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ABSTRACT

Cebus capucinus monkeys search for licorice-scented *Pipers* to use as an insect repellent (Brown 1996). Bioassays measuring mosquito larvicidal activity of six *Piper* plants were completed in Monteverde, Costa Rica to see if scent is a reliable cue. Overall, there was no relationship between strength of scent and larvicidal activity (Linear Regression F=0.064, P<0.05). The species in the study that exhibited the highest larvicidal activity was *Piper auritum*, a species that contains the essential oil safrole and has a strong licorice odor. *Piper auritum* and *Piper marginatum* (the only species *C. capucinus* is reported to use) both had a licorice-like scent and together had significantly higher larvicidal activity than the four species tested that lacked the licorice-like scent (Independent Samples T-test t=2.74, DF=15, P<0.05). Altogether these data show that the quality of *Piper* scent can indicate insect repellent ability but strength cannot. Thus, it makes sense that *C. capucinus* use scent cues to find effective plant repellents as specific scents can indicate larvicidal essential oils.

RESUMEN

Los monos de la especie *Cebus capucinus* buscan plantas del género *Piper* con esencia a regaliz y lo utilizan como repelente para insectos. Bioensayos midiendo el efecto larvicida en seis plantas de *Piper* se realizó en Monteverde, Costa Rica para ver si el olor es una pista confiable. Sobre todo, no hubo relación alguna entre lo fuerte del olor y el efecto larvicida (F= 0.064, p <0.05). La especie en este estudio que exhibenun mayor efecto larvicida fue *Piper auritum*, esta especie contiene el aceite esencial safrol y un fuerte olor a regaliz. *Piper auritum* y *P. marginatum* (la única especie reportada como usada por los monos) tienen un fuerte olor a regaliz y juntas tienen significativamente un mayor efecto larvicida que las otras cuatro especies que carecen de olor a regaliz (t=2.74, DF=15, P<0.05). Estos datos sugieren que la calidad de la esencia de *Piper* puede indicar habilidades como repelente de insectos pero no la fuerza. Así, tiene sentido que *C. capucinus* utilice la esencia como una pista para encontrar repelentes efectivos y que estas esencias pueden indicar aceites esenciales con efectos larvicidas.

INTRODUCTION

Many plants of the tropical rainforest have medicinal value due to their vast array of secondary metabolites which function in herbivore defense (Balandrin e*t al.* 1985). Since the 1940's, 48% of all cancer drugs have been derived from natural plant products (Cragg and Newman 2007). Some non-human animals also use plants for medicinal purposes. Zoopharmacognosy is a term that refers to non-human species selecting and using plants for disease treatment or parasite protection. This activity has been documented in many different animals including dusky-footed wood rats (*Neotoma fuscipes*), coatis (*Nasua narica*), ants, and several primates (Raman & Kandula 2008).

The *Piper* plant genus (family Piperaceae) is distributed pantropically and has characteristic attributes, one of these being a peppery "Ranalean" odor (Gentry 1993), given by essential oils of the plant. *Cebus capuchinus* monkeys look for *Piper marginatum* by smell in order to rub the leaves on their fur as insect repellent (Baker 1996). It is hypothesized that *C.*

capuchinus preferentially choose *P. marginatum* over other *Pipers* because of the licorice-like odor (Baker 1996) that comes from the essential oil safrole found in its leaves (Andrade *et al.* 2008). Humans also use the essential oils of *Piper* spp. in traditional medicine as an insect repellent and to relieve skin problems, sore muscles, and swelling (Bernhardt 2008).

Bioassays measuring mosquito larvicidal activity of *Piper* spp. elucidate which species have adult mosquito repellent capabilities. Mosquito larvicidal activity varies across the *Piper* genus with at least 611 active ingredients from approximately 110 species identified as having larvicidal activity (Dyer *et al*. 2004), though not every species has the same larvicidal effectiveness. For instance, *P. nigrum* and *P. retrofractum* have been isolated from groups of *Piper* spp. as being more toxic to mosquito larvae than other species of *Piper* (Park *et al*. 2002 & Chansang *et al.* 2005 respectively). The fact that *C. capucinus* prefers a licorice-scented *Piper* species (Baker 1996) could suggest that *Pipers* with this particular scent have high larvicidal activity. Additionally, it is possible that the strength of *P. marginatum* could be a cue to the monkeys, as the strength could reflect essential oil concentration in the leaf.

