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Development and Characterization of Nanoemulsions Incorporating Tuna Fish Oil

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***Abstract* – Tuna fish oil (TFO) possesses various health-promoting and nutritional benefits due to the presence of eicosapentaenoic acid (EPA) and docosahexaenoic acids (DHA). The foremost limitation in its application in broad-spectrum is its instability when exposed to the environment. In the present study, tuna fish oil was encapsulated in nanoemulsions stabilized by purity gum ultra (PGU), that is, octenyl succinic anhydride (OSA) modified starch. Different concentrations (1, 1.5 and 2%) of PGU were used to prepare tuna fish oil loaded nanoemulsions to select the best and optimum concentration of PGU. For that reason, the high energy input technique (Ultrasonic homogenization) was used to prepare the nanoemulsions in the current study. The freshly prepared nanoemulsions were then characterized in terms of size, polydispersity index (PDI), zeta potential (ZP) and rheological properties as well as observed the particle size by confocal microscopy. Results of freshly prepared nanoemulsions presented that as concentrations of PGU increased, a gradual increase in size (226 to 259 nm) was reported, whereas the trend for ZP (– 34.2 to – 31.4 mV) exhibited reverse pattern. Besides, all nanoemulsions showed shear thinning behavior. Furthermore, confocal results depicted that the oil droplets were successfully encapsulated within the nanoemulsions. All nanoemulsions were subjected to 4 weeks of storage at room temperature for the evaluation of physical stability. After 4 weeks, a gradual increase in size and PDI for all samples was monitored, whereas ZP values were decreased to some extent. On the other hand, all nanoemulsions had displayed shear thinning behavior even after 4 weeks. The confocal microscopy image of prepared nanoemulsion (1.5% PGU) confirmed very fine and evenly distributed oil droplets as compared to other formulations after a storage period. According to our findings, prepared nanoemulsion (1.5% PGU) showed negligible changes after one-month storage. Thus, such nanoemulsions can be developed and incorporated into dairy and beverage products.**

***Keywords*- Tuna Fish Oil, Purity Gum Ultra, Nanoemulsion, Storage Stability, Rheology.**

I. INTRODUCTION

In recent years, functional foods have governed much more attention of consumers to fulfill their demands regarding safer, healthier and nutritious diets. Omega-3 (ω-3) polyunsaturated fatty acids (PUFA), particularly, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are being used as fascinating functional ingredients in different pharmaceutical and food products because of their putative bioactivities to reduce several human disorders such as cancer, diabetes, allergy, cardiovascular diseases, immune response, inflammation, hypertension, renal disorder and mental illness [1-3]. Additionally, they are proved to be more advantageous for the growth of the eye and brain in infants [4]. The major causes of death in developing countries are cardiovascular diseases, cancer, and diabetes, which can be overcome by the consumption of EPA and DHA. Tuna fish oil is the richest source of ω-3 polyunsaturated fatty acids including DHA and EPA. On the other hand, it is easily oxidized when comes in contact with the environment. This is a major constraint that limits its application on the large

scale because it may produce harmful effects such as unpleasant flavor, safety, nutritional value, functional characteristics and several quality issues of food products as well as also affect consumer health, sensory evaluation and acceptability of market [5, 6].

To overcome such hurdles, lipid-based drug delivery system such as nanoemulsion, which is a thermodynamically stable system comprising water, oil and a surfactant having the size of droplets in nano-scale offers promising ways to enhance the solubility and stability of bioactive compounds [7, 8]. Due to their nanoparticle size range and thermodynamic stability, nanoemulsions showed a variety of advantages as compared to other delivery systems [9]. Several preparation approaches and fabrication materials govern the quality characteristics and application of fabricated nanoemulsions. In order to develop the nanoemulsions, high-energy techniques, that is, microfluidization, high-pressure homogenization, and low frequency (20 kHz) ultrasonication are being widely used for this purpose. Ultrasonication has gained much attention from researchers in recent times owing to receive the status of "green technology'' because it has high efficiency and less instrumental requirements as well as provides fine droplet size of nanoemulsions [10].

