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Effectiveness of essential oils from *Citrus sinensis* and *Calendula officinalis* and organic extract from fruits of *Maclura pomifera* as repellants against the wolf spider *Rabidosa punctulata*

Garrett Owen

ABSTRACT

The essential oils (EO's) of *Citrus sinensis*, *Calendual officinalis*, and *Maclura pomifera* were extracted via either steam distillation or organic extraction and tested for their repellency effect on the wolf spider, *Rabidosa punculata*. Essential oil repellency was tested in a Y-maze fumigation test and a filter paper contact test. The data collected was subjected to statistical analysis; the results from the binomial test of the fumigation trials data suggest no significant repellant activity of the fumes of any of the EO's extracted. Although, *Citrus sinensis* EO's presented hope for further studies. Results from the Wilcoxon rank sum test of the contact trials data showed *Calendula officinalis* as an effective deterrent against *Rabidosa punctulata* while the other two EO's showed no significant effects. The isolated EOs from each plant were analyzed using GC/MS to identify the major compounds present. Results from the GC/MS showed d-Limonene to be the major component of *Citrus sinensis* at 92.56% while major components of *Maclura pomifera* were (1S)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene at 18.96%, 3-Carene at 17.05%, Cedrol at 16.81%, and a-Terpinyl acetate at 5.52%. It was concluded that d-Limonene is a common ingredient in many insect repellants, but exists as a component of a mixture of several chemicals. This could possibly point toward further investigation of citrus EO's as an active component of a mixture of several EO's for an effective natural spider repellant. The results of this study also point toward potential evidence of the identification of spider olfactory organs as the same sensilla used for taste.

Keywords: *essential oils, natural insect repellants, fumigation trials*

INTRODUCTION

Essentials oils (EO's) are plant produced substances that are volatile, fragrant, and typically presented in liquid form (Bassolé 2012). The composition of these oils are quite complex and consist of many hydrocarbon compounds of which are said to give them their essence and unique properties. A few of the common classes of compounds present in many essential oils are alcohols, terpenes, aldehydes, esters, and ketones. The properties of essential oils vary, but it is possible that common characteristics among many EO's include attracting or repelling insects. (Devi et al. 2015). Other natural biological functions of these chemicals include protecting against predators, inhibiting seed germination, and communication between other plants (Hanif et al. 2019).

While the exact time of the discovery of EOs is unclear, documents suggest they were used as early as the ninth century (Hanif et al 2019). Essential oils can be extracted from a variety of plant raw materials, including leaves, stems, peels, and flowers. Since many EOs own a characteristic odor, they are commonly used in our everyday lives in materials such as cosmetics, toiletries, and perfumery. In addition, they have been long recognized for their antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Bassolé 2012).

In recent years, as the popularity of EOs has begun to rise, scientists have begun to perform various studies with them. One reason that EOs have become a popular scientific research topic is because of the need to find natural pesticides and pest repellants that are eco-friendly. Since synthetic chemicals are known to cause residual toxicity and raise resistant races of the insect pests, it seems logical to turn to natural resources as pesticides or repellants since they have been present in nature for thousands of years and present many different biological activities (Upadhyay et al. 2018). A great example is a study done by Gopal and Benny (2018), in which they tested to see if EOs from *Elletaria cardamom*, *Merremia vitfolia*, and *Peperomia pellucida* were effective deterrents against different insect orders that coexist with humans from day to day. The EOs tested were found to be effective toxins against the specific insects. Several different studies have been done on insects and results seem to differ between specific types of oils. Another example is a study done in 2018 by a group of scientists who studied the repellant activity of different EOs mixed into corn-starch based thixogel. (Nasrul et al. 2018). The EOs were found to significantly improve repellant activity. A third example is a study done by scientists in 2012 on the repellant properties of several essential oils against a species of ants (Scocco et al. 2012). Results showed that all essential oils at many different concentrations were repellent to Argentine ants. Although several studies have been conducted on different pests, there seems to be a lack of research on eco-friendly repellents for arachnids.