This study looks at six different species of *Piper* in order to elucidate the correlation between the scent of the leaves and the larvicidal activity. The type of scent and the strength of the scent should give an indication of the essential oil composition of the leaf and could provide information about the potential repellent uses of different *Piper* species.

METHODS

Plant Collection

Six *Piper* species were collected and identified from Monteverde, Costa Rica in the Premontane Moist Forest Holdridge life zone at 800-1450 m. Mean annual rainfall is 2000-4000 mm with a mean annual temperature between 17-24°C (Haber 2000). Young fully opened leaves were collected (between 5-7 leaves) to ensure the highest possible secondary metabolite concentration.

Scent Quantification

Piper leaves from each species (approximately five) were washed with water to remove dust and then ground with a mortar and pestle. The leaves were allowed to soak in water for 24 hours in a ratio of 50 g wet leaf weight: 1 liter water (methods adapted from Chansang *et al.* 2005). The filtrate from each species was diluted in 2-fold serial dilutions from 50 g/L to 0.75 g/L with water and stored in capped vials. A panel of 10 human subjects quantified the strength of the scent by smelling the vials from the weakest to the strongest concentration (smelling water in between each concentration) and indicating when the scent was first detectable. The subjects were also asked to describe the odors of each species in order to group the species into licorice scents and non-licorice scents. Species described by over half of the participants as having a licorice-like scent were recorded as possessing a licorice scent.

Mosquito larvae bioassay

Mosquito larvae (Culicidae) were obtained from bromeliad tanks and small, shallow pools of water in Monteverde and kept for up to two weeks in Tupperware containers of the water and leaf matter from which they were obtained. Ten 3rd and 4th instar mosquito larvae were added to 15 mL of water in a 50 mL plastic cup along with 0.5 g of ground-up *Piper* leaf picked from the plant on the same day as the test (a concentration of 0.03 g crude *Piper* extract/mL water). Twenty-four hours later, the number of dead larvae was counted; a larva whose appendages did not move upon touching with a toothpick constituted a dead organism. A mosquito larvae cup of 10 individuals with no treatment served as the control for each species. Five tests were completed for each species of *Piper*.

RESULTS

Scent Classification and Quantification

Of the six *Piper* species, two species were classified as licorice-scented while the other four species had a sweet, "Ranalean" scent (Table 1).

TABLE 1. A description of six *Piper* species collected from Premontane Moist Forest in Monteverde, Costa Rica. Scent descriptions were determined by a concensus from a 10-person panel.

Number	Species	Description of Leaf	Description of Scent
1	*P. friedrichsthalii*	Small, elongated, drip tip	Peppery, ranalean
2	*P. methysticum*	Large, rounded, rough texture	Sweet, ranalean
3	*P. auritum*	Very large and long, asymmetrical petiole, hairy texture	Licorice
4	*P. amalago*	Small, very thin, smooth, drip tip	Sweet, ranalean
5	*P. aequale*	Small, drip tip, tough leathery texture	Sweet, ranalean
6	*P. marginatum*	Very large heart-shaped, drip tip	Licorice

The scent strengths varied among the different *Piper* species (Kruskal Wallace Test, H=30.9, DF=5, $P<0.05$). *Piper auritum* had the lowest mean detectable scent concentration (indicating a strong scent strength) of 3.025% +/-1.9% and was significantly lower than all other species except for *P. amalago*(Post hoc multiple comparisons, $P<0.05$) which had a mean detectable scent concentration of 6.4% +/-3.9%, although this scent was described as sweet and not licorice-like. However, the mean detectable concentration of *P. amalago* was only significantly lower than *P. aequale*(Post hoc multiple comparisons, $P<0.05$), the *Piper* with the largest mean detectable scent of 33.75% +/-27.0 (indicating a weak scent strength). This detectable concentration is about 5 times greater than *P. amalago* and over 10 times greater than *P. auritum*. *Piper friedrichstalii* had a mean detectable scent of 21.35% +/-16.5%, *P. methysticum* had a mean detectable scent of 13.75% +/- 6.5%, and *P. marginatum* had a mean detectable scent of 16.9% +/-14.7%, none of which differ from one another. All of the means had large standard deviations, a measure of the variability in the smell capabilities of human test subjects.

