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Original Article

Toxicity of fixed oil and crude extract from sa-dao-thiam, *Azadirachta excelsa* (Jack) seed kernel to *Aedes aegypti* (L.)

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Abstract

The larvicidal activity of various concentrations of fixed oil and crude extract from sa-dao-thiam, *Azadirachta excelsa* (Jack) seed kernel was assayed on an *Aedes aegypti* (L.) test population under controlled laboratory conditions. Concentration levels of responses at 24, 48, 72, and 96 hrs were evaluated. The LC_{50} values of the fixed oil and the crude extract were 403.6 and 518.7 ppm, respectively. One hundred percent mortality in 24 hrs-post treatment was achieved at 2,000 and 4,000 ppm for the oil and crude extract, respectively. It suggested that the oil is more toxic to *Ae. aegypti* larvae than the crude extract. Further investigation suggested the occurrence of molting inhibition of *Ae. aegypti* larvae by the fixed oil and crude extract as indicated by the small number of emerged adults. In addition, histological study suggested that damages on the epithelial cells of the midgut could result from the effects of the oil and crude extract. Hypertrophy and degeneration of the epithelial cells were observed, resulting in a presence of some cytoplasmic material in the alimentary canal. Further studies should be taken into account to identify their stability and residual activity of these products under field conditions.

Keywords: *Azadirachta excelsa*, *Aedes aegypti*, toxicity, development, histology

1. Introduction

The incidence of dengue has grown dramatically around the world in recent decades. WHO currently estimates that there may be 50 million dengue infections worldwide every year (WHO, 2009). The dengue virus is transmitted by *Aedes aegypti* (L.), an effective vector that preferentially feeds on humans and is often found in and near human dwellings (Gubler, 1997). Despite considerable research over many decades, an effective and commercially available dengue vaccine is not yet available, and prevention of this disease remains entirely dependent on vector control. Most vector control relies exclusively on using synthetic insecticides, which are commonly used in homes; but there is an important downside to this, as it could be an important cause of insecticide resistance in the house-haunting mosquito (Paeporn *et al.*, 2003; Ponlawat *et al.*, 2005; Jirakanjanakit *et al.*, 2007; Thanispong *et al.*, 2008).

A number of alternative methods have been extensively studied to control the spread of this disease, including the use of plant-derived compounds (Perich *et al.*, 1994; Nagpal *et al.*, 1995; Pathak *et al.*, 2000; Singh *et al.*, 2002; Albuquerque, 2004). Sa-dao-thiam, *Azadirchta excelsa* (Jack) is one such compound. This plant is believed to be native Indonesia, Malaysia, Papua New Guinea, the Philippines, and

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Vietnam, but to be exotic to Singapore and Thailand (Orwa *et al.*, 2009). In Thailand, it is widely grown in the southern Provinces, from Chumphon to Narathiwat Province (Denrungruang *et al.*, 1995). The fixed oil and crude extract of the *A. excelsa* seed kernel have been found to have insecticidal activities against many insect species (Butpha, 2000; Palintorn, 2000; Sritungnanta, 2005; Mustafa and Al-Khazraji, 2008). Its principal active ingredient is marrangin (azadirachtin L), which has multiple effects on the development of insects (Orwa *et al.*, 2009); the active insecticidal components are 1-isopentanoic acid-3-acetylazadirachtol, azadirachtin L and 11*b*-hydroxyazadirachtin H (Kanokmedhakul *et al.*, 2005). Bioassays testing leaf, bark, and seed extracts of *A. excelsa* against insects have shown greater activity than against *A. indica* (Schmutterer and Doll, 1993). Most of the studies previously mentioned have focused on the toxic effects of the test compounds, while few have examined the histological effects. In addition, few published documents regarding larvicidal activity on *Ae. aegypti* of the fixed oil and crude extract from *A. excelsa* are available.

In this study, the toxic and histological effects of fixed oil and crude extract from *A. excelsa* were assayed on the fourth larval stage of *Ae. aegypti* under controlled laboratory conditions. Mortality was determined at 24, 48, 72, 96, and 120 hrs post-treatment.

