Efficacy of Neem Seed Kernel Extract on Oviposition Deterrence of *Bactrocera dorsalis* (Hendel) and *Bactrocera correcta* (Bezzi) on Guava Plantation

ประสิทธิภาพของสารสกัดเมล็ดสะเดาต่อการยับยั้งการวางไข่ของแมลงวันผลไม้และแมลงวันผลฝรั่งในแปลงปลูกฝรั่ง

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บทคัดย่อ: แมลงวันผลไม้ *Bactrocera dorsalis* (Hendel) และแมลงวันผลฝรั่ง *Bactrocera correcta* (Bezzi) เป็นสาเหตุสำคัญที่ทำให้เกิดความเสียหายต่อไม้ผลอย่างมาก จึงได้ทำการประเมินประสิทธิภาพของสารสกัดสะเดาที่สกัดด้วยตัวทำละลายต่าง ๆ ความเข้มข้น ความคงทน และความทนทานต่อแสงแดด ต่อการยับยั้งการวางไข่ของแมลงวันผลไม้ขึ้น ผลการทดสอบสารสกัดสะเดาที่สกัดด้วยสารทำละลาย 7 ชนิดที่ความเข้มข้นร้อยละ 20 พบว่าเอทิลอะซิเตท เมทิลแอลกอฮอล์ และเฮกเซนเอทิลอะซิเตตผสมกับเมทานอลมีประสิทธิภาพมากที่สุด อย่างไรก็ตาม สารสกัดสะเดามีความคงทนอยู่ได้เพียงวันเดียวภายใต้สภาพแปลง การเพิ่มความเข้มข้นจากร้อยละ 20 เป็น 50 ไม่ได้ยับยั้งการวางไข่ของแมลงได้อย่างสมบูรณ์ หรือเพิ่มความคงทนของสารสกัดสะเดาจากเอทิลอะซิเตทหรือเมทานอลแต่อย่างใด นอกจากนี้การใช้สารช่วยเพิ่มความทนทานต่อแสงแดด คือ 8-hydroxyquinoline และ tert-butylhydroquinone นั้น แม้ว่ามีประสิทธิภาพในการยับยั้งการวางไข่ แต่ไม่ได้เพิ่มความทนทานของสารสกัดสะเดาจากเอทิลอะซิเตตที่ความเข้มข้น 20% ภายใต้สภาพแปลง การสลายตัวเมื่อถูกแสงยังคงเป็นอุปสรรคสำคัญต่อประสิทธิภาพของสารสกัดสะเดาที่ใช้ในสภาพแปลง

คำสำคัญ: แมลงวันผลไม้ แมลงวันผลฝรั่ง สารสกัดสะเดา การยับยั้งการวางไข่

Abstract: Oriental fruit fly, *Bactrocera dorsalis* (Hendel) and guava fruit fly, *Bactrocera correcta* (Bezzi) causes significant damage to the fruit crops. The field efficacy of different neem extracts, concentrations, persistence and photostability of neem extracts on oviposition deterrence were evaluated. Amongst the seven different neem extracts tested at 20% concentration, ethyl acetate, methanol and hexane+ethyl acetate+methanol extracts were most effective. However, the persistence of the neem extract was only one day under field condition. The increase in concentration from 20 to 50% neither provided complete deterrence nor enhanced the persistence of ethyl acetate or methanol neem extract. Moreover, the use of photostabilizer, 8-hydroxyquinoline, and tert-butylhydroquinone although effective, did not enhance the persistence of 20% ethyl acetate neem extract under field condition. The photo degradation of neem extracts remains a substantial impediment to field efficacy.

Keywords: *Bactrocera dorsalis*, *Bactrocera correcta,* fruit fly, neem, oviposition deterrence

Introduction

Fruit flies (Tephritidae, Diptera) are an economically important insect pest of over 4,000 species causing significant damage to the fruits and vegetable crops. Amongst many fruit flies, *Bactrocera dorsalis* (Hendel) and *B. correcta* (Bezzi) are one of the most widely distributed and destructive pest to agriculture (Drew and Raghu, 2002; Singh, 2003). It is highly polyphagous with multiple overlapping life cycles occurring in the tropics and seasonal appearance in the temperate zones (Shi *et al*., 2005; Ye and Liu, 2005). This fruit flies damage fruits by laying eggs inside the pulp where the larva feeds and makes it completely inconsumable or unmarketable. The damage ranges from 57 to 92.5% in fruit crops (José *et al*., 2013) with an estimated annual economic loss of about US$ 3-15 million (Vargas *et al*., 2008).

