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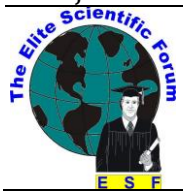

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RESEARCH ARTICLE

Development of Saponin-based Nano Emulsion Formulation from Selected Medicinal Plants to Control *Aphis gossypii*

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ABSTRACT

Aphis (*A.*) *gossypii* is an important agricultural pest because of its polyphagous nature. It leads to substantial losses in agricultural crops by causing either direct damage to its hosts as a phloem-feeder or indirectly by serving as vectors of many important plant viruses. Botanical aphicides represent a sustainable alternative to combat *A. gossypii* due to their biodegradability and overall safety. In this study, a saponin based nano-emulsion formulation from selected medicinal plants with Termul 1284 and rapeseed oil were prepared and evaluated against *A. gossypii* for their efficacy under laboratory and glasshouse conditions. Results revealed that all three prepared formulations were effective in suppressing aphid population under laboratory and glasshouse conditions. However, the highest repellency (73%) and mortality percentage (100%) with shortest LT_{50} (24.32 h) and LC_{50} (1391 $mg.L^{-1}$) values were recorded for Termul 1284/ rapeseed oil/ *Clidemia hirta* extract (TR3CH). TR3CH revealed shortest LT_{50} (55.5 h) and lowest LC_{50} (1480 $mg.L^{-1}$) values in glasshouse bioassay as well. TR3CH nano-emulsion formulation overall performed better than all other tested nano emulsion formulations, therefore, it could be considered as an eco-friendly alternative approach in pesticides technology.

INTRODUCTION

Aphis (*A.*) *gossypii*, also known as cotton aphid or melon aphid, is the most common and widely distributed aphid specie throughout the world (Rostami et al., 2012; Wang et al., 2016). It is an important agricultural pest because of its polyphagous nature as it attacks around 900 plants species from 116 families; ranging from agronomical to horticultural crops to ornamental plants (Blackman and Eastop, 2000; Ma et al., 2019). *A. gossypii* cause either direct damage to its hosts as a phloem-feeder by instigating plant leaves to curl and drop prematurely (CABI, 2020), or indirectly by serving as vectors of many important plant viruses such as mosaic and crinkle viruses (Selvarajan et al., 2006; Ghosh et al., 2014) and supporting the development of fungal sooty mold on its honeydew (Jaharlal et al., 2016; CABI, 2020). To manage this damaging pest, the most preferred and widely practiced method is chemical control through synthetic insecticides because of their rapid action (Uzair et al., 2018). But, due to broad-spectrum properties of synthetic insecticides, they have huge adverse effects on non-target organisms including beneficial insects and humans along with their role in environmental pollution and ozone depletion (Hassaan and Nemr, 2020). However, plant-derived pesticides are reported to be relatively safe for the humans and their environment than synthetic pesticides because of their short residue effects and easy degradation (Isman, 2006; Uzair et al., 2018). Many plants derived bio-active compounds have been tested and proved to be effective against various insect pests of agricultural crops (Adel et al., 2000; El-Wakeil, 2013). One of these compounds is saponin that is widely distributed in monocotyledonous and dicotyledonous angiosperms. Saponins directly disturb the reproduction and growth of the insect pests because of their deterrent or repellent activities (Mokhtar, 2016; Singh and Kaur, 2018; Roonjho et al., 2020). Moreover, they also increase the mortality of target pests by lowering their food intake due to less digestibility and toxicity of the food eaten (Adel et al., 2000; Singh and Kaur, 2018). Plants selected in this study possess various medicinal properties and are widely used in many traditional medicines. *Clidemia hirta* commonly known as soap-

bush is a perennial shrub with a coarse texture, it is being used in many traditional medicines to treat the irritation, bacterial infection, diarrhea, venom fever, skin infections and heart and stomach burns (Binggeli, 2005; Dianita et al., 2011; Abdellaoui et al., 2014). *Antidesma cuspidatum* may grow into two forms, either as tree or shrub and rich in antioxidant, cytotoxic, thrombolytic, antiradical, anti-diabetic, anti-dysenteric, antimicrobial, anticoagulant, anticancerous, sudorific and antihypertensive properties, hence, are widely used in medicines to treat different human disorders throughout the world (Rahmani et al., 1985; Islam et al., 2018). *Porterandia anisophylla* is a forest tree belonging to family *Rubiaceae* which is well-known for its medicinal properties to treat various ailments including ulcers, asthma, jaundice, leprosy, antibacterial, hyperacidity, antioxidant, cough, and fever (Parthasarathy et al., 2009; Astalakshmi and Ganapathy, 2017). The selected plants are considered to be rich in phytochemicals such as saponins, alkaloids, flavonoids and tannins etc. (Borges et al., 2009; Abdellaoui et al., 2014: Islam et al., 2018). Chemical analysis of selected plant materials revealed the strong presence of saponin and confirmed their aphicidal effects of methanolic extract on *A. gossypii* in laboratory condition (Roonjho, 2021). However, plant extracts in unformulated form are less stable and difficult to handle (Mokhtar, 2016). Micro-emulsions are thermodynamically stable, isotopically clear dispersions of two immiscible liquids such as oil and water stabilized by the interfacial film of any surfactant and/or co-surfactant. They are known to improve the solubility, stability, and efficacy of bioactive compounds. Therefore, the aim of this study was to formulate saponin based nano-emulsion formulation from selected medicinal plants to control *A. gossypii*.

