales, Blacksburg, VA). Mated females (5–15 d old) were maintained under a photoperiod of 12:12 (L:D) h at 27°C and 80% RH with a cotton pad moistened with 10% aqueous sucrose solution. Forty-eight hours before being used in bioassays, the nulliparous females had access to only water-moistened pads, and 24 h before testing access to water was removed.

Chemicals. The SS220 and deet used in the tests were at least 99% pure chemically according to capillary gas-liquid chromatography (GLC). Deet was obtained from Morﬂex, Inc. (Greensboro, NC). SS220 (95% stereochemical purity) came from the Chemicals Affecting Insect Behavior Laboratory (Beltsville, MD) where it had been synthesized previously (Klun et al. 2003). Catnip oil was purchased from Health and

Feeding Deterrent Effects of Catnip Oil Components Compared with Two Synthetic Amides Against Aedes aegypti

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ABSTRACT Recently, catnip, Nepeta cataria L. (Lamiaceae), essential oil has been formulated and marketed as an alternative repellent for protection against biting arthropods by several vendors. We isolated the major active components of catnip oil, E,Z- and Z,E-nepetalactone, and quantitatively measured their antibiting efﬁcacy compared with the repellents N,N-diethyl-3-methylbenzamide (deet) and chiral (1S,2′S)-2-methylpipеридинил-3-циклогексен-1-карбоксамид (SS220) against the yellowfever mosquito, Aedes aegypti (L.), by using an in vitro assay and human volunteers at 24 nmol compound/cm² (cloth or skin). Of all compounds tested in an in vitro assay, SS220 ranked as the most effective, whereas catnip oil and the nepetalactone compounds did not differ signiﬁcantly from each other or from deet. However, in human volunteer bioassays, neither E,Z and Z,E-nepetalactone nor racemic nepetalactone deterred mosquito biting as effectively as SS220 or deet. All compounds differed signiﬁcantly from the control. We conclude that catnip oil and nepetalactone isomers are signiﬁcantly less effective than deet or SS220 in deterring the biting of Ae. aegypti.
**Feeding Deterrent Effects of Catnip Oil Components Compared with Two Synthetic Amides Against Aedes aegypti**

**U.S. Army Center for Health Promotion and Preventing Medicine-South, Fort McPherson, GA, 30330**
Herbs (Philomath, OR). E,Z- and Z,E-nepetalactone isomers used in the assay were efficiently isolated from catnip oil (Chauhan and Zhang 2004) and were 99% pure chemically and 95–98% pure stereochromically according to capillary GLC. The structures of nepetalactone isomers (Fig. 1) were confirmed by gas chromatography (GC)-mass spectroscopy and nuclear magnetic resonance spectral analysis (Eisenbraun et al. 1980). Racemic nepetalactone was formulated by mixing 1:1 ratio of E,Z- and Z,E-nepetalactones, and homogeneity was confirmed by GC.

**In Vitro Bioassay.** The assay system consists of a six-well blood reservoir with each of the 3 by 4-cm wells containing 6 ml of human blood cells, water bath warmed (38°C) and covered with a collagen membrane. The blood-membrane unit simulates a human host for mosquito feeding, and antibiting activity of standard repellent compounds measured in the in vitro K&D module system are known to be comparable with activities observed when tested on the skin of human volunteers (Klun et al. 2005). The advantage of this assay is that mosquitoes can be tested much more quickly and without the burden of soliciting human volunteers or concerns about the potential toxicity of compounds being evaluated. Treatments in 110 μl of ethanol were each randomly applied to six 4 by 5-cm areas of organdy cloth and (after air drying) positioned over the membrane-covered blood. K&D modules containing five mosquitoes per cell were positioned over the treated cloth and exposed to the treated surface for 3 min. The number of mosquitoes feeding (proboscis inserted through cloth and collagen membrane into blood) in the exposure period was recorded. The compounds used for these tests were deet, SS220, racemic nepetalactone, E,Z- and Z,E-nepetalactone at 24 nmol each/cm² cloth and catnip oil equivalent to 24 nmol E,Z-nepetalactone/cm². In one block of tests, we used 180 mosquitoes to compare the effects of catnip oil, deet, SS220, and a control (ethanol). In a second block, we used 90 mosquitoes to compare deet, SS220 racemic nepetalactone, E,Z- and Z,E-nepetalactone, and a control. Results from these blocks of tests were combined for the analysis.

