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Original article (Orijinal araştırma)

Formulation and physicochemical characterization of neem oil nanoemulsions for control of *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae) and *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae)

Sitophilus oryzae (L., 1763) (Coleoptera: Curculionidae) ve *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae)'un kontrolünde neem yağının nano emülsiyonlarının ve fizikokimyasal tanımlanması ve formülasyonu

Marziyeh CHOUPANIAN[1*] **Dzolkhifli OMAR[1]**

Abstract

This report describes the development of environmentally-benign nanoemulsion formulations for control of adult *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae) and *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae). The formulations were emulsions of neem [*Azadirachta indica* A. Juss (Sapindales: Meliaceae)] oil, nonionic surfactant (alkylpolyglucoside or polysorbate 80), and water using low-energy emulsification method. Four formulations were chosen and physicochemical characterization showed a particle size range of 208-507 nm in diameter. Bioassays were conducted using food and filter paper impregnation methods. The formulation comprised of polysorbate surfactant with 2.0 mL/kg azadirachtin caused a 100% mortality of *S. oryzae* adults after only 24 h of exposure, with the food impregnation method. The toxicological studies carried out at Universiti Putra Malaysia toxicology laboratory between November 2014 and December 2015 indicated that *S. oryzae* adults were more susceptible than *T. castaneum* adults to nanoemulsion formulations including 2.0 mL/kg azadirachtin according to median lethal times.

Keywords: Biopesticide, nanoemulsion formulation, neem oil, *Sitophilus oryzae, Tribolium castaneum*

Öz

Bu çalışmada, *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae) ve *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) erginlerin mücadelesinde kullanmak için çevre dostu nano emülsiyon formülasyonlarının geliştirilmesini amaçlanmıştır. Emülsiyon formülasyonu, düşük enerjili emülsiyonlaşma yöntemi kullanılarak neem yağından [*Azadirachta indica* A. Juss (Sapindales: Meliacear)], iyonik olmayan yüzey aktif maddeden (alkilpoliglukosid veya polisorbat 80) ve sudan oluşturulmuştur. Dört formülasyon seçilmiş ve fizikokimyasal çapı 208-507 nm olan partikül boyutu aralığı ile karakterize edilmiştir. Biyoassay çalışmaları, besine ve filtre kağıdına emdirme yöntemleri kullanılarak yürütülmüştür. Besine emdirme yöntemi ile, 2.0 mL/kg azadirachtin konsantrasyonlu polisorbat yüzey aktif maddeden oluşan formülasyona sadece 24 saatlik uygulamada *S. oryzae* erginlerinin %100 ölümü gerçekleşmiştir. Kasım 2014 ile Aralık 2015 tarihleri arasında Universiti Putra Malaysia toksikoloji laboratuvarında yürütülen toksikolojik çalışmalar, *S. oryzae* erginlerinin ölüm zamanlarının medyan değerlerine göre 2.0 mL/kg neem yağı içeren nano-emülsiyon formülasyonlarına *T. castaneum* erginlerinden daha duyarlı olduklarını göstermiştir.

Anahtar sözcükler: Biyopestisit, nanoemülsiyon formülasyonu, neem yağı, *Sitophilus oryzae, Tribolium castaneum*

[1] Universiti Putra Malaysia, Faculty of Agriculture, Department of Plant Protection, 43400, UPM Serdang, Malaysia
* Corresponding author (Sorumlu yazar) e-mail: m.choupanian@gmail.com
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Introduction

The rice weevil, *Sitophilus oryzae* (L., 1763) (Coleoptera: Curculionidae) is a major primary pest, and the red flour beetle, *Tribolium castaneum* (Herbst, 1797) (Coleoptera: Tenebrionidae) is a major secondary pest of stored grain-based products, including maize, rice and wheat, particularly in the tropic regions (Howe, 1965; Gonzalez et al., 2013; Wagan et al., 2016). These pests can cause considerable damage to stored grains and completely destroy kernels. Moreover, the product quality is affected by presence of eggs and dead insects, and holes on the grains (Lu & He, 2010). According to the global economic estimation, the related costs of pests to stored food stuffs could reach to about 500 million USD per year (Domínguez Umpiérrez & Marrero Artabe, 2010).

One of the most common control measures in stored-product insect pest management is chemical control, which is mainly dependent on the use of synthetic insecticides, such as fumigants [mostly phosphine (PH_3) or methyl bromide (MeBr)], residual contact insecticides such as organophosphates and pyrethroids (Kljajic & Peric, 2006; Islam et al., 2010). Since these chemicals are easy-to-use and cost-effective, their intensive and repeated use has resulted in several problems, such as the development of insecticide resistance, effectiveness of the ecosystem and toxic effects on humans and mammals (Bell & Wilson, 1995). Many researchers have been seeking for new alternatives to replace synthetic pesticides such as biological control, control by natural plant products (inert dusts and essential oils), behavioral control using insect pheromones and microbial control, to control insect pests (Mazzonetto & Vendramim, 2003). In recent years, one of the alternatives to control insect pests is to use bio insecticides including natural substances derived from the plants in forms of powders, extracts and oils. The secondary metabolites of these plants, known as botanical pesticides, have repellent, antifeedant, growth inhibition and fumigant effects on insect pests (Weaver et al., 1994).

