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Stability and pre-emergence herbicidal potential of citronella (*Cymbopogon nardus*) essential oil-based nanoemulsion during storage

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Abstract Natural herbicides from essential oils (EOs) are widely used in sustainable weed control. A citronella (*Cymbopogon nardus*) EO-based nanoemulsion was fabricated for using as a natural herbicide. It was created using a high-energy emulsification method in a microfluidizer. A gas chromatograph-mass spectrometer (GC-MS) was employed to determine the chemical composition of the citronella EO. The major components of the EO were 33.59% citronellal, 21.42% geraniol, 11.23% citronellol and 4.38% limonene. The citronella EO was formulated to nanoemulsion with a nonionic surfactant mixture (Smix). The Smix at hydrophilic-lipophilic balance (HLB) 14 consisted of Tween 60 (91.2% w/w) and Span 60 (8.8% w/w). The droplet size of the nanoemulsion decreased from 78.6 to 35.2 nm with an increasing number of microfluidization cycles, from 1 to 3 cycles at 15000 psi. The optimal number of microfluidization cycles was 3, which produced the smallest droplet size. The effect of stability storage on droplet size and herbicidal activity of the nanoemulsion was investigated for 10, 20, 30 and 60 days. Droplet size increased with storage time through to 60 days (from 35.2 to 55.2 nm). The herbicidal activity of the nanoemulsion at concentrations of 62.5, 125 and 250 μL L^{-1} was determined on *Amaranthus tricolor* L. During 60 days of storage, the inhibitory effect percentage on seed germination and seedling growth decreased slightly. Also, the inhibition of seed imbibition and α-amylase activity showed non significantly changed with storage time. Our findings provide essential information for using and storing citronella EO nanoemulsion. The results revealed that the nanoemulsion could be stored at 4 ℃ for at least 60 days without phase separation occurring. The results show that the citronella EO-based nanoemulsion can be used as a natural herbicide and long shelf life.

Keywords: Bioherbicides, Nanotechnology, Microfluidizer, Storage stability

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Introduction

Weeds are one problem in an agriculture field. They compete with crop plants for plant growth resources (Charoenying *et al*., 2022). There are various kinds of weed management methods, such as manual, chemical, mechanical and biological ones. Among those methods, chemical herbicides are widely used in field crops (Chotsaeng *et al*., 2019). However, the use of herbicides can damage the environment and human health. For sustainable agriculture, natural herbicides are produced and used as weed control alternatives.

Essential oils (EOs) are complex volatile mixtures of components and contain a range of secondary metabolites from plants (Moghimi *et al*., 2016). EOs as bio-herbicides are environmentally and human-friendly because of their volatile characteristics. They do not remain in the environment after application (Hazrati *et al*., 2017). Citronella (*Cymbopogon nardus*) EO is well-documented as a natural herbicide in the available literature (Lins *et al*., 2019; Somala *et al*., 2022). Applications of EOs such as citronella EO pose many challenges for product developers due to the weaknesses of EOs when they are exposed to humidity, light, heat or oxygen (Kaur *et al*., 2021). Hence, EOs need to be formulated carefully if their herbicidal activity will be used as a potential advantage. Nowadays, many researchers have been developing and formulating natural herbicide-containing EO nanoemulsions using low- and high-energy emulsification methods to reduce particle size. High-energy emulsification methods are the most popular nanoemulsion preparation methods and included are ultrasonic and microfluidization techniques (Mahdi Jafari *et al.*, 2006). A nanoemulsion consists of an EO, water and emulsifying agents, and has a small particle size of between 20 -200 nm (Mustafa and Hussein, 2020). One outstanding benefits of nanoemulsions are kinetically stable over a long period of time, a property is mainly active due to the small droplet size of the nanoemulsion (Gupta *et al.*, 2016).

