

Formulation and Characterization of *Caesalpinia decapetala* Seed Oil Nanoemulsion: Physicochemical Properties, Stability, and Antibacterial Activity

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Abstract

Nanoemulsions contain acceptable O/W or W/O dispersions, with droplet sizes ranging from 100 to 500 nm. In the current study, we formulated O/W nanoemulsion using *Caesalpinia decapetala* seed oil, Tween 20, and Tween 80 surfactant through ultrasonic and spontaneous emulsification methods. *C. decapetala* is a climbing shrub thorny tree. *C. decapetala* seed oil contains different chemical constituents predominantly unsaturated fatty acid and has antibacterial, and antioxidant activity. Then physicochemical properties of the prepared nanoemulsion were evaluated using different methods. As a result, the prepared nanoemulsion came to be 132.56 ± 0.49 - 290.033 ± 1.95 nm average particle size, 0.028 ± 0.038 - 0.3006 ± 0.04 polydispersive index, -32.27 to -58 mV zeta potentials, 1.334 - 1.380 refractive index, 75 - 90.81% percent of transmittance, 4.38 - 6.5 pH value, 3.922 – 5.2468 mPa.s viscosity value with spherical shape, excellent physical stability and also had good oxidative stability of the molecule. Subsequently, gram-positive and gram-negative bacterial strains were employed to assess the nanoemulsion's antibacterial efficacy. Therefore, the results indicate that *C. decapetala* seed oil nanoemulsion has excellent antibacterial activity on both gram-positive and gram-negative bacteria strains. This nanoemulsion that was formulated using Tween 80 had higher inhibition zones like 13.5, 13, 12, and 11 mm than the other methods on *S. aureus*, *E. coli*, *S. pyogenes*, *P. aeruginosa* respectively. Generally, smaller particle size, polydispersive index, stable surface charge, and low value of viscosity indicate that formulated nanoemulsion had better stability and bioavailability activity for antibacterial activity.

1. Introduction

An emulsion is referred to as a heterogeneous system that always contains two immiscible stages: a fluid stage (an outer stage) and a droplet (scattered stage). Emulsions are the dispersion of fat or oil into water (O/W) or the opposite. A liquid acquires a distinct flavor and texture when fat is emulsified into it. Because a typical emulsion's molecule size increases steadily over time and partitions at gravitational force, these emulsions are thermodynamically unstable¹. The proportions of the oil droplets in an emulsion is its most obvious physical feature. For these droplets to not be categorized as a colloidal suspension, their size must be greater than 0.1 micrometers. In reality, the majority of commercial emulsions contain particles with diameters ranging from roughly 0.2 μm to 100 μm ².

There are two types of emulsion these are single and double emulsion. W/O or O/W emulsions are single emulsions that, are synthesized by a single-step process and use a single surfactant³. Double emulsions are W/O/W or O/W/O emulsions. They are generated in two steps using two different surfactants: one hydrophobic for maintaining the interface and the other hydrophilic for the oil globule interface. The latter is created by lowering the shear to stop the internal droplets from rupturing, while the primary oil in water is produced through high-shear techniques³. There are three types of emulsions based on time: semi-permanent, permanent, and temporary. A straightforward vinaigrette is an illustration of a temporary emulsion, whereas mayonnaise is a permanent emulsion. The ability of an emulsion to resist

changes in its properties over time is known as emulsion stability⁴. Food products have a significant impact on the apparent quality of an emulsion. These factors include droplet size and distribution, specific gravity, stability, and rheology characteristics. To achieve emulsion dependability, adding the hydrocolloids to the watery stage can produce particular rheological characteristics⁵. Generally, a schematic representation of water- in-oil and oil-in -water emulsion can be described in (Fig. 1).

Emulsions with droplet sizes in the nanometer range, usually ranging from 1 nm – 1000 nm, are known as nanoemulsions. The typical average particle size range is 100–500 nm. According to⁷, the particles can exist in two different forms: water-in-oil and oil-in-water, with water or oil serving as the particle's core. According to⁸, nanoemulsions, which have a diameter of approximately 100 nm, are kinetically stable droplets of one liquid phase distributed in another immiscible phase. An interfacial layer of surfactant reduces the surface tension between the two immiscible liquids, stabilizing the dispersed phase. NEs are characterized by their good transparency, high surface area, exceptional stability, and tunable rheology that make an actual alternative for drug delivery, cosmetics, food, as well as pharmaceutical industries⁹. To improve the kinetic stability of emulsions, stabilizers such as emulsifiers, weighting agents, ripening inhibitors, and texture modifiers (thickeners and gelling agents) are commonly used¹⁰. Emulsion instability causes droplets to float to the top, cohesiveness between droplets, and ultimately creaming and separation. In general, nanoemulsion has more advantages than micro-emulsion since it is made with a lower concentration of surfactants comprised between 3–10% may be enough and is ideal for effectively delivering active ingredients through the skin¹¹. Therefore, the current study aims to formulate a *C. decapetala* seed oil-in-water nanoemulsion, and stabilized by two surfactants, such as Tween 20 and Tween 80 by varying concentrations of specimen seed and surfactant. In order to get stable nanoemulsion ultrasonic and spontaneous emulsification methods are used. The formulated nanoemulsion was characterize using FT-IR, pH, oxidative stability, refractive index, present transmittance, average particle size, polydisperse index, surface charge, and physical stability for analysis of antibacterial activity.

There are two key techniques used to create NEs: high-energy methods and low-energy methods¹². High-energy techniques break large droplets down to a size of approximately 100 nm using excess energy (~ 108 W/kg)⁸. High-energy methods, with their brute force technique, offer a safe technique to synthesize nanoemulsions with droplet phase volume fractions up to 40%¹³. Nevertheless, the use of excess shear makes them incompetent and vulnerable to heat effects. Examples of high-energy techniques are high-pressure homogenizers, microfluidizers, and ultrasonication. In contrast, low-energy techniques exploit the small interfacial tension characteristics of a system to reduce droplet size with energy input that can be achieved by approximately 103 W/kg magnetic stirrer and provide an easy and accessible route to make nanoemulsions without the use of excessive shear. Low-energy emulsification techniques include spontaneous emulsification, phase inversion temperature, and phase inversion composition. Oil, surfactant, water, and co-surfactant are the main constituents involved in the preparation of nanoemulsions. Cationic, anionic, non-ionic, and zwitterionic are under the classifications of charge-based surfactants. Lauric arginate and biosurfactants are two examples of cationic and anionic

surfactants, respectively¹⁴. The food and pharmaceutical industries use a diversity of non-ionic surfactants, including sugar esters, monoglycerides, polyoxyethylene sorbitan fatty acid esters (tweens), and sorbitan fatty acid esters or spans¹⁵. Nanoemulsions can destabilize in any of the conceivable ways; Ostwald ripening is typically responsible for the initial growth of droplets. Nevertheless, as droplet size increases, flocculation and coalescence become more significant.

2. Materials and methods

2.1. Specimen collection, identification and oil extraction

C. decapetala seeds, were collected from the Addis Ababa Science and Technology University campus Akaky Kaliti subcity, Kilinto, Addis Ababa, Ethiopia. The plant specimen with voucher number, YN001/2023 was identified by Melkamu Wondaferah (botanist) in the National Herbarium Department of Plant Biology and Biodiversity Management, College of Natural Sciences Addis Ababa University Sami, Adam park, King George VI St, Addis Ababa, Ethiopia. After collecting the seeds, the seed cover was removed from the pulp manually, then crushed with a mortar and pestle, and stored in a plastic container at room temperature. The extraction process was carried out based on¹⁶ with minor modifications with the help of an automatic soxhlet extractor (Bucher, v16, Germany). The laboratory activity of the current study was carried out in the Addis Ababa Science and Technology University laboratory.

2.2. Chemicals, reagents, and instruments

C. decapetala seed oil, all analytical-grade chemicals and reagents such as polyethylene glycol sorbitan monooleate (Tween 80, Tween 20), polyethylene glycol 4000, acetone, deionized water, Mueller-Hinton agar, 2,2-diphenyl-1-picrylhydrazyl, methanol, standard bacterial strains such as *Streptococcus pyogenes* (*S. pyogenes*, ATCC 19615), *Staphylococcus aureus* (*S. aureus*, ATCC 29737), *Escherichia coli* (*E. coli*, ATCC 25922), and *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 27853), were sourced at the Ethiopian Public Health Institute in Addis Ababa, Ethiopia. from the Traditional and Modern Medicine Research Directorate Laboratory, mortar and pestle, DLS (Malvern Zetasizer, Malvern, UK), sonicator (intelligent ultrasonic processor, NINGBO SJIAAB Equipment Co. LTD., SIIA-950W), digital refractometer (A.KRÜSS Optronic Digital Refractometers T-Model, Germany), UV-Vis (Jasco V-770, Japan), FT-IR (Nicolet TM iS50 FTIR spectrometer), TEM (EOL JEM-F200, and Japan), Modular Compact Rheometer (MCR 102 SN82129736), benchtop centrifuge (Centurion Scientific Ltd C2006), digital pH meter (CPH-102), and hotplate were the main chemicals and instruments required in this study.

2.3. Evaluation of physicochemical properties of *C. decapetala* seed oil

The physicochemical parameters of *C. decapetala* seed oil were assessed using standard procedures. Specific gravity, refractive index, acid value, iodine value, saponification value, unsaponifiable matter, and viscosity were among these parameters. These analyses provide essential information about the quality and characteristics of the seed oil.

2.4. Synthesis of *C. decapetala* seed oil nanoemulsion

2.4.1. Ultrasonication emulsification

Nanoemulsion was formulated based on¹⁷ using oil, Tween 80, and water. Oil-to-surfactant compositions such as 1:0.5, 1:1, 1:1.5, 1:2, 1:3, 1:4, and 1:5 were prepared, and 1.5 mL of polyethylene glycol 4000 as co-surfactant was thoroughly mixed. The solution was mixed at 750 rpm for 3 minutes with a magnetic stirrer to get uniform oil to the surfactant solution. Then, the total internal phase solution was 100 mL by adding distilled water dropwise to obtain an oil-in-water NE. Then the emulsion was stirred at 750 rpm for 10 minutes using a magnetic stirrer to get a uniform distribution. The emulsions were then sonicated by an ultrasonic instrument with frequencies of 20 to 24 kHz with a maximum probe diameter of 6 mm for 20 minutes for each composition. The initial coarse emulsions and final nanoemulsions temperature difference should be less than 10°C. The high-energy method such as ultrasonic emulsification was used to generate heat during the long process of emulsification. This generated heat is nullified by the insertion of the sample container in an ice beaker to minimize the temperature¹⁸. According to the above technique, Table 1 and Figure. 2 show the ratio of oil to Tween 80 with co-surfactant and water for the formulation of nanoemulsion by using the ultrasonication emulsification method.

