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Physiochemical Characterization of Nanoemulsion Formulation of Phenazine and their Antifungal Efficacy against *Ganoderma Boninense* PER71 *in vitro.*

Kai Wei Lee[1], Dzolkhifli bin Omar[2], Khalina bt Abdan[3], and Mui Yun Wong[1, 2]*.

[1]Institute of Tropical Agriculture, Universiti Putra Malaysia, Serdang, 43400, Malaysia.
[2]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, 43400, Malaysia.
[3]Department of Biological and Agricultural Engineering, Universiti Putra Malaysia, Serdang, 43400, Malaysia.

ABSTRACT

Nanoemulsions have attracted much attention due to their applications especially in plant protection field. The aim of this study is to develop and characterize oil-in-water (O/W) phenazine extract nanoemulsions for controlling *Ganoderma boninense* PER71. To this purpose, a phase diagram (PD) was constructed based on non-ionic surfactant Tween80, oil carrier E2126 and water, and 30 % crude phenazine extract as active ingredient by low-energy method. After constructing PD, six formulations were examined for its thermostability and stability over time as primary screening. Four formulations were selected to proceed in physiochemical characterizations. The characterized formulations have a mean droplet size ranging from 130.54 to 309.9 nm with polydispersity index varied between 0.32-0.97. The larger drop size (309.9 nm) shifted to a smaller size of 130.54 nm with decrease in the concentration of oil carrier Emereen 2126. The zeta potential of all formulation is yet stable with the value ranged from -11.8 to -16 mV. The surface tension was around 30.82 to 30.88 mN/m. The fungicidal effect of the formulated phenazine nanoemulsion was checked against *G. boninense* PER71. Nanoemulsion with 174.43 nm size was obtained at a ratio 5:5:90, and it was found to be stable in terms of polydispersity index (0.6), zeta potential (-16.0 mV) and surface tension (30.88 mN/m), and effective in controlling *G. boninense* PER71 at 70.74 %. This is the first time that a phenazine extract nanoemulsion has been reported. The results obtained might corroborate to the application of phenazine extract nanoemulsion as potential candidate for controlling *G. boninense* PER71.

Keywords: Droplet size; polydispersity index; zeta potential; surface tension;

Corresponding author

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INTRODUCTION

Nanotechnology has emerged as a promising area for development of products in a wide range of applications. The usages could be seen in research publications, which can be classified as carbon nanomaterials, non-carbon nanomaterials and nanoporous. Nanoparticle is in group of carbon nanomaterials together with nanopowder, nanocomposite and nanostructure, these are applied in numerous domestic nanotechnology-enabled products of which the following is worth mentioning such as in food, personal care, cosmetics, pharmaceutical, cleaning formulations and most importantly in agrochemical (Tanthapanichakoon *et al.,* 2014). Formulation of crop protection products in agrochemical industries are developed, general types of formulation are emulsified concentrates (EC), wettable powder (WP), solution (SL), granules (GR), water dispersible granules (WG), microcapsule (CS), emulsion (EW) and microemulsion (ME) (Lim *et al.,* 2012).

Nanoemulsion are submicron emulsion with a nanometric-scaled droplet, enhance solubility and dissolution properties of poorly water soluble substrates, they are referred as miniemulsion of ultrafine emulsion and have small droplet size ranged from 20 to 200 nm (Fernandes *et al.,* 2014; Forgiarini *et al.,* 2001) or more broader range of 20-300 nm (Anton and Vandamme, 2009). They are thermodynamically stable and translucent dispersion. It can be oil-in-water (O/W), water-in-oil (W/O), and oil-in-oil (O/O), having a lower surfactant concentration than microemulsions (Shafiq *et al.,* 2007; Anjali *et al.,* 2012). Nanoemulsion was identified as promising delivery systems, O/W emulsions is water-dilutable to replace volatile organic compounds, good alternatives for toxic and non-renewable chemicals such as petroleum oil adjuvant, development of nano-products appear to solve this main problem, enhancing water solubility and bioavailability, and nano-size is crucial for biofungicide penetration, thus considerably more environmentally benign and economically viable (Margulis-Goshen and Magdassi, 2013).

Most emulsion-based formulation products are formulated in concentrates to reduce disposal of container, space storage and transportation cost (Lim *et al.,* 2012). The formulation concentrates consist of oil carrier and active ingredient, and may contain high surfactant content (10-30%) to stabilize formulation in nanoemulsion system (Narang *et al.,* 2007). Surfactant such as Tween80 and Tween20, is commonly used in emulsification and stability of the emulsion (Tadros, 2013).

Oil palm (*Elaeis guineensis*) is the most profitable oil-bearing crop. One of the important factors contributing to significant economic losses of oil palm is Basal Stem Rot (BSR) caused by white rot fungus *Ganoderma boninense*, which can destroy up to 80 % of palms over repeated planting cycles (Paterson *et al.,* 2009). Phenazine extract nanoemulsion could be a promising technology pragmatically in suppressing *G. boninense*, the causal agent of basal stem rot disease in oil palm. Phenazine compounds are commonly known as secondary metabolites and it has excellent fungicidal properties. The formulation of this system may have many advantages compared to conventional methods to suppress *G. boninense* PER71 in vitro.

