



## Research article

## Simple formulation and characterization of double emulsion variant designed to carry three bioactive agents

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## ABSTRACT

Multiple emulsions are thermodynamically stable systems that mark applications in various fields including drug delivery systems. They allow enhanced availability of drugs, greater absorption, and present reduced toxicity, among other desirable properties. In this work, we aimed to formulate a unique double emulsion (O1/W + W1/O2/W/W) with three bioactive components viz. *Ocimum tenuiflorum* oil, *Cocos nucifera* oil and crystalline *Cinnamomum camphora*. Three surfactants with different HLB values viz. Tween-20, Tween-80 and Triton X-100 were used for the emulsification process. The method followed was simple as compared to current methods employed for formulating multiple emulsions. Formulation was characterized using techniques of bright field microscopy, Dynamic Light Scattering (DLS), High-Resolution Transmission Electron Microscopy (HR-TEM) and Fourier-transform infrared spectroscopy (FTIR). Image processing tools were also used to characterize the formulation, which reliably cross-verified the observations from conventional characterization techniques. The potency of individual components of emulsion was compared with the prepared double emulsion model by testing the activity on two pathologically relevant bacterial strains: *Fusobacterium nucleatum* (FN) and *Porphyromonas gingivalis* (PG).

## 1. Introduction

Multiple emulsions are a system of soft materials which possess drop in drop configuration, giving them a huge scope for practical applications [1]. They differ from simple emulsions in having additional dispersed phases as continuous phases [2, 3]. Simple emulsions are homogenous systems and exist in two forms: W/O and O/W [3]. On the other hand, single system in multiple emulsions may contain both forms of simple emulsion simultaneously [4]. They are termed 'emulsions of emulsion' as the dispersed phases of multiple emulsion droplets contain smaller droplets [4]. In multiple emulsions, currently, only double emulsions are exploited for practical applications. Double emulsions are a type of multiple emulsions, where the outer phase contains both oil and water in the dispersed phase which is either oil or water [5]. This brings us to two possibilities- W/O/W and O/W/O, considering there are only two different phases: water and oil [6]. With advanced technologies, researchers have revealed different types of double emulsions: W/O/W, O/W/O, W/W/O, W/O/O, O/W/W, O/O/W [5, 7, 8]. The widely used

traditional methods for production of multiple emulsions are 'two-step method' and 'phase inversion method' [2]. Current advanced technologies such as microfluidics have given various templates of multiple emulsions; however, as and when the complexity of multiple emulsions increases, the design of microfluidics apparatus becomes more challenging as more capillaries of lesser diameter need to be introduced [5]. Also, there are considerable challenges to improve the stability [4]. This made us explore simpler methods to formulate multiple emulsions, which can be followed by anyone without a need for expensive and specialized equipment.

Multiple emulsions are more prone to destabilization compared to simple emulsions due to the presence of thermodynamically unstable interfaces, hence, requirement of two emulsifiers is necessary for the formation of stable multiple emulsions [9]. One should select emulsifiers based on the Hydrophobic-Lipophilic balance (HLB) values [10]. Emulsifiers of different HLB values should be used for the stabilization of multiple emulsions [10].

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**Table 1.** Details for formulation steps formulation.

Steps	Emulsions	Components	Ratio	Total volume	rpm and duration
Step 1 O1/W	O1/W	<i>Ocimum tenuiflorum</i> essential oil and Tween 20	3:1	25ml	1000rpm for 30 min
Step 2 W1/O2/W	W1/O2	1 mg/ml <i>Cinnamomum camphora</i> and <i>Cocos nucifera</i> essential oil	5:6 with Tween 80	25ml	550 rpm for 15 min
	W1/O2/W	W1/O2 and water	5:7 with tween 20	50ml	350 rpm for 15 min
Step 3 W1/O2/W + O1/W/W	W1/O2/W + O1/W/W	O1/W and W1/O2/W + O1/W/W	equal volume with Triton X-100	50 ml	250 rpm for 20 min

The single dispersed phase in simple emulsions limits their broader applications over multiple emulsions [1]. Multiple emulsions have been utilized in the food industry to protect the active food component by encapsulation and helping in anti-oxidation reactions [11]. They have also been reported to aid in enhancing the fatty acid profile of the food [12]. Multiple emulsions can work as drug delivery agents enhancing penetration and availability of drug [13, 14, 15]. Moreover, it is reported that the emulsions can be used to prevent the oxygen deficiency which is likely to be caused in patients during surgery [16].

Multiple emulsions, help in dissolving both hydrophilic and lipophilic polymers and drugs. As multiple emulsions contain both oil phase and water phase, this eliminates the requirement of common solvent for dissolving both hydrophilic and lipophilic polymers and drugs [17]. Oliveri et al. showed that W/O/W emulsions can be used in cosmetics, such as creams and ointments. In this case, the emulsions protect the active substances, and facilitate the expected fractional release upon topical applications [18]. In one of the studies, Amphotericin B was sonicated into nanoemulsions; this resulted in enhanced drug deposition and drug retention during pulmonary drug administration. Encapsulation of vitamins like A, D2, E, K1 in emulsions to increase their efficacy is also one of the major application of emulsions [19].

Keeping in view the multitude of applications of multiple emulsions and the limitations mentioned by us in the preceding section, our focus was to devise a simple strategy to formulate a stable multiple emulsion model (a double emulsion variant, in this case) that would be able to carry more than two bioactive agents or drugs at a time. Additionally, finding efficacy of the formed emulsion was necessary, for which we performed antibacterial assays on two pathologically relevant bacterial strains.

## 2. Materials and methods

### 2.1. Materials

Essential oils of *Ocimum tenuiflorum*, *Cocos nucifera*, and crystalline *Cinnamomum camphora* of therapeutic grade were purchased from Deve Herbes, India. Surfactants Tween-20, Tween-80, and Triton-X-100 were purchased from HiMedia, India. Bacterial strains *Fusobacterium nucleatum*, and *Porphyromonas gingivalis* were kindly provided by Mr. Sourangshu Chakraborty, CBCMT, Vellore Institute of Technology, Vellore.

### 2.2. Methods

#### 2.2.1. Formulation of emulsion

We attempted to introduce O/W emulsion and W/O/W emulsion inside the continuous phase of multiple emulsions. To achieve this formulation, we used essential oils for oil phase and active component dissolved in water for inner water phase. For our understanding we abbreviated our final formulation as O1/W + W1/O2/W/W, and we named it as ‘double emulsion variant’.