In recent work of ours, Somala *et al*. (2022) showed that citronella EO-based nanoemulsion with a non-ionic surfactant mixture (Tween 60 and Span 60) at hydrophilic-lipophilic balance (HLB) 14 was stable for a long time at 4 ℃. Nanoemulsion showed pre-emergence herbicidal activity that completely inhibited seed germination and seedling growth of *Echinochloa cruss-galli* L. They are only evaluated the droplet size during storge. It is interested to investigate the efficiency of the herbicidal activities of citronella EO-based nanoemulsion during storage. The study aimed to prepare and investigate a citronella EO-based nanoemulsion against *Amaranthus tricolor* L. during storage for 10, 20, 30 and 60 days.

Materials and methods

Essential oil (EO) and chemical materials

Citronella EO (*Cymbopogon nardus*), Tween 60 and Span 60 were purchased from Chemipan Corporation Co., Ltd. (Bangkok, Thailand).

Identification of citronella EO constituents by gas chromatography/mass spectrometry (GC/MS)

The citronella EO components were identified using gas chromatography in conjunction with mass spectrometry (GC-MS). The EO was diluted in ethyl acetate. An Agilent 6890 N gas chromatograph with an Agilent 5973 mass detector was used for analysis. Percentage composition was investigated which based on GC peak area and retention time.

Preparation of citronella EO nanoemulsion

Citronella EO-based nanoemulsion was formulated according to Somala *et al*. (2022), who used a high-energy emulsification method for nanoemulsion formulation from citronella EO involving a microfluidizer processor (Microfluidics, Newton, MA, USA) at 25 ℃ and 15000 psi. The nanoemulsion consisted of 2% EO : 2% surfactant mixture (Smix) : 96% deionized (DI) water. The Smix was a mixture of Tween 60 and Span 60 at HLB 14. The obtained nanoemulsion was stored at 4 ℃ for further experimentation.

Droplet analysis

The droplet size (z-average) and polydispersity index (PI) of the nanoemulsion was evaluated using a dynamic light scattering (DLS) technique by Nanoplus 3, MICROMERITICS, Japan. The sample was diluted at 1:9 with DI water to avoid the multi-scattering effect. The measurements were computed using the nanoPlus program in five replications.

Storage study of citronella EO nanoemulsion

The nanoemulsion was stored at 4 ℃ for 10, 20, 30 and 60 days in glass bottles. Droplet size, PI and herbicidal activity were investigated during storage.

Herbicidal activity of citronella EO nanoemulsion

Germination bioassays

The inhibition effect of the nanoemulsion on seed germination and seedling growth was evaluated by Petri dish bioassay. The nanoemulsions were prepared at concentrations of 62.5, 125 and 250 µL/L of EO. Five millilitres of each treatment solution were added to a 9-cm-diameter Petri dish with germination papers. Twenty seeds of *A. tricolor* were placed on the germination papers. Each dish was sealed with Parafilm and kept in a growth chamber at 26±2 ℃ and 12/12 h light/dark. Water served as the control. Germination count and seedling lengths (cm) were recorded after 7 days.

Seed imbibition

The inhibitory effects of the nanoemulsion on the seeds of *A. tricolor* was investigated. Treatment solutions consisted of 62.5, 125 and 250 µL/L EO. Water was used as a control. Seed imbibition was carried out according to Turk and Tawaha (2003). 100 seeds of *A. tricolor* were weighed (W_1) and soaked in the treatment solution for 5, 10 and 15 h. After incubation, the seeds were washed and weighed (W_2). Percentage of seed imbibition was calculated as follows:

$$\text{Seed imbibition (\%)} = [(W_2 - W_1)/W_1] \times 100$$

α-Amylase activity assay

After the seed imbibition experiment, the seeds were used for α-Amylase activity assay using the dinitrosalicylic acid (DNS) method as reported by Sadasivam and Manickam (1996). The seeds were grained with 4 mL of 0.1 M $CaCl_2$ and centrifuged at 10,000 rpm for 20 min at 4 ℃. The supernatant was stored at 4 ℃. The reaction consisted of 1 mL of supernatant and 1 mL of 0.1% starch solution. Then, the reaction solution was incubated at 37 ℃ for 15 min. After incubation, 1 mL of DNS regent was added into the reaction solution tube. The solution was boiled at 100 ℃ for 5 min. Finally, the absorption at 560 nm was measured using a UV/Vis spectrophotometer (Thermo Fisher Scientific, USA) and the enzyme activity assay was calculated and expressed as µmol maltose/min/g(FW).