MATERIALS AND METHODS

Development of nano-emulsion formulation

The methanolic leave extracts of *C. hirta*, *P. anisophylla* and *A. cuspidatum* were prepared as described in previous study (Roonjho, 2021) and used as active ingredient to develop the nano emulsion formulation. After evaluation of various surfactants and oils through preliminary miscibility test, Termul 1284 (KC Chemicals, Malaysia) and ethoxylated rapeseed oil (KLK Oleo, Malaysia) were selected as surfactant and oil respectively and used for the construction of ternary phase diagram. The ternary phase diagram was constructed using aqueous titration method (Aboofazeli and Lawrence, 1994; Shafiq et al., 2007). Total eleven combinations of surfactant and oil (w/w) i.e. 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90 and 0:100 were used in the experiment. Appropriate amounts of surfactant, oil and water were weighed for a total of 0.5 g into 7 ml culture tube with cap. The three components were mixed using vertex mixer (VELP, Italy) to get equilibrium. The surfactant and oil mixtures were dropwise diluted with water, vortexed and then centrifuged for 30 min at 3500 rpm. Amount of water in which the phase transition appeared was derived from weight measurement to calculate the percentage of oil, surfactant and water. Each sample was assessed visually for spontaneous emulsification on the basis of clarity, stability and transparency. Mixture in which transition occurred was used to determine the isotropic phase. The ratio of water, oil and surfactant obtained was marked on three apices of ternary phase diagram and results were used to plot ternary phase diagram using Chemix version 3.5 phase diagram plotter (Chemix, UK). From the constructed phase diagram system, several points were selected near to the water axis. The preference was given to water axis during point selection due to the polar nature of active ingredient which is easily soluble in water as compared to oil. The percentage of oil was fixed at 5% and percentage of surfactants ranged from 15-25% with increment of 5%. The selected points were formulated as per their respective percentage with each active ingredient (crude extract) and subjected to stability and thermal stability tests, zeta potential, particle size measurements and surface tension analysis as follows:

Characterization of nano-emulsion formulation Stability and thermal stability tests

The selected formulations were centrifuged at 3500 rpm for 30 min (Shafiq et al., 2007; Mokhtar, 2016). The obtained formulations were further processed for thermostability test at room temperature (26±2 °C) with 60-80% relative humidity (RH) for 3 months and at 54±1 °C for 28 days. The higher temperature (54±2 °C) is prescribed by the Food and Agriculture Organization (FAO) as a standard evaluation method for the stability test of agrochemical products in the tropical climate (Chen et al., 2000). The formulations retain transparent one-phase appearance after each test indicated the stability of emulsion (Flanagan et al., 2006).

Zeta potential measurement

The formulations were diluted in distilled H_2O at 5000 $mg.L^{-1}$ concentration in a falcon tube and gently mixed by inverting the tube to measure their zeta potential. About 1 mL of sample was pipetted into 1 cm^2 of quartz cell and placed into a Zetasizer Nano-ZS (Malven, UK). The analysis was done based on the standard procedure of Zetasizer Nano-ZS user manual with three replications.

Particle size measurement

The particle size measurement was also done through Zetasizer Nano-ZS following the method similar to Zeta potential measurement.

Surface Tension analysis

A 60 mL sample of each formulation was prepared in distilled H_2O in glass tubes and the surface tension

analysis was carried out using Du Nuoy ring method on Attension Force Tensiometer (Sigma 700; KSV, Finland). Before sample measurement, the tensiometer was calibrated using deionized H_2O with surface tension of 72 $mN.m^{-1}$. This study followed the standard procedure of KSV instrument.

Cultivation of eggplant and rearing of *A. gossypii*

Eggplant, *Solanum melongena* was cultivated continuously to be used in bioassay studies and rearing of *A. gossypii*. The *A. gossypii* were morphologically identified through Dino-lite digital microscope and reared in insect proof cages as described previously (Roonjho, 2021).

Laboratory bioassays

Toxicity of prepared saponin based nano-emulsion formulations against *A gossypii* under laboratory conditions (24±1 °C with a 12: 12h L: D photoperiod and 65±10% RH) was evaluated through repellency and mortality bioassays.

