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Efficacy of Single Love Garlic Xtract on Self Nanoemulsifying Drug Delivery System (SCGE-SNEDDS) on Cytokine Expression *In Vitro*

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ABSTRACT

Garlic has several bioactive lipophilic, volatile and found low bioavailability that reduces the effectiveness of its active compounds. A novel drug delivery system is required to preserve the bioactive and enhance solubility. Self-Nanoemulsifying Drug Delivery System (SNEDDS) can load extract that is formed from carrier oil (VCO), surfactant (Tween-80), and co-surfactant (Glycerol). The study aimed to investigate the responses, characterization, cytotoxicity and efficacy of SNEDDS of single clove garlic (*Allium sativum* L.*)* on the expression of TNFα, IL-1β, and IL-10 in vitro. The response test of SCGE-SNEDDS includes emulsification time, % transmission, pH and grade emulsion. The characterization of SGCE-SNEDDS was analyzed using particle size, PDI, and zeta potential. The toxicity test was determined with MTT assay and expression of cytokine TNF-α, IL-1β and IL-10 was determined using CLSM. The characteristics response emulsification time is 55.77 ± 6.69 seconds, % transmittance value of 78.86 ± 0.23%, and pH of 6.83 ± 0.72. The average particle size was 82.14 ± 18.12, Z-Average 0.38 ± 0.02 and zeta potential -33.07 ± 0.45 mV. The toxicity assay was determined in the 3T3-L1 cell line using MTT. SCGE-SNEDDS have a good response and characteristics, which are non-toxic, and have high cell viability. SCGE-SNEDDS formula potentially decreased the expression of TNF-α and increased anti-inflammatory cytokine IL-10 cytokines in 3T3-L1 cells.

Key words: SNEDDS, Single clove Garlic Extract (SCGE), VCO, Cytokine

INTRODUCTION

Obesity is a major issue faced by numerous countries in the world. Obesity has been linked to inflammation. Inflammation is believed to be related to cells and tissues that respond to internal stimuli, metabolic products, and external factors, such as bacteria and viruses (Kaneko *et al*., 2019). Innate and adaptive immunity systems are also responsible for responding to inflammatory mechanisms through pro-inflammatory cytokine regulation in blood, interleukin-1 (IL-1), IL-6, interferon-γ and tumor necrosis factor alpha (TNF-α) (Del Valle *et al*., 2020; Liu *et al*., 2009). Because of their pivotal role in the recruitment and activation of neutrophils, TNF-α and IL-1 are identified as markers of inflammation and organ damage. TNF- α may also regulate IL-1β production (Del Valle *et al*., 2020; Ogawa *et al*., 2014). Several studies indicate that adipose tissues and cells could release various adipokines and inflammatory factors (Cheng *et al*., 2019). Aside from macrophages, adipocytes and preadipocytes could produce proinflammatory cytokines. A range of studies uses undifferentiated 3T3-L1 cells to model inflammation (Renovato-Martins *et al*., 2020; Zhou *et al*., 2008).

Garlic (*Allium sativum* L.) has been reported to have an immunomodulatory activity that triggers T cell proliferation, interleukin expression, and antioxidant activities. It contains organosulfur, saponins, phenolics, flavonoids, and polysaccharides (Metwally, 2009; Shang *et al*.,

2019). Single clove garlic (*Allium sativum* L.) is commonly used to treat several diseases. Single clove garlic generally grows in an unfavorable environment that induces abnormalities of growth, thus it only has one bulb (Lestari & Rifai, 2019).

Garlic is commonly consumed as fresh garlic, cooked garlic, oil, powder, or extracted garlic (Shang *et al*., 2019). Garlic extract has a strong aroma and some of its components are lipophilic and volatile such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide, diallyl tetra sulfide, and ajoene (Abiko *et al*., 2021; Ferioli *et al*., 2020; Petrovska & Cekovska, 2010). Garlic extract has a low bioavailability that reduces the effectiveness of its active compounds (Torres-Palazzolo *et al*., 2018). Due to these limitations, drug delivery strategies must be developed to boost the penetration activity of compounds, and drug targeting strategies must be used to control drug release. Nanoparticles are an example of a drug-delivery mechanism that can be used.

Numerous nanotechnology-based drug delivery systems are currently being developed. For example, a nano-sized particle-based delivery system can be utilized as a nanoemulsion (Occhiutto *et al*., 2020). Co-surfactant and surfactant systems maintain a dispersion of two immiscible liquids called a nanoemulsion (Zorzi *et al*., 2015). Self-Nanoemulsifying Drug Delivery System (SNEDDS) is a stable nanocarrier made of oil, surfactants, cosurfactants, liquid active compounds, or homogenous emulsions. It is intended to shield drugs' active ingredients from enzyme breakdown (Izham *et al*., 2019).

