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Insecticidal Activities of Compounds from Sweet Flag (*Acorus calamus*) against Red Imported Fire Ants *Solenopsis invicta* (Hymenoptera: Formicidae)

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Abstract

Due to public health and environmental issues, alternatives of synthetic pesticides have been researched for a long time. We evaluated the toxicity and repellency of the sweet flag (*Acorus calamus* L.) powder and two bioactive compounds (α-asarone and β-asarone) against workers of the red imported fire ant (RIFA), *Solenopsis invicta* Buren under laboratory conditions. Sweet flag powder applied at 1 mg/cm^2 or more provided 100% ant mortality within 18 hours, and repelled almost 97% of ants within one hour. Beta-asarone was the faster acting compound against RIFA compared to α-asarone and sweet flag powders. The LT_{50} values inclined exponentially with the increase in the application rate of the test items. On the other hand, repellency did not increase with the application rate of the test items, but did increase with the exposure time. Based upon the results of this study, α-asarone and β-asarone, as well as sweet flag powders could be another alternative tool to control the RIFA.

Introduction

Due to public health and environmental issues, alternatives of synthetic pesticides have been researched for a long time. Many plants and plant-based products have showed good efficacy against numbers agricultural and urban insect pests. Among those, sweet flag (*Acorus calamus* L.) has been reported to be effective against various agricultural and urban insect pests (Rahman & Schmidt, 1999; Hasan et al., 2006; Park et al., 2003; Melani et al., 2016). Sweet flag is native to Asia and Europe and can now be found in most countries as well. In ancient Asian medicine it is used as a "rejuvenation" for the brain and nervous system, and as a remedy for digestive disorders (Balakumbahan et al., 2010).

The red imported fire ant (RIFA), *Solenopsis invicta* Buren, native to South America is also distributed in New Zealand, Australia, China, Taiwan and many other countries (Ascunce et al., 2011; Kafle et al., 2011). After practicing chemical control of RIFA for decades, now USA is moving towards the Integrated Pest Management (IPM) of RIFA. The IPM treatment consisted of the release of two biological control agents, the decapitating phorid fly, *Pseudacteon tricuspis* Borgneier, and a pathogen of fire ants, *Thelohania solenopsae* Knell, Allen and Hazard including the application of granular fipronil. IPM practices have resulted in the significant control of RIFA over a time (Oi et al., 2008).

While searching for the alternative chemical control of RIFA, many studies has been conducted. The indigenous cinnamon (*Cinnamomum osmophloeum* Kanehira) leaf essential oil and trans-cinnamaldehyde had an excellent inhibitory effect in controlling RIFA (Cheng et al., 2008). Similarly, the toxicity and repellency of the clove powder, clove oil and bioactive compounds of clove oil (*Syzygium aromaticum* (L.) Merrill & Perry) reported that eugenol, eugenol acetate, as well as β-caryophyllene and clove oil were very effective against RIFA (Kafle & Shih, 2013).

Essential oil from mint oil and wintergreen are also reported as being toxic to fire ants (Appel et al., 2004; Tang et al., 2013). Bioactive compounds from *Tephrosia vogelii* Hook. f. (α-pinene, thujene, d-limonene), *Artemisia annua* L. (cineole, d-camphor, α-terpineol, l (-)-borneol) and *Pronephrium megacuspe* (Bak.) Holtt. (phenol-3-O-beta-D-glucoside) are reported as repellents and toxicants to fire ants (Li et al., 2014; Zhang et al., 2014; Huang et al., 2016).

Similarly, essential oils of *Cymbopogon nardus* (L.) Rendle, *Cinnamomum osmophloeum, Ilex purpurea* Hassk., *Capsicum annum* L., *Mentha longifolia* (L.) Huds., *Cedrus deodara* (Roxb.) G. Don, *Cinnamomum camphora* (L.) J. Presl., *Artemisia annua*, *Eucalyptus globulus* Labill. and *Artemisia argyi* H. Lév. and Vaniot are also reported as repellents against fire ants (Wang et al., 2012; Tang et al., 2013; Wang et al., 2014).

