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# Fish oil based vitamin D nanoencapsulation by ultrasonication and bioaccessibility analysis in simulated gastro-intestinal tract

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ABSTRACT

Recently, nanoemulsions have been employed for different applications including food and drug industries for efficient nutrient delivery system. In this study, vitamin D (a lipophilic molecule) was encapsulated in fish oil for higher oral bioavailability. The oil-in-water nanoemulsion was formulated by ultrasonication technique with a droplet size range of 300–450 nm and a shelf life of more than 90 days. The influence of oil, water and surfactant concentration was investigated by phase diagram. The formulated nanoemulsion had encapsulation efficiency in the range of 95.7–98.2%. Further, nanoemulsion passed through simulated gastro-intestinal tract revealed an increased bioavailability than non-encapsulated vitamin. Thus, the formulation can be used as a drug delivery vehicle for various lipophilic compounds. Till date, no one have fabricated an efficient nano-vehicle for the delivery of vitamin D as well as analyzed the efficient delivery system in simulated GI-tract, this is first of its kind study in this regard. This can be scaled up further after analyzing the safety aspects.

## 1. Introduction

Nanoemulsions are kinetically stable liquid dispersions with its droplet size ranging from 50 to 500 nm. Recently, nanoemulsions are of great interest in food and pharmaceutical industries as an efficient delivery system for drugs and various lipophilic compounds [19,10]. Fig. 1 depicts the main role of how nanotechnology improves the bioavailability of a drug. They have an edge over conventional delivery systems as it shows high stability against coalescence or phase separation [19]. It also enhances the bioavailability and absorptive capacity of lipophilic compounds. Oil in water (O/W) nanoemulsion formulations are more common within the commercial industry as they have more potential benefits over microemulsions. Good understanding of the physiochemical properties of food nanoemulsions would give us an effective formulation technique to improve its application in food industries [22].

Vitamin D is one among the vital and essential vitamins mainly for the development of bone and teeth. Vitamin D3 (cholecalciferol) is synthesized by our skin when exposed to sunlight. However, a number of individuals are vitamin D deficient. This may be due to a number of factors including lack of exposure to the sun, extensive use of sun cream, poor dietary intake and dietary restrictions such as veganism or lactose intolerance. A vitamin D deficiency may lead to bone disease, such as rickets or osteomalacia. Although, there is a growing interest in food or beverage fortified with vitamin D, there are a number of challenges in fortification. These include poor water solubility; chemical degradation whenexposed to light, oxygen, or elevated temperatures; and variable oral bioavailability [11,12]. Thus, in this study, we aimed to encapsulate fat soluble vitamin (vitamin D) with fish oil using ultrasonication technique in order to enhance its bioavailability (see Fig. 1).

Since nanoemulsions are thermodynamically unstable, an amount of energy is required either in the form of agitation or mechanical disturbances to initiate the emulsification process. Since low energy approaches have certain disadvantages such as it requires large amount of surfactant, very precise control of physicochemical parameters and are not applicable to large-scale industrial processes, high energy approaches are favoured for emulsification. Also, they are more prone

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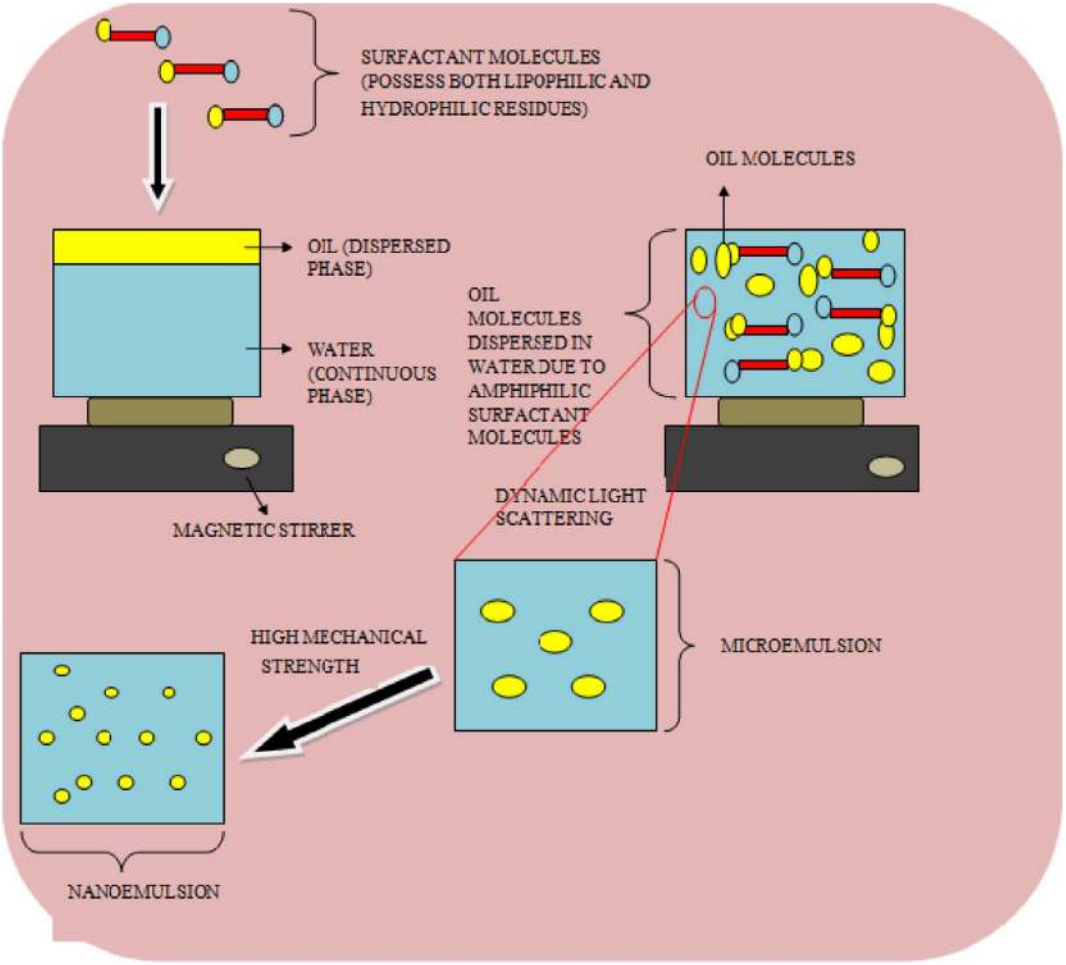


**Fig. 1.** Schematic representation of the role of surfactants (consisting of lipophilic residues, yellow in color and hydrophilic residues, blue in color) in the formation of emulsions using a magnetic stirrer. It can be noted that lipophilic residues get attached to the oil molecules and hydrophobic residues to the water molecules thus, breaking the surface tension between the oil (dispersed phase) and the water (continuous phase) and further resulting in the formation of emulsion. High-energy techniques (ultrasonication) are used to breakdown the size of emulsion particles to nano-range which can be observed using dynamic light scattering.

to coalescence and creaming and have no control in determining particle size. Ultrasound method is generally preferred as some of the characteristics such as particle size distribution and stability of emulsions can be controlled by varying the parameters. It works on the principle of cavitation phenomena. Ultrasound initiates emulsification by generation of droplets in the acoustic field and then creates intense turbulence and microjets during asymmetric cavity collapse. This causes the larger droplets to break into smaller droplets which disperse into the continuous phase. Nanoemulsions produced with ultrasonication overcomes the stability problem by slowing down the Ostwald ripening rate. Moreover, the emulsification process can be enhanced by optimizing power and sonication time [1,4,15].

