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Clove Oil Nano Emulsion Formulation as an Inhibitor of Urediniospores Germination of *Hemileia vastatrix*, the cause of Coffee Leaf Rust Disease

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Abstract

Leaf rust disease caused by *Hemileia vastatrix* causes early shedding of coffee leaves and reduction of the formed flowers quantity leading to the decrease of coffee (*Coffea* spp.) productivity. Farmers control the disease using synthetic fungicides, which can harm consumer health and pollute the environment. Disease control using clove oil is environmentally friendly and safe for human health, but its solubility and effectiveness are relatively low. The clove oil nano emulsion formulation can increase the effectiveness of essential oil-based botanical fungicide. The results of the study obtained the formulation using clove oil (5 and 7.5%), Tween 80 (10 and 15%) surfactant, MCT (5%), glycerol (10 and 15%) co-surfactant could produce clove oil nano emulsions with smaller sizes and high stability. Six clove oil nano emulsion formulation were clear color or without sediment, tiny droplets 16.25–24.89 nm and had a typical mono dispersion graph with a PDI value < 0.2, containing a homogeneous and evenly distributed particle population. Formulas F1, F3 and F4 at a concentration of 5% had higher inhibition of urediniospores germination *H. vastatrix* compared to formulas F2, F5 and F6 at the same concentration. The inhibition percentage of all formulations was 58.64–100%, and formulations at a concentration of 5% had an effectiveness of 58.67–94.67%. It can be tested further on infected coffee plants on a larger scale. © 2023 Friends Science Publishers

Keywords: Clove oil; Eugenol; Nano emulsion; Urediniospores

Introduction

The disease that often attacks coffee plants is coffee leaf rust (*Hemileia vastatrix*). This disease causes symptoms of shedding leaves and the number of flowers decreases, so productivity decreases. Leaf rust has been reported since 1869 in Ceylon (Sri Lanka), then around 1920, spread to most African and Asia countries. In Indonesia, leaf rust disease is reported to attack coffee plants in 14 provinces, with an attack area of 32,825 hectares throughout Indonesia (Agricultural Data Center and Information System 2021). Disease attacks on coffee plants cause a significant reduction in crop production of around 20–40% per year (Worrall *et al.* 2018).

The limited knowledge of farmers and the extent of existing land causes disease management relies on synthetic fungicides that results in high production costs and can leave residues in products that can harm consumer health and pollute the environment. Alternative control method can be utilized by biofungicide. Biofungicides are fungicides which their active ingredients come from plants or other organic materials that are efficacious in controlling disease infestation on plants (Sharf *et al.* 2021; Javaid *et al.* 2023). Biofungicides are gaining popularity worldwide to manage crop diseases (Javaid and Khan 2016; Khan and Javaid 2022).

Essential oils are known for their antimicrobial potential against various pathogens (Ferdosi *et al.* 2021). However, weakness of the antimicrobial active ingredients of essential oils is their low solubility in water because they are hydrophobic. Hence, their concentration is low in the solvent phase, and their effectiveness as an antimicrobial is lower (Ghosh *et al.* 2014). Essential oil nano emulsion can be a solution to overcome this problem. Antimicrobial nano emulsions are highly stable oil-in-water emulsions of nanometer-sized droplets that act against viruses, fungi, and bacteria (Pant *et al.* 2014). According to Miastkowska *et al.* (2020), nano emulsion can increase the antifungal activity of oil tested against plant-pathogenic fungi.

Nano emulsion based biofungicide provides disease control options with minimal risk to humans and is environmentally friendly (Um-e-Aiman *et al.* 2021). Nano emulsion biofungicide can transform global agriculture in

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terms of food protection. Biofungicide in the orm of nano emulsion has the advantage of being able to reduce the concentration of the active substance without reducing its biological effectiveness as an antifungal (Miastkowska *et al.* 2020), small particle size, more effective, high solubility, and low toxicity (Kumar *et al.* 2021), the ability to control disease on the target, increase crop productivity, product quality, consumer acceptance and efficient use of resources (Scortichini 2022). Based on the description above, a study was conducted to formulated a biofungicide derived from clove oil in nano emulsion to control coffee leaf rust.

