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Original Research Article

Self-nanoemulsifying drug delivery system (SNEDDS) prepared from *Begonia medicinalis* and *Moringa oleifera* ethanol extracts

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Abstract

Purpose: *To investigate the activity of self-nanoemulsifying drug delivery system (SNEDDS) containing B. medicinalis and M. oleifera ethanolic extracts.*
Method: *The SNEDDS was prepared using propylene glycol (40 %), Tween 80 (50 %), and isopropyl myristate (10 %). Transmittance percentage, particle size, polydispersity index, and zeta potential were evaluated. In vitro immunostimulant activity was measured by assessing the macrophages' phagocytic capacity and proliferation of lymphocyte cells using MTT assay. In vivo immunostimulant activity was determined by measuring the number (%) of leukocytes, neutrophils, and lymphocytes in Wistar male rats over 14 days following administration of the SNEDDS after induction with Staphylococcus aureus.*
Results: *The SNEDDS formula of combined extract of B. medicinalis and M. oleifera demonstrated polydispersity index (0.46 ± 0.00), particle size (24.4 ± 0.1 nm), zeta potential (-37.73 ± 0.32 mV) and percentage transmittance (86.81 ± 0.16 %). In vitro and in vivo tests revealed that SNEDDS significantly increased macrophage phagocytic capacity, number of leukocytes, neutrophils and lymphocytes over 14 days of treatment ($p < 0.05$) following induction with S. aureus.*
Conclusion: *Self-nanoemulsifying drug delivery system of combined ethanol extract of B. medicinalis and M. oleifera demonstrates stable physicochemical properties, and increases macrophage phagocytic activity, leukocytes, neutrophils and lymphocyte count.*

Keywords: *Begonia medicinalis, Moringa oleifera, SNEDDS, In vitro, In vivo*

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INTRODUCTION

Begonia medicinalis and *Moringa oleifera* are two examples of tropical plants that flourish in Indonesia. Native to Central Sulawesi, *B. medicinalis* is a plant with established uses as an immunostimulant, antioxidant, antiviral, and anticancer agent. *M. oleifera* plants have proven antioxidant, antibacterial, antifungal, antihypertensive, antidiarrheal, anticancer, antidiabetic, antihyperglycemic, anti-inflammatory, analgesic, and immunostimulant activities [1-4]. Phytochemical screening of *B. medicinalis* herbs and *M. oleifera* leaf extracts contained flavonoids, steroids, terpenoids, saponins, tannins, and polyphenols [4,5]. Historically, the immune system has been enhanced through the use of medicinal plants.

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Previous studies have demonstrated that combined extracts from *B. medicinalis* herb and *M. oleifera* leaves exhibited immunomodulatory effects by increasing macrophage phagocytic activity and the production of cytokines TNF-α and IFN-γ [5]. This effect may be influenced by the presence of saponin and flavonoid metabolites [4,6]. Plants rich in flavonoids and saponins have been identified as immunostimulants, as they promote the proliferation of T-cell-derived lymphocytes, which subsequently activate phagocytic cells to trigger phagocytic responses [7,8]. However, both plant extracts have low bioavailability due to their poor water solubility and sensitivity to environmental factors such as light, pH, and temperature [9].

One potential solution to enhance their bioavailability is to incorporate these extracts into self-nanoemulsifying drug delivery systems (SNEDDS). Thus, this study investigated the activity of self-nanoemulsifying drug delivery system (SNEDDS) containing *B. medicinalis* and *M. oleifera* ethanolic extracts.

EXPERIMENTAL

Materials

Begonia medicinalis (leaf and stem parts) was obtained from Toddopuli, Soyojaya District, North Morowali and *Moringa oleifera* (leaf part) was obtained from Sibedi Village, Sigi Regency, Central Sulawesi, Indonesia. Plant identification was carried out at the Laboratory of Plant Biosystematic, Department of Biology, Faculty of Mathematics and Natural Sciences, Tadulako University, with specimen numbers BM12010624 and MO13010624, respectively. Isopropyl myristate, tween 80, propylene glycol, Na-CMC 0.5 %, ketamine, and xylazine were procured from Sigma Aldrich.

Extraction

Begonia medicinalis (leaves and stems) and *M. oleifera* (leaves) were extracted by maceration using 70 % ethanol for 3 - 5 days. The collected filtrate was then evaporated by a rotary evaporator and stored till further use.

