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Development of Nanoemulsion containing *Centella Asiatica* Crude Extract as a Promising Drug Delivery System for Epilepsy Treatment

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Abstract: Preparations of products containing herbal extracts have grown by leaps and bounds, hitting the pharmaceutical industries due to the natural healing approach. *Centella asiatica* L. (pegaga) *(C. asiatica)* is a famous plant commonly served as a salad in Asian diets. It contains various phytoconstituents, which plays a vital role in the treatment of various illness. For example, in the treatment of epilepsy, crossing the blood-brain barrier (BBB) has been a challenge (even as parenteral application) as not all the drugs were able to pass through the membrane and produce a maximum therapeutic effect to the targeted site of action. Thus, a nanoemulsion formulation containing *C. asiatica* crude extract needs to be developed to penetrate the BBB. This study aims to formulate a nanoemulsion containing crude extract of *C. asiatica* leaves for epilepsy treatment. Nanoemulsion was prepared by using low energy emulsification method. The particle size, polydispersity index (PDI), and zeta potential of *C. asiatica* crude extract nanoemulsion were found to be at 57.86 ± 0.03 nm, 0.50 ± 0.03, and -26.50 ± 0.03 mV, respectively. The formulation remained physically stable at different storage temperatures (4, 25, and 45 °C) for 90 days. The particle size observed by transmission electron microscopy (TEM) was shown to be at 50 nm, which correlated well with the Zetasizer analysis. The cytotoxicity study, conducted on formulated *C. asiatica* nanoemulsion towards Vero cell line and 3T3 cell line, showed that the IC_{50} value indicated that it is nontoxic (>500 µg/ml). *C. asiatica* nanoemulsion was found to be stable based on the good evidence of physicochemical properties, and the IC_{50} value indicates significance for future in vivo and in vitro studies based upon the route of administration.

Keywords: *Centella asiatica*; nanoemulsion; cytotoxicity study.

1. Introduction

Epilepsy is a chronic brain disorder that affects people worldwide [1]. According to Falco-Walter *et al.* [2], epilepsy is a transient occurrence of signs or symptoms due to abnormal excessive or synchronous neuronal activity in the brain, and this definition was updated by

International League Against Epilepsy (ILAE) [3]. About 50 million people globally have epilepsy. This number has therefore signified the world's most common neurological diseases.

Adding to the statistics, low- and middle-income countries have the highest percentage of people with epilepsy, approximately 80% [1,4]. Specifically, it is estimated that epileptic patients make up 1% of the overall Malaysian population, with roughly 230,000 diagnosed cases affecting individuals of all races, ages, and gender [1]. A survey study shows that the prevalence of lifetime epilepsy in Malaysia is 7.8 per 1000 persons [3]. Antiepileptic drugs (AEDs) such as phenytoin and phenobarbital are used to treat epilepsy to reduce the frequency and severity of the seizure without causing an adverse effect [5]. Unfortunately, drugs used to treat epilepsy have poor bioavailability and turn ineffective due to drug resistance [6,7]. Some newer AEDs, such as levetiracetam, are less prescribed due to their serious side effects [8]. Currently, AEDs are prescribed for safer and better patient tolerability only.

For older drugs, the serious side effects are that it affects the central nervous system (CNS), and other side effects such as hepatotoxicity, encephalopathy, and gingival hyperplasia have made it less prescribed by a physician compared to newer AEDs [9]. Besides that, some older drug like phenytoin also has an enzyme-inducing effect that contributes to the negative impact on bone metabolism. Poor bone metabolism will result in reduced bone density and increased risk of fracture [5]. Generally, the disadvantage of modern drugs used for the treatment of epilepsy is clear, and therefore other alternatives such as traditional medicine should be looked for.

Traditional herbal medicine does play a vital role in the treatment of epilepsy [10]. Traditionally, *Centella asiatica (C. asiatica)* has been used as an Ayurvedic remedy in the treatment of epilepsy [11,12]. This plant can be widely found in China, Japan, Italy, Sri Lanka, Iran, India, Madagascar, America, Australia, South Africa, Indonesia, and Malaysia [13,14]. *C. asiatica* enhances the function of the nervous system and can be dissolved in methanol, ethanol, and water [13]. An *in vivo* study conducted by Deka *et al*. [15] has proven that the crude extract of *C. asiatica* possesses antiepileptic activity. Another study has been reported for the phytochemical screening of *C. asiatica* aqueous extract contains triterpenes and flavonoids, which are responsible for anticonvulsants and other pharmacological activity [16,17]. It is concluded that the bioactive compound from those extracts can be used to treat epilepsy and to control seizures [18]. Secondary metabolite compounds present in the plant, such as terpenoids, demonstrate poor absorption because they are unable to cross lipid membranes specifically to the blood-brain barrier (BBB) and have large molecular sizes, which results in the loss of efficacy in clinical trials [19,20]. The major obstacle in treating epilepsy is the penetration of the drug across BBB [21]. Thus, nanoemulsion for parenteral delivery can be developed as the particle size of the drug used will be smaller and easily pass through the BBB [22].

Nanoemulsion is an emulsion having a droplet size below 200 nm. Nanoemulsion consists of oil, water, and surfactants [6]. The large interfacial area in nanoemulsion promotes the transport of drug or active ingredient (for example, a targeted compound from the crude extract) to the targeted site. Incorporating this active ingredient in oil will increase the permeability of the compound through passive diffusion. It increases delivery to the brain by improving lipid solubility [23]. Thus, a nanoemulsion-based delivery system containing *C.asiatica* crude extract would improve biodistribution and stability by determining physicochemical characterization for clinical practice.

2. Materials and Methods

2.1. Materials.

Acetone, methanol, and Tween 80 were purchased from Merck Chemicals (Germany). Virgin coconut oil (Cocos Nucifera), soybean oil, safflower seed oil, sunflower seed oil, olive oil, and polyethylene glycol 300 were purchased from Sigma-Aldrich (St.Louis, USA). Palm oil and flaxseed oil were purchased from Essentials Wholesale (Malaysia). Deionized water was purified using a Milli-Q water system (EMD Millipore, Billerica, MA, USA) Thermo scientific. All chemicals were analytical-grade reagents. The cells used for this study were obtained from the Institute of Bioscience, UPM.

2.2. Plant collection.

Fresh leaves of *C. asiatica* were purchased in Sri Serdang, Malaysia, and were authenticated by a pharmacognosist from Forest Research Institute Malaysia (FRIM). A voucher specimen was deposited at (PID 010119-01, FRIM). The collected leaves were washed with tap water and dried under the shed below 40 °C for 5 days, and then the dried leaves were pulverized to a coarse powder using a mechanical blender.

2.3. Extraction.

20 g of *C. asiatica* leaves were soaked separately in 600 ml of methanol and acetone for 7 days with continuous shaking. After 7 days of extraction, it was filtered using a vacuum pump aspirator and evaporated to dryness using a rotatory vacuum evaporator (Rotavapor R-210, Buchi, Switzerland). The yield of the crude extract was weighed and kept in a desiccator until further analysis. The percentage of extraction yield was expressed as shown in Equation 1.

$$\text{Percentage yield (\%)} = \frac{\text{Amount (g) of crude extract obtained}}{\text{Amount (g) of dried coarse powder}} \times 100 \quad (1)$$

2.4. Qualitative analysis of the crude extracts of C. asiatica leaves.

The crude samples undergo several phytochemical tests to identify the major constituent present in this species.

2.4.1. Terpenoid test.

2.4.1.1. Salkowski test.

About 5 ml of the sample was mixed with 2 ml of chloroform in a test tube to which 3 ml of concentrated sulphuric acid was carefully added through the sides to form a layer. If reddish-brown color appears at the interface, it indicates the presence of terpenoids.

2.4.2. Saponin test.

In a test tube, 1 ml of the sample was taken and mixed properly with 5 ml of distilled water and vigorously shaken; if stable foam appears, it indicates saponins' presence.

2.4.3. Carbohydrates and glycosides tests.

2.4.3.1. Benedict's test.

To 1 ml of the sample, benedict reagent was added and heated in a water bath for 2 min. The formation of a red precipitate indicates the presence of carbohydrates and glycosides.

2.4.4. Alkaloids test.

In a test tube, 1 ml of the sample was mixed with 2 ml of Wagner's reagent. If reddish-brown colored precipitate formed indicates presences of alkaloids. The same method was used by replacing Wagner's reagent with Mayer's reagent. If a white/creamy precipitate formed, it indicates the presence of alkaloids.