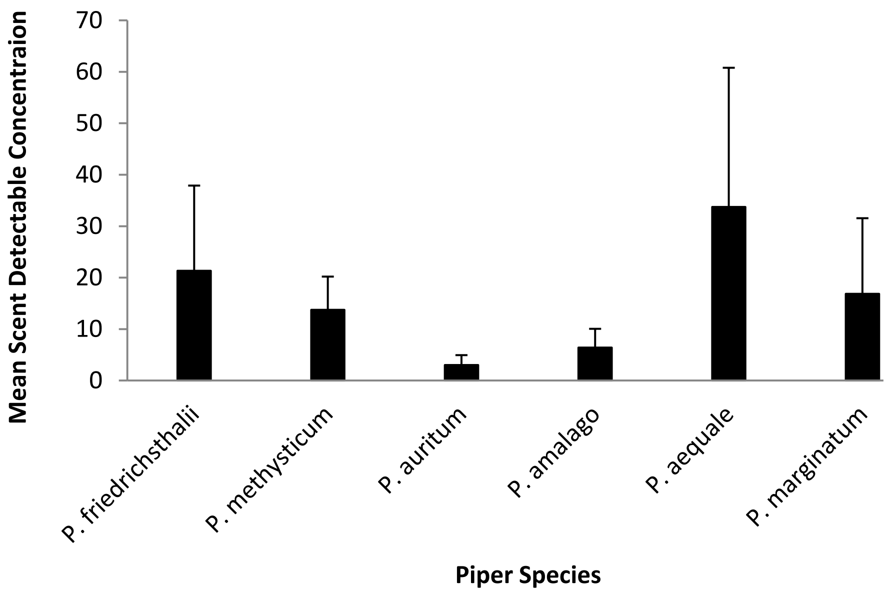

FIGURE 1. The scent detectable concentration as a percentage of pure solution (50 g crude *Piper* extract/L) for each *Piper* species differed among six *Piper* species in Monteverde, Costa Rica (Kruskal Wallace, H=30.9, DF=5, P<0.05). Low detectable concentrations indicate strong scents. Differences are found between *P. auritum* and all species except for *P. amalago*, and between *P. amalago* and *P. aequale*(Multiple Comparison Post Hoc Test, P<0.05).

Larvae Bioassays

There is differential larvicidal activity across the six *Piper* species (Kruskal Wallace Test, H=23.9, DF=5, P<0.05). However, the only significant difference in mean larvae mortality is between *P. auritum* and the plants which exhibited no larvicidal activity (*P. methysticum* and *P. amalago*, Post hoc multiple comparisons, P<0.05). *P. auritum* showed a mean mortality percentage of 88 +/- 11 % (Figure 1). *Piper friedrichstalii* had the second highest mean mortality proportion of 64 +/-18%. *Piper aequale* and *P. marginatum* had mean mortality proportions of 28 +/-16% and 32 +/-33% respectively which is between one-half and one-thirds the mean mortality proportions of *P. friedrichstalii* and *P. auritum*. An interesting observation is that *P. marginatum* has an extremely high standard deviation. In contrast to the other tests, the testing of *P. marginatum* was completed with a leaf that was 3 days old. It is also interesting to note that *P. friedrichstalii* had the second highest larvicidal activity even though it did not possess a licorice scent or a strong scent. None of the control tests yielded larvae mortality.