Surfactants and co-surfactants facilitate emulsion enhancement with active compounds. The oil phase will dissolve lipophilic extract elements (Ujilestari *et al*., 2018a). Virgin coconut oil (VCO) is the best carrier oil in the SNEDDS system. VCO is a product of coconut kernel that is often used in pharmaceutical and food industries and has a stable chain at a high temperature (Binsi *et al*., 2013). Inhibition of pro-inflammatory cytokines of IL-1β, IL-6, IL-8, and TNF-α was observed in the application of Genkwanin-contained SNEDDS (Dou *et al*., 2018). SNEDDS Brucine extracts have the potential to inhibit pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL-10, TNF-α, and IFN-γ (Yin *et al*., 2021). However, there are no studies on the effect of SNEDDS with garlic extract on pro-inflammatory and anti-inflammatory cytokine. Therefore, the current study aimed to investigate the effectiveness of SNEDDS of single clove garlic (*Allium Sativum* L*)* extract with VCO carrier oil phase on the expression of IL-1, IL-1β and TNF-α in vitro.

MATERIAL AND METHODS

The making of SCGE-SNEDDS used the following materials, VCO (MAYANG, Semarang, Indonesia), Tween 80 (sigma Aldrich, Germany), Glycerol (Merck, Germany), single clove garlic from Ngadas Village, Malang Regency, double destilated water (Ikha Pharmindo, Indonesia). 3T3-L1 pre-adipocyte cell line were obtained from the Laboratory of Structure, Development, and Physiology of Animals, Biology Department, Science and Mathematics Faculty, Universitas Brawijaya. The culture cells reagent included Dulbecco's Modified Eagle's Medium (DMEM) (Gibco®, USA), Penicillin-Streptomycin, Fetal Bovine Serum (FBS) (Gibco®, USA), Trypsin (Gibco®, USA) and Trypan Blue (Gibco® USA). Sodium Ethylenediaminetetraacetic acid (Na.EDTA), Sodium Phosphate Anhydrates (Na_2HPO_4), Sodium dihydrogen phosphate (NaH_2PO_4) Sodium Chloride (NaCl) from Nacalai Tesque Inc., Japan. Deionized water (DI water) (Otsuka®, Indonesia). Methylglyoxal (Sigma Aldrich, Germany), MTT reagent (Nacalai Tesque Inc., Japan). IL-10, IL-1β, TNF-α antibody from Santa Cruz Biotechnology Inc., USA. Dimethyl sulfoxide (DMSO) (Merck, Germany), Paraformaldehyde (PFA)(TCI, Japan), Bovine Serum Albumin (BSA) (BioWest, France). Triton X (Merck, Germany), Rhodamine (abcam, USA), and fluorescein isothiocyanate (FITC) secondary antibody (abcam, USA). Tools used in the research included HORIBA SZ-100 Particle Size Analyzer, Transmission Electron Microscope (TEM) (Tecnai 200 kV D2360 SuperTwin, Japan), spectrophotometer UV-VIS (Libra S11/12 Visible & UV Spectrophotometers, UK), magnetic stirrer (Thermolyne cimarec®2, USA), ultra turrax (IKA©-WERKE), sonicator (IWAKI Ultrasonic Cleaner Japan), Confocal Laser Scanning Microscopy (Olympus FV1000, Japan), and Inverted microscope (Olympus IX53, Japan).

The Extraction of Single Clove Garlic Extract (SGCE)

Single clove garlic was obtained from Ngadas Village, Malang Regency. Samples were chopped and dried in a shaded place at room temperature. The extract was done using the maceration process refer to Lestari *et al*. 2021 with modification. One hundred grams of single clove garlic was extracted using 70% ethanol with a ratio of 1:3 and then put

into the shaker water bath for 1x24 h at a temperature of 37°C. The extraction results were evaporated using a rotary evaporator.

SNEDDS Preparation and Drug Loading

The process was conducted by weighting the VCO oil phase (0.546 g): Tween 80 surfactants (3.180 g) : Glycerol co-surfactants (1.273 g). Surfactants and co-surfactants (mixture A) were homogenized using ultraturrax for 5 min and stirred for 15 min. One hundred µg of single clove garlic extract (SCGE) was gradually mixed with VCO in the beaker glass and stirred until homogeneous. The mixture of SCGE and VCO (mixture B) was also homogenized using ultraturrax for 5 min before stirring once again for 15 min. Mixture A and B were homogenized using the ultraturrax for 5 min. The mixture homogeneity was stirred for 15 min and put in the shaker water bath at 500 rpm, 45°C, 15 min. The mixture was stirred again for 15 min and allowed to sonication for 15 min. The SNEDDS system mixture was then rested for 24 h until stable. The system was observed using a response test.