2.4.5. Flavonoids test.

2.4.5.1. With concentrated sulphuric acid.

About 1 ml of the sample was dissolved in a few drops of concentrated sulphuric acid in a test tube. A yellow color indicates the presence of flavonoids which disappear after some time.

2.4.5.2. With sodium hydroxide.

In a test tube, 1 ml of the sample was added to 2 ml of the 10% aqueous sodium hydroxide was later added to produce a yellow coloration. A change in color from yellow to colorless on the addition of dilute hydrochloric acid indicated the presence of flavonoids.

2.4.6. Steroids test.

In a test tube, 1 ml of the sample was taken and dissolved in 5 ml of chloroform, and then the equal volume (5 ml) of concentrated sulphuric acid was carefully added through the sides of the test tube. If the upper layer turns into a red color and the sulphuric acid layer turns yellow with slight green fluorescence, which indicates the presence of steroids.

2.4.7. Protein and amino acid tests.

2.4.7.1. Biuret test.

In a test tube, 0.5 mg of extract equal volume of 40% NaOH solution and two drops of 1% copper sulfate solution was added. The appearance of a violet color indicates the presence of protein.

2.4.7.2. Ninhydrin test.

About 0.5 mg of extract was taken, and 2 drops of freshly prepared ninhydrin reagent (0.2%) were added and heated. The appearance of pink or purple color indicates the presence of proteins, peptides, or amino acids.

2.5. Quantitative analysis of the crude extracts of C. asiatica leaves.

2.5.1. Gas Chromatography-Mass Spectroscopy (GC-MS) measurement.

The GC-MS analysis was carried out by using GCMS QP2010 Plus SHIMADZU (Japan) instrument. 0.3 µL of the sample was injected at the sample holder. This analysis used a high polar fused silica capillary column consisting of Zebron ZB-FFAP (30 m x 0.20). The column oven and injection temperatures were set at 50 and 250 °C, respectively.

2.5.2. Liquid Chromatography-Mass Spectroscopy (LC-MS) measurement.

Liquid Chromatography-Mass Spectroscopy (LC-MS) analysis was performed by Ultra-High-Performance Liquid Chromatogram (UHPLC) from Thermo Scientific Dionex Ultimate 3000 Series Waltham (MA, USA). It is well equipped with a Mass Spectrometer (MS) from Thermo Scientific Q Exactive Focus (Waltham (MA, USA). The water acquity UPLC BEH column (1.70 µm x 2.10 mm x 100.00 mm) was set up at 40 ˚C for the analysis. The mobile phase consisted of solvent A (deionized water + 0.1% formic acid) and solvent B (acetonitrile + 0.1% formic acid) with gradient elution. The solvent gradient was started at 95% A and 5% B for 0.45 min and followed by 0% A and 100% B for the next 34 min and was finalized by 95% A and 5% B for another 4 min. The sample (1 µL) was injected with 0.15 ml/min of flow rate.

2.5.3. Fourier Transform-Infrared Spectroscopy (FT-IR) measurement.

The FTIR analysis of the crude extract was carried out by KBr (Potassium bromide) method using the FTIR (Perkin Elmer, FTIR 1760) method. The crude extract was dried and prepared in a powdered form for analysis. KBr extract pellets were prepared for analysis by applying pressure. The infrared transmittance spectral data was obtained with a scanning range of 500-4000 cm^{-1}.

2.6. Solubility study of C. asiatica leaves crude extract.

The solubility of *C. asiatica* leaves crude extract was investigated with different oils (olive oil, soybean oil, flaxseed oil, safflower seed oil, sunflower seed oil, palm oil, and virgin coconut oil). The crude extract (0.2% w/v) was added to the 5 ml of olive oil. The mixture was stirred using a magnetic stirrer until the crude sample dissolved in the olive oil. The mixture was then homogenized using a vortex mixer (VTX-3000L Mixer Uzusio, LMS, Japan) and was centrifuged (EBA 200, Hettich Zentrifugen, Germany) at 4000 rpm for 15 min. The phase separation was observed visually and recorded. These steps were repeated by adding crude extract sample up until 1% w/v. The selection of oil was based on the highest percentage of crude extract that could be dissolved in it.

2.7. Construction of the ternary phase diagram for oil/surfactant/water system.

The oil phase was prepared by dissolving 0.2 g of crude extract in 5 g virgin coconut oil. The mixture (crude extract and virgin coconut oil) and Tween 80 were weighed in eleven screw-cap glass tubes, separately at the different ratios between 0:100 and 100:0, respectively (total weight was 0.5 g). The mixture was then homogenized using a vortex mixer and was continuously centrifuged at 4000 rpm for 15 min. Water (5% w/w) was added to each test tube

and continuously vortexed to be homogenized and then centrifuged. These methods were repeated by adding water (10, 20, 30, until 90% w/w). Ternary phase diagrams were constructed by using the Chemix School v3.50 Software (Arne Standnes, Norway).

2.8. Preparation of nanoemulsion containing crude extract of C.asiatica leaves.

Nanoemulsion containing crude extract of *C. asiatica* leaves was prepared by low energy emulsification. This method was carried out by phase inversion composition technique (PIC), where the aqueous phase was added dropwise into the oil phase. The oil phase consists of surfactant (Tween 80) and oil (virgin coconut oil). The nanoemulsion composition was selected (Table 1) in the isotropic region from the ternary phase diagram of virgin coconut oil/Tween 80/water system. It was prepared with a slight change by adding 1% of α-tocopherol [24] to the emulsion to prevent lipid peroxidation. α-tocopherol (1%) was added in the oil phase, and the mixture was homogenized using a magnetic stirrer at 600 rpm for 15 min. Tween 80 (11% w/w) was then added into the mixture and continuously homogenized. Water (86% w/w) was added dropwise while stirring. The mixture was then stirred at 800 rpm for 2 h.

Table 1. Composition and percentage of *C. asiatica* nanoemulsion.

Composition	**Percentage (% w/w)**
Virgin coconut oil	1.996
Crude extract	0.004
Tween 80	11.000
α-tocopherol	1.000
Water	86.000

2.9. Physicochemical characterization of nanoemulsion.

2.9.1. Particle size and polydispersity index measurement.

The particle size and polydispersity index (PDI) of the formulated nanoemulsion were measured by using Zetasizer (Nano ZS, Malvern Instrument Ltd., UK) with dynamic light scattering technique (DLS) at an angle of 173° at room temperature (25 ± 0.5 °C). The required amount of samples was diluted with deionized water (1:10) before it was injected into the sample cell to make a mean average measurement using intensity distribution. The measurement was repeated in triplicates.

2.9.2. Zeta potential measurement.

The freshly formulated nanoemulsion was diluted with deionized water (1:10) and was injected into a folded capillary cell (DTS 1070, Malvern Instruments, UK). The surface charge was determined by measuring the electrophoretic mobility of the dispersed particles in a charged field. The measurement was measured at room temperature (25 ± 0.5 °C) using Zetasizer (Nano ZS, Malvern Instrument Ltd., UK).

2.9.3. pH measurement.

The pH of the formulation was measured by using Delta 320 pH meter (Mettler-Toledo, Switzerland) at room temperature (28 ± 1.0 °C). Before measurements, the pH meter was calibrated with three pH standard buffer solutions (pH 4.00, 7.00, and 10.00). The average value of pH from three readings was taken as the result.

2.9.4. Conductivity measurement.

The formulation's conductivity was measured using Conductometer (Mettler-Toledo, Switzerland) at room temperature (28 ± 1.0 °C). Conductivity measurements allow the interpretation of free ions in the formulation. A high value of conductivity results in the determination of the aqueous phase as the continuous phase, in brief, oil-in-water (O/W) emulsion, and a low value of conductivity results in water in oil (W/O) emulsion.

2.9.5. Morphology study.

The morphology of the formulated nanoemulsion was visualized using Transmission Electron Microscopy (TEM, JEOL JEM-1400 Flash, USA). The sample was diluted with deionized water and then dropped to 300-mesh formvar-coated copper grids and negatively stained with 1.00 wt % uranyl acetate for 5 min. Whatman filter paper was used to dry the excess liquid, and the sample was dried at room temperature before measurement. The acquired digital images were processed with Adobe Photoshop® software.

