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Bio-efficacy of *Annona squamosa*, *Azadirachta indica* and *Artocarpus heterophyllus* against fall armyworm

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Abstract

Fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera Noctuidae), is one of the serious global pests that causes high economic losses in crop production, mainly in maize plants. As natural plant products are becoming more and more interested in protecting agricultural crops today, the aim of the present investigation was conducted to evaluate the insecticidal action of *Annona squamosa* L., *Azadirachta indica* A. Juss and *Artocarpus heterophyllus* Lam. crude ethanolic extracts against fall armyworm. Affectivity was based on the mortality test of the egg as well as its second and third larval stages. All extracts showed high ovicidal activity and exhibited 100% mortality against the eggs of fall armyworm. All of the eggs were remained completely dried out and unhatched three days following treatment with the 10 mg mL^{-1} dose. In this study, three different type of bioassays, namely ingestion bioassay, topical bioassay and residual bioassay were carried out. At ingestion bioassay tested with second instar, LC_{50} value of ethanolic extracts of *A. squamosa*, *A. indica* and *A. heterophyllus* were 4.66 mg mL^{-1}, 3.78 mg mL^{-1} and 4.04 mg mL^{-1}, respectively. For third instar, LC_{50} value of ethanolic extracts of *A. squamosa*, *A. indica* and *A. heterophyllus* were 17.64 mg mL^{-1}, 14.12 mg mL^{-1} and 15.70 mg mL^{-1}, respectively. In topical bioassay treated with 25 mg mL^{-1} of all plant extracts, 95-100% mortality was detected in second instars and 85-95% mortality was noted in third instars. In residual bioassay treated with 25 mg mL^{-1} of extracts, 48-67% mortality was observed in second instars and 6-15% mortality was observed in third instars. The later in their growth stages the larvae are, the higher their chance of surviving when treated with crude extracts. The crude ethanolic extract of *A. squamosa* had moderate insecticidal activity. *A. heterophyllus* showed high insecticidal activity and it could be the good candidate to be developed as sources of botanical insecticides for the management of fall armyworm since its effect was comparable with that of a well-known plant, *A. indica*, for its insecticidal activity.

Key words: *Annona squamosa*, *Artocarpus heterophyllus*, *Azadirachta indica*, fall armyworm, insecticidal.

Introduction

Fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera Noctuidae), is the invasive migratory pest native to tropical and subtropical America (Sparks, 1979). It feeds on the leaves, stems and reproductive parts of more than 350 plant species (Ganiger *et al*., 2018) and causes major damage to the cultivated crops, including maize and rice (Montezano *et al*., 2018). In 2016, it was first reported in West and Central Africa, then quickly invaded to Indian subcontinent (Gurrola-Pérez *et al*., 2018), African countries, India, Myanmar, Thailand, other Asian countries and Yunnan Province in western China (Sun *et al*., 2021). At the end, it becomes the world threat in agriculture. Extensive use of chemical insecticides for control of fall armyworm has created problems related to the emergence of insect resistance to several classes of insecticide (Goergen *et al*., 2016), adverse effects on non-target insect species (Desneux *et al*., 2007), biodiversity loss (Aguoru *et al*., 2015) and human health related challenges such as carcinogenesis (Damalas and Eleftherohorinos, 2011). Hence, evaluation of new products on the effectiveness in controlling this pest is necessary.

In recent years, natural plant compounds have drawn the attention of the researchers as strong sources of novel insecticides. Botanical pesticides have been used in agriculture for at least 2000 years in Asia and the Near East (Thacker, 2002) and increased interest because of their efficacy, degradability, and physiological activity (Isman, 1999). They are seen as viable alternatives in agriculture since they are less hazardous to the environment and people's health than conventional chemical insecticides. A range of plant species derivatives, including leaves, flowers, fruits, seeds, barks, and roots, produce a wide variety of secondary metabolites that are repellent or toxic to insect pests (Besmer *et al*., 2021). In the last thirty years, researchers have been focused on the use of plant-derived materials as a potential source for value goods like commercial biopesticides (Souto *et al*., 2021). It has been proven that numerous plants have insecticidal properties against a variety of insect pests (Jbilou *et al*., 2006; Satti *et al*., 2010; Diabaté *et al*., 2014; Ahmed *et al*., 2020). The substances such as terpenoids, flavonoids, alkaloids, glycosides, esters and fatty acids extracted from nature plants have important ecological activity in nature, such as repellent, insecticidal, antifeedant, growth inhibitors, oviposition inhibitors, ovicides, and growth-reducing effect on a variety of insects (Wafaa *et al*., 2017; Souto *et al*., 2021). An integrated insect management program has advocated using certain botanical pesticides because they limit the use of synthetic insecticides, only affect the target insects, and provide a safe environment and uncontaminated food (Wafaa *et al*., 2017).