For the formulation of double emulsion variant, we introduced an additional step in the conventional two step formulation method. *Ocimum tenuiflorum* essential oil was used as O1 for O1/W emulsion. *Cinnamomum camphora* solution was used as W1 for W1/O2/W emulsion. *Cocos nucifera* was used as O2 for W1/O2/W. Formulation of the double emulsion variant was done precisely in three steps and the details are as follows:

##### Step 1: formulation of oil in water (O1/W) emulsion

For the O1/W emulsion formulation, we used the technique followed by Ghosh et al. 2013 with a slight modification [20]. *Ocimum tenuiflorum* essential oil and Tween 20 were taken in a 3:1 ratio for 25ml of Milli-Q water. This mixture was stirred using a magnetic stirrer at 1000 rpm, for 30 min at room temperature.

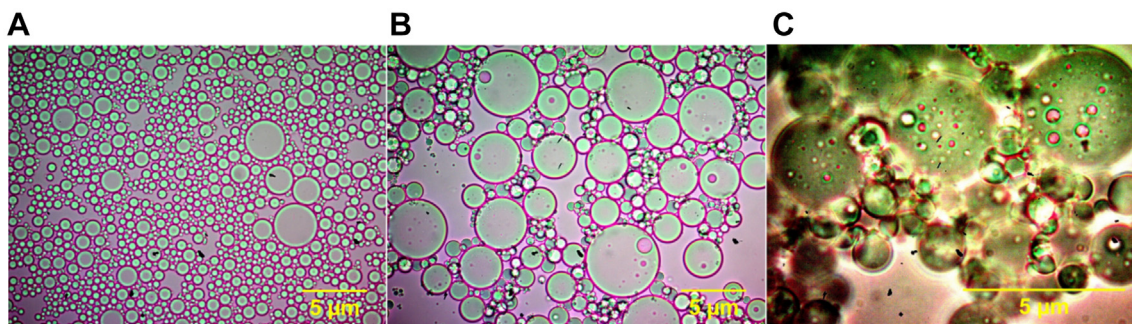
##### Step 2: formulation of W1/O2/W emulsion

Solution of 1 mg/ml *Cinnamomum camphora* crystals was constituted in Milli-Q water. We aimed to make this solution as an internal water phase (W1) of W1/O2/W emulsion. The formulation technique was followed from Khadem et al. [21] with slight modifications. W1/O2 emulsion was first formulated, where, W1 and *Cocos nucifera* essential oil were taken in 5:6 ratio and emulsified using 1ml Tween 80 for the total volume of 25ml. This mixture was stirred using a magnetic stirrer at 550 rpm for 15 min at room temperature.

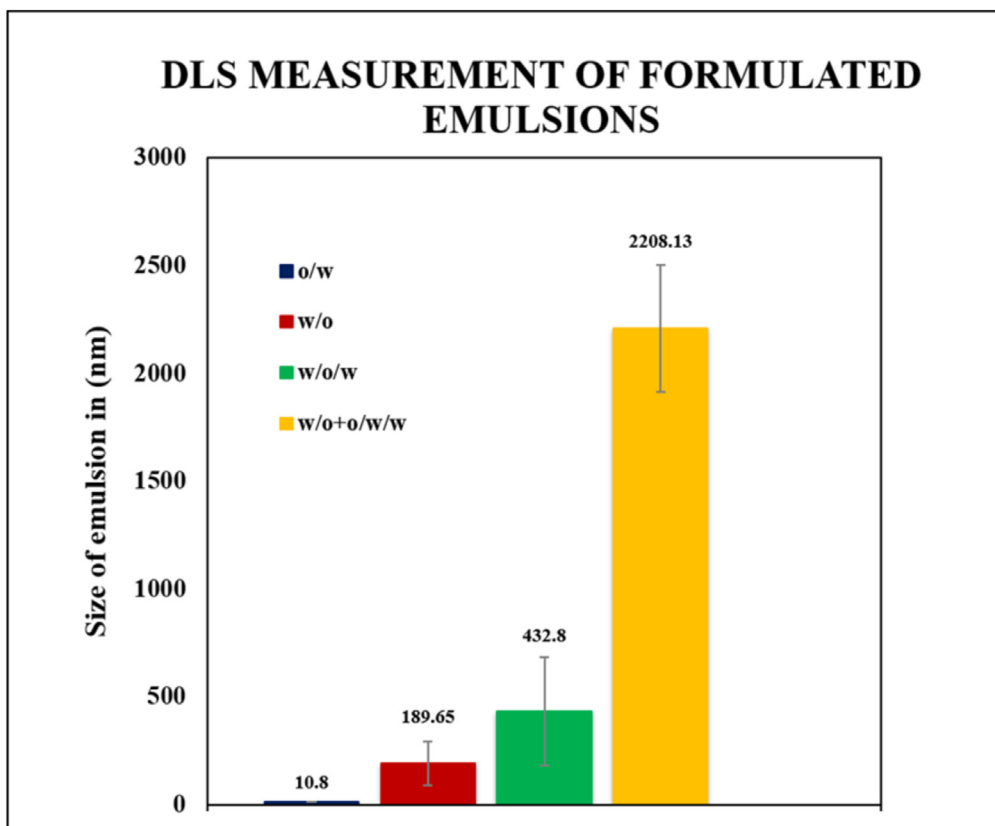
Prepared W1/O2 emulsion was further used to make W1/O2/W. This was obtained by mixing W1/O2 and Milli-Q water in 5:7 ratio by using 1ml Tween 20 for the total volume of 50ml. This mixture too, was stirred using a magnetic stirrer 350 rpm for 15 min at room temperature.