Formulation	CD oil	Tween 80(S)	PEG 4000 (CS)	Ratio oil to S	Water	The appearance of prepared NE
NE1	1mL	0.5 mL	1.5mL	1:0.5 mL	97 mL	Milky
NE2		1 mL		1:1 mL	96.5 mL	Milky
NE3		1.5 mL		1:1.5 mL	96 mL	“
NE4		2 mL		1:2 mL	95.5 mL	“
NE5		3 mL		1:3 mL	94.5 mL	“
NE6		4 mL		1:4 mL	93.5 mL	“
NE7		5 mL		1:5 mL	92.5 mL	“
Note = CD: <i>Caesalpinia decapetala</i> , CS: Co-Surfactance, NE: nanoemulsion, and S: surfactant						

Table 1: Ratio of CD oil, Tween 80, co-surfactant, and water for nanoemulsion synthesis

Additionally, nanoemulsion was formulated using Tween 20 surfactant based on¹⁸ with slight modifications. Various ratios of oil and Tween 20 surfactant were mixed, such as 1:0.5, 1:1, 1:2, 1:3, and

1:4, and water was added at different volumes. The solution was blended at 750 rpm for 10 minutes with a magnetic stirrer to get a uniform distribution, then sonicated by an ultrasonicator with frequencies of 20 to 24 kHz for 20 minutes with 750 W maximum power output. Based on this, Table 2 and Figure. 2 describe the formation of nanoemulsions using oil, Tween 20, and water as core materials with different ratio.

Formulation	CD oil	Tween 20	Ratio oil to S	Water	Appearance of NE
NE1	1 mL	0.5 mL	1:0.5 mL	98.5 mL	Milky
NE2		1 mL	1:1 mL	98 mL	Milky
NE3		2 mL	1:2 mL	97 mL	“
NE4		3 mL	1:3 mL	96 mL	“
NE5		4 mL	1:4 mL	95 mL	“
Note= CD for <i>Caesalpinia decapetala</i> , NE for nanoemulsion, and S for surfactants					

Table 2: Ratio of CD oil, Tween 20, and water for synthesis of nanoemulsion

2.4.2. Spontaneous emulsification

Nanoemulsion was formulated by spontaneous emulsification based on the¹⁹ method with some modifications. The homogeneous organic solution was formulated by mixing 400 mg oil and 86 mg lipophilic surfactant (polyethylene glycol 4000) in 40 mL acetone (water-miscible solvent). 136 mg Tween 80 and 80 mL of water were mixed to create the homogenous aqueous phase. After that, the organic phase was added to the aqueous phase while being stirred magnetically. This caused the organic solvent to diffuse into the external aqueous phase, forming nano-droplets that instantly formed the O/W emulsion. For thirty minutes, the magnetic stirring was kept up to allow the system to come to equilibrium. By letting the water-miscible solvent evaporate under low pressure for 45 minutes, the solvent was eliminated. Oil was dispersed in a homogenous solution and hydrophilic surfactant²⁰. Of these, Figure.3 shows the formation of nanoemulsions by using Tween 80 and co-surfactant through spontaneous emulsification methods.

2.5. Characterization of nanoemulsion (NE)

2.5.1. Droplet size and particle size distribution (PSD)

The typical particle size and polydisperse index of NE were assessed using a dynamic light scattering instrument. This method determines the size distribution of small droplets in suspension, ranging from 3 nm to 5 μm²¹. To ensure accurate droplet size measurement via dynamic light scattering, the specimen should be transparent and appropriately diluted. The test was conducted at 25°C with a 173⁰ scattering

angle¹⁷. A 10% v/v solution was prepared to mitigate multiple scattering effects caused by high-quality fractions.

2.5.2. Surface charge

The zeta potential of the nanoemulsion was assessed using dynamic light scattering, following the technique designated by¹⁷ with some modifications. The Zeta potential of the NE was measured by prepared accurately, a 10% v/v solution.

2.5.3. The refractive index of NE

The refractive index of NE was analyzed by a digital refractometer. Some drops of nanoemulsion were placed on the slide of the digital refractometer at 25°C, and the value was then recorded²².

2.5.4. Percent transmittance of NE

The percent transmittance of the formulated NE was analyzed using UV-Vis spectroscopy. This method, inspired by the work of²³, underwent a minor modification. One mL of the formulated nanoemulsion was diluted 100 times using methanol. The diluted sample was then analyzed at a wavelength of 260 nm, with methanol as the blank.

2.5.5. Determination of pH value of NE

A calibrated pH meter was employed to determine the pH of the nanoemulsions at 25⁰C. The electrode was inserted directly into the formulated nanoemulsion. The digital pH meter was used to determine the pH value of the nanoemulsion based on the technique described by²⁴ by preparing the sample, (1:10 v/v) of nanoemulsion with water in a beaker and homogenizing the solution. The electrode was then inserted directly into the homogenized solution of the sample. All the readings were taken in triplicate. pH is a serious parameter to get a stable nanoemulsion because any pH deviation can influence the whole stability of the nanoemulsion.

2.5.6. Physical stability of NE

The stability of the formulated nanoemulsion was assessed using a Benchtop centrifuge based on the¹⁸ method with a minor adjustment. In this test, 15 mL of the nanoemulsion was placed in a centrifuge vial and centrifuged at 10,000 rpm for 30 minutes. Additionally, the intrinsic stability of the formulated nanoemulsion was investigated by storing it at room temperature. During this storage period, the sample was detected for slight signs of creaming formation or phase separation. Furthermore, changes in droplet diameter were monitored at various time intervals.

2.5.7. Rheological properties of NE

Viscosity is an essential parameter of nanoemulsions. The Modular Compact Rheometer device was used to measure the rheological properties of the formulated nanoemulsions, with minor modifications based on the²⁵ method. With the use of a sample holder cup plate and a rheometer spindle, the rheology

property of the nanoemulsion was determined at 40°C temperature at a shear rate of 0.01 to 1000 s⁻¹ in the presence of torque using 20 mL of nanoemulsion sample.

2.5.8. Determination of oxidative stability of NE

The peroxide value of the nanoemulsion was investigated following the National Food Safety Standards and the technique outlined by²⁶, with minor adjustments. 1.5 mL of isooctane /isopropanol (3:1 v/v) mixture and 0.3 mL of nanoemulsion were centrifuged for five minutes at 10,000 rpm. Next, 0.2 mL of the transparent upper solvent layer was mixed with 2.8 mL of methanol/butanol (2:1 v/v) and 30 µL of thiocyanate/ferrous iron solution. 15 µL of 3.94 M ammonium thiocyanate solution and 15 µL of 0.072 M ferrous iron solution were mixed to create the thiocyanate/ferrous iron solution. 25 mL of BaCl₂ solution (0.132 M BaCl₂ in 0.4 M HCl) and 25 mL of 0.144 M FeSO₄ solution were mixed to create the ferrous iron solution. After 20 minutes, UV-VIS spectroscopy was used to measure the samples' absorbance at 510 nm against a blank. For the liner curve, a standard solution of Fe (III) at 1 mg/mL of stock solution was prepared. Then the peroxide value of the nanoemulsion was calculated based on the²⁷ formula and expressed as meqO₂/Kg nanoemulsion.

$$PV = \frac{Abs}{55.84 \times w} \times \frac{1}{b}$$

Where: w = sample weight (g); Abs = absorbance; 55.84 = atomic weight of Fe³⁺; b = the slope of the Fe (III) calibration curve.

2.5.9. Morphology of NE

The nanostructure and morphology of a prepared nanoemulsion were investigated using TEM. The methodology followed the description by²⁸. After diluting the nanoemulsion by 1:100 using deionized water, 50 µL was passed through a 0.22 µm filter membrane. Then a portion of the sample was cast-mounted onto 200 mesh formvar-coated copper grids and stained with a two percent solution of uranyl acetate (UA) or phosphotungstic acid. Afterward, excess liquid was removed, and the synthesized specimens were dried using Whatman filter paper at 25 °C. Finally, a microscope was employed to analyze the prepared slide at an accelerated voltage of 120 kV.

2.5.10. FT-IR analysis of NE

The infrared spectrum of the formulated nanoemulsion was analyzed using a Fourier transform infrared spectrophotometer. FT-IR investigation was used to identify the functional groups that are present in nanoemulsion and also detect the interactions between them. A small drop of formulated nanoemulsion was placed on FT-IR pate. The spectra of nanoemulsion were subjected in the wavenumber range of 4000 – 500 cm⁻¹.

2.5.11. Antibacterial activity of NE

A formulated nanoemulsion and four bacterial strains were utilized to assess the antibacterial activity of the nanoemulsion sample. Two bacterial strains were from Gram-positive (*S. aureus* and *S. pyogenes*),

while the other two bacterial strains were from Gram-negative (*E.coli* and *P. aeruginosa*). The methodology, as outlined by²⁹, was presented to investigate the antimicrobial activity of the nanoemulsion, with minor adjustments. Specifically, 8g of Luria-Bertani (LB) media and 20g of agar powder were dissolved in 1000 mL of deionized water and autoclaved at 121⁰C for 20 minutes to sterilize the culture media. Subsequently, the mixture was cooled to 55°C and stored in the dark at 4°C for future experiments. The bacterial cultures were grown at 37°C for 24 hours using an LB broth medium, as previously mentioned. The final bacterial population was standardized to 0.5 McFarland turbidity equivalent to 1.5×10^7 CFU/mL. For the antibacterial assay, 0.1 mL of the bacterial suspension was placed on each plate filled with LB agar. Next, various volumes (100, 75, and 50 mg/mL) of the nanoemulsion were applied to sterile discs, covered with filter papers, and incubated for 24 hours at 37°C. Finally, the inhibition zones of samples on tested bacteria were measured.