A wide variety of plants possess broad insecticidal activity and have been commonly used in traditional agricultural applications in many parts of the developing countries (Nair and Kavrekar, 2017). Previous research reported that the leaf extract of *Annona squamosa* L. contains alkaloids, protein, amino acid, carbohydrate,

Table 1. Sampled plants of Myanmar.

Species	Myanmar common name	Family	Parts used
Annona squamosa	Awzar	Annonaceae	Leaves
Azadirachta indica	Thamar	Miliaceae	Leaves
Artocarpus heterophyllus	Pein-nawe	Moraceae	Leaves

glycosides, phytosterols, tannins, phenolic compounds etc. and showed the potent insecticidal activity against the storage pest *Sitophilus oryzae* (L.) (Kumar *et al*., 2010). *Azadirachta indica* A. Juss also have various modes of action against insects such as repellent (Parugrug and Roxas, 2008; Djenontin Tindo *et al*., 2012), antifeedant (Akhtar *et al*., 2008; Pavela, 2009; Pinheiro and Quintela, 2010; Gadi, 2017; Kurniati *et al*., 2018), toxic (Bello *et al*., 2014; Nishan and Subramanian, 2015; Zanuncio *et al*., 2016), growth inhabitant (Nisbet, 2000; Nathan *et al*., 2006; Akhtar *et al*., 2008), fecundity suppression (Nisbet *et al*., 1996) and sterilization (Linton *et al*., 1997; Mulla and Su, 1999; Bansal *et al*., 2010). As *A. indica* extracts are not harmful to plants or humans, they can also be used to control the insects in gardens and around dwellings (Gadi, 2017). *Artocarpus heterophyllus* Lam. is a species of tree of the mulberry family and native to Western Ghats of India, Malaysia and also found in central and eastern Africa, south-eastern Asia, the Caribbean, Florida, Brazil, Australia, Puerto Rico and many Pacific Islands (Rahman *et al*., 1999). It also has important source of compounds that are useful in fever, boils, wounds, skin diseases, convulsions, diuretic, constipation, ophthalmic disorders and snake bite etc (Prakash *et al*., 2009).

In order to manage pest problems and enhance sustainable production by finding some natural sources of botanical origin with insecticidal potential against *S. frugiperda*, our study aims to clarify the insecticidal activity of ethanol extracts isolated from *A. squamosa*, *A. indica* and *A. heterophyllus*. The results of laboratory tests on ovicidal and larvicidal properties of plant extracts were discussed.

Materials and methods

Insect rearing and maize crop planting

S. frugiperda larvae were first collected in the field of maize cultivation land, Mandalay region, Myanmar on January 2020. The larvae were maintained as a stock colony in the Entomology Lab at the Department of Biotechnology Research (DBR) in Myanmar under constant temperature (27 ± 2 °C), relative humidity (70 ± 5%), and 16L:8D photoperiod. Freshly harvested maize plants and freshly ground maize seeds were fed to the larvae during their development. The bioassays were carried out in the Entomology Lab of DBR with the same condition of the rearing. For insect diets, maize crops were planted in plastic pots. For bioassay experiments, two maize crops were planted in each 500 ml plastic drinking cup.

Plant sample extraction

Plant samples were collected from Saw-ye village, Sinkaing Township, Mandalay region, Myanmar, and identification of tested samples was carried out by an authorized botanist of Mandalay University, Myanmar. All extracts were produced only from plant leaves (table 1). The samples were dried at room temperature. After completely drying, the samples were ground in an electric mill and stored in plastic bags. Then, 100 g of crushed plant material from each sample was added to the glass container containing 1.0 L of alcoholic solution (95% ethanol). The containers were placed at room temperature and left for 1 month. The extracted plant materials were filtered using filter paper, and the solvent was removed using a rotary evaporator. The concentrated plant extracts were dried and stored in the refrigerator for further experiments.