Response Test of SCGE-SNEDDS

Emulsification Time

Fifty µL of SCGE-SNEDDS was emulsified in 5 mL double distilled water and then homogenized using vortex. The emulsification time was calculated after the SNEDDS were homogenous and perfectly dissolved (Pratiwi *et al*., 2017).

pH Test

The emulsion pH was observed using pH meter. The optimum pH was 6-8 (Pratiwi *et al*., 2017).

Percent of Transmittance Test

Fifty microliter of SCGE-SNEDDS was emulsified in 5 mL double distilled water. The mixture was mixed with a vortex until homogenous. The percent transmittance was measured using a spectrophotometer UV-VIS at 650 nm and double distilled water as the blank solution. Good nanoemulsions exhibited a percentage of transmittance of more than 90% (Pratiwi *et al*., 2017).

Visual observation of emulsion grade

Fifty microliter of SCGE-SNEDDS was emulsified in 5 mL aquabidest and then measured using the following criteria (Czajkowska-Košnik *et al*., 2015; Izham *et al*., 2019). Grade A: Rapidly forming emulsion (within 1 min), clear or bluish; Grade B: Rapidly forming emulsion (within 1 min), slightly less clear and bluish tint white; Grade C: Smooth milky emulsion (within 2 min); Grade D: Slowly forming emulsion (> 2 min), dull, greyish, and slightly greasy; Grade E: Poor or minimal emulsification with large oil droplets surface.

Characterization of SCGE-SNEDDS

SCGE-SNEDDS characterization was conducted by measuring Z-average, PDI, and Zeta Potential using a particle size analyzer. Morphological characteristic was then carried out using visualization with TEM. The emulsion used for the PDI, Z-average, zeta potential, and TEM characterization was in a ratio of 1:1000. Good SNEDDS was indicated by PDI < 0.5, homogenous distribution, 200 nm in system size, and a zeta potential value > +30 mV or < -30 mV (Ujilestari, *et al*., 2018b).

Cytotoxicity Test of SCGE-SNEDDS and Determination of IC_{50}

The 3T3-L1 pre-adipocyte cell lines were obtained from the Laboratory of Structure, Development, and Physiology of Animals, Biology Department, Science and Mathematics Faculty, Brawijaya University. The 3T3-L1 cells were cultured in a complete medium consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal bovine serum, 1% penicillin-streptomycin. The 3T3-L1 cells were cultured in a 25 cm^2 Tissue Culture (TC) flask and incubated in the incubator with 5% CO_2 at 37°C until ± 80% confluency and then harvested.

About 5 x 10^3 cells were transferred into 100 µL culture media in each 96-well plate with the number of well needed. The cells were washed with 100 µL of PBS 1x in each well. Each *well* was added with 100 µL of culture media that contained SCGE-SNEDDS according to the following concentration (n=3): 62.5, 125, 250, 500, 1000, 2000, and 4000 µg/mL for each formulation and incubated for 24 h. After incubation, the culture media was removed. The cells were washed with 100 µL PBS 1x in each well. About 100 µL MTT solution was added to each 96-well plate, except for the blank samples. The cells were incubated for 2-4 h in the incubator until purplish formazan crystals were formed. If the formazan had clearly formed, 100 µL of DMSO was added to stop the reaction. The well plate was covered with aluminium foil, rested for 30 min and then put into the shaker for 5 min. The absorbance of each samples was measured using a microplate reader at 595 nm.

Induction of Inflammation

The 3T3-L1 cells were induced with methylglyoxal before SCGE-SNEDDS treatment (Chotimah *et al*., 2015). The 3T3-L1 cells were maintained in the 25 cm^2 TC flask until ± 80% confluency. Cells were then harvested, and the number of total cells was calculated. Suspended cells from the flask were moved to a 15 mL centrifuge tube and centrifuged for 4 min at 2500 rpm. The pellet was added 1 mL of culture media, and then 20 uL of suspension was placed in a centrifuge tube. The cells aliquoted mixed with 20 uL trypan blue. The total cell numbers were calculated with dispersed 20 uL of mixed cells and trypan blue into a hemocytometer. Cells ($1x10^4$) were transferred into 300 µL culture media in each 24-well plate where the coverslip was already in the well. The cell condition was observed with an inverted microscope to identify cell distribution. The cells were incubated in the incubator for 24 h. Cells were treated with several SCGE-SNEDDS concentrations, including 62.5, 125, and 250 µg/mL, after the cells returned to their normal condition. After the treatment, cells were washed with 100 µL PBS and added with 100 µL culture media containing 5 µL/mL methylglyoxal and incubated in the incubator for 24 h to induce inflammation.