Although a number of nature-based products could be toxic or repellent to RIFA, there are no reports on the repellency and mortality of sweet flag powder and the bioactive compounds of sweet flag against the RIFA. Therefore, a series of studies were conducted to determine the toxicity and repellency of sweet flag and two bioactive compounds (α-asarone and β-asarone) from sweet flag against RIFA under laboratory conditions.

Materials and methods

Source of S. invicta

Solenopsis invicta polygyne colonies were collected from Hsinchu County, Taiwan. The field collected ants were separated from soil using the methods reported by Chen (2007) and reared under laboratory conditions for at least one week prior to conducting the experiments at ambient temperature and relative humidity, 27 ± 1°C and 50 ± 3% RH, respectively, under a 14:10 LL:DD photoperiod (Kafle et al., 2010; Kafle & Shih, 2013). This study was conducted in Laboratory of Extension Entomology and Science Education, Department of Entomology, National Taiwan University, Taipei, Taiwan.

Sweet flag powder and bioactive compounds

Dried sweet flag tubers were bought from local market in Butwal, Nepal and washed with tap water and dried under room conditions for 48 h and then ground to a fine powder (< 0.8 mm). Alpha-asarone and β-asaroneare are the most common bioactive compounds of the sweet flag (Lee et al., 2002; Yao et al., 2007; Liu et al., 2013) and were purchased from Sigma-Aldrich (Sigma-Aldrich China, Inc., China). A 10% stock solution of each bioactive compounds was prepared separately using methyl alcohol as solvent and stored at 5ºC for future uses.

Toxicity tests

To determine the toxicity of the sweet flag powder, α-asarone and β-asarone against the RIFA, methods reported by Cheng et al. (2008) and Kafle and Shih (2013) were applied with some modifications.

Finely grounded sweet flag powder was used during this study. Beakers (9 cm dia.) were used for the toxicity tests of the test materials. The inside vertical wall of each beaker was coated with a Teflon emulsion to prevent the RIFA from escaping (Kafle & Shih, 2013).

Sweet flag powder was dusted on the bottom surface inside the beaker and then 20 RIFA workers were transferred into each beaker. The 0.021 g, 0.064 g, 0.191 g, 0.573 g and 0.785 g of sweet flag powder was sprayed on the beaker those were equals to the five concentrations (0.33, 1, 3, 9 and 12 mg/cm^2). For the control treatment, the beaker only contained ants but no sweet flag powder.

To determine the toxicity of α-asarone and β-asarone, compounds were sprayed according to the tests on the inside bottom surface of the beaker. Then kept under an exhaust hood until the sprayed chemical had dried. Then, 20 RIFA workers were transferred into each beaker containing a test chemical. The 0.21 ml, 0.64 ml and 1.91 ml of stock solution of α-asarone and β-asarone applied separately on each beaker those were equal to the three concentrations (0.33, 1 and 3 mL/cm^2). The beakers for the control treatment were only sprayed with solvent.

Ant mortality was determined by counting and removing dead ants every 30 min for 24 h. During the tests, only water was provided to the fire ants.

Repellency tests

To determine the repellency of sweet flag powder, α-asarone and β-asarone against RIFA, methods reported by Appel et al. (2004) and Kafle and Shih (2013) were applied with some modifications. The repellency of each test item was determined using 9 cm dia. glass Petri dishes. The inside of upper vertical wall of each Petri dish was coated with a Teflon emulsion to prevent the ants from escaping.

The sweet flag powder was distributed uniformly over one-half of the bottom of each Petri dish, and the other bottom half remained untreated. The sweet flag powder was tested at four concentrations (0, 0.33, 1 and 3 mg/cm^2) to get those concentrations, 0 g, 0.012 g, 0.032 g and 0.095 g of sweet flag powder was applied separately per Petri dish. and ten worker ants were tested in each treatment. The ants were able to move freely between the treated and untreated surfaces. Each Petri dish containing ants were then uncovered and exposed to the air. The number of ants on each side (sweet flag powder treated and the untreated glass) of each Petri dish were counted every 10 min for 60 min after which the ants were released into the dishes.