Neem [*Azadirachta indica* A. Juss (Sapindales: Meliaceae)] oil is a broad spectrum botanical pesticide commonly used for stored grain protection worldwide (Medeiros et al., 2007; Costa et al., 2014; Choupanian et al., 2017). In a recent study, the contact toxicity of neem oil nanoemulsions, were evaluated against *S. oryzae* and *T. castaneum* adults by filter paper impregnation method (Choupanian et al., 2017). In order to understand better the performance of the nanoemulsion formulations when applied by different methods, it is also relevant to study the effect of the product when mixed with the commodity. It is known that insecticidal efficacy of neem oil is affected by its application method (Gahukar, 2014).

Therefore, the aims of the study were to (a) evaluate the insecticidal properties of four neem oil nanoemulsion formulations in management of two common insect pests of stored products, *S. oryzae* and *T. castaneum* adults by two different application methods (filter paper and food impregnation method) and (b) evaluate the median lethal time (LT_{50}) of the nanoemulsion formulations against the target pests.

Material and Methods

Chemicals

The nonionic surfactants, APG (Agnique® MBL 510H), and Polys (Tween 80) were supplied by Cognis Oleochemicals (M) Sdn Bhd (Selangor, Malaysia) and Duchefa Biochemie (Haarlem, The Netherlands), respectively. Neem oil was received from VM Consolidated (M) Sdn Bhd (Seri Kembangan, Malaysia) and Neemix® (EC formulation) was provided from Zeenex AgroScience (M) Sdn Bhd (Kuala Lumpur, Malaysia).

Insects

The initial populations of the rice weevil (*S. oryzae*) and the red flour beetle (*T. castaneum*) were obtained from the Entomology Laboratory of Universiti Putra Malaysia in January 2015. *Sitophilus oryzae* and *T. castaneum* were cultured in clear plastic containers of rice grain and wheat germ, respectively, under laboratory conditions of 27±1ºC, 75±1% RH and 12:12 h L:D photoperiod. Adult insects, 7-14 d old, were used for the experiments.

Preparation and characterization of the neem oil nanoemulsions

Pseudoternary phase behavior study was conducted in previous study in October 2014 (Choupanian et al., 2017). Four formulations were selected from the stable region of the emulsion system based on the features of being optically clear and single-phase, and substantially stable at 25°C for 30 d (Flanangan et al., 2006). The selected formulations were physicochemically characterized with respect to stability (at room temperature for a period of 90 d), thermostability (at 54ºC for a period of 14 d), particle size, and zeta potential analysis in November 2014.

Toxicity of the neem oil nanoemulsions using food impregnation method

The toxicity of the formulated neem oil nanoemulsions against the adults of tested insect species was studied using food impregnation method according to Talukder & Howse (1994) with some modifications in March 2015. The nanoemulsion formulations were diluted to four azadirachtin concentrations (5.0, 6.0, 7.5 and 10.0 mL/kg). One mL of each formulation was loaded into 5 g disinfested rice grain for *S. oryzae* and broken wheat grain for *T. castaneum*, to bring the amount of azadirachtin in the food media to 1.0, 1.2, 1.5 and 2.0 mL/kg, where Neemix® and neem oil alone were used as positive control and distilled water as negative control. The treated food media were air-dried to evaporate the solvent and placed into glass vials. Afterwards, 20 adults of *S. oryzae* and *T. castaneum* were released into rice kernel and broken wheat grain, respectively. The experiment was repeated five times, and adult mortality was recorded after 24, 48 and 72 h of exposure.

Toxicity of the nanoemulsion formulations using filter paper impregnation method

The contact toxicity of the nanoemulsion formulations was evaluated using filter paper impregnation method (Hameed et al., 2012) in March 2015. The nanoemulsion formulations were diluted to four azadirachtin concentrations (5.0, 6.0, 7.5 and 10.0 mL/kg) and then 1 mL of each formulation was loaded into a filter paper (5 cm ø), to bring the amount of azadirachtin on the filter paper to 2.6, 3.1, 3.8 and 5.1 mL/m^2. The treated filter papers were left at room temperature for 5 min to evaporate the solvent and then, were placed into Petri dishes. Twenty adults of *S. oryzae* or *T. castaneum* were released into each set of treatment and the Petri dishes were covered with lid so that the adults could not escape. The experiment was repeated five times and adult mortality was recorded after 24, 48 and 72 h of exposure.