In past decades, nanoemulsions have gained immense interest in water-soluble pesticide of various kinds of active ingredient (Wang *et al.,* 2007), research has highlighted tremendous improvement on physical characteristics application and biological performance. Yet, biofungicide nanoemulsion formulation with phenazine crude extracts, characteristics and its functionality in suppression on fungal pathogen have not been reported.

In this work, nanoemulsion of biofungicide formulation containing phenazine crude extracts as active ingredient was developed. Currently, there is a need to determine the optimum conditions in which these formulations are formed. Therefore, objectives of the present study were to formulate and characterize nanoemulsions contain phenazine crude extracts and to evaluate its efficacy in suppressing *G. boninense* PER71 *in vitro*.

MATERIALS AND METHODS

Study site

The *in vitro* experiment was conducted in Laboratory of Pest Management, Institute of Tropical Agriculture (ITA), Universiti Putra Malaysia (UPM). The present study was carried out under laboratory condition (Temperature 26 ± 2 ^{o}C, RH 65 ± 5%). The glasshouse in vivo experiment was conducted in

Glasshouse 2D in Field 2, UPM, it was carried out under ambient condition (Temperature 28 ± 2 ^{O}C, RH 65 ± 5 %).

Ganoderma isolates and Host Plants

The *G. boninense* PER71 was obtained from Ganoderma and Diseases Research for Oil Palm (GANODROP) Unit, Malaysian Palm Oil Board (MPOB) and oil palm seedlings were supplied by Sime Darby, Banting.

MATERIALS AND CHEMICALS

Tween80 (Sigma Aldrich, USA) was used as surfactant and E2126 was gifted from Emery oleochemicals (Malaysia) as oil carrier in nanoemulsion formulation. The crude phenazine was used as active ingredient and it was extracted from broth culture of three days *P. aeruginosa* UPM P3 by using benzene, lyophilized by freeze drier.

Construction of Phase Diagram (PD)

PD was constructed according to the methods described by Ahmad *et al.* (2012). Initially, 10 % of crude phenazine were dissolved in surfactant Tween80 with continuous vortex and mixed with oil carrier E2126, samples were shaken till it attain equilibrium, subsequently titrated with water. The aqueous phase volume ranged, yielding 99 compositions in which surfactant/ oil carrier ratio (SOR) ranged from 10:90; 20:80; 30:70; 40:60; 50:50; 60:40; 70:30; 80:20 and 90:10. After each titration, samples were homogenized by vortex and centrifuged at 3500 rpm for 15 minutes at room temperature. The compositions phase separation after mixture were inspected visually through uniformity and transparency, then it was used to construct PD that indicate isotropic and anisotropic area of compositions in three axis ternary phase diagram.

Preliminary Selection of pre-formulations

From the constructed PD, compositions were selected from isotropic region of PD and processed by adding 30 % (w/v) of active ingredient (A.I.) - crude phenazine. In preliminary screening, A.I. loading may incur phase separation to the compositions, thus those compositions of remain single phase after A.I. loading were selected as pre-formulations and subsequently subjected to thermostability and stability for secondary screening.

Thermostability and stability test

Nanoemulsion thermostability were evaluated by centrifugation at 3500 rpm (Eppendolf, Hamburg, Germany) for 15 min and kept at incubator at 54 ^{O}C for 2 weeks and at 28 ^{O}C for 2 months. For stability test, centrifuge at 3500 rpm for 30 min and kept at room temperature for four weeks, as prescribed by the Food and Agriculture Organization (FAO), as a standard evaluation for agrochemical products to show the stability in tropical climate (Chen *et al.*, 2000). The pre-formulations were evaluated based on phase separation and transparency. In secondary screening, pre-formulations which show single phase domain were selected for formulation characterization.

Formulations characterization

Dynamic light scattering (DLS) was monitored at a 90 ^{O} and at temperature of 25 ^{O}C to examine mean droplet diameter and polydispersity index (PdI) of samples by using Zetasizer Nano ZS (Malvern instruments, Malvern, UK). All values reported correspond to the mean ± standard deviation (SD) of three measurements of each formulation. Polydispersion index (PdI), calculated by the device, reflects the homogeneity profile of droplets diameter.

Zeta potential of formulations were determined by electrophoretic mode using Zetasizer Nano ZS (Malvern instruments, Malvern, UK), with a laser reader of 633 nm and the temperature of 25 ^{O}C. Formulations 5:5:90; 10:5:85; 10:10:80 and 10:40:50 were added to the folded capillary cell (vial) and each sample was measured in triplicates for each parameter. The electrical charge on oil droplets in the emulsion

was determined under holder temperature of 25 ^{O}C and electrical voltage 3.9 V. Surface tension of the formulations were measured with Nima Dynamic surface tensiometer equipment (Model DST 9005, Nima Technology Ltd. England) based on immersing technique by Du Nuoy ring, it was performed at 25 ^{O}C.