Formulation and characterization of SNEDDS

Preparation of SNEDDS combination of *B. medicinalis* herb and *M. oleifera* leaves extract (50 mg: 50 mg) was done by mixing the extract with isopropyl myristate as oil, tween 80 as surfactant, and propylene glycol as co-surfactant (1:5:4) using an ultrasonicator (model 300 131 V/T, USA). Transmittance, particle size, polydispersity index, and zeta potential of the SNEDDS were evaluated following previous method [9].

In vitro immunostimulant activity

Isolation and culture of macrophage cells

A male BALB/c mouse was anesthetized with ketamine-xylazine mixture and positioned on its back, after which the abdominal skin was incised. The peritoneal cavity was cleaned with 70 % alcohol, and 10 mL of cold Roswell Park Memorial Institute (RPMI) media was injected into it. The mouse was gently patted for 3 min to distribute the media. Using a syringe, peritoneal fluid was aspirated from the cavity cautiously selecting fluid from the lean area away from the intestines. This aspirate was transferred to a centrifuge tube and centrifuged at 1200 rpm for 10 min. The entire procedure was performed in a laminar airflow hood to ensure macrophage isolation and prevent contamination.

After discarding the supernatant, the pellet was resuspended in 3 mL of complete RPMI containing 10 % fetal bovine serum (FBS). The cell count was determined using a hemocytometer, and the cells were resuspended in complete RPMI to achieve final density of 2.5 x 10^6 cells/mL. This cell suspension was placed into a 24-well plate with round coverslips, with each well containing 200 µL (5 x 10^5 cells). The cells were incubated in 5 % CO_2 incubator at 37 °C for 30 min, after which 800 µL of complete RPMI was added, and the cells were incubated for another 24 h [10,11].

Macrophage phagocytosis activity test

Macrophage cells were isolated, incubated for 24 h, and washed with RPMI media. Subsequently, 500 µL of each preparation, consisting of a SNEDDS base and a combination of extracts from *B. medicinalis* herb and *M. oleifera* leaves at concentrations of 1000, 500, 250, 125, and 62.5 µg/mL, were added to 3 wells. Control wells for the media contained 1 mL of media, while cell control wells remained untreated and incubated for an additional 4 h. Thereafter, the media was removed, and the wells were rinsed with complete RPMI to eliminate non-adherent cells. Then, 200 µL of latex was added to each well and incubated in a 5 % CO_2 environment at 37 °C for 1 h.

The petri dish were rinsed twice with PBS, fixed with methanol for 30 s, and allowed to dry.

Coverslips were stained with 10 % Giemsa for 20 min, after which the petri dish was rinsed 3 to 4 times with distilled water and dried at room temperature.

The preparations were examined under a microscope (x40 magnification) to evaluate phagocytic activity, with macrophages appearing purple and clear latex particles visible. Phagocytic activity was quantified using the phagocytosis index and capacity as shown in Eqs 1 and 2 [10,11].

Phagocytosis capacity = $(M_2/M_1)100$ …… (1)

where M_2 is the amount of phagocytosing macrophage and M_1 is the amount of counted macrophage (100).

Phagocytosis index = (P_2/P_1) …… (2)

where P_2 is the amount of phagocytosed latex and P_1 is the amount of active macrophage (100).

Isolation and culture of lymphocytes

A male BALB/c mouse was initially anesthetized with a ketamine-xylazine mixture and positioned on its back. The abdominal skin and peritoneal cavity were opened to extract the spleen for cell isolation. The spleen was aseptically placed in a petri dish containing 10 mL of RPMI media, which was then pumped into the spleen to release lymphocytes into the media. This cell suspension was transferred to a 10 mL centrifuge tube and centrifuged at 1,500 rpm for 10 min at 4 °C. The resulting pellet was resuspended in 5 mL of Tris-buffered ammonium chloride to lyse the red blood cells, and the mixture was allowed to sit at room temperature for 15 min or until it turned slightly yellow. Thereafter, RPMI media was then added to bring the total volume to 10 mL, and the mixture was centrifuged again at 1,500 rpm for 10 min at 4 °C.