Ovicidal bioassay

The efficiency of ethanolic plant extracts was evaluated on each egg mass of *S. frugiperda*. First, the leaves of the maize plant laid with the egg masses of *S. frugiperda* were collected from rearing insect cages. Then, a leaf part (≅ 25 mm length) containing each egg mass of *S. frugiperda* were put into individual sterile polystyrene cell culture dish (6.0 cm diameter × 1.5 cm height). Before treatment, the eggs per mass were examined under a stereomicroscope and checked to see whether they were intact. Most of the *S. frugiperda* egg masses were irregular in shape, and some were covered with hairs. The number of eggs per mass varies and is approximately 100 to 200 eggs per mass. Then, the eggs were treated with ethanolic extracts of *A. squamosa*, *A. indica* and *A. heterophyllus*. 10 µL of each extract with three different concentrations (10 mg mL^{-1}, 25 mg mL^{-1} and 50 mg mL^{-1}) was dropped to each egg mass. Besides, the Petri dish was sealed with paraffin in order to prevent the escape of the hatched larvae. This experiment was performed three times, and ethanol alone was used as a negative control. Subsequently, the egg masses were observed daily under microscope and checked the condition of hatchery. Finally, data analysis was carried out.

Ingestion bioassay

15 ml polyethylene centrifuge tubes were utilized as the experiment container in this bioassay. First, half of a fresh maize seed was added into the bottom of each 15 ml centrifuge and crushed well. 10 µL of each extract with six different concentrations (2.5, 5, 10, 20, 30 and 50 mg mL^{-1}) was added topically. An individual larva was then put in each centrifuge tube containing treated diet. The cotton ball was moistened with water and blocked at the tip of the tubes. Ethanol alone was used as a negative control instead of plant extracts. The bioassay was three times replicated (20 larvae per replication) and ethanol alone was used as negative control. Only larvae that survived within 12 hours were continued being kept

at 27 °C. After all treated diets had gone, two fresh maize seeds were replaced daily for 7 days. Final mortality data were recorded daily by counting the number of dead larvae and the LC_{50} value for each extract was calculated using Probit analysis (Robertson *et al*., 2017).

Topical bioassay

Direct contact toxicity was performed by topical application. First, each larva was picked from rearing insect cage and put onto a glass Petri dish (9.0 cm × 1.5 cm). Then 10 µL of three concentrations (10, 25 and 50 mg mL^{-1}) of each extract was dropped onto each larva using a pipette. Each treated larva (second instar and third instar) was immediately transferred to each plastic cup planting with two maize plants at 2 weeks of seedling stage. Another plastic cup that had been punctured with several needle holes was inverted on it. The tips of these plastic cups were sealed with paraffin to prevent insects from escaping. For each extract concentration, twenty larvae were employed and three replications were done. Ethanol alone was used as negative control. Final mortality data were recorded daily by counting the number of dead larvae, with mortality data corrected using Abbott's formula (Abbott, 1925).

Residual spraying bioassay

First, two maize seedlings were planted per 500 ml plastic drinking cup for this bioassay. At eighteen days after seedling, maize plants were sprayed with three concentrations (10, 25 and 50 mg mL^{-1}) of plant extract solution and allowed to dry for 1 hour. Once dry, each larva (second and third instar) was placed in the treated plants per plastic cup. To avoid a larva escaping, each treated cup was covered with another empty 500 ml plastic drinking cup, and sealed with paraffin. For each treatment, 20 larvae were used and 3 replications were carried out. Just ethanol was used as a negative control. Daily mortality data was recorded and adjusted using Abbott's formula (Abbott, 1925). At day 4, each live larva was placed into a 500 ml polypropylene plastic drink cup with untreated maize plants.

Results and discussion

Ovicidal activity of nature plant extracts

Three days after treatment with 10 mg mL^{-1} concentration, all eggs were still completely dried and unhatched (figure 1). Their appearance looked as if they had been burned. Some eggs were distorted and shrunk. 100% motility was observed in *S. frugiperda* eggs treated with all ethanolic extracts. In a negative control using ethanol alone, nearly 1% mortality of the eggs was observed (figure 2).