Antifungal efficacy against *G. boninense* PER71 *in vitro*

Antagonistic assay *in vitro* was carried out to study the direct effect of phenazine nanoemulsion formulation on *G. boninense* PER71. The formulations 5:5:90, 10:5:85, 10:10:80 and 10:40:50 were prepared into different concentration (100, 200, 400, 800 and 1000 ppm) where hexaconazole 1000 ppm was used as positive control and distilled water as negative control. Poison agar plates were prepared by spreading 50 µl of each concentration of formulations onto solidified Potato dexterous agar, PDA (Difco Laboratories, Detroit, MI) plate separately. Agar disc (5 mm) containing seven days old fungal culture grown on PDA was placed at the center of poison agar plates. Diameter growth of the fungus was recorded to calculate the percent inhibition in each treatment. This assay was performed in three replicates. The percent inhibition of diameter was measured using the following formula (Meon, 1998).

$$Percentage\ inhibition = \frac{(C - T)}{C} x\ 100$$

Where,
C = colony diameter (cm) of the control
T = colony diameter (cm) of the test plate

Statistical Analysis

Data were expressed as mean ± SD if triplicate samples by one-way of variance (ANOVA) and significant differences between treatments were detected by a Tukey's honestly significant difference (HSD) test. Analyses used the Statistical software JMP (9.0) (SAS institute, Cary, NC). Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Phase Diagram (PD) Analysis

Nanoemulsions were prepared by spontaneous homogenization which is low-energy centrifugation. Ternary PD constructed with mixed surfactant (Tween80), oil carrier (E2126) and water is shown in Fig.1. The pre-formulations (w/v) in the phase diagrams system which was the manipulated variable and 10 % phenazine crude extracts was the constant variable. The formation of isotropic phase, the monophasic optically transparent liquid and multiphase region, biphasic and triphasic compositions were discerned.

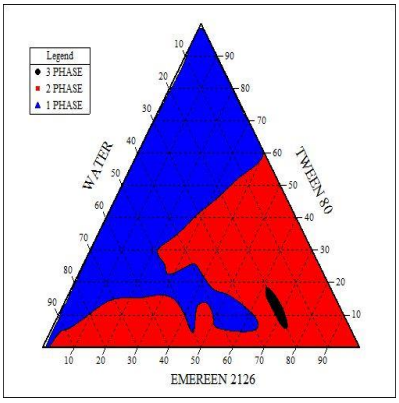

Figure 1 Ternary phase diagram of Tween 80: Emereen 2126 and water, showing the O/W nanoemulsion areas

In this study, Tween80 or Polysorbate80 was selected as nonionic surfactant. It is commonly used as O/W emulsifier to improve the affinity of hydrophobic active ingredient to water by reducing surface tension between aqueous and oil phase thus promoting the formation of conventional liquid emulsions, it also lowering the interfacial tension hence the energy needed to break up droplets into smaller size is reduced. Tween80 also prevent coalescence of newly formed drops (Tadros, 2013).

Emulsions are a class of disperse systems consisting of two immiscible liquids while emulsifier or surfactant is needed to mingle two immiscible liquids. But manipulated variable, the oil carrier and surfactant total content in pre-formulation has high flexibility to partition into oil and water interface. Figure 1 indicates thermodynamically distinct phases can occur at equilibrium, where lines of equilibrium of phase boundaries mark multiple phases can coexist at equilibrium and it thus determine the balance among mixtures of surfactants, oil carrier and water in emulsified systems (Warisnoicharoen *et al.,* 2000). For different materials composition (surfactant, oil and phase concentration) in pre-formulation, it has its own distinctive PD and the selection of optimum conditions is crucial for long-term stability (Komaiko and McClements, 2014).

Tween80 was used in nanoemulsion formulation, although it is a synthetic compound but due to it is not carcinogenic, safe and well tolerated by far. The surfactant Tween80 have been used in low energy methods, the centrifugation able to produce nanoemulsions with smaller mean droplet size, when compared to other surfactant. This could be explained by the nature of tween80 that it lowers surface tension, which is associated to generation of nanoemulsion (Fernandes *et al*., 2014). Besides, Tween80 is used since it is safe to user. In Europe and America, people eat about 100 mg of polysorbate80 in foods per day on average. It is generally used in cosmetic, foods and health products. For instance, Polysorbate80 is used as surfactant in soaps and mouthwash; polysorbate80 in 0.5 % (v/v) concentrations is added to make ice cream smoother and increase resistant to melting (Goff, 1997); In medicine, it is an excipient to stabilize aqueous formulation and influenza vaccines. Moreover, in plant nutrition aspect, it had been proven that it helps in absorbing the pesticide micronutrient for the plants.