In the present study we fabricated stable vitamin D nanoemulsion as an efficient delivery vehicle using ultrasonication technique. These were further characterized for its size and morphological features using dynamic light scattering (DLS), scanning electron microscope (SEM), transmission electron microscope (TEM) and atomic force microscope (AFM). Vitamin D encapsulation efficiency was quantified using high performance liquid chromatography (HPLC). The efficiency and stability of the formulated nanoemulsion was analyzed in simulated gastro-intestinal tract. This is first of its kind study in which ultrasonication approach has been taken for efficient nano-encapsulation of vitamin D in fish-oil. As the absorption of vitamin-D will start in small intestine, in this regard, fish-oil based nanoencapsulated system is novel and efficient delivery vehicle. Efficient delivery has been studied in simulated gastro-intestinal tract. Though, many have analyzed the efficient delivery of nanoemulsions in simulated GI tract but till date, no one have fabricated an efficient nano-vehicle for the delivery of vitamin D as well as analyzed the efficient delivery system in simulated GI-tract, this is first of its kind study in this regard.

## 2. Materials and methods

### 2.1. Materials

Vitamin $D_3$ (Cholecalciferol, 97% purity, MW 384.64 g/mol) and Tween 20 were indented from HiMedia Laboratories Pvt. Ltd., India. Fish oil was obtained from a local store in Vellore, Tamil Nadu, India. All other reagents used were of analytical grade and were used without further purification. Throughout the procedures, double deionized (DI) water was used.

### 2.2. Nanoemulsion fabrication by ultrasonication

Vitamin D nanoemulsion was fabricated as per our previously standardized protocol with some modifications [27]. Briefly, nanoemulsion was fabricated by wash out method followed by ultrasonication. Water phase was added continuously to the mixture of oil phase (fish oil), surfactant (tween-20) and the functional compound (Vitamin D). Tween 20 was used as a surfactant to solubilize vitamin D dissolved in fish oil. The prepared mixture (of oil, surfactant and vitamin) and water were preheated separately at 75 ± 2 °C. Water phase was slowly

added drop by drop to the oil phase containing surfactant and functional compound using magnetic stirrer. To obtain homogeneity throughout the NE,the mixture was centrifuged at 400 rpm for 15 min at 25 °C. Various concentrations of surfactant and oil were used and further the conditions were optimized for stability. The obtained formulation was subjected to ultrasonication at a frequency of 20 kHz for 10 min at 400 W using ultrasonic homogenizer, JY98-IIIDN (Ningbo Scientz, China). It can be noted that, 60000 IUs of vitamin D have been taken in the final volume of fabricated nanoemulsion, which meet the recommended dietary upper limit. The recommended dietary allowance for vitamin D is 600 IUs [14]. As vitamin D is highly sensitive to light, heat and oxygen the fabrication was performed in dark and in ice bath while stirring and sonicating the sample.

The fabricated samples were visually analyzed for stability and the stable ones were considered for further analysis.

### 2.3. Droplet size and size distribution

The droplet size and size distribution of the vitamin D nanoemulsion was determined using dynamic light scattering set-up (Malvern Zetasizer Nano ZS90, Malvern Instruments, UK) at 25 °C with scattering angle 173° excluding the unstable samples. The droplet size was described in terms of nm. Each measurement was carried out in triplicates, and the results were calculated as mean ± S.E.

### 2.4. Phase diagram

Based on the stability and size observations, phase diagram was plotted using origin software (OriginLab Corporation, Northampton, MA, USA). Different regions were plotted for flocculation, phase separation, microemulsion and nanoemulsion accordingly. The stable emulsions in nano range were selected for further analysis.

### 2.5. Viscosity determination

Viscosity determination is carried out using an Ostwald U-tube viscometer [25]. Pure methanol was taken as a reference with a viscosity of 0.507 mPa.s at 30 °C. Measurements were taken in triplicates to ensure accuracy and average values were recorded.

### 2.6. Morphological analysis of nanoemulsion by microscopic techniques

The droplet structural morphology – droplet shape and size – of stable nanoamulsions were analyzed using TEM and AFM.

#### 2.6.1. Transmission electron microscope

The morphology of nanoemulsion was further confirmed using transmission electron microscope (TEM, Morgagni 268, Philips-FEI, Hillsboro, USA) at 80 Kv. One drop of each sample was added to a copper grid and allowed to dry for 2 min. A drop of sodium phosphotungstate (2%, w/v) was set over the nanoemulsion droplet as a negative stain and was allowed to dry before analysis [41].

#### 2.6.2. Atomic force microscope

To examine the detailed morphology of the nanoemulsions, a thin film of the samples were prepared on a glass slide by dropping 100 μL of the sample on the slide, and was allowed to dry. The slides were then scanned using atomic force microscope (NanosurfEasyscan 2, Switzerland).

### 2.7. pH analysis

pH of the formulated nanoemulsions was measured using a pH pen (HI 98108, Hanna).

### 2.8. Quantitative analysis by HPLC

#### 2.8.1. Determination of absorption spectrum

The absorption spectrum of nanoemulsions was recorded between 200 and 400 nm using UV–VIS Spectrophotometer (UV-3600, Shimadzu scientific instruments, Kyoto, Japan). UV scan testing was performed in order to verify the maximum absorption wavelength ($\lambda_{max}$) for vitamin D, fish oil (oil phase), Tween-20 (surfactant) using methanol as solubilizing media.

The objectives of this were to: (i) determine the strongest λ maximum which would then be used for the HPLC method, since different values were reported in the literature [3,28] (ii) quantify the vitamin D by drawing the HPLC calibration curve and (iii) verify possible interfering peaks (overlapping) of the oil phase constituents (which contains ω-3 fatty acids) [38] and of the surfactants on the drug detection peak. Samples were prepared by dissolving known amounts of vitamin D, fish oil, Tween-20 in methanol.

#### 2.8.2. Vitamin D quantification by HPLC

Vitamin D was analyzed using the RP-HPLC method and detected using a UV/Vis detector. The mobile phase was filtered using a 0.45 mm filter (Type HVLP, Millipore) and sonicated for 30 min. The standards and samples were prepared following earlier reports [3,27]. The optimal HPLC conditions determined were as followed: mobile phase: 98% methanol/2% water with 1% of phosphoric acid solution (10% v/v); flow rate = 1 mL/min; λ = 265 nm, injection volume = 15 μL; running time: 15 min. A Ascentis® C18 column (25 cm × 4.6 mm) was used. The calibration curve was linear over a concentration range of 100–500 ng/ml.

A calibration curve was plotted using the measured retention time and peak area for each concentration using which the unknown concentration of non-encapsulated Vitamin D present in our nanoemulsion was determined.

#### 2.8.3. Determination of encapsulation efficiency

The encapsulation efficiency of vitamin D in the nanoemulsion was determined by the method established earlier for HPLC analysis. Nanoemulsion was subjected to centrifugal forces by submitting samples to 5000*g* for 30 min at 4 °C (Remi laboratory instruments, India). 4 mL was withdrawn from the pellet and diluted using isopropanol. Subsequently, the samples were filtered using a microfilter of size (0.45 μm) and injected in the HPLC (as described above) for the estimation of vitamin D acetate concentration [27].

### 2.9. Antioxidant analysis

Anti-oxidant activity test of the vitamin D nanoemulsion was undertaken to check whether it acts as an antioxidant, if incorporated in food. To accomplish this, DPPH (α, α-diphenylβ-picrylhydrazyl) radical scavenging assay was performed as per the protocol used by the researchers [9]. In this assay, 10 mL of DPPH (0.001 M) was prepared using methanol. 2 mL of NE is mixed with 4 mL of DPPH solution. The control contained 2 mL of methanol instead of NE. Both the test and the control were incubated in dark for 30 min. to allow the reaction to occur. After this, absorbance was checked at 517 nm. Free radical scavenging activity or antioxidant activity was calculated using formula:

$$\text{Antioxidant activity}(\%) = (C-S)*100/C$$

where S = Absorbance of sample, C = Absorbance of control.