Examination of TNF-α, IL-1β, and IL-10 expression

The TNF-α, IL-1β, and IL-10 expression in the 3T3-L1 cells were evaluated using the Immunocyto Chemistry (ICC) method. About $1x\ 10^4$ cells were seeded into 24 well plates and divided into six groups (n=3): control treatment group (C), MG (methylglyoxal), Single Clove Garlic extract + MG (SCGE), 62.5 µg/mL SCGE-SNEDDS + MG (SCGE-SNEDDS 1), 125 µg/mL SCGE-SNEDDS + MG (SCGE-SNEDDS 2), and 250 µg/mL SCGE-SNEDDS + MG (SCGE-SNEDDS 3). The cells were incubated with 5% CO_2 for 24 h at 37°C. After incubation, the cell media were aspirated, and cells were washed 3 times with PBS 300 µL.

Cells were fixed with 100 µL of 4% paraformaldehyde (4% PFA) for 5 min and then washed with 100 µL PBS. The cells were incubated with 100 µL 0.5% Triton-x for 30 min and 5% BSA for 30 min. The solution of Triton-x and BSA was removed. Each sample was incubated with the following antibody: TNF-α primary antibody, IL-1β primary antibodies and IL-10 primary antibodies overnight. The cells were washed with 100 µL of PBS for 3 times. The cells were incubated for 1 h with rhodamine-conjugated secondary antibody for TNF-α and TRITC-conjugated secondary antibody for IL-1β and IL-10, and then washed with 100 µL of PBS for 3 times. The coverslip was carefully removed. The TNF-α expression of the 3T3-L1 cells was immediately observed using Confocal Laser Scanning Microscope (CLSM) at the Central Laboratory of Life Sciences, Brawijaya University.

RESULT AND DISCUSSION

Response Test of SCGE-SNEDDS Formulation

Characterization and responses are essential examinations for SNEDDS because they can directedly affect the in vitro tested characteristics (Buya *et al*., 2020). The SCGE-SNEDDS formulations were evaluated for emulsification time, percent transmittance and pH of emulsion. SNEDDS formula can form emulsions automatically and spontaneously after direct contact with gastrointestinal fluids. Following a light stir in water media, SNEDDS would turn into an oil emulsion in smooth water. By lowering the surface tension between the oil and water surfaces, the formulation's surfactants helped turn the oil phase into very small particles (Baloch *et al*., 2019).

The result of emulsification time suggests that the SCGE-SNEDDS emulsified in approximately 1 min (Table I). High quality SNEDDS emulsification time was less than 1 min (Eid *et al*., 2014). When spontaneous emulsification occurs in the digestive tract, the faster the emulsion time, the more quickly drugs will be absorbed since the absorption surface area is larger. The emulsification process occurred after a light stir in water media, SNEDDS would turn into oil emulsion in very smooth water. The value of SCGE-SNEDDS was considered in a nanodispersion (Table I) (Nasr *et al*., 2016). Transmittance is often represented as a percentage, where the measurement result represents the amount of light that passes through the sample. The emulsification rate can be monitored by the enhancement of transmittance value and the correlation between the percentage of transmittance and nanoparticle droplet size (Buya *et al*., 2020). The 100% of transmittance indicates that produced emulsion's droplets are already nanometer-sized (Khan *et al*., 2015; Nasr *et al*., 2016). The pH value of SCGE-SNEDDS falls within the optimal range of 6-8, meeting the required specifications for SNEDDS formulations (Table I).

Table I. The response of SCGE-SNEDDS with VCO carrier oil phase

No	Type of Test	Result
1.	Emulsification time	55.77 ± 6.69 s
2.	% transmittance	98.86 ± 0.23 %
3.	Emulsion pH	6.71 ± 0.50

Table II. PSA Results of SCGE-SNEDDS characterization with VCO carrier oil phase

No	Data	SCGE-SNEDDS Average ± SD
1.	Z-Average (nm)	82.14 ± 18.12
2.	PDI (Polydispersity Index)	0.38 ± 0.02
3.	Zeta Potential (mV)	-33.07 ± 0.45

When SNEDDS is applied to the skin, pH testing is essential to determine their safety because if the pH level is too low, it may irritate the skin. Additionally, a high pH might cause scaly skin (Pratiwi *et al*., 2017).

The formulation resulted was homogenous, transparent, and slightly yellowish. The appearance of the formed emulsion was homogeneous and in a bluish category. The appearance of the emulsion is in grade A but the emulsification time is more than one minute. Grade A and Grade B formulations ensure the formation of nanometer-sized nanoemulsions when dispersed in gastrointestinal fluids (Senapati *et al*., 2016). The appearance is regarded with a percentage transmission. The clear appearance indicated transmittance of SNEDDS is close to 100% and is related to particle size (Bali *et al*., 2011).