Several control techniques were developed, however, their use was limited because of the high dispersal potential, multi-generation ability, dynamic population pattern and vast continental environment (Chen and Ye, 2007; Chen *et al*., 2006; Shi *et al*., 2005; Ye and Liu, 2005). Besides, the conventional chemical control poses a serious threat to both human and the environment. As complete fruit fly control was difficult, numerous research explored plant bioactive on oviposition deterrence. In many *in vitro* studies, neem seed kernel extract (NSKE) was the most effective in deterring oviposition compared to other plant extracts (Areekul *et al*., 1988; Thakur and Gupta, 2013). It was also more effective than the commercial neem as it contained other compounds besides azadirachtin (Silva *et al*., 2012; Singh and Singh, 1998). Although the complete deterrence was mostly achieved at 20% concentration, the efficiency varied with concentrations and extraction solvents used. The persistence of neem extract was only one to three days when exposed to direct sunlight (Ahmed, 2015; Caboni *et al*., 2006) but its half-life of azadirachtin could be retained for 30-44 days using 8-hydroxyquinoline and tert-butylhydroquinone stabilizers (Johnson *et al*., 2003).

Besides considerable results, all the studies suggested the need of investigating the neem extracts for field efficacy (Mahmoud and Shoeib, 2008; Silva *et al*., 2013), as the results may or may not translate the same way as achieved under laboratory assay. However, except for few studies on

vegetable, no studies on oviposition deterrence of fruit flies on fruit crops are available. In the absence, the efficacy of neem extracts remained unauthenticated for wide use in the field. Therefore, this study proposed to assess the field efficacy of NSKE on oviposition deterrence of *B. dorsalis* and *B. correcta* on guava. The study specifically evaluated the effect of different neem extracts, concentrations, persistence and effect of photostabilizers under field condition.

Materials and Methods

Experimental site and design

All the experiments were conducted at a commercial guava (Kimchu cultivar) field at Kamphaeng Saen district, Nakhon Pathom province. In all the experiments, mature guava fruits selected randomly were used as an experimental unit. The study used randomized complete block design (RCBD) for the assessment of different neem extracts and persistence levels, 2x4 factorial in RCBD to evaluate two neem extracts with four-concentration levels and 3x4 factorial in RCBD for three photostabilizers with four spray intervals on oviposition deterrence.

Neem extraction procedures

The mature neem seeds were freshly collected from the ground, grown around Kasetsart University, Kamphaeng Saen Campus, de-pulped manually, washed in tap water and dried in an electric dryer at 50 ^{o}C. Dried seeds were decorticated manually and pulverized using an electric blender.

The single neem extract from hexane, ethyl acetate and methanol were performed by mixing neem powder in their respective solvents in three separate beakers. The quantity of neem powder and solvent was dependent on the desired concentration of neem extract used in each experiment. Each mixture was stirred for 12 hours using electric stirrer and equilibrated in ambient room temperature for 72 hours. The mixture was filtered using a fine muslin cloth. The final neem extract was separated from the hexane, ethyl acetate and methanol using rotary evaporator at a reduced pressure and boiling temperature of 69, 77 and 64.5 ^{o}C, respectively. The combined neem extracts (e.g. hexane+methanol or hexane+ethyl acetate+methanol) requiring two or three solvents were extracted using the neem residue (filtrate) from initial extraction by second or third solvent with the same ratio of neem powder to the solvent. Finally, the extracts were combined to make the single extract. The combined extracts were formulated into equivalent concentration as a single extract for the actual field spray.