3. Results

Statistical analysis

All experiments were performed in triplicate, and the results were expressed as the average and standard deviation calculated from these measurements. Analysis of variance (ANOVA) was performed. The physicochemical properties of NE such as stabilities, particle size, poly dispersive index, particle, pH value, FT-IR, and microstructure were characterized for their various application.

3.1. Physicochemical characteristics of *C. decapetala* seed oil

In this study, the percentage yield and physicochemical properties of the extracted *C. decapetala* were calculated and evaluated using several parameters because it is an essential tool in the exercise of quality control of seed oil for its applications, and the results are mentioned in (Table 3).

Table 3
Physicochemical properties of *C. decapetala* seed and seed oil

Parameters	Unit	Experimental value
Yields (w/w)	%	22.06
Physical state at 25 ⁰ C	-	liquid
Freezing point	⁰ C	-5
Boiling point	⁰ C	145
Color	-	Reddish yellow
Odor	-	Disagreeable
Moisture content	wt. %	0.937 ± 0.1103
Specific gravity at 25 ⁰ C	-	0.933 ± 0.0017
Viscosity at 25 ⁰ C and 40 ⁰ C	mPa.S	12.271 and 37.322
Refractive index at 40 ⁰ C	-	1.4765 ± 0.00017
Ash content	wt. %	3.435 ± 0.0722
Saponification value	mg KOH/g oil	188.6667 ± 1.2472
Acid value	mg NaOH/g of oil	1.706 ± 0.212
Peroxide value	meq O ₂ /kg of oil	7.333 ± 0.39969
Iodine value	gl ₂ /100g of oil	90.117 ± 1.10446
Unsaponification matter	wt. %	3.183 ± 0.1078

3.2. Formulation of nanoemulsion

In the current study, O/W NE was synthesized through two techniques. The first technique involved ultrasonic emulsification, utilizing Tween 80 and Tween 20 surfactants. This method operates at higher energy levels. The second technique employed was spontaneous emulsification, which operates at lower energy levels. Ultrasonic emulsification is a rapid and effective method for synthesizing stable nanoemulsions with very small particle diameters and low polydisperse index. We achieved a small droplet size and stable nanoemulsion from different ratios using the ultrasonic emulsification method in the presence of co-surfactant (polyethylene glycol 4000) with a Tween 80 surfactant at 1:1 oil-to-Tween 80 surfactant ratio. The optimized average particle size under this condition was 132.56 ± 0.499 nm. Additionally, optimized a small droplet-size nanoemulsion from different ratios was obtained using Tween 20 at a 1:4 ratio. In this case, the average particle size of the formulated nanoemulsion was 290.033 ± 0.038 nm. Nevertheless, in the spontaneous emulsification method, the average particle size

was greater than the ultrasonic method which was 305.4 nm because no external force was used to minimize the droplets.

3.3. Determination of particle size and polydispersive index

Stability, quality, homogeneity, and dispersibility of nanoemulsions could be determined by droplet size and polydispersity index. In this study, the droplet size and particle size distribution of the nanoemulsion were determined by the DLS technique, with the size distribution profile observed as a single and narrow peak in monodispersed nanoemulsion with the plot of particle concentration with its size based on²¹ methods. Therefore, the result of the droplet size and PSD of formulated *C. decapetala* seed oil nanoemulsion by using Tween 80 and Tween 20 was in the range of 132.5 nm to 434.03 nm and from 290.033 ± 1.955 nm to 376.33 ± 15.36 nm, respectively, as mentioned in Table 4 and Fig. 4 and also in Table 5 and Fig. 5

Table 4
Particle size and PDI of nanoemulsion using Tween 80 with co-surfactant

Formulations	Oil to S (T80) ratio	Z- average (nm)	PDI
NE1	1:0.5	330.5 ± 7.597	0.827 ± 0.2474
NE2	1:1	132.56 ± 0.499	0.404 ± 0.0017
NE3	1:1.5	399.56 ± 2.540	0.353 ± 0.027
NE4	1:2	407.66 ± 1.501	0.236 ± 0.001
NE5	1:3	271.93 ± 4.907	0.596 ± 0.012
NE6	1:4	397.33 ± 1.270	0.517 ± 0.057
NE7	1:5	434.03 ± 4.041	0.028 ± 0.038
Note: S for surfactant, and PDI for polydispersive index			

Formulations	Oil to S ratio	Z- average (nm)	PDI
NE1	1:0.5	341 ± 7.014	0.817 ± 0.077
NE2	1:1	313.66 ± 28.29	0.529 ± 0.0432
NE3	1:2	376.33 ± 15.36	0.553 ± 0.015
NE4	1:3	387.066 ± 8.078	0.499 ± 0.038
NE5	1:4	290.033 ± 1.955	0.3006 ± 0.040
Note: S for surfactant, and PDI for particle size distribution			

Table 5: Particle size and PSD of NE using Tween 20

Figure 5: (A) Particle size and PDI of NE (B) diagram of both particle size and PDI of NE by using oil and Tween 20

The particle size, polydispersive index, and particle charge values of nanoemulsion that were synthesized by spontaneous emulsification were 309.83 ± 6.748 nm, 0.431 ± 0.1215 , and -10.02 ± 0.495 respectively.

3.4. Zeta-potential (Particle charge)

Zeta potential is the physical parameter of nanoemulsion and can be determined by using a DLS instrument. According to standard reported values, the value of zeta potential was greater than + 30 mV or -30 mV indicating good stability and sufficient stability of nanoemulsion³⁰. The results of zeta potential of formulated nanoemulsion NE1, NE2, NE3, and NE4 for using Tween 80 were - 60.43 mV,-32.1mV,-32.06, and 31.23 mV respectively, that shown in Table 6 and Fig. 7. Additionally, the zeta potential value of formulated nanoemulsion using Tween 20 NE1, NE2, NE3, and NE4 were - 44.433 mV, -36.066 mV, -33.466 and - 32.3 mV respectively, shown in Table 7 and Fig. 8. Furthermore zeta potential of nanoemulsion through spontaneous emulsification was - 10.2 mV which proves the formulations are significantly stable.

Table 6
Zeta potential of NE using Tween 80 surfactant and co-surfactant

Formulations	Oil to Tween 80	Zeta potential (mV)
NE1	1:0.5	-60.43 ± 1.27
NE2	1:1	-32.1 ± 1.0535
NE3	1:1.5	-32.06 ± 0.6110
NE4	1:2	-31.23 ± 0.8504
NE5	1:3	-29.233 ± 1.040
NE6	1:4	-26.866 ± 1.101
NE7	1:5	-25.933 ± 0.404

Table 7
Zeta potential of nanoemulsion using Tween 20 surfactant

Formulations	Oil to Tween 20	Zeta potential
NE1	1:0.5	-44.433 ± 1.563
NE2	1:1	-36.066 ± 0.4725
NE3	1:2	-33.466 ± 0.321
NE4	1:3	-32.3 ± 0.345
NE5	1:4	-28 ± 0.173

3.5. The refractive index of nanoemulsion

The average range of refractive index values of formulated nanoemulsion in this study was for Tween 80 with co-surfactant 1.380- 1.35201 and from 1.334 to 1.339 for Tween 20. The refractive index value of nanoemulsion synthesized by spontaneous emulsification was 1.3345.

3.6. Percentage transmittance

To determine the percentage transmittance of nanoemulsion, formulated nanoemulsion should be pure and translucent in appearance. The percent transmittance value of formulated nanoemulsions by using Tween 20 and Tween 80 surfactants at 260 nm was 78.52–88.41%, and 75.05–90.81%, respectively. Additionally, the percent transmittance value of spontaneous emulsification was 85.259%.

3.7. pH value on nanoemulsion

The result of the pH value range of formulated nanoemulsions in this study using Tween 20 and Tween 80 was from 5.2 to 6.5 and from 4.38 to 5.27 correspondingly, and also the pH value of nanoemulsion formulated by spontaneous emulsification was 5.4.

3.8. Viscosity of nanoemulsion

The viscosity values of formulated nanoemulsion through ultrasonication emulsification method using Tween 80 at 6.667min, at 40.09 °C, in the presence of 3.9222 Pa shear stress and 0.20818 mN.m torque was 3.922 mPa.S and using Tween 20 at 6.667min, at 40.13 °C, in the presence of 5.2468 Pa shear stress and 0.27849 mN.m torque was 5.247 mPa.s.

3.9. Stability of nanoemulsion

The result of the physical stability of the formulated nanoemulsion in this study was obtained by centrifuging the sample at 3000 rpm for 10 minutes and checked visually and there was no phase separation even when we added additional 5 minutes.

3.10. Determination of oxidative stability of nanoemulsion

Peroxide values of nanoemulsion samples in this study were obtained from (Fig. 9) calibration curve with a slope (b) of 0.0213 and a coefficient of determination $R^2 = 0.9892$ and the absorbance value of Tween 80 with polyethylene glycol as co-surfactant was 0.83, Tween 20 was 0.74, and SE was 0.658. From this, the result of the peroxide value of nanoemulsions was 14.36 meq O_2 /Kg of NE, 10.14 meq O_2 /Kg of NE, and 6.23 meq O_2 /Kg of NE respectively.

3.11. Transmission electron microscopy of NE

Figure 10 (A–C) shows the microstructure and morphology of NE in the range of 100 to 500 nm measurements. In brief, Fig. 10A shows the morphology of NE formulated using Tween 80 with co-surfactant, and Fig. 10B shows the morphology of NE synthesized using Tween 20 in the absence of surfactant. Additionally, Fig. 10C shows the morphology of NE that was prepared through spontaneous emulsification using Tween 80 surfactant.

3.12. FT- IR analysis of CDONE

FT-IR analysis of nanoemulsions was conducted for all three types of samples and oil as the intensities of the band region extended from 4000 to 500 cm^{-1} . As a result, Fig. 11 was shown the FT-IR spectra of *C. decapetala* seed oil nanoemulsion with crude oil.