Repellency test

Repellency test was performed according to method described by Chen et al. (2017) with minor modifications. The test was conducted to evaluate the repellent action of the selected formulations against *A. gossypii* adults. The repellency experiment was based on a choice test using eggplant leaf discs (50 mm in diameter), divided into two parts by a vein. One half of the leaf disc was dipped in prepared concentration of each formulation (1000, 2500, 5000, 7500 and 10000 $mg.L^{1}$) and other part was treated with water only. Both the treated part and the control parts were air dried to evaporate the solvent completely (Huang et al., 2014). Ten adults of aphid were released with equal numbers (5/5) on both parts of leaf disc and the top of petri dish was covered carefully with modified ventilated petri-dish lid. The number of insects present on control (NC) and treated part (NT) were counted after 1, 3, 6, 12, 18 and 24 hrs. Percent repellency (PR) values were computed by using formula as follows:

$PR = [(NC-NT)/(NC+NT)] \times 100$

Each treatment was replicated ten times as the experiment was arranged in CRD design.

Mortality Bioassay

The mortality bioassay of *A. gossypii* using selected nano-emulsion formulations was conducted based on the guidelines of Insecticides Resistance Action Committee (IRAC), through leaf-dip bioassay method for aphids (IRAC Susceptibility Test Method No. 019). Leaf discs of egg plants (50 mm) were prepared using leaf disc cutter and dipped in the prepared concentrations (1000, 2500, 5000, 7500 and 10000 $mg.L^{-1}$) of each formulation for 10 sec and then left for complete dryness on a towel paper. The leaf discs were then placed upside down in a small petri dish (50 mm diameter) on agar. Ten healthy, 2-3 days old *A. gossypii* adults (black morph) were released carefully on the surface of leaves in each petri dish using fine brush. Leaves dipped in tap water and imidacloprid (18%) at the recommended dose (2.5 mL/ 10L) were used as negative and positive controls, respectively. The experiment was arranged in a completely randomized design (CRD) where each treatment was replicated ten times. The data was observed after 1, 3, 6 and 12 hrs of the application of treatments, whereas subsequent observations were taken after every 12 hrs for three days. Aphids were considered dead if they failed to show any movement when gently prodded with brush.

Glasshouse assessment

The experiment was conducted in glasshouse at Field 15, Faculty of Agriculture, Universiti Putra Malaysia using method described by Pavela (2018) with slight modifications. Four-weeks-old egg plants with two to three true leaves in the pot of 10 cm diameter were placed in insect-proof cages (16 cm x 16 cm x 18 cm). Twenty black morph adult aphids (2–3 days old) were introduced to the plants and allowed for one hour to spread over the plant naturally before application. Five different concentrations (1000, 2500, 5000, 7500 and 10000 $mg.L^{-1}$) of each formulation were prepared and applied on eggplants using a pneumatic sprayer. The numbers of aphids on the plant were counted after 3, 6, 12, 24 hrs of application and subsequently after every 24 hrs up to 120 hrs. Fusilier 18.3SL (Imidacloprid) at five different concentrations (50, 100, 250, 500 and 1000 $mg.L^{-1}$) were used as positive control, while, water was used as negative control. The treatments were arranged in Randomized Complete Block Design (RCBD) and each treatment was replicated ten times.

Statistical Analysis

The results obtained from mortality bioassays were analyzed using Polo Plus computer software to calculate lethal concentration 50 (LC_{50}) and lethal time 50 (LT_{50}) values. However, the data collected from repellency test were analyzed by ANOVA and means were compared using Tukey's HSD test at 0.05 probability level using SAS 9.4 computer software (SAS Institute Inc. 2009).

RESULTS

Ternary phase diagram

The ternary phase diagram comprised of Termul 1284, ethoxylated rapeseed oil and water (Fig. 1) revealed 50% isotropic region. The isotropic region of ternary phase system observed when the ratio of Termul 1284 was more than 15%, ethoxylated rapeseed oil was less than 20% and water was less than 80%. Three points were selected from isotropic region of ternary phase diagram and formulated as per their respective percentage with each crude extract. Total nine formulations were prepared and coded as shown in

Table 1 (TR1CH, TR1PA, TR1AC, TR2CH, TR2PA, TR2AC, TR3CH, TR3PA and TR3AC).

Stability tests

Stability tests results revealed that all the formulations were stable and indicated the presence of nano-emulsion by retaining one transparent phase appearance after centrifugation (Table 2). However, only three formulations TR3CH, TR3AC and TR3PA showed the thermal stability and retained transparent one phase after storage of 28 days at 54±1°C and three months' storage at room temperature (26±1). Therefore, only these formulations processed for further characterization.

Zeta potential

Results revealed that overall the mean zeta potential of all the formulations ranged between -21.6 to -23.0 mV (Table 3). Basically, zeta potential is used for predicting the dispersion stability of a colloidal dispersion that can be separated into two phases i.e., continuous phase and disperse phase. The stability of particles depends on their total potential energy where high zeta potential value indicates high degree of stability of formulation over a long period of time.

Particle size

Mean particle size of all the formulations ranged between 69.4 nm to 88.9 nm (Table 3). The particle size distribution is a numerical value that is also considered as one of the most important physical property to indicate the quality and performance of a formulation. Formulations which possess particle size from 50 nm to 500 nm are classified as nano-emulsions. Therefore, all the formulations tested in this study were grouped as nano-emulsions.