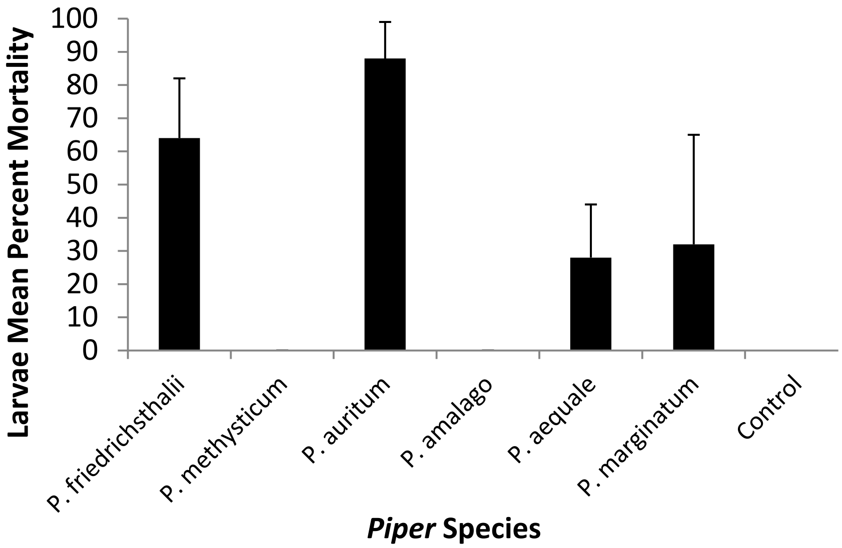

FIGURE 2. Larvae mean proportion mortality differed among six *Piper* species in Monteverde, Costa Rica(Kruskal Wallace Test, H=23.9, DF=5, P<0.05). Post hoc tests show the differences are found between *Piper auritum* and the species with no larvicidal activity (*P. methysticum* and *P. amalago*, Post hoc multiple comparison tests, P<0.05).

There is no correlation between strength of *Piper* scent and mean proportion larvae mortality (Linear Regression, F=0.064, P>0.05). As expected, the *Piper* with the strongest scent (*Piper* 3) also had the highest mean proportion larvae mortality (Figure 3); however, the *Pipers* with the second and third strongest scents showed virtually no larvicidal activity.

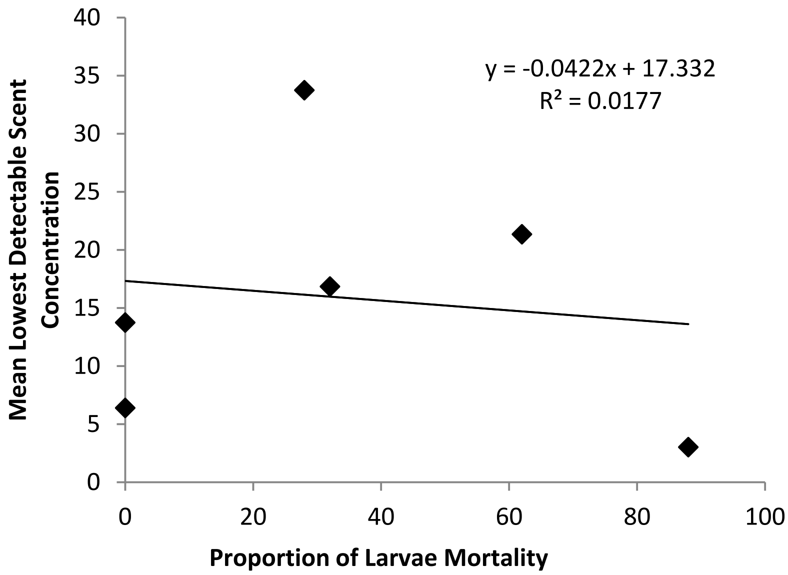

FIGURE 3. Strength of *Piper* scent does not correspond to larvicidal activity among six *Piper* species in Monteverde, Costa Rica (Linear Regression, F=0.064, P<0.05).

A comparison of mean larvae mortality proportions in comparing *Pipers* based on type of smell show that the means vary between *Pipers* with a licorice scent and *Pipers* without a licorice scent (Independent samples T-test, t=2.74, DF=15, P<0.05). The licorice-scented *Piper* plants 3 and 6 had a mean larvae mortality proportion of 0.6 +/-0.37 whereas the other *Pipers* had a combined mean larvae mortality proportion of 0.23 +/- 0.29, almost a three-fold difference (Figure 4).