Lots of previous reports were revealed on the effective kill of *A. squamosa* against lepidopteran larvae (Leatemia and Isman, 2004; Ruiz Hidalgo *et al*., 2018; Vetal and Pardeshi, 2019), *Tribolium castaneum* (Herbst) (Khalequzzaman and Sultana, 2006), *S. oryzae* (Ashokkumar *et al*., 2010), cockroach (Kesetyaningsih, 2012), *Hyalomma anatolicum* Koch (Ilham *et al*., 2014), *Anopheles stephensi* Liston, *Tenebrio molitor* L. and non-targeting organism *Artemia* nauplii (Vivekanandhan *et al*., 2021). According to Singh *et al*. (2011), an aqueous extract of *A. indica* leaves at a dosage of 1.25 mg/ml significantly reduced *Haemonchus contortus* (Rudolphi) (Nematoda Rhabditida) egg embryonation and egg hatching.

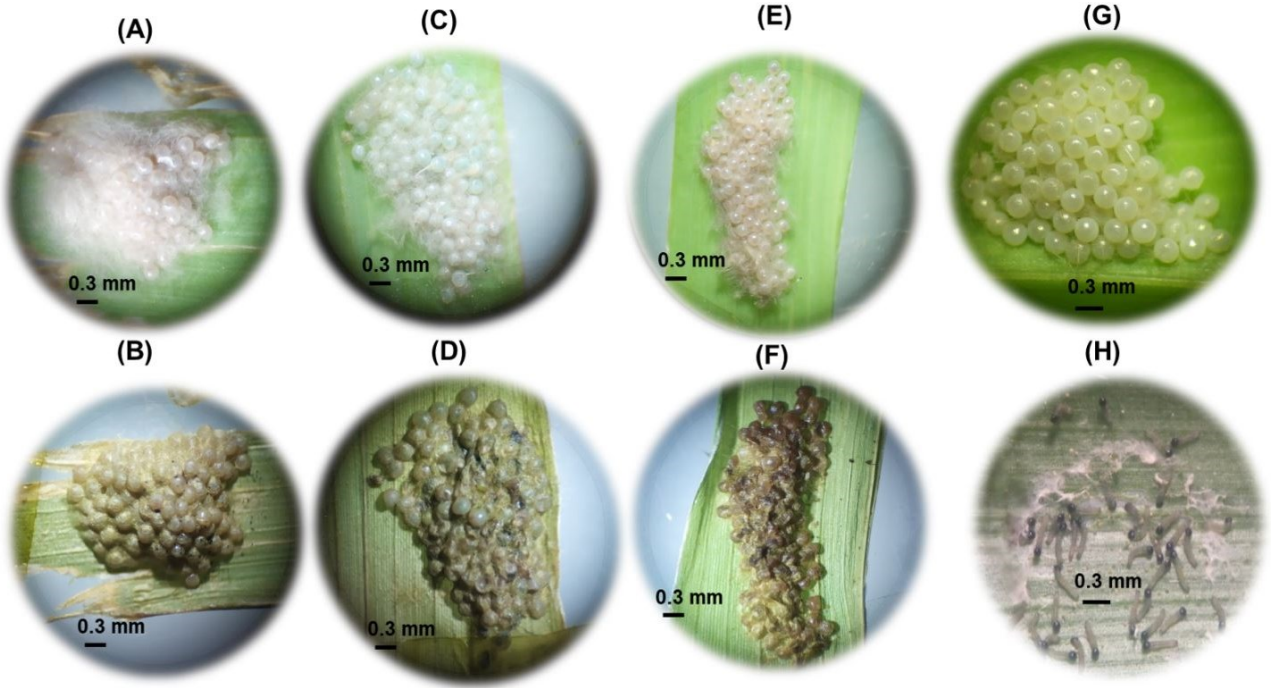

Figure 1. Appearance of *S. frugiperda* eggs per mass under stereomicroscope before and after treatment with the concentration of 25 mg mL^{-1} plant extracts. **(A)** Before treatment with *A. squamosa*, **(B)** after treatment with *A. squamosa*, **(C)** before treatment with *A. indica*, **(D)** after treatment with *A. indica*, **(E)** before treatment with *A. heterophyllus*, **(F)** after treatment with *A. heterophyllus*, **(G)** before treatment with ethanol as negative control, **(H)** after treatment with ethanol as negative control.

