INTL J TROP DRYLANDS
Volume 6, Number 1, June 2022 E-ISSN: 2580-2828
Pages: 39-44 DOI: 10.13057/tropdrylands/t060105

The toxicity of *Annona squamosa* seeds and *Anacardium occidentale* seed shells from East Nusa Tenggara, Indonesia, against cabbage caterpillar (*Crocidolomia pavonana*)

PETRONELLA S. NENOTEK♥, JESAYAS A. LONDINGKENE, RIKA LUDJI, TITIK S. HARINI, MAX JUNUS KAPA, ELIAS S. O. NGURU, EFFY ROEFAIDA, MELIANA KONANIN
Department of Agrotechnology, Faculty of Agriculture, Universitas Nusa Cendana. Jl. Adisucipto Penfui, Kupang 85001, East Nusa Tenggara, Indonesia. Tel./fax.: +62-380-881-580, ♥email: nellanenotek01@gmail.com

Manuscript received: 16 January 2021. Revision accepted: 16 June 2022.

Abstract. *Nenotek PS, Londingkene JA, Ludji R, Harini TS, Kapa MJ, Nguru ESO, Roefaida E, Konanin M. 2022. The toxicity of* Annona squamosa *seeds and* Anacardium occidentale *seed shell from East Nusa Tenggara, Indonesia, against cabbage caterpillar (*Crocidolomia pavonana*). Intl J Trop Drylands 6: 39-44.* The study objective was to determine the effect of methanol extracts of *Annona squamosa* L. seeds and seed coat of *Anacardium occidentale* L. from East Nusa Tenggara, Indonesia, on the *Crocidolomia pavonana* (Fabricius, 1794), the cabbage caterpillar. Bioassay was done using with a residue method in feed for one minute at each concentration of *A. squamosa* seed extract (0.01%; 0.03%; 0.06%; 0.125%; 0.25%; and control) and extract *A. occidentale* (0.06%; 0.125%; 0.25%; 0.5%; 1%; and control). The results showed that the methanol extract of *A. squamosa* seeds was more toxic to *C. pavonana* larvae compared to the methanol extract of *A. occidentale*. The LC_{50} and LC_{95} values of *A. squamosa* seed extract against the tested insects were 0.04% and 0.16%.

Keywords: *Anacardium occidentale, Annona squamosa, Croccidolomia pavonana*, lethal concentrate, toxicity

INTRODUCTION

Farmers' overreliance on synthetic pesticides in agriculture caused negative impacts on various non-target organisms and the environment. For example, it posed serious health risks to humans, damaged biodiversity and the environment, increased bioaccumulation of pesticide residues in the food chain, killed pollinators, polluted water and air, influenced changes in the microbiome to reduce mammalian resistance to pathogens, and the emergence of resistant insect (Mahmood et al. 2016; Tarar 2019; Syromyatnikov et al. 2020; Kalyabina et al. 2021; Riyaz et al. 2022). Mesnage et al. (2021) found 186 pesticide residues from the pyrethroid and organophosphate groups in human urine samples. According to World Health Organization (WHO), every year, there are three million cases of poisoning by synthetic pesticides, which cause the death of about 220,000 people (Mughal 2018). To reduce the negative impact of the unwise use of synthetic pesticides, the United States government has issued laws on the protection of food quality and regulations to reduce the circulation of synthetic pesticides in the market (Dayan et al. 2009). In addition, there is an increasing public demand for food safety and quality (Damalas and Koutroubas 2018). Thus, pest control technology must comply with these standards; one of the control technologies is botanical insecticides. Botanical insecticides have advantages compared to synthetic pesticides. The volatile residue is relatively safe for human health and the natural environment and does not cause insect resistance. Botanical pesticide is an alternative pest control technology that is relatively safe in producing quality food (Damalas and Koutroubas 2020) and causes no environmental harm. Sources of botanical pesticides are secondary metabolites produced from various species of plants such as flavonoids, alkaloids, essential oils, glycosides, ethers, and fatty acids that function as antifeedants, repellents, attractants, inhibit growth and kill the pests and diseases of the plant (Hikal et al. 2017). These secondary metabolites are stored in tissues such as stems, seeds, latex, cell wall, trichome, and seed coat. Furthermore, more then hundreds of plants have been demonstrated in the laboratory as insecticides, including *Annona squamosa* L. and *Anacardium occidentale* L. (Murray 2011; Nenotek and Ludji 2020).