Materials and Methods

Preparation of nano emulsion

Clove oil nano emulsion (CONE) was prepared using modified methods of Komaiko and McClements (2015) and Yuliani and Noveriza (2019). Clove oil (Qiandra, eugenol 73%) was placed into a beaker and mixed with Medium Chained Triglycerides (MCT) and Tween 80 with the treatment composition as shown in Table 1, stirred using a magnetic stirrer at a speed of 750 rpm for 30 min to form an organic phase. Separately, the aqueous phase is prepared by mixing water and glycerol under the same stirring conditions as preparing the organic phase. The nano emulsion was then formed by adding the aqueous phase to the organic phase while stirring for 60 min at a speed of 750 rpm and continued for 60 min at 1000 rpm. The formed nano emulsion was placed into a sample vial for further analysis. Observed parameters included determination of droplet size, measurement of pH and inhibition percentage of urediniospores germination.

Droplet size measurement

Droplet and Polydispersity Index (PDI) measurements used a particle size analyzer (PSA) with dynamic light scattering type. The cuvette was cleaned, and 10 mL of the sample was put into the cuvette. The cuvette containing the sample was inserted into the sample holder and analyzed by the instrument (Yuliani *et al.* 2016).

pH measurement

The pH measurement was carried out at room temperature using a pH meter. Before use, the electrode was calibrated using standard solutions of pH 4 and 7. After the calibration process, the electrode was dipped into the preparation and the pH value of the preparation appeared on the screen (Yuliani *et al.* 2016).

Preparation of spore suspension

Since *H. vastatrix* is an obligate parasite, the urediniospores are collected directly from fresh symptomatic coffee leaves in the field to obtain viable urediniospores inoculum. Urediniospores were collected using a brush and stored in a test tube for 48 h before use. Preparation was carried out using 32 mg of urediniospores suspended in 2 mL of sterile water, then added Tween 80 (0.025%).

Antifungal assay of clove oil nano emulsion formulation

The inhibition test of urediniospores germination was conducted *in vitro*. The experiment used a randomized block design consisting of a combination of CONE formulas (F1, F2, F3, F4, F5 and F6) and several concentrations of formulas and control (0, 5, 10, 15 and 20%). Each concentration was prepared by diluting 5, 10, 15 and 20 mL formulation solution into 100 mL with sterile distillated water. The treatment formulation is presented in Table 3. WA (water agar) germination media is prepared by incorporating 20 grams of agar powder into 1 L of water and sterilizing using an autoclave, after that the WA medium is poured into a petri dish, after which the solid media is sliced with a size of 1.5 cm x 1.5 cm and transferred to on the object glass. Approximately 10 μL *H. vastatrix* urediniospores suspension from each treatment was dripped on a glass slide with WA medium covered with a cover slip and then air-dried. Preparation to incubate for 72 h at room temperature in a tray covered with damp tissue in the dark. Each treatment was repeated three times. Observations using a compound microscope on germinated urediniospores for each treatment. Urediniospores were observed for 24 h. The inhibition percentage of urediniospores germination (PIU) was calculated based on the following formula:

$$PIU = \frac{Uk - Ub}{Uk} \times 100\%$$

PIU is the inhibition percentage of urediniospores, Uk is the total number of germinated urediniospores and Ub is the number of urediniospores observed.

Data analysis

The effect of treatment on the observed variables and analysis of variance was carried out using the STAR (Statistical Tool for Agricultural Research) program. If there were differences, it was continued with Duncan's Multiple Range Test at a 5% level.

Results

Physical properties of clove oil nano emulsion

Six CONE formulas were produced in this study (Table 2). Characteristic of CONE with surfactan Tween 80, glycerol and MCT indicates that the surfactant mixing ratio was optimal to produce eugenol nanoparticles with stable and evenly distributed particle size.

Table 1: Composition of the clove oil nano emulsion formulation

Formula	Clove oil (%)	MCT (%)	Gliserol (%)	Tween 80 (%)	Aquades (%)	Other carrier (%)
F1	5.0	5	10	10	60.0	10
F2	7.5	5	10	10	57.5	10
F3	5.0	5	10	15	55.0	10
F4	7.5	5	10	15	52.5	10
F5	5.0	5	15	10	55.0	10
F6	7.5	5	15	10	52.5	10

MCT (*Medium Chained Triglycerides*)

