Sociobiology
An international journal on social insects

RESEARCH ARTICLE - TERMITES

Repellent Effects of *Annona* Crude Seed Extract on the Asian Subterranean Termite *Coptotermes gestroi* Wasmann (Isoptera:Rhinotermitidae)

MN Acda

University of the Philippines Los Banos, Philippines.

Article History

Edited by
Og de Souza - UFV, Brazil
Received 11 February 2014
Initial acceptance 28 May 2014
Final acceptance 15 June 2014

Keywords
Annonaceae, repellent, acetogenins, *Coptotermes gestroi*, feeding deterrence

Corresponding author
Menandro N Acda
Department of Forest Products and Paper Science, College of Forestry and Natural Resources
University of the Philippines
Los Banos
E-Mail: mnacda@yahoo.com

Abstract

Crude seed extract of three tropical fruits belonging to the family Annonaceae, viz., sweetsop (*Annona squamosa* L.), soursop (*A. muricata* L.) and biriba (*Rollinia mucosa* Baill.) were investigated for their repellent effects on the Asian subterranean termite *Coptotermes gestroi* Wasmann (Isoptera: *Rhinotermitidae*). Results of laboratory feeding bioassay (choice and no-choice) indicated that crude extract of *A. squamosa, A. muricata* and *R. mucosa* had feeding deterrent effects on *C. gestroi*. Termites showed significant avoidance behavior to filter paper treated with extracts of the three Annona species investigated. Soil barrier test revealed that Annona extracts were able to limit penetration of *C. gestroi* in laboratory tunneling test. The results suggest that *Annona* seed extracts may offer an alternative source of natural insecticide against subterranean termites.

Introduction

Plant extracts have been studied in the past as potential sources of botanical insecticides to control a variety of arthropod pests. Their use for pest management has been regarded as an alternative to synthetic chemical insecticides (Logan et al., 1990; Isman, 2006). A large pool of plant based materials belonging to the families Meliaceae, Rutaceae, Asceraceace, Labiateae, Piperaceae and Annonaceae, among others, have been investigated for their insecticidal properties (Jacobson, 1975; Schoonhoven, 1982; Grainge et al., 1984; Arnason et al., 1989; Van Beek & Breteler, 1993). Plant parts used for screening and evaluation include leaves, flowers, roots, stem, fruit peeling, seeds and bulbs. Studies have shown that plant extracts containing limonoids and terpenoids are effective against various pests such as the Colorado potato beetle (Alford et al., 1987), the brine shrimp larva (*Culex quinquefascintus*) (Magadula et al., 2009), the larvae of the mosquitoes *Aedes aegypti* (Hoe at al., 1998) and *Anopheles stephensi* (Saxena et al., 2004), the diamondback moth, *Plutella xylostella* (Leatemia and Isman 2004a; 2005), the cabbage looper *Trichoplusia ni* (Leatemia & Isman, 2004b), the lepidopteran *Spodoptera frugiperda* (Alvarez Colom et al., 2007), the German cockroach (*Blattella germanica*) (Alali et al. 2000), the adult and egg masses of the snail *Biomphalaria glabrata* (Dos Santos & Santa Ana, 2010).

One potential application of botanical insecticides is the control of subterranean termites. Termites are serious structural pests of homes and wood structures in tropical and sub-tropical regions of the world (Lee, 1971). Total worldwide damage and repair costs due to termite activity had been estimated to be about USD 40 billion annually (Rust & Su, 2012). Recent studies showed that plant derivatives such as pyrethrins, terpenoids, azadirachtin and flavanoids have excellent termiticidal activity (Sharma et al., 1994; Cornelius et al., 1997; Chen et al., 2001; Zhu et al., 2001a, b). One of the best sources of botanical insecticides is tropical Annona species, i.e., members of the custard apple family (Annonaceae). Annona species are important sources of fruit juices, frozen pulp, jelly and ice cream in Southeast Asia and thousands of tons of seeds from these fruits are generated annually from commercial processing plants (Isman, 2006, SCUC 2006).

