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# Insecticidal activity of the methanol extract of *Pronephrium megacuspae* (Thelypteridaceae) and its active component on *Solenopsis invicta* (Hymenoptera: Formicidae)

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## Abstract

Baits were used to investigate the toxicity of methanol extracts from the fern *Pronephrium megacuspae* (Thelypteridaceae) on workers of the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). The methanol extract of *P. megacuspae* had toxic effects on workers. With macrergates, the LC50 values of the methanol extract and ethyl acetate fraction were 524.0 and 145.9  $\mu\text{g/g}$ , respectively, after 48 h of treatment and 362.5 and 99.0  $\mu\text{g/g}$ , respectively, after 72 h of treatment. With micrergates, the LC50 values of the methanol extract and ethyl acetate fraction were 321.9 and 90.0  $\mu\text{g/g}$ , respectively, after 48 h of treatment and 235.4 and 79.1  $\mu\text{g/g}$ , respectively, after 72 h of treatment. NMR spectral data revealed that the isolated compound from the ethyl acetate soluble fraction of methanol extract was phenol-3-O-beta-D-glucoside. The LC50 values of this compound with macrergates after 48 and 72 h were 21.6 and 9.7  $\mu\text{g/g}$ , respectively. Similarly, the LC50 values of this compound with micrergates after 48 and 72 h were 3.4 and 2.4  $\mu\text{g/g}$ , respectively. A low dose of methanol extract and ethyl acetate fractions of *P. megacuspae*, and of phenol-3-O-beta-D-glucoside diminished the walking and grasping abilities of *S. invicta* micrergates and macrergates.

Key Words: worker ant; secondary metabolite; fern

## Resumen

Se utilizaron cebos para investigar la toxicidad de los extractos de metanol del helecho *Pronephrium megacuspae* (Thelypteridaceae) sobre las trabajadoras de la hormiga roja de fuego importada, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). El extracto de metanol de *P. megacuspae* tuvo efectos tóxicos sobre las trabajadoras. Con macrergates, los valores de LC50 de la fracción de extracto de metanol y acetato de etilo fueron 524,0 y 145,9 mg/g, respectivamente, después de 48 h de tratamiento y 362,5 y 99,0  $\mu\text{g/g}$ , respectivamente, después de 72 h de tratamiento. Con micrergates, los valores de LC50 de la fracción de extracto de metanol y acetato de etilo fueron 321,9 y 90,0  $\mu\text{g/g}$ , respectivamente, después de 48 h de tratamiento y 235,4 y 79,1 g / g, respectivamente, después de 72 h de tratamiento. Los datos espectrales de RMN reveló que el compuesto aislado de la fracción de acetato de etilo-soluble de extracto de metanol se extrajo con fenol-3-O-beta-D-glucósido. Los valores de LC50 de este compuesto en macrergates después de 48 y 72 horas fueron de 21,6 y 9,7 mg/g, respectivamente. Del mismo modo, los valores de LC50 de este compuesto con micrergates después de 48 y 72 h fueron 3,4 y 2,4  $\mu\text{g/g}$ , respectivamente. Una dosis baja de extracto de metanol y acetato de etilo fracciones de *P. megacuspae*, y de fenol-3-O-beta-D-glucósido disminuyen las habilidades de caminar y de agarre de los micrergates y macrergates trabajadoras de *S. invicta*.

Palabras Clave: hormiga del trabajador; metabolitos secundarios; helecho

*Solenopsis invicta* Buren (Hymenoptera: Formicidae), commonly known as red imported fire ant, is an important invasive species known to cause a painful and persistently irritating sting that often leaves a pustule on the skin. Although this species is native to South America, it was found in Taiwan in 2003 (Zhang et al. 2007) and in mainland China in 2004 (Zeng et al. 2005). Since then, *S. invicta* has rapidly spread in southern China, including Guangdong, Guangxi, Hunan, Fujian, and Jiangxi, occupying at least 71 km<sup>2</sup> (Zhang et al. 2007).