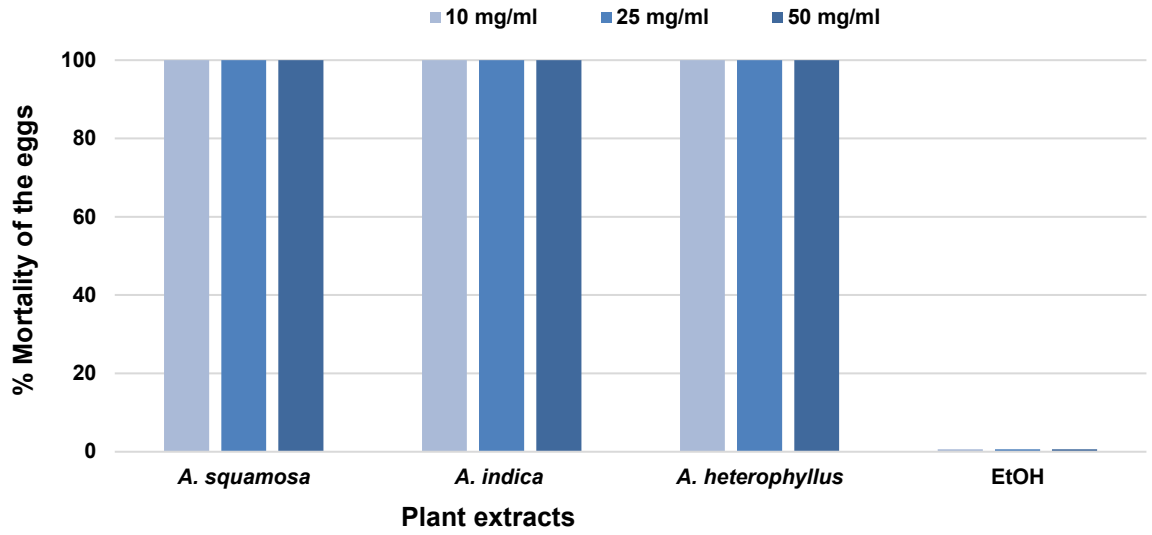

Figure 2. Mortality of *S. frugiperda* eggs after treatment with ethanolic extracts of *A. squamosa*, *A. indica* and *A. heterophyllus*.

Table 2. Probit analysis of dose mortality response of selected plant extracts against the larvae of *S. frugiperda*.

Extracts	LC_{50} (mg mL^{-1})	95%CI	Chi-Square	Intercept	Sig.
Second instar					
Annona squamosa	4.66	3.59-6.03	1.945	-2.018	0.584
Azadirachta indica	3.78	2.88-4.87	0.517	-1.760	0.915
Artocarpus heterophyllus	4.04	3.09-5.23	1.699	-1.809	0.637
Third instar					
Annona squamosa	17.64	13.94-22.13	3.354	-3.932	0.340
Azadirachta indica	14.12	11.24-17.33	1.987	-4.182	0.575
Artocarpus heterophyllus	15.70	12.30-19.64	2.941	-3.758	0.401

Larvicidal activity of selected plant extracts

In the present work, the insecticidal activity of ethanolic plant extracts were evaluated against the immature fall armyworm. At ingestion bioassay, LC_{50} of ethanolic extracts on second and third instars were estimated (table 2). For the second instar larvae, LC_{50} values of ethanolic extracts of *A. squamosa*, *A. indica* and *A. heterophyllus* were 4.66 mg mL^{-1}, 3.78 mg mL^{-1} and 4.04 mg mL^{-1}, respectively. For the third instar larvae, LC_{50} values of *A. squamosa*, *A. indica* and *A. heterophyllus* were 17.64 mg mL^{-1}, 14.12 mg mL^{-1} and 15.70 mg mL^{-1}, respectively. Ethanolic extracts of *A. indica* and *A. heterophyllus* had the lowest LC_{50} values. The larvicidal effectiveness of *A. squamosa* was comparable to that of those two extracts.