### 2.10. Anti-microbial analysis

#### 2.10.1. Zone of inhibition

A comparative antimicrobial study was performed for nanoemulsions using different bacterial strains – Gram positive: *B. Subtilis* and *S.*

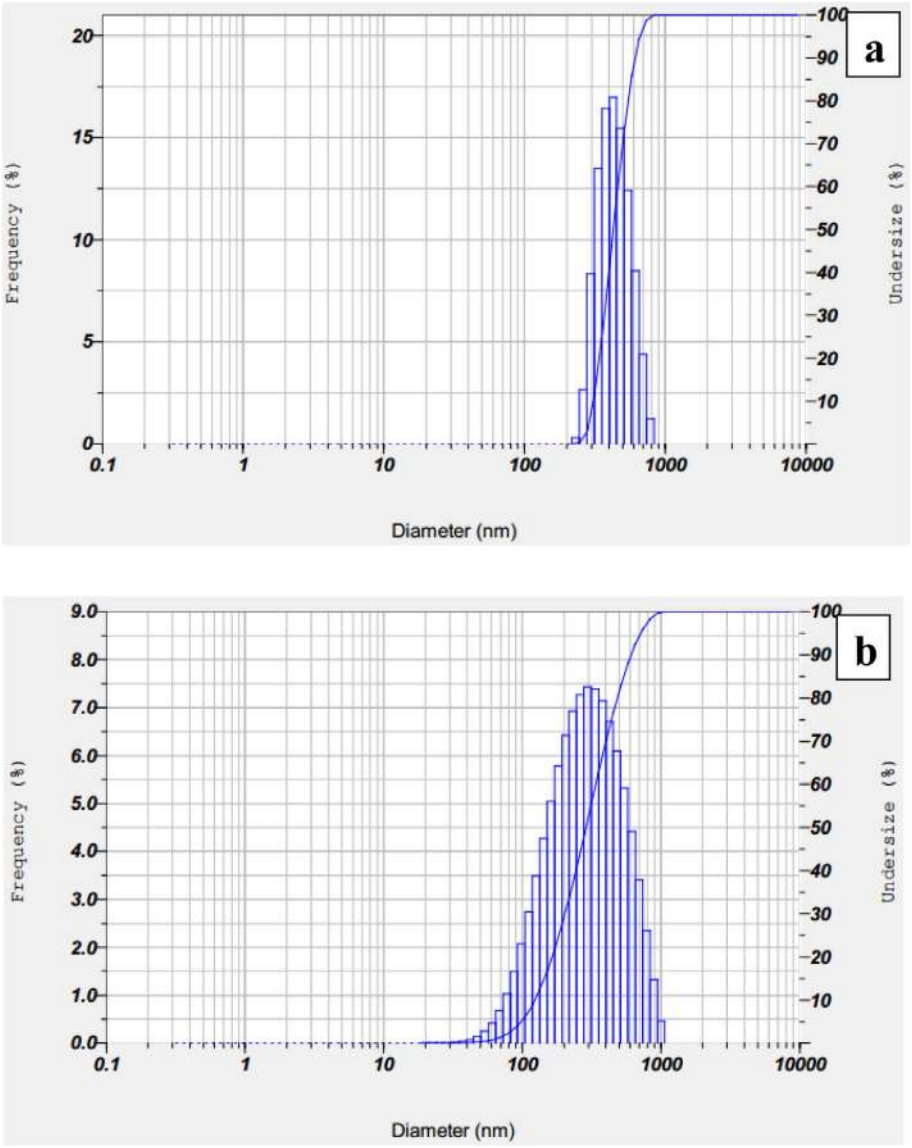


**Fig. 2.** Size distribution graphs for (a) Nanoemulsion containing more organic phase content (z-average: 446.8 ± 115.9 nm) (b) Nanoemulsion containing more aqueous phase (z-average: 332.1 ± 191.9 nm).

*Aureus*; Gram negative: *P. Aeuroginosa* and *E. Coli*. Well diffusion assay – for different volumes and strains – was followed to analyze antimicrobial activity (Ranjan and Chidambaram, 2016). 100 μL of a bacterial suspension containing $10^3$–$10^4$ colony forming units (CFU) $mL^{-1}$ microbial loads was spread uniformly on a nutrient agar plate surface. 20 μL of different concentration of both the nanoemulsions was added in each well. After 24 h of incubation at 32 °C, the average zone of inhibition was measured using a ruler with up to 1 mm resolution. The mean and standard deviation (SD) reported for nanoemulsion with each microbial strain were based on three replicates.

### 2.10.2. Reactive oxygen species generation

*Escherichia coli* was cultured in 5 mL of nutrient broth medium at 25 °C for 24 h. The cultured bacteria were treated with stable nanoemulsions and were incubated for next 48 h. The control had only the suspension culture. The cultured bacteria were centrifuged at 4000 rpm at 4 °C for 10 min to aspirate out the medium and cells and were then re-suspended in phosphate buffer at pH 7.0. The treated samples as well as negative control were treated with 100 μM DCFH2DA for 60 min. Next, centrifugation of the bacterial suspension followed by removal of extracellular media was carried out to remove any excess DCFH2-DA and further washed three times to observe clear background. The fluorescence of oxidized green fluorescent 2′,7′-dichlorofluorescein (DCF) was measured (excitation, 492; emission, 523 nm) using fluorescent microscopy (Zeiss Axiovert 220 M). It can be noted that, $DCFH_2DA$ is reported to be more reactive with nascent oxygen in comparison with other dyes, so it has been selected for this analysis.

### 2.10.3. Scanning electron microscopic analysis to study structural and colony deformation

SEM technique was used to study the effect of anti-bacterial property of nanoemulsion on bacterial strain. For SEM analysis (JEOL JSM-7600F, Japan), samples were prepared on carbon coated copper grids without any conductive coating (gold/platinum sputtering) as it may mask the real morphology of the substrates.

### 2.11. Simulated gastric behavior analysis

To determine the fate of synthesized vitamin D nanoemulsions, they were made to pass through the simulated mouth and gastric phases which were prepared as per the protocol followed by Mayer et al. [21]. Briefly, ten milliliter of saliva fluid was added to an equivalent

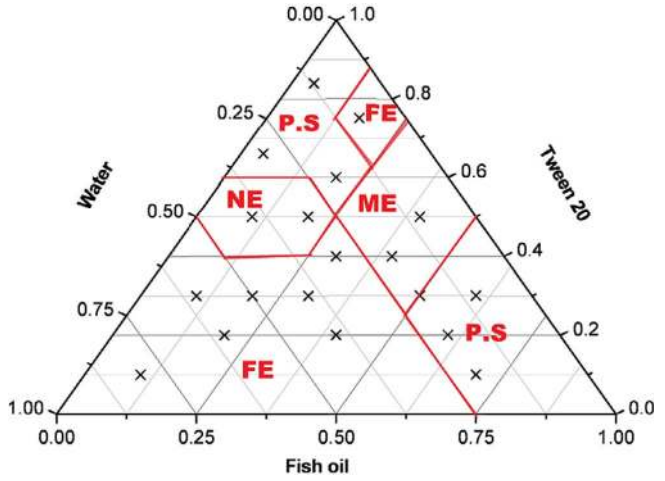


**Fig. 3.** Phase diagram showing regions of stable and unstable samples. This diagram represents combinations of three different components -water, fish oil and tween 20- based upon which further categorization of emulsions is done, into flocculated samples, phase separated samples and stable samples. This representation helps in selecting stable nano-ranged emulsions for further analysis.

nanoemulsion sample and the final mixture was adjusted to pH 6.8, followed by incubating for 10 min. Bolus sample from the mouth phase was further added to simulated gastric fluid (1:1), pH adjusted to 2.5 using 1.0 M HCl and incubated for 2 h. Simulated gastric fluid contained 2 g of NaCl and 7 ml of HCl without any addition of pepsin as prepared by a previous study [23].

## 3. Results and discussion

### 3.1. Nanoemulsion fabrication by ultrasonication

Vitamin D is an oil-soluble vitamin and is sensitive to light, heat and oxygen [12]. Since, they are absorbed only in small intestine it becomes extremely essential to protect them from unfavourable gastric conditions. Therefore, an efficient and controlled nano-delivery system has been established using fish oil. Fish oil was selected in this study, amongst the various other oils as fish oil has high potential to improve the bioavailability of vitamins [29].

