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Evaluation of the efficacy and toxicity of massoia oil nanoemulsion

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Abstract: In order to enhance essential oil's stability and water insolubility, *Massoia aromatica* oil nanoemulsion was formulated and tested on the planktonic growth and biofilm formation of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*; macrophage phagocytosis and on Vero cells viability. Oil in water nanoemulsion formula was optimized by using several solvents and co-solvents composition. The stability test of the formula was conducted by using a six cycle's freeze-thaw technique. Particle size and morphology were analyzed using a particle size analyzer and transmission electron microscopy. Microbial growth, biofilm formation inhibition, and cytotoxicity assays were performed on the optimized formula by using micro dilution methods. Mice macrophage phagocytosis activities against latex and *C. albicans* in the presence of samples were evaluated. Massoia nanoemulsion was obtained as a transparent yellowish emulsion having 99.6-99.9% of transmittance; physically and chemically stable; showed stronger antibacterial and antibiofilm on *P. aeruginosa* and *S. aureus*, moderate to *C. albicans*; no significant different on phagocytic activities. The IC_{50} of massoia oil nanoemulsion and massoia oil towards Vero cells were 35.9µg/mL and 107.5µg/mL respectively. Massoia oil nanoemulsion can protect the stability and decreases the hydrophobicity of the oil, conserve the antimicrobial and immunomodulatory activities, but increases its cytotoxicity.

Keywords: Massoia oil, Nanoemulsion, antimicrobial, cytotoxicity, macrophage phagocytosis.

INTRODUCTION

Microorganisms involved in biofilms are more resistant to host defense mechanism and also to most antimicrobials, and usually become a reservoir responsible for relapse infection (Donlan and Costerton, 2002). In accordance with extensive reported studies on antimicrobial and antifungal potencies of essential oils in general (Baratta *et al.*, 1998; Cowan, 1999; Holetz *et al.*, 2002), our earlier study has revealed the prominent inhibitory effects of that obtained from the bark of *Massoia aromatica* against *P.aeruginosa*, *Staphylococcus aureus* (Pratiwi *et al.*, 2015) and *C. albicans* planktonic growth and biofilms, as well as its potential stimulation on mice macrophage phagocytosis (Hertiani *et al.*, 2016). Despite its potential anti-infective activity, massoia oil as similar to other essential oils exhibits high lipophilicity and unstable properties. Nanoemulsion is an example of pharmaceutical nanoparticles, having a system which emulsified oil in water. The mean of droplet diameters ranges from 50 up to 1000nm. Nanoemulsions offer a better solubility for a larger quantity of drugs having low solubility and yet protect the drugs from degradation. However as the nanoparticles can penetrate cells more efficiently, the potential toxicity may arise (Chime *et al.*, 2014; Suciati *et al.*, 2014).

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MATERIALS AND METHODS

Sample materials

M. aromatica bark was obtained from Sorong, West Papua. Sample identity was confirmed by Pharmacognosy Laboratory, Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia (DR. Djoko Santosa, M.Sc.). Dried pulverized barks were distilled for 6h by steam-hydro distillation to obtain the essential oils. The oil was kept in a light-protected vial and ready for assays and analyses.

Microbial strains and culture conditions

Bacterial stocks (*P. aeruginosa* NCTC 12924 and *S. aureus* ATCC 29213) were inoculated in Luria Bertani (LB) media. Overnight incubation was taken place in a shaking incubator at 28°C for *P. aeruginosa* and 37°C for *S. aureus*. Cultures dilution by 100 fold with LB media were adjusted to OD_{600} 0.1 (approximately 10^8 CFU ml^{-1}) and ready for further assays.

C. albicans ATCC 10231 was inoculated in Soja-Dextrose Broth (SDB). A culture of $5x10^7$ CFU/mL in SDB was used for the assay. The total volume in each well was 200µL which included SDB, cells and samples.

Optimization of nanoemulsion formula

The optimization was performed in two steps. The first step was optimizing the kind and ratio of oil, surfactant and co surfactant composition. After finding the best formula, drug load was optimized.

Surfactant and co-surfactant were weighed and mixed and stirred with a magnetic stirrer, followed by 10min sonication. Meanwhile, distilled water was heated to 70°C. The mixture was stirred again, and heated water was added in drops until a stable emulsion occurs. Afterwards, the mixture was sonicated for 10min, and measured for transmittance at 650nm by spectrophotometer UV-Vis.

Characterization of massoia oil nanoemulsion and stability testing

Clarity / turbidity test

The level of clarity of nanoemulsion of which distilled water was used as a blank. The emulsion droplets reached the nano size when the absorbance of the emulsion is not significantly differed to the water absorbance.

Particle size distribution

Particle size analyzer and mean of nanoemulsion droplet diameter were measured by a Particle Size Analyzer (Horiba Scientific SZ-100). The measurement was performed at *scattering angle* 90 at 25°C.

Nanoemulsion droplet morphology

Morphology of the droplet was observed by Transmission Electron Microscope (JEM 1400). The sample was dripped onto a *copper grid*, and carbon coated for 5s and left dry at room temperature for 24h. Afterwards, the procedure was repeated once, and the sample was put into a *holder* and analyzed in 120kV.

Stability testing

Thermodynamic stability testing was done by left the emulsion on freeze (±4°C) and thaw (±25°C) condition in 5 cycles of 25h each according to Suciati *et al*. (2014) with modification. The transmittance was observed before and after the testing takes place. Besides, the stability of the chemical constituents was observed by comparing the profile of the nanoemulsion before and after the stability test. Further, nanoemulsion was stored for three months at room temperature to observe the physical and chemical stability.

Chemical stability testing was performed by using TLC by using silica gel F_{254} as the stationary phase, toluene : ethyl acetate (93:7v/v) as the mobile phase, in 2µL spotting Detection was performed under 254 and 366nm UV lights and using anisaldehyde-H_2SO_4 as spraying reagent.