E2126 was used as oil carrier to formulate O/W nanoemulsion in this study. It is derived from oil palm kernel, and chemically inert to help formulate agrochemical formulation such as pesticide, seed treatments and micro nutrient formulations. The components of formulation are desirably biological-based and eco-friendly. E2126 is an alternative and natural-based oil carrier to facilitate stewardship. Amongst many types of oil carrier either chemical or organic based. Palm oil-based oil carrier such as E1810, E1820, E1402 and E1407 had been tested in preliminary solubility studies, regarding choice of oil phase and surfactant mix-ability, the phase diagram constructed of each material gave limited single phase domain region, only E2126 gives large single phase domain for points of selection.

The A.I. in formulation was phenazine crude extracts. It was proven 5000 ppm of crude extracts controlled *Ganoderma boninense* PER71 at 89 %, which means 0.5 % of crude phenazine dissolved in 80 % methanol. In formulation, 30 % (w/v) of formulation is A.I. which makes the formulation work in controlling the disease. Maximum 50 % of A.I. is acceptable, but A.I. should be kept as low percentage as possible. Toxicology studied about the adverse effects and chemical substances in excess and beneficial in lower doses (Mulqueen, 2003).

In general, single phase domain is shown in regions of surfactant and oil carrier percentage ranging from 60 % and above, however the formulation texture was much viscous with more surfactant. Thus, the desirable formulations which shows single phase domain, low viscous and watery should have less than 30 % surfactant (Anton and Vandamme, 2012). With this limitation, at surfactant 30 %, the oil carrier percentage was limited to below 20 %; surfactant at 20 %, oil carrier was below 40 %; and surfactant at 10 %, oil carrier was at 10, 40, and ranging 50 to 60 %.

In preliminary pre-formulation screening, twelve pre-formulations from single domain of phase diagram were loaded with A.I. and its phase separation and texture were recorded as shown in Table1. The A.I. was 10 % (w/v) phenazine crude extracts. Preliminary screening was performed to study the solubility of 10 % A.I. in pre-formulations and incorporating A.I. into formulations have found some technological limitations relates to hydrophobic that incur phase inversion and viscosity change. Pre-formulations attained high viscosity and turned into gel whenever Tween80 is more than 20 % and E2126 more than 30 %, thus pre-

formulations no.7 to 12 were omitted in thermostability tests. The successful formulations were subsequently characterized for its enhanced properties.

Table 1: Pre-formulations loaded with active ingredient

Formulation no.	Pre-formulation compositions	Phase status after A.I. loading	
	Tween80: E2126: Water	Separation	Phase Texture
1	5:5:90	No	Watery
2	10:5:85	No	Watery
3	10:10:80	No	Watery
4	10:40:50	No	Watery
5	20:15:65	No	Watery
6	20:20:60	No	Watery
7	20:30:50	No	Gel
8	20:40:40	No	Gel
9	25:20:55	No	Gel
10	25:25:50	No	Gel
11	30:15:55	No	Gel
12	30:20:50	No	Gel

Thermostability and Stability Study

Pre-formulation screenings is a crucial stage to formulate stable nanoemulsion especially by low energy method. Thermostability and stability evaluation are required as fundamental analysis prior to characterizations. Thermostability of formulations was examined under two conditions and different length of periods. Formulations 1 to 6 were undergone incubation at 54 ^{O}C for two weeks. As shown in Table 2, Formulation 1 and 2 were cloudy and fluidly without phase separation, while the rest of formulations showed phase separation.

For thermostability sitting at 28 ^{O}C for 2 months, Formulations 1, 2 and 3 showed cloudy and no phase separation while the rests of formulations were phase separated. For stability test, all formulations were centrifuged at 3500 rpm for 30 minutes; this was used to examine the stability of formulation over speed. All formulations were placed at ambient temperature up for one month, formulations no.1 and 2 showed cloudy and single phase, formulation no. 3 and 4 showed more turbid as milky and remain single phase while the remaining formulations demonstrated phase separation. It is observed that not every formulation produces desirable traits over the whole range of possible compositions. Thus a total of 12 pre-formulation emulsions were prepared using different percentage of Tween80, E2126 and water, only six were subjected to thermostability and stability tests.

Table 2: Thermostability and stability tests on the selected pre-formulations

Formulation No.	Thermostability			Stability
	54 ^{O}C	28 ^{O}C		
1	✓	✓		✓
2	✓	✓		✓
3	✗	✓		✓
4	✗	✗		✓
5	✗	✗		✗
6	✗	✗		✗

✓=stable

✗=not stable

In thermostability and stability tests, no important visual modifications could be observed between storage conditions of formulation 1 and 2 for two months. Formulation No. 5 and 6 showed phase separation

with creaming form, thus these two formulations were omitted in subsequent characterizations. Out of six formulations, only four formulations No. 1, 2, 3 and 4 (5:5:90, 10:5:85, 10:10:80 and 10:40:50) were selected to further in nanoemulsion characterizations. Without phase separation of emulsion over thermostability and stability test is necessary. Nevertheless, most formulations exhibit signs of flocculation 90 days after preparation. The unstable formulations were those with higher surfactant concentration. Similar results were found in a study on the development of β-carotene nanoemulsions, whose stability decreased with increasing concentration of emulsifier (Yuan *et al.,* 2008).