2.10. Stability study: accelerated stability testing.

The stability of the formulated nanoemulsion was examined involving stability under centrifugation test, storage stability at three different temperatures within 90 days (4, 25, and 45 °C), and freeze-thaw cycles.

2.10.1. Centrifugation test.

Triplicate samples were subjected to a centrifugal force at 4000 rpm for 15 min, and observations of phase changes were made for each cycle.

2.10.2. Storage temperature.

The formulated nanoemulsion was kept at three different temperatures (4, 25, and 45 °C) for 90 days. The physical appearance (phase separation) of the nanoemulsion was observed.

2.10.3. Droplet size against time.

The droplet size of the nanoemulsions was measured at different times. The mechanism involved in studying the instability of nanoemulsion is the analysis of Ostwald ripening and coalescence rate.

2.10.3.1. Coalescence rate.

The coalescence-rate analysis was carried out to determine factors that affect droplet size changes over time. The collected droplet size data within the three different storage temperatures were analyzed and calculated in Equation 3.1.

$$\frac{1}{r^2} = \frac{1}{r^0} - \left(\frac{8\pi}{3}\right)wt \qquad (3.1)$$

Based on the equation above, r is the mean radius after time, w is the frequency of rupture per unit of the film surface, and r0 is the value at time t = 0. The graph of 1/ r2 against time was plotted to evaluate the coalescence rate. A linear relationship pattern was predicted for the coalescence rate to occur.

2.10.3.2. Ostwald ripening rate.

The enlargement of nanoemulsion droplet size due to the diffusion of the oil phase through the aqueous phase is the effect of Ostwald ripening. The effect of storage temperature was determined based on the Lifshitz-Slesov-Wagner (LSW) theory as equation (3.2).

$$\frac{dr^3}{dt} = \frac{8}{9}\left[\frac{C(\infty)\gamma VmD}{pRT}\right] \qquad (3.2)$$

2.11. In-Vitro cytotoxicity study.

The cytotoxicity study of the formulated nanoemulsion containing crude extract *C.asiatica* leaves was tested using MTT (3-(4,5-dimethylthiaxol-2-yl)-2, 5-diphenyl-tetrazolium bromide) assay on Vero cell line (green monkey kidney epithelial cells) and 3T3 cell line (mouse embryonic fibroblast cell). Cell culture with a 2 x 103 cells/ml concentration was prepared and plated (100 µl/well) onto 96-well plates. The diluted ranges of sample extracts were added to each well with identified concentrations of 500, 100, 50, 20, 10, 5, and 1 µg/ml further incubated for 72 h. MTT solution was added by the end of incubation samples to the cells and continued for incubation in an incubator for 3 h. After solubilization of the purple formazan crystals using (dimethyl sulfoxide), DMSO was completed, the Density (DO) of the crude extracts was measured using an ELISA reader at a wavelength of 570 nm. The cytotoxicity was recorded as the drug concentration causing 50 % growth inhibition of the tumor cells (IC50 value) using Eq. 3.3

Cell Viability (%)=(Absorbance same (mean))/(Absorbance control (mean)) (3.3)

2.12. In-Vitro permeation study.

The formulated nanoemulsion release across cellulose acetate membrane (molecular weight cut-off between 12,000 and 14,000 Da) was studied using a dialysis bag diffusion technique according to the method used by Harun *et al.* [25]. The cellulose membrane was soaked overnight in the release medium (pH 6.5, 7.4, and 8.5). 3 ml of the formulated nanoemulsion were placed in the cellulose membrane, and both ends of the bags were tied. The dialysis bag was then carefully immersed in beakers containing a mixture of phosphate buffer solution (pH 6.5, 7.4, 8.5). The elution medium was stirred using a magnetic bar at 100 rpm. The receptor medium (1 ml) was withdrawn at different time intervals and replaced with the same volume of fresh media to maintain the sink condition. These samples were analyzed using UV-VIS spectroscopy (UV-1601 Shimadzu spectrophotometer, Japan) at a wavelength of 300 nm. All experiment was carried out in triplicate.

2.13. Statistical analysis.

The student's t-test was used to analyze intergroup differences. Experiments were repeated three times, and data are represented as the mean ± standard deviation. A p-value of less than 0.05 was considered to be statistically significant.

3. Results and Discussion

3.1. Extraction of C. asiatica leaves.

C. asiatica leaves were extracted with organic solvent (methanol and acetone) using a cold maceration technique. Table 2 shows the percentage extraction yield of both methanol and acetone extract. Based on Table 1, the percentage extraction yield obtained from methanol extract was 33.20% and 15.90% in acetone extract. The results were shown to be in good agreement with the known efficiency of extraction and polarity of solvent (acetone < methanol). Methanol has the highest percentage of extraction yield compared to acetone due to its properties to elucidate most of the phytochemical compounds [26,27]. A study conducted by Yunusa *et al*. [28] shows that a methanol extraction of *Adansonia digitata* stem bark extract contained phytochemicals responsible for anticonvulsant activity. Methanol extract of *Colebrookea oppositifolia* stem has revealed that among the other solvent used (petroleum ether and aqueous), methanol extract has significant potent anticonvulsant activity through GABA mediated mechanism for epilepsy [29]. Another study conducted by Ahmed *et al.* [30], shows that methanol extract of *Albizia chevalieri Harms (Mimosaceae)* leaves revealed anticonvulsant activity in acute and chronic experimental models of epilepsy based on the presence of alkaloids, flavonoids, saponins, steroids, tannins, triterpenes glycosides and cardiac glycosid found in the preliminary study of the leaves. Based on the strong evidence from previous research, methanol extract has been chosen in this study for further analysis.

Table 2. Percentage extraction yield of organic solvents.

Solvents	Weight extract (g)	Percentage yield (%)
Methanol	6.64 ± 0.06	33.20
Acetone	3.18 ± 0.07	15.90

Note: The values were expressed as mean ± standard deviation (n=3)

Preliminary phytochemical analysis was carried out on different crude extracts of *C. asiatica* leaves to identify the presence of phytochemical varieties. Table 2 shows the preliminary phytochemical analysis of *C. asiatica* leaves crude extract for organic solvents. Based on Table 3, terpenoids and flavones were present in both extracts. The chemical test showed that methanol extract's phytochemical groups were the highest (7 phytochemical compounds including terpenoid, saponin, flavones, and steroids), followed by acetone extracts. Research conducted on the methanol extract of *Combretum hypopilinum* stem bark extract executed the presence of steroids, flavonoids, and alkaloids [31]. Preliminary phytochemical analysis of methanol extract of *Acorus calamus* leaves shows the presence of triterpenoids, flavonoids, saponins, and tannins, which were responsible for antiepileptic by potentiating the activity of gamma-aminobutyric acid pathway in the central nervous system [32]. Thus, these finding supports the evidence of methanol extract producing the highest percentage of extraction yield. It can be concluded that methanol was the best solvent choice for the extraction due to the high amount of polar organic compounds such as terpenoids, which is responsible for various pharmacological activities such as anti-inflammatory, antimalarial antibacterial, and antiepileptic [33,34].

Table 3. Preliminary phytochemical analysis of *C. asiatica* leaves crude extract in organic solvents.

Chemical constituents	Types of test	Extract	
		Methanol	Acetone
Alkaloids	Mayer	-	-
	Wagner	-	-

Chemical constituents	Types of test	Extract	
		Methanol	Acetone
Carbohydrates and glycosides	Benedict	+	-
Proteins and amino acids	Biuret	+	+
	Ninhydrin	+	+
Terpenoids	Salkowski	+	+
Saponins	foam	+	-
Flavones	Aq. sodium hydroxide	+	+
	Conc. sulphuric acid	+	+
Steroids	Salkowski	+	+

3.3. Quantitative analysis.

3.3.1 GC-MS analysis.

GC-MS analysis was carried using *C. asiatica* leaves extract in methanol solvent, and the result was presented in Fig 1. Based on the analyzed spectrum, there were 35 phytochemical compounds revealed in methanol extract (Fig 1). The spectrum showed that the extract has 4 major phytochemical compounds (n-hexadecanoic acid, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, 9,12-octadecadienoic acid, and 9,12,15-octadecatrienoic acid). The percentage peak area of the bioactive compounds in methanol extract of *C. asiatica* leaves is shown in (Table 4).

