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The effect of microfludization on characteristics and herbicidal potential of peppermint nanoemulsion on *Amaranthus tricolor*

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Abstract The natural herbicides are friendly environment and human health. Peppermint essential oil nanoemulsion was prepared by a high-energy emulsification method using microfluidization with a non-ionic surfactant Tween 60 and was used to inhibit germination of *Amaranthus tricolor* seeds. Droplet size of the nanoemulsion was reduced by increasing the pressure of microfluidization from 5000 to 20000 psi (from 130.2 to 69.8 nm). The highest pressure (20000 psi) found the smallest droplet size of the nanoemulsion that droplet size, PI value, and zeta potential were 69.8 nm, 0.277, and -44.17 mV, respectively. Also, each pressure formulation (5000, 10000, 15000, and 20000 psi) was evaluated for the pre-emergence herbicidal activities namely inhibition of seed germination and seedling growth, seed imbibition, and α-amylase of *Amaranthus tricolor* seed. The obtained results of herbicidal activities correlated with droplet size that the herbicidal activities increased when increasing the pressure of microfluidization. The nanoemulsion formulation of pressure at 20000 psi treatment solution showed the highest herbicidal activities. Thus, these results may promote the optimized nanoemulsion from peppermint essential oil using a microfluidization method as a natural pre-emergence herbicide to inhibit seed germination and seedling growth of *A. tricolor*.

Keywords: High-energy emulsification, Natural herbicide, Pre-emergence herbicide, Essential oil, Nanotechnology

Introduction

The finding for a sustainable alternative material for natural herbicide production is necessary due to the use of chemical herbicides that may affect to environment and human health (Chotsaeng *et al*., 2019). The natural materials for natural herbicide production should friendly environment without contamination in an ecosystem for sustainable weed management.

Essential oils (EOs) are a complex compound mixture of secondary metabolites obtained from plants that are known for various biological activities (Falleh *et al*., 2021). In literature, the main compounds of EOs were monoterpene

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alcohols or oxygenated monoterpenes which can result in inhibition of weed germination and development. Peppermint essential oil (PEO) was documented that can be used as a candidate for a natural herbicide (Synowiec *et al*., 2019). Campiglia *et al.* (2007) reported that PEO can inhibit seed germination of ryegrass (*Lolium perenne*). In addition, PEO has a strong effect on seed germination of *Erigeron bonariensis* L. and *Araujia sericifera* Brot. (Bellache *et al*., 2022). The use of EO needs to formulate due to their weaknesses when they are exposed to light, humidity, heat or oxygen (Kaur *et al*., 2021). Commonly, PEO-based natural herbicide was formulated into an emulsion with emulsifier agents for convenience applications (Somala *et al*., 2022). In literature, emulsions are fabricated into nanoemulsions to improve stability and activity efficiency (Mustafa and Hussein, 2020). Nanoemulsion is a thermodynamically unstable colloidal dispersion that consists of tiny droplets of one liquid distributed in another immiscible liquid (Uluata *et al*., 2016). Generally, nanoemulsion droplet size is ranging from 20 to 200 nm (Somala *et al*., 2022). One unique advantage of nanoemulsions are kinetically stable over a prolonged duration of time, and their effects are particularly functional because of the tiny droplet size of the nanoemulsion (Gupta *et al*., 2016).

Microfluidization emulsification method for nanoemulsion preparation is incomparable to the different types of traditional homogenization (Yousef *et al*., 2023). A microfluidizer machine operates on the guide of separating a pressure stream into two portions, giving each part via a fine orifice, and running the flows at each other in the heart of the machine. This machine operates a high pressure to drive the outpour stream via microchannels toward the impingement area, which makes a very heightened shearing activity that delivers an exceptional nanoemulsion. Within the interaction chamber, cavitation, along with shear and influence, decreases droplet size (Mahdi Jafari *et al*., 2006). Via this technique, nanoemulsions can be fabricated with small droplet size (100 – 1000 nm) (Villalobos-Castillejos *et al.,* 2018).