LT_{50} determination

The LT_{50} for *S. oryzae* and *T. castaneum* adults at 2.0 mL/kg (in food media) and 5.1 mL/m^2 (on filter paper) of neem oil nanoemulsion, and the controls, was evaluated using the same bioassays, food impregnation method (Talukder & Howse, 1994) and filter paper impregnation method (Hameed et al., 2012), with five replicates. The treatments were checked every 2-3 h to record the number of dead adults. The experiment was done in June 2015.

Statistical analysis

The mortality data were tested for normality using Bartlett's test and, where necessary, transformed using Box-Cox transformation (Osborne, 2010). Repeated measure analysis of variance (ANOVA) was used to analyze mortality data with considering exposure time as the repeated variable (because the same Petri dishes were tested after 24, 48 and 72 h of exposure) and formulation with concentration as the main effects, using SAS 9.2 software (SAS Institute, Carey, NC, USA). Mean values were separated by Tukey's multiple comparison test ($P < 0.05$).

The LT_{50} values of the neem oil nanoemulsions and controls at 2.0 mL/kg (in food media), and 5.1 mL/m^2 (on filter paper) were determined for 50% mortality with confidence intervals of 95% using Probit analysis with PoloPlus software version 1.0 (LeOra Software, El Cerrito, CA, USA) according to the classical maximum likelihood procedure of Finney (1971).

Results

Composition and physicochemical characteristics of the nanoemulsion formulations

Four formulations were selected from the isotropic region of the phase diagram plots with 48:30:22 ($NeemPolys_1$ and $NeemAPG_1$) and 40:27:33 ($NeemPolys_2$ and $NeemAPG_2$) percentage of neem oil, surfactant and water (Table 1). The formulations were visually clear and transparent and physically stable after storage at room temperature (25ºC) for 90 d and subsequently at 54ºC for 14 d, except $NeemAPG_2$. All the selected compositions unequivocally exhibited long term and well stabilized properties. However, $NeemAPG_2$ showed phase separation at 54°C after 14 d. The formulations contained Polys surfactant showed smaller particle size of 208 ($NeemPolys_1$) and 253 nm ($NeemPolys_2$), but the formulations comprised APG exhibited bigger particle size of 328 ($NeemAPG_1$) and 507 nm ($NeemAPG_2$). The data of zeta potential analysis demonstrates that $NeemPolys_1$ and $NeemPolys_2$ comprised of Polys surfactant are more stable (39.1-37.9 mV) than $NeemAPG_1$ and $NeemAPG_2$ containing APG surfactant (32.5-31.3 mV) (Table 1).

Table 1. Physicochemical characteristics of the neem oil nanoemulsions for stability, thermostability, particle size, and zeta potential analysis

Formulation	Component (%) [a]	S [b]	T [c]	Particle size (nm)±SE [d]	Zeta potential (mV)±SE [d]
$NeemPolys_1$	30:48:22	√	√	208±1.2 d	39.1±1.5 a
$NeemPolys_2$	27:40:33	√	√	253±1.6 c	37.9±1.1 ab
$NeemAPG_1$	30:48:22	√	√	328±2.3 b	32.5±1.9 b
$NeemAPG_2$	27:40:33	√	×	507±2.7 a	31.3±1.3 b

[a] Surfactant (Polys or APG): neem oil: water;
[b] stability at 25ºC for 90 d;
[c] thermostability at 54ºC for 14 d;
[d] each value is the mean of three replicates (n = 3),
Within the column means followed by the same letter are not significantly different (Tukey's test, $P < 0.05$); √= stable; ×= not stable.

Bioassay

Mortality of *S. oryzae* and *T. castaneum*, via food and filter paper impregnation methods, were significantly affected by the exposure time, all main effects (formulations and concentrations) and associated interactions (Table 2). There were significant differences between toxicity of controls (Neemix® and neem oil) and nanoemulsions against the tested insect species for both application methods ($P < 0.05$) (Tables 3-6).

After 24 h of exposure, the highest mortality was recorded for *S. oryzae*, with food impregnation method, exposed to 2.0 mL/kg of $NeemPolys_1$, where all exposed adults were dead (Table 3). After 48 h of exposure in both application methods, 2.0 mL/kg (in food media) and 5.1 mL/m^2 (on filter paper) of the nanoemulsion formulations caused over 75% mortality of exposed adults of both insect species (Tables 3-6). After 72 h of exposure in both application methods, all concentration rates resulted in, more than 50% mortality of the exposed adults, and the proportion was lower for filter paper impregnation method (Tables 3-6). The mortality of *S. oryzae* reached to 100% after 72 h of exposure at all nanoemulsion formulation concentrations, with food impregnation method (Table 3).