Field efficacy of different neem extracts

The seven NSKE; hexane, ethyl acetate, methanol, hexane+ethyl acetate, hexane+methanol, ethyl acetate+methanol and hexane+ethyl acetate+methanol were sprayed on 120 mature guava fruits to test the effect of different neem extracts against an untreated control. A 150 mL neem spray solution of each extract of 20% weight by volume (w/v) concentration was prepared with 30 g of neem extract and 10% of Tween 80 surfactant added with water until final volume was obtained. Each guava fruit was sprayed with 5 mL using mist hand sprayer to ensure uniform distribution of neem spray. After 48 hours, all the fruits were harvested and placed in the pupation box in the laboratory at a constant temperature of 25 ^{o}C. The dimension of pupation box was 27 x 19 x 11 cm transparent plastic box. A circular opening (10 cm diameter)

covered with nylon screen was made on the lid for aeration. The pupation box was filled with 1-inch fine sand as pupation medium and one fruit was placed per box. After the pupation, the sand was sieved through 1 mm diameter steel sieve to separate pupae from sand and number of pupae per fruit was recorded. Water was added when required to keep pupation medium at the desired moisture level.

All the following experiments followed the same procedure for pupation, oviposition rate calculation and used the same amount of the neem spray per fruit.

Persistence of neem extracts

The combined neem extract prepared from hexane+ethyl acetate+methanol was used to test the persistence for 1, 3, 5 and 7-days, respectively in open field condition. A 600 mL neem spray solution of 20% (w/v) concentration was prepared and sprayed on 120 fruits on the first day. The treated fruits were wrapped with plastic at 1, 3, 5 and 7-days, respectively as per their spray interval. All the fruits were harvested on the eighth day and placed in the insect rearing cage for pupation.

Extract type and concentration of neem extracts

This experiment was conducted to evaluate the efficacy of neem extract from ethyl acetate and methanol at the different concentration of 20, 30, 40 and 50%. A 500 mL stock solution of 70% w/v neem extracts was prepared with 350 g of neem extracts, 10% Tween 80 and water. Accordingly, the final volume of 200 mL each with the concentration of 20, 30, 40 and 50% was prepared by diluting required quantity from a stock solution in the water. On the first day, 120 guava fruits were sprayed. All the fruits were harvested after 48 hours and placed in pupation box.

Photostabilizer and persistence

This experiment compared 8-hydroxyquinoline and tert-butylhydroquinone photostabilizer with normal surfactant (Tween 80) against the persistence duration of 1, 3, 5 and 7-days, respectively. A 400 mL of 20% neem solution was prepared with 5% 8-hydroxyquinoline (20 g), 5% Tween 80 and 80 g of neem extracts added with water to final volume. Similarly, 400 mL of 20% neem spray with 5% tert-butylhydroquinone powder and 400 mL of 20% neem spray with 10% Tween 80 were prepared. On the first day, 180 fruits were sprayed with respective neem spray. The treated fruits were harvested after 1-day, 3-days, 5-days and 7-days of spraying.

Statistical analysis

The analysis of the efficacy of different neem extracts and persistence was subjected to analysis of variance and mean comparison using Duncan's multiple range test and least significant difference respectively. A factorial analysis of variance was used to analyze the main effect and interaction between type of extract, concentration, type of photostabilizer and persistence on oviposition deterrence. The number of pupae per fruit was transformed using logarithmic transformation $\log_{10}(x+1)$ as described by Gomez and Gomez (1984) to satisfy the homogeneity of variance. All the analyses were based on 95% confidence level using IBM SPSS version 20 software.

Results and Discussion

Field efficacy of different neem extracts

The study evaluated the efficacy of seven different neem extracts on oviposition deterrence of *B. dorsalis* and *B. correcta*. As shown in Table 1, all the neem extracts had a significant effect on the

Table 1. Effect of different neem extracts at 20% concentration on oviposition deterrence and fruit fly emergence

Neem extracts (Extraction solvents)	Number of pupae/fruit (Mean)	(%) Oviposition deterrence	Number of adult/fruit	
			B. dorsalis Mean ± SD	*B. correcta* Mean ± SD
Untreated control	43.3[a1]	0	2.7 ± 1.9	57.1 ± 41.6
Hexane	22.3[a]	48	1.9 ± 2.3	19.8 ± 10.1
Hexane + Ethyl acetate	17.8[b]	59	1.4 ± 0.9	14.0 ± 8.7
Ethyl acetate + Methanol	15.0[b]	65	1.0	20.2 ± 9.2
Hexane + Methanol	13.9[b]	68	1.4 ± 1.2	13.6 ± 7.4
Methanol	11.9[c]	72	1.0	21.1 ± 10.1
Hexane + Ethyl acetate + Methanol	09.9[c]	80	1.0	14.6 ± 7.1
Ethyl acetate	07.1[c]	83	1.0	11.6 ± 6.6