*Pronephrium megacuspae* (Thelypteridaceae) is a fern growing in thick forests at an elevation of 130 to 400 m asl. This species is distributed in the provinces of Jingxi, Guangdong, Guangxi, and Yunnan in China, and is also found in Vietnam, Thailand, and Japan. *Pronephrium megacuspae*

contains active compounds that can be used as bioactive agents against pests. For example, the methanol extract of *P. megacuspae* has toxic effects on adults of *Musca domestica* L. (Diptera: Muscidae), on larvae of *Aedes albopictus* Skuse (Diptera: Culicidae), *Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae), *Plutella xylostella* L. (Lepidoptera: Plutellidae), and *Spodoptera litura* F. (Lepidoptera: Noctuidae), and on nymphs of *Myzuspersicae* (Sulzer) (Hemiptera: Aphididae). These toxic effects are attributed to the components of *P. megacuspae* methanol extract, including 7-hydroxy-5-methoxy-6,8-dimethyl-flavanone; 2',4'-dihydroxy-3'-methyl-6'-methoxychalcone; 5,7-dihydroxy-6,8-dimethylflavanone; and 5-hydroxy-6,7-dimethoxyflavone. At 50 mg/L, these compounds have insecticidal activity on *O. furnacalis* larvae (Huang 2008).

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In this study, *P. megacuspe* methanol extract showed good insecticidal activities on red imported fire ant workers. The active compound of *P. megacuspe* on fire ant workers was isolated and identified, and a *P. megacuspe* based bait was prepared. Toxicity and behavioral effects of this compound on fire ants were evaluated. Our results showed that *P. megacuspe* is a novel potential source of insecticidal baits to control fire ants.

## Materials and Methods

### PLANT MATERIAL

Fresh whole plants of *P. megacuspe* were collected in Shiwandashan Mountain of Guangxi Zhuang Autonomous Region of China in April 2006. The plant materials were air dried naturally, ground with a mill to powder form (400  $\mu\text{m}$ ), and stored in hermetically sealed plastic bags at room temperature before extraction.

### EXTRACTION AND ISOLATION

Dried plant extracts were obtained by 3-stage percolation. *Pro-nephrium megacuspe* powder (7.5 kg) was extracted 3 times with 20 L of methanol at room temperature for 72 h. The filtrate was concentrated using a rotary evaporator at 40 °C until dryness. The resulting mixture was re-suspended in 1.5 L water and partitioned 4 times with 1.5 L of ethyl acetate to produce 0.23 kg of dried organic extract. The methanol extract and the ethyl acetate soluble fraction were subjected to bioactivity assays on fire ant micrergates and macrergates, and the latter had the highest potency. The ethyl acetate extract (230 g) was subjected to Si-gel column chromatography (10 cm  $\times$  120 cm, 200 mesh to 300 mesh, 1.2 kg) eluted with  $\text{CHCl}_3$ -MeOH with gradient polarities of 100:0, 20:1, 10:1, 5:1, and 1:1.

### ACTIVE CONSTITUENT OF *P. MEGACUSPE*

The active compound was isolated with thin layer chromatography, column chromatography, gel chromatography, and recrystallization, with different insecticidal activities previously evaluated against other insects (Huang et al. 2012) as follows: (1) toxicity of methanol extract against adults of *M. domestica*, 4th instar larvae of *Ae. albopictus*, and nymphs of *M. persicae*; (2) antifeedant activities of methanol extract against larvae of *P. xylostella*, *O. furnacalis*, and *Pieris rapae* L. (Lepidoptera: Pieridae); (3) toxicity of different fractions of the methanol extract against adults of *M. domestica* and larvae of *Ae. albopictus*; (4) antifeedant activity of the ethyl acetate fraction against 3rd instar larvae of *M. domestica*. The structure of the isolated compound was determined by spectroscopic analyses ( $^1\text{H}$  and  $^{13}\text{C}$  NMR) and by direct comparison with an authentic reference compound.

### ORIGIN AND REARING OF RED IMPORTED FIRE ANT

*Solenopsis invicta* colonies were collected from the suburbs of Guangzhou and maintained in our laboratory for bioassays (Lu et al. 2006; Huang et al. 2007). The ants were fed with a mixture of 10% honey and live insects (*Tenebrio molitor* L.; Coleoptera: Tenebrionidae). A test tube (25 mm  $\times$  200 mm), partially filled with water and then plugged with cotton, was used as a water source. The ants were maintained in our laboratory at  $25 \pm 2$  °C and 80% relative humidity.