Table 2: Characterization of the clove oil nano emulsion formulas

Nano emulsion formula	Condition after storage	Particle size (nm)	PDI	pH	
				24 hours of storage	After storage 1 years
F1	No sediment	21.28	0.079	5.31	4.29
F2	No sediment	22.20	0.066	5.01	4.21
F3	No sediment	16.25	0.058	4.89	4.09
F4	No sediment	18.18	0.059	4.87	4.34
F5	No sediment	21.70	0.121	4.96	4.36
F6	No sediment	24.89	0.116	5.00	4.53

PDI (Polydiversity Index)

Table 3: Effect CONE formulas and several concentration of formulas on the percentage inhibition of *H. vastatrix* urediniospores germination

CONE formula	Concentration (%)	Inhibition (%)
Control	0	4 c
F1	5	90.67 a
	10	90.67 a
	15	100.00 a
	20	100.00 a
F2	5	58.67 b
	10	82.67 ab
	15	98.67 a
	20	98.67 a
F3	5	89.80 a
	10	100.00 a
	15	100.00 a
	20	100.00 a
F4	5	94.67 a
	10	94.67 a
	15	94.67 a
	20	97.33 a
F5	5	76.00 ab
	10	81.33 ab
	15	100.00 a
	20	94.67 a
F6	5	81.33 ab
	10	82.67 ab
	15	85.33 ab
	20	88.00 ab

The same letter shows no significant difference at the 5% level

Clove oil nano emulsion stability

The pH measurement was carried out to determine the acidity of the nano emulsion formulations, which had different compotitions (Table 2) and the stability of the formulations. The results showed that the pH value of the nano emulsion ranged from 4.87–5.31. Formulas F3, F4 and F5 each have relatively lower pH values (4.89, 4.87 and 4.96) than formulas F1, F2 and F6.

All the resulting formulas were perfectly dispersed and showed no segregation, sediment or foam (Fig. 1). The highest level of clarity was obtained in formula F3 compared to other formulas. Furthermore, the F4 and F5 formulas also had clear colors. The low pH value influences the transparent color in the formulas F3, F4 and F5. Then formula F1 was a slightly white color, and formulas F2 and F6 had a white or cloudy color, each with a pH value of > 5. Even after being stored for one year, all formulas did not show discoloration, and no sediment was formed (Fig. 2). However, the results of pH measurements show an increase in acidity (Table 2) but do not affect color stability.

Particle size and polydispersity index (PDI)

Clove oil nano formulation produced droplet size diameter < 100 nm, with the smallest droplet size shown in F3 of 16.25 nm and F4 of 18.18 nm and the largest in formula F6 (Table 2). The addition of different concentrations of Tween 80 affected the decrease in the particle size of the clove oil emulsion nanoparticles in formulas F3 and F4, followed by a decrease in the PDI value.

The analysis of CONE showed that the smallest PDI value in the F3 formula was 0.058, and then sequentially, the F4, F2, F1, F6 and F5 formulas (Table 2). The resulting CONE formulation had a standard mono dispersion or homogeneous particle size shown on the chart, only having a single peak typical (mono dispersion), not polydispersion. It contains a homogeneous and evenly distributed particle population (Fig. 3). Formulas F3 and F4 have small particle population sizes and are homogeneous compared to other formulas. However, formulas F1, F2, F5 and F6 are still included in the criteria with a small and homogeneous particle size population with a PDI value < 0.2 (Table 2).

Antifungal assay

All the formulas and concentrations used in this study significantly inhibited urediniospores germination compared

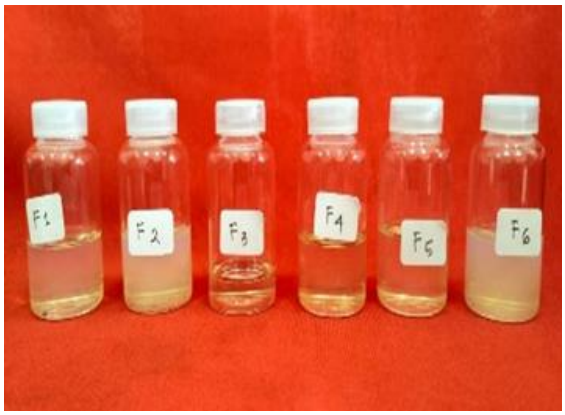

Fig. 1: Clove oil formula after 24 hours of storage. The formulas were perfectly dispersed and showed no separation, sediment, or foam. F1 is slightly white, F2 is white or hazy, F3 has the highest level of clarity, formulas F4 and F5 have transparent colors and F6 is white or hazy

