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Preparation and Evaluation of *In-vitro* Release and Cytotoxicity of *Indigofera zollingeriana* Crude Leaf Extract-loaded Nanoemulsion

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This study aimed to prepare and investigate the *in-vitro* release and cytotoxicity effect of an *Indigofera zollingeriana* crude leaf extract-loaded nanoemulsion formulation. Initially, *I. zollingeriana* leaf extract was extracted and the ternary phase diagram was constructed to select the nanoemulsion formulation. The nanoemulsion was prepared with a combination of oil, surfactant, and water using a low-energy emulsification method. The formulation was characterized according to droplet size, polydispersity, zeta potential, morphology, and physical stability. *In-vitro* release and cytotoxicity were investigated to determine the efficacy of the nanoemulsion. Based on the results, a stable formulation (no phase separation) with a ratio of 5:10:85 (sunflower seed oil: surfactant: water) was obtained with droplet size, polydispersity index (PDI) and zeta potential of 160.5 nm, 0.262 and -31.1 mV, respectively. Morphology study revealed that particles of the nanoemulsion were spherical with diameters ranging from 95.97 to 148.31 nm. *In-vitro* release study showed fast release in the first 3 h at pH 7.4. The percentage cumulative release of the crude extract was obtained by up to 86.50% for 24 h. Cytotoxicity study of the nanoemulsion formulation showed moderate and mild toxicity against lung cancer (A549) and breast cancer (MCF-7) cell lines, respectively.

Key words: *Indigofera zollingeriana*; nanoemulsion; In-vitro release; cytotoxicity

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Indigofera is a genus that comes from the Fabaceae family, which was previously known as Leguminosae. There are three subfamilies of the Fabaceae, which are *Caesalpinioideae*, *Mimosoideae* and *Faboideae*, and can be found in the tropical and subtropical regions of the world. Various species of *Indigofera* have been reported, such as *Indigofera tinctoria, Indigofera trita, Indigofera gerardiana* and *Indigofera oblongifolia*, which are widely distributed in India, Ceylon, South African, Pakistan, Asia, and Africa [1–5].

Many studies have been previously reported on different species of the *Indigofera* genus. For instance, a study conducted by Lubbad *et al.* [6] revealed that *I. oblongifolia* exhibited antioxidant and antimalarial effects on *Plasmodium chabaudi* in mice. Moreover, a study on *I. tinctoria* found that the ethanol extract of this species is able to inhibit Gram-positive bacteria (*Staphylococcus aureus, Streptococcus pyogenes*, and *Bacillus pumilus*), with strong antioxidant activity and cytotoxicity effect [7]. Apart from that, the methanol extract of *I. aspalathoides* could increase the life span of swiss albino mice by decreasing the volume of tumor, which shows that this species has the ability as an anticancer [8].

Although many studies have been conducted on different species of *Indigofera*, there are limited studies on *I. zollingeriana*. According to Hisaeda *et al.* [9], the isolation of *I. zollingeriana* extract showed the presence of flavanol glycosides and megastigmane glucosides. Traditionally, it has been used to treat wounds and cuts by crushing young stems to extract the juice, which is then topically applied to the affected areas [10]. Studies have reported that *I. zollingeriana*, which is widely grown in Vietnam, was found to exhibit cytotoxic effects against HepG2 and Huh7 cells, but not against normal human primary fibroblasts [11].

A nanoemulsion has been described as a system consisting of oil and water stabilized by a surfactant with a particle size below 500 nm [12-13]. It has been applied to the drug delivery system. The development of a nanoemulsion is required to deliver an active natural compound, as well as to maintain the natural structure [14]. There are various studies that have been reported on nanoemulsions, for example, a mangosteen crude extract nanoemulsion which focused on the bioactive compounds and α-mangostin from mangosteen for topical application [15].

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A nanoemulsion containing Rapanea ferruginea (mirsinoic acids as bioactive compounds) was reported could improve topical delivery and anti-inflammatory activity [16]. Secondary metabolites which are found in most plants have low bioavailability and poor water solubility, which lead to low absorption at the targeted sites. Thus, nanoemulsions have been used to overcome the challenges of secondary metabolites and their pharmacological activities [17]. Nanoemulsions are also able to deliver hydrophilic and hydrophobic components simultaneously to targeted sites of the skin [18]. They could also reduce absorption variability, enhance bioavailability of active ingredients, and increase efficacy in delivering active ingredients [19].