Compounds of secondary metabolites of *A. squamosa* and *A. occidentale* have insecticidal properties against pests. For example, hexane seed extract of *A. squamosa* on LD_{50}= 13.98, larvae instar three *Spodoptera litura* (Fabricius, 1775) (Vetal and Pardeshi 2019). Seed methanol extract of *A. squamosa* killed larvae of *Aedes albopictus* (Skuse, 1894) and *Culex quinquefasciatus* (Say, 1823) (Ravaomanarivo et al. 2014). The *A. occidentale* can control the beetle *Callosobruchus maculatus* (Fabricius, 1775) on cowpea (Ileke and Olotuah 2011). Leaves of *A. occidentale* can suppress the development of mosquito larvae (Tripathy et al. 2011). The research by Oparaeke and Bunmi (2006) showed that cashew nut shells killed 100% of *C. subinnotatus* at a concentration of 2.5% and reduced their oviposition.

A. squamosa and *A. occidentale* mostly grow in dryland areas of the East Nusa Tenggara islands. Their waste contains secondary metabolite compounds that can function as botanical insecticides. Their toxicities were tested on the caterpillar *Crocidolomia pavonana* (Fabricius, 1794), one of the important pests of the cabbage. The damage caused by these pests can cause yield losses, so farmers control them with synthetic insecticides such as profenofos. However, profenofos residues attached to cabbage leaves can interfere with human health. Because cabbage leaves are often directly consumed in fresh form and to reduce the negative impact of synthetic insecticides on human health, pest control techniques that are safe for human health and the environment and competitive in the free market are urgently required. Thus, this research was carried out to determine the efficacy of *A. squamosa,* and *A. occidentale* extracts on *C. povonana* larvae.

MATERIALS AND METHODS

Insecticidal plant materials

The experiment was conducted at the Bioscience Laboratory of Universitas Nusa Cendana (for extraction) and Plant Pests, Department of Agrotechnology, Faculty of Agriculture, Universitas Nusa Cendana (for bioassay), East Nusa Tenggara, Indonesia. The materials used as candidate plant insecticides were *A. squamosa* seeds and *A. occidentale* seed coats. Both materials were collected from Salbait Village, West Mollo Sub-district, South Central Timor District, East Nusa Tenggara, Indonesia.

Extraction of plant material

The *A. squamosa* seeds were peeled, and the kernel was collected. Meanwhile, *A. occidentale* was taken from the pseudo-seed coat. Each of these materials was ground using a blender and then sieved through a 0.5 mm mesh. The pulverized *A. squamosa* and *A. occidentale* were macerated separately with methanol for 24 hours at a ratio of 1:5, followed by filtration using a watchman paper No 41. The filtrates were collected in an Erlenmeyer flask. The solvent was evaporated using a rotary evaporator at a temperature of 50°C and a pressure of 240 m bar. The maceration mentioned above was repeated until the extraxt was slightly clear or colorless. The extract was then stored in a refrigerator at a temperature of ± 4°C until it was used for the bioassay.

Preparation of tested insect feed

Pesticide-free cabbage leaves were used for feeding the tested insects and treatment. The cabbage plants were prepared as follows. First, the seeds were sown on the tray. The seedling media consisted of a mixture of soil and compost at 1:1. Two seeds were given to each tray hole at a depth of 1 cm. Second, after the seedlings were 2 weeks old, they were transferred to polybags measuring 20 cm x 20 cm x 20 cm, filled with soil and manure in a ratio of 3:1 (v/v). Each polybag contained one cabbage seed. Cabbage maintenance includes watering, fertilizing, and controlling pests by mechanical means. The cabbage leaves were taken one month after planting and used as feed for the tested insect.

Rearing of tested insects

The tested insect used in this study was the second instar larvae of *C. pavonana*. Rearing of tested insects, following procedures as described by Nenotek (2010) and Prijono and Hassan (1992). Larvae were obtained from cabbage plantations in Tarus Village, East Kupang District, East Nusa Tenggara, Indonesia, then reared in plastic boxes measuring 20 cm x 10 cm x 5 cm by feeding them insecticide-free cabbage leaves. Before pupating, the last instar larvae were transferred to another plastic box which had been given sterile sawdust as a medium for pupating. The pupae were then placed in cages until the emergence of imago. Imago were kept in cages (40 cm x 40 cm x 40 cm). The emerging imago was given a 10% honey solution (absorbed on the cotton) as food. In the cage, pesticide-free cabbage leaves were put in a film bottle filled with water as a place for the imago to lay eggs. The group of eggs contained in the cabbage leaf was removed from the cage and put in another plastic box. Before the eggs hatch, the steamed cabbage leaves are transferred to a plastic box that has been lined with opaque paper and provided with insecticide-free cabbage leaves. Newly hatched second-instar larvae were used for bioassay.