In contrast to the study performed by Komaiko and McClements [16], where food-grade nanoemulsions were synthesized using low energy approach, our research has a major advantage as it does not require high concentration of surfactants for nanoemulsion formulation. This greatly reduces the toxicity of nanoemulsion. In the recent years, many vitamin-based nanoemulsions have been fabricated using high and low energy approaches to find the most suitable emulsifying condition for the production of nanoemulsions. Ultrasonication method has long been used for the preparation of micro/macro emulsions but its use in the preparation of nanoemulsion is still new. Use of ultrasonicators is an emerging technique for the production of stable emulsions with low energy input [35]. Emulsification is the first and the most widely used application of ultrasound in food industries [5]. Ultrasonicators make use of high ultrasonic waves to breakdown large molecules into smaller molecules. Sonicator probe tip, consisting of a piezoelectric crystal for generation of pressure waves is made in contact with the liquid that undergoes homogenization. This results in the generation of mechanical vibration, which causes cavitation [22].

It is extremely essential to consider an appropriate frequency for emulsification process. It has been reported that shear forces generated are quite strong at low frequencies, approximately 20 kHz. On the other hand, studies have shown that higher frequencies (211 kH) do not produce an effective emulsion as the shear forces generated at such frequencies are comparatively weaker. In general, high intensity and low frequency ultrasonic waves are able to produce the most effective emulsions [5]. Ultrasonicators hold some benefits over conventional methods. For instances, ultrasonicators require low energy consumption for emulsion production, less surfactant requirement, and emulsions generated by ultrasound are found more stable. Various parameters affect the production of emulsion including hydrostatic pressure, ultrasonic power, ultrasonic probe position within the liquid, and exposure time [6,5].

A careful analysis of the existing literature indicates the most common operating parameters for ultrasound to be 400 W, 20kH and approximately 10–30 min exposure time. Ultrasonic emulsification has been widely studied under various operating parameters. Researchers have found decrease in emulsion droplet size with increased sonication time.

### 3.2. Characterization of nanoemulsion

#### 3.2.1. Stability analysis

After storing the samples at 25 °C for 72 h, samples with flocculation and phase separation were omitted, followed by further analysis of only the stable samples.

Application of ultrasonication has proved advantageous in keeping the nanoemulsion stable for a longer period of time as no particle aggregation was observed in stable samples even when left undisturbed for more than 45 days. Our samples were found more stable, in comparison with recent studies [11] where, physical stability of nanoemulsion lasted for around 30 days and [13] where, significant degradation of vitamin was observed when exposed to long storage conditions.

#### 3.2.2. Dynamic light scattering – hydrodynamic size and zeta potential evaluation

The hydrodynamic size distributions of all the samples were found in the range of 300–1270 nm. Size distribution graphs of only stable samples in the nano range have been shown in Fig. 2 below. Based on these observations, samples were categorized into nanoemulsions and microemulsions as shown in the phase diagram (Fig. 3).

Guttoff et al. [11] successfully fabricated vitamin D based nanoemulsion of particle size less than 200 nm using low-energy approach (spontaneous emulsification). The production of small particle size can be explained by the fact that the surfactant used in their study was tween 80, which is believed to produce smaller droplets as compared to other surfactants of tween series [11].

#### 3.2.3. Phase diagram

Based on size and visual stability, phase diagram was constructed as depicted in Fig. 3. It can be noted that samples with code NE-5 & NE-11 are the only two nano-ranged, monodispersed stable samples obtained, which were considered for further analysis (will further be mentioned as stable samples throughout the manuscript). Samples coded NE-5 and NE-11 contained higher oil content and lower oil content, respectively.

Earlier researchers have synthesized vitamin D emulsion using lemon oil with particle size diameter of 220 nm [43]. They have obtained smaller droplet size but they have also used homogenization for more than five times which may lead to vitamin degradation during fabrication process. In our experimental design, we have obtained a bit larger size range but in very less energy consumption. Therefore, our study can be considered as energy efficient as it may lead to no or lesser degradation of vitamins.

#### 3.2.4. Viscosity determination

Densities were calculated to be 0.838 g/ml and 0.874 g/ml for nanoemulsions with higher oil content and lower oil content, respectively. Viscosity is also a very important factor for stable and efficient release of bioactives. The viscosity of nanoemulsions is a function of oil concentration, water and surfactant concentration. Viscosities of our stable samples were found to be 0.886 mPa.s and 0.877 mPa.s for

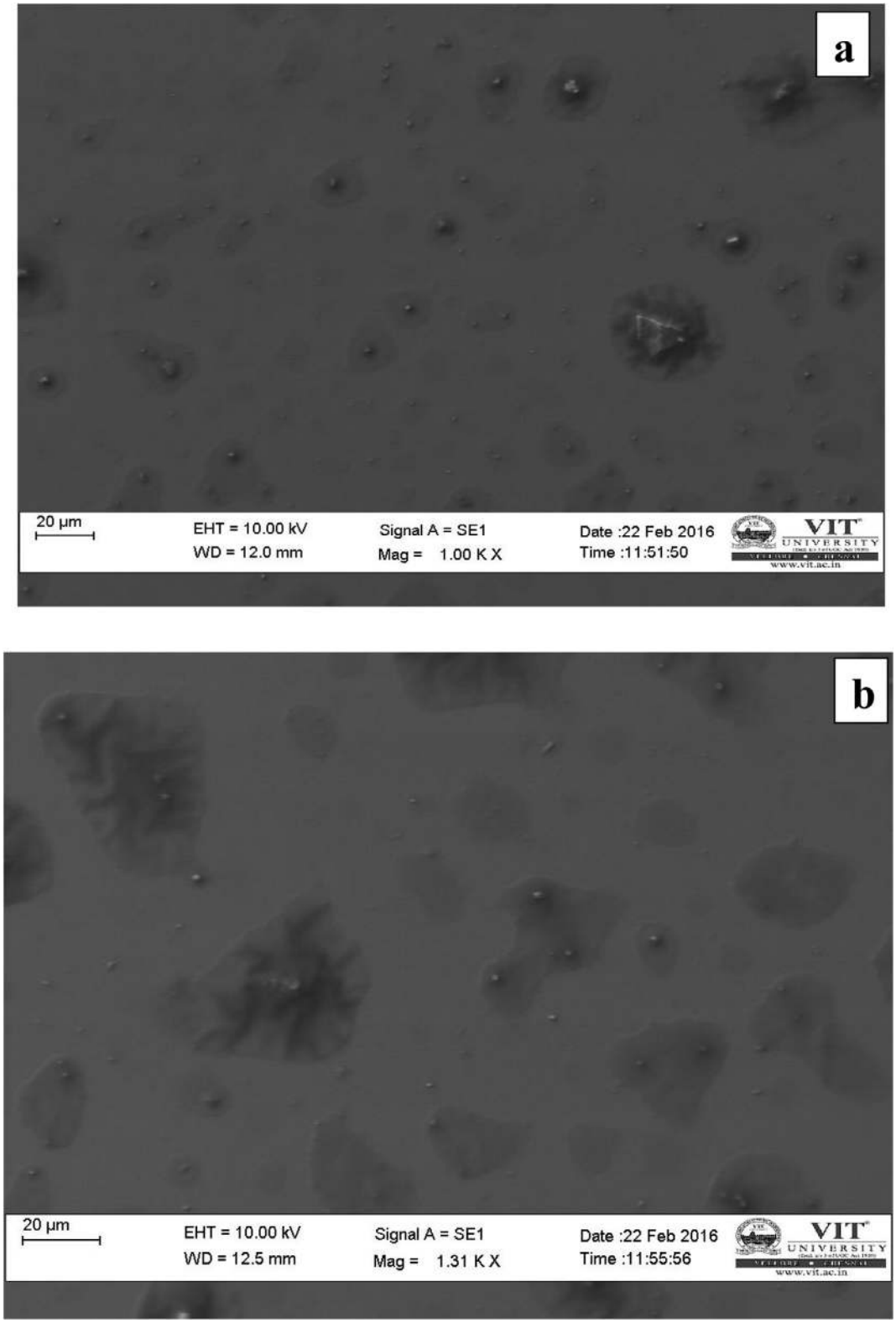


**Fig. 4.** (a) SEM image of nanoemulsion containing higher oil concentration (b) SEM image of nanoemulsion containing lower oil concentration.

nanoemulsions with higher oil content and lower oil content, respectively. It must be noted that, though the difference between both viscosities is minimal but the result is as expected, with a slightly higher viscosity for emulsion containing greater amount of oil content (former nanoemulsion). Higher the oil content present in the emulsion, higher will be its viscosity and vice versa [19].