The present study aimed to prepare and characterize the physicochemical characteristics of an I. zollingeriana crude extract-loaded nanoemulsion formulation, which is not being explored yet. Then, the in-vitro release and cytotoxicity of the nanoemulsion formulation were evaluated.

MATERIALS AND METHODS

Materials

Sunflower seed oil was obtained from Sigma-Aldrich (St Louis, USA). Castor oil was purchased from Fluka (Switzerland). Ethanol (95.0% purity) was purchased from System Chemicals (Shah Alam, Malaysia). Emersense® AM 8205, as a non-ionic surfactant, was a gift from Emery Oleochemicals Sdn. Bhd. (Teluk Panglima Garang, Malaysia). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) was purchased from Merck Chemical (Darmstadt, Germany). All the chemicals used were of analytical grade. Water was deionized using Milli-Q, Thermo scientific.

Plant

Fresh *I. zollingeriana* leaves were collected from the Malaysian Agricultural Research and Development Institute (MARDI) in Kluang, Johor, Malaysia. The voucher specimen number PID260718-24 was deposited at the Herbarium of Forest Research Institute Malaysia (FRIM).

Extraction of I. Zollingeriana Leaf Extract

Fresh leaves (1.00 kg) of *I. zollingeriana* were air-dried and ground into a fine powder. The fine powder (30.00 g) was soaked in ethanol for 4 days before being filtered and concentrated using a rotary evaporator (Rotavapor R-210, Buchi, Switzerland) to obtain the crude extract. The selection of ethanol was based on a preliminary study (not shown here). The crude extract was stored at -20°C until further analysis.

Solubility Study of the I. Zollingeriana Crude Extract

The solubility of the *I. zollingeriana* crude extract was investigated with different oils (castor oil and sunflower seed oil). Initially, the crude extract (0.5% w/v) was mixed with castor oil using a magnetic stirrer for 15 mins. The mixture was continuously homogenized using a vortex mixer for about 1 min and then centrifuged at 4000 rpm for 15 mins. The appearance was observed and recorded. These steps were repeated by increasing the percentage of the crude extract (at 0.10% w/v intervals) until 2 layers were obtained. The experiment was repeated for sunflower seed oil. The highest percentage of *I. zollingeriana* crude extract soluble in oil was selected as the oil phase.

Construction of the Ternary Phase Diagram

The oil phase was prepared by dissolving the crude extract (0.9% w/w) in sunflower seed oil. Mixtures of the oil phase and surfactant (Emersense® AM 8205) were weighed at different ratios from 0:100 until 100:0 in eleven screw-cap glass tubes, separately. The mixtures were homogenized using a vortex mixer for about 1 min at 3150 rpm (VM-300, Gemmy Industrial CORP-Taiwan) and centrifuged using a Hettich centrifuge (Model EBA, Germany) at 4000 rpm for 15 mins. Water (5.0% w/w) was added dropwise into each glass tube and then vortexed and centrifuged again at 4000 rpm for 15 mins. The appearance of the resultant solutions was observed and recorded, where a transparent or translucent solution was considered as isotropic (L_i), a milky solution was considered as homogenous (H_m), while a solution with a separation layer was considered as multilayer (M_L). These steps were repeated by increasing the percentage of water. A ternary phase diagram was constructed using Chemix School v3.50 software. Based on this ternary phase diagram, a formulation was selected in the water-based region (homogenous, H_m) for the nanoemulsion formulation.

Preparation of the Selected Nanoemulsion Formulation

The crude extract (0.9% w/w) was added to sunflower seed oil (Table 1) and homogenized using a magnetic stirrer for 30 mins. Once the crude extract completely dissolved, the surfactant was added into the oil phase mixture. Deionized water was then added dropwise into the mixture while stirring and homogenized using an overhead stirrer (IKA@RW 20 Digital, Nara, Japan) at 300 rpm for 4 h. The samples were wrapped with aluminium foil and kept at room temperature for further analysis.