In recent years, nanoemulsions have fetched much attention due to their advantages such as high efficiency, low laboratory requirements, low price and usage of friendly materials with the ecosystem. Oil-in-water (O/W) nanoemulsion is an effective delivery system to carry lipophilic compounds. The oil phase covering the bioactive compound and aqueous phase holding the emulsifier, are homogenized and further treated to get the size in the nm range. Thus, the prepared formulation enhances the stability, antioxidant activity and improves the bioavailability of the food functional compounds [11-13].

The use of native starches is not welcomed due to their few drawbacks such as they may produce a rubbery paste and cohesive during the heating process and undesirable gels after the cooling process [14]. Mostly food manufacturing industries prefer to use modified starches as an emulsifier over the native starches because of their good behavioral characteristics. In recent studies, utilization of octenyl succinic anhydride-modified starches (OSA-MS) being stabilizers and emulsifiers has successfully been gained many interests and being used for fabrication of oil-in-water (O/W) nanoemulsions by food and pharmaceutical industries due to their better stability, ionic strength and a wide range of pH [8, 15, 16]. Purity gum ultra (PGU) is newly developed OSA-MS that provides beneficial properties such as low viscosities, high oil loading capacities and resistance to oxygen [17-19]. The present study aimed to investigate the effect of different concentrations of PGU on the size, zeta potential, polydispersity index (PDI) and rheological properties for tuna oil nanoemulsions.

II. Materials and Methods

1.1. *Raw Material and Chemicals*

Tuna fish oil (TFO) was gifted by Novosana (Taicang) Ltd. (Suzhou, Jiangsu, China). Purity Gum Ultra was purchased from Ingredion China Limited (Shanghai, China). Nile red dye was obtained from Sigma Aldrich Co. (Deisenhofen, Germany). Double distilled water was used for all experiments. All other chemicals used in experiments were of analytical grade.

1.2. *Nanoemulsions Preparation*

Nanoemulsions, stabilized by different concentrations (1, 1.5 and 2 % w / v), were prepared by adopting [10]

method, with some modifications. To develop aqueous suspensions, different concentrations (1, 1.5 and 2% w/v) of PGU were dispersed into double distilled water and kept stirring overnight at room temperature to ensure its complete hydration. Coarse emulsions of TFO were prepared after proper mixing of aqueous phase (90%) and oil phase (10%) with the help of high-speed homogenizer (Ultra-turrax, Germany) at 15,000 rpm for 3 minutes at room temperature. After that, 40 mL samples of coarse emulsions were processed through high-intensity sonication at the frequency of 20kHz using a 1200W ultrasonic processor (JY98-IIIDN, 20 kHz, volume processing capacity: 50-1000mL, Ningbo Scientz biotechnology Co., Ningbo, China). Applied power (40%) and time (13 min) were applied in this study. In order to avoid the overheating, work and rest time of the sonication were set at 5s and 7s, respectively. For keeping samples cool and maintaining temperature, an ice jacket was used throughout in preparation of all samples.

1.3. *Determination of Mean Droplet Size and Polydispersity Index (PDI)*

After the preparation of nanoemulsion, the mean droplet size and polydispersity index (PDI) of all samples were analyzed with a dynamic light scattering technique (DLS) (Zetasizer Nano ZS laser diffractometer, Malvern Instruments Ltd., Worcestershire, UK) at room temperature. Samples were diluted 200-folds and mixed well to avoid multiple light scattering effects. The refractive index of TFO and water was 1.45 and 1.33 respectively. The polydispersity is the measure of stability and homogeneity of nanoemulsion samples. Less than 0.3 PDI values of nanoemulsions showed long-term stability and narrow size distribution. All measurements for fresh and stored samples were performed in triplicate.

1.4. *Determination of Zeta Potential (ZP)*

The zeta potential of nanoemulsions was measured by dynamic light scattering (Zetasizer Nano ZS laser diffractometer, Malvern Instruments Ltd., Worcestershire, UK) at room temperature. All nanoemulsion samples were diluted 200 times with double distilled water and placed in disposable zeta cell. In general, when ZP values of nanoemulsion are high (positive or negative) repulsive forces dominate on attractive forces that produce high stability. Samples were agitated well and the number of runs maximum 100 and minimum 12 were chosen while analyzing the experiments. All measurements were carried out in triplicate at room temperature.