Characterization of SCGE-SNEDDS Formulation

Characterization of SCGE-SNEDDS is crucial to assess the stability of the formulation of the SCGE-SNEDDS system. The SCGE-SNEDDS formulation demonstrates that its particle size falls within the optimal range for effective SNEDDS, which is 20-200 nm (Table II). Drug release rate and average velocity are correlated with particle size. The surface area for drug absorption increases with droplet size in an emulsion. The SCGE-SNEDDS formulation was in the <200 nm; hence, it has reached a good formulation (Izham *et al*., 2019). The nanosize in the formed SNEDDS droplet was ideal at 20-200 nm (Kazi *et al*., 2019, 2020; Khan *et al*., 2015). Due to the decreased particle size and higher surface area, the SNEDDS formulation generally dissolved in vitro more rapidly than the original drug. Drug release rate and average velocity are correlated with particle size. Nanosize in the SNEDDS droplet will increase lymphatic absorption, determining the drug efficacy (Buya *et al*., 2020; Kazi *et al*., 2020; Khan *et al*., 2015). Tween-80 as surfactant content is reported to decrease the value of the Tween-80 PDI (Hidajat *et al*., 2020).

The polydispersity Index (PDI) value less than 0.5 indicated that the particles were homogeneously dispersed (Table II) (Ali & Hussein, 2017). A low PDI value suggests a monodisperse emulsion with high stability, whereas a high PDI value indicates low stability (Polychniatou & Tzia, 2014). When producing pharmaceutical or food-grade products, PDI is crucial for the physical characteristics that must be paid attention to. Materials used in making SNEDDS can influence mass nature, product performance, the capability of process, stability, and appearance of final products. In formulating a lipid-based bioactive carrier system, PDI analysis illustrates the degree of non-uniformity of the particle size distribution (Danaei *et al*., 2018).

The synthesized SCGE-SNEDDS indicated stability, as the zeta potential value was greater than -30 mV, which helps reduce agglomeration (Table II). Emulsions are regarded as stable if their positive or negative zeta potentials are greater than 30 mV (Eid *et al*., 2014). Zeta potential directly affects surface stability and mobility, suggesting electrostatic repulsion in the formed droplet groups. The electrostatic force of the emulsion can assess the extent of the formed SNEDDS system stability (Zhang *et al*., 2015). The non-ionic surfactants may be responsible for the negative value of the zeta potential. A more negative value than 30 mV is considered ideal for making the emulsion stable since the same negative load will cause droplets to repel each other, avoiding aggregation and destabilization (Galvão *et al*., 2018).

The SCGE-SNEDDS droplets were in nano-size and round shape, indicated by a scale of ±50 nm (Figure 1B). However, some of the SCGE-SNEDDS droplets showed agglomeration (Figure 1A). The agglomeration suggests instability in the produced SCGE-SNEDDS system. The size of the dispersion droplet observed through TEM provides information on the particle shape that could affect drug solubility. This factor is often ignored in various emulsion tests that mostly assume ball-like particle shapes (Kazi *et al*., 2019). Aggregation observed in the TEM image could happen due to being dispersed in water (Izham *et al*., 2019). However, the aggregation in the TEM test result did not support data of zeta potential measurement result that indicated a stable value. This has not previously been reported. According to Kazi *et al*., (2019), increasing the concentration of non-ionic surfactants can result in a better self-emulsifying system that renders the interface negatively charged, increasing the stability of the formulation and lowering the aggregation rate.

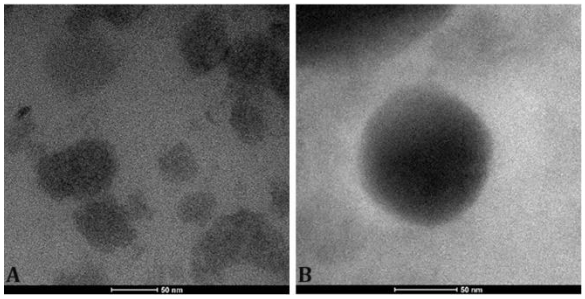

Figure 1. The morphological observation of SCGE-SNEDDS droplets using TEM analysis. A. agglomeration from SCGE-SNEDDS droplets; B. The shape of SCGE-SNEDDS droplets.