Physicochemical Characterization of the Nanoemulsion

Fourier Transform Infrared Measurement

Fourier Transform Infrared (FT-IR) spectroscopy was used to confirm either the functional groups of the crude extract were still present or not in samples of the *I. zollingeriana* crude extract-loaded nanoemulsion formulation. The results were analyzed using an FT-IR spectrometer (Perkin Elmer, Spectrum 100, United Kingdom). The wavenumbers used were in the range 500 cm^{-1} to 4000 cm^{-1}. The sampling technique used was attenuated total reflectance (ATR), which enabled the samples to be examined directly in the liquid state.

Droplet Size, Polydispersity Index (PDI) and Zeta Potential Measurement

Droplet size, PDI, and zeta potential values of the selected nanoemulsion formulation containing *I. zollingeriana* crude extract were measured by using Zetasizer (Nano ZS, Malvern Instrument Ltd., UK) at room temperature (25.0 ± 0.5 °C). Droplet size and PDI were measured using a dynamic light scattering technique at a scattering angle of 173°. The measurement of the electrophoretic mobility of dispersed particles in a charged field was calculated by using Henry's Equation, as in Eq.1. The samples were prepared by dilution with deionized water. All measurements were repeated in triplicate.

$$U_e = \frac{2\varepsilon\zeta f\,(\kappa\alpha)}{3\mu} \quad (1)$$

Transmission Electron Microscopy Measurement

The morphology of the selected nanoemulsion was observed using a Transmission Electron Microscope (TEM, Hitachi H-7100, Japan). The sample was prepared by dilution with deionized water. The diluted sample was then dropped into a 300-mesh formvar-coated copper grid and negatively stained with 2.0% w/w of uranyl acetate. Excess liquid was removed using a Whatman filter paper and the sample was dried at room temperature before being observed by TEM. The acquired digital images were processed with Adobe Photoshop® software (Adobe, San Jose, CA).

pH Measurement

The pH of the sample was measured using Delta 320 pH meter (Mettler Toledo, Columbus, OH, USA).

Stability Study

The stability of the nanoemulsion was investigated by centrifugation force at 4000 rpm for 15 mins. The storage stability of the nanoemulsion was studied at different temperatures (4.0, 25.0 and 45.0°C) within 90 days.

In-Vitro Release Study

The *in-vitro* release study of the selected nanoemulsion formulation was performed in a mixture of ethanol and phosphate buffer solution (ratio 3:7 v/v, pH 7.4) as a release medium. The dialysis bag diffusion technique was used to study the diffusion of the nanoemulsion formulation across cellulose acetate membrane (molecular weight cut-off between 12 and 14 kDa). The cellulose membrane was soaked overnight in the release medium. 3.00 ml of the nanoemulsion was placed into the cellulose membrane and the ends of the bags were tied and carefully immerse in the release medium. The elution medium was stirred at 100 rpm using a magnetic stirrer. The receptor medium (1 ml) was withdrawn at different time intervals and replaced with the same volume of fresh medium to maintain the sink condition. The samples were then analyzed using a UV-Vis spectrophotometer (UV-1601 Shimadzu Spectrophotometer, Japan) at the wavelength of 250 nm. Known concentrations of the *I. zollingeriana* crude extract, with the concentrations of 1.00 - 250.00 µg/ml (R^2=0.9978), were used to determine the crude extract release. All experiments were carried out in triplicate.

In-Vitro Cytotoxicity Study

The cytotoxicity of the nanoemulsion formulation was determined by using MTT assay (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) on lung cancer (A549) and breast cancer (MCF-7) cell lines. Cell cultures with a concentration of 2 × 103 cells/ml were prepared and plated (100 µL/well) onto 96-well plates. Diluted samples with identified concentrations of 500.00, 200.00, 100.00, 50.00, 20.00, and 1.00 µg/ml were tranferred into individual wells, and incubated for 72 h. MTT solution was added at the end of the incubation, and the plates were further incubated for 3 h in the incubator. Once solubilization was completed, the optical density (OD) of the samples was measured by using an ELISA microplate reader (Los Angeles, CA, USA) at the wavelength of 570 nm. Percentage of cell viability of the treated cells with various concentrations of the samples compared to the negative control (untreated cells) was calculated from the absorbance values using Eq. 2. This experiment was carried out in triplicate. The cytotoxicity was recorded as the concentration causing 50.0% growth inhibition of the lung cancer cells (IC_{50}). The results were interpreted as follows: IC_{50} < 1.0 µg/ml was considered as highly toxic; IC_{50} = 1.0 - 10.0 µg/ml was considered as toxic; IC_{50} = 10.0 - 30.0 µg/ml was considered as moderately toxic; IC_{50} = 30 - 100 µg/ml was considered as mildly toxic; and IC_{50} < 100 µg/ml was considered as nontoxic [20].