1.5. *Determination of Rheological Properties*

The Rheological behavior of fresh and stored TFO loaded nanoemulsions were calculated by adopting a method of [20] using the Discovery Hybrid Rheometer (DHR-3, TA-Instruments, New Castle, DE, USA) having plate and cone geometry (cone angle 2, cone diameter 40mm and gap 48μm). For measurements, ~1 ml of nanoemulsion samples were loaded between the plate fixture and cone. All viscosity measurements were calculated at room temperature by steady-state flow diagram and shear rate having range 0s-1 to 500s-1. Experimental flow curves were fitted to a power-law model.

$$\eta = K\gamma n\text{-}1 \quad (1)$$

In the above-mentioned equation, η is the viscosity (Pa. s), where K is the constant (Pa.sn), γ is the shear rate (s-1), n is the flow behavior index. All measurements were taken at room temperature in triplicate.

1.6. *Confocal Laser Scanning Microscopy (CLSM) Analysis*

All fresh and stored nanoemulsion samples were analyzed using the Zeiss LSM 710 confocal microscope (Le-

-ic, Heidelberg, Germany) by the following method of [21] with some modifications. Briefly, Nile red dye, an oil-soluble fluorescent dye was dissolved in methanol with 1mg/ml concentration, and in order to stain the oil phase of nanoemulsions, 5µl from Nile red prepared solution was added into 50µl nanoemulsion samples. Stained nanoemulsion samples were placed on slide and coverslip were applied without air bubbles. Nile red was excited at 543nm and all images were taken by Zeiss LSM 710 at 40 magnifications to illustrate the structural changes before and after storage.

1.7. *Statistical Analysis*

All samples were analyzed in triplicate. Data are presented in mean values ± standard deviations and further examined for a significant difference $p<0.05$ by using IBM SPSS statistics 20.0 software package (IBM, Armonk, NK).

III. Results and Discussions

3.1. *Size and PDI of Fresh and Stored Nanoemulsions*

Nanoemulsions were characterized for their mean droplet diameter (MDD) before and after storage. According to the results presented in Fig. 1a, indicating that with the increasing concentration of the wall material, a gradual rise in MDD of nanoemulsions was observed. [22, 23] also observed the same phenomenon in MDD with the increasing concentration of modified starch. All nanoemulsions size was in the nanometric range [23]. The nanoemulsion containing PGU 2% showed the highest MDD of (259 ± 7.77 nm). Polydispersity index is a significant feature which governs the distribution of particles in a colloidal system. The narrow range of PDI offers more stability to the system. The PDI results presented in Fig.1a, indicating that the nanoemulsions were stable. Furthermore, the nanoemulsion with PGU 1.5% concentration showed lowest PDI and more stability.

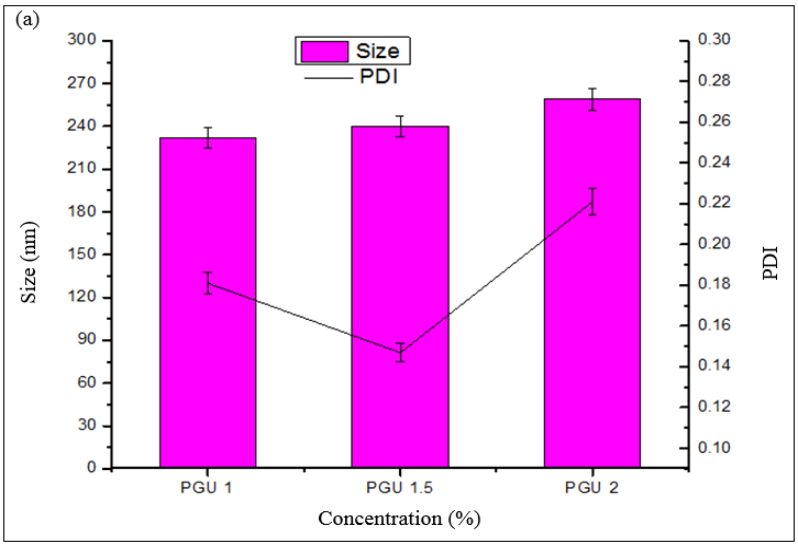


Fig. 1. (a) Effect of Different Concentrations (1, 1.5 and 2%) of PGU on the Size and PDI of Nanoemulsions.