**In Vivo Bioassay.** In conducting this bioassay, we followed guidelines established by the National Institutes of Health involving human subjects and protocols approved by the Human-Use Review Board of the WRAIR. SS220, deet, and nepetalactone have abundant safety databases (Harney et al. 1978, Massoco et al. 1995, Klun et al. 2003) that permitted experimentation by using human volunteers. Repellent activity of deet, SS220, racemic nepetalactone, and its Z,E- and E,Z-isomers was measured using methods described by Klun and Debboun (2000) and Klun et al. (2003). The bioassays were done in a walk-in incubator (27°C and 80% RH) in ambient fluorescent light from 0730 to 1030 hours. The bioassay consisted of six treatments: deet, SS220, nepetalactone racemate, E,Z-nepetalactone, E,Z-nepetalactone, and a control. Using a skin-marking template and a washable-ink marker, skin areas representing 3 cm by 4-cm floor openings of the K&D module were outlined on the outer, top, and inner thigh positions of a volunteer’s leg. Six areas to be treated with stoichiometrically equivalent amounts of each compound and control were assigned randomly. All treatments were pipetted onto a 4 by 5-cm rectangular area extending 0.5 cm beyond the marks designating the rectangular area of the volunteers’ skin in 55 μl ethanol/treatment. Treating outside template marks ensured that areas beneath each K&D module cell contained no untreated skin. The control was 55 μl of ethanol alone applied to 20 cm² of skin, whereas 55-μl ethanol solutions of compounds were each applied to volunteers’ skin at 24 nmol compound/cm² skin. Each of six adjacent cells in the K&D modules were provided with five female mosquitoes randomly selected from cages containing ≥200 adults. The K&D module was positioned over the treated skin areas with the cells aligned over with the marked and treated areas of skin. At time 0, sliding doors between the cell and skin were opened. For the next 2 min, the number of females biting (proboscis in-
serted into skin and/or observed blood-engorged females) within each of the cells was recorded. The trial was concluded by closing the sliding doors. Individual mosquitoes were scored as having either fed or not fed during a trial. In total, 3,120 mosquitoes were used for this experiment, with 120–180 mosquitoes used on each of the six volunteers.

In in vitro and in vivo assays, all compounds were tested at a standard dose of 24 nmol compound/cm² (cloth or skin) because we wanted to specifically compare the performance of catnip and its nepetalactone isomers at a dose that was stoichiometrically equivalent to a dose of deet or SS220 known to suppress A. aegypti biting by at least 80% in a similar testing situation (Klun et al. 2005).

Statistical Methods. We used the Glimmix macro, which iteratively uses PROC MIXED (SAS Institute 1999) to analyze the data sets by using human volunteers, fitting a generalized linear mixed model with a logit link (McCullagh and Nelder 1989). This macro uses a weighted least squares approach to accommodate for the dependency of the variance on the mean that occurs when data are binomial. In this model, estimates for the dependent variable, logit \( p \) = \( \log \left( \frac{p}{1 - p} \right) \), where \( p \) is the (true) proportion of nonbiting mosquitoes, depends on both fixed (compound) and random (volunteer, where each volunteer acts as a block) effects. This approach is essentially identical to that described in Klun et al. (2003). Here, we estimate the following model: logit \( (p_i) = \tau_i + u_j \), where \( p \) is the proportion of nonbiting mosquitoes; \( i \) indexes the control, deet, SS220, or one of the nepetalactone treatments (\( \tau_i \)); and \( j \) indexes the volunteers (\( u_j \)). Degrees of freedom were estimated using the approximation described by Kenward and Roger (1997). The error rate for multiple comparisons was controlled using the “simulate” option in PROC MIXED, which resamples from the appropriate multivariate \( t \) distribution and assumes the covariance parameters are fixed (not estimated), which may produce slightly liberal results. However, software to estimate generalized linear mixed models and exactly control multiple comparison error rates is not currently available. An alternative in SAS is the use of PROC MULTTEST (which yielded identical treatment groupings); however, its ‘strata’ grouping variable seems designed only for fixed effects (we consider volunteer a random effect), so arguably it also gives liberal results for our data.

Because the random effect of human volunteer is not present for the in vitro data, a reduced model with only fixed effects was estimated using PROC LOGISTIC (SAS Institute 1999). Statistical comparisons of the compounds were made using PROC MULTTEST with the step-down permutation \( p \) adjustment option to the Cochran–Armitage test.

Results and Discussion

Table 1 summarizes the in vitro results, on the original scale with 95% confidence intervals. Compound ordering was similar to that obtained using human volunteers (Table 2). Catnip oil and the nepetalactone compounds did not differ significantly from each other or from Deet (all adjusted 0.0001–0.1). All compounds differed significantly from the ethanol control (all adjusted 0.0001–0.0002). Of all compounds tested using this assay, SS220 was ranked highest (similar results were obtained in Klun et al. 2003) and differed significantly from all other compounds (all adjusted 0.0001). As in the assay using human volunteers (Table 2), biting deterrence of catnip oil and the nepetalactone compounds fall between the control and deet and SS220.