The research finding aimed to fabricate PEO-based nanoemulsion using microfluidization method at pressures of 5000, 10000, 15000 and 20000 psi. PEO-based nanoemulsion was evaluated droplet characteristic and herbicidal potential on *Amaranthus tricolor*.

Materials and methods

Preparation of PEO nanoemulsion

PEO was used as a bioactive material for natural herbicide fabrication in nanoemulsion formulation. Tween 60 was used as an emulsifier agent. Firstly, PEO was mixed with Tween 60 using a magnetic stirrer for 5 min. Then, deionized water was added to the mixture and continuously stirred for 5 min. The obtained coarse emulsion was fed into a microfluidizer (Microfluidics, Newton, MA, USA) at different pressures of 5000, 10000, 15000, and 20000 psi for a cycle. The obtained solutions were stored at room temperature for further experiment.

Characteristics of PEO nanoemulsion

Droplet size, PI value and zeta potential of PEO nanoemulsion were evaluated using a dynamic light scattering (DLS) technique. PEO nanoemulsion was diluted with deionized water (1:9) to avoid scattering influence.

Herbicidal activities

Germination bioassay

The inhibition effect of PEO nanoemulsion on seed germination and plant development of *A. tricolor* was determined using Petri dish bioassay. PEO nanoemulsion treatments were prepared at different pressures of 5000, 10000, 15000 and 20000 psi and PEO concentrations of 200, 400 and 800 μL/L. Two germination papers were placed in Petri dishes. 5 ml of each treatment solution was added to the Petri dishes. Then, 20 seeds of *A. tricolor* were placed onto the germination papers. All dishes were incubated in a growth chamber for 7 days at 27 ± 2 °C and 12 h light/day. After 7 days, germination count and root and shoot lengths were measured.

Seed imbibition

PEO nanoemulsion was determined the inhibition of seed imbibition. The nanoemulsion treatments were prepared at different pressures of 5000, 10000, 15000 and 20000 psi and PEO concentrations of 200, 400 and 800 μL/L. Seed imbibition was performed following the method of Turk and Tawaha (2003). The tested seeds were weighed (W1) and soaked in the nanoemulsion and incubated in dark box for 15 h. Then, the tested seeds were washed and weighed (W2) again. Seed imbibition (%) was calculated as follows:

$$\text{Seed imbibition (\%)} = [(W2 - W1)/W1] \times 100$$

α-Amylase activity

After seed imbibition assay, the tested seeds were grained with 4 mL of 0.1 M $CaCl_2$. The obtained solution was centrifuged at 10000 rpm, 4 °C for 20 min.

The supernatant was kept at 4 °C. α-Amylase activity in the tested seeds was measured as reported by Sadasivam and Manickam (1996) using the dinitrosalicylic acid (DNS) method. The supernatant was mixed with 1% soluble starch solution and incubated at 37 °C for 15 min. Then, the solution was added by 1 mL of DNS reagent and boiled at 100 °C. Ultimately, the absorption at 560 nm was recorded.

Results

The effect of pressure of microfluidization on characteristics of PEO nanoemulsion droplet

Microfluidization emulsification method was used for PEO-based nanoemulsion fabrication. PEO nanoemulsions with different pressures of microfluidization (5000, 10000, 15000 and 20000 psi) presented small droplet size below 200 nm (Table 1). After microfluidization process, droplet size of the nanoemulsion was reduced from 130.2 to 69.8 nm by increasing the pressure from 5000 to 20000 nm. Also, PI value of the nanoemulsion decreased from 0.171 to 0.136 when increasing the pressure from 5000 to 15000 psi. However, the lowest PI value was found at the pressure of 15000 psi. Zeta potential of the nanoemulsion increased negatively from -26.31 to -44.17 when the pressure was from 5000 to 20000 psi.

The nanoemulsion with microfluidization at the pressure of 5000 and 10000 psi appeared white milky. However, the nanoemulsions at the pressures of 15000 and 20000 appeared a blue tint and were without a phase separation (Figure 1).

Table 1. Effect of the pressure of microfluidization on characteristics of droplet of PEO nanoemulsion.