After discarding the supernatant, the pellet was washed twice with RPMI media to eliminate any remaining red blood cells, resulting in white pellets. The lymphocyte pellets were then suspended in complete RPMI media, and cell counts were performed using a hemocytometer to a concentration of 1.5 x 10^6 cells/mL. Finally, 100 µL of this cell suspension was placed into a 96-well plate with RPMI complement, and the plate was incubated in 5 % CO_2 incubator at 37 °C for 24 h [2,10].

Lymphocyte cell proliferation assay

Lymphocytes at 1.5 x 10^6 cells/mL were distributed into wells of a 96-well microplate and incubated overnight in a 5 % CO_2 incubator. Sample preparations, including test samples in the form of SNEDDS, SNEDDS base, and combined extracts, were diluted to concentrations of 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 µg/mL. Each well received 100 µL of diluted preparations, with three replicates for each concentration. Control wells for cells and media were filled with 100 µL of media and left untreated. All wells were incubated at 37 °C for 48 h in 5 % CO_2 environment [12].

After incubation, the media was removed, and 10 µL of (3-(4-dimethylthiazol-2-yl)2-5-diphenyltetrazolium bromide; MTT) solution was added to each well. The MTT solution was prepared by mixing 1 mL of MTT with complete media to a final volume of 10 mL. The wells were then incubated for additional 4 h at 37 °C, allowing living cells to react with MTT and produce a purple color. Following this, 50 µL of stop reagent (10 % sodium dodecyl sulphate (SDS) in 0.1 N HCl) was added to each well, and the microplate was covered with aluminum foil to shield it from light exposure. The plate was then incubated at room temperature for 24 h, and absorbance was read using an enzyme-linked immunosorbent assay (ELISA) reader at 550 nm [2,11].

***In vivo* immunostimulant activity**

A total of 40 male Wistar rats (weighing between 160 and 300 g) were acclimated for 7 days under standard laboratory conditions (12 h light-dark cycle, unrestricted access to feed and water). Ethical approval was obtained from the Islamic University of Indonesia (approval no. 37/Ka.Kom.Et/70/KE/VII/2023). The rats were randomly divided into eight groups (n = 5 each): Group 1 was control group (no treatment), group 2 was negative control, group 3 received stimuno (4.5 mg/kg as positive control), group 4 received SNEDDS base as a base control, group 5 received a suspension of a combination of *B. medicinalis* herb and *M. oleifera* leaf extracts (100 mg/kg each), group 6 received SNEDDS combination of *B. medicinalis* extract and *M. oleifera* leaves (50 mg/kg each), group 7 received SNEDDS combination at 100 mg/kg each, and group 8 received SNEDDS combination at 150 mg/kg each.

Leukocyte, neutrophil and lymphocyte activity

On day 1, blood was drawn in all groups of rats, and treatment continued for 14 days according to the test sample of each group. On day 15, blood was taken from all groups and a suspension of *S.aureus* bacteria was administered through the intraperitoneal to each test group except control. The rats were anesthetized using a combination of ketamine-xylazine. On day 16, blood was taken from all groups of rats through the orbital sinus. Blood samples were collected into microtubes that had been filled with 0.1 % EDTA to prevent coagulation. The number of leukocytes, neutrophils, and lymphocytes was counted using a hematology analyzer.

Data analysis

The data were analyzed using Statistical Packages for Social Sciences (SPSS, version 22.0, IBM, Armonk, NY, USA), and Microsoft Excel. Measurement data were analyzed using one-way ANOVA at 95 % confidence level and post hoc Tukey analysis if the data is normally distributed and homogeneous. If the data are not normally distributed or homogeneous, the Kruskal-Wallis test and Mann-Whitney test were used at 95 % confidence level.