Surface tension

The mean surface tension for all formulations ranged between 34.88 $mN.m^{-1}$ to 35.41 $mN.m^{-1}$ (Table 3), which were much lower compared to the surface tension of water (72 $mN.m^{-1}$). According to Laplace's law, surface tension is the elastic propensity of liquids which makes them to obtain the least surface possible area possible, hence, it is considered as base for the formulation of liquid droplets.

Laboratory bioassays

Repellency bioassay: The repellency percentages of saponin based nano-emulsion formulations on *A. gossypii* are given in Table 4. After 24 hours, a significant difference ($P<0.05$) was observed in the repellency percentage of aphids treated with different concentration of various treatments. The highest repellency percentage of *A. gossypii* was observed in 10,000 $mg.L^{-1}$ of TR3CH (73%) which was significantly different from remaining treatments. At 10,000 $mg.L^{-1}$ concentration, TR3AC and TR3PA revealed 60 and 59% respectively. These both treatments were not significantly different from each other. Moreover, the lowest repellency percentage 4.9 and 5.3% was observed in TR3AC and TR3PA at 1000 $mg.L^{-1}$ concentration. Furthermore, all the plant materials showed repellent properties towards *A. gossypii* even at their lowest concentration i.e. 1000 $mg.L^{-1}$.

Mortality bioassay

The mortality percentages of *A. gossypii* after 24, 48 and 72 hrs of the application of five different concentrations of prepared nano-emulsion formulations are shown in Table 5. According to results, maximum (82%) aphid mortality was recorded in positive control at 24 hours. Moreover, all the nano-emulsion formulations showed aphicidal properties even at their lowest concentration i.e., 1000 $mg.L^{-1}$. After 24 hours, the highest mortality percentage of aphids (36%) was observed in TR3CH at 10000 $mg.L^{-1}$ concentration, which was significantly different ($P<0.05$) from TR3AC (29%) and TR3PA (28%). Whereas, the lowest mortality percentage (14%) after 24 hours was observed in 1000 $mg.L^{-1}$ concentration of TR3AC and TR3PA.

After 48 hrs, positive control imidacloprid revealed 100% aphid mortality. Among the nano-emulsion formulations, the highest mortality percentage (76%) was observed in TR3CH at 10000 $mg.L^{-1}$. After 72 hrs of treatment, the mortality percentage in TR3CH reached to 100%, while, mortality percentage in TR3AC and TR3PA at 10000 $mg.L^{-1}$ was 96 and 95%, respectively. No mortality was observed in untreated negative control throughout the experiment.

Table 6 reveals LC_{50} and LT_{50} values of prepared nano emulsion formulations under laboratory condition. According to results, the lowest LC_{50} value of 1391 $mg.L^{-1}$ (1144-1629) was recorded at 72 hrs in TR3CH, whereas, the highest LC_{50} value 72,044 $mg.L^{-1}$ (27991-104903) at 24 hrs was recorded for TR3PA. After 24 hrs, TR3CH and TR3AC nano-emulsion formulations exhibited the lowest LC_{50} values of 58918 $mg.L^{-1}$

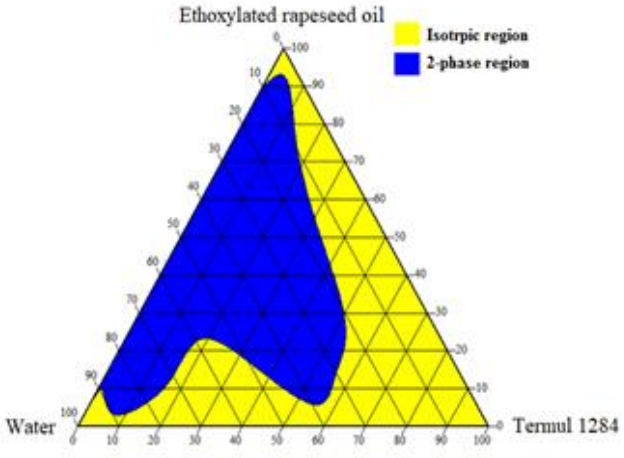

Fig. 1: Ternary phase diagram system of Termul1284/ ethoxylated rapeseed oil/water

Table 1: Ratio of the mixture and type of active ingredient in each formulation

No.	Codes	Compounds	Proportion	Active ingredient
1	TR1CH	Termul1284/rapeseed oil/water	5:15:80	*C. hirta*
2	TR1PA	Termul1284/rapeseed oil/water	5:15:80	*P. anisophylla*
3	TR1AC	Termul1284/rapeseed oil/water	5:15:80	*A. cuspidatum*
4	TR2CH	Termul1284/rapeseed oil/water	5:20:75	*C. hirta*
5	TR2PA	Termul1284/rapeseed oil/water	5:20:75	*P. anisophylla*
6	TR2AC	Termul1284/rapeseed oil/water	5:20:75	*A. cuspidatum*
7	TR3CH	Termul1284/rapeseed oil/water	5:25:70	*C. hirta*
8	TR3PA	Termul1284/rapeseed oil/water	5:25:70	*P. anisophylla*
9	TR3AC	Termul1284/rapeseed oil/water	5:25:70	*A. cuspidatum*