##### Step 3: formulation of W1/O2/W + O1/W/W emulsion

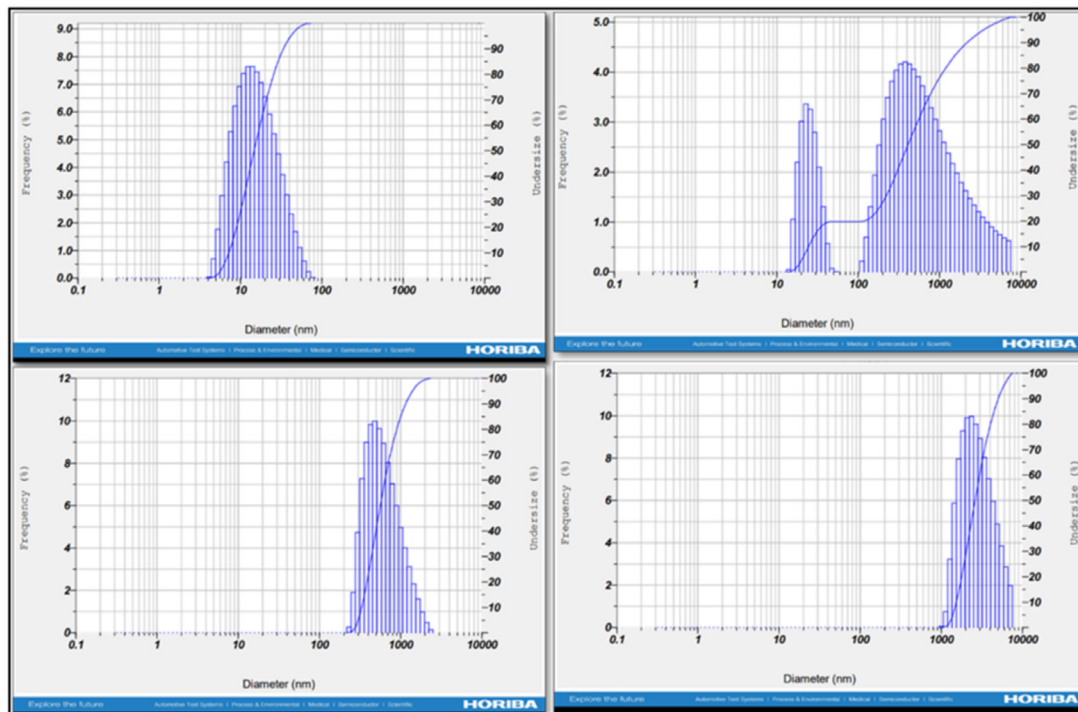
For the final double emulsion variant, an equal volume of O1/W emulsion and W1/O2/W emulsion were added making it 50 ml and emulsified using 1 ml Triton X-100. The mixture was stirred using a magnetic stirrer at 250 rpm for 20 min at room temperature. Step by step details for formulating the double emulsion variant are summarized in Table 1.



**Figure 1.** A: Bright field microscopic images of W1/O2 emulsions, B: Bright field microscopic images of W1/O2/W emulsions, C: Bright field microscopic images of W1/O2/W + O1/W/W emulsions.

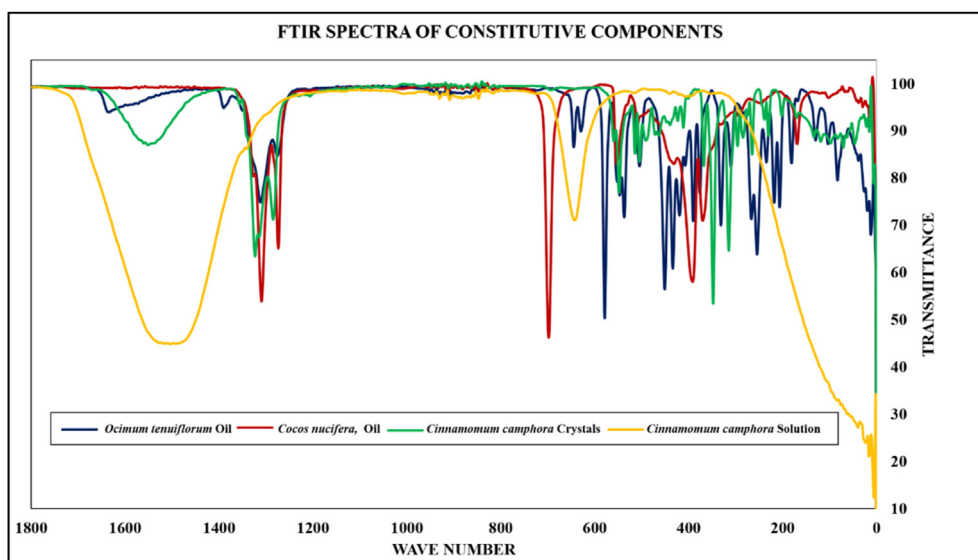


A

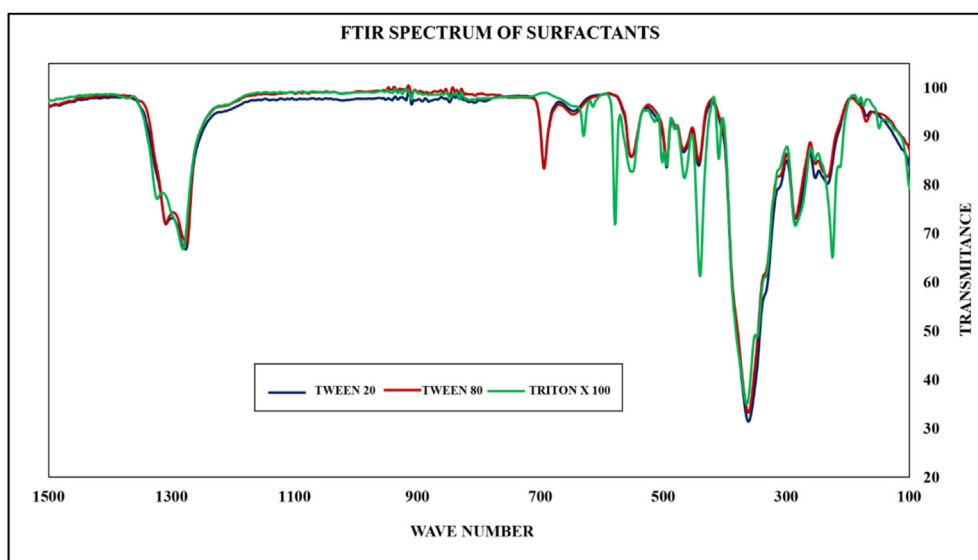


B

**Figure 2.** A: The plot shows the mean hydrodynamic radii of all the formulated emulsions, mean of the triplicates was considered with standard errors, B: The plots showing polygraphs of all the formulated emulsions. Left top corner is the DLS micrograph of O1/W; Right top corner is the DLS micrograph of W1/O2; Left bottom corner is the DLS micrograph of W1/O2/W; Right bottom corner is the DLS micrograph of W1/O2/W + O1/W/W.



**Figure 3.** The spectrum shows distinctive peaks in the fingerprint region of different constituents used in the formulation that are *Ocimum tenuiflorum* oil, *Cocos nucifera* oil, *Cinnamomum camphora* crystals and *Cinnamomum camphora* solution.



**Figure 4.** The spectrum shows the distinctive peaks in the fingerprint region of surfactants used as emulsifiers in the formulation that are Tween 20, Tween 80 and Triton-X-100.

### 3. Characterization of emulsion

#### 3.1. Bright field microscopy

A binocular CH-20i bright field microscope (Magnus, India) was used to observe emulsions at every step of preparation, at magnifications of 100X, 400X, and 1000X. Images were captured using AxioCam ERC 5s (Carl Zeiss, Germany).

#### 3.2. Dynamic Light Scattering

Hydrodynamic sizes and zeta potential of the formulation were measured on a SZ 100 Nanoparticle/Zeta Potential Analyzer (Horiba, India). For DLS, the dynamic range of 0.3 nm–8  $\mu$ m was selected. Measurements were taken in triplicates, and the obtained average hydrodynamic size of formulated emulsions was calculated. Values are represented as Mean  $\pm$  SE.