Pressure (psi)	Characteristics of nanoemulsion droplet		
	Size (nm)	PI value	Zeta potential (mV)
Coarse emulsion	N/A	N/A	N/A
5000	130.2 a	0.171 b	-26.31 a
10000	123.7 b	0.150 c	-39.48 b
15000	123.8 b	0.136 c	-41.81 bc
20000	69.8 c	0.277 a	-44.17 c

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

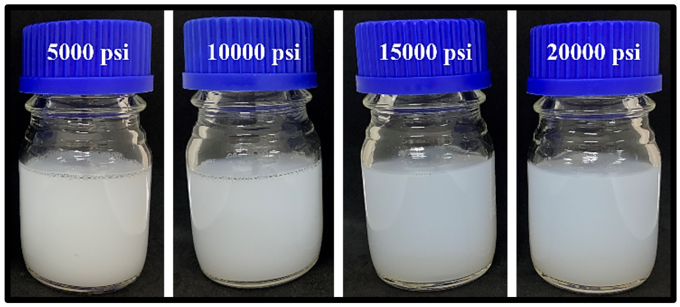

Figure 1. The microfluidized PEO nanoemulsions2u at different pressures of 5000 – 20000 psi

Inhibition effect of PEO nanoemulsion at different pressures of microfluidization on seed germination and seedling growth of A. tricolor

The microfluidized PEO nanoemulsions at pressures of 5000, 10000, 15000 and 20000 psi were determined for the inhibition potential on seed germination of *A. tricolor*. In, the inhibition effect level of PEO nanoemulsion increased when increasing the concentrations from 200 to 800 µL/L (Figure 2A). At seven days after treatment, the level of inhibition effect of all nanoemulsion formulations at a concentration of 200 and 400 µL/L are non-significant inhibition. At 20000 psi, seed germination was inhibited by 82.5% for PEO nanoemulsion concentrations of 800 µL/L relative to control which is the highest inhibition level.

The inhibition effect on seedling growth (shoot and root length) of the nanoemulsion also increased when increasing in a dose-dependent manner (Figure 2B and 2C). As a result, the inhibition effect of nanoformulations on shoot and root length was non-significant inhibition in each concentration. At a concentration of 800 µL/L, the nanoemulsion at a pressure of 20000 psi showed the highest root inhibition level (59.92% over control) significantly different from the course emulsion (without microfluidization) and 5000 psi.

Inhibition effect of PEO nanoemulsion at different pressures of microfluidization on seed imbibition and α-amylase activity in A. tricolor

Seed imbibition and α-amylase activity in *A. tricolor* was inhibited by the treatment of PEO nanoemulsion at different pressures in a concentration-dependent manner. PEO nanoemulsion showed non-significant differences in the inhibition in all pressures at concentrations of 200 and 400 µL/L compared to control (Table 2). At a concentration of 800 µL/L, the nanoemulsions showed a significant inhibition compared to control except for a pressure of 10000 psi.