Statistical analysis

All experiments were performed using a completely randomized design (CRD). Data are expressed as mean value ± standard deviation (SD). Mean values were compared by Tukey's multiple range tests (p<0.05).

Results

The composition of citronella EO

The chemical composition of EO from citronella was analysed by GC-MS. The major components of the EO were 33.59% citronellal, 21.42% geraniol, 11.23% citronellol and 4.38% Limonene.

The effect of number of cycles of microfluidization on droplet size of citronella EO nanoemulsion

Citronella EO was prepared into a nanoemulsion using a high-energy emulsification method with a microfluidizer machine to produce nanoemulsion droplets in the nanoscale. The effect of the cycling number of microfluidization on droplet size and PI value of the nanoemulsion was studied at a pressure of 15000 psi by passing the emulsion through the device for 1 to 3 cycles. the droplet size of the nanoemulsion decreased from 78.6 to 35.2 nm as the number of cycles of microfluidization was increased from 1 to 3. Also, PI value became narrower in droplet size distribution, narrowing from 0.265 to 0.165 as shown in Table 1). The nanoemulsion solution made with a microfluidizer at 1 cycle appeared milky and without a separated phase (Figure 1). A transparent appearance was observed for the nanoemulsion formulation prepared with the microfluidizer at 2 and 3 cycles.

Table 1. The effect of number of cycles of microfluidization at 15000 psi on droplet size and PI value of citronella EO-based nanoemulsion

The number of cycles	Droplet size (nm)	PI value
1	78.6 ± 0.38 a	0.265 ± 0.006 a
2	48.7 ± 0.11 b	0.234 ± 0.009 b
3	35.2 ± 0.19 c	0.165 ± 0.011 c

Means ± standard deviations.
Means with different letters within a column are significantly different ($p < 0.05$).

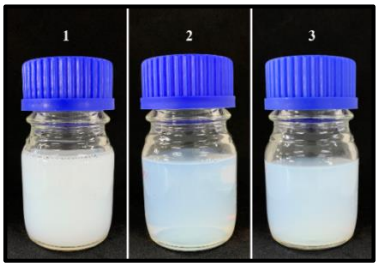

Figure 1. Citronella EO-based nanoemulsion prepared using microfluidizer at 1-3 cycles of microfluidization

The effect of storage time on droplet of citronella EO nanoemulsion

The change in droplet size and PI value as functions of storage time for the nanoemulsions stored at 4 ℃ are shown in Table 2. After preparation, the droplet size and PI value of the nanoemulsion were 35.2 nm and 0.162, respectively. The nanoemulsion was stored in glass bottles for 10, 20, 30 and 60 days. The droplet size significantly increased to 52.8 nm during 10 days of storage. After 10 days, the droplet size changed slightly from 52.8 to 55.2 nm. The PI value decreased with storage time. After 60 days, phase separation was not found in the nanoemulsion.

Table 2. The effect of storage on droplet size and PI of citronella EO-based nanoemulsion during 0 – 60 days

Storage time (days)	Droplet size (nm)	PI
0	35.2 ± 0.19 d	0.162 ± 0.011 a
10	52.8 ± 1.04 c	0.094 ± 0.010 bc
20	53.8 ± 0.26 bc	0.102 ± 0.008 b
30	54.2 ± 0.89 ab	0.076 ± 0.010 c
60	55.2 ± 0.41 a	0.079 ± 0.010 c

Means ± standard deviations.
Means with different letters within a column are significantly different ($p < 0.05$).