Bioassay

Both extracts of *A. squamosa* and *A. occidentale* were tested at different concentrations. *A. squamosa* extract was tested at a concentration of 0.01%; 0.03%; 0.06%; 0.125%; 0.25%; and control while that of *A. occidentale* extract was 0.06%; 0.125%; 0.25%; 0.5%; 1%; and control. Each treatment was three replicates. Each concentration of each extract was diluted with a mixture of agristic, acetone, and methanol (15:5:4, final concentration 1.2%), then diluted with distilled water to the desired volume. The control solution consisted of aquadest and a mixture of methanol, acetone, and agricultural.

Each treatment was tested on the second instar *C. pavonana* larvae using the residue method on cabbage leaves. Pesticide-free cabbage leaf pieces measuring 4 cm x 4 cm were immersed in the extract for one minute, then dried on opaque paper. The cabbage leaves were placed in a Petridish (9 cm x 9 cm), lined with tissue, then ten instars II larvae of *C. pavonona* (which had just molted) were added to the Petridish. The larvae were given treatment or control feed for 2 days, then replaced with untreated feed for up to 6 days after treatment (DAT). Observations were made 24 hours after treatment by counting the number of dead larvae. Dead larvae were removed from the cup. Mortality monitoring was continued until 6 DAT.

Data analysis

The observed variables were the symptoms of larval mortality and the number of dead larvae in each treatment. Mortality data were analyzed using the POLO PC probit analysis to determine the LC_{50} and LC_{95} toxicity values (LeOra Software 1987).

RESULTS AND DISCUSSION

Toxicity of *A. squamosa* and *A. occidentale* seed extracts against *C. pavonana* larvae

Compared with *A. occidentale* seed extract, *A. squamosa* seed extract is an effective botanical insecticide against *C. pavonana* larvae. The mortality rate of *C. pavonana* larvae was 58% at the concentration of 0.06% *A. squamosa* seed extract, reaching 96%-100% at the concentration of 0.125-0.25%. On the other hand, the mortality of tested insects caused by *A. occidentale* extract did not reach 50%, even at a concentration of 1% (Table 1).

The mortality of *C. pavonana* larvae treated with *A. squamosa* and *A. occidentale* seed extracts started after 24 hours or a day after treatment. On the first day, the mortality of *C. pavonana* larvae reached 100% at a concentration of 0.25%. At a concentration of 0.125%, mortality reached 96% and 100% on the 5th day after treatment (DAT). All 2nd instar larvae died at these two concentrations.

The mortality of 2nd instar larvae of *C. pavonana* reached 34% at a concentration of 0.06%. Larvae mortality increased by 44%, 50%, and 58% on the 2nd, 3rd, and 4th DAT. No larvae died on the 5th and 6th DAT. At the lowest concentration, mortality was 2%, and there was no mortality increase until the observation's end. At this concentration, the live test insects reached instar IV on the 5th and 6th DAT. The same thing was found at a concentration of 0.06%. The mortality development of *C. pavonana* larvae treated with *A. squamosa* seed extract is shown in Figure 1A.

The mortality of *C. pavonana* larvae occurred more at a concentration of 0.5% given *A. occidentale* seed coat extract, which was observed on the first day after treatment. The larvae mortality continued to increase until the 3rd DadAT, after which there was no increase in mortality. At a concentration of 1%, mortality was lower than that of 0.5%. Larvae mortality reached 3%, observed on the first DAT at that concentration. Larvae mortality increased continuously from 2-5 DAT. Larvae mortality reached 18% on the 5th DAT, and there was no increase in mortality on the 6th DAT.