Open access journal: http://periodicos.uefs.br/ojs/index.php/sociobiology
ISSN: 0361-6525

Seeds of *Annona* species yield upon extraction a mixture of long fatty acid derivatives known as acetogenins (Chang et al., 1998; Polo et al,. 1998; Pettit et al., 2008). Annonaceous acetogenins are widely reported for its high insecticidal, molluscicidal and nematicidal properties (Rupprecht et al., 1990; McLaughlin et al., 1997; Isman, 2006). However, limited studies have been conducted on the efficacy of annonaceous acetogenins against subterranean termites. The present paper reports on the repellent effects of the crude seed extracts of *Anonna squamosa* L., *A. muricata* L. and *Rollinia mucosa* Baill. on the Asian subterranean termite *Coptotermes gestroi* Wasmann (Isopetra: *Rhinotermitidae*).

Materials and Methods

Plant Extracts

Ripe fruits of sweetsop (*A. squamosa*), soursop (*A. muricata*) and biriba (*R. mucosa*) were purchased from several market locations in Laguna province, Philippines. The fruits were harvested from small farms in the area cultivated by local farmers free of applied insecticide. Extraction of seeds to separate bioactive crude components was based on the method described by Leatemia and Isman (2004a). Briefly, seeds from various locations were pooled, washed with water, air dried and ground in a Wiley mill (1.0 mm). One hundred gram of each ground samples were extracted in 200 mL of 95% ethanol (5x) over 5 days by soaking. The suspension was stirred at intervals to facilitate uniform extraction. The supernatants were filtered (Whatman #1) and concentrated in a rotary evaporator under vacuum (35ºC). The concentrated extracts were re-suspended in 95% ethanol then transferred to pre-weigh vials. The ethanol was dried in a fume hood and yield determined by re-weighing the vials to determine extract weight.

Termites

Secondary nests of three active field colonies of the Asian subterranean termite *C. gestroi* were collected from infested buildings in the University of the Philippines Los Banos campus. *C. gestroi* (formerly known as *C. vastator* in the Philippines) is a major structural pest of wood structures in Southeast Asia (Acda, 2004; Lee et al., 2007). The nests were placed in black garbage bags, transported to the laboratory and placed inside 100 liter plastic containers with lids kept in a room at 25° C for three days. Distilled water was sprayed on the sides of the container to keep the relative humidity above 80%. Mature worker (pseudergates beyond the third instar as determined by size) and soldier termites were separated from nest debris by breaking apart and sharply tapping materials into plastic trays containing moist paper towels. Termites were then sorted using a soft bird feather and used for bioassay within one hour of extraction and segregation.

Feeding Test

A no-choice and choice feeding bioassay was performed to determine toxicity and repellency of crude *Annona* seed extracts on the Asian subterranean termite *C. gestroi*. Dry crude extracts were used to prepare stock solutions in 95% ethanol. The no-choice feeding test was conducted using Petri dishes (5.5 cm in diameter) containing sterilized moist sand (1 mm) and a 2.54 x 2.54 cm filter paper (Whatman #1) impregnated with 80 µL of extract solution (12.4 µL/cm^2). Extract concentrations tested were 0, 1, 5, 10, 20% (w/v). Concentrations used were based on preliminary screening. Filter paper wetted with distilled water served as control. Fifty worker termites plus 5 soldiers of *C. gestroi* from three active field colonies were placed in each dish. Experimental units containing the termites were placed in an incubator maintained at 28°C and 85% relative humidity and force-fed on treated paper for 14 days. After the prescribed exposure period, percent mortality was determined by examining the experimental units for dead or moribund termites. Workers were considered moribund when they no longer walk or stand when probed with forceps. Dead or moribund workers were recorded and removed from each experimental unit daily. Termite mortalities were corrected by Abbott's formula (1925). The test was replicated three times for each colony with a total of nine replicates for each concentration. The amount of filter paper consumed by the termites was determined by estimating the loss in surface area (mm2) of treated paper. Paper consumption reported was the average of the evaluation of three individuals. Data from the feeding test were fitted in a completely randomized design and evaluated by analysis of variance (ANOVA) using Statgraphics Centurion 16.1 software (2010). Treatment means were separated by Tukey's Honest Significance Difference (HSD) test ($\alpha = 0.05$).

