TOXICITY OF *Azadirachta indica* AND *Piper sarmentosum* EXTRACT MIXTURE FORMULATIONS AGAINST *Nilaparvata lugens* (HEMIPTERA: DELPHACIDAE) IN PADDY FIELD

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ABSTRACT

Infestation of *Nilaparvata lugens* on rice in Malaysia is not as heavy compared to other Asian countries, but still lower the rice yield production. *Nilaparvata lugens* is developing resistance against chemical pesticide, therefore an alternative approach such as the application of botanical pesticide is potential to control the *N. lugens*. The aims of this research were to determine the synergistic activity in combination of *Azadirachta indica* and *Piper sarmentosum* emulsion formulations extract against *Nilaparvata lugens*. Three formulations were developed were coded as F1, F2, and F3. The F1 was selected as the best formulation because it in range of good zeta potential value (-48.3 mV) and viscosity value (100.1 mPa/s), the lowest particle size (202.5 nm), remained homogenous for centrifugation and stability test, polydispersity index (PDI) value indicated it is in ideal monodisperse phase (0.359), low surface tension (31.3 mN/m) and low pH (3.65). The lethal concentration, LC_{50} and sub lethal, LC_{10} and LC_{25} were determined from nymph mortality bioassay test on third instar *N. lugens* nymphs. Mortality rate of F1 and F2 were 0.011 mg/l and F3 was 0.031 mg/l. The value of lethal and sublethal concentration were used in a test against female *N. lugens* to determine the effect of formulations on fecundity of *N. lugens*. There were significant differences between formulations on the number of eggs produced after treatments. The lowest egg produced from F1 with 128.11 number of eggs. It can be concluded that all formulations showed promising result in controlling *N. lugens*.

Keywords: Synergism, *Azadirachta indica*, *Piper sarmentosum*, *Nilaparvata lugens*, emulsion formulation

ABSTRAK

Infestasi *Nilaparvata lugens* ke atas tanaman padi di Malaysia tidak seterok negara Asia lain tetapi masih menunjukkan penurunan hasil pengeluaran padi. *Nilaparvata lugens* menunjukkan kerintangan terhadap racun kimia, oleh itu pendekatan alternatif seperti aplikasi racun perosak botani berpotensi dalam mengawal *N. lugens*. Matlamat penyelidikan ini adalah untuk

menentukan gabungan aktiviti sinergistik bagi ekstrak formulasi emulsi *Azadirachta indica* dan *Piper sarmentosum* terhadap *N. lugen*s. Tiga rumusan telah dibangunkan dan dikodkan sebagai F1, F2, dan F3. F1 dipilih sebagai formulasi terbaik kerana ia berada dalam julat nilai potensi zeta yang baik (-48.3 mV) dan nilai kelikatan (100.1 mPa/s), saiz zarah terendah (202.5 nm), kekal homogenik untuk ujian sentrifugasi dan kestabilan, nilai PDI menunjukkan ia berada dalam fasa sebaran mono yang ideal (0.359), tegangan permukaan yang rendah (31.3 mN/m) dan pH rendah (3.65). Kepekatan maut, LC_{50} dan sub-maut, LC_{10} dan LC_{25} ditentukan hasil daripada ujian bioasai terhadap nimfa *N. lugens* instar ketiga. Kadar kematian F1 dan F2 ialah 0.011 mg/l dan F3 ialah 0.031 mg/l. Nilai kepekatan maut dan sub maut digunakan dalam ujian terhadap *N. lugens* betina untuk menentukan kesan formulasi terhadap kesuburan *N. lugens*. Terdapat perbezaan yang ketara antara formulasi pada bilangan telur yang dihasilkan selepas rawatan. Telur yang paling rendah dihasilkan daripada F1 dengan 128.11 biji telur sahaja. Dapat disimpulkan bahawa semua formulasi menunjukkan hasil yang memuaskan untuk mengawal *N. lugens*.