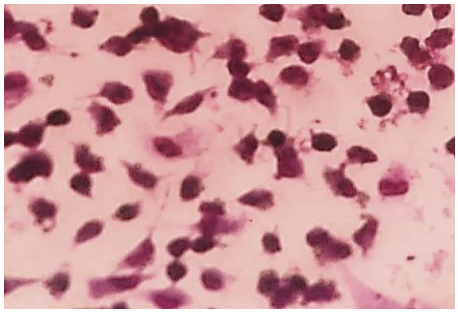
RESULTS

Characteristics of SNEDDS

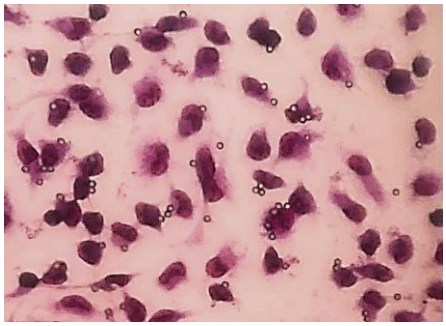
The formulated SNEDDS had transmittance value of 86.81 ± 0.16 %, polydispersity index (PDI) of 0.46 ± 0.00, particle size of 24.4 ± 0.1 nm and zeta potential of -37.73 ± 0.32 mV (Table 1). This suggests the effectiveness of SNEDDS in enhancing drug delivery [13,14].

Macrophage phagocytotic activity

Macrophages treated with the test samples exhibited higher phagocytic activity towards latex compared to control group (Figure 1). This difference is evident in both the number of active macrophages and the quantity of latex they phagocytosed. Mean phagocytic activity increased as the concentration of the extract combination rose from 62.5 µg/mL to 1000 µg/mL, with the highest activity observed at 1000 µg/mL. Also, the phagocytic activity of the SNEDDS base remained stable across different concentrations, indicating that it acts as a neutral carrier without pharmacological effects. Combination of the two plant extracts exhibited significantly higher phagocytic activity compared to control groups, highlighting its potential as an immunostimulant ($p < 0.05$; Table 2).



(a)



(b)

Figure 1: Macrophage phagocytosis activity (40× magnification). (a) Control group (b) Study group

Lymphocyte cell proliferation activity

The SNEDDS formula demonstrated strong stimulation of cell proliferation, with a stimulation index (SI) greater than 2 at 250 µg/mL (2.981 ± 0.044) and 500 µg/mL (4.649 ± 0.104). Furthermore, combination of *B. medicinalis* and *M. oleifera* extracts along with SNEDDS base, showed significantly greater SI at 500 µg/mL, and among samples at the same concentration. Also, the SNEDDS formula, SNEDDS base, and combined extracts showed significant differences in SI below 500 µg/mL (Table 3). This suggests that these formulations effectively stimulate lymphocyte proliferation, particularly at higher concentrations.

Table 1: Characteristics of SNEDDS formulation

Sample	Transmittance (%)	Particle size (nm)	Polydispersity index	Zeta Potential (mV)
SNEDDS	86.81±0.16	24.4 ±0.10	0.46 ±0.00	-37.73 ±0.32

Table 2: Macrophage phagocytosis activity and phagocytosis index of SNEDDS

Conc. (µg/mL)	Phagocytosis Capacity (%)			Phagocytosis Index		
	Combined extract	SNEDDS base	SNEDDS formula	Combined Extract	SNEDDS base	SNEDDS formula
1000	80.00±8.72[a]*	40.33±4.16[a]	58.67±9.29	1.66±0.40[a]*	0.7±0.16	0.92±0.13[a]*
500	77.33±5.03[b]*	43.33±5.13[b d]	62.00±2.00[d]	1.37±0.20[b]*	0.67±0.23	0.95±0.15[b]*
250	69.00±11.27[c]	45.67±6.43	67.33±5.51[c]	1.32±0.09[c]*	0.75±0.09[cf]	1.2±0.26[f]*
125	65.00±9.54	48.00±8.00[e]	71.33±4.04[e]*	1.21±0.29[d]*	0.90±0.16[d]*	1.31±0.48*
62.5	63.67±7.57	49.67±9.07[f]	81.33±7.23[f]*	0.96±0.25[e]	0.91±0.19[eg]*	1.62±0.37[g]*
Control cell	46.67±10.69	46.67±10.69	46.67±10.69	0.67±0.06	0.67±0.06	0.67±0.06
DMSO Control	46.00±7.55	46.00±7.55	46.00±7.55	0.66±0.10	0.66±0.10	0.66±0.10

*P < 0.05 compared to control cells and DMSO in the same sample. [abcdef]P < 0.05 between samples with the same concentration