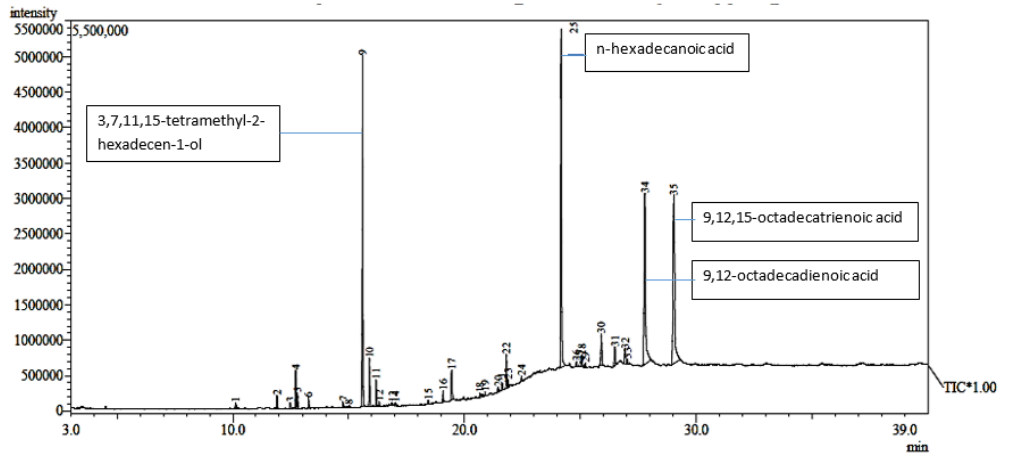
Table 4. The percentage peak area of the bioactive compounds in methanol extract of *C. asiatica* leaves.

Extract	Phytochemical Compound	Retention Time (min)	Area (%)
	9,12,15-octadecatrienoic acid	29.12	27.95
	9,12-octadecadienoic acid	27.84	20.48
Acetone	n-hexadecanoic acid	24.20	17.07
	3,7,11,15-tetramethyl-2-hexadecen-1-ol	15.62	7.35
	Phytol	21.81	1.92
	Oleic acid	26.94	1.21
	Octadecanoic acid	26.50	1.12
	Andrographolide	19.83	0.40
	Octanoic acid	16.97	0.07
Methanol	n-hexadecanoic acid	24.17	21.57
	9,12,15-octadecatrienoic acid	29.03	21.05
	9,12-octadecadienoic acid	27.78	18.29
	3,7,11,15-tetramethyl-2-hexadecen-1-ol	15.60	16.00
	Phytol	21.80	1.56
	Oleic acid	26.93	1.29
	Octadecanoic acid	26.47	1.51
	Linoleic acid ethyl ester	25.06	0.86
	Octanoic acid	21.89	0.31

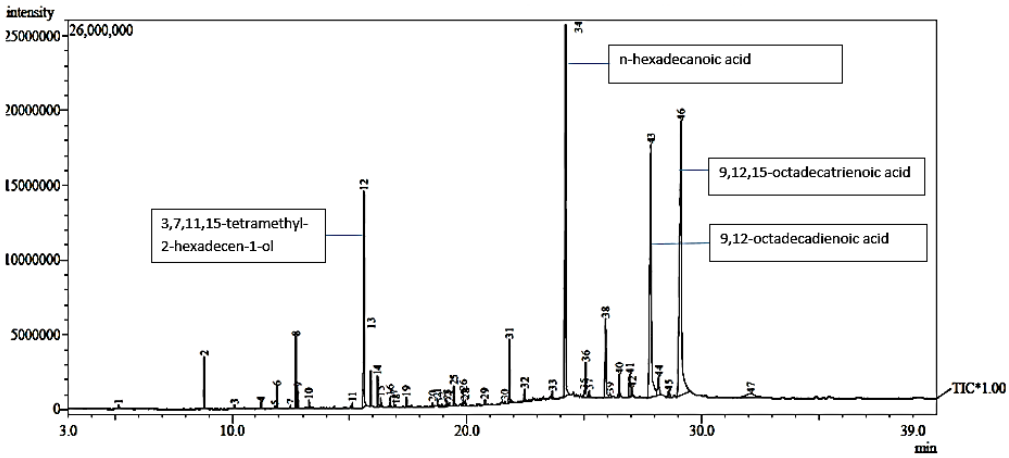
Table 4, fatty acid was found to be released in methanol extract. Fatty acids were essential for brain function and normal neurotransmission [19]. One of the saturated fatty acids found dominantly in all the extracts is n-hexadecanoic acid, also known as palmitic acid [35]. Palmitic acid does possess biological activity such as anti-cancer, antimicrobial and antioxidant [36-40]. Looking examples of unsaturated fatty acid (PUFA) found in this analysis are 9, 12, 15-octadecatrienoic acid (α-linoleic acid) and 9, 12-octadecadienoic acid (linoleic acid). Studies have reported that both of these PUFA raise the composition of unesterified fatty acids in the brain and elevate resistance to pentylenetetrazol-induced seizures [41]. The presence of linoleic acid has also shown potential activity such as anti-inflammatory and antidiabetic activities [42]. GC-MS analysis of palm kernel nut oil revealed the presence of dodecanoic acid, hexadecanoic acid, myristic acid octanoic acid. The combination of palm kernel nut oil with octanoic acid revealed the anti-seizure activity by delaying the onset of seizure from induction to the first seizure time [43].

Another major phytochemical compound obtained was 3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol, also known as phytol. The presence of phytol does indicate the activity such as antimicrobial, diuretic, antioxidant, and anti-inflammatory [44]. A study conducted by Costa *et al.* [45] has stated that phytol can produce an anticonvulsant effect by blocking mortality rate and dose-dependent behavior during the first hour in the acute phase of seizures. Other compounds such as decanoic acid act as a non-competitive antagonist at therapeutically relevant concentrations in a voltage and subunit-dependent manner, which shows that the crude extract of *C. asiatica* does possess anti-seizure effects [46]. Adding to the point, the properties of decanoic acid, which can readily cross the blood-brain barrier, support the evidence of anti-seizure activity [47]. Besides, another compound that is revealed through this analysis was octadecanoic, which is widely known as caprylic acid. This compound has the properties to enhance penetration across the blood-brain membrane [48].

A study conducted by Rath *et al.* [49] on methanol extract of aloe vera leaves shows that the GC-MS analysis revealed antioxidant and anti-inflammatory compounds such as phytol, alpha-tocopherol, caryophyllene, n-hexadecanoic acid, hexadecanoic acid methyl ester, squalene, and 9,12,15-octadecatrienoic acid methyl ester which possess pharmacological activities related to a neurodegenerative disorder such as antianxiety, antiepileptic, sedative-hypnotic and neuroprotective potential in neurodegenerative models. These activities have high prospects significantly in the treatment of neurodegenerative disorders.



(a)



(b)

Figure 1. GC-MS chromatograms of similar compounds found in (a) methanol; (b) acetone, crude extracts of *C.asiatica* leaves.

3.3.2. FT-IR analysis.

The FT-IR spectrum of methanol extract is shown in Fig 2. The data on the possible functional group based on peak values are tabulated in Table 4. The extract shows a characteristic absorption band at the wavelength range of 3365.78-3396.54 cm^{-1}, which indicates the presence of hydroxyl group (O-H). The wavelength range of 1413.82-1454.33 cm^{-1} and 2933.73 cm^{-1} indicated the presence of saturated alkanes (C-H). Carboxylic acid (C=O) was in the range of 1631.78cm^{-1}. Amides (C-N) were also found to be present in methanol extract at the range of 1359.82 cm^{-1}, whereas ether (C-O) at 1 049.28 cm^{-1}. The result was correlated with the secondary metabolites compounds found in the preliminary phytochemical analysis and GCMS analysis.

Table 4. Functional group based on peak values in both extracts.