2. Materials and Methods

2.1 Plant material and extarction method

The seeds of *Azadirachta excelsa* (Jack) (Meliaceae) were collected from Rattapum District, Songkhla Province of Southern Thailand in May 2007 and authenticated by Dr. K. Sridith, Department of Biology Faculty of Science, Prince of Songkla University. After being exposed to direct sunlight for a few days, the seed coats were removed and the seed kernels were ground. The product was macerated with *n*-hexane for seven days at room temperature prior to filtration. Seed cakes were repeatedly extracted seven times. All filtrates were combined and the solvents were evaporated with a rotary evaporator. The residue from the evaporator was poured into an evaporator dish, and then placed in a hot-water bath at 50°C until the remained solvents were completely removed. The final product obtained from this process was the "fixed oil". Sa-dao-thiam seed cakes were marcerated using methanol following the same process as that of *n*-hexane extraction and the product was referred to "crude extract". Two final products were stored in a refrigerator at +15°C until use. Yields of the fixed oil and the crude extract obtained from this study were 53.4% and 19.3%, respectively.

These two products have different properties. The fixed oil is a concentrated light yellow liquid possessing a relatively high viscosity, whereas the crude extract is a dark brown liquid with a relative low viscosity. In addition, the crude extract quickly spreads on water surface, but the fixed oil does not spread on water as readily.

2.2 Mosquito test population

A test population of *Ae. aegypti* was used in this study. The immature stages were collected in May 2008 from Bonwure Community of Muang District, Songkhla Province. All development stages were reared in a temperature-controlled insectary at 27±2°C and 80±5% relative humidity, using a rearing method described by Laojareonsuk (2002). Teneral adult males and females were identified to species and provided cotton pads soaked with 10% sugar solution. Following free mating, between Day 2 and 5, post-emergence female mosquitoes were allowed to feed on a guinea pig. An oviposition site containing tap water and filter paper were placed in the cages for egg deposition following 2-3 days ovarian development. The F_1 were utilized for testing.

2.3 Bioassay test and data analysis

Different concentrations of fixed oil and crude extract were tested on the fourth larval stage of *Ae. aegypti*. The larvae were exposed to 200, 400, 600, 800, 1,000, 2,000, and 3,000 ppm of the fixed oil, and 200, 400, 600, 800, 1,000, 2,000, 3,000, 4,000 and 5,000 ppm of the crude extract. All tests were conducted in a 500-ml plastic bowl containing 200 ml of de-chlorinated tap water. A control set containing tap water without fixed oil and crude extract was also done. Each trial included twenty larvae per bowl, replicated five times for each concentration. A half gram of chicken meal per bowl was given as a food source of larvae. Larval mortality was recorded at 24, 48, 72, 96, and 120 hours after exposure. Corrected mortality percentage was performed at 24 hours by using the Abbott's formula. The LC_{50} values were estimated from dosage-mortality regression using probit analysis (Raymond, 1985). The pupal and adult stages developed from the survived larvae treated with the fixed oil and the crude extracts were also recorded.

2.4 Histological study

Dead larvae caused by fixed oil and crude extract were fixed in 10% formalin. The tissues were dehydrated with ethyl alcohol for 5 hours, after which they were placed in xylene for tissue clearing. They were then embedded and blocked by paraplast and sectioned with a microtome. The staining was used according to the routine stain method of Hematoxylin & Eosin (H&E). The observation on the midgut tissue was investigated under a compound microscope. Untreated larvae were investigated in the same manner.

3. Results and Discussion

3.1 Larvicidal activity of sa-dao-thiam fixed oil and crude extract

The mortality and LC_{50} values of *Ae. aegypti* larvae after exposure to various concentrations of sa-dao-thiam

fixed oil and crude extract for 24 hours are shown in Tables 1A and B, respectively. Both had significant larvicidal activity against *Ae. aegypti* larvae. The mortality rates increased with increasing concentration. However, the fixed oil was more toxic to larvae than the crude extract. The LC_{50} value of the fixed oil was 403.6 ppm, whereas that of the crude extract was 518.7 ppm (Table 1B). This finding suggests that *n*-hexane extract contains more substances, which had better larvicidal activity against *Ae. aegypti* larvae than that of methanolic extract. This result agrees with the finding of Singh *et al.* (2006), who found that hexane extracts of *Momordica chanrantia* Linn. (Cucurbitaceae) showed more potent larvicidal activity against larvae of *Anopheles stephensi*, *Culex quinquefasciatus* and *Ae. aegypti* than the crude extract. It is possible that *n*-hexane extracts contain different compounds than methanolic extracts. The chemical composition in these two fractions of sa-dao-thiam seed should be further elucidated.