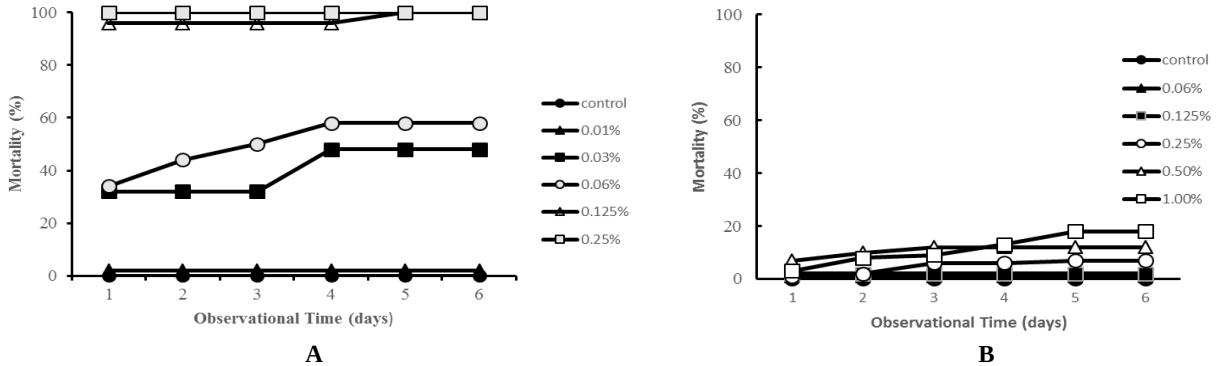
At a concentration of 0.06% larvae mortality reached 1% and 2% at a concentration of 0.125%. Furthermore, no larvae died until the end of the observation. Treatment larvae that did not die successfully developed into IV instars on the 5th and 6th DAT. In control, no larvae died and developed into four instars on the 4th DAT. The mortality development of *C. pavonana* larvae treated with *A. occindentale* seed coat extract is shown in Figure 1B.

The *C. pavonana* larvae decreased growth and development after being treated with *A. squamosa* seed oil and *A. occidentale* seed coat extract. Symptoms of poisoning began to appear 24 HAT, such as decreased feeding activities and moving slower, body size smaller or shrinking compared to the control as a result of not eating, and lack of fluids, the color of the cuticle of the larvae changed from green to cream, the dead larvae were dark in color.

The death of the tested insects was thought to be due to the biological activity of secondary metabolites that can affect behavior and physiology, inhibit and damage body tissues and ultimately result in the death of *C. pavonana* larvae. There is also the possibility that secondary metabolites are antifeedants which causes the larvae not to eat and lack energy which can interfere with the physiological metabolism of the tested insects.

Table 1. Mortality of *C. pavonana* larvae treated with *Annona squamosa* seed extract and *Anacardium occidentale* cashew seed shell

Extract type	Concentration (%)	Mortality (%)*
A. squamosa	Control	0
	0.01	2
	0.03	48
	0.06	58
	0.125	96
	0.25	100
A. occidentale	Control	0
	0.06	2
	0.125	4
	0.25	12
	0.50	18
	1.00	36



A **B**

Figure 1. Daily mortality development of *C. pavonana* larvae treated by extracting (A) *A. squamosa* seeds and (B) *A. occidentale* seed coat

Previous studies revealed two main compounds in *A. squamosa* seeds, i.e., polyphenolics and acetogenins, that inhibited growth and development and killed the tested insects (Bhattacharya and Chakraverty 2016). Polyphenolic compounds consist of flavonoids, saponins, tannins, and alkaloids (Al-ghazzawi 2019; Ma et al. 2019). Saponin works as an antifeedant (Sang et al. 2019), flavonoids interfere with the respiratory system of insects (Justino 2017), and tannins are contact poisons that can reduce amylase and protease activity in digestive enzymes so that protein absorption is disrupted. The effect is lethal due to the disruption of nutrient absorption (Mappau et al. 2018). If the tested insects survive, the growth of larvae will be disrupted and inhibited. Flavonoids and alkaloids contained in *A. squamosa* seeds can kill *A. albopictus* and *C. quinquefasciatus* larvae, with LC_{50} values of 0.5% -1% for larvae and 1% -5% for adults (Ravaomanarivo et al. 2014) so that polyphenic compounds are suspected of interfering with and inhibiting the behavior of *C. pavonana* larvae.

The compounds of the acetogenin group are annonain, squamosin, annonacin, asimicin, cohibinsin, squamostatin-A, and bullatacin (Chen et al. 2012). In addition, Acetogenin also contains squamocin I, squamocin II, squamocin III, and squamoxinone-D, which inhibit cancer cells in mammalian groups (Miao et al. 2015). Squamosin concentrations are abundant in *A. squamosa* (Isman 2006)

The death of *C. pavonana* larvae is suspected to be due to the action of compounds from the acetogenin group by blocking the production of ATP energy in the mitochondria so that the energy supply is blocked and cut off, which causes the tested insects to become weak and eventually die. In addition, the acetogenin group inhibits electron transfer in complex I by blocking the bonds between NADH-ubiquinone oxidoreductase in mitochondrial oxidative phosphorylation, causing a reduction in the amount of ATP (adenine triphosphate) (González-Coloma et al. 2002; Yabunaka et al. 2003) so that metabolism is disrupted because it does not get energy, which can cause insects to experience ATP deficiency, poisoning, and death.