Spiders contain a variety of sensory systems. (Foelix 1996). In this study, we focus on chemoreception. Chemoreception could be olfactory or taste-by-touch. The olfaction sense, or the spiders' ability to detect odor EO molecules in the air, will be tested through a fumigation test in which the potential repellant will be presented in the form of a gas at a low concentration. Although the site of the olfaction nerves remains unknown, it is known that spiders use this sense in many instances including during courtship (Foelix 1996). Spiders contain small, microscopic-sized hairs on the distal ends of their walking legs that allow them to determine chemical properties of a substrate by barely touching a substrate (Foelix 1996). This sensory system, taste-by-touch, will be tested through a contact test in which the spiders come into physical contact with the essential oils themselves. Since the sex-pheromones of spiders undoubtedly cause a chemical response to spiders, there is hope that similar, volatile chemicals such as essential oils of citrus fruit, marigold flowers, and hedge apples will elicit a chemical response as well.

This study aims to explore the potential repellant properties of EOs from three plants, navel oranges (*Citrus sinensis*), marigold flowers (*Calendula officinalis*), and hedge apples (*Maclura pomifera*) against a species of wolf spiders, *Rabidosa punctulata*. This type of wolf spider is one of the more common cursorial spiders in the Midwest, yet little research has been done considering repellants that may help keep them out of our homes.

MATERIALS AND METHODS

For the study navel oranges were purchased from the local Walmart. The oranges were peeled. Marigold flowers were hand-picked from the McPherson College flower beds. Hedge apples were collected from the Turkey Creek Golf Course in McPherson, Kansas. The orange peels and marigold flowers were cut into approximately 1cm x 1cm pieces. The hedge apples were cut into approximately 1 inch by 1inch cubes. Between 600 g – 1,000 g amounts of each plant material was subjected to steam distillation.

Essential oils are extracted using a variety of techniques, such as distillation and cold press. In this specific study, steam distillation was used to extract the oils form marigold and citrus oils. In steam distillation, a pot of water is placed on a hot plate. The pot of water is connected to the distilling container which holds the material of which oil is to be extracted. The steam from the water will rise and enter the container with the orange peels and ass steam is passed through the packed bed, it condenses in the first element before going to the next (Masango 2003). As the steam from the boiling water passes through the container with the plant material, the increased temperature and pressure helps the release of the molecules of EOs from the oil pockets in the plant (Valderrama 2018). The EOs and water are eventually collected in a piece of glassware used for separating mixtures.

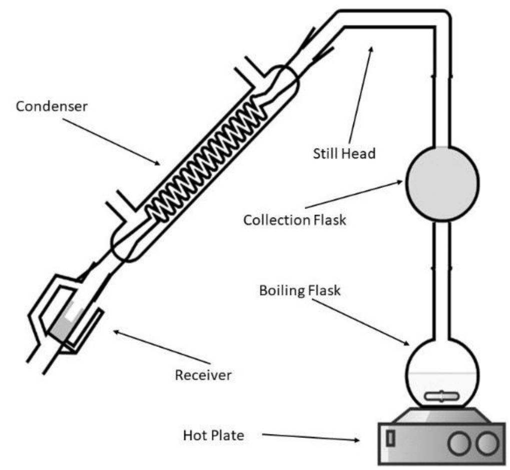

Figure 1. Steam distillation apparatus.

With orange peels, a total of two distillations were performed and approximately 3 milliliters of citrus oil was extracted. After four distillations of marigold flowers, approximately 2mL of EOs was collected. However, steam distillation of hedge apples did not produce any EOs. Since the hedge apples did not produce any EOs, the aqueous liquid collected in the still pot from the steam distillation was extracted into 600 ml of dichloromethane (300mL x 2). The organic solvent was removed using a rotary evaporator to isolate 500 mg of solid material. The EOs from the citrus fruit and the marigold flowers and the isolated material from the hedge apples were tested in the fumigation and contact trials.

Spider Collection & Behavioral Trials

All spiders (N = 132) used in this study were collected at night between the dates of September 5, 2020 and September 9, 2020 in McPherson County, KS. The spiders were housed in small cubicle cages, one spider per cage, and fed three baby crickets once per week. Water was supplied to the spiders through a small wick in the bottom of the cage that was constantly submerged in water.