Mean data were transformed using $Log_{10}(x+1)$ before analysis of variance

[1]Mean values followed by different lowercase superscript letters in the same column are significantly different ($P<0.05$)

oviposition deterrence as compared to untreated control. The ethyl acetate, hexane+ethyl acetate+methanol and methanol extract showed the significant effect as compared to other extracts and control treatment ($P < 0.05$). The mean number of pupae per fruit was the lowest in ethyl acetate (7 pupae per fruit) and the highest average eggs laid was in hexane extract treated fruits (22 pupae per fruit). Hexane+ethyl acetate+methanol showed the significant effect as compared to other combined extracts. The ethyl acetate deterred the highest oviposition (83%) while hexane extract was the lowest (48%). The number of adult fruit fly emergence per fruit was higher for *B. correcta* (98%) as compared to *B. dorsalis* (2%) in guava.

Although all neem extracts have shown a significant effect, the efficacy differed with the polarity of extraction solvents. The ethyl acetate (mid-polar solvent) and methanol (polar) neem extract showed higher efficacy, while hexane (non-polar) was significantly lower. This may be due to the extraction of higher limonoid compound from the neem seed kernel by the ethyl acetate and methanol, which has both polar and non-polar group. Limonoids such as azadirachtin, nimbin and salannin found in neem are considered a mid-polar compound, which is more soluble in the mid-polar and polar solvents (Melwita and Ju, 2010). This finding corroborated with the study by Singh and Singh (1998) where the neem extracted with acetone, a mid-polar solvent, provided a complete deterrence against *B. dorsalis* and *B. cucurbitae* on guava. This was also in conformity with finding where polar ethanolic extract showed higher efficacy over the non-polar hexane extract and neem oil at a lower concentration (Singh and Singh, 1998). Similarly, methanolic of dichloromethane *Melia azedarach* extract was effective in deterring oviposition of *B. dorsalis* on tomato fruits (Srinivasan *et al*., 2015). Furthermore, ethanolic extract of neem reduced egg laying by 93% in *Bactrocera tau* and by 93.6% in *Bactrocera cucurbitae* as compared to other plant extracts (Thakur and Gupta, 2013). Several studies showed that crude neem extracts had higher oviposition deterrence efficacy and attributed it to the complimentary effect of salannin and nimbin beside

azadirachtin in enhancing the efficacy (Chen *et al*., 1996; Singh and Singh, 1998). This was also emphasized in a study where they found that hexane extract of *Tephrosia vogelii* herb which has rotenone as active constituent significantly deterred the egg laying by maize weevil, *Sitophilus zeamais* in stored maize as compared to acetone extract, ethanol extract and neem oil. They suggested that other compounds besides rotenone are responsible for the deterrence because hexane (non-polar) cannot extract rotenone, which is highly soluble in polar solvents (Koona *et al*., 2007). Therefore, in this study salannin and nimbin may have contributed in providing higher oviposition deterrence. Hexane+ethyl acetate+methanol extract also showed a similar effect as ethyl acetate or methanol, which might be due to the presence of a larger amount of the neem extracts. Nonetheless, ethyl acetate or methanol may be suggested for the neem extraction over hexane+ethyl acetate+methanol, as it requires three solvents and more time for the extraction.

The major fruit fly infesting guava was *B. correcta followed* by *B. dorsalis*. Perhaps the *B. correcta* may have oviposited earlier, which prevented *B. dorsalis* to oviposit on the same fruit. The female fruit flies usually release host-marking pheromones after oviposition to reduce host competition from other fruit flies, however, some fruit flies tend to reuse the same site (Prokopy *et al*., 1999). Similarly, in this study, *B. dorsalis* reused the same site for oviposition because of limited fruits available, which resulted in the emergence of both the fruit flies.

Persistence of neem extracts

The neem extracts have shown a significant effect on the oviposition deterrence in the earlier experiment. This study thus evaluated the persistence of 20% ethyl acetate neem extract under open field condition. There was a significant difference in the mean number of pupae per fruit in each spray interval (P<0.05). The mean oviposition rate increased from zero pupa per fruit in the 1-day interval to 67 pupae per fruit in the 7-days spray interval. The efficacy of neem extract was only one day under open field condition (Table 2).