Functional group	Frequency (cm^{-1})	
	Methanol Extract	Acetone Extract
Phenol (O-H)	3365.78	3385.70
Alkanes (C-H)	2933.73, 1413.82-1454.33	2924.09-2854.65
Carboxylic acid (C=0)	1631.78	1735.93-108.93
Amides (C-N)	1359.82	-
Ether (C-0)	1049.28	1035.77
Aromatic rings (C=C)	-	3010.88

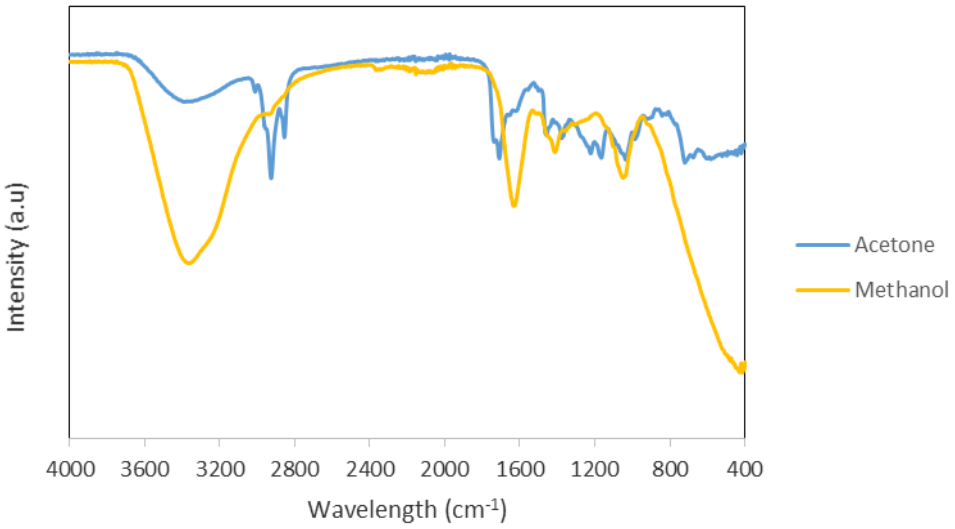


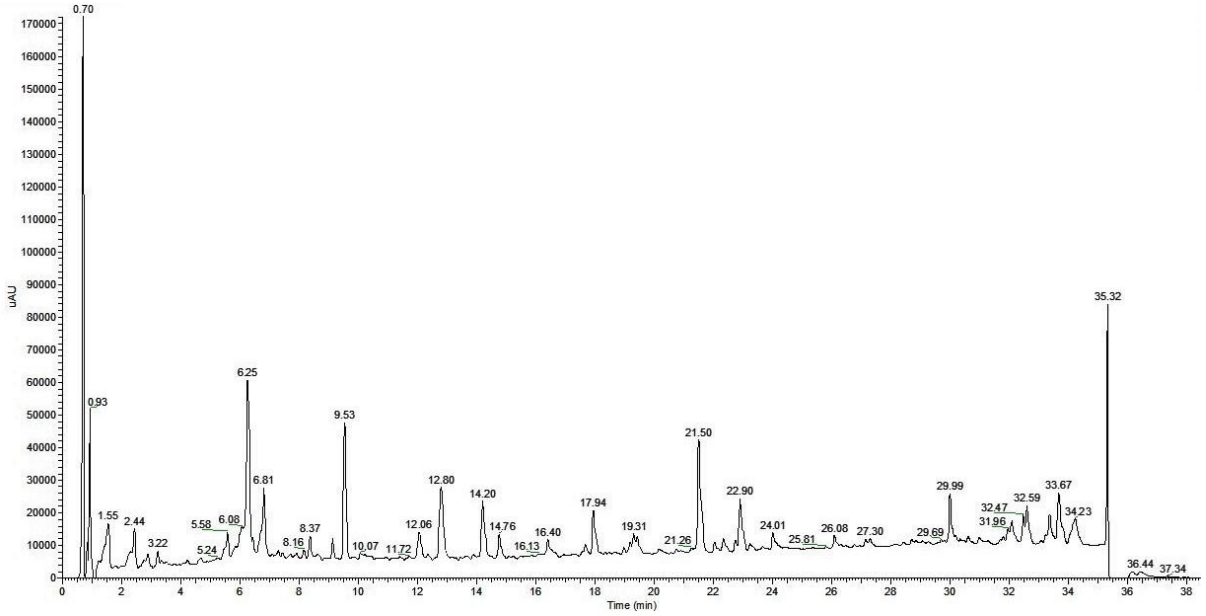
Figure 2. FT-IR spectra for methanol and acetone extract of *C.asiatica* leaves.

3.3.3. LC-MS analysis.

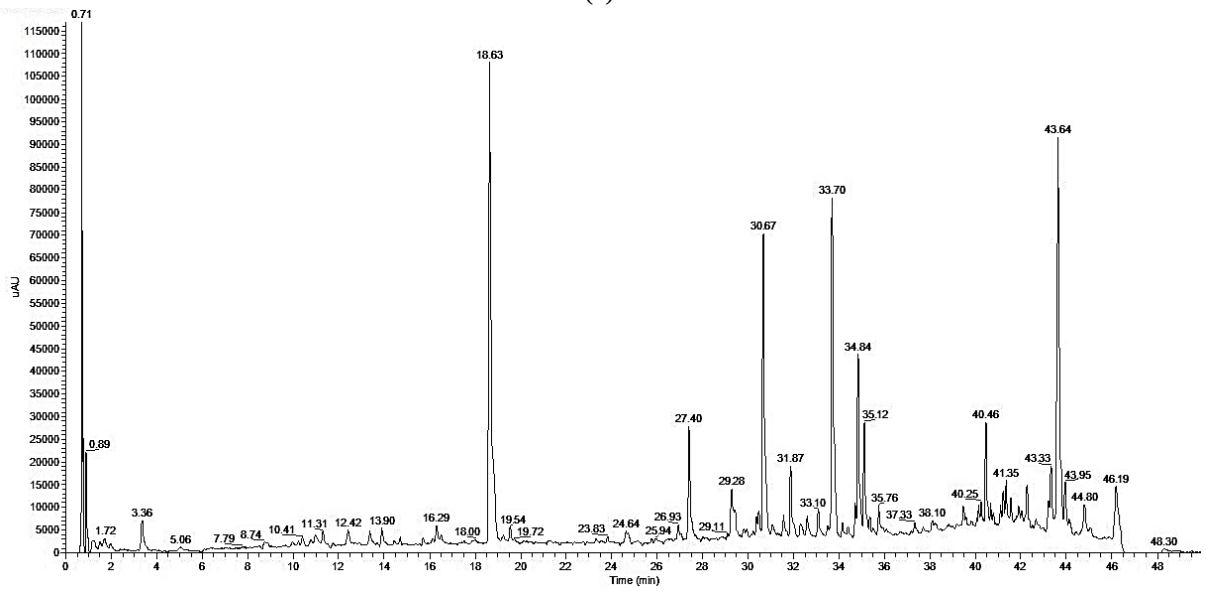
LC-MS analysis has been interpreted, and the vast compound has been found in methanol extract of *C. asiatica*. The presence of compounds together with their protonated molecular ions was interpreted using the PubChem database. Phytochemical compounds such as polyunsaturated fatty acid and saturated fatty acid were found in this analysis at different retention times (min) and molecular weight. Fig. 3 shows the LC-MS spectrum for methanol extract. Palmitic acid was found with a molecular weight of 256.43 g/mol with a retention time of 20.33 min and linoleic acid at 19.79 min with a molecular weight of 280.45 g/mol. Other phytochemical which was found to be responsible for biological activity are rutin, luteolin, and chlorogenic acid, which were found at the retention time of 5.20, 9.53, and 6.79 min, respectively.

Studies have reported that chlorogenic acid is a flavonoid compound that has the potential to act as an anti-depressant [50]. It has also shown a good protective effect against

enzymes such aminolevulinate dehydratase and acetylcholinesterase in the streptozotocin-induced rat in behavioral change [51]. The ability of chlorogenic acid to activate the voltage-gated channels has benefits in neuropathic and inflammatory pain by reducing the neuron's excitement [52]. Zaixiang *et al*. [53] have reported a study on the mechanism action of chlorogenic acid as an antibacterial agent that acts by killing pathogenic bacteria strain by provoking irreversible permeability changes in the cell membrane. Adaze *et al.* [54] have stated that rutin's ability to act as a neuroprotective effect could be due to its other biological properties such as antioxidant, anti-apoptotic, and anti-inflammatory.



(a)



(b)

Figure 3. LC-MS spectrum of (a) methanol; (b) acetone crude extracts of *C. asiatica* leaves.