3.13. Antibacterial activity

The results in Fig. 12 showed that *C. decapetala* seed oil nanoemulsion had an antibacterial effect against all studied bacterial agents. 100 mg/mL concentration CDONE that was synthesized using Tween 20, Tween 80, and spontaneous emulsification gave inhibition zones 11, 11.5, 10, and 11; 13.5, 12, 13, and 11, and also 12.5, 12, 11, and 10.5 mm in diameter against *S. aureus*, *S. pyogenes*, *E. coli*, and *P. aeruginosa* respectively. 75 mg/mL concentration CDONE that was synthesized using Tween 20, Tween 80, and spontaneous emulsification gave inhibition zones 9.5, 10, 9, and 9.5; 12, 11, 12, and 10.5, and also 10, 10, 9.5, 9 mm in diameter against *S. aureus*, *S. pyogenes*, *E. coli*, and *P. aeruginosa* respectively. 50 mg/mL concentration CDONE that was synthesized using Tween 20, Tween 80, and spontaneous emulsification gave inhibition zones 8, 8, 8.5, and 8; 10, 9.5, 10.5, and 9, and also 8, 8.5, 8, 7.5 mm in diameter against *S. aureus*, *S. pyogenes*, *E. coli*, and *P. aeruginosa* respectively.

4. Discussion

Physicochemical characteristics of *C. decapetala* seed oil

Physicochemical properties, and quality of *C. decapetala* seed oil for its applications were investigated as the results mentioned in Table 3. The yield of *C. decapetala* seed oil was 22.06%, with 188.678 mgKOH/g of oil saponification value, 37.322 mPa.S viscosity at 40⁰C, 0.9333 specific gravity at 25⁰C, 1.476 refractive index at 40⁰C, 1.706 mg NaOH/g of oil acid value, 7.333 meq O_2 /kg of oil of peroxide

value, 90.1167 gI₂/100g of oil iodine value were the major results of seed oil. According to FAO/WHO³¹, the physicochemical properties of *C. decapetala* seed oil results were acceptable and comparable results.

Particle size and polydisperse index

As mentioned in Table 4 and Fig. 4 the droplet sizes of the nanoemulsion that was synthesized using Tween 80 were: NE1 was 330.5 ± 7.597 nm, NE2 was 132.56 ± 0.499 nm, NE3 was 399.56 ± 2.540 nm, NE4 was 407.66 ± 1.501 nm, NE5 was 271.93 ± 4.907 nm, NE6 was 397.33 ± 1.270 nm, and NE7 was 434.03 ± 4.041 nm. Consequently, from this formulated nanoemulsion, NE2 had the smallest average droplet size and was more stable than the others in Tween 80.

Additionally, as stated in Table 5 and Fig. 5 the average droplet sizes of nanoemulsions synthesized using Tween 20 were: NE1 was 341 ± 7.014 nm, NE2 was 313.66 ± 28.29 nm, NE3 was 376.33 ± 15.36 nm, NE4 was 387.066 ± 8.078 nm, and NE5 was 290.033 ± 1.955 nm. Therefore, from these formulated nanoemulsions, NE5 had the smallest average droplet size and was more stable than the others in Tween 20. The average particle size of NE that was formulated by spontaneous emulsification was 305.4 nm, with a 0.345 PDI mentioned in (Fig. 6). The PDI describes the homogeneity of nanoemulsions range was 0 to 1, 0 stands for monodisperse system, and 1 stands for a polydisperse particle dispersion. The PDI of the seed oil nanoemulsions in the present work were in the range of 0.028 ± 0.03811 to 0.827 ± 0.247 using Tween 80 and from 0.3006 ± 0.040 to 0.817 ± 0.077 using Tween 20, which represents the acceptable monodisperse distribution of droplets of seed oil nanoemulsion. Generally, nanoemulsions formulated by ultrasonic emulsification using Tween 80 with co-surfactant were more stable than nanoemulsions formulated using Tween 20 surfactant and spontaneous emulsification. As a result by average particle size and polydisperse index values of formulated nanoemulsion were in the range of average particle size of nanoemulsion³².

Zeta-potential (Particle charge)

The particle charge value provides a sign of the possible stability of the colloidal system. Surface charge is calculated as zeta potential value which is determined through a particle's electrophoretic mobility of an electrical field. As the charged particles repel one another and this force overrides the inclination to aggregate, the higher the ZP, the more probable it is that the suspension will be stable. It can give important details about how particles behave in foams, emulsions, and nanocellulose aggregation, which should be minimal for effective use as a reinforcing agent³³.

High zeta potential emulsions (-41 to -50 mV) are electrically stable, while low values (-11 to -20 mV) tend to coagulate, which could result in poor physical stability. The stability of a system is determined by the relative strengths of the repulsive and attractive forces¹⁷. Generally, the standard reported values of these physical parameters say that zeta potential greater than + 30 mV or -30 mV indicates good stability and sufficient stability of nanoemulsion³⁰. As mentioned in Table 6 and Fig. 7 and Table 7 and

Fig. 8 the optimized zeta potential value of formulated nanoemulsion was from – 31.23 mV to -60.43 mV. Therefore, in this study nanoemulsions that were formulated using Tween 80 had higher zeta potential values and were more stable than the other formulated nanoemulsions.

The refractive index of nanoemulsion

A material's refractive index is a measurement of how light moves through it. Light travels more slowly at higher refractive indexes, which results in a proportion greater change in the direction of light within the material. For instance, the light-scattering characteristics of sesame oil are determined by its refractive index, which stands at 1.47³⁴. The medication was isotropic, as shown by the nanoemulsions' refractive index value of 1.32. Therefore, the average range of refractive index value of formulated nanoemulsion using two methods and two surfactants in this study was 1.380–1.352 the result of refractive index value of formulated nanoemulsion comparable to refractive index value of sesame seed oil.

Percentage transmittance

To determine the percentage transmittance of nanoemulsion, it should be pure and translucent in appearance. As surfactant concentration increases in the formulation of nanoemulsion, the result of nanoemulsion has clear and transparent properties³⁵. Generally, based on the results of the percentage transmittance of formulated nanoemulsions using ultrasonication and spontaneous emulsification have a clear, and transparent appearance indicating the isotropic nature of the drug.

pH value of nanoemulsion

Nanoemulsion stability and properties were affected by pH value, from this, the accepted pH values ranging of nanoemulsion is from 4.2 to 5.8, making the nanoemulsion non-irritant for pharmacological and skin purposes³⁶. In this study, all pH values of formulated nanoemulsions by ultrasonication and spontaneous emulsification that read at room temperature were acceptable in the range of previous work and similar to the work described by ³⁶.

Viscosity of nanoemulsion

The viscosity of the nanoemulsion was analyzed using a rheometer instrument. The viscosity values of formulated nanoemulsion through ultrasonication emulsification method using Tween 80 was 3.922 mPa.S and using Tween 20 was 5.247 mPa.s. Therefore the viscosity of formatted nanoemulsion in this work was the acceptable range of nanoemulsion³⁷.

Stability of nanoemulsion

The samples exhibiting significant stability and other desired attributes are selected for additional analysis. This study investigated intrinsic stability by keeping the emulsion at 25 °C. After that, watched any phase separation or creaming formation. Additionally, the variation in droplet diameter was examined at various time intervals¹⁷. Physical observations of the developed formulations' visual

appearance revealed clear and transparent nanoemulsions free of turbidity. Therefore, the physical stability of the formulated nanoemulsion in this study was no phase separation even when we added 5 minutes.

Determination of oxidative stability of nanoemulsion

The peroxide value of the nanoemulsion was analyzed by co-oxidizing the synthesized sample from Fe (II) to Fe (III) to form a reddish Fe (III)-thiocyanate complex. High peroxide values of nanoemulsion are a sign of a rancid nanoemulsion. As shown in Fig. 9, the peroxide value of nanoemulsion with Tween 80 was higher than the peroxide value of Tween 20, and nanoemulsion formulated by spontaneous emulsification. Therefore the obtained peroxide value of nanoemulsion in this study was acceptable to a general rule of PV that should not be above 10–20 meqO₂/kg sample to avoid the rancidity flavor of the sample³⁸.

Transmission electron microscopy of NE

The microstructure and morphology of NE prepared by ultrasonic emulsification using Tween 20, and Tween 80 surfactants and spontaneous emulsification methods were determined by TEM. Figure 10 (A–C) shows the microstructure and morphology of NE in the range of 100 to 500 nm measurements. The droplet size of the NE from the DLS imaging was 132.56 ± 0.499 – 387.066 ± 8.078 nm range. The result relating to particle size achieved by TEM analysis was associated with the result of particle diameter achieved using a droplet size analyzer (DLS). Therefore, the morphology of NE confirmed the spherical shape with some aggregation because it took some time until detection by TEM after formulation.

FT- IR analysis of CDONE

FT-IR analysis was used to identify the functional groups associated with the lipid they contain and their path of attachment of oil with surfactants, and co-surfactants in nanoemulsion for antibacterial purposes. As Fig. 11 shows spectra of *C. decapetala* seed oil nanoemulsion with crude oil the -OH group was indicated in the range from 3300–3500 cm⁻¹ in the nanoemulsion and crude oil. The -C = C- bond which was the functional group present in the Tween 20 and Tween 80 molecules indicates wavenumber ranges from 1620–1670 cm⁻¹. The wavenumber range from 1050–1200 cm⁻¹ indicates -C-O- bond from non-deteriorated molecules of oil particles. The band at 3008 cm⁻¹ can indicate the degree of unsaturation of seed oil, the band at 2922 cm⁻¹ indicates C-H stretching vibration of cis double bond within unsaturated fatty acyl ester, at 2854 cm⁻¹ observed -CH₂- stretching, the band around at 1742 cm⁻¹ can be indicated carbonyl carbon stretching, the band at 1459 – 1373 cm⁻¹ indicated C-O stretching vibration such as COOR, the band at 1150 – 400 cm⁻¹ for all the oils indicating the C-O stretching vibration for example, in triacylglycerol, and also cis -CH = CH- bending out of plane and CH₂ rocking were observed.

Antibacterial activity

Antibacterial activity of nanoemulsion was investigated in this work, because nanoemulsion particles are thermodynamically interested in fusing via lipid-containing organisms, and NE as antibacterial activity is a novel and hopeful revolution. The pathogen's negative charge and the emulsion's positive charge are attracted to each other electrostatically, improving the fusion. Both of the active ingredients release energy that causes the pathogen's lipid membrane to become unstable, which leads to cell lysis and death. A broad variety of action-defined bacteria, such as *Salmonella*, *S. aureus*, and *E. coli*, are present in the nanoemulsion. The known difficulty in growing antimicrobial-resistant strains practiced with the use of current agents due to the widespread, and sometimes inappropriate use of antibiotics, disinfectants, and antiseptics, encouraged the application of nanoemulsion to examine as the antibacterial agent ³⁹. Inhibition zones of all three types of samples against four tested bacteria were closed to the inhibition zone of our reference (amoxicillin).