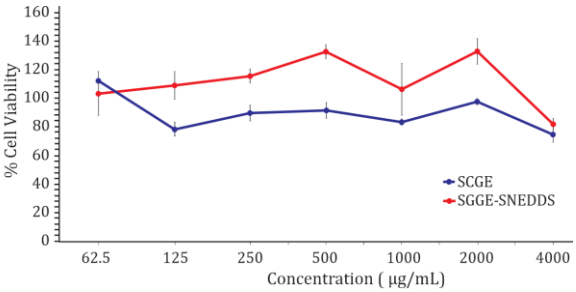

Figure 2. The viability of 3T3-L1 cells after being treated with SCGE and SCGE-SNEDDS

Viability of 3T3-L1 cells after treated with SCGE-SNEDDS formulation

The cytotoxicity assay will ensure that the SNEDDS is safe to use and expected to decrease proinflammatory factors in the body. All the data were taken for a good and optimal SNEDDS formula. Higher cell viability was observed on 3T3-L1 cells after being treated with 2000 µg/mL SCGE-SNEDDS (133.06 ± 9.13 %) (Figure 2). While the lowest cell viability was found in 3T3-L1 cells after being treated with 4000 µg/mL SCGE-SNEDDS (82.30 ± 4.05 %). In general, the viability of the 3T3-L1 cells after being treated with SCGE-SNEDDS was high (>80%). While the cell viability of 3T3-L1 after being treated with SCGE was >70%.

The highest viability of 3T3-L1 cell was observed at a concentration of SCGE of 62.5 µg/mL (112.35 ± 2.02 %), whereas the lowest was found in 4000 µg/mL of SCGE (74.79 ± 4.81 %). The cell viability criteria were 90% viability (no toxicity), 60-90% viability (low toxicity), 30-60% viability (moderate toxicity), and < 30% viability (severe toxicity) (Tabari *et al*., 2017).

A perfect drug delivery system maximizes therapeutic effects while minimizing toxicity. Low toxicity could enhance drug efficiency, reduce concentration without losing efficacy, and fewer side effects (Milhomem-Paixão *et al*., 2017), SNEDDS still showed purple formazan crystal expressed by mitochondria. It was indicated that SCGE and SCGE-SNEDDS had low toxicity. It was demonstrated by forming a large number of formazan crystals, indicating a healthy cell. Gruhlke *et al.*, 2017 stated that garlic extract had low toxicity to NIH-3T3 fibroblast cells (Gruhlke *et al*., 2017). Vemuri *et al.*, (2018) also elucidated that garlic extract is non-toxic to normal cells and toxic to Human carcinomas cell lines such as A549 and SCC-9 cells. Flavonoids, as one of the bioactive of garlic, are identified as having low toxicity to 3T3-L1 cells (Kim *et al*., 2020). Flavonoids in the flavonol type that can be found in *Allium* species are related to a modulatory effect on oxidative stress and inflammation. The compound can regulate the amount of ROS in cells through a variety of mechanisms such as nuclear factor erythroid 2-related factor 2 (Nrf2), which is a transcription factor for cellular antioxidant. Nrf2 regulates inflammation through the direct regulation of antioxidant enzymes and pro-inflammatory genes and crosstalks with nuclear factor-κB (NF-κB) (Kothari *et al*., 2020).