For the repellency evaluation of the bioactive compounds, the same process as for sweet flag powder was followed, but the test compounds were sprayed on the bottom surface inside the Petri dish using pipettes and dried under the exhaust hood before 10 ants were transferred to the 9 cm Petri dish containing the test compound. Since the mortality of α-asarone and β-asarone at 1 mL/cm² were significantly higher than 0.33 mL/cm² but statistically similar to 3 mL/cm², therefore only the repellency of 1 mL/cm² was evaluated for both test items against fire ants. To get the 1 mL/cm² concentration of test compounds for this test 0.32 ml of 10% stock solution sprayed on half of the Petri dish (9 cm dia.). The Petri dishes for the control study were only sprayed with solvent.

The toxicity tests of sweet flag powder were replicated four times, and the toxicity and repellency of the bioactive compounds (α-asarone and β-asarone) were replicated three and five times, respectively each time using ants from different colonies. Total 20 worker ants were used for toxicity tests and 10 worker ants were used for repellency tests. The studies were conducted under ambient temperature and relative humidity, averaging 27 ± 1°C and 50 ± 3% RH, respectively, under a 14:10 LL:DD photoperiod.

Data analysis

Means were compared using Tukey's HSD test, and the lethal time (LT_{50}), which is the time (hours) required for 50% of the ants to die, were estimated by probit analysis (SAS, 2015).

Results

Toxicity of sweet flag powder

Five different concentrations of sweet flag powder were evaluated against fire ants. At 24 h after treatment (HAT), all the treatments had killed a significantly higher number of ants than the control (F = 154.62, df = 5, p = 0.001). Beakers containing 12 mg/cm² of sweet flag powder had the lowest LT_{50} values among all the treatments. When the amount of sweet flag powder was increased from 0.33 mg/cm² to 12 mg/cm², the LT_{50} value was reduced 7.7 folds and mortality of the ants increased from 87.5% to 100% at 24 HAT (Table 1).

Repellency of sweet flag powder

When three different concentrations of sweet flag powder were evaluated for 1 h for its repellency against fire ants, significantly more ants were observed on the untreated half of the Petri dishes than the sweet flag powder-treated half of the Petri dishes at all the concentrations. However, at the control treatments, the numbers of ants on both halves were not significantly different (F = 33.39, df = 3, p = 0.001). The mean repellency of ants ranged from 97.1% to 98.8% when the application rate of sweet flag powder was increased from 0.33 mg/cm² to 3 mg/cm² (Table 2).

Table 1. Toxicity of sweet flag powder against *Solenopsis invicta* workers.

Application rates	No. of ants died (Mean ± SE)[1]	LT_{50}[2] (h)
0.33 mg/cm²	17.5 ± 1.5a	15.61
1 mg/cm²	20 ± 0a	7.99
3 mg/cm²	20 ± 0a	5.53
9 mg/cm²	20 ± 0a	2.66
12 mg/cm²	20 ± 0a	2.03
0 mg/cm²	0.75 ± 0.25b	

[1] Means within the same column followed by the same letter are not significantly different (p < 0.05) (Tukey's HSD test, SAS, 2015).
[2] LT_{50} values (h) were determined by probit analysis (SAS, 2015).

Table 2. Repellency of sweet flag powder to the *Solenopsis invicta* workers.

Application rates	No. of ants (Mean ± SE)[1]	
	Treated area	Non-treated area
0.33 mg/cm²	0.13 ± 0.13bB	9.87 ± 0.13aA
1 mg/cm²	0.29 ± 0.29bB	9.71 ± 0.29aA
3 mg/cm²	0.29 ± 0.17bB	9.71 ± 0.17aA
0 mg/ cm²	4.46 ± 0.64aA	5.54 ± 0.64bA

[1] Means within the same column (lower case) and same row (upper case) followed by the same letter are not significantly different (p < 0.05) (Tukey's HSD test, SAS, 2015).