It is not exactly clear what the mechanism of death is, but two main pathways have been suggested, through suffocation and through direct absorption through the larval skin (Green, 1925; Djouaka *et al.*, 2007). There are two characteristics of fixed oil, possessing suffocation and absorption through the larval cuticle, could explain the greater toxicity of the oil on *Ae. aegypti* larvae than the crude extract. In another recent study, Djouaka *et al.* (2007) found that *Anopheles* larvae exposed to petroleum products (kerosene, petrol, and engine oils) were mostly killed by direct contact toxicity and not by suffocation. Green (1925) explained that death of the larvae was attributed to suffocation by creating a barrier on the water surface.

Percent mortality increased with increasing time of exposure at concentrations of less than 1,000 ppm of the fixed oil and 3,000 ppm of the crude extract (Figures 1A and 1B). The lowest concentration required to reach 100% mortality with the fixed oil was lower than the crude extract. The fixed oil attained 100% mortality at 800.0 ppm after 72-hours of exposure (Figure 1A), whereas the crude extract at 2,000 ppm reached 100% mortality after 96-hours of exposure (Figure 1B).

Previous studies comparing the LC_{50} of fixed oils and crude extracts obtained from various plants also showed different LC_{50} values. The LC_{50} values of the fixed oils obtained from *Cymbopogon nardus*, *C. flexuosus, C. martinii, Lavadula officinalis, Mentha arvensis, Racinus communis, Eucalyptus globulus, Eugenia caryophyllus, Melia azedarach, Cannabis sativae,* and *Ipomoae cairica* Linn. with the larvae of *An. stephensi* were 105.4, 91.4, 100.0, 83.6, 83.8, 113.0, 98.5, 96.5, 88.5, 27.0, and 120.0 ppm, respectively (Chavan and Nikam, 1982; Kumar and Dutta, 1987; Thomas *et al.*, 2000). The LC_{50} values of methanolic extracts of *Vitex* spp against *Cx. quinquefasciatus* ranged from 41.4-212.5 ppm (Kanna-

Table 1A Percent mortality of *Aedes aegypti* (L.) larvae after exposure to various concentrations of sa-dao-thiam fixed oil and crude extract for 24 hours.

Concentration (ppm)	Percent mortality (Means±SEM)[1/]	
	Fixed oil	Crude extract
0	0.0±0.0	0.0±0.0
200	32.0±3.0	22.0±8.8
400	43.0±4.6	46.0±4.0
600	62.0±5.8	51.0±5.3
800	69.0±5.7	57.0±9.9
1,000	84.0±3.3	75.0±8.2
2,000	100.0±0.0	84.0±5.1
3,000	100.0±0.0	97.0±2.0
4,000	NT[2/]	100.0±0.0
5,000	NT	100.0±0.0

[1/]Means of 5 replications, [2/] NT= not tested, SEM= Standard error of the mean

Table 1B Susceptibility data of sa-dao-thiam fixed oil and crude extract based on dose/mortality relationships tested against the fourth larval stage of *Aedes aegypti* (L.).

	LC_{50} (95% confidence interval, ppm)	Slope	Chi-square
Fixed oil	403.6 (285.7-563.7)	2.4±0.4	14.3
Crude extract	518.7 (412.2-647.7)	2.1±0.2	12.9

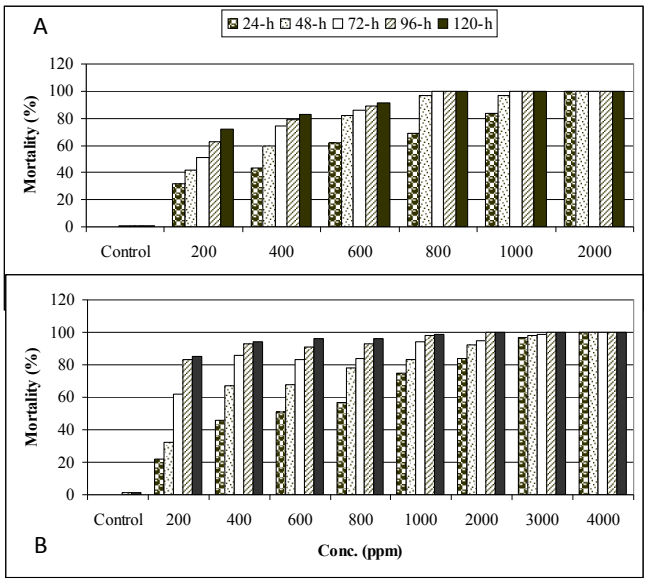

Figure 1. Mortality of *Aedes aegypti* (L.) larvae after exposure to various concentrations of sa-dao-thiam oil (A), and sa-dao-thiam crude extract (B) for 24, 48, 72, 96, and 120 hours.