Table 3: Lymphocyte cell proliferation stimulation index

Concentration (µg/mL)	Stimulation Index		
	Combined extract	*SNEDDS base*	*SNEDDS formula*
500	2.668±0.049[a]*	2.432±0.101[ae]	4.649±0.104[e]*
250	1.722±0.112[b]*	1.333±0.089[bf]	2.981±0.044[f]*
125	1.281±0.083[c]*	0.885±0.107[cg]	1.700±0.138[g]*
62.5	1.081±0.015[d]	0.574±0.076[dh]	1.288±0.187[h]
31.25	0.581±0.030*	0.442±0.144[i]	0.864±0.125[i]*
15.625	0.499±0.107	0.368±0.017[j]	0.486±0.054[j]
7.8125	0.339±0.026	0.354±0.044	0.357±0.017
Cell Control	1.000±0.046	1.000±0.046	1.000±0.046
DMSO Control	0.943±0.150	0.943±0.150	0.943±0.150
Media Control	0.000±0.039	0.000±0.039	0.000±0.039

*P < 0.05 vs control cells and DMSO in the same sample. [abcdefghij]P < 0.05 vs samples at the same concentration

Leukocytes, neutrophils and lymphocyte activities

The SNEDDS combination of *B. medicinalis* and *M. oleifera* extracts at 50 mg/kg showed significantly higher leukocyte (15.94 x 10^3/µL; Figure 2 A), neutrophil (5.90 x 10^3/µL; Figure 2 B), and non-significant lymphocyte (10.04 x 10^3/µL; Figure 2 C) count after 14 days compared to control group ($p < 0.05$) particularly highlighting the effectiveness of treatment as an immunostimulant (Figure 2).

DISCUSSION

The obtained SNEDDS formula was investigated as an immunostimulant by measuring the *in vitro* macrophage phagocytosis, lymphocyte cell proliferation and *in vivo* leukocyte, neutrophil, and lymphocyte activity in male Wistar rats. Macrophages serve as primary phagocytic cells in the immune system, playing a crucial role in defending against foreign pathogens through phagocytosis in both innate and adaptive responses. The phagocytic ability is assessed using latex particles, where the phagocytosis index indicates the number of particles engulfed by 100 active macrophages, and phagocytosis capacity reflects the number of macrophages capable of consuming these particles.

Fluorescent yellow latex spheres signify successful phagocytosis, while empty macrophages indicate inactivity. An increase in both phagocytosis index and capacity suggests enhanced macrophage activity [10]. Also, lymphocyte proliferation occurs in response to antigen stimulation, where lymphocytes specifically recognize foreign antigens and mediate humoral and cellular immunity. This proliferation is evaluated using MTT assay which measures the color intensity of formazan crystals with an ELISA reader. This reflects cell viability and proliferation. The results are expressed as a stimulation index (SI), where values below 2 indicate no effect, 2-3 suggest weak activity and values above 3 denote active stimulation of lymphocyte proliferation [2].

Findings from this study indicated that lower SNEDDS concentration (62.5 µg/mL) demonstrated enhanced macrophage phagocytosis activity, suggesting that the SNEDDS formulation improves solubility, bioavailability, and absorption due to its small particle size [15]. The phagocytosis index also reflected values greater than 1 indicating strong immunostimulant properties, particularly at lower concentrations [16].