Among the nanoemulsion formulations, $NeemAPG_2$ showed lower toxicity than other formulations in some cases, but despite showing less mortality there was trend toward enhanced efficiency throughout the assay. The mortality on the control treatment with neem oil and Neemix® were significantly lower than that of the nanoemulsion formulations. Neem oil exhibited the lowest mortality in each assessment and also on the total mortality. Mortality of *S. oryzae* and *T. castaneum* for neem oil alone treatment, after 72 h of exposure and all concentration rates, ranged between 8.2 and 33.5%, and 0 and 27.7%, respectively, via application of food impregnation method (Tables 3 and 4). A similar trend for the application of filter paper impregnation method was observed by mortality range of 0 to 21.7% (*S. oryzae*) and 0 to 15.5% (*T. castaneum*) (Tables 5 & 6).

Table 2. Parameters of repeated measure ANOVA for main effects and associated interactions for mortality of *Sitophilus oryzae* and *Tribolium castaneum* adults, through application of food and filter paper impregnation methods (error *df* = 360)

Source	*df*	Food impregnation method				Filter paper impregnation method			
		S. oryzae		*T. castaneum*		*S. oryzae*		*T. castaneum*	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Exposure time	2	6.35	0.019	8.73	0.005	6.73	0.009	5.95	0.012
Formulation	5	63.17	0.000	32.04	0.000	11.77	0.001	10.89	0.001
Concentration	4	18.76	0.000	24.54	0.000	14.27	0.001	13.88	0.001
Exposure time × formulation	10	1.52	0.167	2.87	0.009	5.39	0.000	8.22	0.000
Exposure time × concentration	8	6.25	0.000	4.90	0.000	6.22	0.000	9.23	0.000
Formulation × concentration	20	6.87	0.000	8.01	0.000	6.80	0.000	11.08	0.000
Exposure time × Formulation × concentration	40	53.37	0.000	40.74	0.000	42.50	0.000	25.75	0.000

Table 3. Mean mortality (%±SE) of *Sitophilus oryzae* adults exposed for 24, 48 and 72 h on rice kernels treated with four nanoemulsion formulations of neem oil, Neemix® and neem oil (crude extract) at five concentrations

Exposure time (h)	Formulation [a]	Mortality (%)±SE [b]				
		Concentration (mL/kg)				
		0.0	1.0	1.2	1.5	2.0
24	$NeemAPG_1$	0.0±0.0a	15.0±1.1c	42.2±0.4c	80.0±0.7bc	88.0±0.8b
	$NeemAPG_2$	0.0±0.0a	0.0±0.0d	29.4±0.3d	73.2±0.4c	81.2±0.4b
	$NeemPolys_1$	0.0±0.0a	26.2±0.4a	76.3±0.6a	95.0±0.4a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	19.3±1.2b	57.2±0.7b	88.7±0.2ab	96.3±0.4a
	Neemix®	0.0±0.0a	0.0±0.0d	0.0±0.0e	13.5±0.4d	21.8±0.7c
	Neem oil	0.0±0.0a	0.0±0.0d	0.0±0.0e	0.0±0.0e	0.0±0.0d
48	$NeemAPG_1$	0.0±0.0a	46.2±0.4b	87.6±1.2b	100.0±0.0a	100.0±0.0a
	$NeemAPG_2$	0.0±0.0a	34.0±0.5c	63.2±1.3c	100.0±0.0a	100.0±0.0a
	$NeemPolys_1$	0.2±0.3a	86.3±0.3a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	59.2±1.2b	100.0±0.0a	100.0±0.0a	100.0±0.0a
	Neemix®	0.0±0.0a	0.0±0.0d	0.0±0.0d	20.3±0.6b	34.7±0.4b
	Neem oil	0.0±0.0a	0.0±0.0d	0.0±0.0d	8.2±0.8c	18.5±0.6c
72	$NeemAPG_1$	0.3±0.9a	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	$NeemAPG_2$	0.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	$NeemPolys_1$	0.4±0.7a	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.2±0.5a	100.0±0.0a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	Neemix®	0.0±0.0a	12.5±1.3b	25.2±0.6b	35.4±0.4b	52.5±0.7b
	Neem oil	0.0±0.0a	8.2±0.5c	17.5±0.5c	24.5±0.8c	33.5±0.6c

[a] For component percentage of the formulations coded in this column, refer to Table 1;
[b] Each value is the mean of five replicates (n = 5);
For each exposure time, within each concentration (columns), means followed by the same letter are not significantly different (Tukey HSD test at $P < 0.05$).