### PREPARATION OF RED IMPORTED FIRE ANT BAIT

Bait material (700  $\mu\text{m}$  to 1500  $\mu\text{m}$  particles) was prepared with maize powder, peanut oil, fishbone powder, and carbohydrates in ac-

cordance with previously described methods (Kafle et al. 2010; Zhang 2013). Methanol extract, ethyl acetate fraction, or compound 26 (Kazuma et al. 2000; see Results section) was dissolved in a small volume of acetone. The solution was mixed uniformly with the bait. Methanol extract, ethyl acetate fraction, and compound 26 based baits were prepared after acetone had volatilized completely.

### INSECTICIDAL TOXICITY BIOASSAY

A toxicity bioassay was performed in accordance with a previously described method (Zeng et al. 2006). Worker ants, a water-filled test tube, and prepared baits were placed in a disposable plastic cup (top diameter, bottom diameter, height: 62 mm, 40 mm, 60 mm); the vertical wall of this cup was coated with a fluon emulsion. Each treatment was replicated 3 times, and each replicate included 10 worker ants. Acetone-based bait was used as a control. The worker ants were maintained at 24 to 26 °C and 60 to 80% relative humidity.

### WALKING ABILITY OF WORKER ANTS

Worker ants were treated as described for the toxicity bioassay. The walking behavior of worker ants was observed with previously described methods (Zhang et al. 2007; Li et al. 2014). Each treatment was replicated 3 times, and each replicate included 10 worker ants. In brief, worker ants were placed on A4 paper. We considered that an ant could walk when it walked continuously for 10 s without falling. Walking ability was quantified as follows: walking rate = number of worker ants possessing walking ability/number of worker ants per replicate  $\times$  100.

### GRASPING ABILITY OF WORKER ANTS

Worker ants were treated as described for the toxicity bioassay. The grasping ability of worker ants was observed in accordance with a previously described method (Li et al. 2014). Each treatment was replicated 3 times, and each replicate included 10 worker ants. Worker ants were placed on A4 paper; after 10 s, the paper was gently turned over at 180°. We considered that an ant could grasp when it did not fall off the paper. Grasping ability was quantified as follows: grasping rate = number of worker ants possessing grasping ability/number of worker ants per replicate  $\times$  100.

### STATISTICAL ANALYSES

The proportions of ants dying, walking, and grasping the substrate were transformed to arcsine square root values for analysis with ANOVA. Data were expressed as means  $\pm$  SE.

## Results

### CHEMICAL STRUCTURE OF *P. MEGACUSPE*

The compound that we extracted appeared as a yellow needle-shaped crystal; the characteristics of the compound were the same as those of compound 26, phenol-3-O-beta-D-glucoside (Kazuma et al. 2000). Analysis by electrospray ionization mass spectrometry revealed quasi-molecular ion peaks at 448  $[\text{M}+\text{H}]^+$  consistent with those of a compound with the molecular formula  $\text{C}_{21}\text{H}_{20}\text{O}_{11}$ . This formula was confirmed by high-resolution electrospray ionization mass spectrometry.  $^{13}\text{C}$  NMR data (150 MHz,  $\text{C}_6\text{D}_6\text{N}$ ) were listed as follows:  $\delta_c$  157.3 (C-2), 135.1 (C-3), 178.8 (C-4), 157.6 (C-5), 99.9 (C-6), 166.0 (C-7), 94.6 (C-8), 162.9 (C-9), 105.3 (C-10), 122.0 (C-1'), 131.9 (C-2', C-6'), 116.1 (C-3', C-5'), 161.7 (C-4'), 104.0 (C-1''), 76.2 (C-2''), 78.6 (C-3''), 71.5 (C-4''),

78.1 (C-5"), 62.6 (C-6"). The compound was identified as phenol-3-*O*-beta-D-glucoside (compound 26).