Antimicrobial susceptibility testing, a micro dilution method (CLSI, 2007)

Cultures of *S. aureus* and *P. aeruginosa* (5mL in LB broth) and *C. albicans* in SDB, prepared as previously described, were incubated for an additional 2h and diluted to reach $5x10^5$ CFU ml^{-1}. Assays were taken place on sterile flat-bottom 96-well polystyrene micro titer plates and used Mueller Hinton (MH) broth medium (SDB for *C. albicans*). Controls were prepared as follows, negative controls (cells + media), positive controls (cells + media + antibiotic - streptomycin or nystatin for *C. albicans*), vehicle controls (cells + media + MeOH), and media controls. All plates were incubated overnight at 37°C (*S. aureus*) or 28°C (*P. aeruginosa*) or 37°C 48h (*C. albicans*). Readings of the optical density were conducted at 595nm. All tests were performed in triplicate. The formula from Pirbalouti *et al.* (2010) was used to determine the MIC of the EOs (11):

Inhibition % = [(ODc –ODt) / ODc] ×100

ODc is the OD_{595} of the negative control at 24h post-inoculation, and ODt is the OD_{595} for the tested samples tested at 24h or 48h post-inoculation. The essential oils concentration caused growth inhibition of microbes by at least 50% was considered as the MIC_{50} (Pirbalouti *et al*., 2010)

Biofilm formation inhibition assay

(Pratiwi *et al*., 2015; Hertiani *et al*., 2016) A 5µL culture *S. aureus* or *P. aeruginosa* or *C. albicans* (10^7 CFU mL^{-1}) was put into each well of 96-well polystyrene flat-bottom micro titer plates containing a solution of medium and tested samples. Negative controls were prepared by addition of 100µL TSB medium for assay on *S. aureus*, while on *P. aeruginosa*, M63 medium was used with added supplements, i.e. 20% casamino acid, 20% glucose and 1mM $MgSO_4$ for. SDB was used for assay on *C. albicans*. The positive control was prepared as 1mg mL^{-1} streptomycin in the medium.

Following overnight incubation at 28˚C (*P. aeruginosa*) or 48 hours at 37˚C (*S. aureus* and *C. albicans*-mature phase), the wells were poured off. After 3 times rinsing with distilled water, the plates were left to dry at room temperature for 10min. Staining was conducted by adding 125µL crystal violet 1%, left for 15min. After the staining was being discarded and rinsed with tap water to eliminate excess stain, 200µL ethanol was added to the wells, and the solution was transferred to other flat-bottom 96-well plate. Optical density at 595nm was measured, and the results were used to determine the % inhibition and the minimum biofilm inhibitory concentration, MBIC values. The inhibition percentage was calculated as the average of OD of the control wells in comparison to that of the sample wells, as defined by the following formula:

[(ODcontrol – Odsample) / ODcontrol] x 100

Effect on phagocytosis activity of macrophages

Measurement of phagocytosis activity was conducted by using three μm latex beads ($2.5\times10^6 mL^{-1}$ suspended in PBS) and *C. albicans* $6.25x10^6$ CFU/mL in SDB). After 24h incubation, the culture of peritoneal macrophages in wells equipped with cover slips was washed twice with RPMI 1640 and then added to a serial dilution of samples ranging from 10-40μg/mL. Incubation was taken place in a 5% CO_2 incubator at 37°C for 4h. After rinsing the cells three times with PBS, suspension of latex beads (200μL/well) was added. Further incubation was performed in 5% CO_2 incubator at 37°C for 30min. The latex beads were removed by rinsing the cells three times with PBS. Following fixation with 300μL/well methanol for 1min and then poured off, the cover slip was allowed to dry, and 300 μL Giemsa dye 10% v/v was added and left for 20 minutes. After the dye was being discarded, rinsed with distilled water and dried, the macrophage phagocytic index was counted under light microscope. The phagocytic index was calculated according to the following formula (Syamsudin *et al*., 2008).

$$\left(\frac{\text{Total number of active macrophage in each 100 macrophages}}{\text{Number of macrophages engulfed cells or beads}}\right)$$

The animal handling has been approved by the Ethical Clearance Commission for Preclinical-Studies of the LPPT-UGM under Nr. 217/KEC-LPPT/II/2015 (The Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Indonesia)

Cytotoxicity assay

Vero cells were cultured at 37°C under a humidified atmosphere containing 5% CO2 in $25cm^2$ plastic culture flask containing M199 medium of which 10% FBS, 100U/mL penicillin, and 100μg/mL streptomycin were supplemented. Culture medium was removed when the cell reached 90% confluence. Trypsin EDTA was dispensed to the cell cultures for detaching cells from the flask. The cells suspension is having a density of 1×10^4 cells/well in 100μl medium and incubated overnight in a CO_2 incubator at 37°C. Afterwards, 100μl of each serial dilution samples (unformulated massoia oil and its nanoemulsion) was dispensed into each well. After 24h of the incubation period, the media were removed from the plate. Cell viability was identified using MTT reagent [3 (4, 5-dimetyltiazol-2- yl) -2.5-diphenyl tetrazolium bromide]. One hundred microliters MTT was dispensed into each well. Following 4h incubation, 100μl stopper solution (10% SDS) was added (Sakurazawa and Ohkusa, 2005). After 24 incubation, the optical density was measured by micro titer plate reader at a wavelength of 595nm and calculated as follows:

Percentage of viable cells = [(OD Treatment– blank) / (OD control-blank)] x 100%

RESULTS

Massoia oil was obtained as a clear yellow oil having a distinguishing sweet-coconut like aroma with 0.3% v/w recovery calculated from the dried pulverized bark.

Nanoemulsion formulation and nanoparticles characterization analysis

After exploring of several oil possibilities, we found that a nanoemulsion basis formula using virgin coconut oil (VCO) showed a better appearance than the test using olive oil. The result of screening for finding the best basis composition has recommended the formula B as the chosen formula (table 1 and fig. 1). The addition of the massoia oil resulted a good nanoemulsion as exhibited by a transmittance value of 90% (table 2).

The clarity of nanoemulsion can be measured by determining its transmittance. The smaller the particle size, the fluid will be more transparent. The transmittance of the chosen formula was measured at 650 nm of which distilled water was set as blank (T=100%). The results showed a transmittance of 99.95%±0.06% for massoia oil (table II). Transmittance value of a formula which is approaching 100% indicates that the formula tested is clear and transparent (Bali *et al.*, 2010).

