The physical and chemical analysis of nanoemulsions from extract rodent tuber mutant plant (Typhonium flagelliforme Lodd.)

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Abstract. Typhonium flagelliforme Lodd. is well known as rodent tuber herbal plant has been successfully irradiated by using gamma rays irradiation technique to increase the bioactive compounds. Despite the potential of rodent tuber mutant plants, the idea came up to develop functional food formulations into nanoemulsion systems. Nanoemulsion composed of extract, glycerol, DMSO, tween 80, and water in different concentrations. The rodent tuber mutant plant extract and DMSO were formulated into nanoemulsion with a fixed concentration of 0.1% of the weight of the formula, the emulsifier was used tween 80 with a concentration of 0.1% and glycerol with a concentration of 0%, 2.5%, 5%, 7.5%, 10% of the weight of the formula while the solvent used is distilled water with a final volume 10 mL of the weight of the formula. This study aimed to determine the physical and chemical characteristics of nanoemulsion prepared at various concentrations and volume ratios of rodent tuber mutant plant extract and surfactant. The analysis of the component of the nanoemulsions was conducted using Gas Chromatography-Mass Spectrometry (GC-MS). The alkane group found in the nanoemulsion sample, such as eicosane (5.96%). It can be concluded that the best extract and surfactant concentrations are using glycerol at 0%. The droplet sizes of 0% glycerol concentration had 111.6 nm and a polydispersity index of 0.541 compared with other concentrations. Nanoemulsion at 0% of glycerol showed a low zeta potential value of -19.36 mV. The destabilization of nanoemulsion after 24 h was dominated with creaming appeared, as shown in negative zeta potential. This shows that the formulation is not stable enough for a certain period of time even though it has a nanoparticle size. The development of nanoemulsion optimalization is a prospective approach to solve the stabilization problem.

1. Introduction
Rodent tuber (Typhonium flagelliforme Lodd.) is an herbal medicinal plant that has an anticancer activity that has the potential as a source of raw material for anticancer drugs. The main problem of rodent tuber has vegetative propagation and low production of bioactive compounds. One of the potential and promising for increasing bioactive compounds is through the mutation of rodent tuber plant, which has been carried out through a combination of in vitro biotechnology (somaclonal
variation) and gamma-ray irradiation of somatic cell populations. This research is to increase the content of rodent tuber bioactive compounds, especially anticancer compounds.

Rodent tuber mutant plant was produced, which contained higher anticancer compounds than the mother plants. Besides that, the rodent tuber mutant plants had new anticancer compounds that are not found in the mother plants based on GC-MS analysis [20, 21, 22].

Rodent tuber extracts have been known to encourage apoptosis in several cancer cells in vitro. Rodent tuber extract with ethanol fraction has been shown to be effective in inhibiting the growth of breast cancer cells T47D [14], rodent tuber mutant plant extract also can inhibit MCF-7 of breast cancer cell more effective than compared to the mother plant [23], inhibiting cell proliferation human T4-lymphoblastoid cancer [10, 11], and can inhibit the growth of NCI-H23 cell culture in non-small cell lung carcinoma [7].

During this time, the consumption of rodent tuber herbal plants for alternative treatments for cancer is done by consuming fresh plant juice. Rodent tuber mutant plant needs to be processed into functional beverage products and to make it easier for consumers to consume rodent tuber plants, and still get the benefits of the ability of bioactive compounds as anticancer.

Nanoemulsion has been recognized many potentials in various applications such as pharmaceutical, cosmetic, and food industry [1, 16, 18]. Nanoemulsion can be characterized by specifying as small droplet size, kinetically stable transparent, or translucent colloidal dispersion systems of oil, water, and surfactant [30]. The application of nanoemulsion can be suitable for use as a transdermal drug delivery system and also for cosmetics [28]. Nanoemulsion has tiny droplet size in the range of 20-500 nm. The advantages of nanoemulsion technology can penetrate through the rough skin, enhance penetration of the active ingredients, and the bioavailability of lipophilic drugs [17, 29, 26]. Nanoemulsion has a great promise formulation for delivery as pharmaceuticals [33].

In this study, nanoemulsions of rodent tuber mutant plant extract were carried out using the homogenization method. The homogenization process to reduce particle size needs to be done to obtain a stable emulsion [4]. The working principle of the homogenizer is to reduce the size of the grain by grinding large particles, to produce particles that are smaller than the previous size. In general, the emulsification process increases the emulsion, but the combination with emulsifiers or stabilizers will result in smaller emulsion grain sizes, making it more stable [9]. Recently, the addition of glycerol concentration increasingly in the aqueous phase was found to decrease the droplet size of oil-in-water emulsions produced using by homogenizer [35]. The addition of small amounts of glycerol 1.5% (w/w) was found significantly increase the storage stability of oil-in-water emulsions [34].