In order to test the effectiveness of the essential oils as deterrents, a y-maze fumigation test was conducted. This maze was constructed by attaching 3 pieces of polyvinyl chloride (PVC) pipe, each 5 inches long, into the shape of a y. This type of maze is called a bifurcation maze, meaning that it has two branches, hence the name Y-maze. This type of maze gives immediate results, is very straight forward, and cost-effective (Czaczkes 2018). The results from this test are one of two possibilities. Either the spider does pick the side with the EOs, or doesn't pick the side with the essential oil.

The constructed PVC Y-maze laid flat on a table. At the end of each branch of the Y there was a filter paper that had been taped to fit the end of the pipe as if it were a cap. On one side, the filter paper had a pipette drop of water and the other side a pipette drop of essential oil. In order to account for bias, a dice was rolled to determine which side the EOs would be placed. At the end of each branch, behind the filter paper, a brushless DC 24V fan was placed in order to fumigate each branch with the fumes of the specific liquid. The fans were attached to the same alkaline 6V Energizer battery to ensure equal air flow. Then, at the end base of the Y-maze a spider was placed. As the spider made its way up the base of the Y, it came to a screen half way up the base. Another screen was added behind the spider to "trap" it in this area. These screens prohibited the spider from walking up a chosen branch. At this point, each spider was given 60 seconds to acclimate to the fumes of each branch. After 60 seconds, the screen was removed and the spider choose a branch to continue walking through. If upon removal of the foremost screen, the back screen was gently tapped until the spider chose to walk up the maze. After each trial, the maze was wiped clean with 70% ethanol. A total of 30 tests were ran for each oil, except for hedge apple EOs due to the small amount of organic extract.

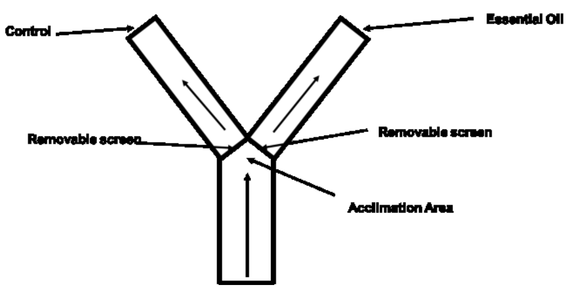

Figure 2. Y-maze apparatus.

The second test to test repellent activity of EOs was a contact test. In this set up, there was a circular dish with a diameter of 25 centimeters for the arena. On one side of the dish a filter paper that had been sprayed three times with a solution of EOs that was diluted 50 times with hexane was laid. On the other side, a filter paper that had been sprayed with the control solvent, hexane, was laid. Once the filter papers were placed properly, the spider was dropped into the arena in the center where it was held in a vile for 60 seconds. This was the acclimation period. I then observed the spider for 3 minutes. The amount of time that each spider spent on either side was recorded for further data analysis. This reaction to the filter paper allowed me to observe whether or not the EOs solution repelled the spiders by contact.

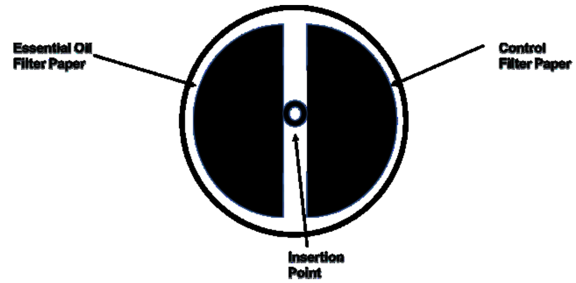

Figure 3. Contact trial arena.

A binomial test was conducted on the data from the y-maze, and a Wilcoxon rank sum test, due to the suggested violation of the assumption of normality, was used to interpret the contact data. The binomial test allowed me to test the null hypothesis that there was no repellent activity and that the probability of the spider choosing either branch was the same. In the one Wilcoxon rank sum test, the sample mean, or average time spent on the oil-saturated filter paper, was compared to the null hypothesis value of 1 minute and 30 seconds.