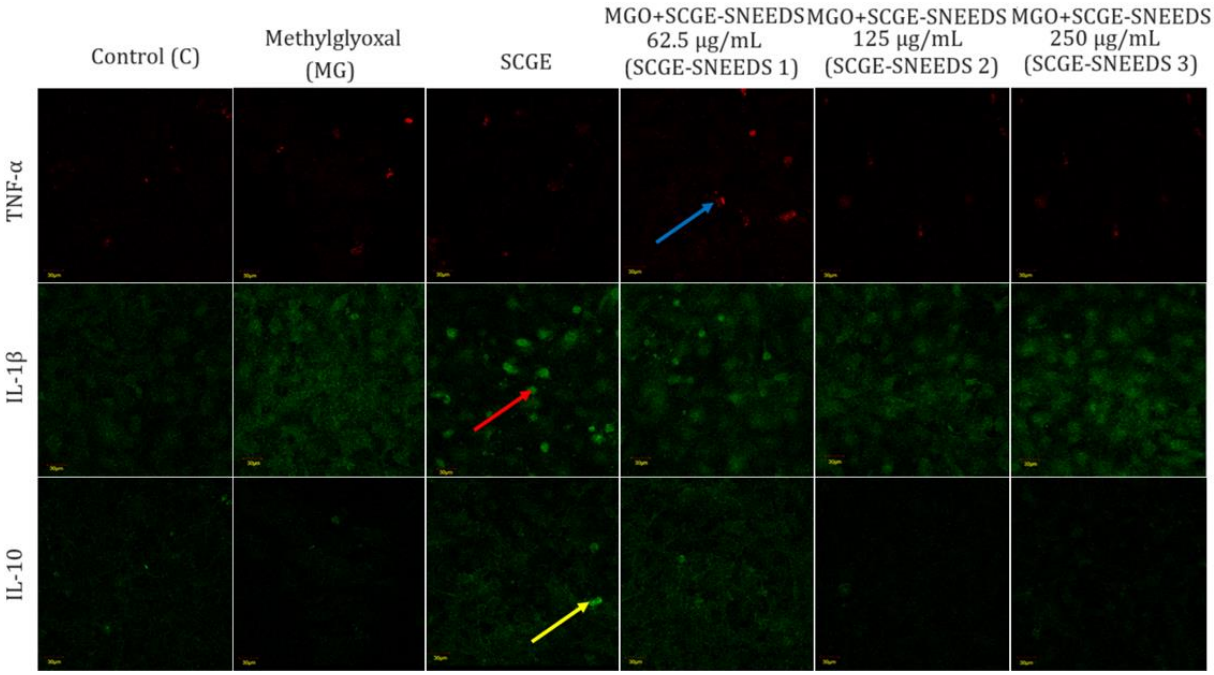

Figure 3. The expression of TNF-α, IL-1β, and IL-10 cytokines in 3T3-L1 cells after Methylglyoxal induction. The intensity of fluorescent in each sample were observed using CLSM.
Note: ↑. TNF-α (red), ↑. IL-1β (green), and ↑. IL-10 (green)

The low toxicity of SNEDDS might be caused by surfactant content. Several research found that non-ionic surfactants were low toxicity for the cells. Tween-80 and glycerol in the components of SNEDDS used are non-ionic surfactants (Abdulredha *et al*., 2019; Polychniatou & Tzia, 2014). So, it might be the reason why in this research, the SNEDDS non-toxic for 3T3-L1. As the comparison SNEDDS in study by Alghananim *et al.*, (2020), with a surfactant of Tween-80, was also relatively non-toxic for K562 cells.

Formulation test of SCGE-SNEDDS on IL-1β, TNF-α, and IL-10 expression in 3T3-L1 cells

Methylglyoxal (MG) activates inflammatory pathways and stress responses through SAPK/JNK signalling (Wang *et al*., 2021). The activation mechanism consists of P-PKCβ, which will activate NOX4 and increase ROS in the cells. High ROS will activate MAPKs via p-P38, p-ERK, and p-JNK, followed by activation of p-NF-κB and P65; thus, it will increase gene expression and proinflammatory cytokine secretion of TNF- α and IL-1β (D. Kim *et al*., 2021; Li *et al*., 2006). The expression of TNF-α in the control group was not significant ($p>0.05$) with MG treatment group, indicated by the reduced intensity of fluorescent (Figure 3). Interestingly, SCGE significantly decreased ($P<0.05$) TNF-α expression compared to the control and MG group (Figure 4A). SCGE was not significant compared with SCGE-SNEDDS 2 and SCGE-SNEDDS 3. SCGE-SNEDDS 1 had the highest TNF-α expression. SCGE-SNEDDS could decrease TNF-α expression at a concentration of 125 µg/mL (SCGE-SNEDDS 2). This new finding is supported by the report of Lee *et al.*, 2012 which showed decreases in TNF after garlic extract treatment; however, the extract was not made in SNEDDS. Several studies have demonstrated organosulfur compounds, such as alliin and allicin in garlic, can inhibit TNF-α-induced inflammation by decreasing ROS levels (Hall *et al*., 2017; M. Zhang *et al*., 2017).

The induction of MG in 3T3-L1 cells significantly ($p<0.05$) triggers the production of IL-1β compared to the control group (Figure 4B). However, SCGE and SCGE-SNEDDS treatments could not decrease IL-1β expression in the 3T3-L1 cells. Our finding showed that SCGE treatment increases the level of IL-1β (Figure 4B).