The parameters used to determine the particle size distribution of nanoparticles system is Polydispersity Index (PI). PI value ranges between 0-1. The smaller the value of PI indicates that the particle size distribution in the system occurs as more uniform nanoparticles (Galindo-Rodriguez *et al*., 2004).

As shown in fig. 3, the PI values of massoia oil nanoemulsion particles were 0.391 suggesting a uniform nanoparticles size distribution. Particle size distribution data indicates that the size of the nanoparticle samples ranged between 7-194 nm with an average particle size of 20.8nm (fig. 2). By using transmission electron microscopy (TEM) imaging, we found out that the nanoparticles of massoia oil have a spherical shape with sizes ranging from 100-200nm (fig. 3). Transmission electron microscopy (TEM) can be used to analyse the morphology of nanoemulsion. However, it could not be used for an accurate observation and for measuring with certainty the diameter of nanoemulsion tested. This is in contrast to the measurements conducted using PSA that could indicate the particle size distribution and the average droplet diameter of nanoemulsion particles.

The thermodynamic stability test was performed using a six cycle's freeze-thaw method. The formula stability was evaluated based on the organoleptic, separation and turbidity which were observed macroscopically, while transmittances were determined by a spectrophotometer (table 3), and a TLC profile of the nanoemulsion profiles were also analysed by comparing before and after treatment (fig. 4).

Table 1: Nanoemulsion formulation using VCO

Formula	Tween80 & PEG400			VCO (gr)	Aquadest	Morphology	Transmittance (650 nm)
	Tween80 (S)	PEG400 (K)	K+S				
A	16.67	8.33	25	5	70	Cloudy white emulsion formed	3.4 %
B	17.33	8.67	26	4	70	Clear yellowish nanoemulsion formed	91.5 %
C	18	9	27	3	70	Foggy micro emulsion formed	76.8 %

To determine the significance of differences in transmittance measurement before and after freeze thaw test, statistical tests using Paired-Samples T Test was performed. The results of statistical data processing showed that the nanoemulsion transmittance before and after freeze thaw tests differed significantly ($P<0.05$).

In addition to be able to maintain nanoemulsion shape at different and extreme temperatures, a formula tested must also show capability in maintaining the stability of the active substance. The result at figure 5 showed that before and after five cycles of thermodynamic stability test, the active material content in the massoia oil nanoemulsion formula remained stable, as can be shown from the Rf values of spots samples before and after freeze thaw tests, which show similar characteristic to spot of C10 massoia lactone.

The massoia oil nanoemulsion was considered as stable while the transmittance shows no significant different with the blank after three months. Based on TLC-densitometry, C-10 massoia lactone content on the massoia oil nanoemulsion was calculated as 3.4% ± 0.3% w/v.

Influence of massoia oil nanoemulsion on P. aeruginosa, S. aureus and C. albicans planktonic growth and biofilm formation

We observed inhibition activity of massoia oil nanoemulsion and the unformulated oil against *P. aeruginosa* and *S. aureus* using a micro dilution method. Growth inhibition values of massoia oil on bacterial strains tested are shown in figs. 6 and 7. The essential oils used in this study showed more than 90% bacterial growth inhibition against *P. aeruginosa* and *S. aureus*, at the highest concentration tested (0.225 % v/v).

The massoia nanoemulsion showed higher antibacterial and antibiofilm activity against *P. aeruginosa* and *S. aureus* compared to unformulated oil ($P<0.05$) (figs. 5, 6). The values were higher compared to nanoemulsion blank or the oil alone, whereas at the lowest concentration tested (0.075% v/v) massoia oil nanoemulsion showed capability in inhibit as much as 77.64% of the growth of *P. aeruginosa,* and 74.43% against *S. aureus* (fig. 5).

The nanoemulsion showed higher activity towards biofilm formation of the microbial tested. At the lowest concentration tested (0.075% v/v) massoia oil nanoemulsion showed capability in inhibit as much as 47.77±0.03% of the biofilm of *P. aeruginosa,* and 50.72±0.03 % against *S. aureus* (fig. 6).

Candida albicans biofilm inhibition of massoia oil nanoemulsion was not significantly different with the unformulated oil. However, the antibiofilm activity decreased following biofilm maturity. The percentages of 24h-old-biofilm inhibition following massoia oil nanoemulsion application and massoia oil at concentration 750µg/mL; 1,500µg/mL; 2,250µg/mL were found as follows, 64.2%±3.4%; 68.9%±0.9%; 69.7%± 0.5% and 54.1±2.4%; 63.0%±2.2%; 66.3%±0.7% respectively. The percentages of 48h-old-biofilm inhibition of massoia-oil-containing-nanoemulsion and massoia oil at concentration 750µg/mL; 1,500µg/mL; 2,250µg/mL were 42.0%±1.1%; 48.0%±0.5%; 45.5%± 1.5% and 19.5%±2.3%; 28.6%±9.5%; 42.5%±1.5% respectively (fig.7). The phagocytosis activities of the massoia-oil-containing-nanoemulsion against latex and *Candida albicans* were not significantly different with the unformulated massoia oil. The IC_{50} of massoia-oil-nanoemulsion and massoia oil towards Vero cells were observed at 35.9µg/mL and 107.5µg/mL respectively.

Effect of massoia oil nanoemulsion and massoia oil on phagocytosis activity of macrophages

Results of the nonspecific and specific testing can be observed in fig. 8. Non-specific testing refers to the activity of macrophage against latex. The phagocytosis activity of macrophage treated with Massoia nanoemulsion indicated that phagocytic index was not significantly different to the unformulated oil. However,

treated samples resulted in a significantly different phagocytosis activity in comparison to control cells at the highest concentration (40mg/mL) (fig. 9, 10). On the other hands, the specific testing resulted in a significant difference phagocytic index ($P<0.05$) comparing both samples and control cells (fig. 10). Phagocytic index following massoia oil nanoemulsion application appeared to be higher than the nanoemulsion base, but the difference was not statistically significant ($P<0.05$). Massoia lactones contained in both samples suspected to play a role in activating macrophage. Compounds with lactone group are known to increase the phagocytic activity of macrophages. Nanoemulsion base and DMSO at the highest concentration showed phagocytic activity. Nanoemulsion base contains a VCO, Tween 80 and PEG-400. Phagocytic activity of macrophages by that components are unknown yet. DMSO at low concentrations (0.1-0.5%v/v) reportedly demonstrated anti-inflammatory activity in Caco-2 cells in vitro (Hollebeeck *et al*., 2011).