Table 2: Stability and thermal stability of prepared nano emulsion formulations

Formulations	TR1CH	TR1PA	TR1AC	TR2CH	TR2PA	TR2AC	TR3CH	TR3PA	TR3AC
Centrifugation	✓	✓	✓	✓	✓	✓	✓	✓	✓
26 ± 1 °C	×	×	×	×	×	×	✓	✓	✓
54 ± 1 °C	×	×	×	×	×	×	✓	✓	✓

√ Stable; x Unstable

Table 3: Characterization of saponin based nano emulsion formulations

Formulations	Particle size (nm)	Surface tension ($mN.m^{-1}$)	Zeta potential (mV)
TR3CH	79.0	35.41	-22.5
TR3AC	88.9	34.88	-21.6
TR3PA	69.4	35.05	-22.0

Table 4: Repellency percentage (Mean±SE) of saponin based nano-emulsion formulation on *A. gossypii* after 24 hrs.

Formulations	Concentrations ($mg.L^{-1}$)				
	1000	2500	5000	7500	10000
TR3CH	$11.4±2.6^{a}$	$18.8±2.9^{a}$	$34.9±0.3^{a}$	$59.4±2.5^{a}$	$73.2±2.7^{a}$
TR3AC	$4.9±1.9^{b}$	$9.3±1.7^{b}$	$18.1±2.7^{b}$	$47.4±1.9^{b}$	$60.1±2.9^{b}$
TR3PA	$5.3±2.5^{b}$	$10.1±2.1^{b}$	$19.3±2.9^{b}$	$46.3±2.4^{b}$	$59.1±2.9^{b}$

Means with same letters within column are not significantly different (P>0.05).

Table 5: Percentage mortality (Mean±SE) of *Aphis gossypii* treated with selected nano-emulsions at 24, 48 and 72 hours.

Time	Conc. ($mg.L^{-1}$)	Treatments				
		TR3CH	TR3AC	TR3PA	CNT POS	CNT NEG
	1000	$21±1.5^{b}$	$14±2.1^{c}$	$14±2.7^{c}$	$82±2.1^{a}$	$0±0.0^{d}$
	2500	$23±1.6^{b}$	$16±1.7^{c}$	$15±2.3^{c}$	$82±2.1^{a}$	$0±0.0^{d}$
24 h	5000	$26±1.3^{b}$	$17±2.3^{c}$	$19±1.6^{c}$	$82±2.1^{a}$	$0±0.0^{d}$
	7500	$30±2.6^{b}$	$18±1.9^{c}$	$20±1.4^{c}$	$82±2.1^{a}$	$0±0.0^{d}$
	10000	$36±1.8^{b}$	$29±2.2^{c}$	$28±2.6^{c}$	$82±2.1^{a}$	$0±0.0^{d}$
	1000	$32±2.1^{b}$	$26±1.6^{c}$	$25±2.5^{c}$	$100±0.0^{a}$	$0±0.0^{d}$
	2500	$41±1.3^{b}$	$32±2.6^{c}$	$32±2.2^{c}$	$100±0.0^{a}$	$0±0.0^{d}$
48 h	5000	$52±2.6^{b}$	$42±2.1^{c}$	$41±1.6^{c}$	$100±0.0^{a}$	$0±0.0^{d}$
	7500	$62±2.1^{b}$	$46±1.3^{c}$	$53±1.8^{bc}$	$100±0.0^{a}$	$0±0.0^{d}$
	10000	$76±1.9^{b}$	$64±2.1^{c}$	$69±1.3^{bc}$	$100±0.0^{a}$	$0±0.0^{d}$
	1000	$48±1.9^{b}$	$42±2.2^{c}$	$41±1.9^{c}$	$100±0.0^{a}$	$0±0.0^{d}$
	2500	$56±1.3^{b}$	$47±2.3^{c}$	$46±2.1^{c}$	$100±0.0^{a}$	$0±0.0^{d}$
72 h	5000	80v1.3^{b}	$65±1.3^{c}$	$67±1.5^{c}$	$100±0.0^{a}$	$0±0.0^{d}$
	7500	$97±2.3^{a}$	$77±2.1^{b}$	$78±1.9^{b}$	$100±0.0^{a}$	$0±0.0^{c}$
	10000	$100±0.0^{a}$	$96±2.1^{b}$	$95±1.9^{b}$	$100±0.0^{a}$	$0±0.0^{c}$

Means with same letters within rows are not significantly different (P>0.05).