All samples were then stored at room temperature for one-month to check their stability related to time and temperature. All nanoemulsions were then physically visualized, there was no phase separation and creaming observed. The increase in size was 12 to 22 nm for the stored emulsions. The nanoemulsion stabilized with PGU 1.5 % showed a minimum increase in size, whereas nanoemulsion stabilized by 2 % PGU showed the highest increase in size. After one-month storage, MDD and PDI results of all nanoemulsions are presented in Fig. 1b.

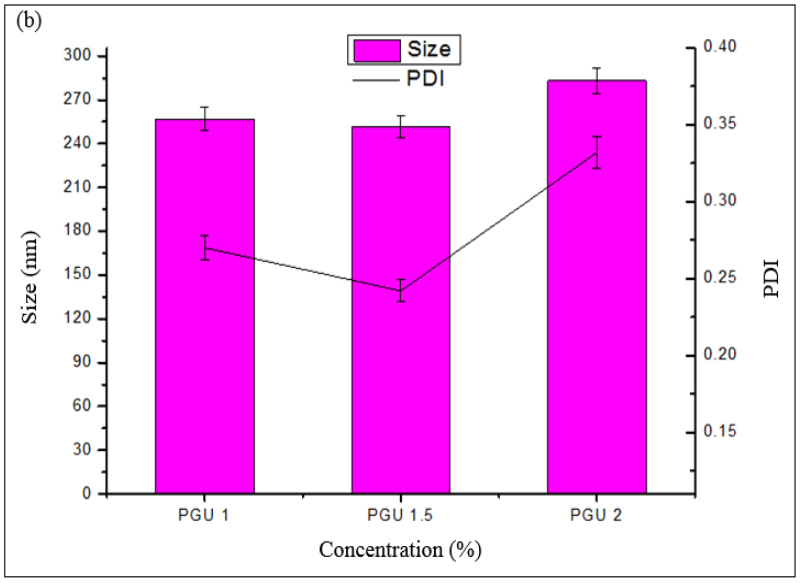


Fig. 1. (b) Effect of Storage Time and Temperature on the Size and PDI of Nanoemulsions Stabilized by Different Concentrations (1, 1.5 and 2%) of PGU.

3.2. *Zeta Potential of Fresh and Stored Nanoemulsions*

Zeta potential has a great influence on the stability of dispersed oil droplets, therefore it is very important to know about the ZP values of the samples. Higher ZP values mean longer the stability of the system. Ranging from very high positive to very high negative values of ZP is considered better for the system because higher ZP shows the better electrostatic repulsion between the dispersed particles. ZP values ranged from (-31.4 ± 0.942 mV) to (-34.2 ± 1.026 mV) for the fresh emulsions as presented in the Fig. 2a. With the increasing concentration of PGU, a decline trending ZP was observed. OSA-MS possesses negative charge due to the presence of a carboxylic group, that's why our values show ZP in the negative [24]. Results of this study are in accordance with previous following reports that revealed higher negative ZP values of nanoemulsions emulsified by OSA-MS [20, 25, 26].

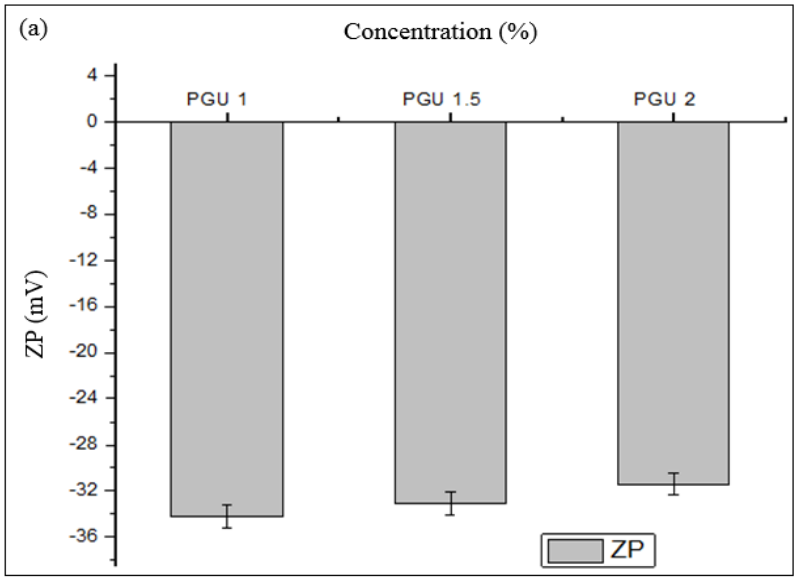


Fig. 2. (a) Effect of Different Concentrations (1, 1.5 and 2%) of PGU on the ZP of Fresh Nanoemulsions.