5. Conclusions

A stable oil-in-water (O/W) nanoemulsion was formulated using *C. decapetala* seed oil and surfactants, including Tween 20 and Tween 80, in the presence of a co-surfactant through spontaneous and ultrasonic emulsification methods. Our investigation focused on optimizing surfactant concentration to enhance stability, minimize droplet size, achieve narrow particle size distribution, and ensure appropriate zeta potential, viscosity, pH, and refractive index values. The nanoemulsions exhibited excellent physical stability over 50 days at room temperature, with no phase separation observed and good oxidative stability indicated by the peroxide value. Droplet sizes ranged from 132.56 nm to 309.83 nm, with corresponding polydispersity indices. Antibacterial assays against various strains, including *S. aureus*, *S. pyogenes*, *E. coli*, and *P. aeruginosa*, demonstrated significant activity. This study presents the first instance of producing a *C. decapetala* seed oil nanoemulsion with ultrasonically achieved low particle size. The findings suggest the potential for novel nanoemulsion formulations with enhanced stability. The demonstrated antibacterial efficacy against both gram-positive and gram-negative bacteria underscores the nanoemulsion's potential for biomedical applications and drug delivery systems.

Abbreviations

CD: *Caesalpinia Decapetala*; CDO: *Caesalpinia Decapetala* oil; CDONE: *Caesalpinia Decapetala* Oil Nanoemulsion; DLS: Dynamic Light Scattering; FT-IR: Fourier Transform Infrared Spectroscopy; HLB: Hydrophilic-Lipophilic Balance; NE: Nanoemulsion; O/W: Oil- in-Water; PEG: Polyethylene glycol; PSD: Particle Size Distribution; S: Surfactant; SE: Spontaneous Emulsification; SOR: Surfactance to Oil Ratio; TEM: Transmission Electron Microscopy

Declarations

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data generated or analysed during this study are included in this published article

Conflicts of Interest: The authors declare no conflicts of interest.

