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NANOEMULSIONS OF POMEGRANATE SEED OIL: PREPARATION, CHARACTERIZATION, AND BIOACTIVITY SCREENING

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ABSTRACT

Punicic acid, a major fatty acid of pomegranate seed oil (PSO), exhibits various health-promoting biological activities, including antioxidant, anti-inflammatory, anticancer, and cardioprotective effects. This study aimed to formulate PSO nanoemulsions using two low-energy methods: Emulsion phase inversion (EPI) and EPI combined with sonication (EPI-sonication). Pseudo-ternary phase diagrams were constructed to identify stable zones. The optimal EPI formulation yielded nano-sized droplets (197.57 ± 0.86 nm) with stability up to 7 days. Incorporation of sonication further reduced droplet size (93.00 ± 1.11 nm) and enhanced stability at a lower surfactant-to-oil ratio of 1.0. Antioxidant assays (DPPH and ABTS) revealed superior activity in the EPI-sonication formulation (IC_{50} = 0.88 and 0.47 mgOE/mL, respectively), compared to the EPI method (IC_{50} = 1.01 and 0.62 mgOE/mL, respectively). Antibacterial evaluation indicated selective inhibition against *Escherichia coli*. These findings highlight the potential of PSO nanoemulsions as stable carriers for bioactive delivery in functional food or nutraceutical applications.

Keywords: Nanoemulsion, punicic acid, emulsion phase inversion (EPI), sonication, antioxidant activity, *E. coli* inhibition.

1. INTRODUCTION

The growing popularity of healthy diets has driven increased consumption of functional foods—nutrient-rich products containing bioactive compounds that contribute to disease prevention and overall well-being. Among these, *punica granatum* (pomegranate) seed oil has attracted attention due to its high content of polyunsaturated fatty acids (notably punicic acid), polyphenols, flavonoids, and antioxidants. These constituents exhibit potent biological activities, including anticancer, anti-inflammatory, antibacterial, and antioxidant effects [1]. However, the hydrophobic nature of PSO limits its solubility, bioavailability, and applicability in food and pharmaceutical formulations [2].

Nanoemulsion technology offers a promising strategy to overcome these limitations. By reducing droplet size to the nanoscale (20–200 nm), nanoemulsions enhance the solubility, absorption, and stability of lipophilic compounds such as PSO [3]. For instance, the antioxidant capacity of PSO has been reported to improve significantly post-nanoemulsification, with DPPH scavenging activity increasing from approximately 43% to over 70% [4]. The improved performance is attributed to the increased surface area of nano-sized droplets and more efficient interaction with free radicals. Nanoemulsions can be prepared using high-energy methods—such as high-pressure homogenization, high-shear mixing, or ultrasonication—which require specialized equipment and energy input [5]. Alternatively, low-energy methods like emulsion phase inversion (EPI) rely on compositional changes or variations in water/oil ratios to induce phase transitions and form stable emulsions. Although low-energy methods are simple, cost-effective, and suitable at the laboratory scale, they are less commonly applied in industrial contexts [6].

Previous studies have successfully utilized the EPI to formulate nanoemulsions for hydrophobic bioactives such as vitamin E and omega-3 fatty acids [6], [7]. When combined with sonication, the EPI can produce emulsions with smaller droplet sizes and improved uniformity while reducing energy

requirements [6]. However, limited research has compared nanoemulsions produced solely by the EPI with those formed by EPI combined with sonication, especially in the context of PSO. This study aims to study the formulation of PSO nanoemulsions using both the EPI and the EPI-sonication methods. The effects of oil concentration, surfactant-to-oil ratio, and processing conditions on nanoemulsion characteristics, such as particle size, viscosity, and stability, were evaluated. Additionally, the biological properties of the resulting nanoemulsions, including antioxidant and antibacterial activity, were assessed. The findings contribute to a deeper understanding of PSO nanoemulsion systems and their potential applications in functional food and pharmaceutical products.

2. MATERIALS AND METHODS

2.1 Materials and chemicals

Pomegranate seed oil was purchased from Shams Natural Oils (Moscow, Russia). The peroxide value, acidic value, and iodine value of studied oils were determined, as 5.5±0.47 (meq O2/kg), 0.75±0.10, and 90.74±1.34, respectively. Tween 80, Span 80, lecithin, oleic acid, 1-1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), ampicillin and cefotaxime were acquired from Fisher Scientific (USA).