This study was conducted to determine the physical characteristics of nanoparticles and chemical compounds in the nanoemulsions. The nanoemulsions were prepared at various concentrations and volume ratios of rodent tuber mutant plant extract and surfactant, in order to obtain process conditions in obtaining rodent tuber mutant plant nanoparticles with high monodispersity and stability. The purpose of this study was to investigate the possibility of using glycerol as a cosolvent in the formulation of rodent tuber mutant extract produced using a low-energy phase inversion. In particular, this study aimed to understand how glycerol influences the formation, properties, and stability of these nanoemulsion formulas. The parameter used to determine the level of uniformity is the polydispersity index value of the particle size distribution, while the parameter to determine stability is the potential value of zeta.

The utilization of a superior mutant plant of rodent tuber as a basis in the formulation of functional drinks has never been done. This research activity is one of the series of activities that are expected to provide opportunities to be applied in the food industry scale, especially to obtain health drink formulations as the basis for developing superior functional drinks that contain bioactive compounds with high anticancer activity.
2. Material and Methodology

2.1. Preparation of extract from rodent tuber mutant plant

The rodent tuber mutant plant KB 6-1-2 was dried and macerated in 96% ethanol overnight. The solvent was removed after it was filtered through Whatman filter paper No. 1. The supernatant was collected and evaporated to dryness under the rotary evaporator. The concentrated extract was collected and used for emulsion preparation.

2.2. Preparation of nanoemulsion

Nanoemulsion was prepared using a low-energy phase inversion or homogenization technique according to a method described by [15]. This technique involves the formation of the water-in-oil emulsion and the phase inversion into an oil-in-water emulsion. Rodent tuber mutant plant extract (0.1% of the final emulsion) was placed into a beaker and mixed with DMSO (0.1% of the final emulsion) until dissolved then added tween 80 (0.1% of the final emulsion). Subsequently, the concentration of glycerol was added with 0%, 2.5%, 5%, 7.5%, 10% under magnetic stirring at 750 rpm. While stirring distilled water was added for the final volume of emulsion 10 mL of the weight of the formula for 30 min to form an organic phase. Experiments were done in two replications. The nanoemulsion formed was placed into sample bottles for further use of storage studies.

2.3. Measurement of particle size and zeta potential analysis

Emulsion droplet size was measured using a Malvern ZetaSizer (Nano-ZS, Malvern Instruments, Malvern, UK). The emulsion sample in 2 drops was placed into a cuvette and diluted in 2 mL water for particle size measurement. Sample for zeta potential measurement in 25 µL was placed into a capillary cell and diluted in 2 mL water. The particle size was measured as Z-average applying Stokes-Einstein relation with its corresponding polydispersity index (PI). Measurement of particle size and zeta potential of each sample was taken three times.

2.4. Morphological of nanoemulsion

Morphology and structure of nanoemulsion were examined with transmission electron microscopy (TEM). The nanoemulsions were diluted to 0.05 wt % in HCl solution of pH 2.0. A drop of the diluted sample was deposited onto a 5 nm thick carbon support film on a copper grid (400 mesh). The excess was removed after 15 s using a piece of filter paper. Electron micrographs were made using TEM (G2F20, FEI TechnaiTM, USA) operated at 120 kV.

2.5. GCMS analysis

Nanoemulsions of formula 1 & 2 (F1 & F2) and rodent tuber mutant plant extract KB 6-1-2 were injected into the GC column. Injection volume was 5µl with a 5:1 split ratio, and the injection temperature was 250°C. Helium was used as carrier gas with a velocity of 0.8 µl per min. The column temperature was set at 70°C with 5°C per min. The temperature reached 200°C. It remained constant for 1 min and then will be increased at the rate of 20°C per min until the temperature reached 280°C. The temperature remained constant for another 28 min. The mass spectrometer was operated in electron impact ionization mode with 70 eV voltage. The compounds were identified by comparing their relative retention time and mass spectra with those the National Institute Standard Technique (NIST) database with ≥ 90% fit factor.