Rutin has also shown antidiabetic activity by improving glycemic status by inhibiting intestinal carbohydrate absorption and stimulating pancreatic insulin secretion [55]. Luteolin, another flavonoid found in this extract, has been reported by Lin *et al.* [56] to possess anti-inflammatory and anti-cancer action due to its redox and estrogen regulating properties. Other than that, the main compound that belongs to *C. asiatica* leaves is asiatic acid, and it was found at a retention time of 14.22 min with a molecular weight of 488.70 g/mol. In general, asiatic

acid is a pentacyclic triterpenoid and plays a significant role in pharmacological activities, including anti-hypertensive, antioxidant, anti-inflammatory, anti-tumor, and neuroprotective. Besides that, these activities are supported by the abilities of asiatic acid to produce effects on enzymes, cell signaling cascades, growth factors, and receptors responsible for the vast pharmacological activities [11].

3.4. Solubility studies.

3.4.1. Selection of oil phase.

Table 5 shows seven types of oil used to determine the solubility of *C. asiatica* methanolic extract. The percentage of crude extract used was increased up to 1.0% (w/v) to demonstrate the maximum concentration of crude extract dissolving in oil. Based on the solubility screening, methanolic extract of *C.asiatica* leaves was able to dissolve at 0.2% (w/v) in virgin coconut oil without any precipitation compared to other oils, as shown in Table 4. Thus, virgin coconut oil was chosen as the oil phase to dissolve the methanolic extract of *C.asiatica* leaves.

Table 5. The solubility of oil in 0.2 % crude extract of *C. asiatica.*

Types of oil	**0.2 % of crude extract solubility physical observation**
Olive oil	Insoluble
Soybean oil	Insoluble
Flaxseed oil	Insoluble
Safflower seed oil	Insoluble
Sunflower seed oil	Insoluble
Palm oil	Insoluble
Virgin coconut oil	Soluble

Note: Soluble - homogeneous, Insoluble – contains a precipitate.

Based on the observation, virgin coconut oil was chosen for further analysis and was selected as the oil phase. Tween 80 has been chosen as a surfactant to bind with the oil phase due to the HLB value (15) and is used to coat the nanoemulsion surface to allow them more permeable towards the blood-brain barrier (BBB) [57]. Surfactants are important to minimize the surface tension between two different surfaces.

3.4.2. Phase behavior of virgin coconut oil/Tween 80/water system.

Fig. 4 shows the phase behavior of virgin coconut oil/Tween 80/water system, which comprises three different isotropic regions (F1, F2, and F3), obtained at a maximum of 0.2% *C.asiatica* crude extract used. All those three regions represent different percentage values of the oil phase, surfactant, and aqueous phase, as shown in Table 6. Particle size analysis was carried out for all three different regions. Based on the results, F2 shows the smallest particle size (57.86 nm), followed by F1 (107.4 nm) and F3 (125.9nm). The particle size was found in a suitable range of nanoemulsions.

F2 was selected as a formulation composition as it reveals the smallest particle size compared to the other points, which may lead to good stability. Alpha-tocopherol was then added to the chosen F2 formulation to enhance the biological stability of the formulation [58]. Besides that, studies conducted by Mehvari *et al.* [59] reveal that the addition of α-tocopherol has improved seizure control and reduced oxidative stress. Adding a small amount (1%) of α-tocopherol did not alter the particle size. The particle size was shown to be at ± 57.6 nm.

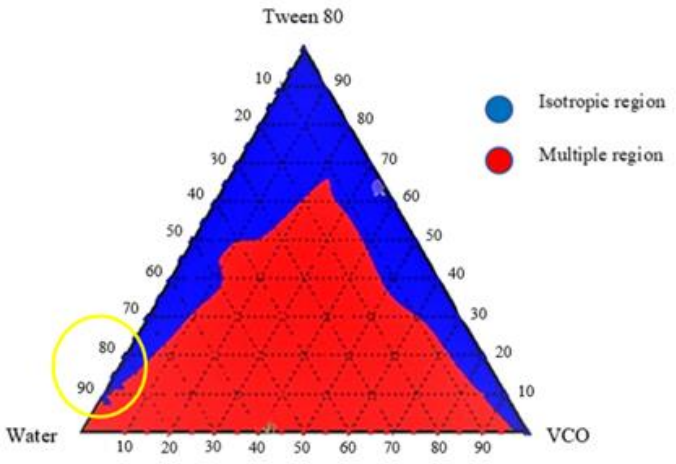


Figure 4. Phase behavior study of virgin coconut oil/Tween 80 and water system.

Table 6. Different percentage values of the oil phase, surfactant, and aqueous in three different regions.

	Formulation (% w/w)		
	F1	**F2**	**F3**
Composition			
Water	85.000	87.000	90.000
Virgin coconut oil	2.994	1.996	1.996
Crude extract	0.006	0.004	0.004
Tween 80	12.000	11.000	8.000
Physicochemical characterization			
Droplet size (nm)	125.4 ± 0.41	57.6 ± 0.31	107.8 ± 0.46
Polydispersity Index (PDI)	0.416±0.01	0.50±0.005	0.48 ± 0.004
Zeta Potential (mV)	-24.14 ± 0.30	-25.60 ± 0.21	-27.00 ± 0.29

Note: Values were expressed as mean ± standard deviation (n=3)

3.5. Physicochemical characterization.

3.5.1. Mean Droplet Size, Zeta Potential, and Polydispersity Index (PDI) analysis.

The mean droplet size, zeta potential, and polydispersity index (PDI) of *C. asiatica* nanoemulsion were at 57.60±0.31 nm, -26.5 Mv, and 0.40, respectively. The formulated nanoemulsion was found to be in the range below 200 nm [21,60]. This was also favorable with the suitable range targeted to the blood-brain barrier, which is to be below the range of 100 nm to achieve high penetration across the barrier. The stability of a nanoemulsion is measured by the electrokinetic potential of a particle in a solution via zeta potential measurement. The potential zeta range, which falls between +30 mV and lower than −30 mV, formed a stable nanoemulsion. Looking into the polydispersity index (PDI), the PDI of the formulated nanoemulsion was 0.40, which shows a good monodisperse system.

Roselan *et al.* [61] have stated that a PDI value near 0 indicates a monodisperse system, whereas a PDI value nearing 1 indicates a polydisperse system. Zanela da Silva Marques *et al.* [62] has also reported that a PDI value <0.4 shows a monodisperse system; meanwhile, a PDI value >0.5 gives a polydisperse system which may affect the organoleptic characteristics of the formulated nanoemulsion. Thus, both statements reported show a good agreement of a PDI range from 0 to 1 and are comparable with the results obtained. These parameters obtained show a good application to be used in the treatment of epilepsy. The parameters were found in good agreement due to the method of formulating the nanoemulsion, which is by using low energy emulsification method where it requires less energy as it utilizes the chemical energy formed in the system [63].

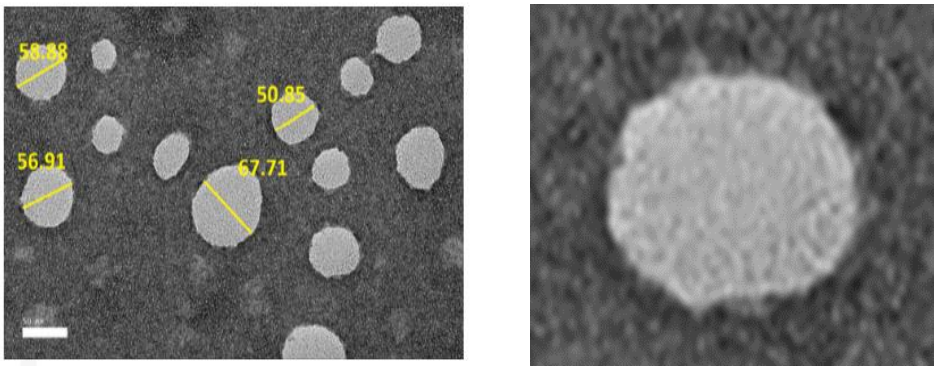
3.5.2. pH and conductivity measurement.

The pH value of the freshly formulated nanoemulsion containing crude extract of *C.asiatica* was 4.00. The pH range of 4.0 to 7.0 is usually suitable for topical application. Therefore, slight modification has been done by adding sodium chloride to obtain a suitable pH value for parenteral application, above 8.0. The formulated nanoemulsion containing crude extract of *C.asiatica* was developed as a promising drug delivery system in the treatment of epilepsy. Yet, the route of administration could be decided according to the pH value for any novel preparation in the future. The pH value could be altered according to the administration site [64]. In this study, the pH was altered to 8.3 with the future purpose of parenteral application. The addition of sodium chloride to the nanoemulsion produces droplet size (60 nm) within the acceptable range of 20 nm to 200 nm.