Bacillus subtilis (ATCC 6633), *Staphylococcus aureus* (ATCC 13709), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 15442) were used.

2.2. Pomegranate seed oil nanoemulsion fabrication

2.2.1. Emulsion phase inversion method

The emulsion phase inversion (EPI) method was performed following the procedure described by Ostertag et al [6] with minor modifications. Initially, a water-in-oil (W/O) emulsion was formed by mixing the surfactant and oil using a magnetic stirrer for 30 mins. Subsequently, the aqueous phase was added to the W/O mixture at a controlled flow rate of 2 mL/min while stirring was maintained for 30 minutes.

The influence of various surfactants on the formation and characteristics of PSO nanoemulsions was systematically investigated. Four emulsifiers (Tween 80, Span 80, soy lecithin, and oleic acid—were employed in the formulation. The oil concentration was maintained at a constant 3%, while the surfactant concentration was varied between 1% and 8%. Additionally, emulsions with varying surfactant-to-oil ratios (SOR) of 0.5, 1.0, 1.5, 2.0, and 3.0, and oil contents of 0.5%, 1.5%, 3.0%, 4.5%, and 6.0% were prepared and analyzed.

2.2.2. Emulsion phase inversion with sonication method

A pre-nanoemulsion system was first prepared using the EPI method as mentioned in 2.2.1. The resulting coarse emulsion was then subjected to a sonic probe to produce the final nanoemulsion. To investigate the stability of pomegranate seed oil nanoemulsions, emulsions were prepared with varying SOR (0.5, 1, 1.5, 2, 3), oil contents (0.5, 1.5, 3, 4.5, 6, 9%), sonication amplitudes (20, 30, 40, 50, 60%), and sonic durations (4, 6, 8, 10, 12 min).

All resulting emulsions were evaluated for viscosity, stability, and particle size.

2.3. Pseudo-ternary phase diagram construction

The pseudo-ternary phase diagram is a valuable tool for understanding the phase behavior of formulations comprising oil, water, and emulsifiers. In this study, the diagrams were constructed for two methods. Pomegranate seed oil and Tween 80 served as the oil phase and surfactant, respectively. Six SORs were evaluated (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0). For each formulation, pomegranate seed oil was mixed with Tween80 according to the designated SOR and stirred for 15 min using a magnetic stirrer (5000 rpm, 25 ^{o}C). Distilled water was then added dropwise at a rate of 2 mL/min until reaching a final volume of 100 mL, followed by an additional 15 min of stirring. In the EPI-sonication method, the coarse emulsion obtained from the EPI process was further sonicated at 50% amplitude for 10 min.

2.4. Analytical method

2.4.1. Particle size measurement

Particle size, zeta potential, and polydispersity index (PDI) were determined using a nanoparticle analyzer (SZ–100 Nanoparticle Series, Horiba Scientific) based on the dynamic light scattering (DLS) method. Pomegranate seed oil nanoemulsions were prepared using the selected formulation in three independent batches. The optimal formulation was identified based on the resulting droplet size, zeta potential and PDI.

2.4.2. Viscosity measurement

The viscosity of the nanoemulsion system was measured using a Brookfield viscometer (Brookfield, USA), following the method described by Pal [8]. The sample was placed in a test tube, and an appropriate spindle was selected. Parameters were adjusted according to the chosen spindle and rotation speed, and the viscosity values were recorded accordingly.

2.5. Stability determination

2.5.1. Storage stability method

This method was conducted as described in research by Arash [9], with some modifications. The stability of the nanoemulsion system was determined by the layer separation method over time (visual test). The observation is based on the mechanism by which the process of nanoemulsion droplets moving downward (sedimentation) or upward (creaming) due to their density being higher or lower than the surrounding liquid is called delamination. The nanoemulsion system, formed by EPI and EPI-sonication, will be placed in a 50 mL measuring cylinder and left to stand at room temperature. The samples will be continuously observed over time to determine the moment of phase separation. Whether the sample is stable or not will be determined by the phase separation time (1h, 12h, 1 day, 3 days, 5 days, 7 days) at 25 ^{o}C.