### 3.3. Morphological analysis of nanoemulsion by microscopic techniques

#### 3.3.1. Scanning electron microscopy

SEM produces image of the sample by scanning it with a focused beam of electrons. SEM was performed to understand the nanoemulsion droplet morphology (Fig. 4a and b). The shape of nanoemulsion droplets was found to be spherical as depicted in Fig. 4a and b, which

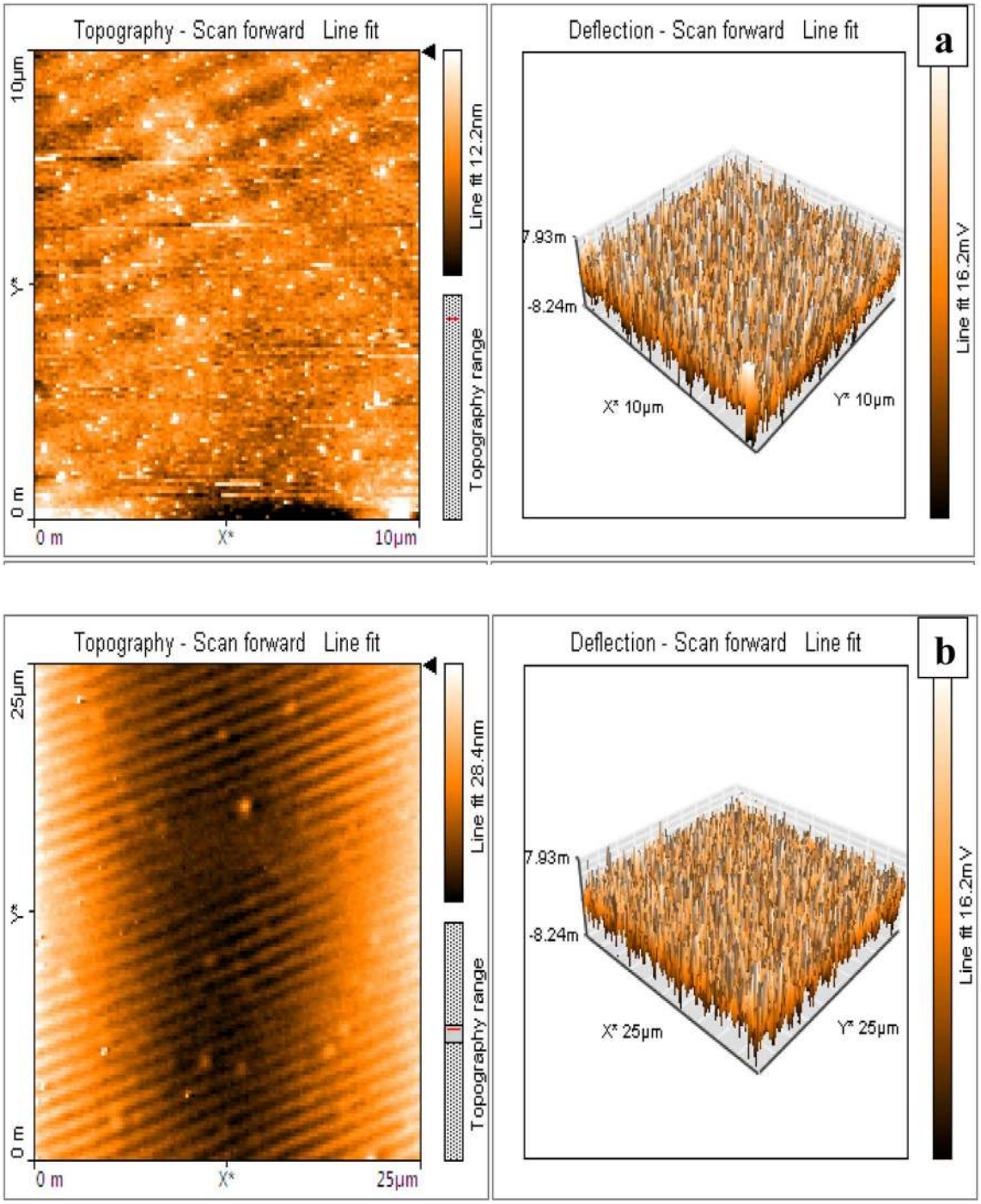


**Fig. 5.** (a) AFM image of nanoemulsion containing higher oil concentration (b) AFM image of nanoemulsion containing lower oil concentration.

was further confirmed with TEM and AFM.

*3.3.2. Atomic force microscopy*

This technique was used to determine the surface morphology of nanoparticles and samples were prepared by pouring a drop of nanoemulsion on a glass surface and allowing it to dry. The result of AFM analysis demonstrated spherical shape of nanoemulsions (Fig. 5a and b).

*3.3.3. Transmission electron microscopy*

TEM analysis confirmed spherical shaped emulsion droplets. The shape of nanoemulsion droplets was found to be spherical with a distinct zone surrounding each droplet, indicating the encapsulation of vitamin D molecules as shown in Fig. 6a and b.

*3.4. pH analysis*

The pH of the formulated stable nanoemulsions was found to be 4.2 after refrigeration and 4.1 at the room temperature. It can be noted that the change in temperature did not cause any significant change in the pH of nanoemulsions and were found to be acidic in nature, which allows them to be used in the fortification of drinks of pH range 4–5. Our results are in agreement with the results obtained by Bhushani et al. [2] as they observed variation of mere < 0.3 in the pH value of

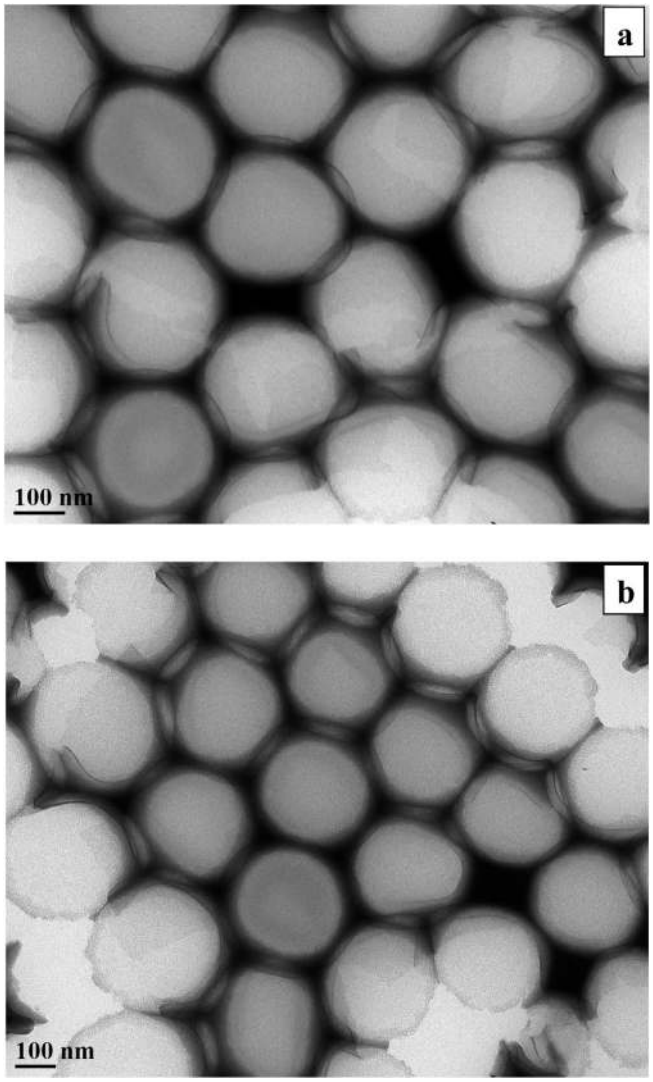


**Fig. 6.** (a) TEM image of nanoemulsion containing higher oil concentration (b) TEM image of nanoemulsion containing lower oil concentration.

nanoemulsion at different temperatures and also, when stored for 5 days undisturbed.