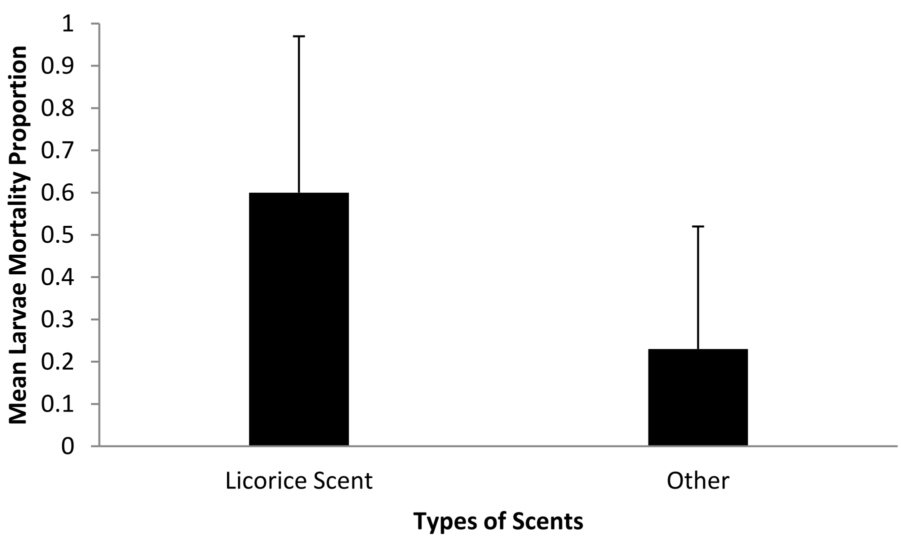

FIGURE 4. The mean larvae mortality proportions differ significantly between *Pipers* possessing a licorice scent (2 species) and those lacking a licorice scent (4 species) in Monteverde, Costa Rica (Independent samples T-test, t=2.74, DF=15, P<0.05).

DISCUSSION

The six *Piper* species differ in their larvicidal bioassay activity, which is a well-established finding in the *Piper* genus (Dyer *et al.* 2004). *Piper auritum* showed the highest larvicidal activity, which confirms previous findings of *Piper auritum* having high insecticidal activity (Leyva *et al.* 2009). The three other species that showed larvicidal activity also have been previously documented as larvicides (*P. aequale*, Marquis 1991; *P. marginatum*, Autran *et al*. 2008; and monoterpenoids found in *P. friedrichsthalii*, Lee 1997), though no studies have compared these specific species. It is interesting that in this study *P. marginatum* (the species used by *C. capucinus*) did not show significantly greater larvicidal activity than the control. The high variability in mean percent larvae mortality could be explained by possible leaf degradation in certain parts of the leaf since the tests were not completed the same day as leaf collection. This is congruent with a study showing loss of larvicidal activity as a function of *Piper* fruit age (Chansang *et al*. 2005).

Strength of the scent did not indicate larvicidal activity, but instead type of scent was much more important in determining larvicidal activity. This supports the hypothesis that certain types of odors (licorice-like odors) can confer repellent ability (Brown 1996). These data point to the difference in chemical functions of the essential oils in the *Piper* genus; though the plants are phylogenetically related, their secondary metabolite defenses are not equal. Chemical structures of essential oils found in *Piper* include alkaloids/amides, lignans, terpenes, propenylphenols, steroids, kavapyrones, chalcones, flavones, flavanones, and piperolides (Dyer *et al*. 2004), indicating the vast array of chemical defense mechanisms found in *Piper* spp. The scent types of *Piper* species can indicate the chemical composition of the essential oils; thus, it makes sense that scent type rather than scent strength is a better indicator of larvicidal activity. For instance, the second and third strongest scented plants did not show larvicidal activity, indicating that though their essential oil content is high, their chemical defense abilities do not impact mosquito larvae.

The licorice scent present in *P. auritum* and *P. marginatum* is due to safrole, the main essential oil present in both species (Andrade *et al.* 2008 and Hansel *et al*. 1975). Not surprisingly, safrole is already used commercially as a precursor to piperonal butoxide, an insecticide (Tozzi 1998). In regards to the efficacy of safrole as a potential natural insecticide, there are mixed reports. Safrole has also been implicated as a weak hepatocarcinogen in rats (Liu *et al*. 1999) and is banned as a food additive by the FDA (FDA 2010); however, one study showed that while carcinogenic metabolites were found in the urine of rats, no carcinogenic metabolites were found in the urine of humans, bringing to question the actual carcinogenicity (Benedetti and Broillet 1977). Perhaps concentration or chemical modification of safrole could provide a new mosquito repellent product.

Regardless, it is interesting to find that the licorice scent that *Cebus capucinus* search for as insect repellent is indeed indicative of larvicidal essential oils. *Cebus capucinus* monkeys have not been previously recorded as using *P. auritum* even though it has a strong licorice odor and high larvicidal activity. However, it is likely that this has to do with limited research; the fur-rubbing behavior was observed in monkeys at the Curú Refugio de Vida Silvestre in the Nicoya Peninsula of Costa Rica (Brown 1996), a location not recorded in the distribution of *P. auritum* (INBio 1997). Thus, it is probable that in cases where the two co-exist, *C. capucinus* use *P. auritum* as insect repellent. Further observations of fur-rubbing behavior could confirm this conjecture and could also give further insight into other possible larvicidal plants.