Cytotoxicity assay

Results of the cytotoxicity assays on Vero cells (fig. 11) showed an increased toxicity of the oil as a nanoemulsion in comparison to the unformulated oil as showed by the IC_{50} value of massoia nanoemulsion as 35.9mg/mL and massoia oil as107.5mg/mL.

Table 2: Formula of Massoia oil nanoemulsion

Component	Value
Tween 80	17.333 g
PEG 400	8.667 g
Massoia oil	3 g
VCO	1 g
Distilled water	70 g
Total	100 g
Transmittance value	99.9 %

Table 3: The transmittances of nanoemulsion before and after stability test

Sample	Formula	Before	After
Massoia oil	I	99.5 %	99.83 %
	II	99.5 %	99.89 %
	III	99.5 %	99.99 %

DISCUSSION

According to Rali *et al.* (2007) there are three massoia lactone which has some different in carbon chain side. Massoia lactone has a simple molecular structure with 10, 12 and 14 carbon chain components so that each one is called by the C-10, C-12 and C-14 massoia lactone (Rali, 2007).

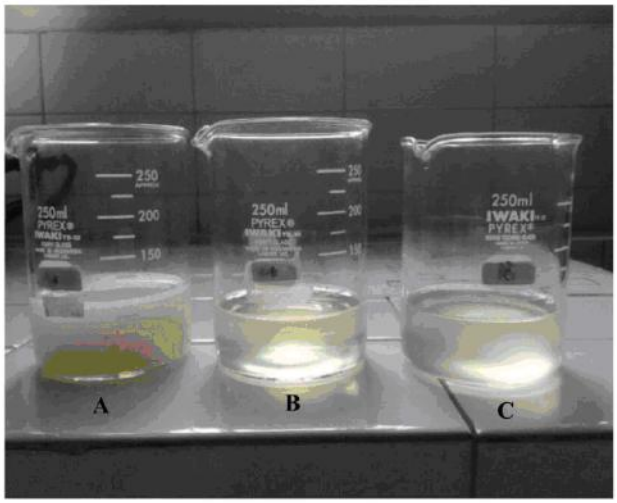


Fig. 1: Formula (massoia oil and VCO) turbidity test result. A:formula A, B : formula B, C: formula C as shown at table 3.

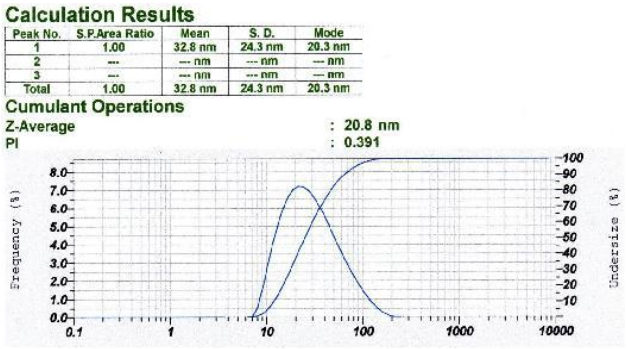


Fig. 2: PSA result for massoia oil nanoemulsion particle size.

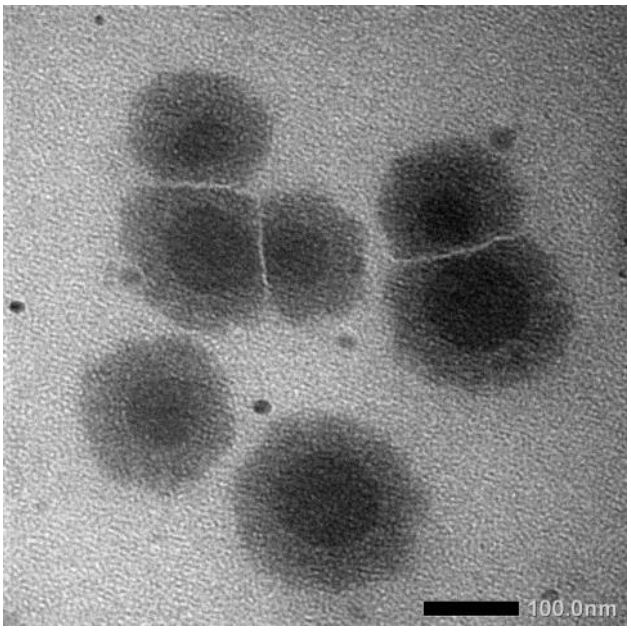


Fig. 3: TEM analysis of massoia oil nanoparticles.

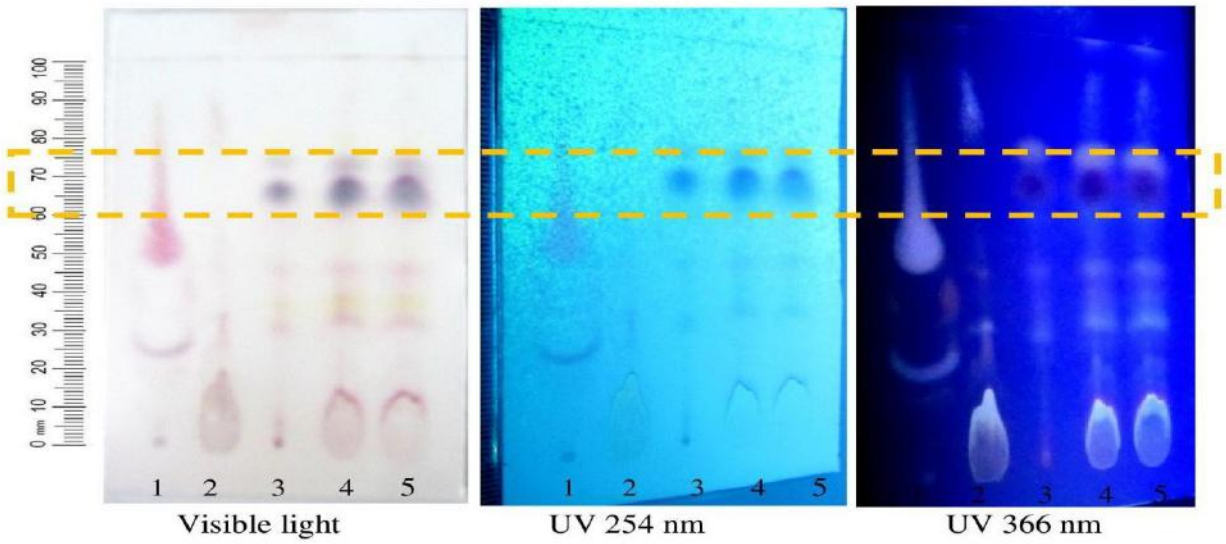


Fig. 4: Massoia oil nanoemulsion TLC profile under visible light (a), and under UV 366nm (b). 1. VCO, 2. Formula (blank, without oil), 3. massoia oil 3% v/v in toluene, 4. Formula after stability test, 5. Formula before stability test.