thasan *et al.*, 2007). Ethanolic extracts of three indigenous Thai plants, *Thevetia peruviana, Pueraria mirifica*, and *Butea superba* were found to have LC_{50} values of 1,023.7, 1,386.1, and 259.1 ppm against the fourth larval stage of *Ae. aegypti* (Lapcharoen *et al.*, 2005). Azmi *et al.* (1998) reported that the LC_{50} value of neem leaf extracts with the late third instar larvae of *Cx. fatigans* was 390.0 ppm. Wandscheer *et al.* (2004) reported the LC_{50} of ethanolic extracts of *M. azedarach* and *Azadirachta indica* with *Ae. aegypti* in laboratory conditions gave LC_{50} values at 25°C and 30°C of 1,660.0 and 1,520.0 ppm, respectively for *M. azedarach* and 440.0 and 630.0 ppm, respectively for *A. indica*.

3.2 Effects of sa-dao-thiam on development of *Ae. aegypti* larvae

The accumulative pupation percentages of mosquito larvae when treated with the fixed oil and the crude extract of sa-dao-thiam were very small compared to the control (Table 2 and 3). At 200 ppm, these values were 2.0% and 13.0% for the fixed oil and the crude extract, respectively, compared with 66.0% and 72.0% for the controls, respectively (Tables 2 and 3). This suggests that both fixed oil and crude extract from sa-dao-thiam delayed the development of *Ae. aegypti* larvae to the pupal stage. At the same concentrations, there was a very low emergence of adults, only 2.0% and 3.0%, from larvae exposed to the fixed oil and the crude extract, respectively, while similar percentage figures for pupae emerged to adults for the controls were 51.0% and 52.0%, respectively (Table 2 and 3). Ndione *et al.* (2007) found that fixed oil and powder products of neem (*A. indica*) seed kernel showed a strong inhibition on the development of *Ae. aegypti* larvae to pupal and adult stages. El hag *et al.* (1999) found that extracts of *A. indica*, *Rhazya stricta* and *Syzygium aromaticum* influence larval development by reducing pupation and inhibiting adult emergence. They also observed that there was no further development of the first instar to the second instar larvae of *Cx. pipiens* after being subjected to a 400.0 ppm methanol extract of *R. stricta.*

3.3 Histological study

Under histological examination, the guts of the untreated larvae appeared more or less normal, with a normal layer of gut epithelial cellsgut, muscle and adipose fabric (Figure 2A). Both fixed oil and crude extract did their work in the midgut of the *Ae. aegypti* larvae, as can be seen in Figures 2B and C, respectively, with major signs of damage to the midgut epithelial cells. The epithelial cells of the fixed oil-treated larvae were slightly hypertrophied, and some cells were degenerated and released into the alimentary canal (Figure 2B). Serious damage was noted in the epithelial cells in the crude extract-treated larvae, resulting in liberation of cytoplasmic content into the alimentary canal (Figure 2C). However, muscle and adipose tissue seemed to be unaffected with either treatment. The results of our study suggest that widespread use of this insecticide would reduce the mosquito population. Earlier studies have suggested a mechanism for such results – for example, the ingestion of *Bacillus sphaeri-*

Table 2. Accumulative percentages of pupation and adult emergence counted from a total of 100 larvae exposed to various concentrations of sa-dao-thiam fixed oil for 24, 48, 72, 96, and 120 hours

	Concentration (ppm)				
	Control	200	400	600	800
24-hrs					
Pupation (%)	16.0	0.0	1.0	0.0	1.0
Emergence (%)	0.0	0.0	0.0	0.0	0.0
48-hrs					
Pupation (%)	26.0	1.0	1.0	0.0	1.0
Emergence (%)	5.0	0.0	1.0	0.0	0.0
72-hrs					
Pupation (%)	39.0	2.0	1.0	0.0	1.0
Emergence (%)	20.0	1.0	1.0	0.0	0.0
96-hrs					
Pupation (%)	56.0	2.0	1.0	0.0	1.0
Emergence (%)	34.0	2.0	1.0	0.0	0.0
120-hrs					
Pupation (%)	66.0	2.0	1.0	0.0	1.0
Emergence (%)	51.0	2.0	1.0	0.0	0.0

Remarks: There were no pupae and adults emerged at concentrations of 1,000, 2,000 and 3,000 ppm.