Altogether this study provides a good example of nonhuman animals locating and using plants for medicinal purposes, and it points to the value of allowing animals of the rainforest to guide our learning about plants of the rainforest.

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LITERATURE CITED

Balandrin, M. F., J. A. Klocke, E. S. Wurtele, and W. H. Bollinger. 1985. Natural plant chemicals: Sources of industrial and medicinal materials. Science 228, 1154-60.

Baker, M. 1996. Fur rubbing: Use of medicinal plants by capuchin monkeys (Cebus capucinus). American Journal of Primatology 38: 263-270.

Benedetti, M., A. Malnoë, and A. Broillet. 1977. Absorption, metabolism and excretion of safrole in the rat and man. Toxicology 7, 69–83.

Bernhardt, E. Medicinal Plants of Costa Rica. pp. 85. Zona Tropical Publication. China.

Chansang, U., N. Zahiri, J. Bansiddhi, T. Boonruad, P. Thongsrirak, J. Mingmuang, N. Benjapong, M. Mulla. 2005. Mosquito larvicidal activity of aqueous extracts of long pepper (*Piper retrofractum*) from Thailand. Journal of Vector Ecology 30: 195-201.

Cragg GM and Newman DJ. 2007. Natural Products as Sources of New Drugs over the Last 25 Years. Journal of Natural Products 70, 461-477.

Dyer, L.A., J. Richard, and C.D. Dodson. 2004. Isolation,synthesis, and evolutionary ecology of *Piper* amides. In: L.A. Dyer and A.D.N. Palmer (eds.) A model genus for studies of phytochemistry, ecology, and evolution. pp.117-139. Kluwer/Plenum. New York.

FDA. 2010. Safrole: CFR- Code of Federal Regulations Title 21. Retrieved from http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=189.180.

Gentry, A. 1993. Piperaceae. In: Gentry, A. A Field Guide to the Families and Genera of Woody Plants of Northwest South America (Colombia, Ecuador, Peru*)*. pp. 683-684. University of Chicago Press. Chicago.

Haber, W. 2000. Plants and Vegetation. *In* Nadkarni, N. and N. Wheelwright. Monteverde, pp. 90. Oxford University Press, New York.

Hansel, R., A. Leuschke, and A. Gomez-Pompa. 1975. Aporthine-type alkaloids from *Piper auritum*. Lloydia 38, 529-530.

INBio. 1997. List of Specimens of *Piper auritum.* Retrieved from http://www.inbio.ac.cr/bims/k03/p13/c045/o0234/f01525/g008580/s027005.htm.

Lee, S., R. Tsao, C. Peterson, and J. Coats. 1997. Insecticidal activity of monoterpenoids to western corn rootworm (Coleoptera: Chrysomelidae), twospotted spider mite (Acari: Tetranychidae), and house fly (Diptera: Muscidae). Journal of Economic Entomology 90: 883-892.

Leyva, M., M. Marquetti, J. Tacoronte, R. Scull, O. Tiomno, A. Mesa, and D. Montada. 2009. Actividad larvicida de aceites esenciales de plantas contra de *Aedes aegypti*. Rev Biomed 20, 5-13.

Marquis, R. 1991. Herbivore fauna of *Piper* (Piperaceae) in a Costa Rican wet forest: Diversity, specificity, and impact. In: P. Price, Lewinsohn T., Fernández G.,and Benson W. (eds.). Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate Regions. pp. 177-199. J. Wiley and Sons. United States.

Park, I., S. Lee, S. Shin, J. Park, and Y. Ahn. 2002. Larvicidal Activity of Isobutlyamindes Identified in Piper nigrum Fruits against Three Mosquito Species. Journal of Agricultural Food Chemistry 50: 1866-1870.

Raman, R. and S. Kandula. Zoopharmacognosy: Self-Medication in Wild Animals. Resonance March 2008: 245-253.

Tozzi, A. 1998. A Brief History of the Development of Piperonyl Butoxide as an Insecticide Synergist. In: D. Jones (Ed.) *Piperonyl Butoxide*: *The Insecticide Synergist. Pp. 1-5.* San Diego.