### 3.5. Quantitative analysis

#### 3.5.1. Determination of encapsulation efficiency

As per the formula followed by Nandita et al. [27], encapsulation efficiency was found to be 95.7% and 98.2% for the samples containing higher oil content and lower oil content, respectively. It must be noted that the nanoemulsion with higher oil concentration will have a thicker layer of oil molecules over vitamin D particles but the number of vitamin D particles per oil molecules will be less and thus, would exhibit lower efficiency as compared to the latter nanoemulsion, which had lower but sufficient oil molecules to encapsulate vitamin D particles.

Estimation of encapsulation efficiency depends upon the encapsulation method, type of oil, surfactant and the compound being encapsulated [24].

Encapsulation efficiency of vitamin D nanoemulsion was found to be higher in this study as compared to nanoliposomal encapsulation technique used by Mohammadi et al. [24], even though the base technique used for both studies was sonication [24].

Encapsulation efficiency reported by Morais and Burgess [26]; 99.72%, is similar to data obtained by Nandita et al. [27]; 99.65% and this similarity can be attributed to the fact that the same compound (vitamin E acetate) has been encapsulated in both studies. These results showed high encapsulation efficiency, which suggest that nanoemulsions can be used to fortify food products and to prolong their release into the targeted sites.

### 3.6. Antioxidant analysis

Oxidation of fats results in the release of free radicals (Reactive Oxygen Species) which can cause severe damage to the cells and overall aging. Vitamin D is a membrane antioxidant that elevates the production of other powerful natural antioxidants such as, superoxide dismutase and also, activates enzymes that are involved in inhibiting lipid peroxidation [40]. Long has been the myth of omega-3 series of fish oil increasing the free radical amount in the body prevented people to take enough advantage of fish oil's antioxidant property. However, researchers around the world have been experimenting to unmask this deceptive myth. In a study, researchers treated human aortic endothelial cells (HAECs) to omega-3s, omega-6s and saturated fats and found far lesser radicals formed when treated with omega-3s [36]; these results are in agreement with the study performed by Lagarde et al. [17].

Where platelets were exposed to eicosapentaenoic acid (omega-3 fatty acid).

Another study demonstrated that the supplementation of eicosapentaenoic acid and docosahexaenoic acid (both components of omega-3 fatty acids) does not increase oxidation of plasma proteins and thus, proves that omega-3 fatty acids are completely safe [37].

Also, the experiment performed by Erdogan et al. [7] clearly suggests that fish oil serves as an anti-oxidant. In this present study, a DPPH assay was performed to analyze the anti-oxidant property of our synthesized nanoemulsions containing fish oil and vitamin D. The anti-oxidant property was found to be 60% and 34.3% for the nanoemulsions containing higher oil content and lower oil content, respectively. Our results came out as expected, for the reason that former nanoemul-

**Table 2**
Well diffusion assay for nanoemulsion with lower oil content (measurements in cm).

| Nanoemulsion concentration | 40 μL | 80 μL | 120 μL | 160 μL |
|---|---|---|---|---|
| Bacterial Strain | Zone of Inhibition | | | |
| *P. Aeruginosa* | 1.2 ± 0.031 | 1.3 ± 0.022 | 1.6 ± 0.038 | 1.6 ± 0.036 |
| *B. subtilis* | 1.7 ± 0.042 | 2.4 ± 0.017 | 1.9 ± 0.074 | 2.0 ± 0.073 |
| *S. aureus* | 2.0 ± 0.063 | 2.0 ± 0.042 | 2.2 ± 0.044 | 2.1 ± 0.025 |
| *E. coli* | 1.0 ± 0.052 | 1.1 ± 0.043 | 1.3 ± 0.052 | 1.5 ± 0.058 |

**Table 1**
Well diffusion assay for nanoemulsion with higher oil content (measurements in cm).

| Nanoemulsion concentration | 40 μL | 80 μL | 120 μL | 160 μL |
|---|---|---|---|---|
| Bacterial Strain | Zone of Inhibition (cm) | | | |
| *P. Aeruginosa* | 1.0 ± 0.093 | 1.13 ± 0.057 | 1.48 ± 0.071 | 1.54 ± 0.068 |
| *B. subtilis* | 1.5 ± 0.037 | 1.5 ± 0.072 | 1.7 ± 0.022 | 2.0 ± 0.084 |
| *S. aureus* | 1.1 ± 0.053 | 1.7 ± 0.64 | 1.6 ± 0.037 | 1.8 ± 0.053 |
| *E. coli* | 1.0 ± 0.047 | 1.0 ± 0.085 | 1.2 ± 0.024 | 1.3 ± 0.041 |

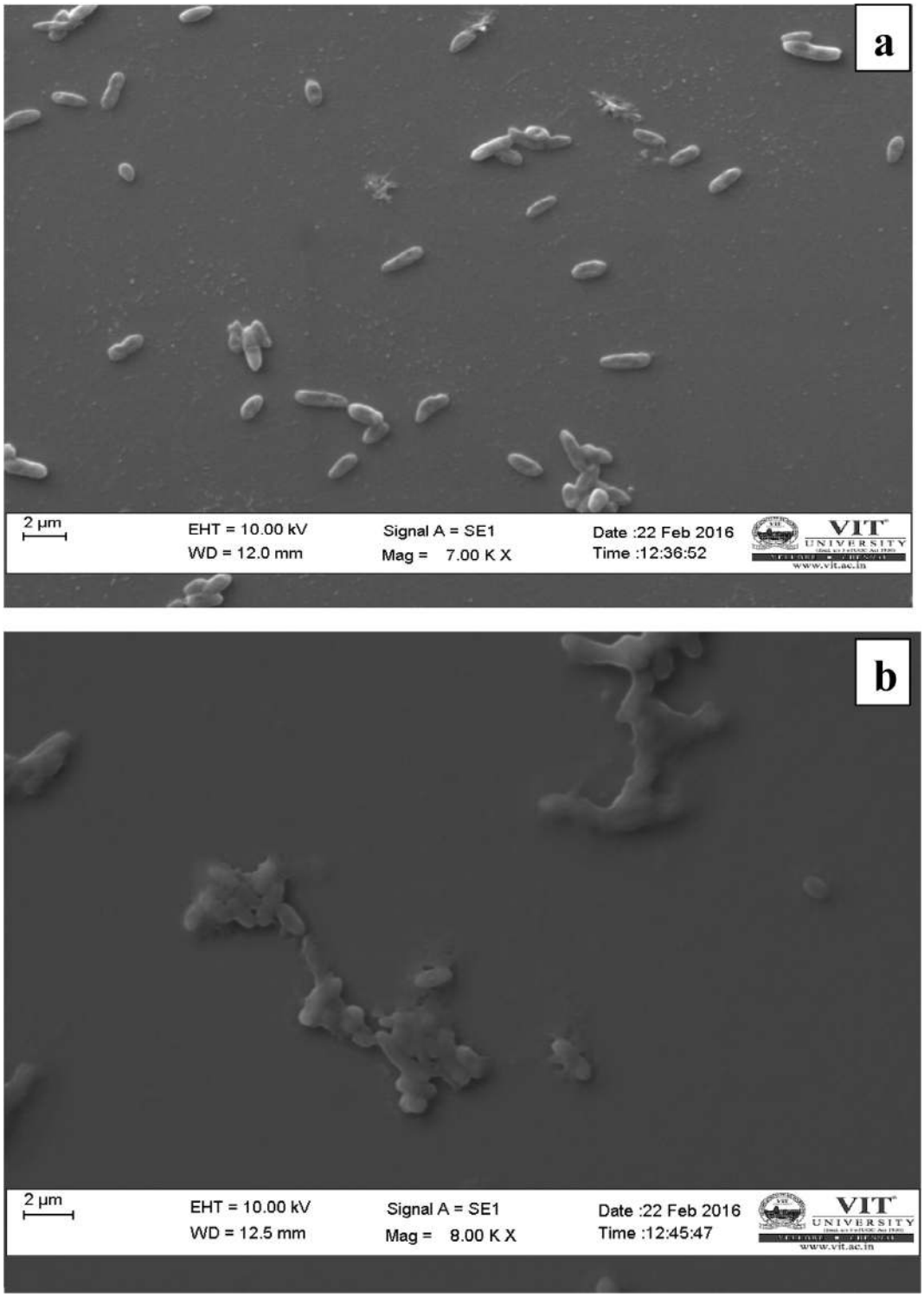


**Fig. 7.** SEM representation of (a) rod-shaped *E. coli* (b) treated *E. coli* cells with our nanoemulsion, indicating damage to the cell membrane of *E. coli* bacteria.

sion had twice the amount of fish oil than the latter, when the amount of vitamin D contained in both the emulsions was same. This can be explained by the fact that fish oil has anti-oxidant property which suggests that greater the fish oil content, greater will be the anti-oxidant property of the synthesized emulsion.