Kata kunci: Sinergi, *Azadirachta indica*, *Piper sarmentosum*, *Nilaparvata lugens*, formulasi emulsi

INTRODUCTION

Nilaparvata lugens (Stål), brown planthopper (BPH) is a planthopper species under order Hemiptera and family Delphacidae. It has brown-coloured wings of adult and creamy white nymph. *Nilaparvata lugens* is an important sap-sucking pest in East and Southeast Asia especially in temperate and tropical regions that caused serious damage by directly feeding on the plant phloem which leads to browning, drying, and wilting of the plant (Hu et al. 2014; Tang et al. 2010; Zhu et al. 2020). The most used control method of *N. lugens* is by using chemical control but recently, *N. lugens* was found to build resistance against chemical pesticide. The high level of resistance to imidacloprid resulted in chemical control failure and great yield loss in 2005 (Wang et al. 2008). The constant and unsystematic use of one insecticide has resulted in the quick development of insecticide resistance and exhaustion of most insecticide alternative in many rice-growing country (Chen et al. 2013). Therefore, it is important to explore other common practices to control *N. lugens*.

Piper sarmentosum also called wild betel or in Malay name known as ‘kaduk, is plant species from order Piperales and family Piperaceae (Maizatul & Nor Farahiya 2018). It is a perennial herb that have a creeping rhizome and striped stem that grow up to 40 cm. The heart-shaped and alternate leaves are light to dark green in colour with a waxy surface (Sharifah Farhana et al. 2016). It can be found in tropical areas of Southeast Asia, Northeast India, and South China.

Azadirachta indica also known as neem is an evergreen plant from order Sapindales and family Meliaceae. It is the most common medicinal plants that has got worldwide attention because of its medicinal and insecticide properties (Sonal & Pankaj 2014). *Azadirachta indica* usually grow up to 12 to 15 metres with tall and straight trunk and long spreading branches forming a broad round crown; it has rough dark brown bark with wide longitudinal fissures separated by flat ridges. The leaves are compound that alternating with one another. The leaves are pinnate with short petioles, each comprising 5 to 15 leaflets and have many flowered panicles, usually in the leaf axils (Hashmat et al. 2012). The aims of this research are to determine the synergistic activity combination of *A. indica* and *P. sarmentosum* emulsion formulations extract against *N. lugens* in Malaysia’s paddy field.

MATERIALS AND METHODS

Collection and Rearing of *Nilaparvata lugens*

Nilaparvata lugens was collected from paddy fields in Tanjung Karang, Selangor, Malaysia (3°27'42.8"N 101°13'13.4"E) and brought to Toxicology Laboratory in Faculty of Agriculture, UPM to be reared at room temperature 28^{o}C±2 with 70 to 80% humidity for 12 hours daylight and 12 hours dark. Seedlings from untreated rice of MR-297 varieties were used for treatment and as host plant.

Extract of *Azadirachta indica,* and Collection and extraction of *Piper sarmentosum*

Azadirachta indica extract containing 3% concentration neem oil was purchased from Agrow Synergy (M) Sdn Bhd. Dried leaves of *P. sarmentosum* were collected from Besut, Terengganu, Malaysia (5°42'10.7"N 102°29'20.3"E) and were extracted by soaking them in 1L hexane, filtered using vacuum pump and concentrated using rotary evaporator.

Bioassay of Crude Extract Mixture

There were nine treatments from mixture of *P. sarmentosum* and *A. indica* extract at mass ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1. Bioassay was performed by exposed *N. lugens* nymph with the mixture with combination ratios (*P. sarmentosum*: *A. indica*) of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1, which were coded as T1, T2, T3, T4, T5, T6, T7, T8 and T9, respectively. Two treatments from single crude extract of *P. sarmentosum* and *A. indica* coded as T10 and T11, as the positive control and deionized water as negative control. Each treatment was diluted with hexane into 0.05%, 0.1% and 0.5% concentration.

Twenty milliliters of mixture solution were sprayed to rice plant via surface contamination and the plants were left to dry. Five third instar *N. lugens* nymphs were transported into the cylinder using suction device. Each treatment had five replications. Treatments were observed for 72 hours at 28^{o}C±2 and mortality rates were recorded at 24, 48 and 72 hours after treatment.