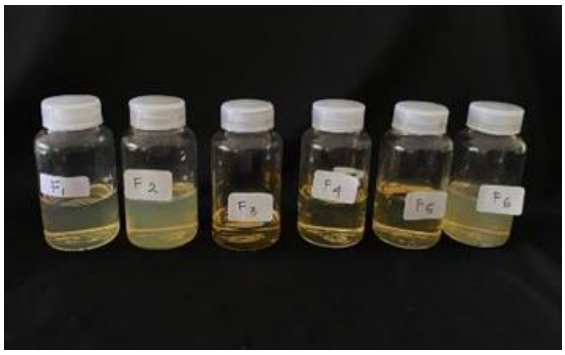

Fig. 2: Clove oil nano emulsion after being stored for one year visually, each formula did not show any discoloration and no sediment was formed

to the control. The inhibition percentage resulting from all formulations treatments ranged from 58.64–100% (Table 3). Increasing the concentration of the CONE formula from 5 to 20% increased the inhibition of urediniospores germination *H. vastatrix*, but there was no significant difference between formulas concentrations except for the F2 formula. Formulas F1, F3 and F4 at the lowest concentration (5%) were more effective in inhibiting urediniospores germination of *H. vastatrix* than to other formulas (F2, F5 and F6) at the same concentration. In the results of the linear regression analysis, it was seen that the particle size influenced the inhibition rate of urediniospores, which was 13.21% (Fig. 4). The obtained linear regression equation is y = 124.86–1.6391x. The equation shows that each increase in the particle size level of 1 nm will cause a decrease in the inhibition rate of urediniospores germination by 1.6391%. It indicated that CONE effectively inhibited urediniospores germination.

Discussion

The nano emulsion formulation was made using a low-energy emulsification technique using phase inversion. In the phase inversion mechanism, nano emulsions form through two stages: the formation of water in oil (w/o) emulsion, which then reverses the phase to o/w. A w/o emulsion is formed when water is added to the mixed phase between the oil and the emulsifier.

The size of the emulsion produced from nano emulsion technology with low energy methods is influenced by the composition system (ratio and type of surfactant, oil-in-water ratio), emulsion preparation (additives, stirring speed) and environmental conditions (temperature) (McClements 2013). Ghosh *et al.* (2014) state that selecting the surfactant type, concentration and optimal emulsion time will result in a smaller particle size and more excellent stability. This condition is related to the role of surfactants Glycerol and Tween 80, which results in smaller particle sizes and higher stability. Glycerol is a factor that affects droplet size and PDI nano emulsion (Chong *et al.* 2018).

The low pH value affects the transparant color of the formulas F3, F4 and F5. According to Liu *et al.* (2019), nanoparticles produced under acidic pH conditions are more stable. Yuliani *et al.* (2016) stated that the pH value of the nano emulsion was affected by temperature, type and concentration of the extract used, type of surfactant, other fillers, storage, aqueous phase and oil phase.

One of the advantages of nano emulsions is that they have kinetic stability (McClements 2012). Due to the tiny particle size and the minor surface tension between the oil and water molecules, nano emulsions have almost no tendency to agglomerate or sediment, reducing the possibility of creaming or sedimentation formation. As a result, nano emulsions are much more stable than other emulsion systems and more translucent than conventional microemulsions and emulsions (McClements 2012). In addition, the absence of sediment is due to the small particle size of a nano emulsion system not affected by gravity (Sutradhar and Amin 2013). Therefore, spraying using a sprayer will not block the nozzle.

The average particle size and polydispersity index (PDI) indicate the quality that determines a formula's safety, stability, efficacy and *in vitro* and *in vivo* behavior (Danaei *et al.* 2018). These parameters are significant physical characteristics when manufacturing products. The characteristics of nanocarriers with small particle sizes can affect bulk properties, stability, product performance, encapsulation efficiency, bio-distribution, final product appearance and cellular uptake (Bahari and Hamishehkar 2016; Maherani and Wattraint 2016).