### 3.7. Anti-microbial analysis

#### 3.7.1. Zone of inhibition

It is a well-known fact that vitamin D possess anti-microbial property and serves to boost our immune system against several infectious agents. Long before the development of antibiotics, various sources of vitamin D (sunlight and cod liver oil) had been used widely for the treatment of tuberculosis [20]. Another study reveals that vitamin D induces activation of human anti-microbial peptide genes and thus, enhances our immune function [39]. This is supported by the demonstration of vitamin D-antimicrobial peptide pathway revealed by Gombart [8].

It can be observed from the results obtained in well diffusion assay (Tables 1 and 2) that our nanoemulsions were found most effective at

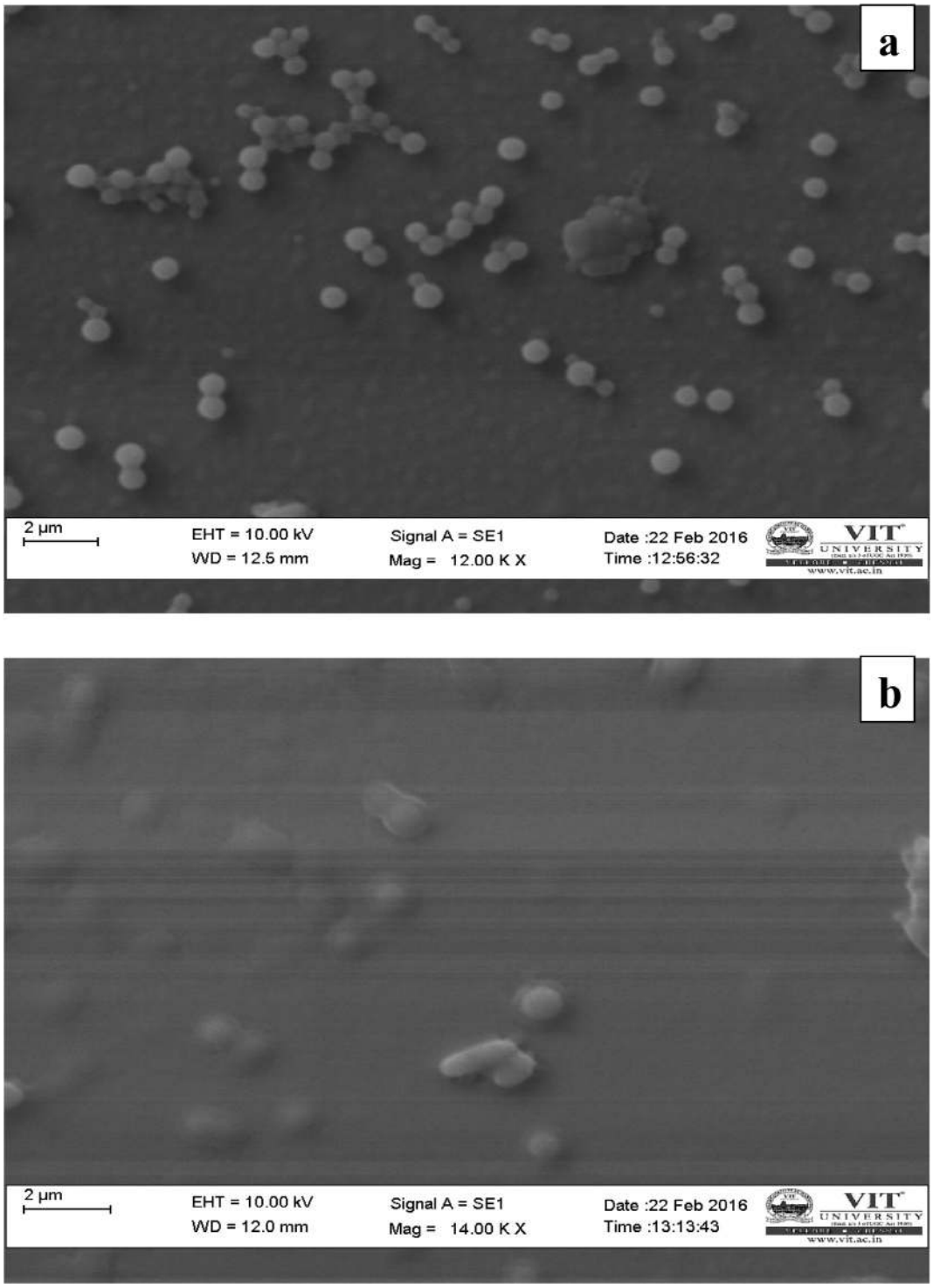


**Fig. 8.** SEM representation of (a) round-shaped *S. aureus* (b) treated *S. aureus* cells with our nanoemulsion.

controlling the growth of *S. aureus* bacteria (exhibiting maximum zone of inhibition) and least effective on the growth of *E. coli* bacteria (exhibiting minimum zone of inhibition). On comparing the anti-microbial property of both nanoemulsions, it was found that nanoemulsion containing lower oil concentration had higher anti-microbial property. This can be justified by the fact that smaller the particle size of nanoemulsion, greater will be the penetration ability of its particles into the microbes, which would thus increase the anti-microbial property of nanoemulsion.

### 3.7.2. Scanning electron microscopy

The nanoemulsion treated samples – *E. coli* and *S. aureus* bacterial strains – were analyzed using SEM and, the damage caused by nanoemulsion to the bacterial outer structures was also visualized. It could be observed from Fig. 7a and b that the nanoemulsion ruptured the cell membrane of the *E. coli* bacteria, indicating its microbial toxicity towards it. However, not much visual damage was seen on *S. aureus* strain (Fig. 8a and b). Hence, it can be assumed that the major damage caused by our nanoemulsion on this particular bacterial strain is internal with a possibility of DNA damage. To the best of our knowledge, SEM analysis for vitamin D nanoemulsion was not explored