The effect of storage time on herbicidal activity of citronella EO nanoemulsion

The nanoemulsion was evaluated for the effects of storage on herbicidal activity on germination, seedling growth, seed imbibition and α-amylase activity. These herbicidal activities increased when increasing concentration of 62.5, 125 and 250 µL/L. At the concentration of 250 µL/L, seed germination and seedling growth were completely inhibited at 0, 10, 20 and 30 days of storage. However, the inhibition effect slightly changed when increasing the time to 60 days (Figure 2A). The seed germination was inhibited by 98.75% at 60-day storage. In addition, the nanoemulsion also affected the seedling growth of *A. tricolor* (Figure 2B and 2C). Shoot and root length were suppressed by the nanoemulsion with a dose-dependent response.

The inhibition effect of the nanoemulsion on seed imbibition and α-amylase activity of *A. tricolor* seed were also determined during storage. Figure 3A-C showed that seed imbibition was not significantly different in every storage time in each soaking time (5, 10 and 15 h). Also, α-amylase activity was not significantly different when soaked the seed in the nanoemulsion for 5 and 10 h (Figure 4A and 4B). However, α-amylase activity significantly decreased when treated the nanoemulsion at a concentration of 62.5 and 125 µL/L for 15 h (Figure 4C).

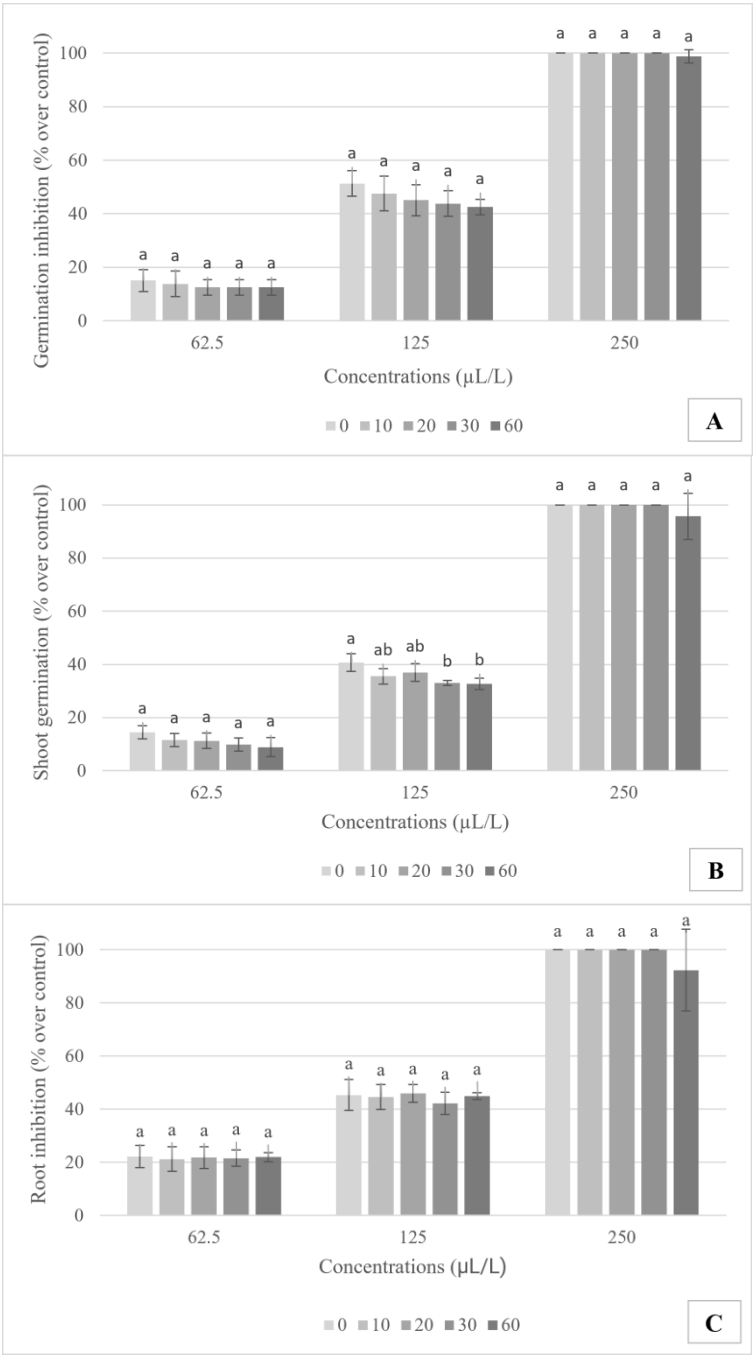

Figure 2. The effect of storage time of citronella EO-based nanoemulsion on seed germination (A), shoot (B) and root (C) length of *A. tricolor* for 60 days. Error bars represent standard deviation of mean

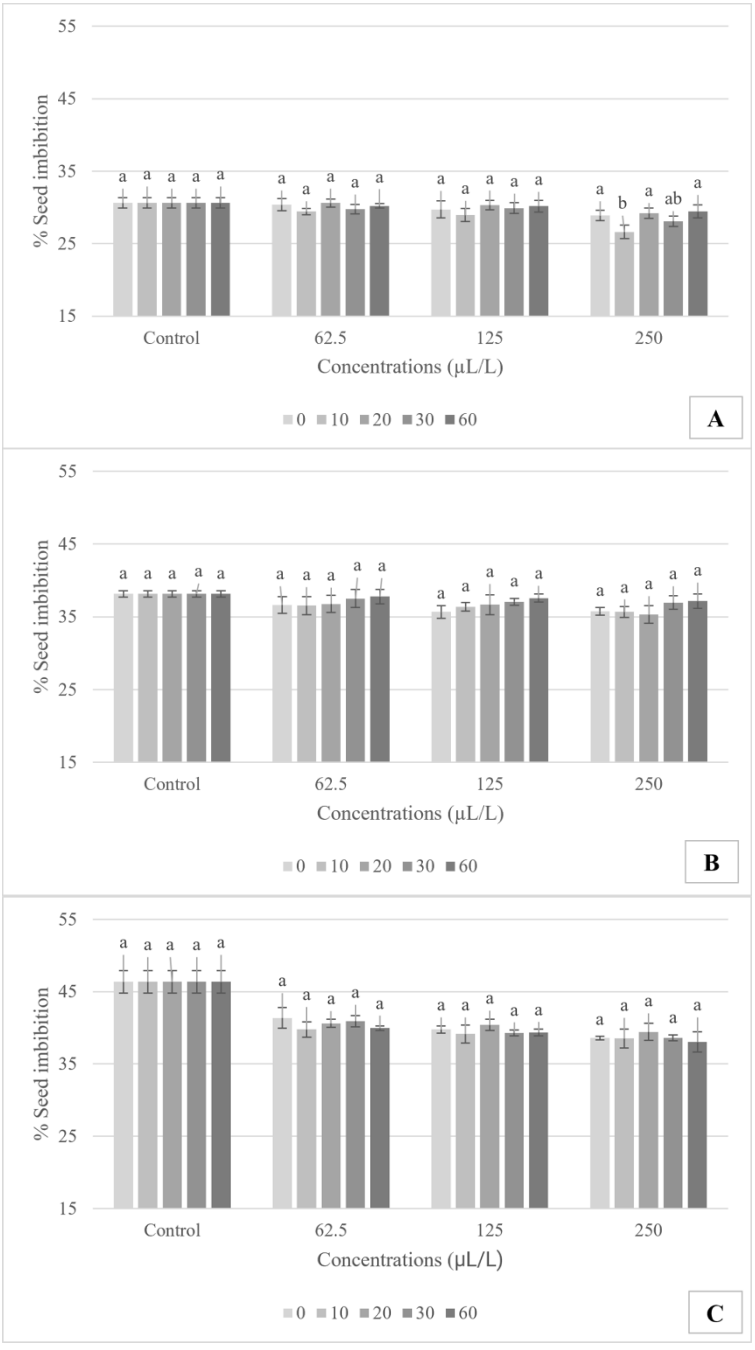

Figure 3. The effect of storage time of citronella EO-based nanoemulsion on seed imbibition of *A. tricolor* at 5 (A), 10 (B) and 15 h (C) after soaking in treatment solution. Error bars represent standard deviation of mean