The addition of concentrations of Tween 80 at formulas F3 and F4 have caused droplet sizes smaller than other formulas, followed by a decrease in PDI values. In line with the research of Dhivya *et al.* (2019) that the addition of a surfactant concentration of 50% can reduce the droplet size of *Acorus calamus* oil up to 43.17%, which is spontaneously emulsified. Thus, proving that the surfactant concentration affects the particle diameter. The results of Pathak *et al.* (2013) stated that the droplet size will decrease

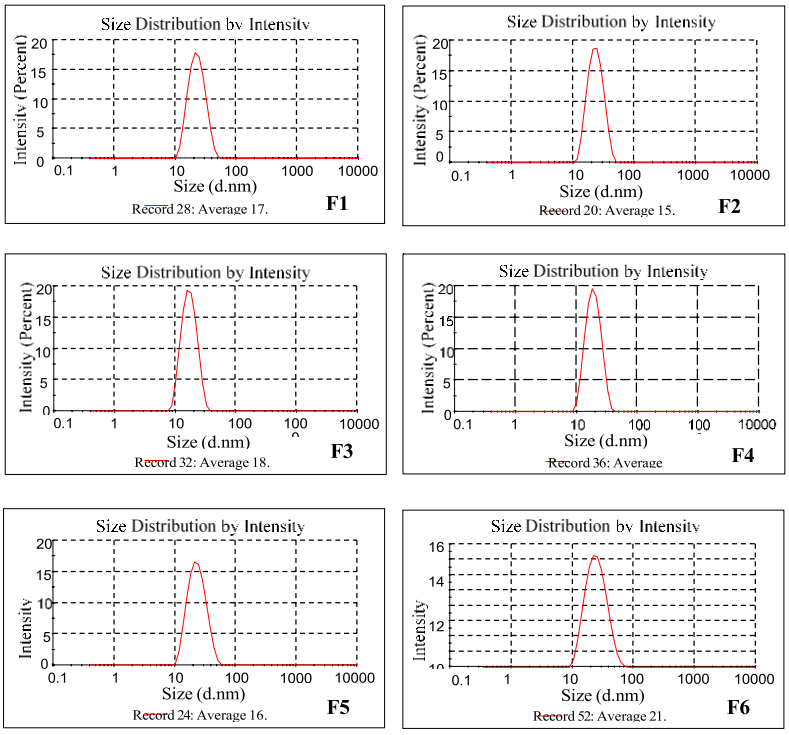

Fig. 3: Graphical representation of the particle size of clove oil formulas F1, F2, F3, F4, F5 and F6. The representation of these clove oil formulas from the particle size chart shows a mono dispersion sample (one peak) containing a homogeneous particle population

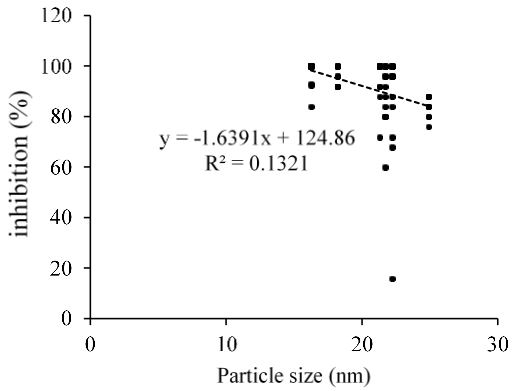

Fig. 4: Linear regression curve of the relationship between particle size and inhibition percentage of *H. vastatrix* urediniospores germination

as the ratio of surfactant to cosurfactant increases.

Particle size distribution is a physicochemical characteristic of nano emulsion that defines it ability to accumulate in plant tissues. Therefore, safe, stable and efficient nanocarrier formulation requires homogeneous (mono dispersion) particles of a certain size nanocarrier. The composition of the nanocarrier and the nature of the solvent and co-solvent used require reasonable consideration to control the particle size distribution during its preparation (Bulbake *et al.* 2017; Dong *et al.* 2017; Mozafari *et al.* 2017). After preparation, the resulting nano emulsion must be analyzed physicochemical to ensure its suitability. The parameters measured were nanoparticle size and PDI.

The PDI value reflects a system's uniformity level, whereas the more minor the PDI value, the distribution of particles in a mono-dispersion system is more uniform (Luo *et al.* 2017). The size distribution algorithm lies between the two extreme PDI values 0.05–0.7, based on the standard documents ISO 13321:1996 E and ISO 22412:2008 (Sakho *et al.* 2017). Nanoparticles with a PDI value > 0.7 have a wide distribution and are thought to contain large droplets or clusters that can settle slowly (Danaei *et al.* 2018; Azmy *et al.* 2019). An index with a value less than 0.05 is seen with a highly monodispersed standard. The polydispersity index (PDI) shows the diversity of a dispersed system and the PDI value ranges from 0 to 1.