Table 4. Mean mortality (%±SE) of *Tribolium castaneum* adults exposed for 24, 48 and 72 h on broken wheat grains treated with four nanoemulsion formulations of neem oil, Neemix® and neem oil (crude extract) at five concentrations

Exposure time (h)	Formulation [a]	Mortality (%)±SE [b]				
		Concentration (mL/kg)				
		0.0	1.0	1.2	1.5	2.0
24	$NeemAPG_1$	0.0±0.0a	5.5±0.4b	27.0±0.7b	59.5±0.4bc	73.5±0.8ab
	$NeemAPG_2$	0.0±0.0a	0.0±0.0c	17.2±0.4c	51.2±0.8c	68.5±0.4b
	$NeemPolys_1$	0.0±0.0a	12.5±0.9a	51.5±0.6a	73.5±0.9a	86.2±0.8a
	$NeemPolys_2$	0.0±0.0a	7.5±0.7b	34.5±0.9b	68.7±0.5ab	80.7±0.5ab
	Neemix®	0.0±0.0a	0.0±0.0c	0.0±0.0d	8.5±0.4d	15.7±0.8c
	Neem oil	0.0±0.0a	0.0±0.0c	0.0±0.0d	0.0±0.0d	0.0±0.0d
48	$NeemAPG_1$	0.0±0.0a	31.5±0.6c	70.0±1.1b	87.5±0.6b	100.0±0.0a
	$NeemAPG_2$	0.0±0.0a	0.0±0.0d	48.2±0.7c	76.7±0.5c	95.0±0.7a
	$NeemPolys_1$	0.0±0.0a	72.5±0.4a	88.2±0.4a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	41.0±1.8b	79.5±0.6b	93.7±0.4a	100.0±0.0a
	Neemix®	0.0±0.0a	0.0±0.0d	0.0±0.0d	15.5±0.9d	27.7±0.4b
	Neem oil	0.0±0.0a	0.0±0.0d	0.0±0.0d	0.0±0.0e	9.8±0.3c
72	$NeemAPG_1$	0.3±0.5a	63.7±0.8c	100.0±0.0a	100.0±0.0a	100.0±0.0a
	$NeemAPG_2$	0.0±0.0a	44.0±0.4d	79.5±0.4b	100.0±0.0a	100.0±0.0a
	$NeemPolys_1$	0.2±0.2a	91.5±0.6a	100.0±0.0a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	78.7±1.3b	100.0±0.0a	100.0±0.0a	100.0±0.0a
	Neemix®	0.0±0.0a	6.2±0.4e	14.7±0.8c	23.5±0.6b	46.5±0.6b
	Neem oil	0.0±0.0a	0.0±0.0e	9.6±0.5d	19.7±0.4b	27.7±0.3c

[a] For component percentage of the formulations coded in this column, refer to Table 1;
[b] Each value is the mean of five replicates (n = 5);
For each exposure time, within each concentration (columns), means followed by the same letter are not significantly different (Tukey HSD test at $P < 0.05$).

Table 5. Mean mortality (%±SE) of *Sitophilus oryzae* adults exposed for 24, 48 and 72 h on filter paper treated with four nanoemulsion formulations of neem oil, Neemix® and neem oil (crude extract) at five concentrations

Exposure time (h)	Formulation [a]	Mortality (%)±SE [b]				
		Concentration (mL/m^2)				
		0.0	2.6	3.1	3.8	5.1
24	$NeemAPG_1$	0.0±0.0a	0.0±0.0a	10.2±0.4b	24.5±0.7bc	46.7±0.7b
	$NeemAPG_2$	0.0±0.0a	0.0±0.0a	0.0±0.0c	15.7±0.8c	37.5±1.2c
	$NeemPolys_1$	0.0±0.0a	0.0±0.0a	21.2±0.6a	42.5±1.3a	65.2±0.7a
	$NeemPolys_2$	0.0±0.0a	0.0±0.0a	16.2±0.5ab	31.7±0.5ab	52.2±0.9ab
	Neemix®	0.0±0.0a	0.0±0.0a	0.0±0.0c	0.0±0.0d	0.0±0.0d
	Neem oil	0.0±0.0a	0.0±0.0a	0.0±0.0c	0.0±0.0d	0.0±0.0d
48	$NeemAPG_1$	0.0±0.0a	16.5±0.8c	49.5±0.9c	79.2±0.6b	100.0±0.0a
	$NeemAPG_2$	0.0±0.0a	0.0±0.0d	32.2±1.4d	61.5±0.8c	82.5±0.6b
	$NeemPolys_1$	0.0±0.0a	42.2±0.4a	78.2±0.8a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	27.2±1.5b	64.5±0.6b	100.0±0.0a	100.0±0.0a
	Neemix®	0.0±0.0a	0.0±0.0d	0.0±0.0e	6.5±0.9d	17.5±0.6c
	Neem oil	0.0±0.0a	0.0±0.0d	0.0±0.0e	0.0±0.0d	0.0±0.0d
72	$NeemAPG_1$	0.0±0.0a	32.2±0.4c	59.5±0.5b	100.0±0.0a	100.0±0.0a
	$NeemAPG_2$	0.0±0.0a	21.5±0.6d	47.2±0.4b	100.0±0.0a	100.0±0.0a
	$NeemPolys_1$	0.0±0.0a	56.2±0.8a	81.5±0.7a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.4±0.2a	41.5±0.6b	73.5±0.5a	100.0±0.0a	100.0±0.0a
	Neemix®	0.0±0.0a	0.0±0.0e	7.75±1.5c	16.5±1.9b	25.8±1.4b
	Neem oil	0.0±0.0a	0.0±0.0e	0.0±0.0c	11.5±1.3c	21.7±1.7b

[a] For component percentage of the formulations coded in this column, refer to Table 1;
[b] Each value is the mean of five replicates (n = 5);
For each exposure time, within each concentration (columns), means followed by the same letter are not significantly different (Tukey HSD test at $P < 0.05$).