$$\%\ \text{cell viability} = \frac{\text{absorbance of sample}}{\text{absorbance of control}} \times 100\% \quad (2)$$

Table 1. Solubility of different concentrations of the crude extract in castor oil and sunflower seed oil

Concentration of crude extract (% w/v)	Castor Oil	Sunflower Seed Oil
0.5	Soluble	Soluble
0.6	Soluble	Soluble
0.7	Soluble*	Soluble
0.8	Not Soluble	Soluble
0.9	-	Soluble*
1.0	-	Not Soluble

Note: *maximum solubility of the crude extract in oil

Statistical Analysis

The results from the cytotoxicity analysis are presented in mean ± standard deviation. Statistical analysis was conducted with the two-way analysis of variance (ANOVA) by using Prism Software GraphPad® Prism version 7.00. The results were considered significant if the P values were < 0.05.

RESULTS AND DISCUSSION

Solubility of the I. Zollingeriana Crude Extract in Oils

Table 1 shows that the solubility of the crude extract in sunflower seed oil (0.90% w/v) was higher than the solubility of the crude extract in castor oil (0.7% w/v). Sunflower seed oil is a rich source of polyunsaturated fatty acids, linoleic acid (approximately 50.0%) [21] compared to castor oil (1-5%). Linoleic acid in sunflower seed oil, which contains 2 unsaturated fatty acids, exhibits anticancer, anti-obesity [22], and anti-inflammatory [23] activities. Thus, sunflower seed oil was selected as the oil phase for further studies.

Ternary Phase Diagram of the Sunflower Seed Oil/ Surfactant/Water System

Figure 1 shows the ternary phase diagram of the sunflower seed oil/surfactant/water system, which consists of three different regions: isotropic (L_i), homogenous (H_m), and multilayer (M_L) regions. From the results, it showed that when the surfactant (Emersense® AM 8205) increased, solubilization of water decreased, which could form the L_i region; then transformed to the H_m region, which extended from the surfactant vertex towards the water vertex. The maximum amount of the oil phase that could be solubilized was 15.0% w/w by using 10.0% w/w of the surfactant, which is the suitable range for a nanoemulsion formulation with a stable dispersion.