#### 3.3. Fourier-transform infrared spectroscopy

Individual components viz. essential oils of *Ocimum tenuiflorum* and *Cocos nucifera*, crystalline *Cinnamomum camphora*, surfactants used as emulsifiers Tween-20, Tween-80 and Triton-X-100, formulated emulsions O1/W, W1/O2, W1/O2/W, and W1/O2/W + O1/W/W were all subjected to FTIR analysis on IR affinity 1 FTIR spectrophotometer (Shimadzu, Japan). Results were analysed based on the spectrum obtained.

#### 3.4. High-Resolution Transmission Electron Microscopy

W1/O2/W + O1/W/W was observed under Tecnai F20 HR-TEM using a field emission of 200 kV S/TEM with an X-TWIN lens and high brightness field emission electron gun. Samples were prepared by incubating freshly prepared W1/O2/W + O1/W/W with 1% Uranyl acetate for 20 min. A drop of sample was added onto the grid, followed by air drying for 30 min. They were observed and imaged thereafter.



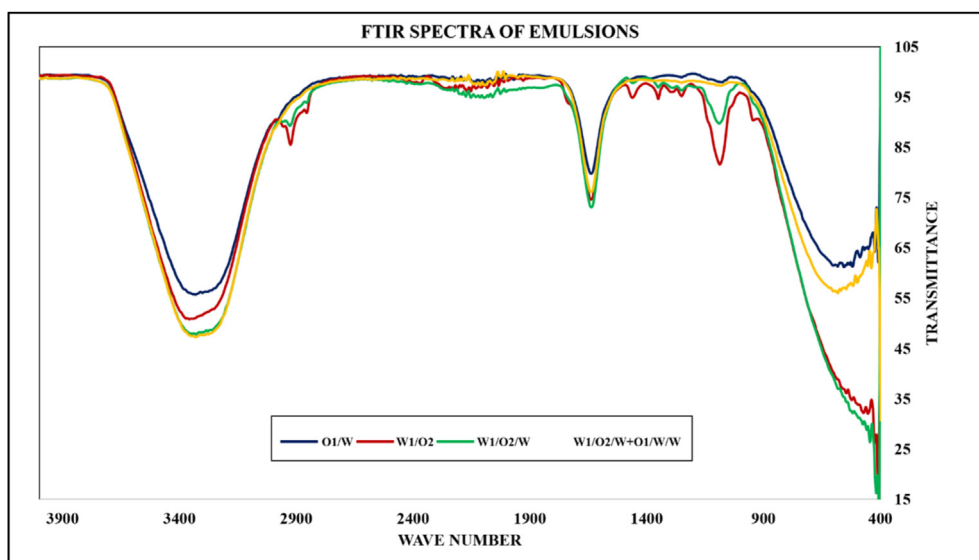


Figure 5. The spectrum shows the distinctive peaks fingerprint region of constituents and surfactants in lesser intensity.

### 3.5. Image processing

Image processing was done using Python 3.7 and its packages Open CV, Skimage, Scipy, Numpy, and Matplotlib to segment out the emulsions and to confirm whether the final O1/W + W1/O2/W/W emulsion is formed or not.

#### 3.5.1. Segmentation of images

We have developed a novel method of contour detection wherein we have used Sobel edge detection, markers, and watershed segmentation algorithm to threshold the segmented image and find the contours, to detect all the emulsions separately (even if they are overlapping).

The Sobel edge detection method was used to find the edges of the emulsions. The Sobel technique of edge detection for image segmentation finds edges using the Sobel approximation derivative [22]. It was then passed through the filter2D function of open CV with a 2D floating-point kernel in order to smoothen the images. The images were changed back to unsigned integer 8 type for further computations. Markers were used to mark out low and high intensities for W1/O2, W1/O2/W, and O1/W emulsions. These threshold values were set manually as they yielded better results compared to other values we checked with lying within the range (0–255). The Watershed segmentation method was used on the 2D filtered images based on the marker values. The watershed algorithm can segment the image into several homogeneous regions which have the same or similar gray levels [23]. Scipy's binary-fill-holes method was used to further exclude the areas with low intensities and fill the segments with zeroes. Binary thresholding was done on the segmented images for values above zero and contours were detected from the thresholded image [24]. For the secondary and tertiary emulsions, the small and overlapping contours were excluded due to their less area which can produce false results on computation.

#### 3.5.2. Detection of W1/O2 emulsion

For every contour, the centroid had been calculated. To get the internal pixels we traced all the pixels from the centroid to each boundary pixel. It was checked that if there were white pixels (intensity 250 above) in every centroid to boundary path which would mean that the contour has a white layer inside it which is the expected primary emulsion. Every contour having this layer was taken in the count.

#### 3.5.3. Detection of W1/O2/W emulsion

Apart from the segmentation, another thresholding was done to detect all low-intensity pixels (dark pixels) by the inverse binary threshold method [25]. We use the centroid to boundary path tracing method to check for independent dark pigments (the expected primary

emulsion) inside the contours. This was done by checking for black, white, and black pixels in a row. For contours having the expected primary emulsions inside it were taken in the count.

#### 3.5.4. Detection of O1/W emulsion

Here we did the segmentation as well as the dark pixels detection by inverse binary thresholding. We also threshold the bright pixels that have values close to 255 (white). We then use the centroid to boundary path tracing method to find independent dark pigments (the expected primary emulsions) and independent white pixels (the detached white pixels are the expected new phase that has been introduced) as we have done for the secondary emulsions. The contours having both of these were taken in the count.

## 4. Anti-bacterial activity of formulated emulsions

### 4.1. Revival of bacterial strains

Lyophilized cultures of *Fusobacterium nucleatum* and *Porphyromonas gingivalis* were revived by plating them on blood agar medium. A loop full of activated strains was further inoculated in tryptic soya broth followed by incubation in an aerobic chamber for 48h.

### 4.2. Disc diffusion assay

Antibacterial activity of formulation was tested by standard disc diffusion assay. In this method, 200  $\mu$ l of O1/W, W1/O2, W1/O2/W, and W1/O2/W + O1/W/W emulsions were added on sterile discs, respectively. These discs were placed on Mueller Hinton agar plate which was freshly streaked with the strains *Fusobacterium nucleatum* and *Porphyromonas gingivalis* using sterile swabs. Apart from the test samples (final formulation), individual constituents of the emulsion were also tested and served as positive controls as they are already known to be antimicrobial agents. Discs incubated with sterile water served as negative control. The experiments were conducted in triplicates. Zone of inhibition around each disc was measured and results are presented as Mean  $\pm$  SE.