Construction of Ternary Phase Diagram

Eleven mixture ratios of surfactant, oil and water were homogenized by vortex mixer to achieve equilibrium before being centrifuged at 3500 rpm for 15 minutes. Each mixture was visually evaluated based on clarity, stability, and transparency to determine their phase. The results obtained from the mixture were used to plot ternary phase diagram (TPD), which was constructed using Chemix School software which then revealed an isotropic region.

Bioassay of Bioinsecticide Formulation

All formulations were diluted with deionized water by serial dilution into 0.5%, 0.1%, 0.05%, 0.025%, 0.0125% and 0.005% concentration, with deionized water served as control treatment. The treatments were replicated three times. Bioassay was performed using similar method as previous experiment which aim to determine the working concentrations that resulted mortality 10, 25 and 50%. Lethal and sublethal concentration (LC_{10}, LC_{25}, LC_{50}) of formulations were used on *N. lugens* nymphs to determine effect of the formulation on fecundity of *N. lugens* female adult.

Determination of Lethal and Sublethal Concentration

All formulations were diluted into previous LC_{10}, LC_{25}, and LC_{50} concentration and bioassay performed as previous method. Each treatment was repeated four times. The newly emerged adults were paired based on the treatments with five pairs per treatment and transferred into

plastic cup consisting of rice seedlings for oviposition. The adults were transferred into different cups every two day until the female died. The number of newly hatched nymphs emerged from each cup were recorded. Observation for each cup was terminated when no nymphs emerged during the last four consecutive days.

Data Analysis

Data from bioassay was analyzed using Probit analysis to obtain lethal and sublethal concentration. Data collected was analyzed in SAS as a Complete Randomized Design (CRD) with an analysis of variance (ANOVA). Fecundity was calculated based on number of newly hatched nymphs.

RESULTS

Toxicity, Lethal Concentration, and Synergistic Effect of Treatments

Based on result shown in Table 1, treatments T1, T2, T3, T4, T8, T10 and T11 produced significant differences 24 hours after treatment. However, treatments T5, T6, T7 and T9 were not significant with each other. At 48 hours, treatments were significantly different unless T5, T6 and T9 and which showed no significant differences between each other. While the result showed no significant differences of T1, T2, T3, T4, T5, T6, T7, T8 and T9 compared to control T10 and T11 after 72 hours. Exposure time had significant effects on the assessment of toxicity, resulting in different interpretations of toxicity depending on the exposure time used (Sara et al. 2000). Study by Hwang et al. (2009) on the evaluation of neem and matrine extract toxicity against six main pests and on natural enemies found out that the mixture insecticidal effect above was 95% control value against plant hopper, cotton aphid and Diamondback Moth and 68.1% on palm thrips. While acute toxicity study carried out by Ugwah-Oguejiofor et al. (2019) concluded that *Caralluma dalzielii* aqueous extract tested against rats and mice via oral route unsuccessfully produce toxicity and lethal to the test subjects.

Lethal concentration values in Table 1 range with the highest mortality can be seen on T9, followed by T6, T7, T5, T8, T3, T1, T4 and lowest was T2 with value of 0.007, 0.014, 0.014, 0.015, 0.021, 0.026, 0.033, 0.033 and 0.041%, respectively. Lethal concentration for positive control were 0.021% and 0.026% for *P. sarmentosum* and *A. indica*, respectively. This showed that for single crude treatment, *P. sarmentosum* caused higher nymphs mortality compared to *A. indica*.

Interaction characteristic in Table 1 were interpreted from CompuSyn software showed that three formulations have synergistic effect, T6 (0.61), T7 (0.63) and T9 (0.33). The T1, T2, T3 and T4 showed antagonistic effect with combination index (CI) value of 2.45, 3.26, 1.77 and 2.67, respectively. The T5 and T8 each gave value of 1.26 and 0.95 which indicated as additive effect.