The acetogenin group (anonnain and squamosin) works in the body of insects as contact poisons and stomach poisons (Khair and Noraida 2019). As a contact poison, the poison enters the insect's body through cuticles, natural openings, sensory with antenna, and tarsi. Furthermore, the poison spreads throughout the body through the bloodstream, which will, in turn, poison and damage the body. Toxins also enter and disrupt the nervous system through the trachea, thus damaging the body tissue of the test insect because the information obtained is disturbed in the trachea system. Finally, toxins that enter through the hemolymph will interfere with the cholinesterase enzyme so that the nerves do not function due to the accumulation of acetylcholine and receive excessive or continuous signals (Mustika et al. 2016).

Acetogenin works as a stomach poison in the mesenteron that is carried by eating; da Silva Costa et al. (2016) showed that squamosin's effect could change the brush border's position and vacuolize the apical cytoplasm of cells in the digestive system of *Aedes aegypti* (Linnaeus, 1762) larvae so that the possibility of these changes causes the changes in water balance. In addition, squamosin also plays a role in reducing the expression of the V-ATPase gene in the digestion of *A. aegypti* larvae. As a result, it can interfere with nutrient absorption, electrolytic ions, and nutrient transport. Furthermore, Squamosin damages the AQP4 gene so that there is no balance of cell osmosis and damages the cell walls of the middle intestine. This phenomenon may have occurred in the digestive system of *C. pavonana* larvae so that the 2^{nd} and 3^{rd} instar larvae consuming *A. squamosa* seed extract could damage their digestive system. Therefore, it is suspected that one of the squamosin control mechanisms is to damage the digestive cell walls of *C. pavonana* larvae, resulting in the death of more larvae in *A. squmosa* extract as compared to *A. occidentale* seed coat extract.

The seed coat of *A. occidentale* was extracted with methanol as a solvent in the form of oil. The oil contains alkaloids, flavonoids, phenolics, cardonals, and tannins (Paiva et al. 2017; Aga 2018). Other compounds in the seed coat of *A. occidentale* are steroids, triterpenoids, xanthoproteins, volatile oils, and emodins that can inhibit the development of pathogens *Aspergillus flavus, Aspergillus niger, Fusarium* sp., and *Curvalaria* sp (Kannan et al. 2009). At a 60.36 mg/ml concentration, phenol can kill 50% of *Sitophilus oryzae* (A.Hustache, 1930) imago, while cardanol can kill 50% of *A. aegypti* larvae at a concentration of 0.0023 ppm 72 HAT of exposure (Buxton et al. 2017). Flavonoids and tannins inhibit the proliferation of cancer cells (Tietbohl et al. 2017). In another study, it was explained that the growth of *Trichoderma* sp. and *Gliocladium* sp. was inhibited after being treated with 2.5% CNSL oil (Bande et al. 2018). In this study, CNSL oil applied to *C. pavonana* larvae did not have a significant effect compared to *A. squamosa* oil. Still, the role of several compounds such as alkaloids, flavonoids, phenols, steroids, triterpenoids, xanthoproteins, essential oils, and emodin was able to kill larvae *C. pavonana* by 36% at the concentration of %. It is suspected that each secondary metabolic compound in CNSL acts at their respective target sites but is not synergistic in suppressing the growth of *C. pavonana* larvae.

Table 2. Estimating the toxicity parameters of *Annona squamosa* seed extract and *A. occidentale* cashew seed shell to *Crocidolomia pavonana* larvae

Extract type	a±SE	*b* ± SE	LC_{50} (CI 95%) (%)	LC_{95} (CI 95%) (%)
A. squamosa	3.84±0.42	2.85 ± 0.31	0.04 (0.03-0.05)	0.16 (0.11-0.31)
A. occidentale	0.40 ± 0.15	1.32 ± 0.27	2.02 (0.99-17.83)	34.99 (6.71-10216.00)

Note: Description: a = probit regression intercep, b = the slope of the probit regression, SE = Standard error, CI = confidence interval