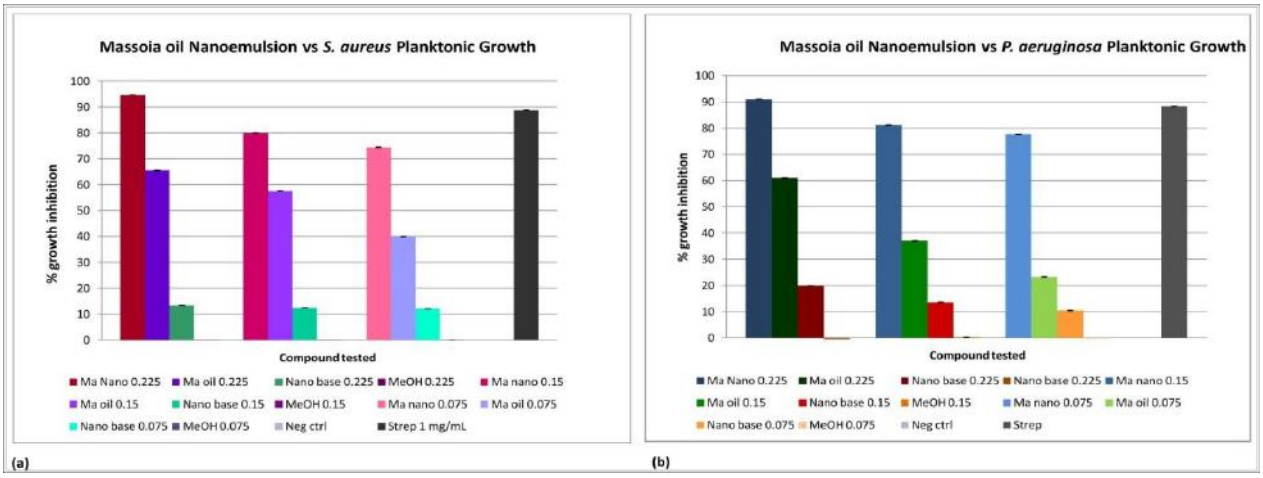


Fig. 5: Massoia oil nanoemulsion antibacterial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

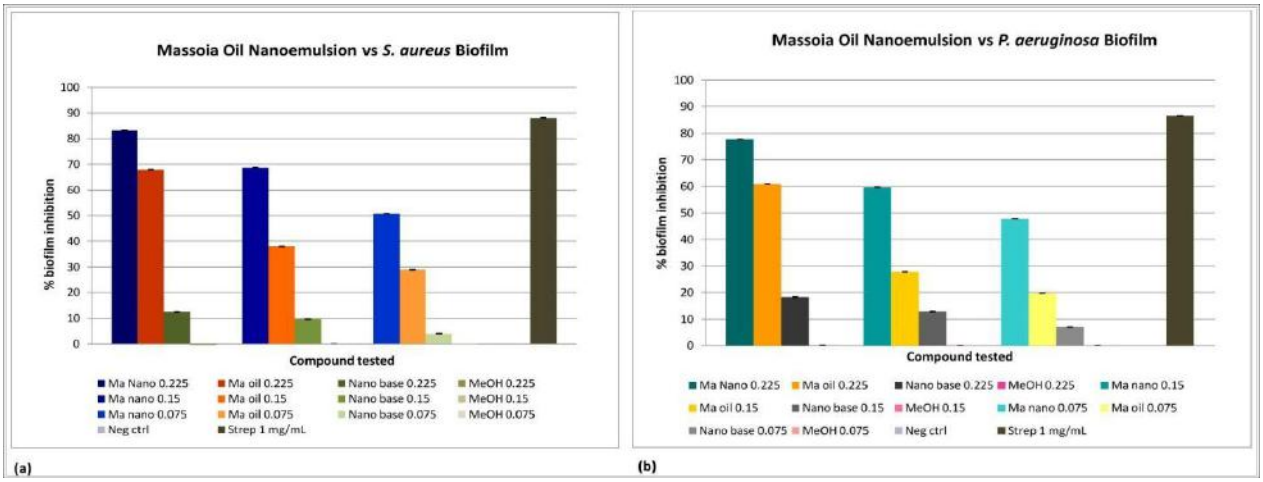


Fig. 6: Massoia oil nanoemulsion antibiofilm activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