(21835-82040) and 69821 $mg.L^{-1}$ (27499-101287), respectively. After 48 hrs of application, the same trend was observed in all treatments with the lowest LC_{50} value of 3426 $mg.L^{-1}$ (2732-4206) recorded in TR3CH. Similarly, after 72 hrs of exposure, TR3CH also exhibited the lowest LC_{50} value of 1391 $mg.L^{-1}$ (1144-1629), followed by 1644 $mg.L^{-1}$ (1284-1990) and 1766 $mg.L^{-1}$ (1406-2115) in TR3AC and TR3PA respectively. The shortest LT_{50} value of 24.32 hrs (22.40-26.42) was recorded for TR3CH at 10,000 $mg.L^{-1}$, whereas the longest LT_{50} value of 79.93 hrs (71.20-92.79) was observed at 1000 $mg.L^{-1}$ in TR3PA. The lowest concentration tested for all treatments in the experiment was 1000 and overall lethal time at this concentration was lower than 80 hrs for all treatments. On the other hand, the highest concentration used was 10,000 $mg.L^{-1}$ and overall lethal time at this concentration was less than 32 hrs.

Table 6: LC_{50} (mg.L^{-1}) and LT_{50} (hrs) values of tested nano-emulsion formulations against *A. gossypii* in laboratory

Formulations	Time	LC_{50}	Limits	Chi^2	Con.	LT_{50}	Limits	Chi^2
TR3CH	24 hrs	58918	21835-82040	6.98	1000	73.53	65.0-85.44	36.20
	48 hrs	3426	2732-4206	12.48	2500	60.32	54.25-68.15	36.35
	72 hrs	1391	1144-1629	35.77	5000	43.20	39.57-47.45	43.76
	-	-	-	-	7500	32.37	29.74-35.34	76.33
	-	-	-	-	10000	24.32	22.40-26.42	73.65
TR3AC	24 hrs	69821	27499-101287	7.862	1000	79.75	71.03-92.43	32.34
	48 hrs	5565	4505-7163	12.91	2500	67.12	61.21-75.06	18.55
	72 hrs	1644	1284-1990	27.94	5000	55.03	51.37-59.41	17.80
	-	-	-	-	7500	41.21	37.84-45.10	58.37
	-	-	-	-	10000	31.30	28.80-34.06	53.91
TR3PA	24 hrs	72044	27991-104903	13.61	1000	79.93	71.20-92.79	18.49
	48 hrs	6425	5074-8783	11.35	2500	69.93	63.21-79.07	30.14
	72 hrs	1766	1406-2115	32.76	5000	55.41	50.59-61.39	46.41
	-	-	-	-	7500	40.85	37.71-44.40	54.25
	-	-	-	-	10000	30.83	28.50-33.37	62.35
Imidacloprid at recommended dose			-	-	-	12.08	11.23-13.01	77.35

Table 7: LC_{50} (mg.L^{-1}) and LT_{50} (hrs) values of saponin based nano-emulsion formulations against *A. gossypii* under glasshouse conditions

Formulations	Time	LC_{50}	Limits	Chi^2	Con.	LT_{50}	Limits	Chi^2
TR3CH	24 hrs	75995	57522-93150	29.35	1000	123.7	115.1-135.1	32.90
	48 hrs	41421	25548-53800	14.97	2500	104.1	98.3-111.2	31.25
	72 hrs	4967	4301-5801	24.46	5000	84.9	80.9- 89.2	48.92
	96 hrs	2886	2489-3294	41.96	7500	71.2	67.9-74.6	68.20
	120 hrs	1480	1241-1711	52.56	10000	55.5	51.9-58.3	79.79
TR3AC	24 hrs	114350	95987-147296	53.47	1000	170.5	151.6-200.5	40.52
	48 hrs	89833	56129-104378	30.90	2500	152.9	138.5-174.1	42.61
	72 hrs	43428	23836-85646	15.42	5000	136.4	125.1-151.7	31.41
	96 hrs	22503	14970-45235	11.67	7500	113.2	105.8-122.5	32.36
	120 hrs	4924	4253-5764	34.60	10000	92.7	88.1-97.8	46.97
TR3PA	24 hrs	126356	96913-145981	29.51	1000	157.2	142.2-80.1	28.32
	48 hrs	93920	58121-110938	27.41	2500	149.5	136.2-169.1	33.35
	72 hrs	46635	24639-88016	18.01	5000	135.7	124.8-150.6	29.63
	96 hrs	23301	14610-53235	12.65	7500	117.6	109.5-127.9	31.62
	120 hrs	5263	4482-6298	33.65	10000	94.6	89.7-100.2	49.66
	24 hrs	590	532 – 662	24.82	2.5/10L			
	48 hrs	185	172 – 198	33.03				
Imidacloprid	72 hrs	131	125 – 139	10.11		26.3	24.7-27.9	65.22
	96 hrs	104	97 – 112	36.06				
	120 hrs	73	68 – 78	30.84				