ZP of all stored nanoemulsions were then analyzed after a one-month storage period. All nanoemulsions showed a decrease in ZP values as compared to fresh samples (Fig. 2b). The formulation emulsified by 1.5% PGU showed

the lowest decrease in ZP which is ~4.4mV drop after one-month storage. The slight drop in ZP values might be due to the formation of propanal (an intermediate oxidation product), which further converted into propanoic acid and caused a reduction in pH [27].

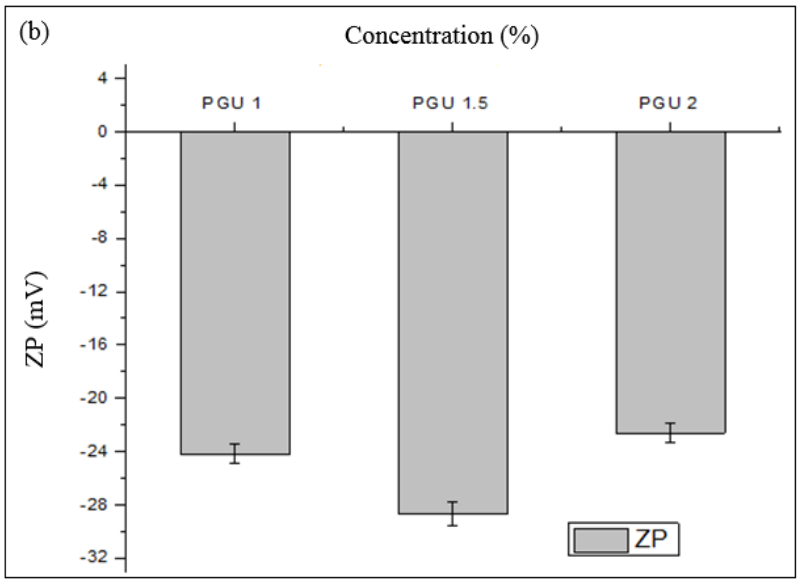


Fig. 2. (b) Effect of Storage Time and Temperature on the ZP of Stored Nanoemulsions Stabilized by Different Concentrations (1, 1.5 and 2%) of PGU.

3.3. *Rheological Properties of Fresh and Stored Nanoemulsions*

Rheological properties of nanoemulsions are very important that links to the stability and final product application. The viscosity of nanoemulsions was assessed under the influence of shear rate. The rheological properties of fresh and stored nanoemulsions (room temperature) emulsified by PGU were analyzed to check the effect of storage time and temperature on their stability. A significant ($p > 0.05$) increase in viscosity was noted for nanoemulsion stabilized by 2% PGU and a direct correlation was observed between MDD and viscosity of nanoemulsions. In short, the nanoemulsion stabilized by 2% PGU showed the highest viscosity as compared to other samples (Fig. 3a).

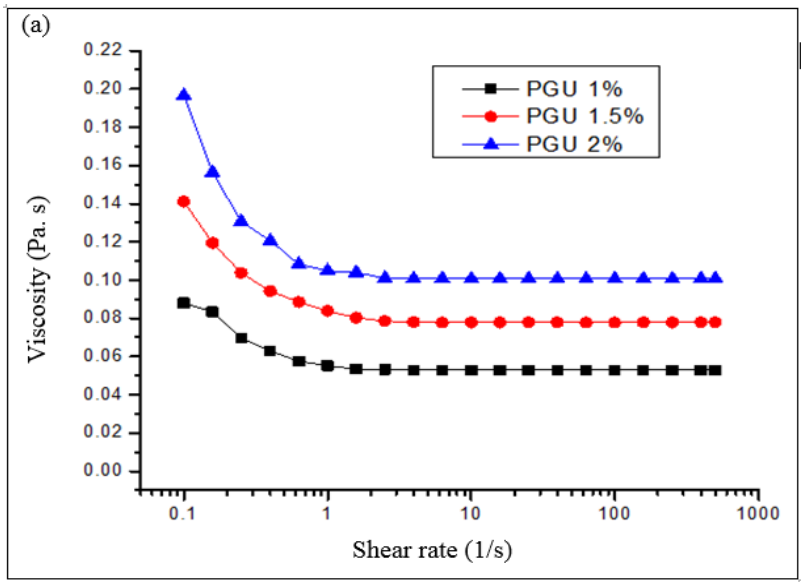


Fig. 3. (a) Rheological Properties of Nanoemulsions Stabilized with Different Concentrations (1%, 1.5%, 2%) of PGU.