Table 1. Toxicity, lethal concentration (LC_{50}) and synergistic effect of treatments against *N. lugens* nymphs

P.sarmentosum: A.indica	**LC_{50} at 72h (%)**	**Mean percentage of dead nymph±SE (%)**			**CI at LC_{50}**	**Effect**
		24h	**48h**	**72h**		
T1 (P:A=1:9)	0.033	$37.33\pm7.00_{de}$	$85.33\pm4.96_{ab}$	$85.33\pm4.96_{a}$	2.45	Antagonistic
T2 (P:A=2:8)	0.041	$28.00\pm6.70_{e}$	$81.33\pm6.31_{b}$	$81.33\pm6.31_{a}$	3.26	Antagonistic
T3 (P:A=3:7)	0.026	$36.00\pm4.45_{de}$	$89.33\pm5.47_{ab}$	$89.33\pm5.47_{a}$	1.76	Antagonistic
T4 (P:A=4:6)	0.033	$56.00\pm6.53_{bcd}$	$85.33\pm5.68_{ab}$	$85.33\pm5.68_{a}$	2.67	Antagonistic

T5 (P:A=5:5)	0.015	$72.00\pm8.00_{ab}$	$92.00\pm2.61_{ab}$	$92.00\pm2.61_{a}$	1.25	Additive
T6 (P:A=6:4)	0.014	$70.67\pm8.02_{ab}$	$94.67\pm2.36_{a}$	$94.67\pm2.36_{a}$	0.61	Synergistic
T7 (P:A=7:3)	0.014	$76.00\pm7.86_{ab}$	$94.67\pm3.07_{a}$	$94.67\pm3.07_{a}$	0.63	Synergistic
T8 (P:A=8:2)	0.021	$65.33\pm8.61_{abc}$	$93.33\pm3.19_{a}$	$93.33\pm3.19_{a}$	0.95	Additive
T9 (P:A=9:1)	0.007	$70.67\pm5.81_{ab}$	$96.00\pm2.14_{a}$	$96.00\pm2.14_{a}$	0.33	Synergistic
T10(*P. sarmentosum*)	0.021	$80.00\pm7.30_{a}$	$93.33\pm3.74_{a}$	$93.30\pm3.74_{a}$	-	-
T11 (*A. indica*)	0.026	$45.33\pm8.39_{cde}$	$92.00\pm3.27_{ab}$	$92.00\pm3.27_{a}$	-	-

Formulation of emulsion

Two phase diagrams consist of surfactant, oil, and water components (Tween 80/ Edenor/ Water produced 35% isotropic region. One point at 15:80:5 have been selected phase diagrams as they showed miscibility with active ingredient to form homogenous emulsion right after being vortexed and centrifuged at 3500 rpm for 15 minutes. Tween 80 surfactant was selected because of its compatibility, relatively low toxicity, and minimal environment impact (Hazreen et al. 2016). Sustainable solvent like palm kernel oil is biodegradable, low toxicity and caused lower environmental effect (Siti Fairuz et al. 2021). The selected point was used to develop formulations which were coded as F1, F2 and F3, which consisted of 14.25% surfactant, 76% carrier (oil), 4.75% of water and 5% active ingredients (*A. indica* and *P. sarmentosum*).

Characterization of emulsion formulation

Based on observation after being centrifuged at ambient temperature at 3500 rpm for 15 minutes, all formulations were physically stable. The formulation was kept at three different temperatures, -20°C and 54°C for 14 days and at 25°C for 60 days for the thermostability test. All formulation at all temperature were also found to be physically stable after the recorded period (Table 2).

The particle size shown in Table 2 were 202.5 nm, 506.9 nm and 611.7 nm for F1, F2 and F3, respectively. The differences between formulation size could be cause by the ratio of active ingredients mixture and type of surfactant used. Ratio of *P. sarmentosum*: *A. indica* mixture for F1 was 6:4, for F2 was 7:3 and for F3 was 9:1.

The zeta potential values for all formulations are -48.3, -59.2 and -53.4 for F1, F2 and F3, respectively (Table 2). There are many views to consider which value ranges of zeta potential indicated the most stability of the nano emulsion. According to Joseph and Singhvi (2019) stated that a greater number of positive and negative values of zeta potential signify good physical stability of particles. The amount of surfactant and oil in formulation can influence the zeta value (Zainuddin et al. 2019). That should explain the almost similar value of zeta potential of F1 to F3 as they all had the same amount of oil and surfactant.