Glasshouse assessment

Table 7 shows the LC_{50} and LT_{50} values of saponin based nano-emulsion formulations against *A. gossypii* under glasshouse conditions. The results revealed that LC_{50} values after 24 hrs of application for all nano-emulsion formulations were higher than 75,000 mg.L^{-1} and after 120 hrs of application, it was below 53,00 mg.L^{-1}. Positive control imidacloprid revealed shortest LC_{50} value of 73 mg.L^{-1} (68-78) at 120 hrs and highest LC_{50} value of 590 mg.L^{-1} (532-662) at 24 hrs. Among the tested nano-emulsion formulations, the lowest LC_{50} value of 1480 mg.L^{-1} (1241-1711) was recorded at 120 hrs in TR3CH, whereas the highest LC_{50} value 126,356 mg.L^{-1} (96913-145981) at 24 hrs recorded for TR3PA. TR3CH exhibited the significantly lowest LC_{50} values throughout the time period. The results also revealed that the shortest LT_{50} value of 26.3 hrs (24.7-27.9) was recorded for imidacloprid at recommended rate which was lower than all tested saponin based nano-emulsions. Among the saponin formulations, the shortest LT_{50} value of 55.5 hrs (51.9-58.3) was recorded for TR3CH at 10,000 mg.L^{-1}, whereas, the longest LT_{50} value 170.5 hrs (151.6-200.5) was observed at 1000 mg.L^{-1} in TR3AC. The lowest concentration tested for all treatments in the experiment was 1000 mg.L^{-1} and overall lethal time at this concentration was lower than 171 hrs for all treatments. On the other hand, the highest concentration i.e., 10,000 mg.L^{-1} showed overall LT_{50} values less than 95 hrs. TR3CH exhibited the significantly shortest LT_{50} values at all tested concentrations.

DISCUSSION

Surfactants used to construct ternary phase diagram was non-ionic. Non-ionic surfactants are known to be the most effective in nano-emulsion formulation because of their low toxicity and ability to stabilize the emulsion against flocculation and coalescence (Salager, 2002; Tadros, 2013, Mokhtar, 2016). According to results, two regions were formed during the process of formulation known as isotropic region and two-phase region. Among these regions, isotropic region is considered as a region of a nano-emulsion where a transparent and clear emulsion is formed without any sedimentation or layer (Mokhtar, 2016). According to Tadros (2013), several breakdown processes lead to instability in an emulsion and includes sedimentation and creaming (Reddy et al., 1980), Ostwald ripening (Taylor, 1998), and coalescence and flocculation (Hubbard, 2004). The percentage of surfactant and oil also play an important role in the performance of any emulsion as it was observed that the emulsions which showed thermal stability over a long period of a time consisted high percentage of surfactant. It is mentioned that one of the major purposes of a surfactant in an emulsion is to enhance the stability of a formulation by reducing the interfacial tension (Burlatsky et al., 2013). According to the Murgich (1996) and Bibette et al. (2002), the major factors that might affect the stability of an emulsion are low viscosity of bulk phase, large droplet size and broad droplet distribution, large volume of dispersed phase and interfacial tension. Thus, only formulations that shown one transparent phase in all tests were preceded for further tests.

It has been suggested that if the value of zeta potential is higher than ±25 mV, then it is enough for an emulsion to produce energy boundaries between particles to avoid unnecessary coalescence (Shanmugam and Ashokkumar, 2014), while, a value more than ±30mV indicates good stability for a formulation (Greenwood and Kendall, 1999; Hanaor et al., 2012). However, the zeta potential value of ±20mV is also desirable in case of a combined electrostatic and steric stabilization (Gupta and Trivedi, 2018). Therefore, the zeta potential values for nano-emulsions ranged between -21.6 mV to -23 mV in this study are in accordance with the findings of Mokhtar (2016), who reported zeta potential value of saponin based nano-emulsion formulations from -10 to -24mV, whereas, Ali (2018) recorded zeta potential value of oil based nano-emulsion formulations from -7 to -39. Wooster et al. (2008) reported that the particle size of an emulsion decreased when surfactant ratio is increased as the emulsion with a smaller particle size is found to be more stable (Tadros, 2013). The droplet size distribution also affects the viscosity of an emulsion because smaller particles dissolve very quickly due to curvature effects and lead to higher suspension viscosities than larger ones. Therefore, the emulsion which possesses particle size from 50 nm to 500 nm can be classified as nano-emulsion (Tadros, 2013; Mokhtar, 2016). The surface tension of all the formulations tested in the study was less than the surface tension of water ($72mN.m^{1}$) as Tadros et al. (2004) mention that lower surface tension in nano-emulsion formulations can increase their penetration, spreading and wetting properties. The lower surface tension of pesticides allows droplet particles to spread and penetrate evenly on surface of leaf with small contact angles in application process (Mokhtar, 2016).

The results obtained from repellency bioassay showed relatively better repellency of saponins treatments against *A. gossypii* than Dolma et al. (2017) who reported 48.6% repellency percentage in *Plutella xylostella* when exposed to tea saponin. The findings are also close to De-Geyter et al. (2011) who reported feeding deterrent activity of 0.97 against pea aphid when exposed to saponin. Furthermore, some previous studies had reported that pea aphids gave preference to the plants with low concentration of saponins for their growth and development than those plants that were rich in saponins (Golawska and Łukasik, 2009; Golawska et al., 2010), because, saponins mainly act as repellent or feeding deterrent and directly affect the growth and reproduction of aphids (Singh and Kaur, 2018). Adel et al. (2000) also reported that saponin lower the food intake in the targeted insects by disturbing the food movement in their gut because of less digestibility and toxicity; hence, increase the mortality among insects.