Table 6. Mean mortality (%±SE) of *Tribolium castaneum* adults exposed for 24, 48 and 72 h on filter paper treated with four nanoemulsion formulations of neem oil, Neemix® and neem oil (crude extract) at five concentrations

Exposure time (h)	Formulation [a]	Mortality (%)±SE [b]				
		Concentration (mL/m^2)				
		0.0	2.6	3.1	3.8	5.1
24	$NeemAPG_1$	0.0±0.0a	0.0±0.0a	0.0±0.0b	19.5±0.6bc	38.5±0.8bc
	$NeemAPG_2$	0.0±0.0a	0.0±0.0a	0.0±0.0b	11.7±1.6c	29.5±1.3c
	$NeemPolys_1$	0.0±0.0a	0.0±0.0a	17.5±1.4a	37.5±1.3a	59.5±1.7a
	$NeemPolys_2$	0.0±0.0a	0.0±0.0a	12.5±0.6a	28.5±0.9ab	45.5±0.8b
	Neemix®	0.0±0.0a	0.0±0.0a	0.0±0.0b	0.0±0.0d	0.0±0.0d
	Neem oil	0.0±0.0a	0.0±0.0a	0.0±0.0b	0.0±0.0d	0.0±0.0d
48	$NeemAPG_1$	0.0±0.0a	11.5±0.5c	41.5±0.8c	70.7±1.5c	90±0.0b
	$NeemAPG_2$	0.0±0.0a	0.0±0.0d	24.5±0.5d	53.5±2.4d	75.5±2.6c
	$NeemPolys_1$	0.0±0.0a	37.5±1.2a	70.8±2.3a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	21.5±1.5b	58.5±1.8b	92.5±0.8b	100.0±0.0a
	Neemix®	0.0±0.0a	0.0±0.0d	0.0±0.0e	0.0±0.0e	10.4±0.8d
	Neem oil	0.0±0.0a	0.0±0.0d	0.0±0.0e	0.0±0.0e	0.0±0.0e
72	$NeemAPG_1$	0.0±0.0a	27.2±1.1bc	53.3±0.8bc	89.5±2.1b	100.0±0.0a
	$NeemAPG_2$	0.0±0.0a	16.5±0.8c	41.5±1.6c	78.3±1.1c	100.0±0.0a
	$NeemPolys_1$	0.0±0.0a	51.5±0.6a	76.0±0.9a	100.0±0.0a	100.0±0.0a
	$NeemPolys_2$	0.0±0.0a	32.7±1.5b	61.5±1.8b	100.0±0.0a	100.0±0.0a
	Neemix®	0.0±0.0a	0.0±0.0d	0.0±0.0d	13.5±1.4d	19.6±1.1b
	Neem oil	0.0±0.0a	0.0±0.0d	0.0±0.0d	0.0±0.0e	15.5±1.9b

[a] For component percentage of the formulations coded in this column, refer to Table 1;
[b] Each value is the mean of five replicates (n = 5);
For each exposure time, within each concentration (columns), means followed by the same letter are not significantly different (Tukey HSD test at $P < 0.05$).

Lethal time determination (LT_{50} and LT_{90})

Results of Probit analysis and lethal time determination are shown in Tables 7 and 8. According to the LT_{50} and LT_{90} values, regardless formulation type and application method, it is concluded that *S. oryzae* adults are more susceptible (LT_{50} 9.6 to 140.4 h) while *T. castaneum* adults (LT_{50} 11.3 to 159.9 h) are more tolerant. The time required for the nanoemulsion formulations to give 50% mortality of *S. oryzae* adults ranged from 9.6 to 17.4 h (food impregnation method) and 17.0 to 32.4 h (filter paper impregnation method). However, LT_{50} values for *T. castaneum* ranged from 11.3 to 23.1 h (food impregnation method at 2.0 mL/kg azadirachtin) and 19.2 to 35.8 h (filter paper impregnation method at 5.1 mL/m^2 azadirachtin). Therefore, it is concluded that food impregnation method is the more effective method for this assay. Generally, the nanoemulsion formulations had lower LT_{50} values compared to neem oil and Neemix® alone as controls. Among the nanoemulsion formulations, $NeemPolys_1$ acted as the most efficient formulation. In all cases, increased susceptibility of both insect species was directly associated with particle size of the formulation and exposure time.