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Figures

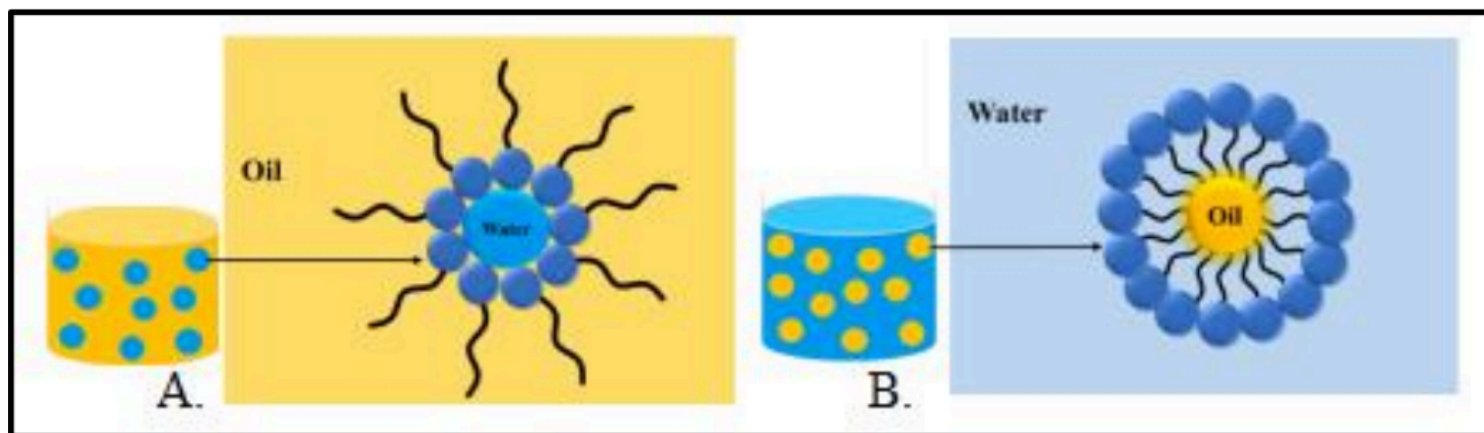


Figure 1

Diagram of (A) W/O and (B) O/W emulsions respectively⁶

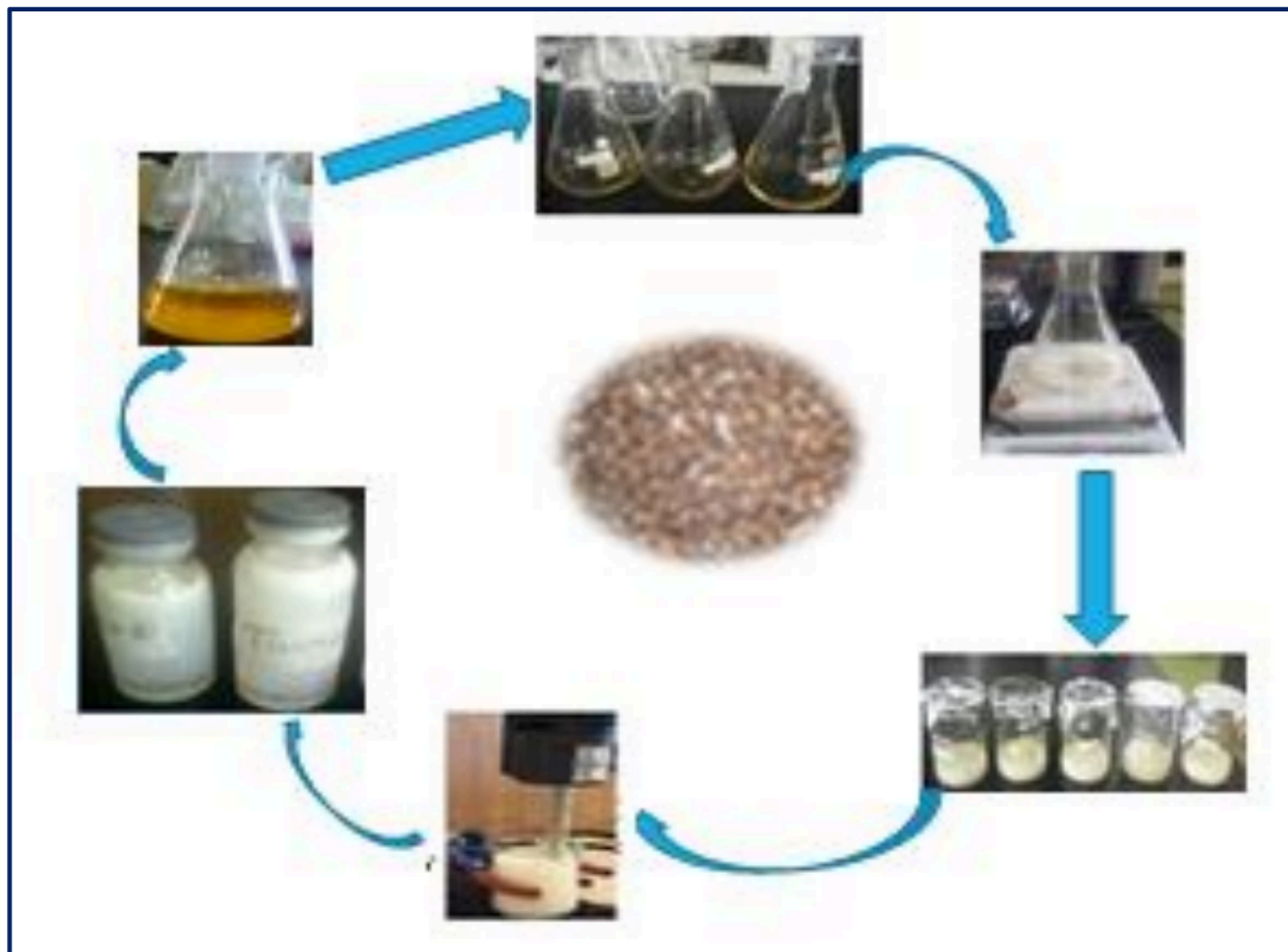


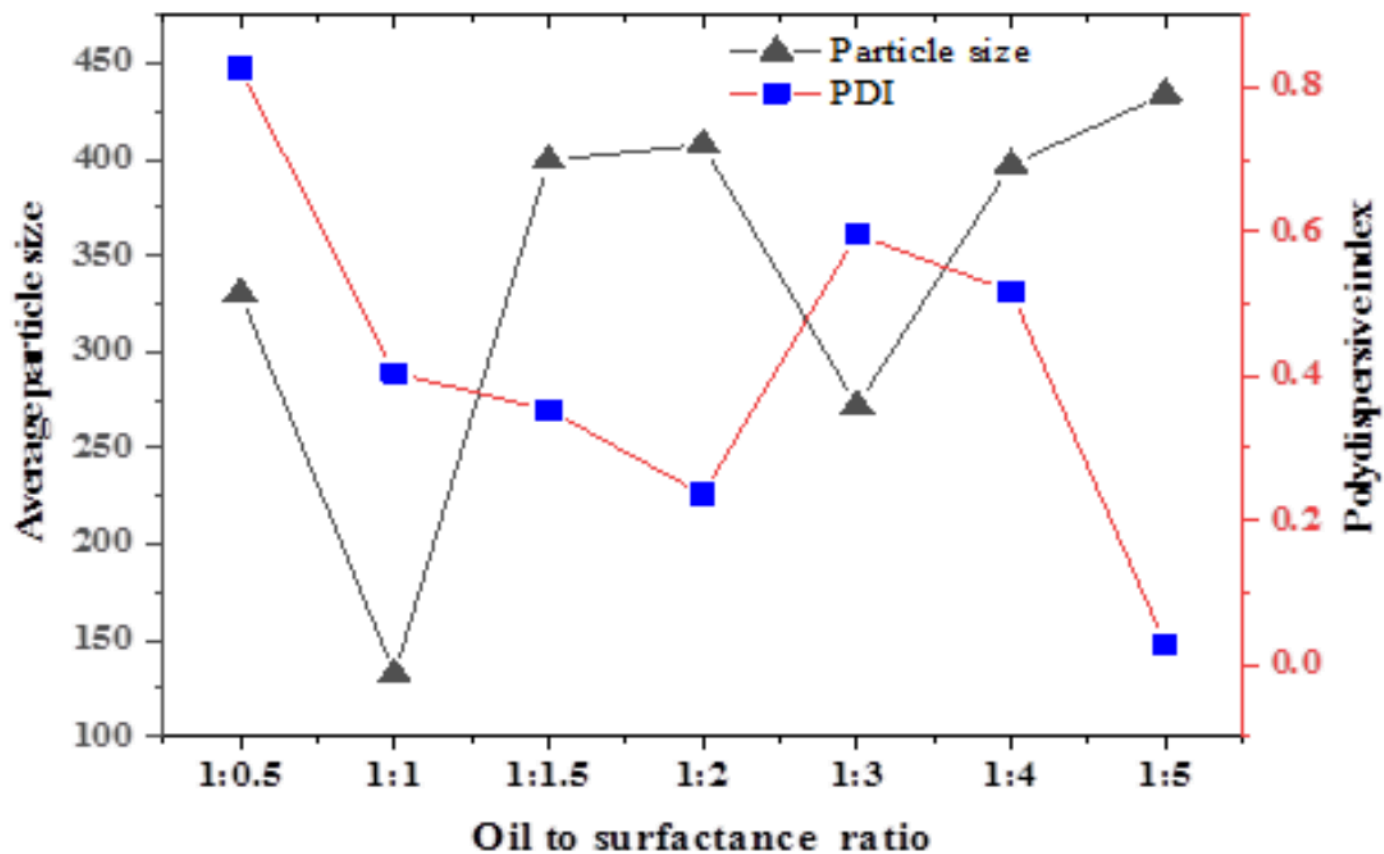
Figure 2

Formation of nanoemulsion by ultrasonication emulsification using T 80 and T 20



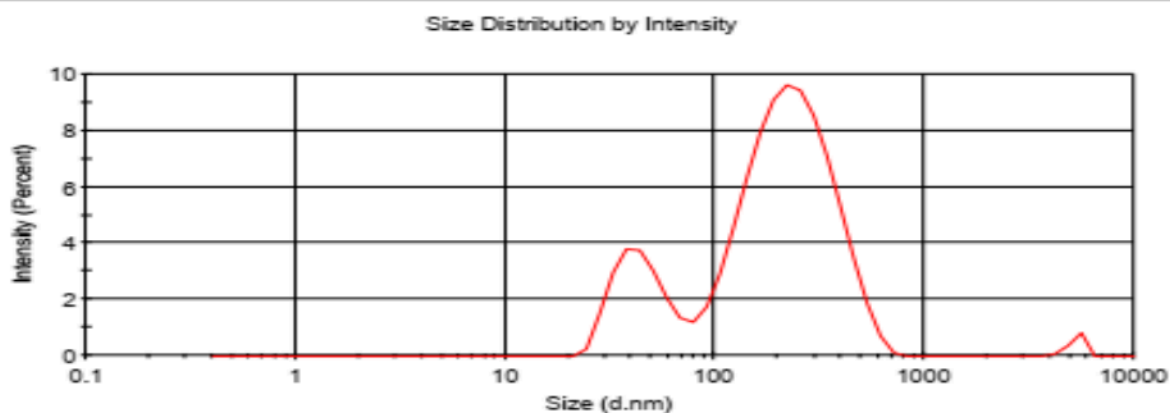
Figure 3

Formation of nanoemulsion by using spontaneous emulsification method



A.

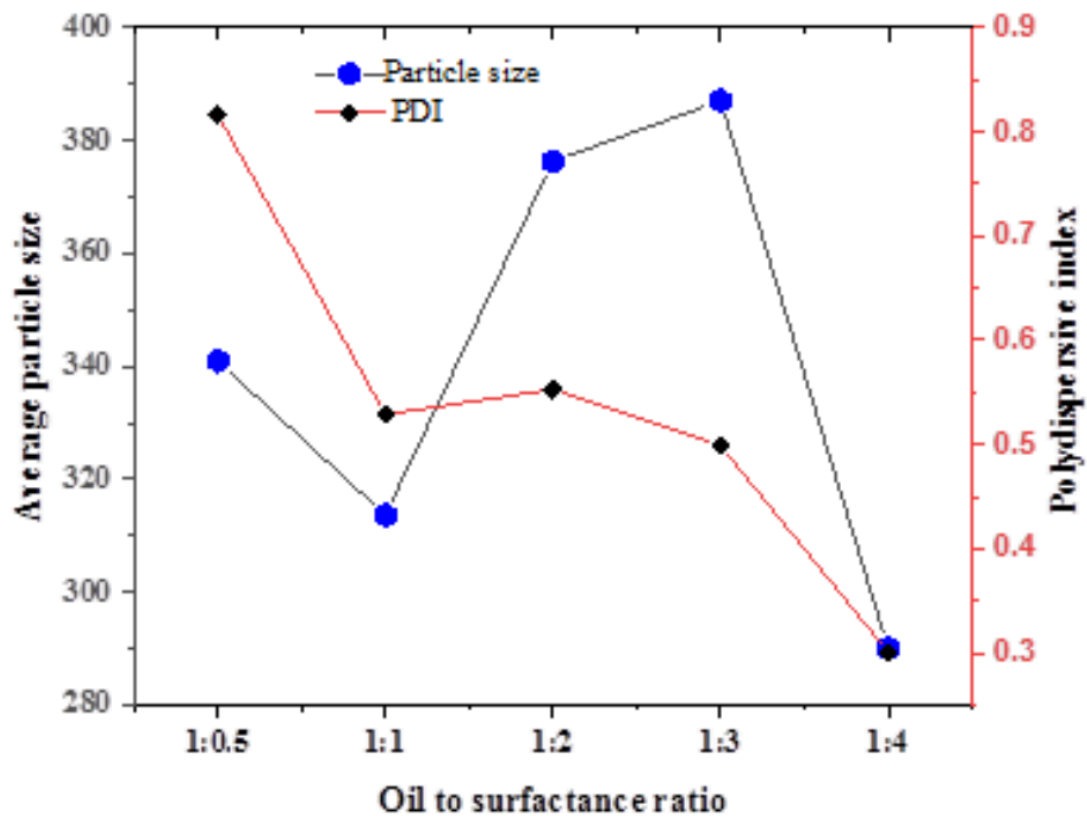
Z-Average (d.nm): 132.8		Size (d.nm):	% Intensity:	St Dev (d.nm):
Pdi: 0.498		Peak 1: 247.0	79.0	112.0
Intercept: 0.891		Peak 2: 45.96	19.7	13.74
Result quality : Refer to quality report		Peak 3: 5251	1.3	442.3



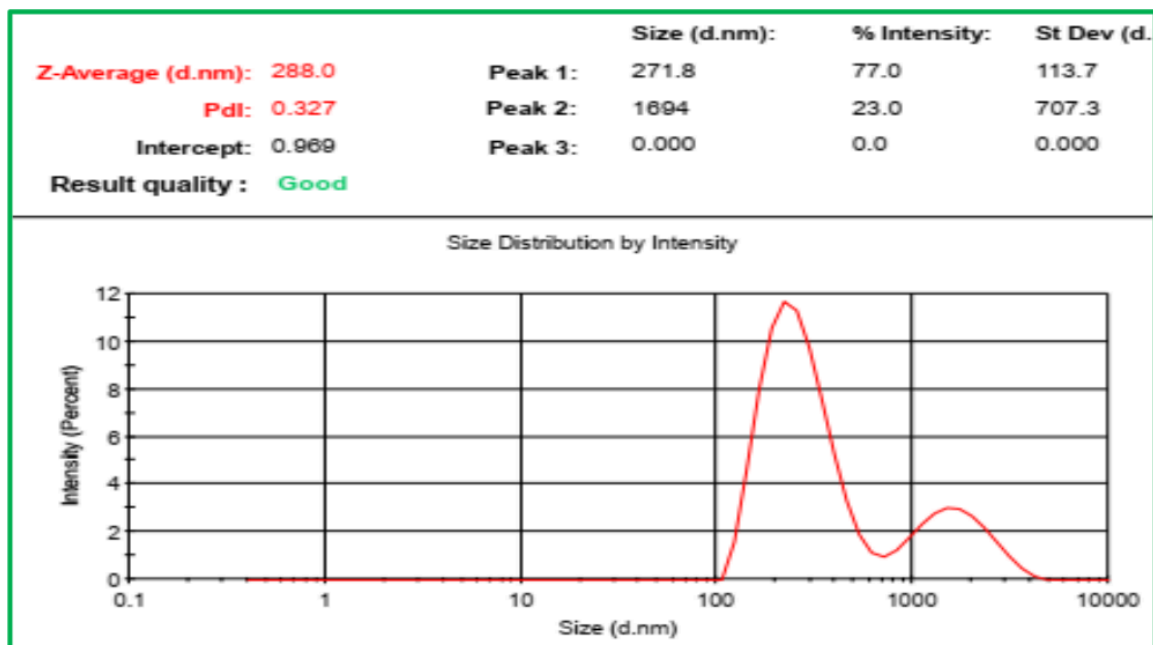
B.

Figure 4

(A) Particle size and PDI of NE (B) diagram of both particle size and PDI of NE using oil and T 80 in the presence of co-surfactant



A.



B.