## EFFECTS ON FIRE ANT WORKERS

The toxicities of methanol extract, ethyl acetate fraction, and compound 26 based baits on red imported fire ant macrergates and micrergates were determined (Tables 1–3). The results showed that all 3 baits had toxic effects on workers. Furthermore, exposure to the 3 baits at low dosages reduced the walking and grasping abilities of both macrergates and micrergates (Figs. 1–4). These effects were enhanced with increasing treatment times.

After 120 h, exposure to 68.7 and 42.0 µg/g ethyl acetate fraction based baits resulted in 42.8 ± 3.6% mortality of macrergates and 29.9 ± 6.8% mortality of micrergates, respectively. At this time, over 72.6% of fire ant workers were walking successfully and nearly half were able to cling to the substrate.

After 120 h, exposure to 7.9 and 3.4 µg/g compound 26 based baits resulted in 31.8 ± 5.2% mortality of macrergates and 21.2% ± 4.5% mortality of micrergates, respectively. There were 62.1% fire ant work-

ers walking successfully at this time, and over 42.8% fire ant workers were clinging to the substrate successfully.

## Discussion

Poisonous baits are among the most effective tools to control fire ants. However, poisonous baits are a threat to human health and the environment because these baits contain high amounts of toxic chemicals (Vogt et al. 2002). Therefore, researchers should strive to develop highly efficient and highly specific poisonous baits that exhibit minimal toxicity to non-target organisms.

We here investigated the potential use of *P. megacuspe* as a pesticide against red imported fire ant. Our results showed that the methanol extract, ethyl acetate fraction, and the identified compound 26 exerted significant insecticidal activities on fire ant workers. These substances also negatively affected the walking and grasping abilities of fire ant workers at low concentrations: although over 80% of fire ant workers remained alive, their walking and grasping abilities were reduced. As treatment times were increased, the effects were enhanced.

**Table 1.** The LC20 and LC50 values of methanol extract, ethyl acetate fraction, and compound 26 (phenol-3-*O*-beta-D-glucoside) from *Pronephrium megacuspe* on red imported fire ant macrergates.

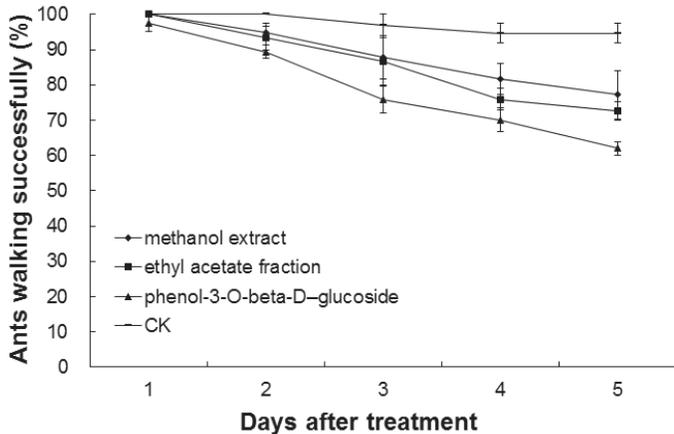
Treatment	Time (h)	Toxicity regression equation	LC20 (µg/g)	LC50 (µg/g)	Confidence interval	Correlation coefficient
Methanol extract	24	$y = 0.4573 + 1.5470$	246.9	864.0	554.4–1,346.5	0.9735
Ethyl acetate fraction	24	$y = 1.5378 + 1.4263$	68.7	267.5	169.8–421.4	0.9891
Compound 26	24	$y = 2.7268 + 1.0519$	23.0	144.9	26.0–808.6	0.8739
Methanol extract	48	$y = 0.3241 + 1.7195$	169.8	524.0	383.6–715.9	0.9910
Ethyl acetate fraction	48	$y = 1.1525 + 1.7778$	49.1	145.9	109.4–194.6	0.9974
Compound 26	48	$y = 3.0894 + 1.4309$	5.6	21.6	13.7–34.2	0.9875
Methanol extract	72	$y = -0.1385 + 2.0078$	138.1	362.5	280.2–469.0	0.9894
Ethyl acetate fraction	72	$y = 1.1838 + 1.9122$	35.9	99.0	75.1–130.5	0.9828
Compound 26	72	$y = 3.4965 + 1.5254$	2.7	9.7	7.0–13.4	0.9859

**Table 2.** The LC20 and LC50 values of methanol extract, ethyl acetate fraction, and compound 26 (phenol-3-*O*-beta-D-glucoside) from *Pronephrium megacuspe* on red imported fire ant micrergates.