The readings of PDI value of FI, F2 and F3 shown in Table 2 were 0.359, 0.485 and 0.563, respectively. According to Malvern, the best PDI value is within the range 0.08 to 0.7 and PDI value more than 0.7 indicates a broadness of particle size. As the value of polydispersity getting higher, the uniformity of the droplet size in the formulation is getting lower (Baboota et al. 2007).

Result in Table 2 showed that all formulations have uniform viscosity which may be due to the same oil content (Baboota et al. 2007). Viscosity is the measure of a substance's resistance to motion under an applied force. Higher energy is required to deform a highly viscous liquid, while lower energy is needed when deforming a less viscous fluid (Wong &

Wong 2013). According to Siti Fairuz et al. (2021), nano emulsion formulation with low viscosity is easier to be mixed and pumped.

Surface tension of all formulations were significantly lower with range from 31.3 to 35.20 mN/m (Table 2). While the pH value of all formulations ranging from the lowest 3.58 followed by 3.65 and 4.19 for F3, F1 and F2, respectively. The result showed the mixture of both extracts have an acidic property. All formulation resulted in pH less than their surfactant, Tween 80 with value 5-7. A study by Maneerat et al. (2013), emulsions prepared at pH 4 were smaller in size, higher viscosity, and more stable after stability test than those prepared at pH 7.

Table 2. Characterization of formulation emulsion

Formulation name	Particle size (nm)	Zeta potential (mV)	Viscosity (mPa/s) at 60 rpm	Stability -20°C, 54°C, 25°C	PDI size	pH value	Surface tension (mN/m)
F1	202.5	-48.3	100.1	/	0.359	3.65	31.3
F2	506.9	-59.2	100.2	/	0.485	4.19	35.0
F3	611.7	-53.4	100.1	/	0.563	3.58	33.0

Bioassay of emulsion formulation against nymph of *N. lugens*

LC_{50} values range from 0.070, 0.083 and 0.117 mg/l for F1, F2 and F3, respectively (Table 3). The result suggested that formulation with smallest difference of mixture ratio (6:4) gave high mortality (F1) than formulation with bigger ratio (9:1) differences (F3).

Table 3. Bioassay of emulsion formulation against nymph of *N. lugens*

Formulation	LC_{10} (%)	LC_{25} (%)	LC_{50} (%)	LC_{50} Lower Limit (%)	LC_{50} Upper Limit (%)
F1	0.011	0.035	0.070	0.045	0.124
F2	0.011	0.042	0.083	0.051	0.160
F3	0.031	0.059	0.117	0.079	0.197

There are significant differences between formulations on the number of eggs produced after treatments. Number of eggs produced for other formulation shown in Table 4 are 128.11, 131.44 and 138.66 for F1, F2 and F3, respectively. According to Schäpers et al. (2017), fecundity depend on the resources a female is able to obtain as a larva and thus increases with adult size and female fecundity variation could thus be connected to both her own size and the size of her pair.

Table 4. Fecundity based on number of egg produced

Formulations	Egg produced (egg)±SE
F1	128.11 ± 6.17^{b}
F2	131.44 ± 6.27^{ab}
F3	138.66 ± 5.58^{a}

There are also significant differences between formulation concentration on the number of eggs produced after treatments. The highest number of eggs produced was from concentration LC_{50} 133.05 eggs, followed by LC_{25} 144.22 eggs and LC_{10} 193.16 eggs. Even though LC_{50} was the highest concentration, but it produced the highest number of eggs, while LC_{10} had the smallest concentration, it produced the fewest egg. Appropriate explanation for this is the generation of *N. lugens* used during the fecundity test. *N. lugens* used were randomly picked from the rearing cages without considering the number of generations.