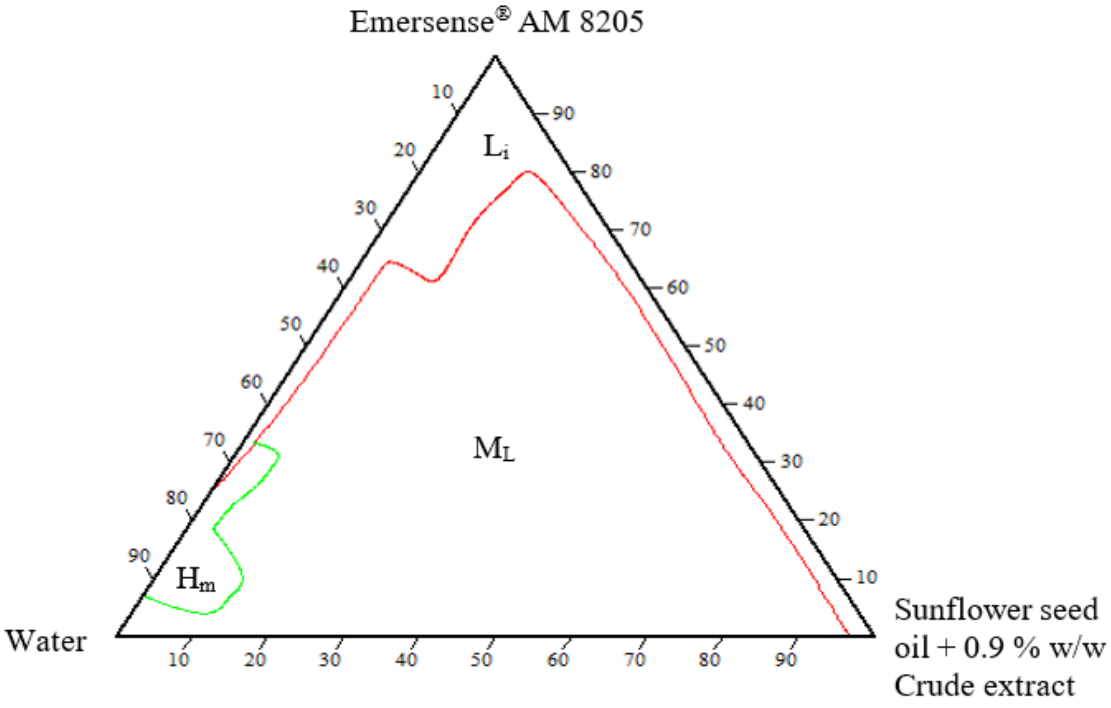

Figure 1. Ternary phase diagram of the sunflower seed oil + crude extract/surfactant/water system, where L_i is an isotropic region, H_m is a homogenous region, and M_L is a multilayer region.

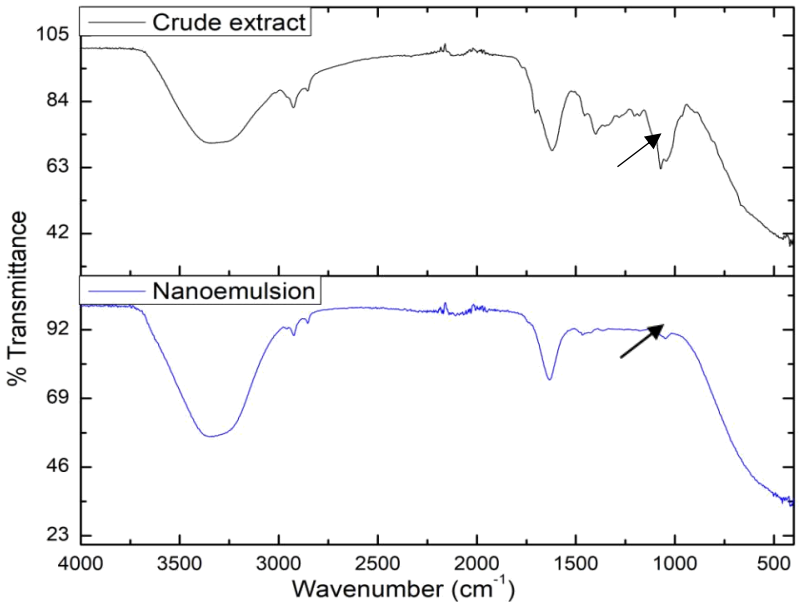

Figure 2. FT-IR spectra of the crude extract and the nanoemulsion formulation containing *I. zollingeriana* crude extract

Physicochemical Characterization of the Nanoemulsion

The FT-IR spectra of the *I. zollingeriana* crude extract and the nanoemulsion containing *I. zollingeriana* crude extract are shown in Figure 2. The O-H stretching vibrations, between 3200 cm^{-1} and 3400 cm^{-1}, indicated the presence of the -OH group. The C-H stretching vibrations were between 2850 cm^{-1} and 2975 cm^{-1}, corresponding to the C-C bonds. The absorbance peaks between 1600 cm^{-1} and 1680 cm^{-1} represented the C=C stretching vibrations, which are indicative of conjugated alkenes. Lastly, the bands in the region between 1020 cm^{-1} and 1200 cm^{-1} showed the existence of the C-O stretching. There was a slight difference between the FT-IR spectra of the *I. zollingeriana* crude extract and the nanoemulsion formulation containing *I. zollingeriana*. This C-O stretching was absent in the spectrum of the nanoemulsion containing *I. zollingeriana*. In the extraction of the *I. zollingeriana* leaf extract, ethanol was used as a solvent. Ethanol is a volatile solvent. The C-O stretching in the FT-IR spectrum of the *I. zollingeriana* leaf extract likely points to the remaining ethanol in the mixture. The disappearance in the FT-IR spectrum of the nanoemulsion containing *I. zollingeriana* crude extract might be due to ethanol had fully evaporated by high temperature.