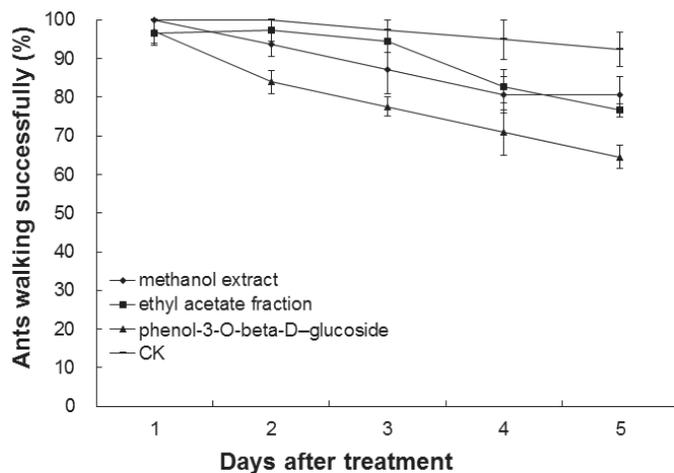
Treatment	Time (h)	Toxicity regression equation	LC20 (µg/g)	LC50 (µg/g)	Confidence interval	Correlation coefficient
Methanol extract	24	$y = -0.0322 + 1.8783$	170.2	477.7	337.3–676.5	0.9825
Ethyl acetate fraction	24	$y = 1.5931 + 1.5799$	42.0	143.4	96.9–212.1	0.9915
Compound 26	24	$y = 3.4141 + 1.4097$	3.4	13.3	7.9–22.6	0.9078
Methanol extract	48	$y = 0.6575 + 1.7317$	105.1	321.9	237.1–437.1	0.9858
Ethyl acetate fraction	48	$y = 1.8207 + 1.6266$	27.4	90.0	65.8–123.2	0.9922
Compound 26	48	$y = 4.3947 + 1.1337$	0.6	3.4	2.2–5.4	0.9920
Methanol extract	72	$y = 0.6048 + 1.8531$	82.7	235.4	178.4–310.5	0.9920
Ethyl acetate fraction	72	$y = 2.1061 + 1.5244$	22.2	79.1	57.2–109.6	0.9848
Compound 26	72	$y = 4.5198 + 1.2536$	0.5	2.4	1.5–4.0	0.9636

**Table 3.** Toxic effects of methanol extract, ethyl acetate fraction, and compound 26 (phenol-3-*O*-beta-D-glucoside) from *Pronephrium megacuspe* on red imported fire ant workers.

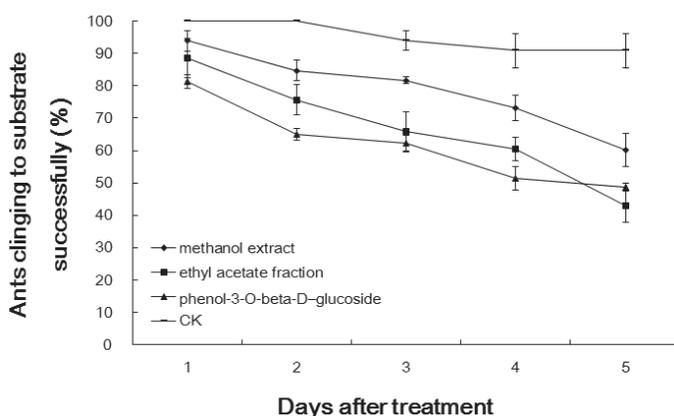
Treatment	Dosage (µg/g)	Worker ant	Corrected mortality (mean % ± SE) over a 5 d period				
			1 d	2 d	3 d	4 d	5 d
Methanol extract	246.9	macrergate	3.3 ± 3.3	6.4 ± 3.2	9.4 ± 0.3	24.8 ± 2.4	28.2 ± 0.9
Ethyl acetate fraction	68.7	macrergate	9.2 ± 0.8	9.2 ± 0.8	11.8 ± 1.8	25.1 ± 7.5	42.8 ± 3.6
Compound 26	23.0	macrergate	7.4 ± 7.4	7.4 ± 7.4	14.4 ± 9.9	28.1 ± 5.9	31.8 ± 5.2
Methanol extract	170.2	micrergate	5.2 ± 2.6	5.2 ± 2.6	7.9 ± 4.8	13.1 ± 7.2	18.5 ± 4.8
Ethyl acetate fraction	42.0	micrergate	6.1 ± 6.1	6.4 ± 3.2	9.7 ± 5.8	29.9 ± 6.8	29.9 ± 6.8
Compound 26	3.4	micrergate	5.6 ± 2.8	5.6 ± 2.8	5.6 ± 2.8	17.9 ± 1.3	21.2 ± 4.5