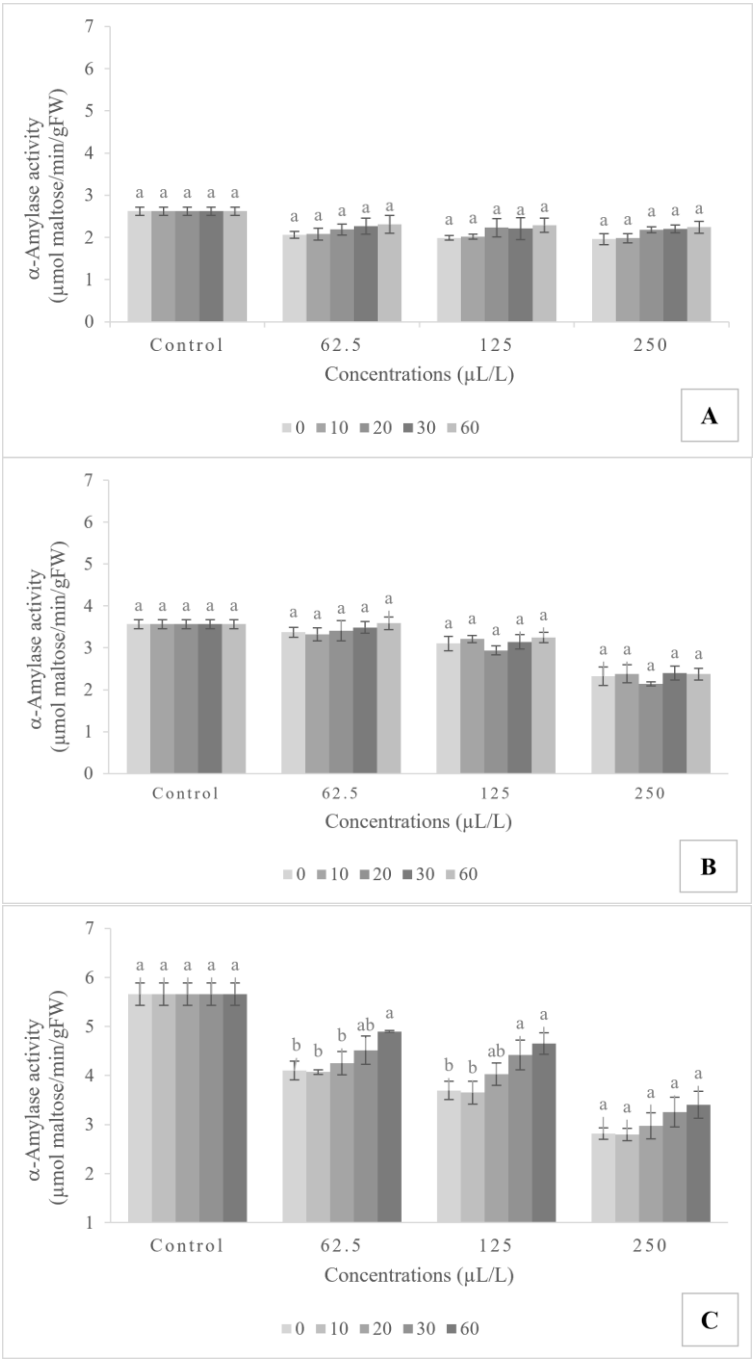

Figure 4. The effect of storage time of citronella EO-based nanoemulsion on α-amylase activity of *A. tricolor* at 5 (A), 10 (B) and 15 h (C) after soaking in treatment solution. Error bars represent standard deviation of mean

Discussion

The research work was determined the effect of storage time on droplet characteristics and herbicidal activity of a citronella EO-based nanoemulsion. Storage time was an essential factor for further application. Hence, the nanoemulsion was monitored for 60 days of storage at 4 ℃.