In topical bioassay, all extracts with a concentration of 50 mg mL^{-1} exhibited the highest 100% mortality of the larvae (data not shown). The mortality of the ethanolic extracts of *A. squamosa*, *A. indica* and *A. heterophyllus* at a concentration of 25 mg mL^{-1} was 86.7%, 88.3%, and 98.3% for the second instar and 95%, 95%, and 100% for the third instar, respectively (figure 3). The survival rate of the fall armyworm was proportionate to the growth stage of its larva in the comparison investigation of the mortality percentage of each extract vs. growth stage (second and third instars). The mortality rate of the larvae following treatment decreases with increasing larval growth stage.

When all extract concentrations were lowered to 10 mg mL^{-1}, the mortalities of the ethanolic extracts of *A. squamosa*, *A indica* and *A. heterophyllus* were 43.3%, 66.7% and 68.3% for the second instar and 23.3%, 53.3% and 66.7% for the third instar, respectively (figure 3). The treated larvae feeding rate on the maize crop was not significantly different with each extract but significantly less than the untreated control. In addition, the mobility of these larvae was sluggish, compared with negative control. The mortality test was monitored until the adult fall armyworms emerged in order to ascertain the impact of the extracts on the later phases of the survivors of the treatment. Although pupae and adults were not treated, subsequent levels of mortality were observed in later stages of the survivors of the treatment (figure 4). Following treatment with *A. squamosa* concentration of 10 mg mL^{-1}, the lifespan response of the third instar larvae was 20% larval death, 25% pupal death, and 55% adult survivor. Similarly, with *A. indica*, it was noted that 50% of larvae died, 20% of pupae died, and 30% of adults survived. In the case of *A. heterophyllus*, 60% of the larvae died, 5% of the pupae died, and 35% of the adults survived.

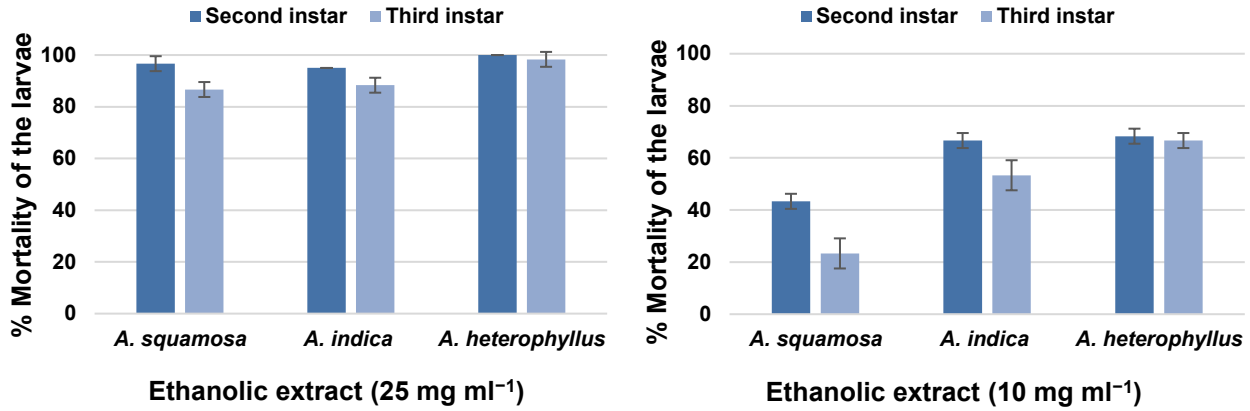

Figure 3. Mortality percentages of the ethanolic extracts of *A. squamosa*, *A. indica* and *A. heterophyllus* using topical bioassay.