Phase separation in emulsion system can be explained by external force such as gravitational. The thermostability and stability test used centrifugal forces to simulate gravitational forces, to examine the formulations' stability over speed, temperature and storage period. When such forces exceed the thermal motion of the droplets, a concentration gradient builds up in the system with the larger droplets (if its density lower than that of medium) moves faster to the top or to the bottom (if its density higher than that of medium) and this leads to sedimentation of emulsion (Tadros, 2013).

Nanoemulsion Characterization

Dynamic light scattering analysis is used to measure droplet size of formulations no. 1 to 4 as shown in Table 3. The formulations with emulsion size 200 nm and below are considered nano-size and above 200 nm is micron-size. The particle sizes were notably different among the formulation no.1 and 2 were nanoemulsion size (d < 200 nm) and no. 3 and 4 were micronemulsion size (d < 500 nm). Formulation no. 3 and 4 contain more oil carrier (more than 10 %, w/v) in compositions had micron-emulsion size, this imply increase in oil content, which may lead to an increase in the viscosity of the disperse phase and consequently increase in flow resistance and restricted droplet break up rate (Tang *et al.,* 2012).

Table 3: Nanoemulsion physiochemical characterizations

Formulation No.	Droplet diameter (nm)	PDI	Zeta potential (mV)	Surface tension (mN/m)
1	174.43±7.25	0.60±0.11	-16.0±0.75	30.88±0.16
2	130.54±6.54	0.97±0.03	-14.1±0.81	30.86±0.02
3	205.2±1.08	0.32±0.01	-11.8±0.38	30.87±0.07
4	309.9±9.89	0.51±0.04	-15.6±0.79	30.82±0.10

Large amount of high-energy is required to deform droplets into smaller size, by high-pressure homogenization, the physical method such as vigorous agitation and higher stress use high-energy emulsification (Tadros, 2013). Based on the generation of mechanical energy through high shear stress, this sophisticated method is more suitable for commercial scale. The current method used is low-energy emulsification or spontaneous emulsification. This is performed on a laboratory scale, attained small droplet size by simple instruments. Owing to the real advantages of this method in terms of formulation yields, potential industrial scale-up and non-aggressive (e.g. fragile active molecules) and the use of organic solvents and the difficulty to implement it on an industrial scale (Anton and Vandamme, 2009; Bouchemal *et al.,* 2004; Kelmann *et al.,* 2007; Dias *et al.,* 2014).

Polydispersity index (PDI) is an indicator of size distribution of particle in emulsion, PDI value close to zero denotes the mono-dispersion system and PDI value close to 1.0 suggesting that the emulsion has a very broad size distribution. Therefore, PDI values lower than 0.2 indicate homogenous populations, while a 0.3 value represents heterogeneity. The desirable characteristics of formulation are the high stability of two immiscible components to stay mixed as one phase emulsion. The acceptable PDI value should be less than 0.7 depending on the sample type (Cheong *et al.,* 2008; Hoeller *et al.,* 2009; Flores *et al.,* 2011). All formulations presented PDI lower than 0.7 except Formulation 2 (0.97), as shown in Table 3.

Zeta potential is a special parameter that should be analyzed to examine the stability of formulations, it is associated to surface potential of the droplets (Bruxel *et al.,* 2012). Ideally, stability is observed when zeta potential value is above ± 25 mV (Barradas *et al.,* 2014). The high stability of formulations with great zeta potential values is associated to repulsive forces that exceed attracting Van der Waals forces, resulting in