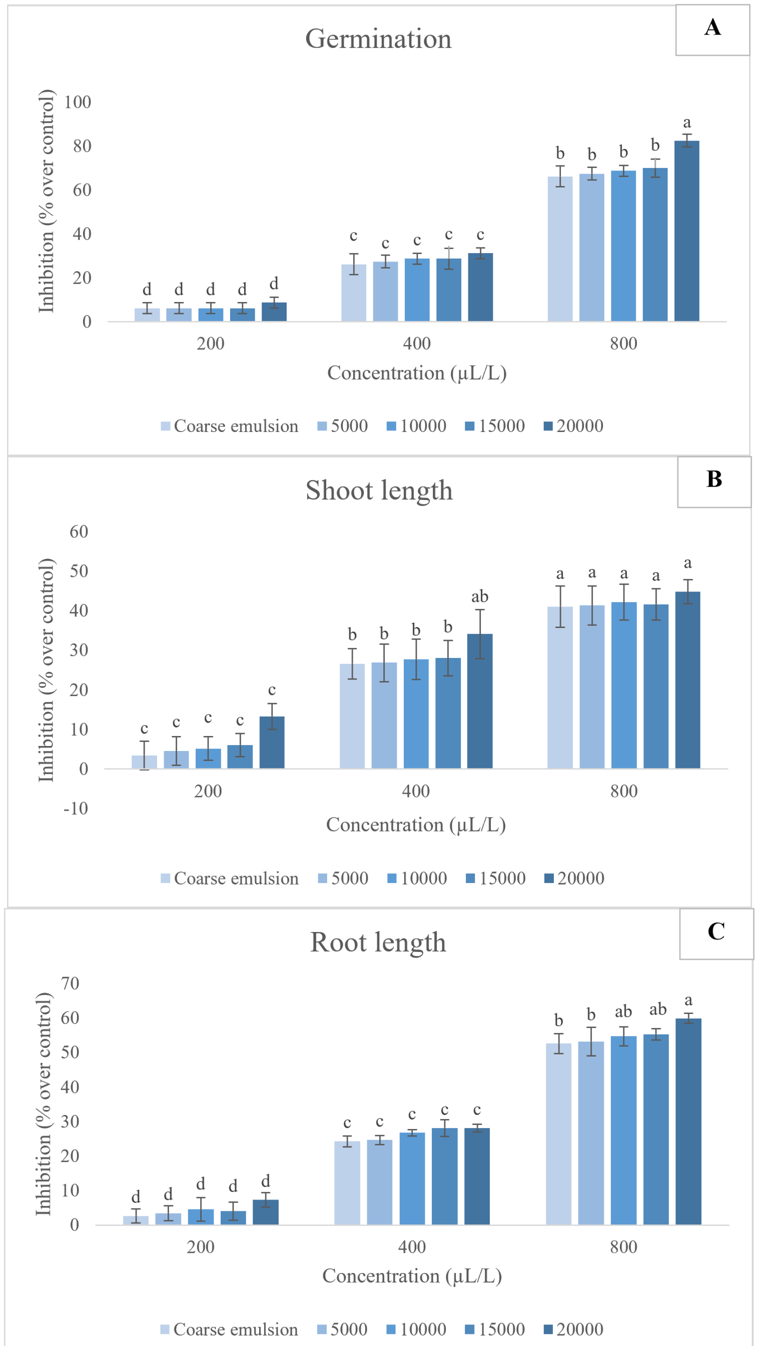

Figure 2. Inhibition effect of PEO nanoemulsion at different pressures on germination and shoot and root growth of *A. tricolor*

The coarse emulsion and the microfluidized nanoemulsion at a concentration of 200 µL/L showed a non-significant different inhibition of α-Amylase activity in *A. tricolor* compared to control (Table 3). At 400 and 800 µL/L, the α-amylase activity was marked to decrease after applying with the nanoformulations and the inhibition level increased with increasing pressure of the formulation.

Table 2. Influence of pressure of microfluidization on seed imbibition inhibition effect of PEO nanoemulsion at different concentrations at 15 h after treatment

Pressure (psi)	Seed imbibition (%)		
	200 µL/L	**400 µL/L**	**800 µL/L**
Control (water)	38.05 ± 0.73 a	38.05 ± 0.73 a	38.05 ± 0.73 a
Coarse emulsion	37.76 ± 1.03 a	36.00 ± 0.56 a	35.21 ± 1.43 b
5000	37.38 ± 2.16 a	36.66 ± 1.34 a	35.31 ± 1.21 b
10000	36.95 ± 0.93 a	36.77 ± 1.16 a	35.67 ± 0.74 ab
15000	37.57 ± 0.56 a	36.64 ± 1.16 a	35.07 ± 0.82 b
20000	37.15 ± 0.99 a	36.33 ± 0.66 a	35.20 ± 0.15 b

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

Table 3. Influence of pressure of microfluidization on α-amylase activity inhibition effect of PEO nanoemulsion at different concentrations at 15 h after treatment