Table 7. LT_{50} and LT_{90} (Lethal Time) (hour) of *Sitophilus oryzae* and *Tribolium castaneum* adults exposed to the formulations at 2.0 mL/kg azadirachtin via food impregnation method

Insect species	Formulation [a]	LT_{50} (lower-upper CL) [b] hours	LT_{90} (lower-upper CL) [b] hours	Slope±S.E	Chi square
S. oryzae	$NeemAPG_1$	14.6 (13.8-15.1)	35.5 (28.8-49.1)	2.7±0.3	4.4
	$NeemAPG_2$	17.4 (16.0-18.2)	42.3 (33.7-59.9)	3.1±0.4	4.5
	$NeemPolys_1$	9.6 (8.1-11.0)	27.7 (22.3-31.2)	3.5±0.4	2.9
	$NeemPolys_2$	11.6 (9.6-13.6)	28.8 (25.1-35.7)	2.8±0.3	3.1
	Neemix®	46.2 (41.3-54.4)	91.8 (84.1-97.3)	1.6±0.2	1.2
	Neem oil	92.2 (79.9-106.4)	156.0 (151.9-182.2)	2.4±0.3	0.8
T. castaneum	$NeemAPG_1$	17.9 (15.7-19.4)	55.4 (41.7-88.1)	2.4±0.3	0.3
	$NeemAPG_2$	23.1 (20.9-25.6)	62.6 (56.0-123.6)	2.5±0.3	2.8
	$NeemPolys_1$	11.3 (9.7-12.0)	35.3 (27.1-54.4)	1.1±0.2	3.6
	$NeemPolys_2$	13.9 (12.4-15.2)	45.1 (34.8-67.0)	2.9±0.3	0.1
	Neemix®	54.7 (52.4-57.6)	109.4 (106.7-126.7)	1.3±0.2	1.2
	Neem oil	112.7 (105.3-129.6)	177.1 (170.0-185.8)	2.2±0.4	4.4

[a] For component percentage of the formulations coded in this column, refer to Table 1;
[b] Upper and lower 95% confidence limits.

Table 8. LT_{50} and LT_{90} (Lethal Time) (hours) of *Sitophilus oryzae* and *Tribolium castaneum* adults exposed to the formulations at 5.1 mL/m^2 via filter paper impregnation method

Insect species	Formulation [a]	LT_{50} (lower-upper CL) [b] hours	LT_{90} (lower-upper CL) [b] hours	Slope±S.E	Chi square
S. oryzae	$NeemAPG_1$	24.9 (21.1-28.0)	71.3 (48.2-81.1)	3.1±0.3	1.2
	$NeemAPG_2$	32.4 (29.3-35.0)	81.3 (53.8-97.3)	4.1±0.3	4.3
	$NeemPolys_1$	17.0 (14.8-19.4)	59.4 (43.8-96.8)	2.4±0.2	2.1
	$NeemPolys_2$	22.4 (19.8-25.6)	68.2 (52.3-101.9)	2.6±0.2	0.4
	Neemix®	68.2 (62.5-73.1)	161.7 (158.6-174.4)	1.2±0.2	0.4
	Neem oil	140.4 (137.2-143.6)	197.9 (193.0-211.8)	2.7±0.5	0.3
T. castaneum	$NeemAPG_1$	29.4 (28.0-31.9)	73.2 (54.4-103.4)	3.1±0.3	7.9
	$NeemAPG_2$	35.8 (32.0-38.4)	80.4 (66.4-106.6)	3.±0.4	0.4
	$NeemPolys_1$	19.2 (16.1-21.8)	61.6 (46.9-93.8)	2.1±0.2	1.6
	$NeemPolys_2$	24.7 (20.9-27.3)	70.2 (68.9-187.3)	2.2±0.2	1.1
	Neemix®	81.7 (74.2-89.0)	172.6 (169.1-173.2)	2.4±0.3	0.2
	Neem oil	159.9 (156.9-162.0)	227.2 (216.0-251.4)	3.7±0.6	0.5

[a] For component percentage of the formulations coded in this column, refer to Table 1;
[b] Upper and lower 95% confidence limits.

Discussion

The emulsion produced for the present study had a particle size range of below 600 nm, which can be considered as a nanoemulsion formulation (Solans et al., 2003; Shafiq et al., 2007). Consistent with Mishra et al. (2014), a reduction in the particle size and turbidity was observed by increasing the surfactant concentration and decreasing the neem oil concentration. The presence of the surfactant in the nanoemulsion formulations created the reduction of interfacial tension at the oil/water interface leading to a drop in the free energy and thus creates a mechanical obstacle to the disambiguation of the droplets, therefore the nano-droplets showed good stability in the emulsion system (Reiss, 1975). Also**,** Chen & Tao (2005) stated that the well stabilized properties of the nanoemulsion formulations is due to the enhanced adsorption of surfactant molecules at the oil-water borders. However, $NeemAPG_2$ showed phase separation at 54°C, which is probably due to a lower surfactant concentration in conjunction with more water content compared to $NeemAPG_1$ comprised the same surfactant. Also, $NeemAPG_2$ with the biggest droplet size of 507 nm demonstrated significantly higher capability to self-assemble as the hydrophobic affinity was enlarged while the solubility in water declined and made the hydrophobic chain to self-organize into larger aggregates (Lin & Lin, 2003).