2.5.2. Centrifugation method

The physical stability of nanoemulsion was evaluated using the centrifugal acceleration method by Joung et al, with some modifications [10]. The nanoemulsions were diluted 100-fold in distilled water and then centrifuged at 5000 rpm for 20 min. An aliquot of the supernatant was withdrawn from the bottom of the tube. Then, the absorbance of the sample was determined at a wavelength of 500 nm. The following formula calculated the centrifugal stability of the nanoemulsions (Ke):

$$Ke = \frac{A - A_0}{A} \times 100$$

A and A_0 were the absorbance values of the diluted nanoemulsion before and after centrifugation, respectively.

2.6. Total polyphenol content determination

A total of 5 g of each sample (PSO and PSO nanoemulsion) was subjected to sequential solvent extraction. Initially, the samples were extracted with 5 mL of methanol (MeOH) and 5 mL of hexane, followed by vigorous mixing for 5 min and incubation for 10 min. The resulting supernatant was collected, and the residual material was subsequently extracted twice more using 10 mL of methanol each time. The combined extracts from all three steps were pooled and adjusted to a final volume of 100 mL, then incubated in the dark prior to further analysis.

The total phenolic content (TPC) of the extracts was quantified using the Folin–Ciocalteu colorimetric method, as previously described by Chatha and Medini [11], [12]. Briefly, 0.5 mL of the extract was mixed with 7.5 mL of distilled water, followed by the addition of 0.5 mL of Folin–Ciocalteu reagent. After 6 min, 1.5 mL of 20% (w/v) aqueous sodium carbonate (Na_2CO_3) solution was added. The final volume was adjusted to 25 mL with distilled water, and the mixture was vigorously agitated. The reaction mixture was then incubated for 90 min at room temperature in the dark. Absorbance was measured at 760 nm using a UV–Vis spectrophotometer. Gallic acid was used as the calibration standard, and the TPC was expressed as milligrams of gallic acid equivalents per gram (mg GAE/g) of oil extract.

2.7. Antioxidant properties

2.7.1. DPPH scavenging activity

The antioxidant activity of pomegranate seed oil and its nanoemulsion was evaluated using the DPPH radical scavenging assay, as described by Xu et al [10]. Samples at various concentrations were mixed with 5 mL of methanolic DPPH solution. After 30 min of incubation at room temperature, the mixtures were centrifuged at 5000 rpm for 20 min to minimize turbidity. The absorbance was then measured at 517 nm. Vitamin C served as a positive control. The control solution was prepared by mixing 1 mL of methanol with 5 mL of DPPH solution. The DPPH radical scavenging activity (%) was calculated using the following equation:

$$DPPH\ Scavenging\ activity\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

The IC_{50} value (50% inhibitory concentration) was evaluated using linear regression analysis by obtaining the Inhibitor percentage at various concentrations.

2.7.2. ABTS scavenging activity

Antioxidant activity was assessed using the ABTS assay, following the method described by Re [13], with slight modifications. ABTS (7 mmol/L) was mixed with potassium persulfate (2.45 mmol/L) in a 1:1 ratio and incubated in the dark at room temperature for 12–16h to generate the $ABTS^+$ radical. Before use, the solution was diluted with methanol to an absorbance of 0.70 ± 0.02 at 734 nm. For the test, 0.5 mL of nanoemulsion at various concentrations was added to 4.5 mL of ABTS solution. The mixture was kept in the dark for 30 min, and absorbance was measured at 734 nm using a spectrophotometer. Vitamin C served as a positive control, while the blank was prepared by mixing 0.5 mL of methanol with 4.5 mL of ABTS solution. The ABTS radical scavenging activity (%) was calculated using the following equation:

$$ABTS\ Scavenging\ activity\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

The IC_{50} value (50% inhibitory concentration) was evaluated using linear regression analysis by obtaining the Inhibitor percentage at various concentrations.

2.8. Antibacterial properties

This method was carried out with slight modifications based on the procedure described by Hadacek et al. [14]. In this study, four microorganisms (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*) were used to evaluate the antibacterial activity of pomegranate seed oil nanoemulsion using the liquid microdilution method to determine IC_{50} and inhibition percentage (IC%) values. The test samples were prepared in sterile culture media across five concentrations, each serially diluted four-fold: 50.00, 12.50, 3.13, and 0.78 mg/mL. Ampicillin was used as the positive control for Gram-positive bacteria, while Cefotaxime served as the reference antibiotic for Gram-negative bacteria.