This study obtained the CONE formulas with a PDI value of < 0.2, which included the standard mono dispersion or homogeneous particle size. As Azmy *et al.* (2019) stated, PDI < 0.6 indicates small and homogeneous droplet size, 0.6-0.7 medium droplet size and > 0.7 contains large droplets and wide/heterogeneous distribution. Shahavi *et al.* (2016) found the optimum conditions of clove oil processed into nano emulsions, can produce a droplet size of 50 nm and a polydispersity index of 0.49. The droplet size and the small PDI value will reduce the occurrence of coagulation due to electrostatic repulsion between particles carrying the same electric charge and make the particles stable. In addition to droplet size, the stability of clove oil is influenced by the type

of emulsifier used, pH and storage temperature.

A higher inhibition of urediniospores germination of *H. vastatrix* by formulas F1, F3 and F4 at the lowest concentration (5%) compared to other formulas was related to the small size of the emulsion nanoparticles and the PDI value < 0.2 in these formulas. However, in almost all formulas (except F2), there was no significant increase in inhibition with the addition of 5–20% formula concentration. A 5% concentration of the CONE formulas in this study had effectiveness ranging from 58.67–94.67%. The study of Hashem *et al*. (2023) also found that the application of CONE at a concentration of 5000 ppm (containing 10% clove essential oil) could inhibit 82.2% of the growth of *Neoscytalidium dimidiatum* blight disease of *Carum carvi* L.

Clove oil contains a component of eugenol which has activity against plant pathogenic fungi (Sameza *et al*. 2016; Sharma *et al.* 2017; Šernaitė *et al.* 2020). Several eugenol derivatives had high antifungal activity, almost comparable to commercial fungicides (Olea *et al*. 2019). There are several mechanisms of action of eugenol compounds in fungi, namely (1) changes in the fluidity and permeability of the fungal membrane by the lipophilic character of the molecules and (2) chemical reactions with unsaturated chains or reduction by enzymes due to the presence of electron-withdrawing solid groups on the fungal aromatic rings.

The CONE toxicity mechanism is due to the active ingredient, eugenol, that enters the membrane and disrupts the fungal cell membrane (Olea *et al.* 2019). The destruction of the eugenol emulsion to the fungal cell membrane can be through the mechanism of inhibiting the biosynthesis of ergosterol and eugenol entering between the fatty acid chains to form a lipid bilayer membrane that changes the fluidity and permeability of the membrane (Abd-Elsalam and Khokhlov 2015).

Nano emulsions have higher antimicrobial activity than essential oils (Abd-Elsalam and Khokhlov 2015; Ali *et al.* 2017). Nanoparticles attack phytopathogens by forming reactive oxygen species (ROS) antioxidants. The ROS formed can damage cell membranes and other cellular components such as fats, proteins, RNA and DNA molecules (Al-Khattaf 2021). The nanoparticles' negative charge and the microbial membranes' electrostatic interaction can stick together. Depolarization of the membrane results in disruption of respiration and membrane permeability, which in turn causes cell death. The spread of nanoparticles causes the formation of gaps around the cell wall (Alghuthaymi *et al.* 2015). Cell membrane damage and cell surface perforation caused by nanoparticles causes inflammation around the cell membrane and the release of cytoplasmic components (Ibrahim *et al.* 2020).

Conclusion

Clove oil nano emulsion formulation using clove oil, Tween 80 surfactant, MCT and glycerol co-surfactant produces small particles and high stability. Six clove oil nano emulsion formulas produced in this study showed not to have lumps or sediment and a droplet size of 16.25–24.89 nm with a typical one-peak particle graph (mono dispersion) containing a homogeneous and evenly distributed particle population. Formulas F1, F3 and F4 at a concentration of 5% had the higher inhibition urediniospores germination of *H. vastatrix* compared to formulas F2, F5 and F6 at the same concentration. Inhibition of urediniospores germination was influenced by the small nano emulsion particle size (PDI < 0.2). The more concentrated CONE applied, the inhibition of urediniospores germination more significant. Further tests can be carried out on infected coffee plants on a larger scale.

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Author Contributions

K, SP and GI planned the experiment, NH and R prepared material, KDS, KKH and YH analyzed data statistically and interpreted data, SP and GI prepared original drafts of preparation for writing, G and YF reviewed and edited.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable to this paper.

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