Figure 5

(A) Particle size and PDI of NE (B) diagram of both particle size and PDI of NE by using oil and Tween 20

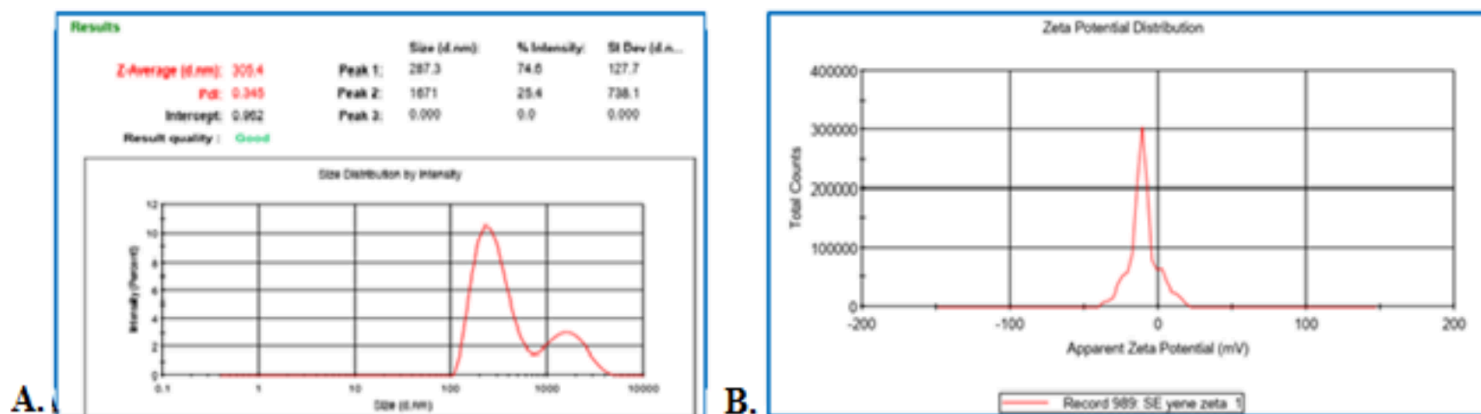


Figure 6

(A) Particle size and (B) zeta potential of NE using oil and T80 surfactant in the presence of co-surfactant syntheses through SE

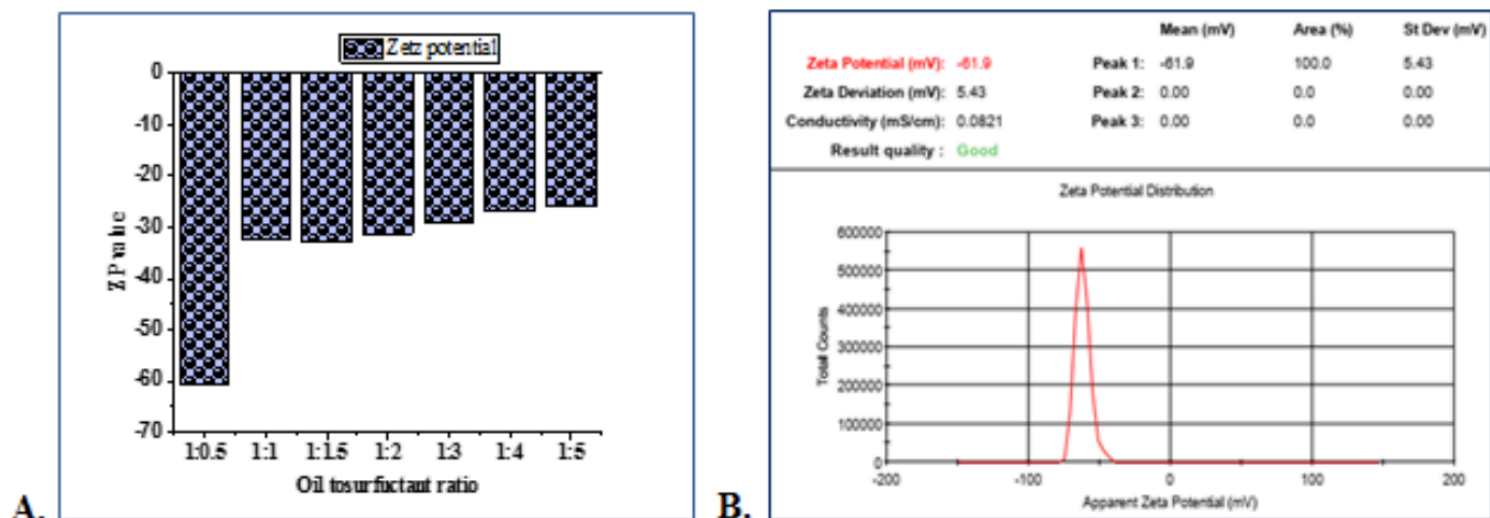


Figure 7

(A) Zeta potentials of all formulated NE and (B) higher zeta potential value of single NE using oil and Tween 80 in the presence of co-surfactant

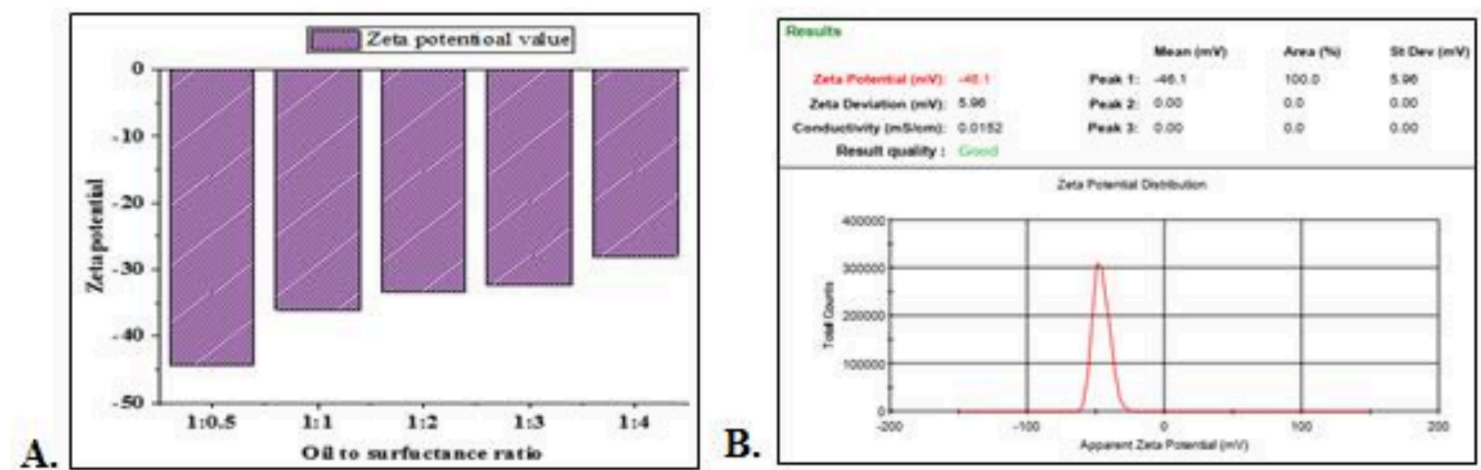


Figure 8

(A) Zeta potentials of all formulated NE and (B) higher zeta potential value of single NE using oil and Tween 20 surfactant

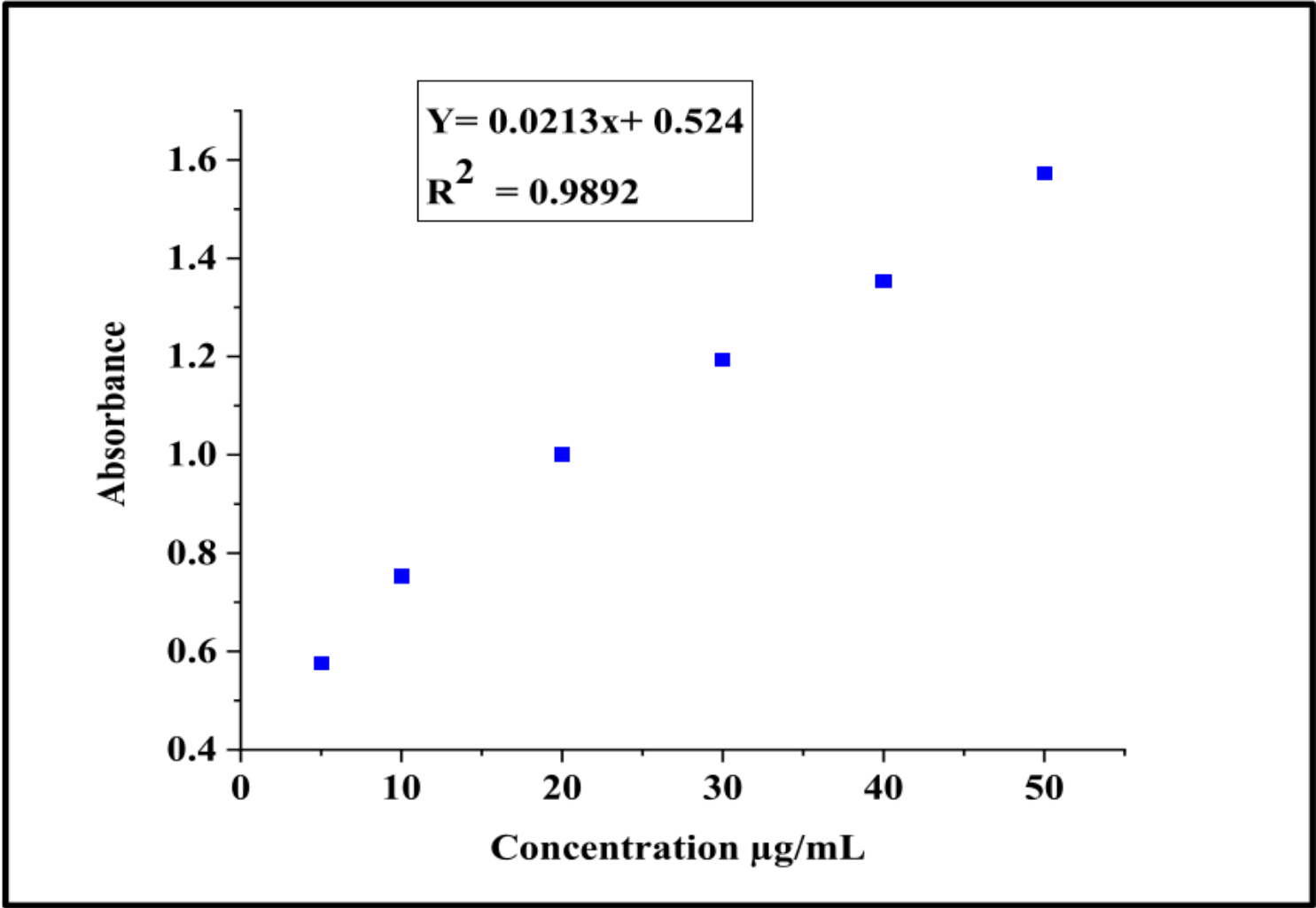


Figure 9

The calibration plot of standard Fe (III) for determination of the peroxide value of NE

