Toxicity and repellency of ethanol extracts of *Annona reticulate* L. seed and leaf against *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae)

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Abstract

Ethanol extracts of *Annona reticulata* L. seed and leaf were evaluated for their toxicity and repellency at 6 concentrations (0.25, 05, 1, 2, 3 and 4%) against adult of *Callosobruchus maculatus*. The toxicity of plant extracts was tested by contact and fumigation bioassay. Mortality was assessed at 24, 48 and 72 h after treatment. The repellent activity of the previous extracts was also studied using the area preference bioassay. The both extracts showed strong insecticidal activity to the insect in both testing methods. In contact bioassay, the LC_{50} values for seed and leaf extracts were 6.18 and 6.81% at 24 h, 4.61 and 6.20 % at 48 h, and 1.61 and 1.73% at 72 h, respectively. In fumigation bioassay, their LC_{50} values were 8.60 and 8.69% at 24 h, 5.76 and 6.68% at 48 h, and 1.54 and 1.71% at 72 h, respectively. Moreover, the both extracts also showed repellent activity against *C. maculatus* in which seed extract was better than leaf extract. The mean repellency for seed extract ranged between class III and V (42.76-84.00%) while that for leaf extract ranged between class II and IV (29.63-72.16%). The repellency rate increased proportionally with the increase of concentration of the extract. These results indicated that *A. reticulata* has potential for integrated pest management programs of *C. maculatus* population.

Keywords: *Callosobruchus maculatus*, *Annona reticulata*, toxicity, repellency, plant extract

1. Introduction

Mungbean, *Vigna radiata* is one of the most importance seed legumes in Thailand, occupying an annual production area of over 300,000 ha (Ngampongsai et al., 2009). Thai people use mungbean seeds for consumption as health foods as well as for religious ceremonies. However, one of the main problems that occurs during storage is the attack of insect pests notably bruchid beetle, *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) (Sanon et al., 2002). Synthetic insecticides have been extensively used for *C. maculatus* control in many years because of their effectiveness and easy application and storage. However, the continuous and excessive use of synthetic insecticides has led to environmental disturbances, insect resistance to insecticides, lethal effects on non-target organisms in addition to direct toxicity to users and consumers (Prakash et al., 2008). Therefore, the development of techniques that would provide more efficient *C. maculatus* control without serious effects on the environment is clearly required.

Among current alternative methods, plant based insecticides have been suggested as alternative sources for insect control because many products are selective to insect pests and have no or little harmful effects on non-target organisms and the environment. In addition, botanical insecticides are easily available and easy to process and use by the small scale farmer (Regnault-Roger, 1997; Viglianco et al., 2008; Vanichpakorn et al., 2010; Abbasipour

et al., 2010). Moreover, they contain mixtures of biologically active substances, which can delay or prevent resistance development (Wang et al., 2007; Pavela, 2009).

The Annonaceae is a large family comprising about 130 genera and 2300 species (Begum et al., 2013). Among them, *Annona reticulata* originates from South America and West Indies and is cultivated throughtout Thailand for edible fruit (Satyanarayana et al., 2013). In addition, *A. reticulata* has been used in the folk medicine of Thailand for treatment of diarrhea, dysentery, scabies, yaws, worm infestation, and constipation. This plant possesses a range of pharmacological activities including analegestic, anti-inflammatory (Chavan et al., 2012; Thang et al., 2013), antidiabetic, anticancer (Pathak and Zaman, 2013) antioxidant, CNS depressant, chemopreventive, anthelmintic activities (Bhalke and Chavan, 2011; Bhale et al., 2011; Chavan, et al., 2014). The seeds, leaves and young fruits also have insecticidal effect (Rajini and Jothi Nisha, 2013). However, few studies have been conducted to evaluate the biological activity of *A. reticulata* against insect pests. Therefore, the aim of the present investigation was to evaluate contact and fumigant toxicity as well as repellent activity of ethanol extracts of *A. reticulata* seed and leaf against *C. maculatus*.