## 5. Results and discussions

### 5.1. Formulation of emulsion

#### 5.1.1. Formulation of oil in water (O1/W) emulsion

In the first step, O1/W formulation with tween 20 as an emulsifier was a transparent and homogenous solution. A similar formulation was

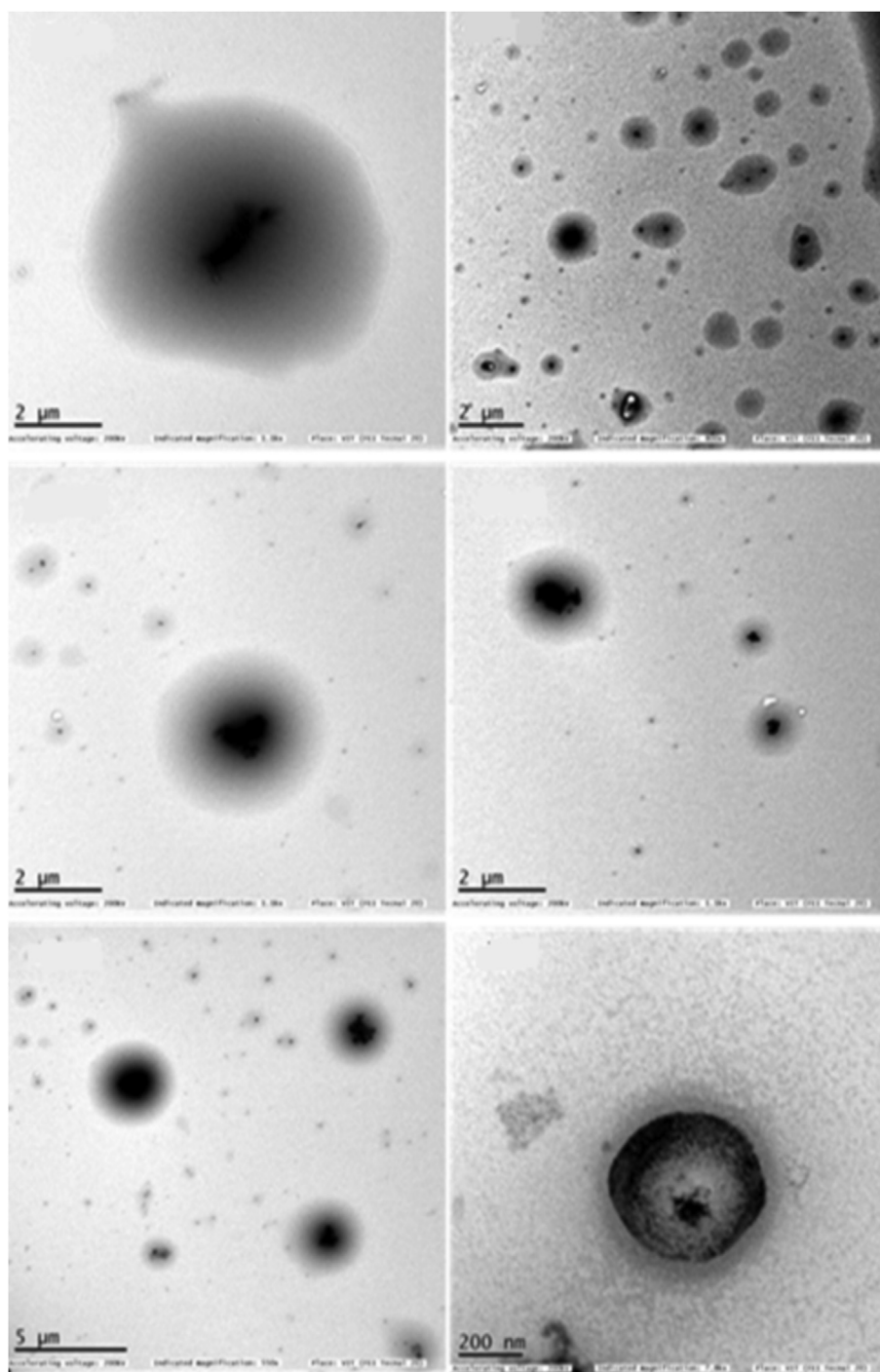
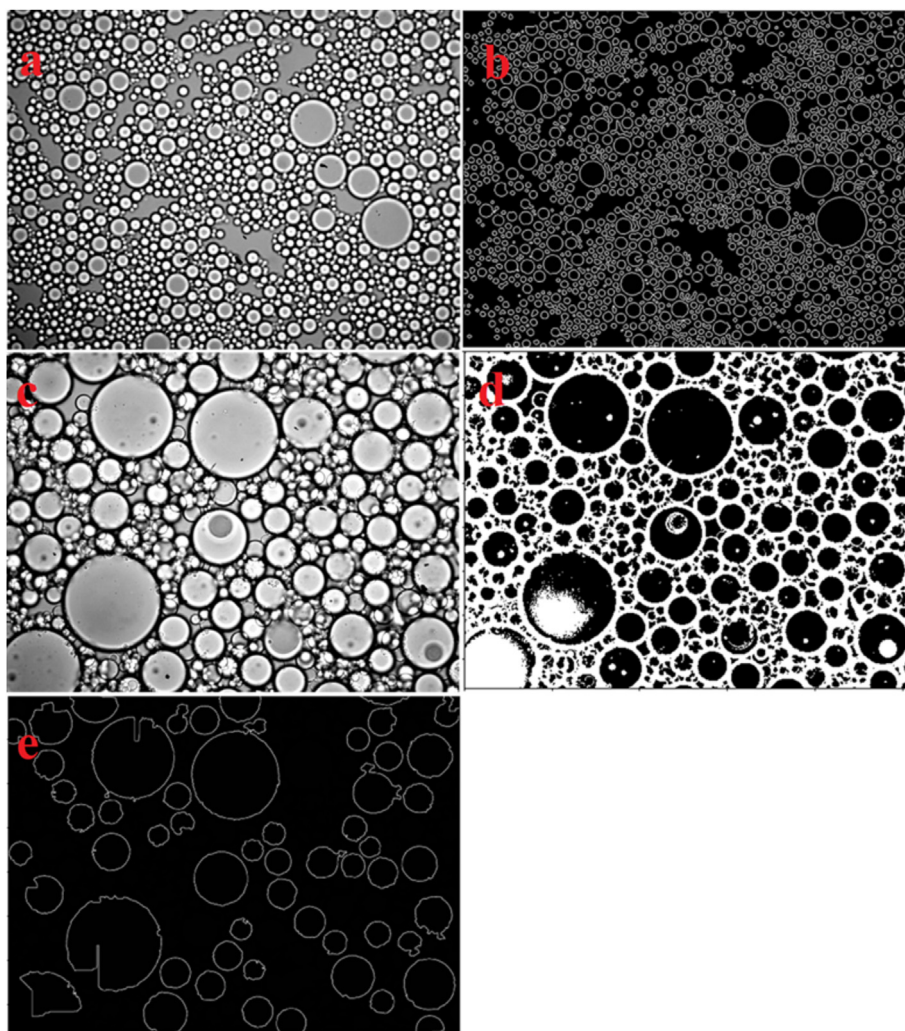