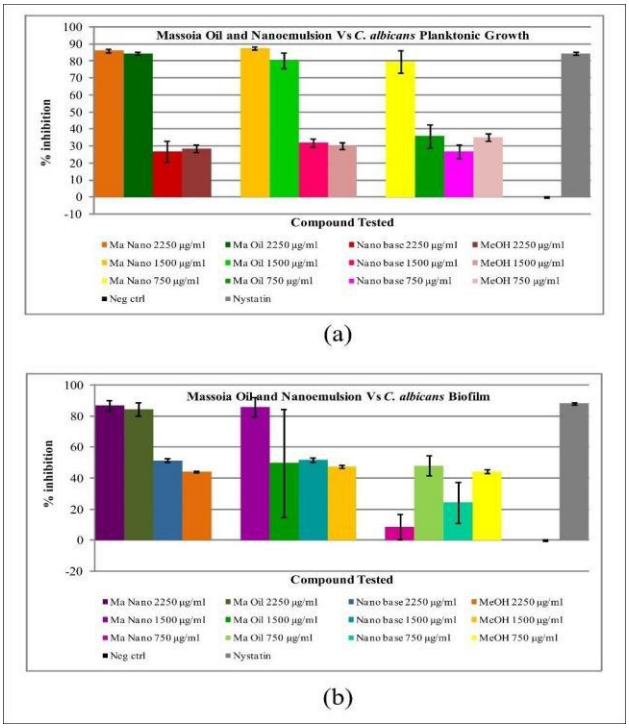
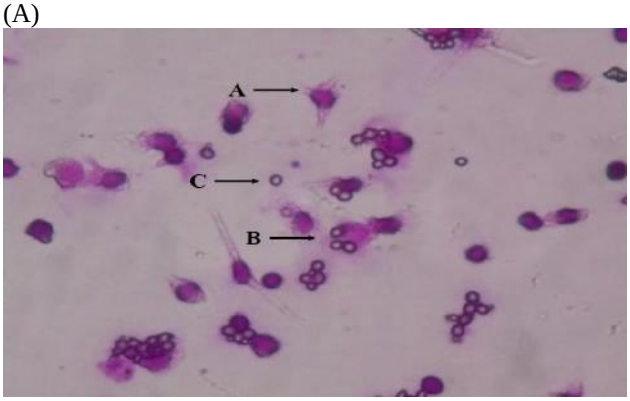


Fig. 7: Massoia oil nanoemulsion antibiofilm activity against *Candida albicans*. (a) planktonic growth, (b) biofilm.

(A)



(B)

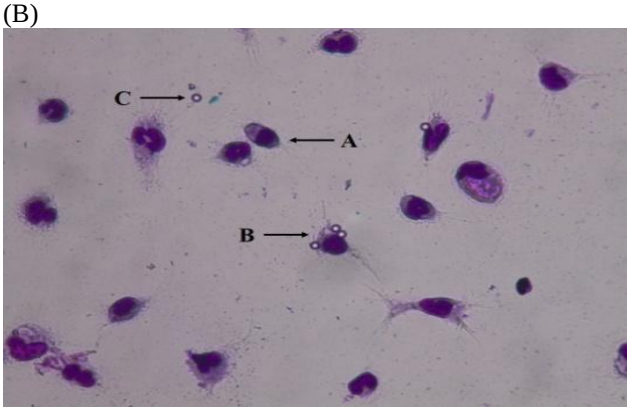


Fig. 8: Macrophages were observed under a light microscope magnification 10x40 times, non-specific testing (a), specific testing (b). A. Inactive macrophage, B. Active macrophage. C. Non engulfed cell.

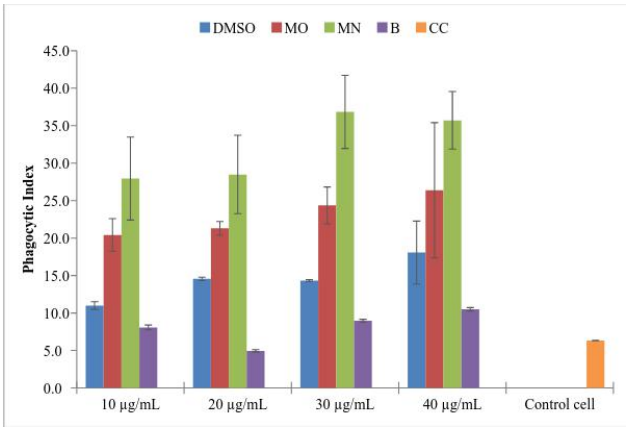


Fig. 9: Phagocytic activity of macrophages against latex. Control cells (CS) and different levels of concentration of DMSO (DMSO), Massoia Oil (MO), Massoia Nanoemulsion (MN), and MN Base (B)

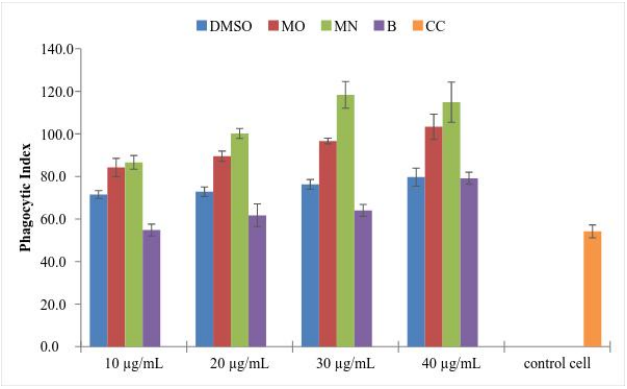


Fig. 10: Phagocytic activity of macrophages against *Candida albicans*. Control cells (CC) and different levels concentration of DMSO (DMSO), Massoia Oil (MO), Massoia Nanoemulsion (MN) and MN Base (B).

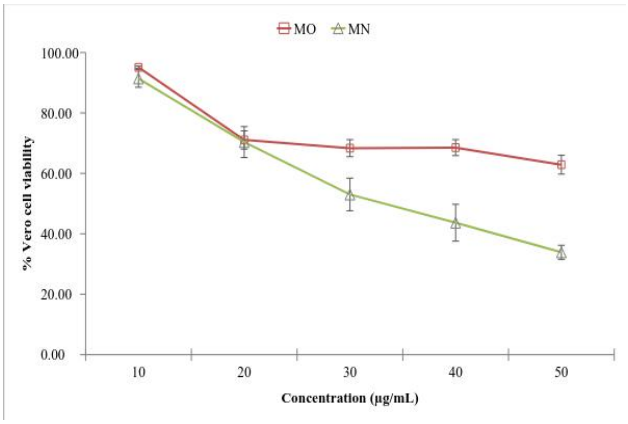


Fig. 11: Vero cell viability after treatment with different levels concentration of massoia oil (MO) and massoia nanoemulsion (MN).

As shown in fig. 3, the PI values of massoia oil nanoemulsion particles were 0.391 suggesting a uniform nanoparticles size distribution. The parameters used to determine the particle size distribution of nanoparticles system is Polydispersity Index (PI). PI value ranges between 0-1. The smaller the value of PI indicates that the particle size distribution in the system more uniform nanoparticles (Gupta *et al.*, 2010).