3. Results and Discussion

The effect of extract and surfactant concentration on nanoparticle formation by varying the concentration of glycerol from 0% to 10%. The purpose of adding glycerol to the nanoemulsion formula was to determine the stability of the solution. The droplet sizes of nanoemulsion were in the range 110.40 – 141.84 nm. The results indicated that the formation of rodent tuber mutant plant nanoparticles succeeded in all concentrations of nanoemulsion, but when it passed within 24 h, micro-
sized particles were rapidly re-aggregated where nanoemulsion, with a marked creaming suspension in the reaction solution, was shown. It is assumed that in all concentrations of glycerol led to unstable nanoemulsion. The particles formed from the electrostatic reaction between the extract and the surfactant are huge and dense so that the groups form aggregates into micro-sized particles. The overall results it can be concluded that the most optimal extract and surfactant concentrations are without glycerol or 0% of glycerol concentration.

The results showed that from all the formulations, only formula 1 (F1) with 0% glycerol concentration had lower polydispersity index value was 0.54 as presented in Table 1. This shows that the formulation is not stable enough for a certain period even though it has a small particle size and particle size distribution. The results of this study were included negative results because the formula without glycerol shows better than the samples with the addition of glycerol. The results were indicated that glycerol does not apply to rodent tuber mutan plant extract, it is possible there must be the addition of other surfactant types that can help the nanoemulsion of rodent tuber mutant extracts be more stable. The results of the characterization using TEM, which showed that the nanoparticles formed were still not uniform, as seen in Figure 1. It can be interpreted that the level of uniformity is quite good because is still far from value 1. The formed nanoparticles have a small size distribution range, or in other words, the level of uniformity is quite good. Zeta potential analysis showed relevant results and strengthened data on the distribution patterns and polydispersity index.

The data of the results obtained through GC-MS analysis of nanoemulsion F1 and rodent tuber mutant plant extract is presented in Figure 1 & Table 2. GC-MS analysis revealed that alkanes and sterols were found predominantly in rodent tuber mutant plant extract KB 6-1-2. Among the minor components identified in the nanoemulsion, eicosane was known to possess anticancer on human carcinoma cell lines [3]. Hexadecanoic acid and octadecanoic acid revealed that rodent tuber mutant plant extract KB 6-1-3-4 has been showed cytotoxic activity on breast cancer cell lines MCF-7 [23]. There are reports on the anticancer and antioxidant activity from nanoemulsion and rodent tuber mutant plant extract KB 6-1-2, as shown in Table 3. The best concentration of nanoemulsion is the F1, which still contains eicosane compounds that have the potential as anticancer compounds. Eicosane compounds were not detected in the nanoemulsion formula which had a glycerol concentration of 2.5% at F2. It was predicted that glycerol had protective properties against alkane compounds in extracts of rodent tuber mutant plant.

Table 1. The physical characteristics of nanoemulsion formula containing with rodent tuber mutant plant extract

| Formula | Nanoeulsion concentration (%) | Mean±SD | Zeta potential (mV) |
|---------|-------------------------------|---------|---------------------|
|         | Extract | DMSO | Tween 80 | Glycerol | Water | Droplet size (nm) | PDI |                     |
| F1      | 0.1     | 0.1  | 0.1      | 0        | 99.7   | 111.55 ± 4.60    | 0.54 ± 0.01 | -19.36 ± 14.21      |
| F2      | 0.1     | 0.1  | 0.1      | 2.5      | 97.2   | 106.40 ± 1.70    | 0.68 ± 0.17 | -24.25 ± 3.75       |
| F3      | 0.1     | 0.1  | 0.1      | 5        | 94.7   | 141.84 ± 55.93   | 0.77 ± 0.04 | -21.75 ± 2.62       |
| F4      | 0.1     | 0.1  | 0.1      | 7.5      | 92.2   | 110.40 ± 8.91    | 0.72 ± 0.10 | -21.90 ± 1.41       |
| F5      | 0.1     | 0.1  | 0.1      | 10       | 89.7   | 136.45 ± 9.12    | 0.68 ± 0.02 | -23.90 ± 2.12       |
| Sample                  | RT  | (%) Peak area | Chemical compound                                      |
|------------------------|-----|---------------|--------------------------------------------------------|
| F1                     |     |               |                                                        |
|                        | 9,427| 1.85          | Propane, 1-methoxy-2-methyl                            |
|                        | 13,571| 2.20          | 2,3-Butanediol                                         |
|                        | 14,199| 89,99         | Dimethyl Sulfoxide                                     |
|                        | 48,848| 5.96          | 1-Eicosane                                             |
| F2                     |     |               |                                                        |
|                        | 14,198| 5.03          | Dimethyl Sulfoxide                                     |
|                        | 26,341| 93.94         | Glycerin                                               |
|                        | 48,827| 1.03          | 1-Decanol, 2-hexyl-                                    |
| Extract ethanol        |     |               |                                                        |
| rodent tuber mutant    |     |               |                                                        |
| plant 6-1-2            |     |               |                                                        |
|                        | 31,912| 8.26          | Hexadecanoic acid, ethyl ester                         |
|                        | 31,968| 22.8          | Hexadecanoic acid                                      |
|                        | 32,568| 1.41          | 9,12-octadecadienoic acid, methyl ester (linoleic acid, methyl ester/ methyl linoleate) |
|                        | 32,954| 6.67          | Ethyl (9Z,12Z)-9,12-octadecadienoate                   |
|                        | 33,03 | 24.29         | 9,12-octadecadienoic acid                             |
|                        | 33,112| 23.4          | (9E,12E)-9,12-octadecadienoic acid                    |
|                        | 33,616| 1.4           | Tricosane                                              |
|                        | 34,064| 0.83          | Z,Z-10,12-hexadecadien-1-ol acetate                   |
|                        | 34,147| 1.84          | 9,12-octadecadienoic acid                             |
|                        | 34,505| 0.56          | Eicosane                                               |
|                        | 35,464| 0.89          | Eicosane                                               |
|                        | 36,263| 1.05          | (6E,10E,14E,18E)-2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene |
|                        | 36,705| 1.71          | Heptacosane, 1-chloro                                  |
|                        | 38,456| 0.83          | Nonacosane                                             |
|                        | 41,063| 1.03          | Stigmasterol                                           |
|                        | 41,152| 0.04          | Stigmasterol                                           |
Table 3. List of compounds was identified as biological activity in nanoemulsions and rodent tuber mutant plant extract KB 6-1-2