Figure 6. High resolution transmission electron microscopy.

obtained by Ghosh et al. but there, the mixture was subjected to sonication and the formulated emulsions were found to be transparent and slightly bluish [21]. Another similar formulation was reported by Sundararajan et al. where they used polysorbate 80 as an emulsifier. Additionally, the oil in their case was extracted from *Ocimum* leaves. Here the formulation yielded a milky solution [26]. For our formulation, we have avoided the use of high energy and avoided overheating of samples as in sonication, by using stirring at higher rpm. This is a viable option for both essential oils and thermolabile drugs which can degrade quickly under such circumstances and lose their efficacy. We obtained a transparent homogenous solution, without much change in the stability. Also, purified essential oil might give a better formulation compared to oil extracted by leaves, due to the interference of plethora of compounds in the latter.

#### 5.1.2. Formulation of W1/O2/W emulsion

Next, W1/O2/W formulation yielded a milky solution for both W1/O2 and W1/O2/W with tween 80 and tween 20 as emulsifiers, respectively. Various combinations of double emulsions are formulated using different oils, and soluble vitamins [27, 28]. In these reports, authors have either heated the surfactants and aqueous phase or have added magnesium, potassium chloride, or azo salts to achieve the final double emulsion formulation. Not many papers were seen which have reported formulation in a combination of *Cinnamomum camphora* solution as internal water phase (W1) and *Cocos nucifera* essential oil as continuous oil (O2) phase. The formulation was achieved by stirring at lower rpm. This prevented unnecessary heating of samples.



**Figure 7.** A: Gray Scale Image of W1/O2 emulsions, B: Contour Detection, C: Gray Scale Image of W1/O2/W emulsions, D: Image Inverse Binarization, E: Contour Detection.

### 5.1.3. Formulation of W1/O2/W + O1/W/W emulsion

Formulation of double emulsion variant (W1/O2/W + O1/W/W) yielded in homogenous solution. To the best of our knowledge, there are a very few studies that report a strategy like ours to formulate a double emulsion variant. Literature studies have shown the possibility of the formulation through microfluidics but the output is not significant [29]. Also, to get W1/O2/W + O1/W/W formulation, the construction of tubes in microfluidics gets complicated as the formulation aims to get distinct phases of both oil/water and water/oil/water emulsions in a single system. Formulation in the present study was obtained by simple conventional emulsification techniques, with modified methodology.

The classic double emulsions are mostly being prepared by 2-step methods using magnetic stirrer [30]. To achieve our interest of double emulsion variant, we added an additional step which made formulation a three-step process. There are not many papers on the formulation of the variant double emulsion using only a magnetic stirrer. The template of similar emulsion, however, has been discussed in few review articles that also state the possible limitations on outcome [8, 31].

## 5.2. Characterization of emulsion

### 5.2.1. Bright field microscopy

Formulations were studied under a bright-field microscope after each step of preparation.

In the first step, in the primary emulsion, no droplets could be seen under lower magnification probably due to formation of micelles; but in higher magnification minute droplets with greenish tinge were observed. Such reports have been published earlier [21]. Since the clear droplets were not visible under a bright field microscope, we could not capture any images for the O1/W emulsion however, the emulsion was formed with *Ocimum tenuiflorum* essential oil as the inner O1 and water as continuous W phase. In Step 2 of the formulation, for W1/O2 emulsions, we observed droplets of various sizes under the microscope. Emulsion droplets had a pinkish tinge. The two phases of W1/O2 were clearly defined [Figure 1A]. Here, *Cinnamomum camphora* solution (W1) as inner phase was emulsified with *Cocos nucifera* (O2) as outer continuous phase. W1/O2 was further emulsified with a secondary water phase to get W1/O2/W. We could observe the encapsulation of W1/O2 droplets in the secondary W phase under the microscope. Encapsulation of W1 by O2 was prominently seen in these droplets [Figure 1B]. The obtained microscopic images were similar to the study done by Vidal et al. [32]. We could see an additional layer around the droplet, which gave it a ring-in-ring appearance. This ring-in-ring pattern makes it double emulsion. In the final step, for O1/W + W1/O2/W/W formulation we observed, a ring-in-ring structure marking double emulsion and an additional unique droplet as the internal phase. Moreover, both were covered by outer continuous water (W) phase [Figure 1C]. These microscopic observations provided a bird's eye view of the emulsions



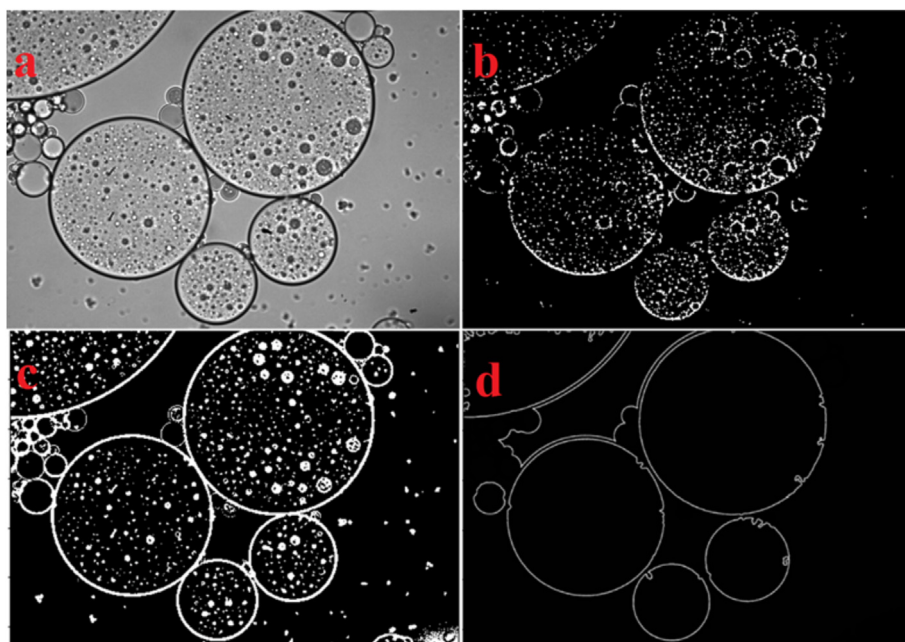


Figure 8. A: Gray Scale Image of W1/O2/W + O1/W/W emulsions, B: Image Binarization, C: Image Inverse Binarization, D: Contour Detection.