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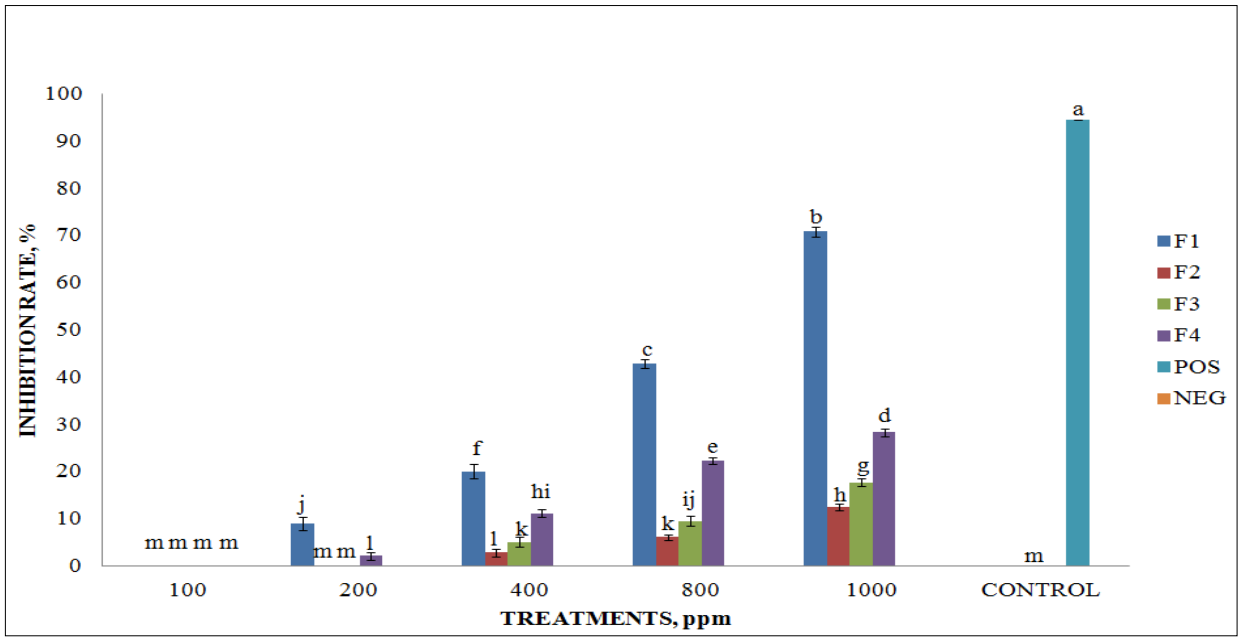
dispersed particles and a deflocculated system (Mahdi *et al.,* 2011.). In Table 3, zeta potential of formulation 5:5:90, 10:5:85, 10:10:80 and 10:40:50 were -16, -14.1, -11.8 and -15.6, respectively. The lower zeta potential values are associated with the non-ionic surfactant used in this study and the presence of acids which can partition at the interface of nanoemulsions (Babchin and Schramm, 2012; Barradas *et al.,* 2014). The distribution of the charged components at the O/W interface may have been influenced not only by composition but also the preparation technique (*Dias et al*., 2014). Zeta potential value of formulation can be adjusted by changing the pH value of sample. To make zeta potential more negative, more alkaline should be added, and to be more positive, acid should be added (Wagner *et al.,* 2003). However, in nanoemulsion formulation, overall the phase separation is the main concern, any changes to the pH value, it may also disturb the other characteristics.

Surface tension is the elastic tendancy of liquid which is responsible for the shape of liquid droplets. Table 3 reported that the four nanoemulsions formulations exhibited low surface tension ranged from 30.82 to 30.88 mN/m, it is more resemblance to surface tension of Ethanol (40 %) which is 29.63 or toluene at 27.73 mN/m. Lower surface tension could increase the amount of excess droplets spread off and could form a thin liquid film thus less deposition for absorption (Ramsey *et al.,* 2005). All formulations showed the lower surface tension compare to water (≈70.2 mN/m) at room temperature. Among the formulations tested, there is no significant difference. Formulation 1 showed highest surface tension and lowest was Formulation 4. Surfactant Tween80 play an important role in increasing the apparent solubility of oil carrier E2126 and effectively reducing the interfacial tension of oil and water in the formulation (Ahmad *et al.,* 2012).

Antifungal efficacy *in vitro*

Percent inhibition of diameter growth of different formulations and its treatments were presented in Figure 2. Hexaconazole at 1000 ppm used as positive control in this study was used according to the recommended dosage for controlling Ganoderma BSR in oil palm. 80 % Methanol used as the negative control. The inhibitory effect on the growth of *G. boninense* PER71 was investigated under four phenazine nanoemulsions and two control treatments by poisonous agar method on PDA plate. A concentration of 1000 ppm of Formulation 1 (5:5:90) yielded the good inhibition against *G. boninense* PER71 at 70.74 % although it failed to completely stop the growth of *G. boninense* (Fig. 2). However, apart from the lowest inhibition against *G. boninense* PER71 recorded by 200 ppm of Formulation 4 (10:40:50), all formulations' inhibition efficacy tested were significantly different ($p < 0.0001$). Concentrations 100 ppm for all formulations showed no effect on the growth of *G. boninense* PER71. At 200 ppm only F1 and F4 had inhibition rate lower than 10 %.

Figure 2: Percent inhibition of diameter growth of different concentration of Phenazine nanoemulsion on Ganoderma boninense PER71



Antibiotics activity of nanoparticles is normally to be size-dependant and smaller sized formulation have higher antifungal activity than larger sized nanoparticles under normal room temperature (Azam *et al.,* 2012) however in this paper Formulation 2 with smallest particle size among the treatments has the lowest inhibition efficacy. Formulation 3 (10:10:80) and 4 (10:40:50) with micron size particle have better inhibition rate than Formulation 2. Thus, particle size of phenazine nanoemulsion couldn't explain inhibition efficacy.