The occurrence of coalescence also increased the droplet size of the nanoemulsion with time, because the liquid film between droplets disrupted and became thin, which led to larger droplet size (Taylor, 2003). Another reason for increasing droplet size is decreasing surfactant/water ratios at fixed amount of oil or increasing of oil/water ratios at constant surfactant (Morales et al., 2003). The particle size and zeta potential characteristics of the nanoemulsions correlated with the type of surfactant. In these circumstances, Polys surfactant preferentially formed better formulations with smaller droplet size and higher zeta potential. The results from zeta sizer analysis confirmed that Polys surfactant acts as the best surfactant compared to APG with less significant difference and thus higher stability (Table 1). As reported in previous studies, the nanoemulsion formulations that contained nonionic surfactants with the smallest particle size were more effective. The lack of phase separation of the nanoemulsion formulations over a long period of time contributed to their extended stability (Solans et al., 2005; Anjali et al., 2012).

The bioassay of the nanoemulsion formulations of neem oil demonstrated toxicity effects on both insect species. The insecticidal effects of the formulations varied with the insect species, concentration of the formulations, exposure time and the method of application. The results of contact toxicity in the filter paper impregnation method showed lower mortality than that with food impregnation method. The possible explanation for these results is the higher absorption of the toxic substance occurs through ingestion of the food into the insect's body. In previous studies (Negahban et al., 2007; Sahaf et al., 2007, 2008; Ogendo et al., 2008; Taghizadeh-Saroukolai et al., 2010), it was found that *S. oryzae* is significantly more susceptible than *T. castaneum,* which is consistent with the results obtained in our experiment. Studies have not previously reported a rapid-acting nanoemulsion formulation of neem oil produced from low-energy emulsification method and low concentrations of azadirachtin for control of stored products insect pests. The effectiveness of neem crude extract with 2.5% azadirachtin against *T. castaneum* has been evaluated by Hameed et al. (2012), which lead to about 46% mortality after 7 d of exposure using filter paper dip method. Among the nanoemulsion formulations, the highest and fastest toxic effect were observed with $NeemPolys_1$ against *S. oryzae* via food impregnation, which justifies the use of Polys surfactant in this formulation as it leads to smaller particle size and therefore more opportunity of the formulation to come in contact with the target insect. Whereas, the lower mortality caused by $NeemAPG_2$ with the biggest particle size indicates that the smaller the particle size, the greater the probability of higher efficacy. This finding is consistent with the studies of Lim et al. (2013) and Asib et al. (2015).

The bioassay results, in most cases, showed that mortality from exposure to the nanoemulsion formulations was not significantly different at 2.0 mL/kg (in food media) and 5.1 mL/m^2 (on filter paper) azadirachtin after 48 h. Therefore, the lethal time experiments were conducted to determine the fastest acting formulation at 2.0 mL/kg (via food impregnation method) and 5.1 mL/m^2 azadirachtin (via filter paper impregnation method). The study showed that oil based nanoemulsion formulations were able to increase the mean mortality rate of *S. oryzae* and *T. castaneum* compared to the commercial EC formulation of neem oil (Neemix®) and crude extract of neem oil. Moreover, the low LT_{50} at 2.0 mL/kg (in food media) and 5.1 mL/m^2 (on filter paper) azadirachtin of the formulated nanoemulsions was due to the

presence of surfactant in the formulations, which increased the opportunity for the toxic substance (azadirachtin) to act more efficiently and stably. Therefore, this study clearly indicates that the formulating of azadirachtin is one of the most effective ways to increase mortality of stored-product insect pests. Generally, these observations evidenced that the formulated neem oil nanoemulsions have suitable properties to be considered as toxic component against insect pests of stored products with rapid and high mortality impacts.

Conclusion

The neem oil, surfactant and deionized water were successfully prepared as nanoemulsion formulation by a low-energy emulsification method. NeemPolys$_1$ containing Polys surfactant with smallest droplet size of 208 nm was the most effective formulation for control of *S. oryzae* and *T. castaneum* adults compared to the other formulations with bigger droplet size. NeemPolys$_1$ also demonstrated the highest mortality against *S. oryzae* via food impregnation method in less than 10 h at 2.0 mL/kg azadirachtin compared to other formulations as well as the conventional emulsifiable formulation (Neemix®) and the unformulated neem oil. Overall, the laboratory studies have shown that the formulated nanoemulsions of neem oil could be a good alternative for the control of the tested species in stored products; however, further research is required to demonstrate the effectiveness of the nanoemulsion formulations on stored products insect pests, in practice through packaging.

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