2. Materials and Methods

2.1. Rearing of Callosobruchus maculatus

C. maculatus adults were collected from naturally infested mungbean seeds. They were reared on sterilized seeds in the laboratory at 26±1°C and 75 % RH under 14: 10 (L: D). The beetles were allowed for mating and oviposition for one week. The insect parents were then removed and the medium containing the eggs were kept in the same condition until adult emergence. Freshly emerged subsequent generations were used for further experiments.

2.2. Plant material and extraction

The air-dried leaves and seeds were ground into fine powder using an electric grinder and screened through an 80-mesh screen. For extraction, 100 g of powdered leaves and seeds was separately extracted by maceration with 500 ml of ethanol at room temperature (26±1°C) for 3 d and filtered. The filtrates were concentrated to dryness by a rotary evaporator under low pressure to obtain the crude extracts. Six concentrations (0.25%, 0.5 %, 1%, 2%, 3% and 4%) of each extract were prepared using acetone as a solvent. The diluted concentration was used for insecticidal and repellent tests.

2.3. Toxicity test

2.3.1. Contact toxicity

An impregnated-filter paper bioassay described by Kim et al. (2003) was adapted to evaluate contact toxicity of ethanol extracts of *A. reticulata* seed and leaf against adults of *C. maculatus*. A filter paper disc of 9 cm diameter was treated with 1 ml of each concentration of tested extracts and allowed to air-dry for 30 min. Then, the treated filter paper disc was placed in Petri dish. Twenty unsexed adults of *C. maculatus* were released on the treated filter paper disc. The control filter paper disc was treated with acetone alone. Each treatment consisted of four replications. Insect mortality was observed at 24, 48, and 72 h after treatment.

2.3.2. Fumigant toxicity

The fumigant toxicity of ethanol extracts of *A. reticulata* seed and leaf was evaluated according to a method described by Michelraj and Sharma (2006). A 250 ml plastic jar with screw lid was used as a fumigation chamber. A filter paper disc of 5 cm diameter was treated with 0.5 ml of each concentration of tested extracts and allowed to air-dry for 30 min. The treated filter paper was

then attached to the under surface of the lid with adhesive tape. Ten adults were transferred to a 10 ml vial and the vial was covered with fine cloth. Four vials containing the insects were placed in the fumigant chamber and considered as four replications. The lid was closed and sealed by adhesive tape to create air tight condition in the chamber. The control consisted of a similar setup but without the extracts. Insect mortality was observed at 24, 48, and 72 h after treatment.

2.4. Repellency test

Repellent activity of ethanol seed and leaf extracts against *C. maculatus* was performed using an area preference bioassay described by Obeng-Ofori et al. (1998). A filter paper disc of 9 cm diameter was divided into two equal parts. The first half was treated with 0.5 ml of each concentration and the control half was treated with 0.5 ml of acetone. After evaporation of solvent, a full disc was remake by attaching the treated half and the control half with clear adhesive tape. Each filter paper disc was placed in a Petri dish. Twenty unsexed adults were released at the center of filter paper disc. Each treatment was replicated four times. The number of insect in each half was recorded at 1, 2, 3, 4 and 24 h after treatment. Repellency rate (%) was calculated by using the following formula from Abbott (1925):

$$\text{Repellency rate (\%)} = \frac{(A-B)}{A} \times 100 \qquad (3)$$

Where A was average number of insects present on untreated portion and B was average number of insects present on treated portion.

2.5. Statistical analysis

One way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were performed on the data to determine significant ($P<0.05$) differences among treatments using software SPSS V17. The LC_{50} values were calculated by probit analysis (SPSS V17). The repellent rate was categorized to repellency class from O to V: class O = >0.01%-<0.10%; class I = 0.10%-20.00%; class II = 20.10%-40.00%; class III = 40.10%-60.00%; class IV = 60.10%-80.00%; class V = 80.10-100.00% (Amin el al., 2000; Roy et al., 2005).