Choice feeding test was performed similar to the no-choice feeding test described above except that two filter papers, one treated with crude extract and the other untreated, were placed in the center 1.0 cm apart. Papers were impregnated with 80 µL of extract solution (0, 1, 5, 10, 20% (w/v). Fifty workers plus 5 soldiers of *C. gestroi* were introduced to each experimental unit and placed in an unlit incubator as described above for 14 days. Two untreated papers wetted with water were used as control. Percent termite mortality was monitored daily. The test was replicated three times for each colony with a total of nine replicates for each concentration. Consumption of treated paper by termites was determined as described above.

Soil Barrier Test

Tunneling and penetration of *C. gestroi* through soil treated with crude *Annona* seed extracts were evaluated using method similar to that described by Su and Scheffrahn (1990) with some modifications. The tunneling units consisted of a 12 cm glass tube (1.5 cm diameter) containing a 5 cm segment of treated soil sandwiched between 2 cm layer of 10% non-nutrient agar. Sterilized loamy soil was thoroughly mixed with the crude seed extract corresponding to 0, 1, 5, 10 and 20% [weight (crude extract)/weight (soil)] concentration. Treated soil was left to stand in a fume hood for 24 hours to evaporate the ethanol solvent. Soil wetted with distilled water served as control. Fifty workers plus 5 soldier termites were introduced into the void space and a piece of moistened filter paper was included to serve as food source. Both ends of the glass tube were sealed with aluminum foil and then placed vertically in an unlit incubator maintained at 28°C and 85% relative humidity. Termites were allowed to tunnel freely for 7 days and cumulative tunneling distance was monitored and recorded daily. The assembly was disassembled after 7 days, number of surviving insects was counted and termite mortality calculated. The test was replicated three times for each colony with a total of nine replicates for each concentration.

Results and Discussion

Feeding Tests

No choice forced feeding of *C. gestroi* on filter paper treated with crude seed extract of *A. squamosa*, *A. muricata* and *R. mucosa* resulted in significant increase ($p < 0.001$) in termite mortality with increasing extract concentration (Fig. 1). Termites feed on paper treated with 1 and 5% crude seed extract of all three Annona species investigated resulting in 23-68% mortalities after 14 days of exposure. Treated filter papers showed termite nibbling along the edges causing about 5.5–12 mm^2 reduction of surface area. However, paper treated with 1 and 5% extract was not sufficient to produce high mortality in *C. gestroi*. In addition, final mortalities at 5% concentration were observed to rise only after 6-7 days after exposure. Mortalities on the first 5 days for the three Annona species were about 8-15%. The reason for this observation is not clear but suggests delayed toxicity of Annona seed extracts on *C. gestroi.* Acetogenins, the bioactive component of the plant family Annonaceae, was reported to act as a slow acting stomach poison against chewing insects (Guadano et al., 2000; Leatemia & Isman, 2004a, b). Further study on the delayed toxicity aspect, however, needs be investigated. Termite mortalities at 10-20% crude extract of *A. squamosa* and *A. muricata* were relatively higher at 87-100% after 14 days of exposure (Fig.1). Papers treated with *R. mucosa* showed lower mortalities (78-85%) at the same extract concentration. However, examination of filter papers treated with 10-20% extract for all three samples revealed that the material was undamaged and not consumed by the termites. In addition, termites partially or completely covered the treated filter papers with sand. Apparently, the insects died of starvation and burying papers in sand could be an attempt to avoid contact with treated material. The results suggest that atleast 10% crude Annona seed extracts had repellent or