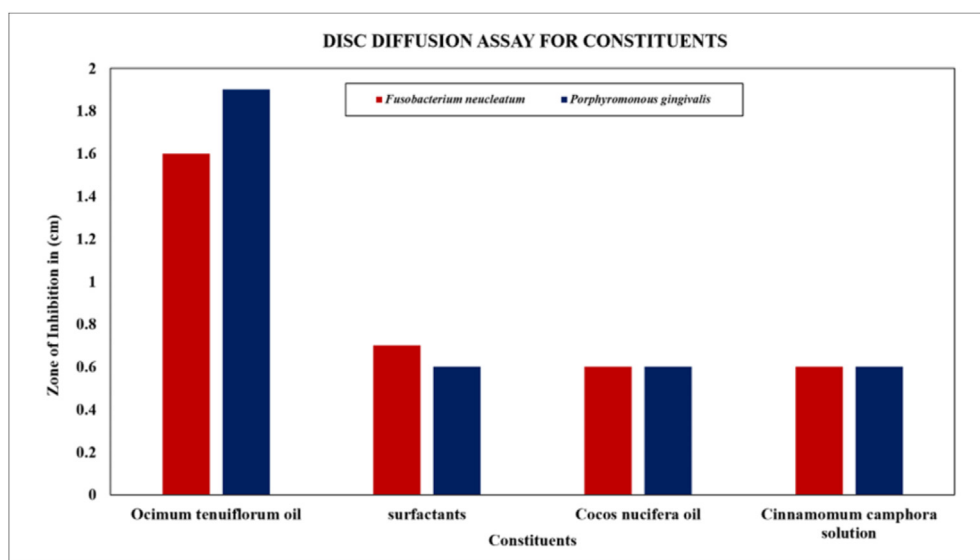


Figure 9. Image contain a plot showing the mean zone of inhibition of both FN and PG, around the discs containing constituents of emulsions with standard errors.

formed. Same images were used for further characterization by image processing.

### 5.2.2. Dynamic Light Scattering

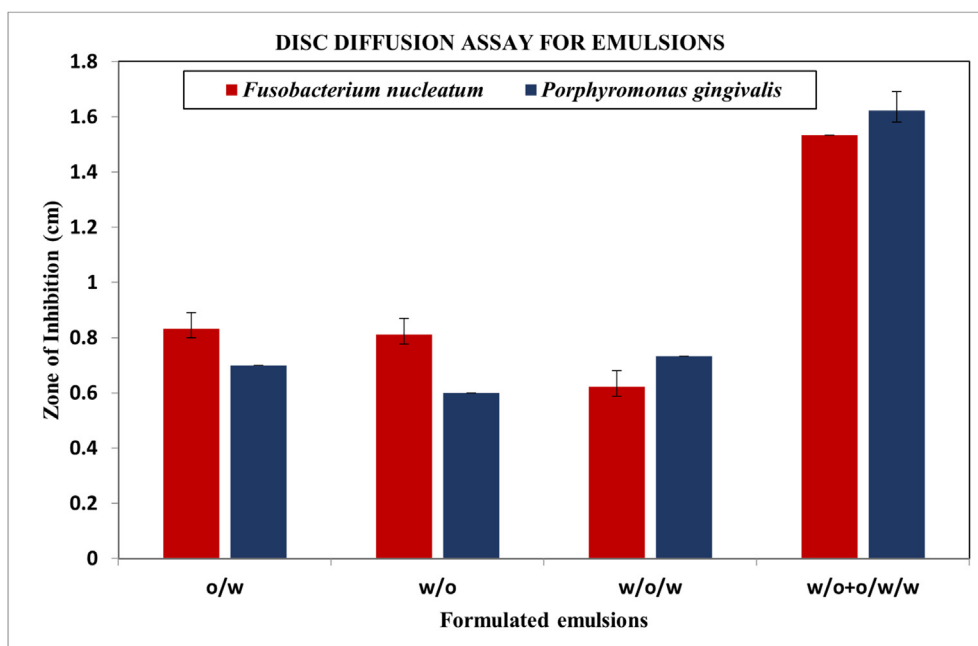
Mean hydrodynamic size of all the formulated emulsions was measured by DLS. O/W emulsions had a mean radius of 10.8 nm with a polydispersity index (PI) of 0.716. W/O emulsion showed a mean radius of 189.65 nm with a PI of 1.721. W/O/W emulsions had a mean radius of (432.8 nm and a PI of 0.036). The final formulation W1/O2/W + O1/W/W had a mean radius of (2208.13 nm with a PI of 1.288). There was an increase in the size of emulsion for every additional phase added [Figures 2A, 2B]. As far as the stability is concerned, zeta potential values of final emulsion were recorded to be  $-2.53 \pm 0.2\text{mV}$  on the day of preparation and,  $-3.17 \pm 0.18\text{mV}$  on the 30th day. Here, it is to be noted that the three non-ionic surfactants used in this study impart steric stability and hence, these values do not mean much. The rule for stability values where it is considered that solutions having values above +25 and

below -25mV are stable, applies only to purely electrostatically stabilized preparations. In our case, not much variation was observed in stability for 30 days but, it has to be improved further. More studies regarding this are in progress.

### 5.2.3. Fourier Transform Infrared spectroscopy

FTIR was performed to confirm the formation of emulsions. First, the constituents were subjected to FT-IR analysis individually. *Ocimum tenuiflorum* essential oil, *Cocos nucifera* essential oil and *Cinnamomum camphora* crystal had their respective fingerprint region with distinctive peaks [Figure 3]. We expected these constituents to be encapsulated in the emulsions resulting in the peaks reducing in their intensity. When we compare the peaks of *Ocimum tenuiflorum* essential oil alone and O1/W, we could prominently make two observations: decreasing intensity of peaks in the fingerprint region and presence of a broad peak at 3400 which corresponds to -OH functional group of water [Figures 3, 4, and 5]. Both the observations infer that the O1/W emulsion is formed. The oil





**Figure 10.** Image contain a plot showing the mean zone of inhibition of FN and PG, around the discs of O1/W, W1/O2, W1/O2/W, O1/W + W1/O2/W/W emulsions with standard errors.