Formulation 1 phenazine nanoemulsion may play a role in limiting the growth of *G. boninense* PER71, but the 1000 ppm concentration was not sufficient to fully inhibit the pathogen. It could, however, slow disease development and if the interaction could be manipulated to increase the concentration of active ingredient, or multiply the application rate to prove its fungitoxic. Numerous papers have been published on the effect of phenazine in others pathogen and plants but not, to date, in *G. boninense* and oil palm nor in nanoemulsion formulation.

CONCLUSION

The nanoemulsion formulation containing E2126, Tween80 and deionized water with active agredient phenazine extracts was successfully optimized by the low-energy method. A nano-sized droplet of 174.43 nm was obtained. Phenazine nanoemulsion with this droplet size was found to be more effective in controlling *G. boninense* PER71 compared with any larger or smaller droplet size. Phenazine nanoemulsion is the green innovation alternative to chemical fungicide in agricultural field, and provide a lower cost, non-toxic and effective agent for development as a biofungicide for BSR disease. To our knowledge, this is the first report of the antifungal activity of crude phenazine compounds in nanoemulsion formulation against *G. boninense* PER71.

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REFERENCES

[1] Ahmad, S. N., Islam, M. T., Abdullah, D. K., & Omar, D. (2012). Evaluation of physicochemical characteristics of microemulsion formulation of rotenone and its insecticidal efficacy against Plutella xylostella L.(Lepidoptera: Plutellidae).*Journal of Food, Agriculture & Environment*, *10*(3&4), 384-388.

[2] Anjali, C. H., Sharma, Y., Mukherjee, A., & Chandrasekaran, N. (2012). Neem oil (Azadirachta indica) nanoemulsion—a potent larvicidal agent against Culex quinquefasciatus. *Pest management science*, *68*(2), 158-163.

[3] Anton, N., & Vandamme, T. F. (2009). The universality of low-energy nano-emulsification. *International Journal of Pharmaceutics*, *377*(1), 142-147.

[4] Azam, A., Ahmed, A. S., Oves, M., Khan, M. S., & Memic, A. (2012). Size-dependent antimicrobial properties of CuO nanoparticles against Gram-positive and-negative bacterial strains. *International journal of nanomedicine*, *7*, 3527.

[5] Babchin, A. J., & Schramm, L. L. (2012). Osmotic repulsion force due to adsorbed surfactants. *Colloids and Surfaces B: Biointerfaces*, *91*, 137-143.

[6] Barradas, T. N., de Campos, V. E. B., Senna, J. P., Coutinho, C. D. S. C., Tebaldi, B. S., e Silva, K. G. D. H., & Mansur, C. R. E. (2014). Engineering Aspects.

[7] Bouchemal, K., Briançon, S., Perrier, E., & Fessi, H. (2004). Nano-emulsion formulation using spontaneous emulsification: solvent, oil and surfactant optimisation. *International journal of pharmaceutics*, *280*(1), 241-251.

[8] Bruxel, F., Laux, M., Wild, L. B., Fraga, M., Koester, L. S., & Teixeira, H. F. (2012). Nanoemulsões como sistemas de liberação parenteral de fármacos.*Quim Nova*, *35*, 1827-1840.

[9] Chen, F., Wang, Y., Zheng, F., Wu, Y., & Liang, W. (2000). Studies on cloud point of agrochemical microemulsions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *175*(1), 257-262.

[10] Cheong, J. N., Tan, C. P., Man, Y. B. C., & Misran, M. (2008). α-Tocopherol nanodispersions: preparation, characterization and stability evaluation. *Journal of Food Engineering*, *89*(2), 204-209.

[11] Dias, D. D. O., Colombo, M., Kelmann, R. G., Kaiser, S., Lucca, L. G., Teixeira, H. F., ... & Koester, L. S. (2014). Optimization of Copaiba oil-based nanoemulsions obtained by different preparation methods. *Industrial Crops and Products*, *59*, 154-162.

[12] Fernandes, C. P., de Almeida, F. B., Silveira, A. N., Gonzalez, M. S., Mello, C. B., Feder, D., ... & Falcão, D. Q. (2014). Development of an insecticidal nanoemulsion with Manilkara subsericea (Sapotaceae) extract. *J. Nanobiotechnology*, *12*, 22.

[13] Flores, F. C., Ribeiro, R. F., Ourique, A. F., Rolim, C. M. B., Silva, C. D. B. D., Pohlmann, A. R., ... & Guterres, S. S. (2011). Nanostructured systems containing an essential oil: protection against volatilization. *Química Nova*,*34*(6), 968-972.

[14] Forgiarini, A., Esquena, J., Gonzalez, C., & Solans, C. (2001). Formation of nano-emulsions by low-energy emulsification methods at constant temperature.*Langmuir*, *17*(7), 2076-2083.