Table 2 shows the physicochemical characteristics of the selected formulation. The droplet size was 160.5 ± 0.13 nm with a PDI value of 0.262 ± 0.14. The PDI value indicated that the formulation was a monodisperse system. The zeta potential value was -31.1 ± 0.05 mV, which indicated that the nanoemulsion was stable due to its high electrostatic repulsion. Besides, it was used to measure the electrokinetic potential of a particle. According to the literature, zeta potential of more than +30 mV or less than -30 mV indicates the stability of a nanoemulsion is greatly improved due to the increasing repulsive forces against flocculation and coalescence predominant between droplets in the nanoemulsion [24].

Surfactant was used to stabilize the formulation by lowering the interfacial tension and repellent forces between the two immiscible liquids, resulting in lower attraction between the molecules [25]. Emersense® AM 8205, which consists of hydrophilic heads and hydrophobic tails, aggregated to form amphiphilic molecules that enable it to self-assemble with sunflower seed oil when interacting in the aqueous environment. It is known for amphiphilic properties between Emersense® AM 8205 and sunflower seed oil. Thus, a stabilized nanoemulsion was formed.

Surfactant plays an important role in the determination of the droplet size and stability of the nanoemulsion [26]. Smaller droplet size could be achieved if the droplet disruption force were bigger than the recoalescence force. During the formation of a small droplet, surfactant could stabilize the droplet based on the Gibbs-Marangoni Effect [27]; which leads to irregular adsorption of surfactant and an environment with only one side was covered by the surfactant. The gradient of surface tension caused by the gradient of surfactant concentration will attract more surfactant towards the uncovered spot, resulting in a fully covered droplet. Thus, the droplet size becomes smaller.

Table 2. Composition and physicochemical characterization of the selected nanoemulsion formulation containing *I. zollingeriana* crude extract

Composition (% w/w)	
Sunflower seed oil	4.95
I. zollingeriana crude extract	0.05
Surfactant (Emersense® AM 8025)	10.00
Deionized water	85.00
Physicochemical characterization	
Droplet size	160.5 ± 0.13 nm
Polydispersity index (PDI)	0.262 ± 0.14
Zeta potential	-31.1 ± 0.05 mV
pH	8.8

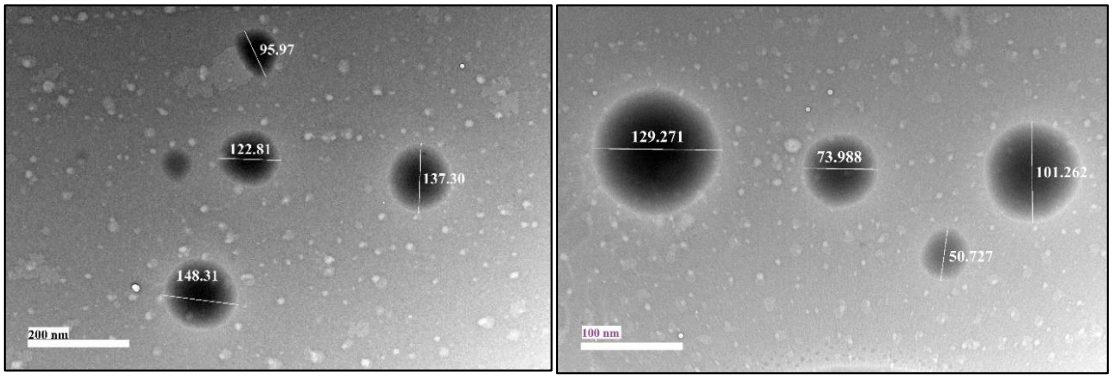

Figure 3. TEM images of the nanoemulsion formulation at (a) 2500× and (b) 5000× magnification.

Figure 3 shows the morphology of the nanoemulsion containing *I. zollingeriana* leaf crude extract. The particle size from TEM analysis showed that it was not significantly different from the particle size obtained from the Zetasizer analysis. TEM analysis showed that the nanoemulsion was spherical. In addition, the nanoemulsion was homogeneously and uniformly distributed without any aggregation.