DISCUSSION

Toxicity, lethal concentration, and synergistic effect of treatments

Due to their high toxicities to *N. lugens*, biopesticide containing matrine, abamectin and azadirachtin were used for sublethal assessments against *C. lividipennis*. Result shown that the reated *C. lividipennis* able to distinguish volatiles released from healthy and *N. lugens*-infested plants indicating that the biopesticides tested did not affect the foraging ability of surviving mirid bugs and azadirachtin was found to decrease the consumption capability of *C. lividipennis* (Dai et al. 2019). While Mayorga et al. (2010) used lethal concentration value to evaluate the lethal effects of 10 Guatemalan plant species extract against *Artemia salina* and *Thamnocephalus platyurus* and also revealed that *T. platyurus* is more sensitive than *A. salina* for detecting toxic effects of plant extracts. Observation by Rawani et al. (2010) revealed that lethal concentration values gradually decreased with the exposed period in crude plant extract bioassay and regression analysis of *S. nigrum* crude extract showed that the mortality rate is positively correlated with the extracts concentration.

Levchenko and Silivanova (2019) stated that the interaction patterns in the insecticide mixtures, either synergistic or antagonistic can depend on the combination of insecticides and their ratio. This is on agreement with Tallarida (2011) which summarized that when synergism is detected, it is almost always dependent on the dose ratio of the combination that is tested and, thus, this information gives an optimal determination of dose combinations.

Result showed that combination ratio with small number of *P. sarmentosum* extract gave antagonistic effect, compared to combination with large number of that extract. Equal combination of both extracts showed an additive interaction (T5). Bioassay conducted by Hematpoor et al. (2017) isolated phenylpropanoids asacirin like and isoasarone from *P. sarmentosum* which showed high toxicity against *Sitophilus oryzae* with lethal concentration 4.7 and 5.6, respectively. Purba et al. (2021) extracted *P. sarmentosum* using methanol, hexane and chloroform solvent and found out that methanolic plant extract had the highest contents of fourteen synergistic compounds. Atangwho et al. (2012) conducted test to evaluate the synergistic antidiabetic action of *Vernonia amygdalina* and *Azadirachta indica* against streptozotocin-induced diabetic rat and the result revealed that the combination lowered blood glucose and maintained a relatively steady level over the study period compared with single extracts.

Characterization of Emulsion Formulation

All formulation were water in oil emulsion and as stated by Asib et al. (2015) the formulations with higher concentration of surfactants relative to oil were more resistant to high temperature. Formulation with higher number of *P. sarmentosum* extract seemed to be larger. This may happen because concentration of *P. sarmentosum* extracts (4%) is more concentrated than *A. indica* oil (3%). Bouchemal et al. (2004) stated that the ideal particle size range of nano

emulsion is within 100-600 nm. According to Marziyeh et al. (2017), droplet size may be affected by the structure of formulation and composition of the emulsion system.

According to Zainuddin et al. (2019), low surface tension permits the formulation to be deposited on the seed surface with lower contact angle and further increased spreading and wetting properties. That is why surfactant is essential in formulation as it helps to stabilize and formed efficient formulation to absorb into the seed coat.

Bioassay of Emulsion Formulation Against Nymph of *N. lugens*

At high and sub lethal doses, *A. indica* oil could inhibit growth and survival, cause mortality, and results in wings and legs anomalies on non-target predator *P. nigrispinus* thus, its use with biological control should be carefully evaluated (Zanuncio et al. 2016).

An increase in longevity of larval, reduction in biomass gain larvae and pupae in addition to affecting the fecundity and fertility of females are the effects from the exposure of *Helicoverpa armigera* to sublethal doses of the active ingredients *Bacillus thuringiensis* and Indoxacarb (Ferreira et al. 2018).

CONCLUSIONS

Overall, the present study managed to developed emulsion formulation from both plant extract combinations which effective in controlling *N. lugens*. Thus, further research is highly recommended to explore the efficacy of plant extracts combination against pests as it has been increasingly important to reduce chemical-based control and consider the use of safer product for organisms and environment. Mass production and further field studies are highly recommended on integrating the formulations in pest management program to make sure it is safe and environment friendly.

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CONFLICT OF INTEREST

None.

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