Estimation of toxicity of *A. squamosa* seed extract and *A. occidentale* cashew seed shell to *C. pavonana* larvae

The relationship between the treatment concentration with plant insecticide extracts and mortality of *C. pavonana* larvae was analyzed using probit analysis. The slope value of *A. squamosa* extract was higher than that of *A. occidentale* (Table 2). This result can explain that adding *A. squamosa* seed extract concentration will increase the mortality of *C. pavonana* larvae higher than that of *A. occidentale* extract at the same concentration. Meanwhile, the intercept value and the slope of *A. occidantale* did not show accurate results, presumably because the secondary metabolic compounds contained in the extract were less effective in poisoning *C. Pavonana* larvae.

The probit analysis showed that the seed extract of *A. squamosa* was more toxic to *C. pavonana* larvae than the seed shell of *A. occidentale* (Table 2). At a concentration of 0.04%. *A. squamosa* extract can kill 50% of *C. pavonana* larvae. Meanwhile, a concentration of 2.02% of *A. occidentale* seed coat is required to kill the same number of tested insects. *A. squamosa* seed extract at a lower concentration (0.16%) killed 95% of *C. pavonana* larvae, but *A. occidentale* oil required a high concentration (34.99%). These results explained that the seed extract of *A. squamosa* was more toxic to *C. pavonana* larvae than the seed coat extract of *A. occidentale*. Based on LC_{50}, *A. squamosa* sees extract is more toxic to *C. pavonana* larvae by 50,5 times than the *A. occidentale* seed coat. Based on the value of the probit analysis, it can be concluded that *A. squamosa* extract was effective for controlling *C. pavonana* larvae because only a small amount of test material is required to kill the test insects. Botanic insecticides are said to be effective if extracted with organic solvents. They can kill ≥80 test insects at the highest concentration of 1% while using water as a solvent at 10% (Dadang and Prijono 2008).

Based on the LC_{50}, the seed extract of *A. squamosa* was more toxic to *C. pavonana* larvae by 50.5 times higher than the seed coat of *A. occidentale*. At the LC_{95} level, *A. squamosa* seed extract was 218.68 times more toxic than *A. occidentale*. The results of Taslimah's research (2014) showed that the LC_{50} value of *A. squamosa* seed extract caused the death of *A. aegypti* larvae by 14.71% or 14.71 ml/100 ml. Furthermore, the seed extract of *A. squamosa* inhibited the feeding activity of *Trichoplusia ni* (Hübner, 1803) larvae (Hubner (DC_{50}= 2.3 mg/mL), inhibited growth (EC_{50}= 38.0 ppm) and killed by feeding method (LC_{50}= 167.5 ppm) (de Cássia Seffrin et al. 2010; Vetal and Pardeshi 2019). Methanol and hexane extract of seeds of *A. squmosa* larvae instar III (Maisng LD_{50}= 13.98 mg/mL; LD_{50}= 22.48 mg/mL) (Vetal and Pardeshi 2019). That illustrates that *A. squamosa* extract has strong insecticidal properties in *C. pavonana* larvae compared to *A. aegypti* larvae, *T. ni* larvae, and larvae of *S. litura*.

The leaves and bark of *A. squamosa* also contain secondary metabolites found in the seeds. Some of the compounds found in the leaves are phenols, alkaloids, flavonoids, and isomeric hydroxyl ketones (Bhattacharya and Chakraverty 2016; Kumar et al. 2021). The compounds found in the bark are Acetogenin and squamocin (Bhattacharya and Chakraverty 2016). The results of this study indicate that the extract of *A. squamosa* is more effective than *A. occidentale* because there is a possibility that the acetogenins and or polyphenolics group of compounds work on different targets. Therefore, the compounds contained in A. squamosa work synergistically. Based on the results of this research and a review of the literature, it is shown that *A. squamosa* has the potential to be developed into an environmentally friendly insecticide product.

In conclusion, *A. squamosa* seed extract effectively controlled *C. pavonana* larvae compared to *A. occidentale* seed extract. The results of probit analysis showed that at concentrations of 0.04% and 0.16% of *A. squamosa* seed extract, it could kill 50% and 95% of *C. pavonana* larvae. On the other hand, while seed coat extract of *A. occidentale* was ineffective in killing larvae *C. pavonana* at a concentration of 1%, it only killed 36% of the tested insects. Therefore, this research shows that *A. squamous* seed extract from dryland in East Nusa Tenggara needs to be developed as an environmentally friendly pest control.

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