Table 2 provides estimates and 95% confidence intervals for the proportion of nonbiting mosquitoes. These values were backtransformed to the original scale from the logit scale for ease of interpretation; significance tests were made on the latter scale. At a dose of 24 nmol/cm² skin, all treatments differed significantly from the control (\( t \)-test, \( df = 24.6 \), all adjusted \( P < 0.0001 \)), deet and SS220 were statistically indistinguishable (\( t \)-test, \( df = 24.6 \), adjusted \( P = 1.00 \)), as were the nepetalactone compounds from each other (\( t \)-test, \( df = 24.6 \), all adjusted \( P = 1.00 \)). SS220 and deet were significantly more effective in reducing biting than any of the nepetalactone compounds (\( t \)-test, \( df = 24.6 \), all \( P < 0.0005 \)). Results of the in vitro and in vivo systems are similar (Tables 1 and 2), although not directly comparable, because the data were not collected concurrently and because of putative in vivo human emanation interactions with test compounds not present in the in vitro assay.

| Table 1. Estimated proportions and their SEs of nonbiting mosquitoes (520 mosquitoes tested per estimate) for assessing in vitro biting deterrence against control, deet, SS220, catnip oil, racemic nepetalactone, \( E,Z \)-nepetalactone, and \( Z,E \)-nepetalactone, at 24 nmol/cm² cloth |
|-----------------|-----------------|---|
| **Compound**    | **Proportion of mosquitoes not biting** | **SE** |
| Control         | 0.39c           | 0.03 |
| Deet            | 0.62b           | 0.05 |
| SS220           | 0.89a           | 0.08 |
| Racemic nepetalactone | 0.57b   | 0.04 |
| \( E,Z \)-Nepetalactone | 0.06b  | 0.02 |
| \( Z,E \)-Nepetalactone | 0.61b  | 0.09 |
| Catnip oil      | 0.59b           | 0.03 |

Numbers followed by the same letter are not significantly different (\( P = 0.0001–0.1 \)) by PROC MULTTEST.

| Table 2. Estimated proportions and their SEs of nonbiting mosquitoes (520 mosquitoes tested per estimate) for assessing biting deterrence against control, deet, SS220, racemic nepetalactone, \( E,Z \)-nepetalactone, and \( Z,E \)-nepetalactone, at 24 nmol/cm² skin in human volunteers bioassay |
|-----------------|-----------------|---|
| **Compound**    | **Proportion of mosquitoes not biting** | **SE** |
| Control         | 0.69c           | 0.06 |
| Deet            | 0.96a           | 0.02 |
| SS220           | 0.96a           | 0.02 |
| Racemic nepetalactone | 0.84b     | 0.05 |
| \( E,Z \)-Nepetalactone | 0.45b  | 0.07 |
| \( Z,E \)-Nepetalactone | 0.53b  | 0.05 |

Numbers followed by the same letter are not significantly different (\( P = 0.0001–0.1 \)) by PROC MULTTEST.
The factors affecting the apparent behavior impact of compounds are dependent upon complex and interactive factors such as environment, insect type or species, exposure time, concentration of the test compounds, or the distance of the test surface from the host-seeking insects (Schreck 1977, Rutledge et al. 1978). Many formulations containing natural products and essential oils have been claimed to be safer and effective alternatives to deet. However, the claimed effectiveness is often unclear because the specific insect behavior being influenced is often ill defined. For example, compounds are often referred to as being repellent; however, the term is used without defining what normal behavior (such as flight orientation, host locating, or feeding) is being adversely changed or influenced by the compound from an insect survival viewpoint. Moreover, the term repellent is often used without a clear understanding of repellent effect. According to Dethier et al. (1960), a chemical that causes insects to make oriented movements away from its source is a repellent, and chemical that inhibits feeding when present in a place where insects would, in its absence, feed is a deterrent. On the basis of these definitions, we suggest that catnip oil may be a more effective repellent than deet as reported by Peterson et al. (2001), because their evaluation was done by olfactometer, and orientation away from the oil was documented for that bioassay system, whereas deet was not as effective in this regard.

Both in vivo and in vitro results of our study indicate that catnip oil components exhibited significantly greater bite deterrence effect than a control. There were no significant differences among the individual nepetalactone isomers nor between them and the racemic mixture, indicating absence of synergistic or antagonist effects. However, racemic nepetalactone and its individual isomers were all significantly less effective than deet or SS220 with reference to deter- cemic mixture, indicating absence of synergistic or nepetalactone isomers nor between them and the ra-

were no significant differences among the individual catnip oil components exhibited significantly more effective than deet or N.N-diethyl-3-methylbenzamide (deet) against mosquitoes. J. Med. En-
tomol. (in press).


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tomol. (in press).

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