Toxicity of the bioactive compounds from sweet flag

Three different concentrations of α-asarone were evaluated against fire ants. At 24 HAT, all treatments had killed a significantly higher number of ants than the control (F = 31.05, df = 3, p = 0.001). Beakers containing 3 mL/cm² α-asarone had the lowest LT_{50} values among all the treatments. When the amount of α-asarone was increased from 0.33 mL/cm² to 3 mL/cm², the LT_{50} value was reduced 3.1 folds and mortality of the ants increased from 50% to 100% at 24 HAT (Table 3).

Three different concentrations of β-asarone were evaluated against fire ants. At 24 HAT, all the treatments had killed a significantly higher number of ants than the control (F = 26.02, df = 3, p = 0.002). Beakers containing 3 mL/cm² of β-asarone had the lowest LT_{50} values among all the treatments. When the amount of β-asarone was increased from 0.33 mg/cm² to 3 mL/cm², the LT_{50} value was reduced 2.9 folds and mortality of the ants increased from 80% to 100% at 24 HAT (Table 3).

When the mortality of fire ants for α-asarone and β-asarone were compared for the 0.33 mL/cm² at 24 HAT, β-asarone killed a significantly higher number of ants than the α-asarone (F = 13.50, df = 1, p = 0.021). The application of 0.33 mL/cm² of α-asarone and β-asarone killed 50% and 80% of the ants, respectively and β-asarone's ant killing speed was 10% faster rate than the α-asarone (Table 3).

When the mortality of fire ants for α-asarone and β-asarone were compared for the 1 mL/cm² of α-asarone and β-asarone at 24 HAT, number of ants killed by α-asarone and β-asarone were not significantly different to each other (F = 0.47, df = 1, p = 0.53). The application of 1 mL/cm² of α-asarone and β-asarone killed 88.4 % and 80% of the ants, respectively and β-asarone's ant killing speed was faster 16% rate than the α-asarone (Table 3).

Table 3. Toxicity of α-asarone and β-asarone against *Solenopsis invicta* workers.

Application rates	No. of ants died (Mean ± SE)[1]		LT_{50}[2] (h)	
	α-asarone	**β**-asarone	**α**-asarone	**β**-asarone
0.33 mL/cm²	10 ± 2.65 b	16 ± 1.53 a	8.45	7.63
1 mL/cm²	16.00 ± 0.58 a	17.67 ±2.33 a	6.98	4.05
3 mL/cm²	20 ±0 a	20 ±0 a	2.77	2.61
0 mg/cm²	3.33±0.88 c	0.33±0.88 b		

[1] Means within the same column followed by the same letter are not significantly different ($p < 0.05$) (Tukey's HSD test, SAS 2015).
[2] LT_{50} values (h) were determined by probit analysis (SAS, 2015).

When the mortality of fire ants for α-asarone and β-asarone were compared for the 3 mL/cm² of α-asarone and β-asarone at 24 HAT, 100% ants were killed by both tests items and β-asarone's ants killing speed was 6% faster rate than the α-asarone (Table 3).

Repellency of the bioactive compounds in sweet flag

When the application rate of 1 mL/cm² of the compounds (α-asarone and β-asarone) were evaluated against fire ants for 1 h, the mean repellency of ants were 92.7% and 96.7% for α-asarone and β-asarone, respectively. However, in the control treatments, the number of ants in both halves was not significantly different ($F = 18.21$, $df = 1$, $p = 0.002$) (Table 4).

Table 4. Repellency of sweet flag powder to the *Solenopsis invicta* workers.

Test items (1 mL/cm²)	No. of ants (Mean ± SE)[1]	
	Treated area	Non-treated area
α-asarone	0.93±0.27bB	9.27±0.27aA
β-asarone	0.33±0.15bB	9.67±0.15aA
Control	5.93±1.23aA	4.07±1.23aB

[1] Means within the same column (lower case) and same row (upper case) followed by the same letter are not significantly different ($p < 0.05$) (Tukey's HSD test, SAS, 2015).

Discussion

Many plant-based products were reported as being effective against fire ants (Appel et al., 2004; Cheng et al., 2008; Wang et al., 2012; Tang et al., 2013; Li et al., 2014; Wang et al., 2014; Zhang et al., 2014; Huang et al., 2016). The present study demonstrated that sweet flag powder is also an effective plant-based product that not only kills ants, but also repels them. Sweet flag powder and its two bioactive compounds also repelled fire ants even at a very low application rate. During this study, the level of repellency of all test items increased with the exposure time.