The formulated nanoemulsion was identified as O/W nanoemulsion as it has a high conductivity value (234 μScm^{-1}) as the water is in the external phase. An emulsion with low conductivity (lower than 10 μScm^{-1}) indicates the emulsion to be a W/O emulsion system, whereas a higher conductivity reveals an O/W μScm^{-1} emulsion system due to the presence of water in the phase [65].

3.5.3. Morphology.

The transmission electron microscopy (TEM) images of the formulated nanoemulsion containing crude extract of *C.asiatica* are shown in Fig. 5. The TEM results have revealed that the formulated nanoemulsion containing crude extract of *C.asiatica* shows a good monodisperse system. In addition, the droplet size was also found to be in the range of 50 to 70 nm. The droplets obtained were spherical without any aggregation. Thus, it can be related that the polydispersity index value for the nanoemulsion was found to be in good agreement with the TEM analysis.



(a) (b)

Figure 5. Transmission electron microscopy (TEM) images of the formulated nanoemulsion containing crude extract of *C.asiatica* (a) on the scale of 50 nm, and (b) magnified spherical shape.

3.6. Stability studies.

3.6.1. Centrifugation.

The formulated nanoemulsion shows a homogeneous mixture after centrifuged at 4000 rpm for 15 min, revealing that the formulation was stable and had good physical stability under normal storage conditions. Table 7 shows the observation based on the centrifugation test at different temperatures.

Table 7. Observation of centrifugation test and storage at different temperatures.

Storage temperature (°C)	Time (days)							Centrifugation Test
	1	7	14	21	30	60	90	
4	√	√	√	√	√	√	√	√
25	√	√	√	√	√	√	√	
45	√	√	√	√	√	√	√	

Note: √ = stable (no separation layer).

The centrifugation test was used to predict the shelf life under normal storage conditions [66]. The formulated nanoemulsion remained at a homogeneous phase after 90 days of storage condition under three different temperatures.

3.6.2. Particle size over time.

The storage stability of CA-NE was found to be stable at different temperatures. Fig. 6 shows the plotted graph of droplet size over the storage of 3 months. The physical observations show no phase separation and color changes for 90 days of storage (Fig. 7). It reveals an increment in the droplet size over time but remained within the range of nanoemulsion (20 - 200 nm). The particle size that falls within the range revealed that the nanoemulsion would be resistant to physical destabilization, flocculation, and creaming [67].

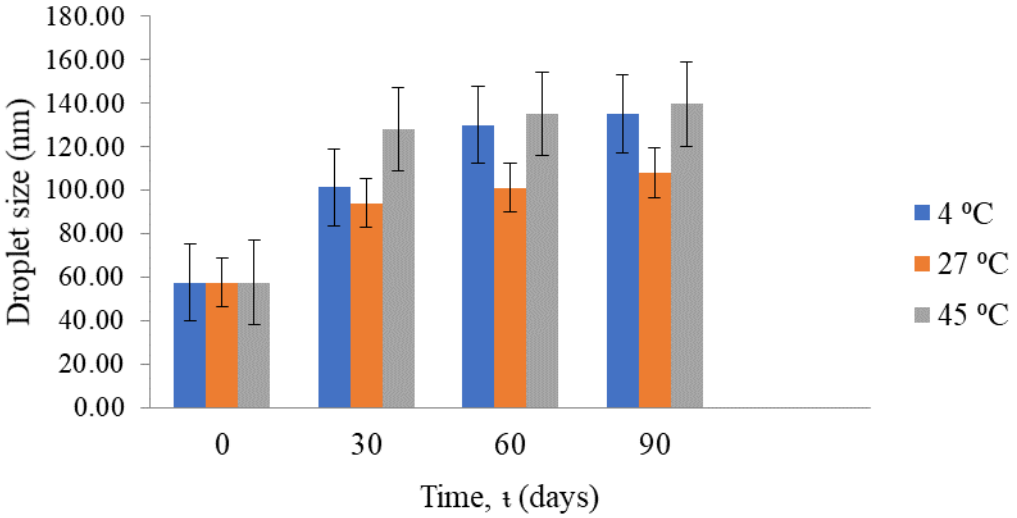


Figure 6. Storage of formulated *C.asiatica* nanoemulsion in 90 days in three different temperatures (4°C, 27°C, and 45°C) against droplet size.

(a) **(b)** **(c)**

Figure 7. Physical observations of formulated *C.asiatica* nanoemulsion at (a) 30 days, (b) 60 days, and (c) 90 days.

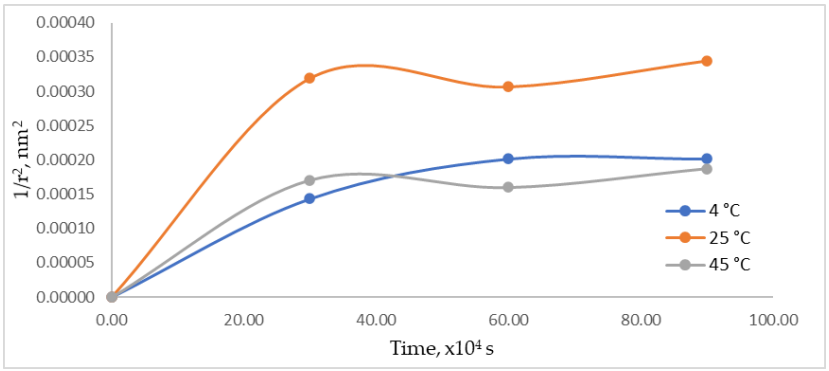
Based on Fig. 6, the droplet size showed increment but was still within the range. Tween 80, which was used as a non-ionic surfactant in the nanoemulsion, has prevented the droplet collision. According to Syed Azhar *et al.* [68], the steric barrier formed on the water interface or oil interface has been a major factor in preventing the droplet collision, enabling the nanoemulsion to maintain the droplet size within the nano range.

3.6.2.1. Coalescence rate.

Coalescence occurs as two or more emulsion droplets fuse upon forming a larger droplet [69]. Fig. 8(a) shows that the graph plotted has no linear relationship, which indicates that the increment of particle size over time is not due to coalescence. According to Sharma *et al.* [70], a small nanoemulsion droplet size can suppress the coagulation or the coalescence of nanoemulsion droplets.

3.6.2.2. Ostwald ripening.

Fig. 8(b) shows that the Ostwald ripening plotted graph over time results in no linear relationship hence the increment that occurred was not caused by Ostwald ripening phenomena. Surrounding temperature is attributed to energy absorption by the particles in the nanoemulsion system, which leads to greater collision and kinetic energy among particles as the energy increases [71]. Roselan *et al.* [61] have also stated that this kinetic energy was possible for small droplet particles to diffuse together, forming larger droplet sizes.



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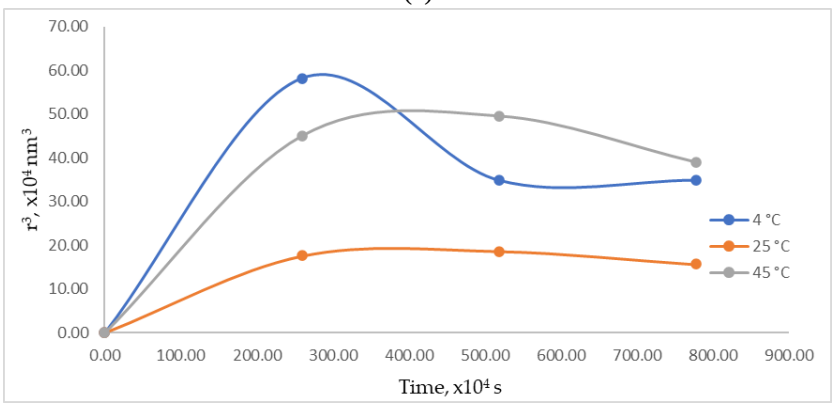


(b)

Figure 8. Coalescence (a) and (b) Ostwald ripening of the formulated nanoemulsions.