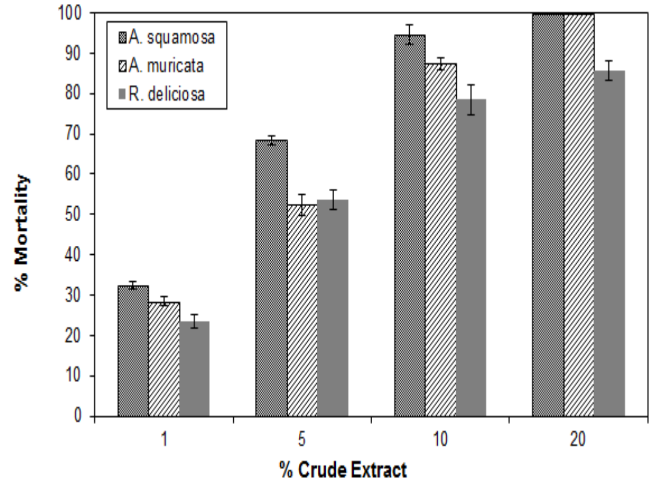

Fig. 1. Mortality of *C. gestroi* in no choice feeding with crude *Annona* seed extracts after 14 days of exposure.

feeding deterrent effects on *C. gestroi*. Toxicity by extract ingestion of treated paper could not be judged in these experiments.

Choice feeding test showed that *C. gestroi* consumed both untreated and treated papers up to 5% extract concentration (Table 1). Termite mortality with papers treated with 1 to 5% extract of *A. squamosa*, *A. muricata* and *R. mucosa* was relatively low at about 3-14% after 14 days of exposure. Paper consumption with untreated and those treated up to 5% extract was about 8-12 mm2 and 6-15 mm2, respectively, indicating healthy feeding on both materials. This would agree with the results of the no choice feeding above that low concentration of Annona seed extracts was not toxic by ingestion to cause high mortality in *C. gestroi* nor repellent to prevent feeding of treated materials. However, termites showed significant avoidance behavior on filter papers treated with 10% extract as indicated by non-consumption of treated papers (Table 1). Termite mortality at 10-20% extract was relatively low at about 11-18% for all Annona species investigated with untreated paper consumption of about 15–36 mm2. Termites also buried or covered treated papers with sand similar to the no-choice test above to prevent contact with the extract. Repellent or anti-feeding effect of plant extracts on *C. formosanus* Shiraki, *Reticulitermes santonensis* De Feytaud, *R. virginicus* Banks such as limonoids (Serit et al., 1992), flavanoids (Ohmura et al., 2000), nootkatone and vetiver oil (Zhu et al., 2001a;b), isoborneol and cedar oil (Blaske & Hertel, 2001; Blaske et al., 2003), matrine and oxymatrine (alkaloids) (Mao and Henderson, 2007), essential oils (Sakasegawa et al., 2003; Simaron et al., 2009), extracts from the heartwood of various hardwoods (Chang et al., 1997; Watanabe et al., 2005; Roszaini et al., 2013) had been reported in the literature. Although the repellency levels reported in this study was relatively high in comparison with synthetic insecticides, the availability of significant amount of cheap raw materials (waste seeds) may offset extraction cost to be commercially feasible. In addition, purified components of

Table 1. Average mortality and paper consumption of *C. gestroi* in choice feeding test with filter paper treated with various concentration of *Annona* seed extract.

% Extract Concentration	*A. squamosa*		*A. muricata*		*R. mucosa*	
	% Mortality	Consumption (mm^2)	% Mortality	Consumption (mm^2)	% Mortality	Consumption (mm^2)
1	8.45 ± 2.13a	10.23 ± 2.65	3.63 ± 1.12a	5.78 ± 1.22	12.03 ± 2.64a	9.34 ± 2.44
Control		12.45 ± 1.83		12.57 ± 3.76		10.25 ± 1.13
5	11.38 ± 1.47a	12.43 ± 0.75	7.48 ± 0.56b	8.26 ± 1.58	14..42 ± 1.47ab	15.12 ± 2.66
Control		8.48 ± 1.27		7.97 ± 2.68		8.573 ± 0.46
10	15.34 ± 1.68b	0	12.34 ± 1.32c	0	11.34 ± 3.17a	0
Control		36.34 ± 3.45		22.53 ± 3.52		30.34 ± 3.55
20	18.56 ± 3.18c	0	15.75 ± 2.75c	0	13.24 ± 3.68a	0
Control		25.45 ± 2.78		15.48 ± 4.35		19.62 ± 2.43

aEach value is the mean of 9 replicates each; numbers within a column followed by the same letter are not significantly different (Tukey's HSD test, α = 0.05).