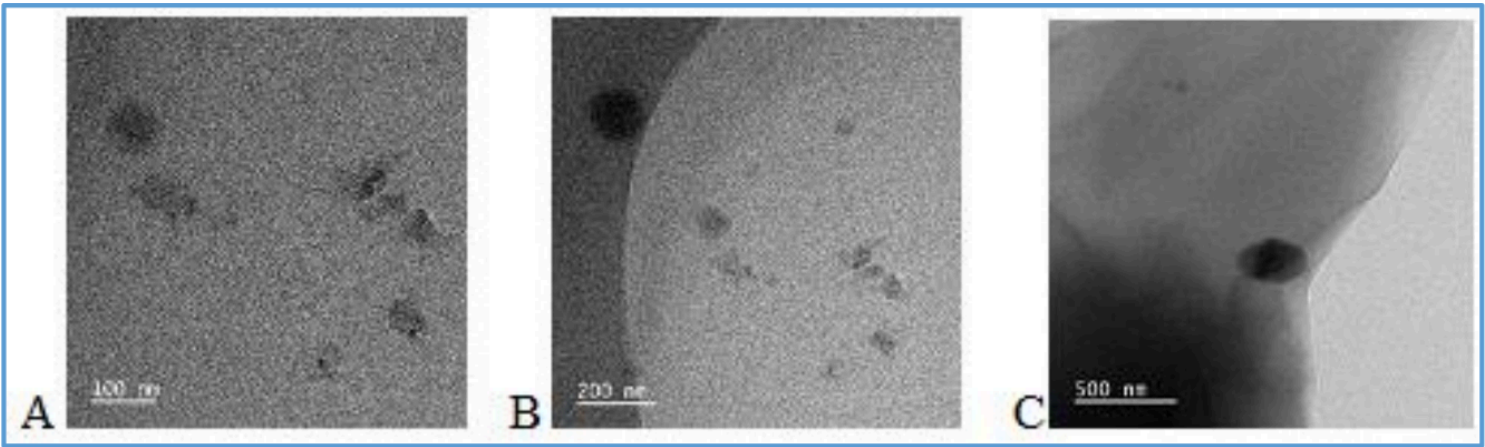


Figure 10

TEM of CDNE (A) NE with T80 surfactant, (B) NE with T20, surfactant, and (C) NE with spontaneous emulsification.

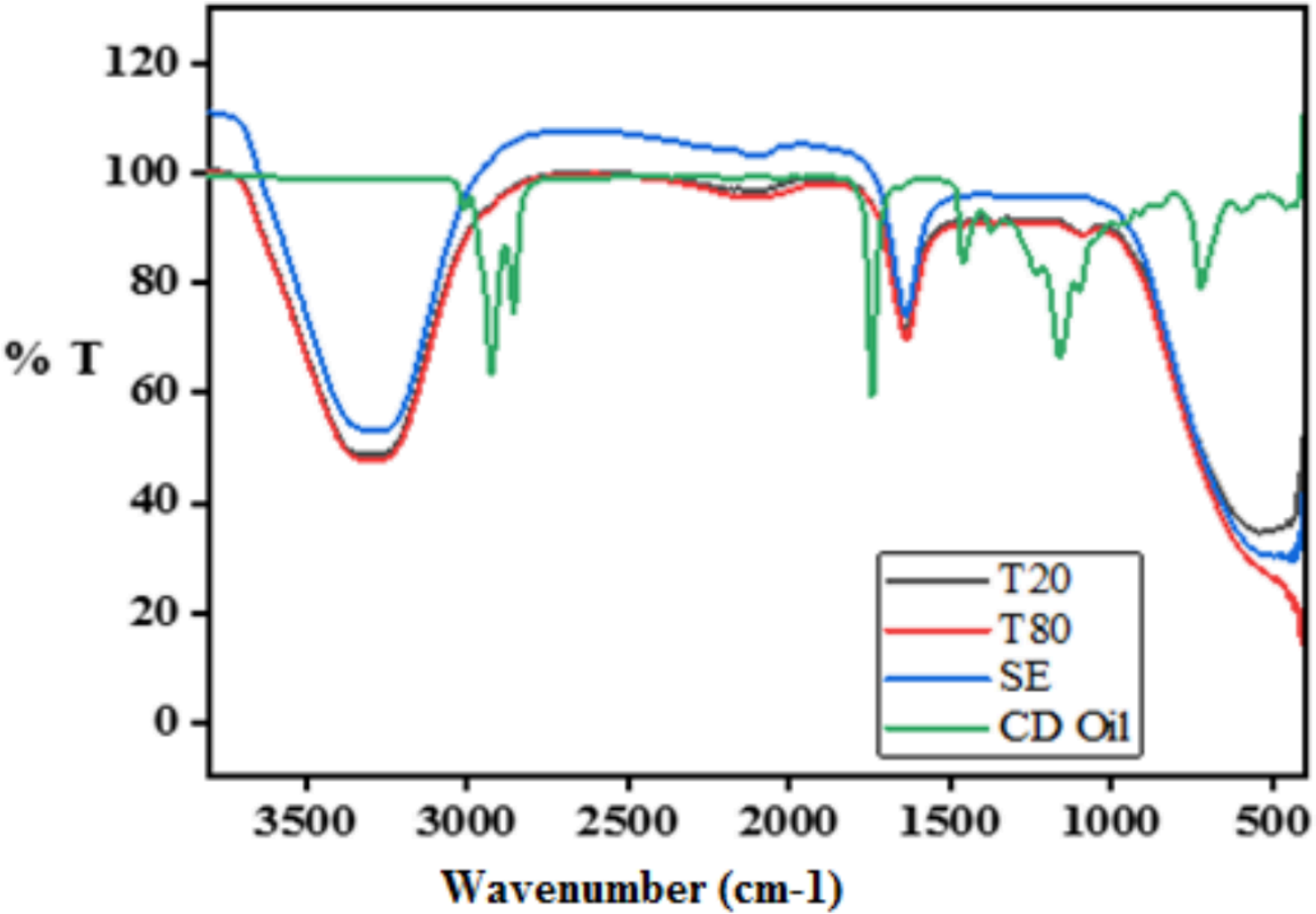


Figure 11

FT- IR analysis of formulated nanoemulsion.

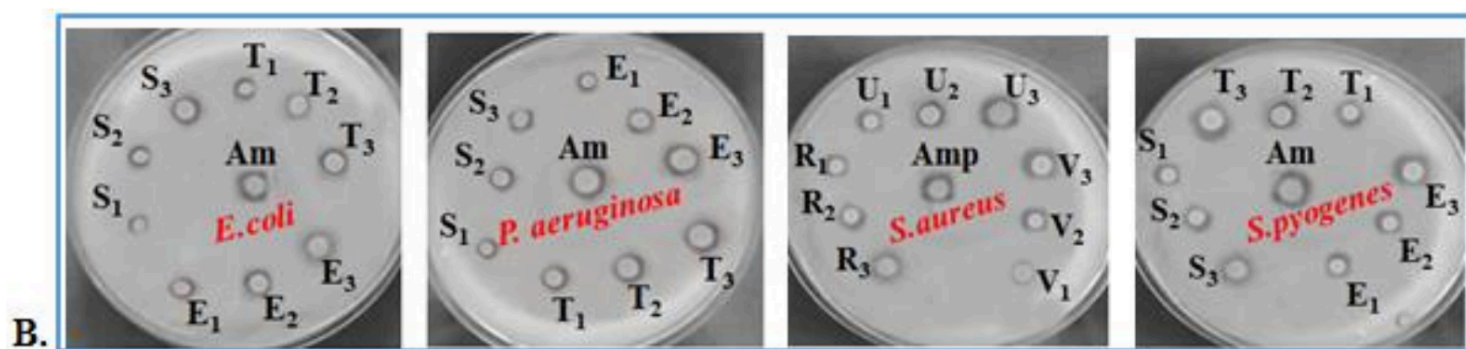
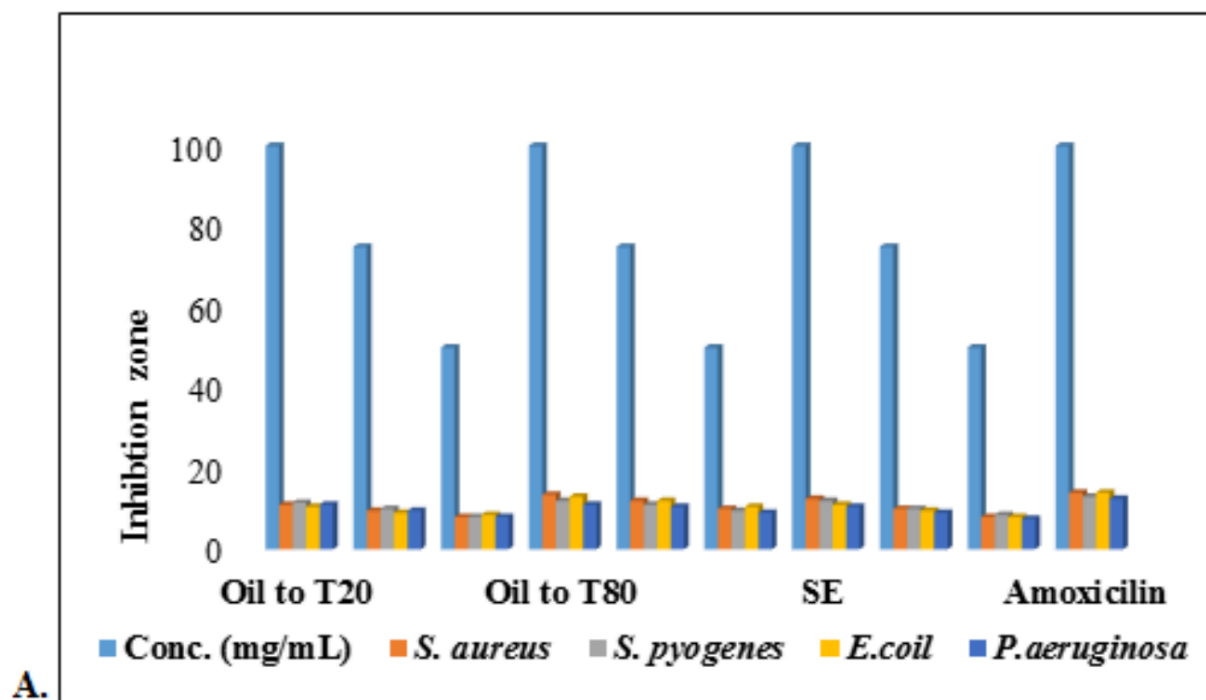


Figure 12

Both (A) and (B) show the inhibition zone of NE in mm against tested bacteria

Supplementary Files

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- [floatimage1.jpeg](#)