The orange and marigold EOs were sent to the University of Nebraska for a GC/MS to be ran on the samples. The instrument used for analysis was a Thermo Scientific Trace 1310 oven coupled to a Thermo Scientific ISQ-LT mass spectrometer (single quadrupole) operated in electron ionization mode.

RESULTS

In the fumigation trials, none of the essential oils were a significant deterrent, to the spiders, although the citrus essential oil provided interesting results suggesting a potential trend that might need further research (Table 1).

Table 1. Y-maze fumigation trial results. N=30 for Citrus and Marigold. N=15 for Hedge.

EO	*Repelled*	*Not Repelled*	*p-value*
Citrus	20	10	0.099
Marigold	16	14	0.856
Hedge	7	8	1.00

In the essential oil contact trials, the Marigold EOs were shown to be an effective repellant against the spiders. The p-value for Marigold was .05<, meaning the null hypothesis is rejected. The remaining p-values can be seen in Table 2.

The results of the GC/MS for the EOs showed d-Limonene to be the major component of navel oranges. The major components of marigold flowers were (1S)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene, 3-Carene, Cedrol and a-Terpinyl acetate)

Table 2. Filter paper contact trial results. Null hypothesis and average time in seconds. N=30 for Citrus and Marigold. N=15 for Hedge.

EO	Null Hypothesis	Average Time	p-value
Citrus	90	76.35±14.3	0.236
Marigold	90	66.67±12.8	0.040
Hedge	90	103.53±21.6	0.881

DISCUSSION

The EOs from navel oranges were determined to be ineffective as a wolf spider repellant, but the fumigation trials did create some hope for possible future studies. In a GC/MS the major component of the oranges was determined to be d-Limonene at 92.56%. D-Limonene is classified under a group of organic compounds commonly found in essential oils called monoterpenes and is common in citrus fruits (Yoon et al. 2007). In fact, there have been previous studies in which the repellent properties of similar citrus fruits are investigated against other arthropods (Camara 2015). When EOs and their compounds were tested for their repellent properties against *Tetranychus urticae*, a type of arachnid, d-Limonene was the most effective repellant among the monoterpene hydrocarbons tested (Camara 2015). Additional studies have shown the repellent activity of specific compounds when mixed with d-Limonene to increase (Nasrul et al 2018.), which offers a possible explanation for the results of this study. Perhaps citrus oil, which is comprised of mostly d-Limone, possesses little spider repellant activity when presented by itself but when presented in the form of a mixture the repellant properties become amplified.

In a similar fashion to the orange EOs, the EOs from marigold flowers showed no evidence of overlap in their ability to repel wolf spiders through fumigation and contact. However, Marigold EOs appeared to effectively repel the spiders in the 30 contact trials. Perhaps this lack of overlap in the data points toward a question that scientists have yet to discover. That is, the distinct line between olfaction sense and taste-by-thouch sense in spider sensory systems (Foelix 1996). It has been found that insects possess fine pores in the walls of olfactory hairs that allow the detection of airborne chemicals. A similar sensilla have yet to be found in spiders (Barth 2002). Where are these organs possibly located? In previous studies it has been suspected that the tip pore sensilla which are used for direct contact reception, are the same sensilla used for olfaction (Ganske and Uhl 2018). Perhaps the results to this study are evidence that the same sensilla hairs are used for both forms of chemoreception, however, the hairs are simply more sensitive to direct contact with chemicals.

A second scenario to consider in terms of how spiders differentiate between gustation and olfaction is the possibility that spiders may use a high order differentiation of tip-pore sensilla based on their position on the body (Ganske and Uhl 2018). Put simply, spiders would be able to use the same sensilla for either gustation or olfaction based on their location on the spider. This hypothesis would confer the results of the repellency of hedge apples, especially. Since the spiders showed to action of being repelled by the EOs of hedge apples, this proposed theory of sensory can be considered.

Overall, the results of this study have provided a possible avenue for further research into essential oils as spider repellants. While only one of the three oils tested showed significant repellant properties, the oils were only tested at one concentration. The dilution of 50 times was randomly chosen due to lack of overall amount of EOs. Perhaps the key to essential oils lies in the concentration of the oil used. Future studies could include beginning with a high concentration and gradually decreasing in order to find the optimal dilution of the EOs.

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