[15] Goff, H. D. (1997). Colloidal aspects of ice cream—a review. *International Dairy Journal*, *7*(6), 363-373.

[16] Hoeller, S., Sperger, A., & Valenta, C. (2009). Lecithin based nanoemulsions: A comparative study of the influence of non-ionic surfactants and the cationic phytosphingosine on physicochemical behaviour and skin permeation.*International journal of pharmaceutics*, *370*(1), 181-186.

[17] Kelmann, R. G., Kuminek, G., Teixeira, H. F., & Koester, L. S. (2007). Carbamazepine parenteral nanoemulsions prepared by spontaneous emulsification process. *International journal of pharmaceutics*, *342*(1), 231-239.

[18] Komaiko, J., & McClements, D. J. (2014). Optimization of isothermal low-energy nanoemulsion formation: Hydrocarbon oil, non-ionic surfactant, and water systems. *Journal of colloid and interface science*, *425*, 59-66.

[19] Lim, C. J., Basri, M., Omar, D., Rahman, M. B. A., Salleh, A. B., & Rahman, R. N. Z. R. A. (2012). Physicochemical characterization and formation of glyphosate-laden nano-emulsion for herbicide formulation. *Industrial Crops and Products*, *36*(1), 607-613.

[20] Mahdi, E. S., Noor, A. M., Sakeena, M. H., Abdullah, G. Z., Abdulkarim, M. F., & Sattar, M. A. (2011). Formulation and in vitro release evaluation of newly synthesized palm kernel oil esters-based nanoemulsion delivery system for 30% ethanolic dried extract derived from local Phyllanthus urinaria for skin antiaging. *International journal of nanomedicine*, *6*, 2499.

[21] Margulis-Goshen, K., & Magdassi, S. (2013). Nanotechnology: an advanced approach to the development of potent insecticides. In *Advanced Technologies for Managing Insect Pests* (pp. 295-314). Springer Netherlands.

[22] Meon, S. (1998). Potential for Biological Control of Sclerotium Foot Rot of Chilli by Trichoderma spp. *Pertanika Journal of Tropical Agricultural Science*, *21*(1), 1-10.

[23] Mulqueen, P. (2003). Recent advances in agrochemical formulation. *Advances in colloid and interface science*, *106*(1), 83-107.

[24] Narang, A. S., Delmarre, D., & Gao, D. (2007). Stable drug encapsulation in micelles and microemulsions. *International journal of pharmaceutics*, *345*(1), 9-25.

[25] Paterson, R. R., Moen, S., & Lima, N. (2009). The feasibility of producing oil palm with altered lignin content to control Ganoderma disease. *Journal of phytopathology*, *157*(11-12), 649-656.

[26] Ramsey, R. J. L., Stephenson, G. R., & Hall, J. C. (2005). A review of the effects of humidity, humectants, and surfactant composition on the absorption and efficacy of highly water-soluble herbicides. *Pesticide Biochemistry and Physiology*, *82*(2), 162-175.

[27] Shafiq, S., Shakeel, F., Talegaonkar, S., Ahmad, F. J., Khar, R. K., & Ali, M. (2007). Design and development of oral oil in water ramipril nanoemulsion formulation: in vitro and in vivo assessment. *Journal of Biomedical Nanotechnology*, *3*(1), 28-44.

[28] Tadros, T. F. (Ed.). (2013). *Emulsion formation and stability*. John Wiley & Sons.

[29] Tang, S. Y., Manickam, S., Wei, T. K., & Nashiru, B. (2012). Formulation development and optimization of a novel Cremophore EL-based nanoemulsion using ultrasound cavitation. *Ultrasonics sonochemistry*, *19*(2), 330-345.

[30] Tanthapanichakoon, W., Chaichanawong, J., & Ratchahat, S. (2014). Review of nanomaterials R&D in 21st century in Thailand with highlight on nanoparticle technology. *Advanced Powder Technology*, *25*(1), 189-194.

[31] Wagner, H., Kostka, K. H., Lehr, C. M., & Schaefer, U. F. (2003). pH profiles in human skin: influence of two in vitro test systems for drug delivery testing.*European journal of pharmaceutics and biopharmaceutics*, *55*(1), 57-65.

[32] Wang, L., Li, X., Zhang, G., Dong, J., & Eastoe, J. (2007). Oil-in-water nanoemulsions for pesticide formulations. *Journal of colloid and interface science*, *314*(1), 230-235.

[33] Warisnoicharoen, W., Lansley, A. B., & Lawrence, M. J. (2000). Nonionic oil-in-water microemulsions: the effect of oil type on phase behaviour. *International Journal of Pharmaceutics*, *198*(1), 7-27.

[34] Yuan, Y., Gao, Y., Mao, L., & Zhao, J. (2008). Optimisation of conditions for the preparation of β-carotene nanoemulsions using response surface methodology.*Food chemistry*, *107*(3), 1300-1306.