The findings from mortality bioassay are supported by previous study conducted by Lebbal et al. (2018) who reported 47% mortality in bean aphid *Aphis fabae* after 24 hrs when treated with 5% saponin from *Thymus algeriensis*. Similarly, Soule et al. (2000) observed 53% mortality after 24 hrs in wheat aphid *Schizaphis graminum* when treated with saponin from *Solanum laxum.* Another study observed 100% mortality of *E. fabea* within 48 hrs of application of saponins from alfalfa (Horber et al., 1974). Moreover, De-Geyter et al. (2011) reported that saponin from *Q. saponaria* against pea aphid *Acyrthosiphon pisum* showed aphicidal effects within 24 hrs and 100% mortality after 72 hrs. The results of the study are also in accordance with Su and Ye (2012) who reported a botanical pesticide formulation containing 10 to 30% tea saponin was effective for the management of *P. xylostella*. The results observed from this study are in line with Rattan et al. (2015) who reported LC_{50} value 0.5 $mg.mL^{-1}$ of saponin from *Clematis graveolens* against *Aphis craccivora*. Similarly, saponin from *Q. saponaria* against pea aphids exhibited LC_{50} value of 0.55 $mg.mL^{-1}$ in no choice bioassay (De-Geyter et al., 2011). Tea

saponin against *A. craccivora* had shown LC_{50} values of 584.6 $mg.L^{-1}$ and 369.6 $mg.L^{-1}$ at 72 and 96 hrs of treatment, respectively (Dolma et al., 2017). The LT_{50} values of tea saponin against *A. craccivora* was obtained as 14.78 hrs and 11.94 hrs at 2000 $mg.L^{-1}$ and 4000 $mg.L^{-1}$, respectively (Dolma et al., 2017). The results obtained from the glasshouse experiment were comparatively slower than the laboratory experiment and the same trend was also observed in positive control imidacloprid. However, Imidacloprid exhibited rapid mortality and lower lethal concentration compared to tested nano-emulsions because synthetic pesticides are known for their immediate effects to kill aphids. But unfortunately, like many other synthetic pesticides, the negative effects of imidacloprid on non-target organism have been already reported and many aphid species have developed the resistance against it (Guo et al., 2020). On other hand, plant derived pesticides are much safer and environment friendly (Nawaz et al., 2016). However, many previous studies have reported that the action of these pesticides is comparatively slower than the synthetic pesticides and required higher concentrations to show rapid effects (El-Wakeil, 2013; Khan and Pathak, 2020). Therefore, the time taken by TR3CH is considerably good and its normal for any botanical pesticide to have longer period to exhibit its effectiveness. It has been observed that all treatment tested in this study could be toxic to *A. gossypii* as their toxicity increased with dose and time in different ways. The toxicity of these nano-emulsions is believed to be the effective due to the action of active ingredient (saponin). There are three phases that can be described the interaction between active ingredient and target organism i.e., exposure phase, toxicokinetic phase and toxicodynamic phase (Mekapati et al., 2005, Stine and Brown, 2006, Mokhtar, 2016). The exposure phase is the phase where the body of targeted organisms come into touch of poison (active ingredient), whereas, in toxicokinetic phase, the compound moves through the body, absorbed into circulatory system and then distributed among tissues. In the meantime, the effects of the poison can be shown in toxicodynamic phase, where the activities of poison started to affect the target molecules and tissues (Mokhtar, 2016). All these phases could be referred to explain the mechanism involved in the aphicidal activities of the saponins observed in this study. Moreover, the mortality of aphids may be due to the formation of complexes with cholesterol which cause ecdysial failure and cellular toxicity in insects (Taylor et al., 2004). It has also been suggested that saponin cause mortality among aphids because of their capability of membrane permeability, hence, they break the internal lining of mucosal cells in the insect gut (Singh and Kaur, 2018). Moreover, saponins can also increase the mortality ratio of the targeted insects by lowering their food intake due to less digestibility and toxicity along with effect on food movement in the insect gut (Adel et al., 2000; Singh and Kaur, 2018). In conclusion,

TR3CH nano-emulsion formulation (Termul 1284/ rapeseed oil/ *Clidemia hirta* extract) performed better than all other tested nano emulsion formulations in laboratory and glasshouse conditions, therefore, it could be considered as an eco-friendly alternative approach in pesticides technology.

Authors' Contribution

RMA conceived the original idea and supervised the project. ARR carried out the experiments, analyzed the results and wrote the manuscript with support of RMA, ASM and NA. ASM and NA co-supervised the project. All authors provided critical feedback and helped to shape the research, analysis, and manuscript. All authors read and approved the final manuscript before publication.

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