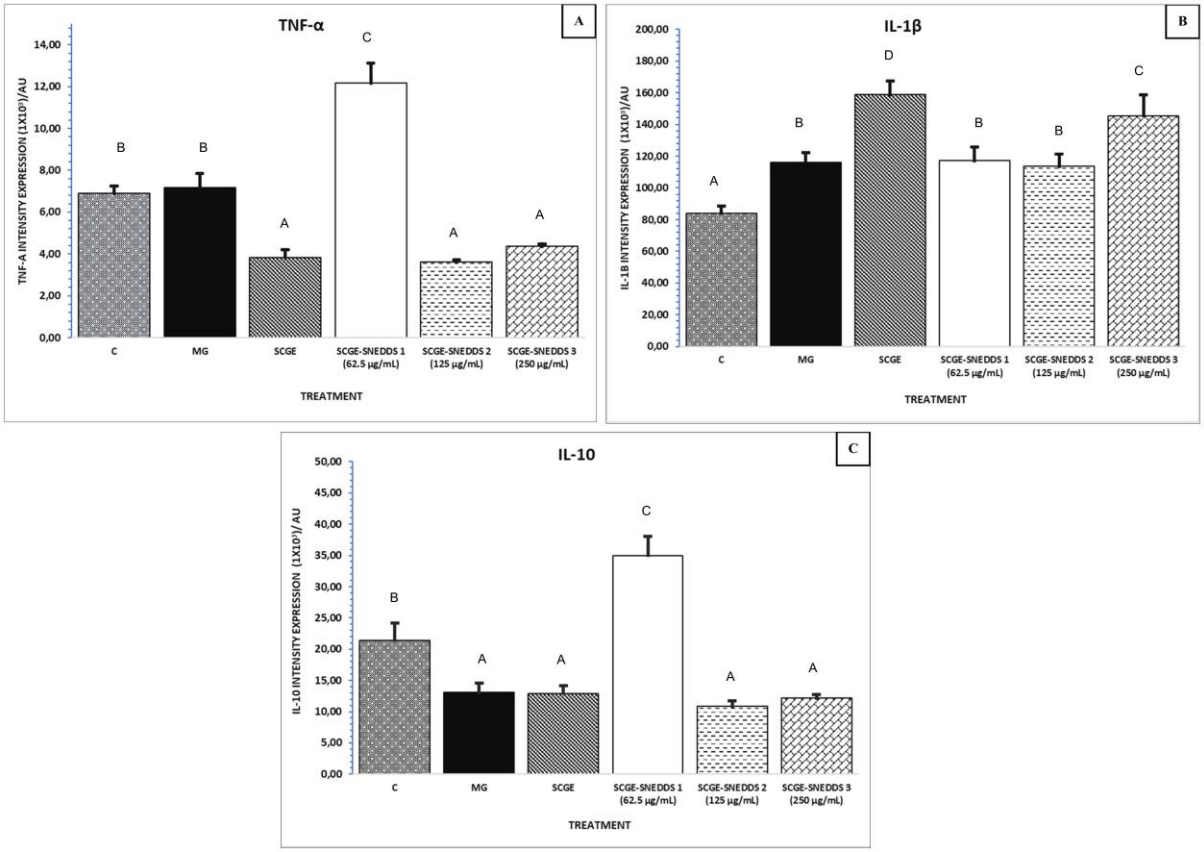

Figure 4. The cytokine intensity of A. TNF-α, B. IL-1β, and C. IL-10 in 3T3-L1 cells after Methylglyoxal induction.
Note: K : Control; MG :Methylglyoxal; SCGE = Single Clove garlic extract; SCGE-SNEDDS 1 = MG + 62.5 µg/mL SCGE-SNEDDS; SCGE-SNEDDS 2 = MG + 125 µg/mL SCGE-SNEDDS; SCGE-SNEDDS 3 = MG + 250 µg/mL SCGE-SNEDDS

SCGE-SNEDDS 1 could enhance ($p<0.05$) IL-10 expresion in the 3T3-L1 cells compared to other treatment group. The decreased intensity of IL-10 was found in SCGE-SNEDDS 2 and 3, SCGE, and MG (Figure 3). The highest IL-10 production in SCGE-SNEDDS 1 did not reduce the production of IL-1β, as indicated in Figure 4B. These results was not in line with Sun *et al.*, 2019 which reported that IL-10 could suppress IL-1β expresion in cells. Several studies have identified IL-10 action inhibits proinflammatory cytokine production in human monocytes through the suppression of NF-kB activation (Dhingra *et al*., 2009; Sun *et al*., 2019). IL-10 cytokines in humans can be regulated by white adipose tissue (WAT) that can limit IL-1β and TNF-α activities as proinflammatory cytokines (Juge-Aubry *et al*., 2005; Wojdasiewicz *et al*., 2014). It is suggested that SCGE-SNEDDS administration does not significantly reduce the expression of cytokines; therefore, further testing on other anti-inflammatory markers is required.

CONCLUSION

SCGE-SNEDDS was a good SNEDDS and stable formulation due to zeta potential value >-30 mV despite the result of the TEM that still indicates an aggregation. The SCGE-SNEDDS droplets were in nano-size and round shape. SCGE-SNEDDS had low toxicity in the 3T3-L1 cells. The administration of 125 and 250 µg/mL SCGE-SNEDDS could suppress TNF-α expression and did not significantly reduce IL-1β and IL-10 expression in inflammatory induced-3T3-L1 cells.

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