phase of O1/W contains *Ocimum tenuiflorum* essential oil. When we observed the spectrum of *Cinnamomum camphora* crystals and *Cinnamomum camphora* solution, a similar observation was made [Figures 3 and 5]. Here the fingerprint region of *Cinnamomum camphora* crystals is completely masked by water in *Cinnamomum camphora* solution. This we had used as W1 for W1/O2 emulsion. When we observed the spectrum of W1/O2, the OH peak corresponding to water at 3400 was observed. There were fewer peaks in the fingerprint region which corresponds to camphor. We could observe few distinctive peaks which marked the fingerprint region of *Cocos nucifera* essential oil [Figures 3 and 5]. These observations made us infer that *Cinnamomum camphora* is still in the soluble form and the process of emulsification did not affect the solubility of *Cinnamomum camphora*. Also, lessening intensity of fingerprint peaks of *Cocos nucifera* essential oil showed that the oil was involved in the emulsification process and most of the components might be interacting with surfactant. When we observed the spectrum of W1/O2/W1, the intensity of W1/O2 further decreased [Figure 5]. This is expected, as an additional water phase is covering W1/O2 making it W1/O2/W. When we observed the spectrum of our final formulation O1/W + W1/O2/W/W we made two observations: no distinctive peaks of constituents were seen in their respective fingerprint region and the OH peak at 3400 was present [Figure 5]. This observation was significant. As this is the third step of formulation, one might expect shear pressure or coalescence [33]. If this were true in our case, then the constituents would have separated from the emulsion revealing characteristic individual peak in the IR spectrum [34]. Absence of this, therefore, indicated that the final formulation contained both O1/W and W1/O2/W internal phase, in the continuous outer water phase, W.

#### 5.2.4. High-Resolution Transmission Electron Microscopy

HR-TEM results confirmed that the formed O1/W + W1/O2/W/W emulsions were in the size range of 200 nm to 5  $\mu$ m [Figure 6]. To confirm and other observations, image processing was employed as a tool to further characterize the emulsion.

#### 5.2.5. Image processing

For W1/O2 emulsions, the centroid to boundary measurement detected the boundary formed by white pixels depicting primary emulsions [Figures 7A,7B]. The centroid to boundary measurement detected

the independent dark pixels first and when the inverse binary thresholding was executed, it detected white pixels. The measurement continued and detected black pixels. The detection of black-white-black pixels in series resembles the formation of W1/O2/W emulsions [Figure 7(C-E)]. Contour detection and thresholding for O1/W + W1/O2/W/W emulsion detected extra white pixels along with the dark pixels giving us a clue of the newly introduced phase [Figure 8(A-D)]. The results from image processing support the formation of W1/O2, W1/O2/W, and O1/W + W1/O2/W/W emulsions. The images had distinguishable intensity differences for every emulsion type. This made image processing techniques to detect double emulsion variant reliable and easy.

#### 5.3. Antibacterial activity of the formulation

A standard disc diffusion assay was performed to examine the antibacterial activity of the formulated emulsion. Here, individual constituents of emulsions were added to different sterile discs and used as a positive control for their known antibacterial property [35, 36, 37, 38]. Zones of inhibition were seen around the discs of positive controls in plates of both bacteria, as expected. A similar zone of inhibition was noted in individual components against a spectrum of bacteria in various studies indicating their antibacterial activity [35, 39, 40]. The mean zone of inhibition in plates of FN around the O1/W emulsion disc was 0.82 cm (0.86 + 0.76+0.86) and for PG it was 0.70 cm (0.70 + 0.70+0.70). Zones of inhibition around W1/O2 in plates of FN were 0.61 cm (0.66 + 0.56+0.63) for PG it was 0.60 cm (0.60 + 0.60+0.60). Zone of inhibition around W1/O2/W in plates of FN were 0.80 cm (0.93 + 0.56+0.93) for PG it was 0.73 cm (0.80 + 0.60+0.60). Zone of inhibition around O1/W + W1/O2/W in the plates of FN were 1.53 cm (1.50 + 1.50+1.60) for PG it was 1.60 cm (1.60 + 1.60+1.60) [Figures 9 and 10]. From these observations we can infer that the formulated emulsion was having antibacterial properties. Interestingly, synergetic effect of final O1/W + W1/O2/W was observed as zones of inhibition kept increasing from simple emulsion to double emulsion to our final formulation (O1/W + W1/O2/W). Another important observation that supported the formation of emulsion was that the zone of inhibition was lesser around the discs of O1/W emulsion compared to *Ocimum tenuiflorum* essential oil, but in the final formulation the zone of inhibition increased [Figure 10]. From this, we inferred that the presence of O1/W in O1/W +

W1/O2/W/W emulsion was successful. No zone of inhibition was observed in discs incubated with sterile water. Results of the assay confirmed the improved activity of final emulsion against the tested bacteria, as compared to the simple emulsions formed in the prior steps. The proposed emulsion can serve as a model system for drug delivery and similar natured drugs can be used instead of oils. This would enable incorporation of three active components in a double emulsion variant. Our interest in using *Fusobacterium nucleatum* and *Porphyromonas gingivalis* arose from the fact that both are involved in multiple pathologies of serious nature. Both are Gram negative anaerobes known to play major role in a range of periodontal diseases including oral cancers [41, 42]. *Fusobacterium* has also been shown to play key roles in adverse pregnancy outcomes, several GI disorders including colorectal cancer and many other conditions like cerebral aneurysm and rheumatoid arthritis to name a few [43]. It is a general observation in many cases that synergistic action of combinational drugs provide better prognosis. In the same context, it might be possible to deliver combination of drugs through complex emulsions, as in this case a double emulsion variant. More extensive studies are required, however, for confirmation of this observation.

## 6. Conclusions and Future directions

In summary, a double emulsion variant was successfully formulated with the methods adopted. From literature studies, we understood that most of the double emulsion formulations resulted in emulsion droplets of micrometre size irrespective of the formulation technique [44]. The current challenge for the researchers in this field is to formulate the double emulsion in nano size, as that may further improve the bioavailability of active agents. Besides, elaborate studies to improve the stability of formulations is also required. Here, image processing and analysis was used as a tool to reliably cross-verify the formation of double emulsion variant, in addition to conventional characterization techniques. As discussed previously, double emulsions are pragmatic and promising candidates for drug delivery applications [45,46]. In this regard, it was interesting to note the improved, synergistic bioactivity of our double emulsion variant, as compared to individual emulsion forming components, in preliminary study conducted on two important microbes. More elaborate studies are required, however, to improve the stability and conclude about the bioavailability, drug retention pattern, and biological activity of the emulsion on different systems. Overall, this work provides a simple template for formulation of complex emulsions. This system can be extended to deliver different combinations of three active principles/ drugs, particularly two hydrophobic and one hydrophilic substance, with improvements stated above.

## Declarations

### Author contribution statement

Hema S.K: Conceived, designed and performed the experiments; Analyzed the data and wrote the manuscript.

Aparajita Karmakar: Performed image analysis and wrote the manuscript.

Raunak Kumar Das: Helped in image analysis and provided bacterial strains

Priyanka Srivastava: Conceived, designed and planned the experiments; Analyzed and interpreted the data; Monitored the overall work and wrote the manuscript.

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## Data availability statement

Data included in article/supp. material/referenced in article.

## Declaration of interest's statement

The authors declare no conflict of interest.

## Additional information

No additional information is available for this paper.

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