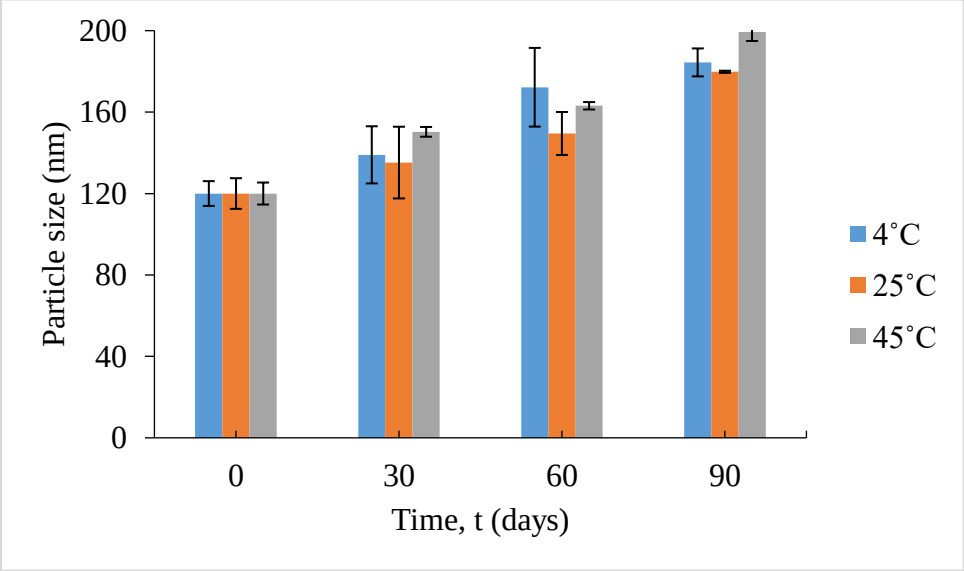

Figure 4. The droplet size of the nanoemulsion containing *I. zollingeriana* crude extract at different storage temperatures against time, t.

Stability analysis of the nanoemulsion

The stability analysis of the nanoemulsion was carried out by subjecting the nanoemulsion to gravitational stress. The nanoemulsion remained homogenous after 90 days with no visual changes or signs of phase separation, which showed good stability of the nanoemulsion. The nanoemulsion was stable due to the small particle size, which was below 200 nm and low interfacial surface tension between the aqueous phase and oil phase [28]. The centrifugation test was used to identify the shelf life of the nanoemulsion against sedimentation or creaming [29].

Figure 4 shows the increment of droplet size at three different storage temperatures within 90 days. From the graph plotted, the particle size remained below 200 nm, which indicated good stability of the nanoemulsion. The increase in droplet size was caused by the chemical alteration, which refers to charged molecules that happened due to the degradation of surfactant or oil phase [30]. The increase in particle size could also be due to the dehydration of the surfactant head, which led to the decrease of interfacial tension and resulting in the increase of interfacial flexibility among the particles [31].

***In-vitro* release analysis**

The efficacy of the nanoemulsion was studied by *in vitro* release study. Figure 5 shows the percent cumulative release at pH 7.4. The study was conducted for 24 h. The nanoemulsion showed fast release in the first 3 h due to the burst effect where the crude extract was dispersed into the surfactant or absorbed onto the droplet surface [32]. The rate of the release for the first 8 h was 69.84 µg ml^{-1} h^{-1} (pH 7.4). Meanwhile, the percent cumulative release up until 8 h was 83.00% and slowly increased up to 86.50 % at 24 h. In-vitro cytotoxicity analysis

Figure 6 shows that the nanoemulsion was able to inhibit the proliferation of both A549 and MCF-7 cell lines after 72 h of treatment. From the results obtained, the cytotoxic effect of the nanoemulsion formulation was moderately and mildly toxic against A549 and MCF-7 cell lines, respectively, and of which the IC50 values of 26.42 µg/ml and 69.50 µg/ml, respectively (Table 3). However, the cytotoxic effects were observed when the nanoemulsion formulation was applied at high concentrations.