Polydispersity index is the ratio between the standard deviation of the mean droplet size that can be used to describe the uniformity of droplet size in a sample (Yuan *et al*., 2008). The smaller the value of polydispersity index, the more narrow the droplet size distribution (Ali *et al*., 2014). The result obtained showed that the droplet nanoemulsion distribution has a particle size below 200 nm. An emulsion droplet size is in the nanometer scale due to the roles of surfactant, co-surfactant, and the methods of forming nanoemulsion. Surfactants have hydrophilic and hydrophobic groups that make it adsorbed at the interface, and thus lowering the voltage between water and oil. PEG400 as a Co-surfactant also has a role in lowering the surface tension. According to Ali *et al*., (2014), the addition of co-surfactant in the form of C3-C8 alcohol chain will keep the surface tension decreases (Haritha *et al*., 2013). The process of forming nanoemulsion which is involving sonication has a role in reducing the size of the nanoemulsion droplet. Nanoemulsion droplet size is very useful in facilitating the active substances to enter the cell and increase penetration across the cell membrane (Martin *et al*., 1990)).

Using transmission electron microscopy (TEM) imaging, we found out that the nanoparticles of massoia oil have a spherical shape with sizes ranging from 100-200nm (fig. 4). The stability of nanoparticles is influenced by particle shape. Particles with a small surface area are not prone to aggregation. This occurs because of the attraction force between particles can be minimised (Tsakalakos *et al.*, 2003). The nanoemulsion droplets also surrounded by surfactant and cosurfactant, thereby minimising inter-phase merger disperse (Brown *et al*., 1969).

Transmission electron microscopy (TEM) can be used to analyse the morphology of nanoemulsion. However, it could not be used for an accurate observation and for measuring with certainty the diameter of nanoemulsion tested. This is in contrast to the measurements conducted using PSA that could indicate the particle size distribution and the average droplet diameter of nanoemulsion particles. From our result, we found out that nanoemulsion characterization using PSA and TEM were mutually supportive.

Based on the experimental results, the transmittance values before and after the freeze thaw test tends to increase. The increasing transmittance probably due to the decreased tension during the process of freezing and thawing that makes the particle size smaller and the nanoemulsion becomes transparent as indicated by the increased value of the transmittance.

Influence of massoia oil nanoemulsion on P. aeruginosa PAO1 and S. aureus planktonic growth and biofilm formation

Despite containing no essential oils nor another active ingredient, nanoemulsion blank showed antimicrobial and antibiofilm activity. This can be due to the influence of its components such as VCO, Tween 80, and PEG 400 which also has antimicrobial activity. An anionic surfactant such as Tween 80 has antimicrobial activity by decreasing the permeability of the cell membrane (Tsakalakos *et al*., 2003). VCO components can also affect the antimicrobial activity nanoemulsion blank. VCO has antimicrobial activity because of hydrophobic lauric acid contained, which easily penetrate through the bacterial's membrane bilayer. Lauric acid from the VCO will disturb the cell membrane integrity, causing the cells to leak (Brown *et al*., 1969).

The difference in activity of oil nanoemulsions against both bacterial tested can also be affected by other components such as VCO. Lauric acid in the VCO is hydrophobic and easily penetrate the membrane bilayer (Tangwatcharin and Khopaibool, 2014). Although several studies have shown that VCO's lauric acid and monolaurin have higher antimicrobial activity against Gram-positive bacteria compared to Gram-negative bacteria, some bacteria were resistant to saturated fatty acids at low levels (Kitahara *et al*., 2004). Therefore VCO, Tween 80, and PEG 400 which are hydrophobic would be easier to penetrate the cell membrane of Gram negative bacteria which is dominated by Gram-positive phospholipids. Nanoemulsion antimicrobial and antibiofilm properties might also be supported by the smaller size of the oil particles, which can facilitate the penetration through the membrane of the prokaryotic cell membranes and fungi, but not the eukaryotic cells of higher organisms (Bila *et al*., 2014). Nanoemulsion droplet size that less than 100 nm are useful to help the active substance to penetrate into microbial cells.

The major compound of the massoia oil is the C-10 massoia lactone, similar to the study reported by Rali (2007). The effects of C-10- massoia lactone towards microbial planktonic growth and biofilms have not been closely inspected yet. However, it is speculated to be involved in membrane disruption, resulting in alterations in membrane permeability and leakage of intracellular materials (Rali, 2007). In the presence of lactone, *Yarrowia lipolytica* loses its cultivability and membrane integrity, and the addition of lactone in the medium provoked a decrease in the concentration of

ergosterol (Ta *et al*., 2010). Since the exact mechanism of antibacterial and antibiofilm activity of massoia oil/massoia lactone remains unclear, a study on microbial cells in response to massoia oil/massoia lactone will be required.

Cytotoxicity assay

The IC_{50} value of massoia-oil-nanoemulsion and unformulated massoia oil was calculated as 35.9mg/mL and 107.5mg/mL respectively. The value indicated that the massoia-oil-containing nanoemulsion is more toxic to Vero cells than the unformulated massoia oil. Nanoemulsion formulation eases the availability of the smaller size of particle size inside the target cells (McClements and Rao, 2011). Toxic materials may cause higher toxicity in line with the increase in the effectiveness of the delivery.

CONCLUSION

Massoia-oil-containing nanoemulsion resulted was physically and chemically stable. Significant activities in the growth inhibition of *P. aeruginosa* and *S. aureus* compared to the unformulated oil were observed. However, the biofilm inhibition of massoia oil nanoemulsion towards *Candida albicans* was not significantly different with the unformulated oil. The antibiofilm activity was observed to be decreased following the maturity of the biofilm. The phagocytic activity of the massoia-oil-containing nanoemulsion against latex and *Candida albicans* was not significantly different with the unformulated oil. Unfortunately, the toxicity of the nanoemulsion towards Vero cells was higher as could be observed by the IC_{50} value of massoia oil nanoemulsion and massoia oil as 35.9µg/mL and 107.5µg/mL respectively.

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REFERENCES

Ali MS, Alam MS, Alam N and Siddiqui MR (2014). Preparation, characterization and stability study of dutasteride loaded nanoemulsion for treatment of benign prostatic hypertrophy. *Iran J. Pharm. Res.*, **13**(4): 1125-1140.