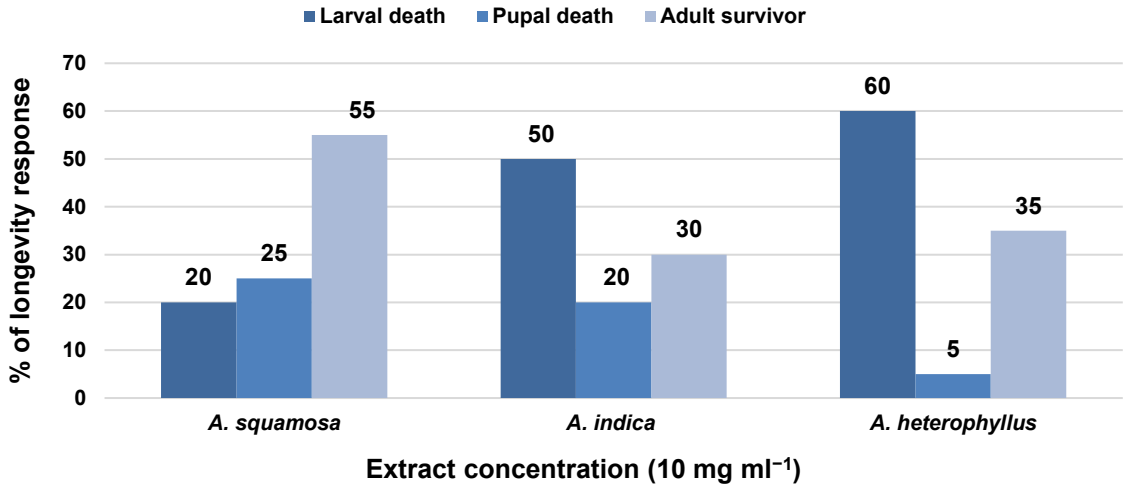

Figure 4. Percentage of longevity response of third instars after treatment with 10 mg mL^{-1} concentration of selected plant extracts.

According to the previous study by Gadi (2017), *A. indica* extract was found to have an adverse effect on eggs, larvae, pupae, and adults, leading to direct mortality in treated stages and some additional mortality in succeeding life stages for individuals who did not die in the treated stage. These long-lasting impacts are probably significantly different in terms of rate and maybe mechanistically from what would have happened if those stages had been treated promptly (Gadi, 2017). Moreover, the increasing concentration of neem oil led to an increase in morphological deformation in the wings, legs, and scutellum, along with mortality (Zanuncio *et al*., 2016).

In residual bioassay, ethanolic extracts of *A. squamosa*, *A. indica* and *A. heterophyllus* at a concentration of 50 mg mL^{-1} caused 80-100% mortality on *S. frugiperda* second instar larva and 48-68% mortality on *S. frugiperda* third instar larva, respectively. At 25 mg mL^{-1} concentration, ethanolic extracts of *A. squamosa*, *A. indica* and *A. heterophyllus* caused 48.3-65% mortality on *S. frugiperda* second instar larva and 6.7-15% mortality on *S. frugiperda* third instar larva, respectively (figure 5). The levels of mortality were lower as the extract concentrations were lower. The extracts from *A. heterophyllus* and *A. indica* showed the highest larval mortality with residual bioassay. Compared with those two extracts, *A. squamosa* had a moderate insecticidal activity.

In residual bioassay, nearly all survivors treated with *A. indica* were found to have significantly reduced growth rates, but only some survivors treated with *A. heterophyllus* and *A. squamosa* showed a significantly reduced growth rate. As azadirachtin substance extracted from *A. indica* interferes with the growth and moulting processes of insects, its ingestion leads to abnormal moults, growth reduction, and increased mortalities (Nisbet, 2000). Along with growth-deterring properties, neem oil significantly delays reproduction in pests (Boulahbel *et al*., 2015). Neem extract also causes lethal toxicity during the pupal stage, which then leads to various morphological deformations such as malformed adults, partial ecdysis, and moult blocking that defers and inhibits adult formation (Boulahbel *et al*., 2015; Chaudhary *et al*., 2017).