Data obtained was tested by using the power-law model and found that all nanoemulsions presented shear thinning behavior under shear rate. There exist three values for n (flow behavior index): n<1 for a shear-thinning fluid, n = 1 for a Newtonian fluid, n>1 for shear thinning fluid. The results of the fresh and stored nanoemulsion are presented in Fig. 3a and Fig. 3b. These results depicted that the nanoemulsions were stable. Current findings are correlated with the previous study conducted on flaxseed oil loaded nanoemulsion stabilized by OSA-MS [28].

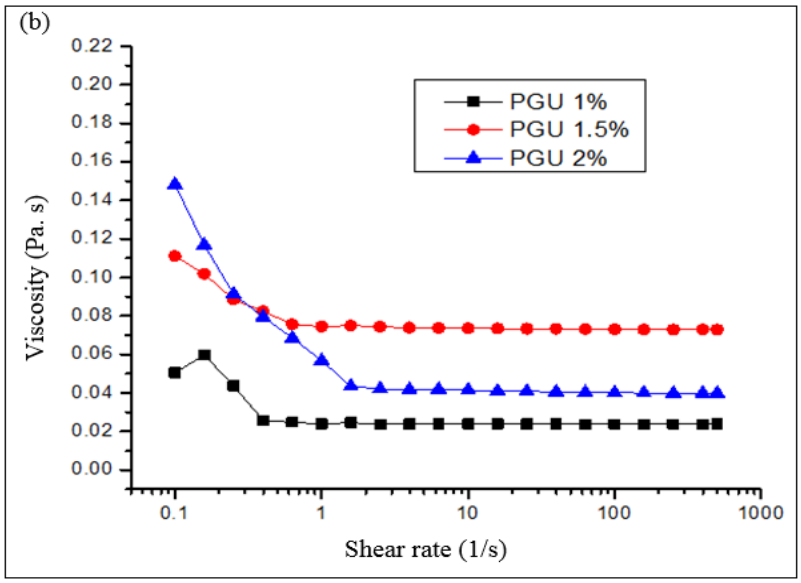


Fig. 3. (b) Effect of Storage Time and Temperature on the Rheological Properties of Nanoemulsions Stabilized with Different Concentrations (1%, 1.5%, 2%) of PGU.

3.4. *Confocal Laser Scanning Microscopy of Fresh and Stored Emulsions*

All the fresh and stored samples were visualized by confocal laser scanning microscopy to check the structural changes (e.g., size and shape) of particles before and after storage. Fresh nanoemulsions showed very fine and evenly distributed oil droplets. All samples presented spherical shapes without any aggregation, which indicated successfully encapsulation of TFO in PGU based nanoemulsions (Fig. 4a). These findings are in accordance with those obtained with dynamic light scattering (Fig. 1a, 1b, and Fig. 2a, 2b).