Bali V, Ali M and Ali J (2010). Study of surfactant combinations and development of a novel nanoemulsion for minimising variations in bioavailability of ezetimibe. *Colloids Surf. B Biointerfac*es, **76**(2): 415-417.

Baratta MT, Dorman HJ, Deans SG Figueiredo AC, Barroso JG and Ruberto G (1998). Antimicrobial and antioxidant properties of some commercial essential oils. *Flavour Fragr.* J., **13**(4): 235-244.

Bila AR, Guccione C and Isacchi B (2014). Essential oils loaded in nanosystem: A developing strategy for a successful therapeutic approach. *Evid. Based Complement. Alternat. Med.*, **2014**: 1-14.

Brown MRW and Winsley BE (1969). Effect of polysorbate 80 on cell leakage and viability of pseudomonas aeruginosa exposed to rapid changes of pH, temperature and tonicity. *J. Gen. Microbiol*., **56**(1): 99-107.

Chime SA, Kenechukwu FC and Attama AA (2014). Nanoemulsion advances in formulation, characterization and application in drug delivery. *In*: Ali DS editor. Application of Nanotechnology in Drug Delivery, InTechOpen, London, UK, pp. 77-111.

Clinical and Laboratory Standard Institute (CLSI) (2007). Performance Standards for Antimicrobial Susceptibility Testing: Seventeenth Informational Supplement. CLSI document M100-S17. Clinical and Laboratory Standard Institute, 940 West Valley Road, Suite 1400, Wayne, PA 19087-1898 USA.

Cowan MM (1999). Plant products as antimicrobial agents. *Clin. Microbiol. Rev*., **12**(4): 564-582.

Donlan RM and Costerton JW (2002). Biofilms: Survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.*, **15**(2): 167-193.

Galindo-Rodriguez S, Allémann E, Fessi H and Doelker E (2004). Physicochemical parameters associated with nanoparticle formation in the salting-out, emulsification-diffusion, and nanoprecipitation methods. *Pharm. Res.*, **21**(8): 14-29.

Gupta PK, Prandit JK and Kumar A (2010). Pharmaceutical nanotechnology novel nanoemulsion-high energy emulsification preparation, evaluation, and application. *T. Ph. Res*, **3**(1): 117-138.

Haritha, Basha SP and Rao PK (2013). A brief introduction to methods of preparation, application and characterization of nanoemulsion drug delivery systems. *Indian J. Res. Pharm. Biotechnol.*, **1**(1): 25-28.

Hertiani T, Pratiwi SUT, Yuswanto A and Permanasari P (2016). Potency of massoia bark in combating immunesuppressed related infection. *Pharmacogn. Mag.*, **12**(Suppl 3): S363-370.

Holetz FB, Pessini GL, Sanches NR, Cortez DA, Nakamura CV and Filho BP (2002). Screening of some plants used in the brazilian folk medicine for the treatment of infectious diseases. *Mem. Inst. Oswaldo Cruz*, **97**(7): 1027-1031.

Hollebeeck S, Raas T, Piront N, Schneider YJ, Toussaint O and Larondelle Y (2011). Dimethyl sulfoxide (DMSO) attenuates the inflammatory response in the in vitro intestinal Caco-2 cell model. *Toxicol. Lett.*, **206**(3): 268-75.

Kitahara T, Koyama N and Matsuda J (2004). Antimicrobial activity of saturated fatty acids and fatty amines against methicillin-resistant *Staphylococcus aureus. Biol. Pharm. Bull.*, **27**(9): 1321-1326.

Martin JF, Paul AB and Martin K (1990). A combined quantum mechanical and molecular mechanical potential for molecular dynamics simulations. *J. Comput. Chem*., **11**(6): 700-733.

McClements DJ and Rao J (2011). Food-grade nanoemulsions: Formulation, fabrication, properties, performance, biological fate, and potential toxicity. *Crit. Rev. Food. Sci. Nutr*., **51**(4): 285-330.

Pratiwi SUT, Lagendijk EL, Weert SD, Hertiani T, Idores R and Hondel CAVD (2015). Effect of *Cinnamomum burmannii* nees ex bl. and *Massoia aromatica* becc. essential oils on planktonic growth and biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. *Int. J. Appl. Res. Nat. Prod.*, **8**(2): 1-13.

Pirbalouti, AG, Moosavi S, Momtaz H, Rahimi E and Hamedi B (2010). Antibacterial activities of the essential oils of some Iranian herbs against *Campylobacter jejuni* and *Campylobacter coli. Adv. Food Sci*., **32**(1): 30-34.

Rali T (2007). Comparative Chemical analysis of the essential oil constituents in the bark, heartwood and Fruits of *Cryptocarya massoy* (Oken) Kosterm. (Lauraceae) from Papua New Guinea. *Molecules*, **12**(2): 149-154.

Syamsudin, Darmono and Kusmardi (2008). The effect of *Garcinia parvifolia* (active fraction) on phagocytosis by peritoneal macrophage during *Plasmodium berghei* in mice. *Research Journal of Immunology*, **1**: 16-20.

Ta TMN, Hoang LC, Thi HP and Tran HD (2010). New insights into the effect of medium-chain-length lactones on yeast membranes. importance of the culture medium. *Appl. Microbiol. Biotechnol.*, **87**(3): 1089-1099.

Tangwatcharin P and Khopaibool P (2014). Activity of virgin coconut oil, lauric acid, or monolaurin in combination with lactic acid against *Staphylococcus aureus. Southeast Asian J. Trop. Med. Public Health*, **43**(4): 969-985.

Tsakalakos T, Ovid'ko IA and Vasudevan AK (2003). Nanostructures: Synthesis, Functional Properties and Applications, Kluwer Academic Publishers, Netherlands.

Sakurazawa T and Ohkusa T (2005). Cytotoxicity of organic acids produced by anaerobic intestinal bacteria on cultured epithelial cells. *J. Gastroenterol.*, **40**(6): 600-609.

Suciati T, Aliyandi A and Satrialdi (2014). Development of transdermal nanoemulsion formulation for simultaneous delivery of protein vaccine and Artin-M adjuvant. *Int. J. Pharm. Pharm. Sci.*, **6**(6): 536-546.

Yuan Y, Gao Y, Zhao J and Mao L (2008). Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions. *Food Res. Int.*, **41**(1): 61-68.