According to our research, *A. heterophyllus* also had growth-deterring properties and similar lethal toxicity like *A. indica*. Insecticidal action of *A. heterophyllus* was also seen against third instar larvae of *Musca domestica* L. (Begum *et al*., 2010). At a sub-lethal dose, the latex

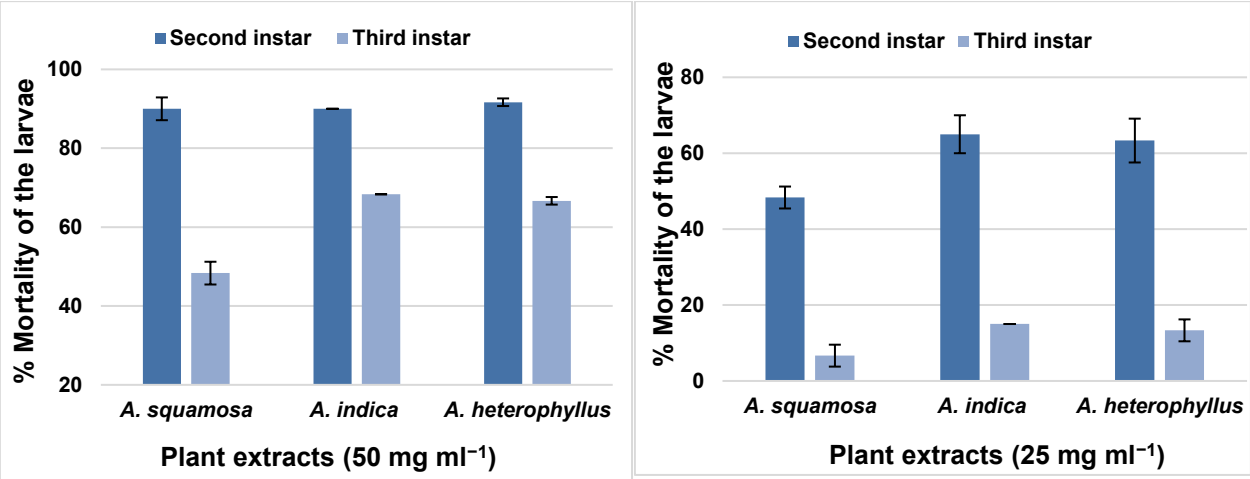

Figure 5. Mortality percentages of the ethanolic extracts of *A. squamosa*, *A. indica* and *A. heterophyllus* using residual bioassay.

extract from *A. heterophyllus* significantly decreased larval weight and had a very high rate of larvae mortality (Upadhyay, 2013). Nucleic acids and protein contents play a significant role in controlling the various activities of cells and are regarded as important biomarkers of the metabolic potential of cells (Nair and Kavrekar, 2017). They reported that *A. heterophyllus* extracts had a reducing effect on the nucleic acid and protein content in the larvae of *Bruchus pisorum* (L.), *Tribolium castaneum* (Herbst), and *Sitophilus oryzae* (L.) (Nair and Kavrekar, 2017).

Moreover, some survivors treated with *A. squamosa* experienced a modest decrease in their rate of growth. Many studies on *A. squamosa* insecticidal effectiveness on different pests have been reported. Vetal and Pardeshi (2019) demonstrated the outstanding insecticidal activity of *A. squamosa* seed extract against *Spodoptera litura* (F.). The leaf extracts of *A. squamosa* at concentrations of 25%, 50%, 75%, and 100% (w/v) showed that they effectively killed *Periplaneta americana* (L.) (Kesetyaningsih, 2012).

The current study also demonstrated that the higher the mortality rate when using topical bioassay or residual bioassay, the younger the larval growth stage. Therefore, the timing of administration at the insect stage is crucial for the successful killing of fall armyworm. The efficiency of *A. squamosa*, *A. indica* and *A. heterophyllus* extracts against *S. frugiperda* eggs and larvae has been demonstrated, and it has been proposed that the plants under study may one day prove useful as natural pesticides for control damages of *S. frugiperda* on maize.

Conclusion

Ethanolic extracts of *A. squamosa*, *A. indica* and *A. heterophyllus* demonstrated a high ovicidal action against fall armyworm. In *S. frugiperda* eggs treated with all ethanolic extracts, 100% mortality was seen. Several eggs were deformed and reduced in size. It was also discovered that all extracts had a negative impact on eggs and larvae, causing direct mortality. Ethanolic extract of *A. squamosa* revealed a moderate larvicidal activity. *A. indica* and *A. heterophyllus* possesses growth-deterring properties and similar significant lethal toxicity against fall armyworm. Hence, *A. heterophyllus* can become an alternative candidate as a natural insecticide. Further studies isolating the specific components responsible for such actions would be necessary for environmentally friendly pest control strategies.

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