The oviposition deterrence efficacy of 20% neem significantly decreases when the number of days exposed to external environment increases. The efficacy of 20% neem on the guava fruit was only one day, which was similar to the finding of Ahmed (2015) where stability of neem extract on the wheat seeds was also one day under direct sunlight exposure. Although the oviposition rate for 3-days was significantly lower than 5-days or 7-days interval, it provided no benefit as single oviposition could

Table 2. Mean number of pupae per fruit at 20% concentration of hexane+ethyl acetate+methanol neem extract at four different spray intervals

Spray Interval (Number of days)	Number of pupae/fruit (Mean)
1-day	0.0[a1, 2]
3-days	19.6[b]
5-days	43.6[c]
7-days	67.2[d]

[1]Mean data were transformed using $Log_{10}(x+1)$ before analysis of variance

[2]Mean values followed by different lowercase superscript letters in the same column are significantly different (P<0.05)

damage the entire fruit. This corroborated with Caboni *et al.* (2006), where the residue of azadirachtin, salannin and nimbin from both commercial neem and crude extract became significantly negligible after three days of spraying on strawberry. Therefore, this experiment clearly showed the reduced efficacy of neem extract under field condition due to photodegradation of neem bioactive by sunlight.

Extract type and concentration of neem extracts

Although 20% neem extract showed a significant effect for one day, it did not achieve complete deterrence in 3-days or 5-days interval, thus this experiment evaluated four levels of concentrations (20, 30, 40 and 50%) extracted with ethyl acetate and methanol solvent on the oviposition deterrence at 48 hours interval. The factorial analysis of variance indicated no interaction between the types of solvents and concentrations on the oviposition deterrence level (P>0.05). There was significant main effect of different concentration levels (P<0.05) and neem extract types (P<0.05). The ethyl acetate extract (grand mean = 77.3, SD = 39.8) was significantly effective than the methanol extract (grand mean = 108.9, SD = 37.5) in deterring oviposition with the increase in concentration (Table 3). There was no significant difference in 20 and 30% of ethyl acetate extract but significant effect was observed in 40 and 50%. Similar efficacy trend was observed in methanol extract but significantly lower. The *B. correcta* showed a higher oviposition rate in both the neem extracts (Table 3).

The ethyl acetate extract was significantly effective in deterring oviposition with the increase in concentration. This could be mainly due to the higher potential of ethyl acetate in extracting required mid-polar to polar compounds responsible for oviposition deterrence, whereas methanol could mostly extract compounds present in the polar state. The past laboratory assay showed the increase in deterrence efficiency with the increase in the concentration of the neem extract (Chen *et al*., 1996; Singh and Singh, 1998; Thakur and Gupta, 2013). A similar trend also occurred under field condition, where the efficacy of both the neem extracts showed the significant effect as the concentration increased from 20 to 50%, but relatively higher concentration was required in the field condition. The 10 and 20% neem extracted with acetone and diethyl ether completely deterred the oviposition of the *B. dorsalis* and *B. cucurbitae* under the laboratory assay (Chen *et al*.,

Table 3. Effect of ethyl acetate and methanol at different concentration levels on oviposition deterrence and emergence rate of *Bactrocera dorsalis* and *B. correcta*

Concentration	Number of pupae/fruit Mean			Number of adult emergence (Mean ± SD)	
	Ethyl acetate	Methanol	Grand mean	*B. dorsalis*	*B. correcta*
20%	91	124	107.8 [ab1]	7.4 ± 5.6	50.8 ± 27.8
30%	114	125	119.6 [a]	17.2 ± 11.2	81.7 ± 32.9
40%	80	96	88.1 [b]	7.9 ± 5.9	45.1 ± 19.6
50%	24	91	57.6 [c]	4.2 ± 5.3	30.7 ± 21.4
Grand mean[2]	77.3[A]	108.9[B]			

[1]Mean values followed by different lowercase superscript letters in the same column are significantly different (P<0.05)

[2]Mean values followed by different uppercase superscript letters in the same row are significantly different (P<0.05)

1996; Singh and Singh, 1998). However, in this study, the ethyl acetate extract did not achieve complete deterrence even at 50% under field condition. This indicates that higher concentration may be required for greater efficacy in the field. The oviposition occurring even at 50% concentration in 48 hours depicts the persistence of the neem residue was reasonably low in the open environment. Therefore, the stabilization of neem compounds using synthetic photostabilizer was essential in spite of increasing the concentration, which may not be attainable (Johnson *et al*., 2003). As discussed earlier, the *B. correcta* dominates the infestation of guava fruit and the overall oviposition rate was relatively higher in methanol treated fruits.