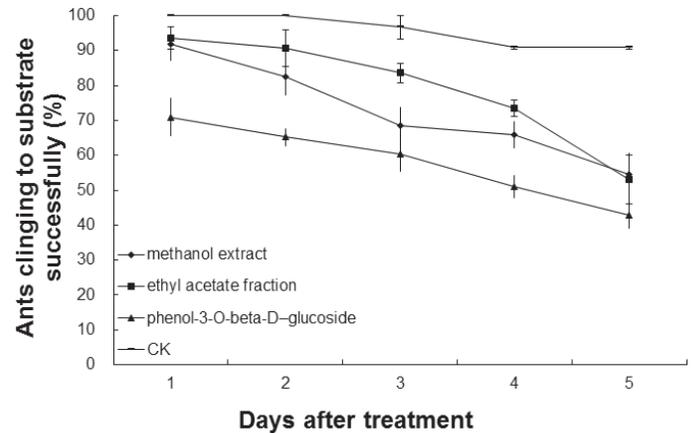
**Fig. 1.** The effect of methanol extract, ethyl acetate fraction, and compound 26 288 (phenol-3-O-beta-D-glucoside) from *Pronephrium megacuspae* on the walking ability of 289 *Solenopsis invicta* macrergates. Each data point represents the mean  $\pm$  SE of 3 replicates. Each 290 replicate contained 10 tested ants. CK = control.



**Fig. 2.** The effect of methanol extract, ethyl acetate fraction, and compound 26 293 (phenol-3-O-beta-D-glucoside) from *Pronephrium megacuspae* on the walking ability of 294 *Solenopsis invicta* micrergates. Each data point represents the mean  $\pm$  SE of 3 replicates. Each 295 replicate contained 10 tested ants. CK = control.



**Fig. 3.** The effect of methanol extract, ethyl acetate fraction, and compound 26 298 (phenol-3-O-beta-D-glucoside) from *Pronephrium megacuspae* on the clinging ability of 299 *Solenopsis invicta* macrergates. Each data point represents the mean  $\pm$  SE of 3 replicates. Each 300 replicate contained 10 tested ants. CK = control.



**Fig. 4.** The effect of methanol extract, ethyl acetate fraction, and compound 26 303 (phenol-3-O-beta-D-glucoside) from *Pronephrium megacuspae* on the clinging ability of 304 *Solenopsis invicta* micrergates. Each data point represents the mean  $\pm$  SE of 3 replicates. Each 305 replicate contained 10 tested ants. CK = control.

These results showed that the toxic effect of the active components of *P. megacuspae* was relatively slow acting. Thus, ant feeding behaviors may not be immediately interrupted, but after some time, the toxicity of these substances could affect the entire ant colony. As a result, the entire colony could be eliminated. This study further revealed that the direct application of *P. megacuspae* methanol extract could control fire ants. In our future studies, the toxic effects of active compounds from *P. megacuspae* combined with synthetic compounds will be evaluated to determine if combined treatments could be used to effectively control fire ants under field conditions.

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