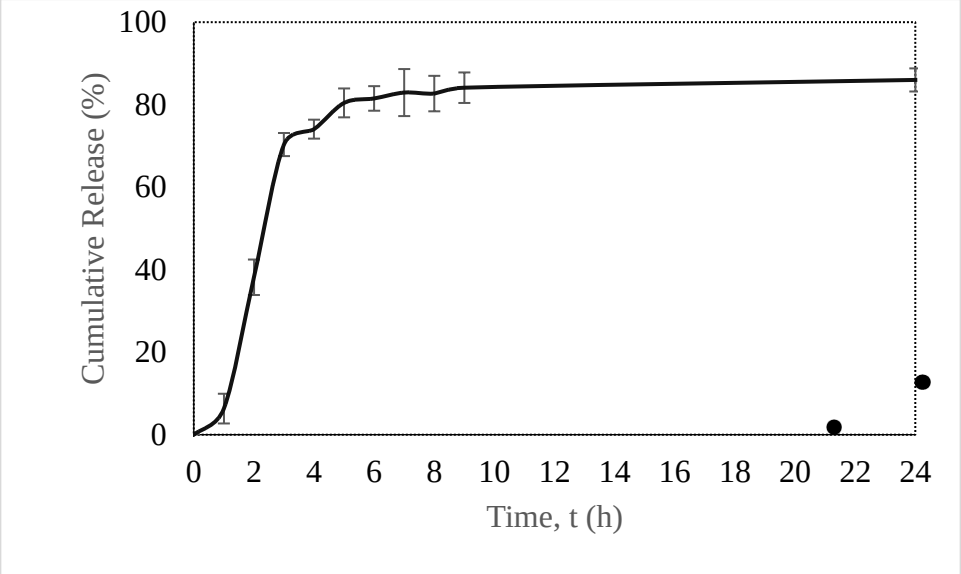

Figure 5. Release profile of the selected nanoemulsion formulation through cellulose acetate membrane using dialysis technique.

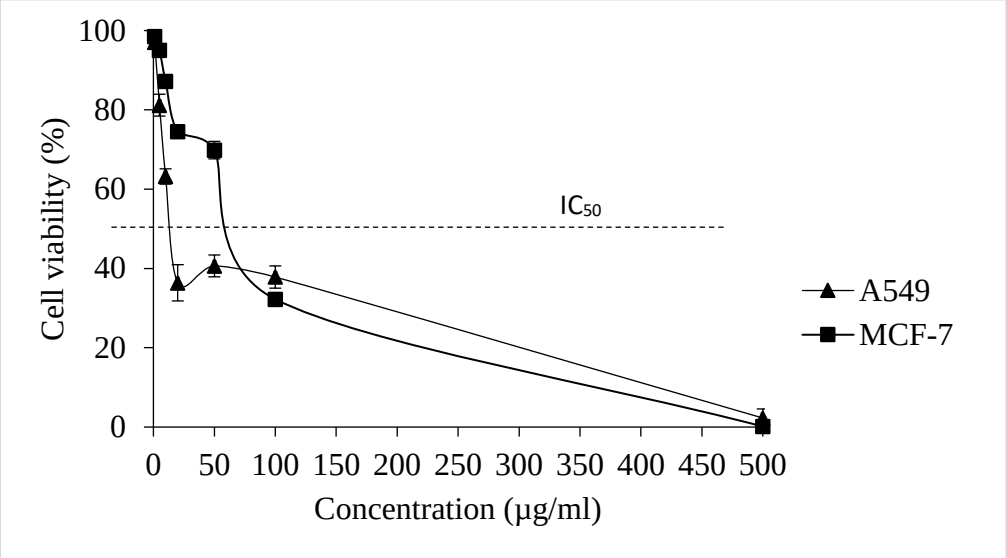

Figure 6. Cytotoxicity effects of *I. zollingeriana* crude extract-loaded nanoemulsion formulation in cancer cell lines (A549 and MCF-7).

Table 3. 50% inhibition of cell viability (IC_{50}) (n=3)

Cell lines	IC_{50} (IC_{50} (µg/ml)
A549	26.42 ± 1.06
MCF-7	69.50 ± 0.98

Note: A549- Human epithelium lung adenocarcinoma; MCF-5 - Human breast adenocarcinoma.

CONCLUSION

An *I. zollingeriana* crude extract loaded-nanoemulsion formulation had been prepared by a low energy emulsification technique. Based on the ternary phase diagram constructed, the selected formulation was successfully prepared with good physical stability against centrifugation test and storage temperatures. The desired physicochemical characteristics including droplet size, polydispersity index, zeta potential, and microscopic analysis were obtained. The *in-vitro* release study showed fast release for the first 8 h and slowly increased up to 86.50% at 24 h. The cytotoxicity effect of the selected nanoemulsion indicated IC_{50} within the range of moderately and mildly toxic against two selected cancer cell lines (A549 and MCF-7, respectively). These results showed that the nanoemulsion formulation containing *I. zollingeriana* crude extract has the potential to be used for pharmaceutical applications and it needs further investigations.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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