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, 0.5, 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 LC50 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 LC50 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; Abbasi pour...
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 2,300 species (Begum et al., 2013). Among them, Annona reticulata originates from South America and West Indies and is cultivated throughout 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 analgesic, 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% r.h. 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):

\[
\text{Repellency rate (\%)} = \left(\frac{A-B}{A}\right) \times 100
\]

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 et 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*.

<table>
<thead>
<tr>
<th>Time</th>
<th>Extract</th>
<th>LC$_{50}$, %</th>
<th>95% CL$^a$</th>
<th>Slope±SE</th>
<th>$\chi^2$(df)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>Seed</td>
<td>6.18</td>
<td>4.69-37.48</td>
<td>3.06±1.18</td>
<td>3.42(4)</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>6.81</td>
<td>4.99-97.43</td>
<td>4.23±1.73</td>
<td>3.04(4)</td>
</tr>
<tr>
<td>48 h</td>
<td>Seed</td>
<td>4.61</td>
<td>3.72-6.45</td>
<td>1.90±0.75</td>
<td>1.90(4)</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>6.20</td>
<td>3.91-69.65</td>
<td>2.16±0.41</td>
<td>8.01(4)</td>
</tr>
<tr>
<td>72 h</td>
<td>Seed</td>
<td>1.61</td>
<td>1.31-2.03</td>
<td>1.31±0.14</td>
<td>6.39(4)</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>1.73</td>
<td>1.38-2.23</td>
<td>1.51±0.14</td>
<td>2.91(4)</td>
</tr>
</tbody>
</table>

$^a$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*.

<table>
<thead>
<tr>
<th>Time</th>
<th>Extract</th>
<th>LC$_{50}$, %</th>
<th>95% CL$^a$</th>
<th>Slope±SE</th>
<th>$\chi^2$(df)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>Seed</td>
<td>8.60</td>
<td>5.29-100.71</td>
<td>1.75±0.61</td>
<td>5.72(4)</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>8.69</td>
<td>5.52-531.05</td>
<td>2.89±1.19</td>
<td>5.52(4)</td>
</tr>
<tr>
<td>48 h</td>
<td>Seed</td>
<td>5.76</td>
<td>4.39-18.43</td>
<td>2.64±0.87</td>
<td>3.54(4)</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>6.68</td>
<td>4.85-33.29</td>
<td>2.90±1.01</td>
<td>5.93(4)</td>
</tr>
<tr>
<td>72 h</td>
<td>Seed</td>
<td>1.54</td>
<td>1.20-2.00</td>
<td>1.06±0.13</td>
<td>1.33(4)</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>1.71</td>
<td>1.24-2.50</td>
<td>0.80±0.12</td>
<td>4.72(4)</td>
</tr>
</tbody>
</table>

$^a$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* (Ohşawa 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/cm² at 30 min, 1,
2, 4, 8, 16 and 24 h 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.

Acknowledgements
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References


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

<table>
<thead>
<tr>
<th>Extract</th>
<th>Conc.</th>
<th>Repellency rate (mean ± SE, %)</th>
<th>Mean Repellency (%)</th>
<th>Repellency class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(% )</td>
<td>1 h</td>
<td>2 h</td>
<td>3 h</td>
</tr>
<tr>
<td>Seed</td>
<td>4</td>
<td>83.99±1.64a</td>
<td>85.62±1.89a</td>
<td>80.81±1.94a</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>78.47±3.47a</td>
<td>74.75±3.21ab</td>
<td>76.79±3.84ab</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>74.75±3.21a</td>
<td>68.75±2.09bc</td>
<td>63.99±4.30bc</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>59.52±2.38b</td>
<td>54.39±2.75d</td>
<td>59.15±4.88cd</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>59.52±2.38b</td>
<td>36.54±3.21ef</td>
<td>54.39±2.75cde</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>46.15±0.00cd</td>
<td>33.33±0.00fg</td>
<td>45.69±4.87de</td>
</tr>
<tr>
<td>Leaf</td>
<td>4</td>
<td>48.90±2.75c</td>
<td>68.75±2.09bc</td>
<td>75.00±0.00ab</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>36.54±3.21de</td>
<td>56.77±4.19cd</td>
<td>68.45±4.25abc</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>36.54±3.21de</td>
<td>54.03±4.95d</td>
<td>68.45±4.25abc</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>33.33±0.00e</td>
<td>48.44±5.66de</td>
<td>54.39±2.75cde</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>21.97±3.79f</td>
<td>36.54±3.21ef</td>
<td>37.74±3.70ef</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>18.18±0.00f</td>
<td>21.97±3.79g</td>
<td>25.76±4.37f</td>
</tr>
</tbody>
</table>

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

1088