Table 3. Accumulative percentages of pupation and adult emergence counted from a total of 100 larvae exposed to various concentrations of sa-dao-thiam crude extract for 24, 48, 72, 96, and 120 hours.

	Concentration (ppm)					
	Control	200	400	600	800	1,000
24-hrs						
Pupation (%)	15.0	3.0	2.0	1.0	1.0	0.0
Emergence (%)	1.0	0.0	0.0	0.0	0.0	0.0
48-hrs						
Pupation (%)	25.0	12.0	4.0	1.0	1.0	0.0
Emergence (%)	6.0	0.0	0.0	0.0	0.0	0.0
72-hrs						
Pupation (%)	35.0	13.0	5.0	1.0	1.0	0.0
Emergence (%)	19.0	2.0	1.0	0.0	0.0	0.0
96-hrs						
Pupation (%)	62.0	13.0	6.0	1.0	1.0	0.0
Emergence (%)	39.0	3.0	1.0	0.0	0.0	0.0
120-hrs						
Pupation (%)	72.0	13.0	6.0	0.0	1.0	1.0
Emergence (%)	52.0	3.0	1.0	0.0	0.0	0.0

Remarks: There were no pupae and adults emerged at concentrations of 2,000, 3,000, 4,000.0, and 5,000 ppm.

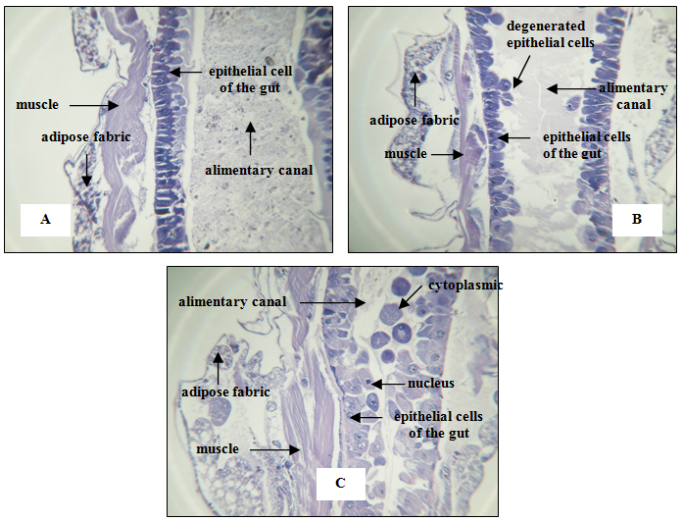

Figure 2. Longitudinal sections in part of the untreated *Aedes aegypti* (L.) larva midgut (A), treated larva with sa-dao-thiam fixed oil showing slightly degenerated epithelial cells of the gut (B), and treated larva with sa-dao-thiam crude extract resulting in serious damage of the epithelial cells of the gut (C).

cus by the larvae of *Cx. pipiens* and *Cx. tarsalis* (Karch and Coz, 1983) and *B. thuringiensis* by the *Simulium pertinax* larvae (Cavados *et al.*, 2004) resulted in a bursting of the epithelial cells, followed by a release of the cytoplasmic material into the alimentary canal. Koua *et al.* (1998) also reported that after treating the *Anopheles gambiae* larvae with aqueous extract of *Persea americana*, most of the epithelial cells in the midgut exploded with a release of cytoplasmic material towards the lumen gut, and degeneration of most cells was observed.

Our results show that the fixed oil of *A. excelsa* has a high potency as a larvicide against *Ae. aegypti*. In field applications, product formulations should be further refined to improve their efficacy. Field trials are needed to investigate the stability and residual activity of the products. In particular, toxicity to non-target organisms in aquatic systems must be investigated and safely precautions taken to minimize unwanted effects before application. Such a result should be achievable, as many studies have found that in general, aquatic insects are at low risk of adverse effects from neem-based insecticides at the expected environmental concentration (EEC) (Kreutzweiser, 1997; Dunkel and Richards, 1998; Kreutzweiser *et al.*, 2000; Scott and Kaushik, 2000).

4. Conclusions

Fixed oil (*n*-hexane extract) obtained from sa-dao-thiam seed kernel has more potent activity as a larvicide against *Ae. aegypti* larvae than the crude extract (methanolic extract). Even at a low concentration of 200 ppm, however, both were effective in delaying larval development to the pupal stage and markedly reduced adult emergence. The mechanism appears to be through causing hypertrophy and degeneration of the epithelial cells of the gut, leading to the death of the *Ae. aegypti* larvae after exposure to these products.

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