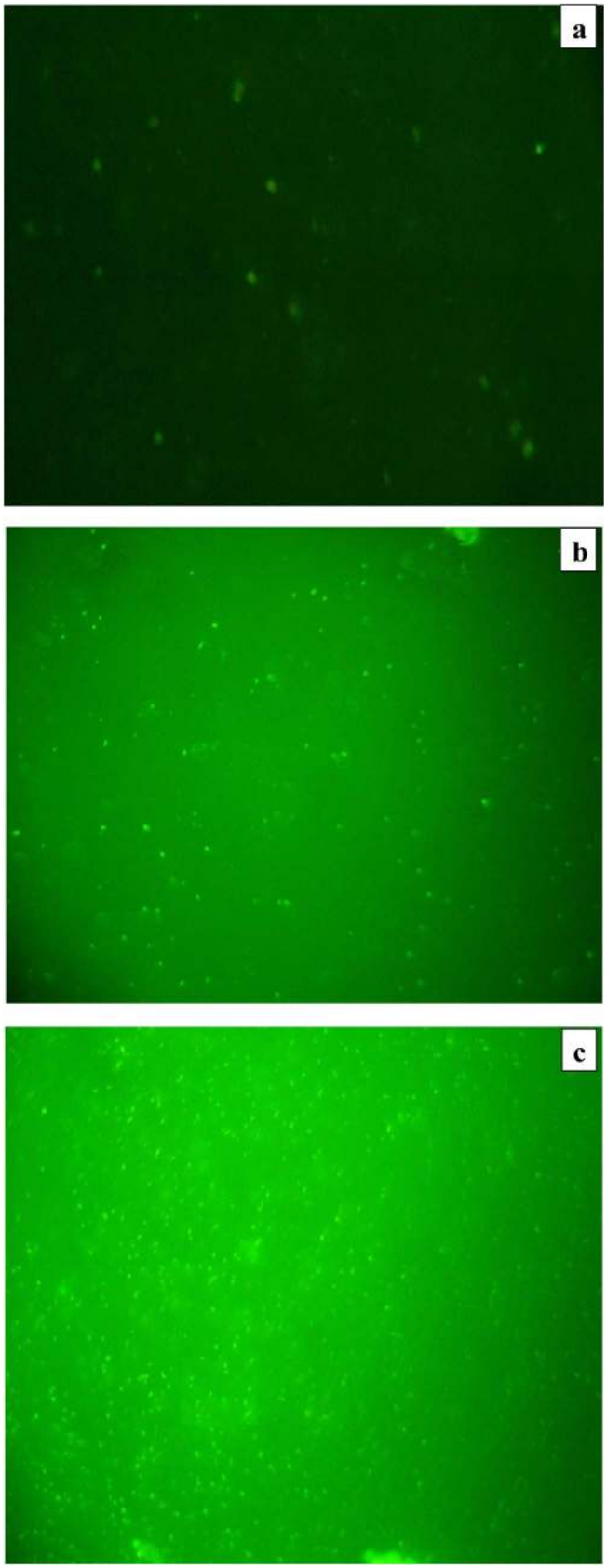


**Fig. 9.** ROS images of bacterial strain (E. coli) under fluorescent microscope (a) untreated bacteria – showing lesser fluorescence depicts lesser stress. Similarly, more stress has been observed when treated with nanoemulsion containing (b) higher and (c) lower oil concentration, respectively under fluorescent microscope. All the images have been taken at 10× magnification.

**Table 3**
Free vitamin D concentration in the stable samples under simulated mouth and gastric phases.

| Analysis stage | Free vitamin D concentration in the sample with higher oil concentration | Free vitamin D concentration in the sample with lower oil concentration |
|---|---|---|
| Before mouth phase | 1284.8 ± 25.8 ng/ml | 530.1 ± 18.3 ng/ml |
| After mouth phase | 399.5 ± 14.8 ng/ml | 765.6 ± 24.7 ng/ml |
| After stomach phase | 13.34 ± 0.16 ng/ml | 30.6 ± 0.21 ng/ml |

until now. To understand the physical damage caused by various synthesized emulsions on different bacterial strains is something that could be explored more in future.

### 3.7.3. Reactive oxygen species generation

In this study, it was observed that our nanoemulsions created stressful conditions for the bacteria, causing them to generate reactive oxygen species (ROS) which then caused damage to the bacterial cells. These damaged bacterial cells reacted with $DCFH_2DA$ dye to give green fluorescent color under fluorescent microscope. The controlled sample showed almost negligible fluorescence as compared to the treated samples due to the absence of emulsion to cause bacterial damage. However, considerable fluorescence was observed in the case of treated samples and thus, supported anti-microbial property of nanoemulsions. The fluorescent microscopic images of negative control and nanoemulsions are depicted in Fig. 9a-c. It was also observed that nanoemulsion containing lower concentration showed greater release of reactive oxygen species as it possessed higher anti-microbial activity as stated above.

### 3.8. Simulated gastric behavior analysis

To understand the effect of gastric conditions on our prepared nanoemulsions and the efficiency of oil molecules to keep vitamin D molecules encapsulated, HPLC was performed before and after each stage of simulated mouth and gastric phases. It must be noted that our basis of result majorly depends upon the free concentration of vitamin D obtained from HPLC analysis, which has been shown in the tabulated form below (Table 3).

It can be noted that earlier many researchers have analyzed behavior or efficient nanoencapsulated delivery system for several compounds such as – β-carotene bioaccessibility using orange oil nanoemulsions [34], cucumin nanoemulsion [31,30], quercetin bioaccessibility in nanoemulsion [32], insulin absorption by polymer based nanoparticles [18] and many have reviewed this delivery systems for nutraceuticals [23,42,33]. Till date, no one have fabricated an efficient nano-vehicle for the delivery of vitamin D as well as analyzed the efficient delivery system in simulated GI-tract, this is first of its kind study in this regard.

With respect to the nanoemulsion with higher oil concentration, it was observed that the amount of free vitamin D molecules reduced by four times of its initial amount when exposed to simulated mouth phase. This could be explained by a possibility that the salivary molecules may have formed micellar complexes with free vitamin D molecules and thus, reduced the free vitamin D amount. After stomach phase, a major reduction of free vitamin D amount was observed, which could be due to the severe action of acidic conditions prevailing in simulated gastric conditions. This shows a continuous trend in the degradation of free vitamin D molecules, which is probably the non-encapsulated vitamin D molecules, without causing damage to the encapsulated vitamin molecules. Another explanation could be that the formation of a thicker layer of oil particles over the vitamin molecules

(due to higher oil concentration in the nanoemulsion system) may have exhibited greater mechanical strength against simulated acidic conditions. This resulted in lower vitamin degradation and thus, showed greater bioavailability of vitamin D molecules under simulated mouth and gastric phases.

On contrary, a slightly different trend was observed with nanoemulsion containing lower oil concentration as the amount of free vitamin D elevated after being passed to the mouth phase. This could be due to the fact that a very less concentration of oil was present in the latter nanoemulsion and thus, had been insufficient to keep vitamin D encapsulated in the mouth phase and therefore, resulted in more release of free vitamin D as opposed to what was recorded with the former nanoemulsion. A similar trend of vitamin D reduction of former nanoemulsion was observed after the stomach stage, showing significant degradation of free vitamin D due to its exposure to acidic conditions. As the oil concentration in latter nanoemulsion was lower, it might have formed just a thin layer of oil over vitamin molecules and thereby, exhibited a much lower mechanical strength against acidic environment and hence, resulted in lower bioavailability of vitamin D in contrast to the former nanoemulsion. It must be understood that this is just an observational analysis and it needs to be further supported by performing several tests, in future. Also, extensive research is required to develop more efficient delivery system in order to increase the bioavailability of poorly soluble vitamins.

## 4. Conclusion

In this study, a large number of nanoemulsion samples were prepared to find out the most suitable emulsifying conditions. Stable samples with particle sizes in the range of 300–450 nm were selected for analysis. HPLC analysis was performed to find out the encapsulation efficiencies, which was calculated to be 95.7% and 98.2% for nanoemulsions containing higher and lower oil concentration, respectively. Further, the stable samples were analyzed carefully for morphological features, anti-oxidant and anti-microbial property, in particular. Nanaoemulsions showed anti-oxidant property of 60% and 34.3% by DPPH analysis. Our synthesized nanoemulsions exhibited anti-microbial property which was analyzed by well diffusion assay. At last, nanoemulsions were passed through simulated mouth and gastric conditions to understand their after-ingestion fate and it observed that our nanoemulsion with higher oil concentration was successful in enhancing the bioavailability of vitamin D by keeping most of the vitamin molecules encapsulated under simulated gastric phase. This is first of its kind study in which ultrasonication approach has been taken for efficient nano-encapsulation of vitamin D in fish-oil. As the absorption of vitamin-D will start in small intestine, in this regard, fish-oil based nanoencapsulated system is novel and efficient delivery vehicle. Efficient delivery has been studied in simulated gastro-intestinal tract. Though, many have analyzed the efficient delivery of nanoemulsions in simulated GI tract but till date, no one have fabricated an efficient vehicle for the delivery of vitamin D as well as analyzed the efficient delivery system in simulated GI-tract, this is first of its kind study in this regard. Fish-oil based nano-vehicles for efficient delivery of vitamin D should be studied further before scaling up to industrial scale and should be analyzed for the safety aspects.

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