The main compounds of citronella EO were citronellal, geraniol, citronellol and limonene, which belong to the monoterpene class. Similarly, the chemical composition of citronella EO found in our work was in agreement with the work of Timung *et al*. (2016). They found that the main compounds in citronella EO were citronellal (55.24%), geraniol (26.29%), and citronellol (13.41%). Meanwhile, Nakahara *et al*. (2013) reported that geraniol (35.7%), *trans*-citral (22.7%), *cis*-citral (14.2%), geranyl acetate (9.7%) and citronellal (5.8%) were the major chemical compounds of *C. nardus* oil. However, the variation of chemical composition depended on several factors such as genetic differences, species, climatic and environmental conditions, and harvest stage.

The production of nanoemulsions requires a large energy input (Llinares *et al.*, 2018). The nanoemulsion is prepared by the microfluidization technique using the microfluidizer machine (type Z of interaction chamber) required 3 cycles of microfluidization, the smallest droplet size was formed. The microfluidizer made small droplet size and a narrow distribution due to high-energy density. Microfluidizer cycling numbers can significantly influence the physical properties of nanoemulsions (Jo and Kwon, 2014).

The nanoemulsion was stored at 4 C °after preparation to determine the effect of storage on droplet size and herbicidal activity of the nanoemulsion during storage of 60 days. After storage, the increase in droplet size was still acceptable because the size was still at the nano scale (< 200 nm). Somala *et al.* (2022) also reported on nanoemulsions made with citronella EO that were stored at 4, 25 and 45 ℃. There are reported that the droplet size of the nanoemulsion was satisfactory over time at 4 ℃ during storage of 28 days. The droplet size became larger, possibly due to the Ostwald ripening process, which is a factor responsible for destabilizing nanoemulsions (Gupta *et al*., 2016; Karthik *et al*., 2017; Rao and McClements, 2012; Somala *et al*., 2022). The mechanism of the Ostwald ripening process involves the growth of particle size due to molecular diffusion of the EO between particles (Wooster *et al.*, 2008).

The nanoemulsion as an herbicide which made from citronella EO was previously studied for weed control potential (Somala *et al*., 2022). However, the herbicidal potential of the nanoemulsion during storage was investigated. The inhibitory effect of the nanoemulsion on germination and seedling growth of *A. tricolor* L. changed slightly during storage. As a result, the inhibitory

potential during storage showed a positive correction between the droplet size of the nanoemulsion and germination and seedling growth inhibitory potential. Furthermore, seed imbibition and α-amylase activity were observed at 5, 10 and 15 h after soaking the seeds of *A. tricolor*. Seed imbibition affected the initial seed germination process of plants. In the germination process, α-amylase is an important enzyme that acts to digest starch, producing nutrients and energy (Liu *et al*., 2018). The effect of storage time on seed imbibition and α-amylase activity did not vary significantly over the 0 to 60 day period, which correlated with germination and seedling growth. The active ingredients were improved by reducing the molecular size when the active ingredient penetrate into the plant is a prerequisite for the efficacy of herbicides formulation (Zainuddin *et al*., 2019). Therefore, the nanoemulsion can store for at least 60 days due to its herbicidal activity still being effective.

In summary, as part of the production of citronella EO-based nanoemulsion as natural herbicides for alternatives to sustainable agriculture by microfluidization technique obtained the natural herbicide with nanoscale droplet and stored for 60 days at 4 ℃. It is confirmed that citronella EO-based nanoemulsion demonstrated storage stability for at least 60 days at 4 ℃, with maintenance of nanoscale droplet size and high potential for herbicidal activity.

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