3. Results and Discussion

3.1. Contact toxicity

The contact toxicity of ethanol extracts of *A. reticulata* seed and leaf against adults of *C. maculatus* was evaluated using the impregnated-filter paper bioassay (Table 1). Both seed and leaf extracts showed contact toxicity against the insect. At 24 h, the seed extract exhibited the strongest insecticidal activity with an LC_{50} value of 6.18%. An LC_{50} value of 6.81% was estimated for the leaf extract. The 48 h LC_{50} value of seed extract was 4.61%, compared to 6.20% for leaf extract. At 72 h, the LC_{50} values were 1.61 and 1.73% for seed and leaf extracts, respectively. Based on the 95% confidence interval of the estimation, there were no significant difference in contact toxicity between seed and leaf extracts. These results indicated that the mortality of the insect increased with the increasing exposure time. The seed extract was more toxic than the leaf extract.

3.2. Fumigant toxicity

The ethanol extracts of *A. reticulata* seed and leaf showed strong fumigant toxicity against *C. maculatus* and insect mortality increased with the increasing exposure time. On the basic of LC_{50} value, there were no significant difference in fumigant toxicity between seed and leaf extracts. However, seed extract was more toxic than the leaf extract. The LC_{50} values for seed

and leaf extracts were 8.60 and 8.69% at 24 h, 5.76 and 6.68% at 48 h and 1.54 and 1.71% at 72 h, respectively (Table 2).

Table 1 Contact toxicity of ethanol extracts of *Annona reticulata* seed and leaf against adults of *Callosobruchus maculatus*.

Time	Extract	LC_{50}, %	95% CL[a]	Slope±SE	χ^2(df)[b]
24 h	Seed	6.18	4.69-37.48	3.06±1.18	3.42(4)
	Leaf	6.81	4.99-97.43	4.23±1.73	3.04(4)
48 h	Seed	4.61	3.72-6.45	1.90±0.75	1.90(4)
	Leaf	6.20	3.91-69.65	2.16±0.41	8.01(4)
72 h	Seed	1.61	1.31-2.03	1.31±0.14	6.39(4)
	Leaf	1.73	1.38-2.23	1.51±0.14	2.91(4)

[a]CL denotes confidence limit.
[b]NS, not significant at P<0.05

Table 2 Fumigant toxicity of ethanol extracts of *Annona reticulata* seed and leaf against adults of *Callosobruchus maculatus*

Time	Extract	LC_{50}, %	95% CL[a]	Slope±SE	χ^2(df)[b]
24 h	Seed	8.60	5.29-100.71	1.75±0.61	5.72(4)
	Leaf	8.69	5.52-531.05	2.89±1.19	5.52(4)
48 h	Seed	5.76	4.39-18.43	2.64±0.87	3.54(4)
	Leaf	6.68	4.85-33.29	2.90±1.01	5.93(4)
72 h	Seed	1.54	1.20-2.00	1.06±0.13	1.33(4)
	Leaf	1.71	1.24-2.50	0.80±0.12	4.72(4)

[a]CL denotes confidence limit.
[b]NS, not significant at P<0.05

Our current laboratory on ethanol extracts of *A. reticulata* seed and leaf against adults of *C. maculatus* revealed that both extracts had strong contact and fumigant toxicity. The results from this investigation are similar to the observation of Rajapakse and Ratnasekera (2008) who reported that ethanol extract of *A. reticulata* leaf had strong contact toxicity against *C. maculatus* with 91% mortality at 72 h. Ahad et al. (2012) also obtained 100% mortality of *C. maculatus* treated with ethanol extract of *A. reticulata* leaf at 3% in 72 h by direct toxicity test. Furthermore, Shin et al. (2010) reported that *A. reticulata* seed extract exhibited insecticidal activity against *Myzus persicae* and *Nilaparvata lugens* with LD_{50} values of 0.45 and 1.42 mg/ml, respectively. Nayak (2014) investigated insecticidal activity of methanol extract of *A. reticulata* leaf against early fourth instar larvae of *Culex quinquefasciatus* and found 100% mortality at concentration of 5 ppm at 48 h.