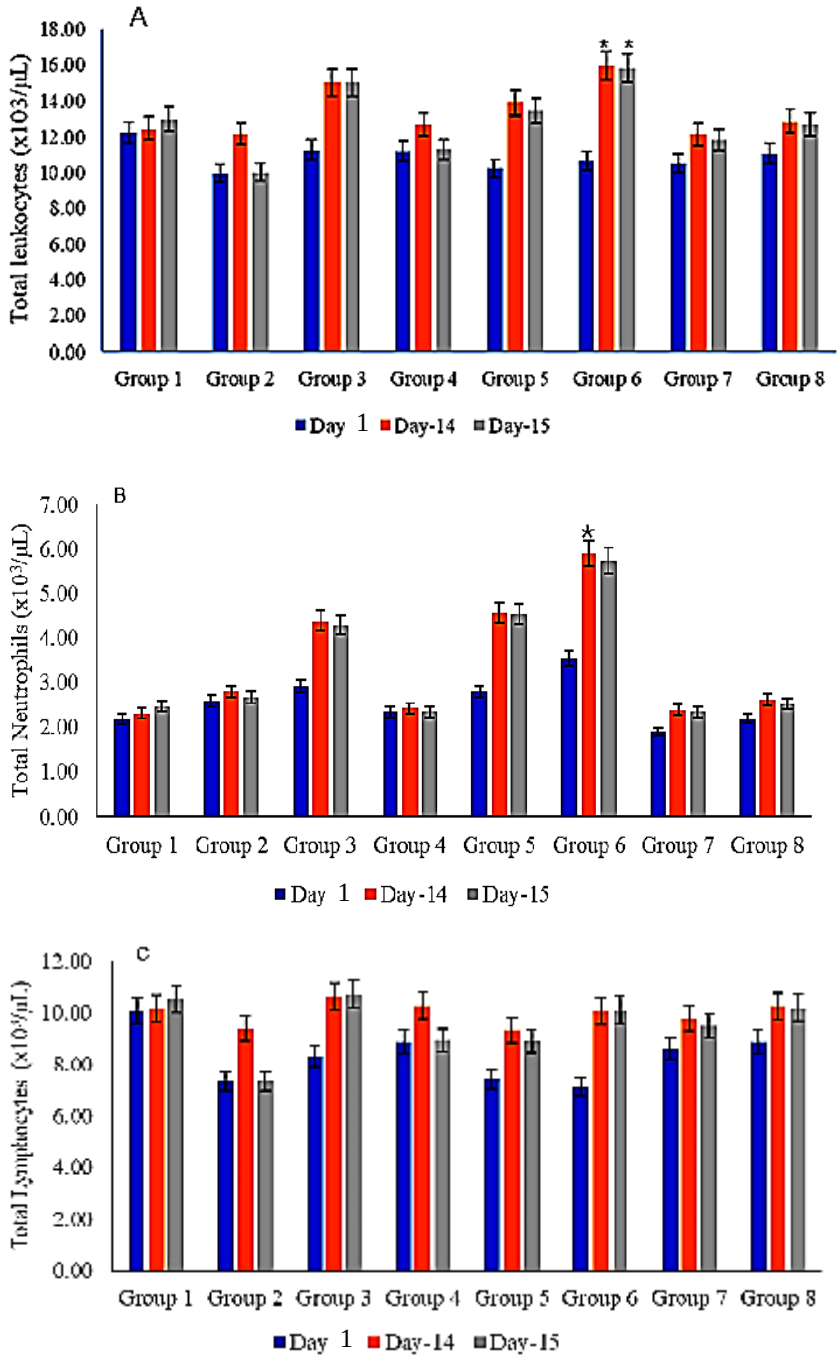


Figure 2: Leukocyte (A), neutrophil (B) and lymphocyte (C) activities on days 1, 14 and 15 (Group 1: control; group 2: negative control; group 3: stimuno 4.5 mg/kg as positive control; group 4: SNEDDS base; group 5: suspension of 100 mg/kg: 100 mg/kg extract combination, group 6: 50 mg/kg: 50 mg/kg SNEDDS extract combination, group 7: 100 mg/kg: 100 mg/kg SNEDDS extract combination, group 8: 150 mg/kg: 150 mg/kg SNEDDS extract combination. *P < 0.05 vs other groups of each cell count

Similarly, neutrophil counts also rose across all groups, with group 6 again showing the most significant increase The flavonoid compounds in the extracts are believed to contribute to this immunostimulatory effect [17].

In examining the immune response in rats induced by *S.aureus*, the number of leukocytes did not significantly increase over 15 s, likely due to a consistent biochemical reaction to the antigen and the migration of leukocytes from the bloodstream to infected tissues. Similarly, neutrophil counts remained stable, as these immune cells are utilized more rapidly than they are produced, with factors such as splenic enlargement and macrophage-mediated clearance further contributing to their reduced numbers. In contrast, lymphocyte counts

significantly increased in the treatment group which may be attributed to flavonoids like kaempferol and quercetin from *Moringa oleifera* leaves. These compounds enhance cytokine production and stimulate the proliferation and differentiation of B and T cells, ultimately supporting antibody production and a robust immune response [5,17].

CONCLUSION

Self-nanoemulsifying drug delivery system (SNEDDS) of combined ethanol extract of *B. medicinalis* and *M. oleifera* demonstrates stable physicochemical properties, increases macrophage phagocytic activity, leukocytes, neutrophils, lymphocytes count, and induces the proliferation of lymphocytes.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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