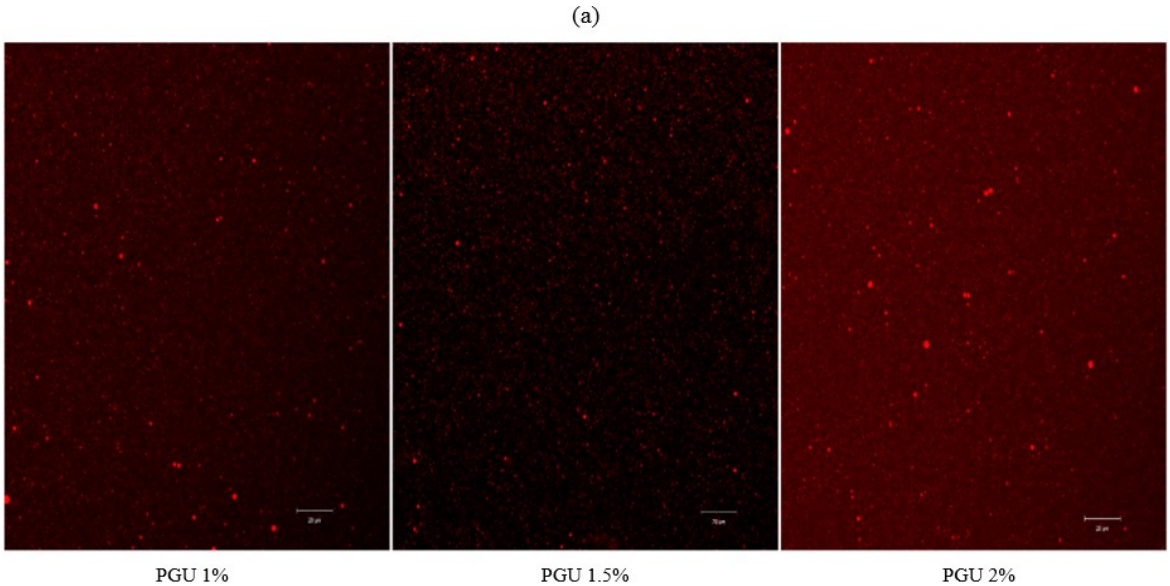
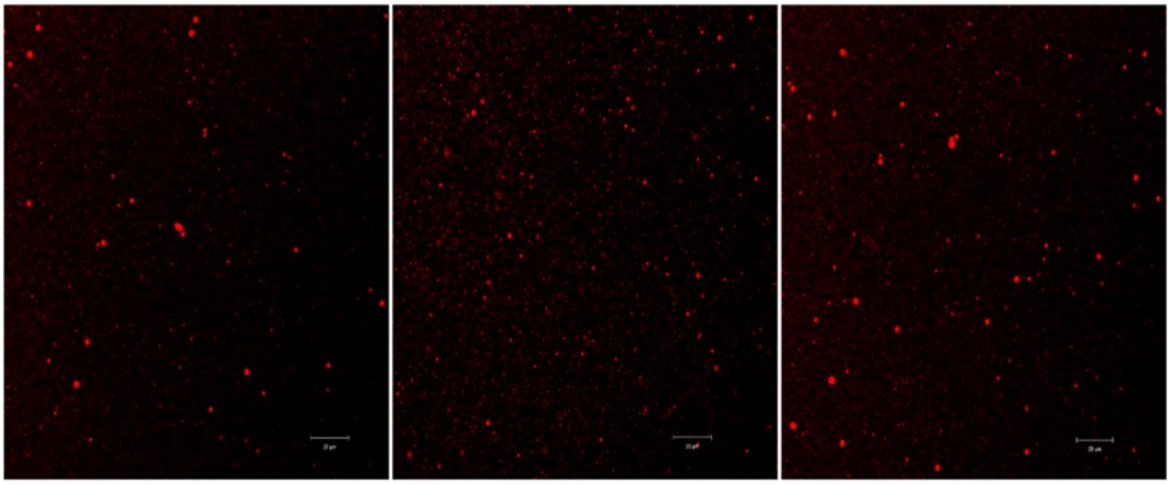


Fig. 4. (a) Confocal Laser Scanning Microscopy Images of the Freshly Prepared PGU (1%, 1.5%, 2%) based Nanoemulsions.

However, due to the small droplet size, it is difficult to observe more details about the oil droplets characteristics. After one-month storage at room temperature, all emulsions showed a slightly larger droplet size. The emulsion stabilized by 1.5% PGU showed the lowest increase in droplet size as shown in Fig. 4b.

(b)



PGU 1% PGU 1.5% PGU 2%

Fig. 4. (b) Confocal Laser Scanning Microscopy Images of Stored PGU (1%, 1.5%, 2%) based Nanoemulsions.

IV. Conclusions

In conclusion, TFO-loaded nanoemulsions stabilized by different concentrations (1, 1.5 and 2%) of PGU were formulated and characterized freshly and after one-month storage. Size distribution results depicted the nano-metric range of all formulations and confocal microscopy images confirmed the TFO encapsulation. All nanoemulsions showed negative ZP, below 1 n (flow index behavior) values and shear thinning behavior. After a one-month storage, all nanoemulsions were found to be stable at room temperature. But, nanoemulsion prepared by 1.5% PGU showed optimum and most favorable results. However, it further can be studied and used in the development of functional foods and beverages.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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