The insecticidal activity of plant extract may be due to the various constituents present in the extract. These compounds may independently or jointly contribute to cause toxic action

against *C. maculatus*. The leaf of *A. reticulata* contains acetogenins, alkaloids, essential oils, flavonoids, phenolic compounds, tannins, glycosides, carbohydrates, saponins, proteins, sterols (Kumar et al., 2008; Satyanarayana et al., 2013; Rout et al., 2013). The main constituents of the seed were acetogenins and alkaloids (Pathak and Zaman, 2013). Annonaceous acetogenins have been shown to possess insecticidal activity. For example, squamosin, an acetogenin isolated from *A. squamosa* seed has been reported to have insecticidal activity against insects including *Plutella xylostella*, *C. chinensis* (Ohsawa et al., 1991), *Aedes aegypti* (Costa et al., 2014). This acetogenin has been found in *A. reticulata* seed (Chang et al., 1998; Pathak and Zaman, 2013). Pandey and Varma (1977) reported that the seed of *A. reticulata* contained an alkaloid, annonaine, which exhibited insecticidal activity against *C. maculatus*. Thus, the toxicity of *A. reticulata* is attributed to the acetogenins and alkaloids. However, it is necessary to investigate the toxicity of other constituents of *A. reticulata* against *C. maculatus*.

3.3. Repellency test

The repellent activity of ethanol extracts of *A. reticulata* seed and leaf at different concentrations was tested against adults of *C. maculatus* using the area preference bioassay (Table 3). There was significant difference among the tested extracts at different hours. Among the six concentrations, the highest concentration (4%) of seed and leaf extracts showed the strongest repellent activity to the insect with mean repellency values of 84.00 and 72.16%, respectively. On the other hand the lower repellency values were obvious at lower concentrations in both extracts (42.76 and 29.63%, respectively at 0.25%). In case of seed extract, the repellent activity increased with the increasing concentration. The higher concentrations (2, 3 and 4%) exhibited strong repellent activity and ranged between class IV and V (69.83-84.00%). The other concentrations gave moderate repellent activity with class III (42.76-58.90%). The repellent activity of leaf extract increased with the increasing concentration and exposure time. Leaf extract at concentration of 2, 3 and 4% showed strong repellent activity against the pest with class IV (60.13-72.16%). Moderate repellent activity (40.31-53.36%) was produced from concentration of 0.5 and 1%. The lowest concentration showed little repellent activity against *C. maculatus* with class II (29.63%).

Previously, many plant extracts have been screened for their repellent activity against *C. maculatus*. Radha and Murugan (2011) reported that leaf extract of *Anisomeles malabarica* at 2% exhibited repellent activity of 73, 65, 62 and 54% at 1, 2, 3 and 4 h after treatment. Udo (2011) tested repellent activity of extracts from *Zanthoxylum xanthoxyloides* and found the highest mean repellency value of 68% from dry bark extract. Fouad (2013) reported that essential oils of *Cinnamomum zeylanicum* at 1 % exhibited moderate repellent activity against *C. maculatus* with mean repellency values of 47.50%.