Annona extracts (i.e. acetogenins) may be more potent against termites and should be further investigated.

Soil Barrier

Untreated and soil treated with 1% crude extract of *A. squamosa*, *A. muricata* and *R. mucosa* were fully breached by *C. gestroi* penetrating the full 5 cm barrier after 7 days in tunneling and penetration tubes (Table 2). However, soil treated with 5% crude extract of *A. squamosa* and *A. muricata* were able to limit termite penetration to about 0.62-1.24 cm during the 7 day exposure period. Extract of *R. mucosa* at 5% showed longer penetration of about 3.3 cm but prevented *C. gestroi* from breaching the barrier. Penetration of soil treated with at atleast 10% extract prevented termite tunneling and penetration of treated soil. No dead termites were found in treated soil. Termite mortality in all concentration tested was relatively low at about 7-16%. The result indicated that Annona crude seed extracts could potentially be used as soil barrier to prevent tunneling and penetration of *C. gestroi*. Apparently, the mechanism involved in preventing penetration of *C. gestroi* appeared to be repellency or avoidance of treated soil. Reduction in penetration and tunneling of subterranean termites were also reported in laboratory trials in sand or soil treated with plant extracts such as isoborneol (Cornelius et al., 1997; Blaske & Hertel, 2001; Blaske et al., 2003), nootkatone and vetiver oil (Maistrello et al., 2001), essential oils (Peterson & Wilson, 2003) and other plant oils (Yoshida et al., 2003; Acda, 2009).

Conclusions

In general, the study showed that ethanolic crude seed extracts of sweetsop (*A. squamosa*), soursop (*A. muricata*) and biriba (*R. mucosa*) had repellent or feeding deterrent effects on the Asian subterranean termite *C. gestroi*. No-choice and choice feeding tests indicated that crude extract of *A. squamosa*, *A. muricata* and *R. mucosa* prevented termite feeding on treated filter paper. Termites showed significant avoidance behavior to filter paper treated with atleast 10% Annona crude seed extract. Soil treated with 5-10% crude extract of the three *Annona* species investigated prevented tunneling and penetration of *C. gestroi*. Termite behavior in both feeding and soil penetration tests indicated that extracts of *A. squamosa*, *A. muricata* and *R. mucosa* seed extracts had anti-feeding or repellent effect on *C. gestroi* indicating

Table 2. Average mortality and penetration distance of *C. gestroi* after 7 days in tunneling tubes containing soil treated with various concentration of *Annona* seed extract.

% Extract Concentration	*A. squamosa*		*A. muricata*		*R. mucosa*	
	% Mortality	Penetration (cm)	% Mortality	Penetration (cm)	% Mortality	Penetration (cm)
0	5.23 ± 0.75a	5.0a	8.45 ± 1.26a	5.0a	8.56 ± 1.13a	5.0a
1	8.34 ± 1.45a	5.0a	9.75 ± 2.52a	5.0a	10.17 ± 1.55ab	5.0a
5	12.43± 1.78b	0.62 ± 0.11b	10.17 ± 1.35ab	1.24 ± 0.68b	14.53 ± 1.43b	3.30 ± 0.74b
10	15.17 ± 2.42c	0b	12.5 ± 2.11b	0.53 ± 0.14c	13.23 ± 2.24b	0.65 ± 0.12c
20	16.26 ± 3.67c	0b	14.01 ± 1.34c	0c	15.18 ± 1.24b	0c

aEach value is the mean of 9 replicates each; numbers within a column followed by the same letter are not significantly different (Tukey's HSD test, α = 0.05).

that these compounds may be potentially useful in the development of an alternative source of natural insecticide or in combination with other control methods against subterranean termites. Purification, identification and testing of bioactive components of seed extracts of Annonaceae species used in this study against termites are now underway.

Acknowledgments

The author wishes to thank the National Research Council of the Philippines (NRCP), Department of Science and Technology for providing funding support for this project; and Ms. Melania Gibe of the Department of Forest Products and Paper Science, University of the Philippines Los Banos for assistance in sample preparation and seed extraction.

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