Photostabilizer and persistence

This experiment evaluated the efficacy of different photostabilizer in enhancing the persistence level of neem extract under field condition. The factorial analysis of variance indicated the interaction between Tween 80 and 8-hydroxyquinoline ($P<0.05$), whereas there was no interaction with tert-butylhydroquinone. There was significant difference between type of photostabilizer ($P<0.05$) and persistence duration ($P<0.05$). The number of pupae per fruit in neem extract with photostabilizer tert-butylhydroquinone (M = 16.8) was significantly lower than the neem extract with 8-hydroxyquinoline (grand mean = 41.2) and Tween 80 (grand mean = 41.4). There was no oviposition in the 1-day interval, while from 3-days onwards, the rate of oviposition increased (Table 4).

In the earlier experiments, although the ethyl acetate neem extract had a significant effect, the efficacy was only one day even with increased concentration. A study reported that the Emulsol N-33 surfactant was found effective in recovering half-life of azadirachtin for 93 minutes under the UV rays (Johnson and Dureja, 2002). Another study showed half-life of azadirachtin was about 59 minutes under UV and 23 minutes under sunlight (Deota *et al*., 2002). Among the several studies on enhancing the persistence of neem extracts, the study by Johnson *et al.* (2003) found that the half-life of azadirachtin was retained for 44 or 36 days using 8-hydroxyquinoline and tert-butylhydroquinone photostabilizer. However, in this study, both the stabilizers provided persistence for only one day, similar to normal surfactant Tween 80. The tert-butylhydroquinone stabilizer showed higher efficacy possibly due to its higher solubility in neem solution.

Table 4. Effect of 20% ethyl acetate neem extract with 5% 8-hydroxyquinoline, 5% tert-butylhydroquinone and 10% Tween 80 on oviposition deterrence at different persistence duration

Neem extracts with photostabilizer and surfactant	Persistence duration (spray interval) Mean number of pupae/fruit				Grand mean
	1-day	3-days	5-days	7-days	
Tween 80	0.0[a]	51.7[a]	56.7[a]	57.2[a]	41.4[a1,2]
8-hydroxyquinoline	0.0[a]	51.1[a]	45.4[a]	68.7[a]	41.3[a]
tert-butylhydroquinone	0.0[a]	11.1[b]	20.2[b]	41.3[b]	16.9[b]
Grand mean	0.00[A3]	37.9[ns]	40.8[ns]	57.5[B]	

[1]Mean data were transformed using $Log_{10}(x+1)$ before analysis of variance

[2]Mean values followed by different lowercase superscript letters in the same column are significantly different ($P<0.05$)

[3]Mean values followed by different uppercase superscript letters in the same row are significantly different ($P<0.05$)

The option to increase the amount of tert-butylhydroquinone above 5% to enhance the persistence is not reasonable, as it showed sign of phytotoxicity on guava fruits. The use of 8-hydroxyquinoline is also not advisable, as it requires alcohol or acetone to dissolve which is phytotoxic to guava fruit. Until promising photostabilizer is studied and identified, the oviposition deterrence of fruit fly using neem extracts may not prove economical and effective in area-wide fruit fly management.

The current study revealed that neem extracts have a significant effect on the oviposition deterrence under field condition. The ethyl acetate neem extract showed higher efficacy as compared to hexane or methanol extract on oviposition deterrence of fruit flies. Despite the efficacy, the persistence of neem extract was only one day with the use of various surfactant or photostabilizer. Thus, the use of neem extract as oviposition deterrence in the open field condition is not advisable as this may necessitate frequent spraying and escalate management and production cost. However, in view of its efficacy, it may be suitable for management of pest and crops through other aspects of insecticidal properties of neem extracts.

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