The sweet flag powder is not only toxic to the fire ants but also for many insect pests as well (Balakumbahan et al., 2010; Patil & Chavani, 2010; Kumar et al., 2015). All these studies have proved that sweet flag powder is an effective tool to control different insect pests. The application of only 0.33 mg/cm² sweet flag powder could control almost 88% of fire ants and if the application rate was increased to 1 mg/cm² the fire ant control rate reached up to 100% within 24 HAT. The LT_{50} values showed that the rate of application of sweet flag powder and the numbers of ants killed were directly correlated (Table 1).

During this study, we also found that applying only 0.33 mL/cm² of α-asarone and β-asarone could control 50-80% % of fire ants and if the application rate increased to 3 mL/cm² the fire ant control rate also increased to 100% within 24 HAT. The LT_{50} values showed that the rate of application of α-asarone and β-asarone and the numbers of ants killed were directly correlated. This study proved that bioactive compounds from sweet flag are toxic to fire ants.

Limited studies have been reported on efficacy of sweet flag powder, oil or its bioactive compounds against ants. Most of the studies were focused on determining the efficacy of sweet flag powder, oil or its bioactive compounds against stored insect pests. Schmidt and Streloke (1994) reported that treatment with only 0.01% sweet flag oil could reduce grain feeding by *Prostephunus truncutus* (Horn) by 50%. Similarly, when Melani et al. (2016) used sweet flag oil against third instar larvae of *S. litura*, the toxicity and antifeedant activity values were 92.5% and 79.3%, respectively, with an LC_{50} value 586.96 ppm. Moreover, when Hasan et al. (2006) applied sweet flag oil vapors against grubs of *Trogaderma granarium* (Everts), they observed that the exposure period is the most important factor affecting the toxic effect of the sweet flag oil rather than the dosage.

Tang et al. (2013) reported that the application rate of essential oils and mortality of fire ants were directly correlated. Higher the application rates of essential oils, higher the mortality rate of fire ants during their study conducted. That similarity was also observed during this study. Huang et al. (2016) and Zhang et al. (2014) also found the similar effects on mortality of *S. invicta* by different bioactive compounds or essential oils during their study.

Both α-asarone and β-asarone are lipophilic in nature (Shenvi et al., 2011). Kafle and Shih (2013) hypothesized that lipophilic compounds were absorbed into the cuticular lipids of the fire ants and then slowly entered into the hemocoel and nervous system. Besides that, it was also assumed that these compounds were absorbed into the tracheal system (Appel et al., 2004; Cheng et al., 2008; Kafle & Shih, 2013). Paneru et al. (1997) reported that both α-asarone and β-asarone were genotoxic at high concentrations in cultured rat hepatocytes and hepatocarcinogenic in pre-weaning mice. Hasan et al. (2006) reported that β-asarone served as a contact and stomach poison. Oh et al. (2004) confirmed that sweet flag extracts inhibit acetylcholinesterase, while Melani et al. (2016) further explained that β-asarone is a contact poison that penetrates the insect body through the cuticle layer towards hemolymph, affecting the nervous system by targeting acetylcholinesterase. Due to a series of physiological and chemical actions and reactions the insect becomes spastic and paralyzed, and then eventually dies. Sharma et al. (2008) reported that when ingested, β-asarone can damage the insect's intestinal wall which can lead to death. Beta-asarone penetrates and disrupts the function of the mesenteron - a tissue layer composed of epithelial cells that absorb nutrients and secrete digestive enzymes in insects.

Both α-asarone and β-asarone displayed delayed toxicity having LT_{50} value at least 2.6 h even for highest concentrations. Alpha-asarone, β-asarone and sweet flag powder have low mammalian toxicity and are nature-based substances. The repellency and toxicity of α-asarone, β-asarone and sweet flag powder to the fire ants could be potentially useful in a comprehensive integrated pest management program.

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