| Compound name                                | Nature of compound | Activity established                                      |
|----------------------------------------------|--------------------|----------------------------------------------------------|
| Eicosane                                     | Alkane             | Antitumor, antioxidant [25, 5, 13]                       |
| Hexadecanoic acid, ethyl ester               | Palmitic acid, ethyl ester | Anticancer [23, 13]                                      |
| Hexadecanoic acid                            | Palmitic acid      | Anticancer [23, 13]                                      |
| 9,12-octadecadienoic acid, methyl ester      | Linoleic acid, methyl ester | Anticancer [23, 13]                                      |
| 9,12-octadecadienoic acid                   | Linoleic acid      | Anticancer [23, 13]                                      |
| Tricosane                                    | Alkane             | Antiproliferative, antioxidant [31, 32]                  |
| Nonacosane                                   | Alkane             | Antiproliferative [6, 31, 32]                            |
| Heptacosane                                  | Alkane             | Antiproliferative [31, 32]                               |
| Stigmasterol                                 | Phytosterol        | Anticancer [2, 27, 6, 8]                                 |

Figure 1. Total ion chromatogram (TIC) of nanoemulsion (F1) extract of rodent tuber mutant plant (A) and extract of rodent tuber mutant plant (B).
Figure 2. The morphological of nanoemulsion containing rodent tuber mutant plants with 0% concentration of glycerol using TEM.

The zeta potential results had negatively charged and varied from -19.36 to -23.90 mV. The smaller zeta potential value, the higher the probability that droplets will approach each other [30]. A low potential zeta value (both positive and negative) causes a reduction in the repelling force between particles to prevent particles from aggregating. Potential zeta from nanoemulsion is generally used to characterize the properties of nanoemulsion surface loading. It reflects the electrical potential of particles, which is influenced by the composition of the particles and the medium that disperses them. In general, particles with a potential zeta value more positive than +30 mV or more negative from -30 mV are predicted to be stable during storage and prevented from particle aggregation [12].Sing & Lillar [24] said that nanoemulsion, which has a potential zeta value above +30 mV or below -30 mV shows a stable colloidal system so that the amount of particle charge can prevent particle aggregation based on the electrostatic resisting repulsion force.

4. Conclusion
The visual observation of 5 formulations, the nanoemulsion was aggregated after 24 h. It can be concluded that the formulation is not stable enough for a certain period even though it has a nanoparticle size and but it was quite good in particle size distribution. The results of this study with 111.55 ± 4.60 particle size, polydispersity index value about 0.54 ± 0.01, and zeta potential value about -19.36 ± 14.21 showed that the best formulations were formula 1 (F1) which is 0% concentration of glycerol. Eicosane as anticancer activity was identified through GC-MS analysis in the nanoemulsion (F1). Further study is needed to develop the nanoemulsion by adding and increasing the surfactants.

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6. References
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