Our results also revealed that both seed and leaf extracts showed strong repellent activity against the insect. This finding agrees with the report of Ahad et al. (2012) who found strong repellent activity of ethanol extract of *A. reticulata* leaf at 0.5, 1, 2 and 3% with mean repellency of 63.89, 66.67, 67.67 and 84.44%, respectively. On the basic of the results, the repellent activity of leaf extract also increased with the increasing exposure time. This can be explained by the fact that the constituents of leaf extract are high molecular weight compounds with low volatility. It is evident from this experiment that time is the main factor for repellency of *C. maculatus* by ethanol extract of *A. reticulata* leaf. Similar results were also found for methanol extract of *Clerodendrum serratum* leaf that showed repellency of 53.3, 73.3, 77.0, 80.0, 80.3, 87.0 and 97.0% for concentration of 0.503 mg/cm^2 at 30 min, 1, 2, 4, 8, 16 and 24h after exposure, respectively on *S. oryzae* adults by using area preference bioassay (Yankanchi et al., 2013). On the other hand, the constituents of seed extract had high

volatility. Thus, seed extract showed strong repellent activity (84.99%) at concentration of 4% at the first hour and also proved highly persistence with mean repellency of 84.00% within 24 h after exposure because of its polar nature. The similar trend was also observed at lower concentrations of seed extract.

4. Conclusions

The ethanol extracts of *A. reticulata* seed and leaf demonstrated strong contact and fumigant toxicity as well as repellent activity against adults of *C. maculatus*. The toxicity and repellent activities of *A. reticulata* extract against the insect depended on several factors including chemical constituents of the extract, plant part, concentration and exposure time. Thus, this plant has excellent potential to provide naturally occurring agents that may utilized for *C. maculatus* control. Further work is in progress to isolate and identify the insecticidal and repellent constituents of this plant. Other areas requiring attention are their persistence in the environment and toxicity to humans as well as the usefulness for commercial application.

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Table 3 Repellent activity of ethanol extracts *of Annona reticulata* seed and leaf against adults of *Callosobruchus maculatus* using area preference bioassay.

Extract	Conc. (%)	Repellency rate (mean ± SE, %)[a] 1 h	2 h	3 h	4 h	24 h	Mean Repellency (%)	Repellency class
Seed	4	83.99±1.64a	85.62±1.89a	80.81±1.94a	82.35±2.85a	87.26±1.64a	84.00±0.97a	V
	3	78.47±3.47a	74.75±3.21ab	76.79±3.84ab	78.88±2.24a	78.68±2.12ab	77.51±1.27ab	IV
	2	74.75±3.21a	68.75±2.09bc	63.99±4.30bc	72.92±2.09ab	68.75±2.09bc	69.83±1.44bc	IV
	1	59.52±2.38b	54.39±2.75d	59.15±4.88cd	64.28±2.38bc	57.14±0.00de	58.90±1.38de	III
	0.5	59.52±2.38b	36.54±3.21ef	54.39±2.75cde	56.77±4.20cd	54.39±2.75e	52.32±2.44e	III
	0.25	46.15±0.00cd	33.33±0.00fg	45.69±4.87de	48.90±2.75de	39.74±3.70f	42.76±1.75f	III
Leaf	4	48.90±2.75c	68.75±2.09bc	75.00±0.00ab	82.35±2.85a	85.82±1.77a	72.16±3.11bc	IV
	3	36.54±3.21de	56.77±4.19cd	68.45±4.25abc	76.94±1.94ab	76.94±1.94abc	63.13±3.72cd	IV
	2	36.54±3.21de	54.03±4.95d	68.45±4.25abc	70.83±2.41ab	70.83±2.41bc	60.13±3.38de	IV
	1	33.33±0.00e	48.44±5.66de	54.39±2.75cde	64.28±2.38bc	66.37±3.65cd	53.36±3.06de	III
	0.5	21.97±3.79f	36.54±3.21ef	37.74±3.70ef	54.39±2.75cde	48.90±2.75ef	40.31±2.87f	III
	0.25	18.18±0.00f	21.97±3.79g	25.76±4.37f	42.49±5.74e	39.74±3.70f	29.63±2.74g	II

[a]Repellency within a column followed by the same letter are not significantly different at P <0.01 by DMRT