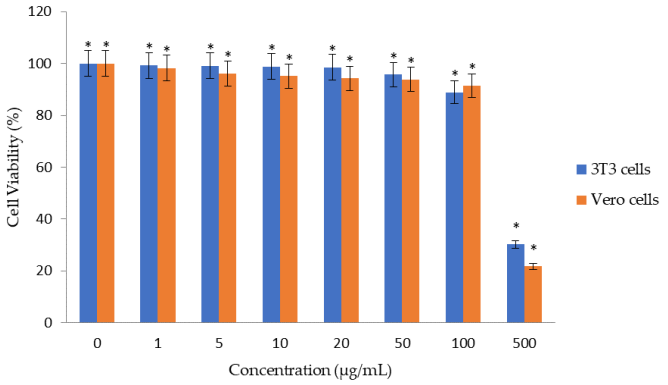
3.7. Cytotoxicity study.

In vitro cytotoxicity screening has been carried for both the methanol extract of *C.asiatica* and formulated nanoemulsion containing crude extract of *C.asiatica* on normal cell lines, including African green monkey kidney (Vero) mouse embryonic fibroblast cell (3T3) for general screening. Vero cells were chosen due to the parenteral administration, as any formulation will pass through the systemic circulation and accumulate in vital organs such as the kidney and liver. This selection of cell lines was also carried out based on a previous study by Vijayarathna & Sasidharan [72]. Table 8 shows the half-maximal inhibitory concentration (IC_{50}) of the methanol extract and nanoemulsion on normal cell lines.

Table 8. The half-maximal inhibitory concentration (IC_{50}) values on normal cell lines.

	IC_{50} (µg/ml)	
	Vero cells	**3T3 cells**
Methanol extract of *C.asiatica*	340.00	365.00
Nanoemulsion containing crude extract of *C.asiatica*	>500.00	

Based on Fig. 9 (a), methanol extract shows cell viability in both normal cell lines with the IC_{50} value of 340.00 µg/ml and 365.00 µg/ml in the Vero cell and 3T3 cell lines, respectively. Interestingly, nanoemulsion formulation showed an IC_{50} value above 500 µg/ml in both normal cell lines (Fig. 9 (b)). An IC_{50} value > 100 µg/ml were classified as least toxic and least effected on cells, whereas IC_{50} value < 100 µg/ml were considered toxic [73]. Since the IC_{50} value of the formulated nanoemulsion shows greater cell viability, it can be suggested for parenteral application in terms of safety and efficacy, which is yet to be further studied.



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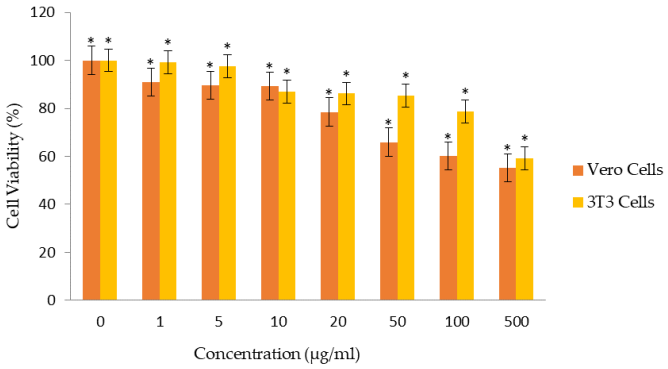

(b)

Figure 9. The cytotoxicity effect of (a) crude extracts of *C. asiatica*, and (b) nanoemulsion containing *C.asiatica* crude extract against Vero and 3T3 cell lines at 72 h treatment.

Statistically significant differences in the various cell lines and the effect on the different types of crude extracts overexposure of 72 h were observed at $p \leq 0.05$. Thus, these results have shown that both the methanol extract and nanoemulsion of *C. asiatica* have significant potential as promising drug delivery in the treatment of epilepsy.

3.8. In vitro permeation study on different pH.

In vitro permeation study has been carried out at two different pH (pH 8.4 and 6.4) for the nanoemulsion formulation containing crude extract of *C.asiatica*. Fig. 10 showed that the cumulative release of *C.asiatica* nanoemulsion at for both pHs was found to be 100%. After 24h, the nanoemulsion formulation containing *C.asiatica* crude extract at pH 8.4 has reached 100% of release and remained constant through 72h. Looking into pH 6.4, the formulation

reaches its maximum efficacy (100%) at 72 h. pH 8.4 shows an intermediate release as the maximum efficacy was reached within 24h, whereas pH 6.4 shows a sustained released pattern throughout the 72 h. According to Bennewitz and Saltman [21], coating with Tween 80 surfactant eventually leads to prolonged release of the nanoemulsion as the surfactant forms a great binding with Apolipoprotein B and E when it comes in contact with blood. This statement was found to be in good agreement with the release of both the pH. The release profile was more favorable in pH 8.4 due to the diffusion that occurs within the nanoemulsion with almost the same pH.

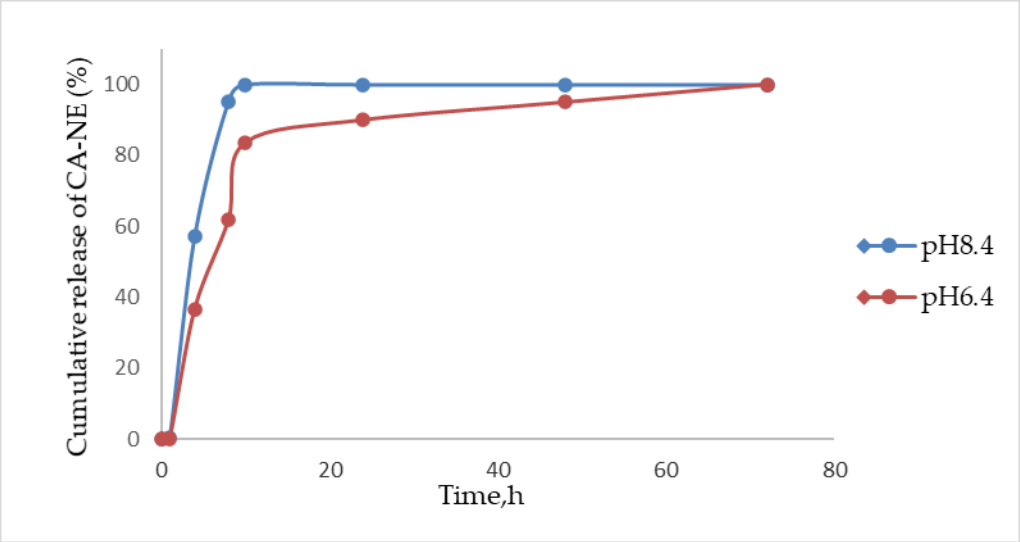

Figure 10. Cumulative release of *C.asiatica* nanoemulsion in two different pH.

4. Conclusions

Various extraction of *C.asiatica* using organic solvents (methanol and acetone) has been studied. Methanol extract reveals the highest percentage of yield (33.20 %) due to the capacity of the solvent to elucidate most of the compounds found in *C.asiatica* leaves. Qualitative analysis of preliminary phytochemical screening has further supported the evidence that the highest percentage yield of methanol crude extract reveals the most phytoconstituent compounds present in *C. asiatica*, mainly terpenoids and saponins, followed by acetone extract and n-hexane extract of *C. asiatica.* GC-MS analysis has revealed the presence of n-hexadecanoic acid, linoleic acid, and phytol, which is found abundantly in both extracts with a different percentage area. In contrast, LC-MS results have shown the presence of the major compound related to epilepsy found in *C.asiatica* leaves which is Asiatic acid, at a retention time of 14.21. FTIR results have shown the presence of various compounds, mainly phenols, that support the presence of the OH group in the extracts. The other claims, as reported, reveal the presence of phytochemicals related to epilepsy treatment, such as terpenoids and flavonoids. The presence of various phytoconstituents has concluded the claims that *C.asiatica* leaves do possess various activities as for anti-epilepsy besides for wound healing, memory enhancer, and anti-cancer. Interestingly, the cytotoxicity of the formulated nanoemulsion showed the least toxicity with the IC_{50} of 500 µg/ml with a greater percentage release profile in pH 8.4. The formulated nanoemulsion containing crude extract of *C. asiatica* leaves proven evidence of good physicochemical characterization properties that enables the formulation to be a promising regimen to be used in further study in a pharmaceutical application, specifically in the treatment of epilepsy.

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Conflicts of Interest

The authors declare no conflict of interest.

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