Pressure (psi)	α-Amylase activity (µmol maltose/min/gFW)		
	200 µL/L	**400 µL/L**	**800 µL/L**
Control (water)	5.03 ± 0.28 a	5.03 ± 0.28 a	5.03 ± 0.28 a
Coarse emulsion	4.52 ± 0.29 ab	4.42 ± 0.30 ab	3.26 ± 0.31 b
5000	4.90 ± 0.02 b	4.65 ± 0.22 ab	3.40 ± 0.27 b
10000	4.10 ± 0.07 b	4.04 ± 0.23 bc	2.98 ± 0.27 b
15000	4.26 ± 0.24 b	3.71 ± 0.19 c	2.82 ± 0.11 b
20000	4.07 ± 0.05 b	3.65 ± 0.24 c	2.80 ± 0.13 b

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

Discussion

The results indicated that the pressures in the microfluidization affected droplet size, PI value and zeta potential of PEO nanoemulsion. Commonly, increasing the pressure of microfluidization would result in emulsions of smaller droplet sizes (Mahdi Jafari *et al.*, 2006). Above results, the microfluidized nanoemulsions found smaller droplet size compared with the coarse emulsion. The smallest droplet size was obtained by microfluidization pressure at 20000 psi similar to Uluata *et al*. (2016). In microfluidization process, the droplets of emulsion are provided to high shear and more elaborate mixing approaches, that decrease droplet size and PI of the emulsion. (García-Márquez *et al*., 2017). This implied that bigger droplets that were not disrupted at more inferior homogenization pressure were disrupted as the pressure was raised because of the more elevated importance of shearing forces produced within the interaction chamber (Goh *et al.*, 2015). PI reveals the uniformity of nanoemulsion. As a result, PI value showed a narrow distribution of nanoemulsion droplet causing a high-energy density. The nanoemulsion with the smallest droplet size had the highest negative zeta potential values. Zeta potential value above the range of ±30 mV verifies that the nanoemulsion is considered stable, defining a heightened energy barrier toward the separated phase (Somala *et al.,* 2022). Our results indicate that increased pressure of microfluidization resulted in more stability in the nanoemulsion.

The inhibition effect of PEO nanoemulsion of various pressures was seen through the germination indices of *A. tricolor*. Previous reports documented that EOs deliver a suitable inhibition potential in a dose-dependent way (Hazrati *et al.*, 2017). In the literature, chemical compounds of PEO were menthol (42.7% of the total) and menthone (25.5% of the total). Menthone intensely impacted seed germination and growth of several plants. And, menthol impacted *Arabidopsis* growth, removed the leaf cuticular coating and changed stomatal structure and operation (Synowiec *et al.*, 2019). In this work, the highest inhibition level of PEO nanoemulsion on germination and plant development was observed in the nanoemulsion with the smallest droplet size. This result may be a positive correction between the nanoemulsion droplet and seed germination inhibition effect.

Seed imbibition is the first step of seed germination process. In process of seed germination, α-amylase is a necessary enzyme that functions to digest starch, delivering nutrients and energy (Liu *et al*., 2018). The present study showed that PEO nanoemulsion at the highest concentration can inhibit seed imbibition. Previously, several researchers reported that EOs affected seed imbibition (Somala *et al*., 2022). Also, α-amylase activity in *A. tricolor* decreased correlation with seed germination similar to Somala *et al*. (2022). They reported that the α-amylase activity was inhibited by *Cymbopogon nardus* L. leaves EO on *Echinochloa crus-galli* seed. This impact could in turn reason seed germination inhibition and cause starch not to be degraded into tiny molecules to resource growth and maturation. The consequences of the current work presented that inhibition of seed imbibition and α-amylase activity is one of the mechanisms of action of PEO nanoemulsion.

In summary, this work has presented that microfluidization emulsification method with high shearing forces is a possible emulsification method to acquire

PEO nanoemulsion, via manipulation of homogenization pressure. PEO nanoemulsion having small droplets with narrow droplet distributions can be produced by optimizing the microfluidization conditions. The nanoemulsion with the smallest droplet size (69.8 nm) was observed under 20000 psi. Herbicidal activities of PEO nanoemulsion were found in all the nanoemulsion formulations. Our results facilitated the application of PEO nanoemulsion as a good alternative for weed control. In addition, PEO may possible be promoted as a sustainable material for natural herbicide production.

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