2.9. Statistical analysis

All experiments were conducted in triplicate. Data were analyzed using Analysis of Variance (ANOVA) with Minitab Statistical Software version 22.3.0. Charts were generated using Microsoft Excel, and pseudo-ternary phase diagrams were constructed using the Ternary Plot online tool (https://www.ternaryplot.com/).

3. RESULTS AND DISCUSSION

3.1. Preparation of pomegranate seed oil nanoemulsion

3.1.1. Selection of emulsifiers for the pomegranate seed oil nanoemulsion

Nanoemulsions are thermodynamically unstable and prone to phenomena such as creaming, flocculation, aggregation, and Ostwald ripening [15]. To enhance stability, emulsifiers with suitable hydrophilic-lipophilic balance (HLB) values are essential [16]. In this study, four emulsifiers—Tween 80 (HLB = 15), Span 80 (HLB = 4.3), soy lecithin (HLB = 3–5), and oleic acid (HLB = 1)—were evaluated for their ability to form stable nanoemulsions of pomegranate seed oil using the phase inversion (EPI) method. The results of emulsion formation and stability are summarized in Table 1.

Table 1. Formation and stability of PSO nanoemulsions using different emulsifiers

Surfactant	Emulsion characteristics
Lecithin	The emulsion exhibited an ivory-yellow appearance and remained stable for up to 12 h. Complete phase separation occurred thereafter across all concentrations (1–5%), with visible clumping observed at 8% concentration.
Span 80	At all tested concentrations (1–8%), the formulation resulted in the formation of suspended gel-like particles, with no stable emulsion formation observed.
Tween 80	A milky homogeneous emulsion was formed and remained stable across all concentrations (1–8%) with no signs of phase separation or clumping.
Oleic acid	Immediate phase separation occurred following emulsification, indicating poor emulsifying capability under the tested conditions.

As shown in Table 1, oleic acid was unable to form a stable emulsion, exhibiting phase separation within 30 min. This behavior is attributed to its predominantly non-polar nature, which renders it more compatible with the oil phase than effective as a primary emulsifier [17]. Span 80, being lipophilic, produced unstable, gel-like aggregates suspended in water, especially at higher concentrations, consistent with its tendency to form water-in-oil emulsions [18]. Soy lecithin showed partial emulsifying capability; however, increasing concentrations (1–8%) led to rising viscosity (from 5.82 ± 0.16 cP to 45.65 ± 2.41 cP) and phase separation within 12–24h. This instability is attributed to vesicle aggregation at high lecithin levels [19].

Tween 80 demonstrated the most promising performance, forming uniform and stable emulsions for up to 3 days with negligible separation, and delaying complete separation until after 7 days. Its amphiphilic structure effectively reduces interfacial tension and facilitates the formation of small, stable oil droplets (<200 nm) [20]. Prior studies by Felix et al. and Zhang et al. further support using Tween 80 in nanoemulsions for food applications [6] [7]. Therefore, Tween 80 was selected as the optimal emulsifier for developing pomegranate seed oil nanoemulsions under the present experimental conditions.

3.1.2. Stability of nanoemulsion by emulsion phase inversion method

The stability of pomegranate seed oil nanoemulsions prepared by the EPI method was evaluated based on oil concentration (0.5–6%) and surfactant-to-oil ratio (SOR). Increasing PSO concentration elevated the viscosity (from 5.26 ± 0.09 to 7.75 ± 0.10 cP) and particle size remained <200 nm across all samples (Figure 1). Although suitable for beverage applications, higher oil content led to reduced stability. At an oil concentration of 6%, phase separation was observed after 3 days of storage. In contrast, emulsions containing 1.5% to 3% pomegranate seed oil (PSO) exhibited greater stability, with no visible separation for up to 7 days. Higher oil concentrations may accelerate Ostwald ripening [21] and lead to increased viscosity due to fat globule agglomeration, ultimately reducing emulsion stability. This destabilization is likely a result of the inability of emulsifiers to stabilize the larger number of dispersed oil droplets adequately [22]. The last separation was observed in the 0.5% sample, likely due to its low oil content and minimal droplet aggregation.

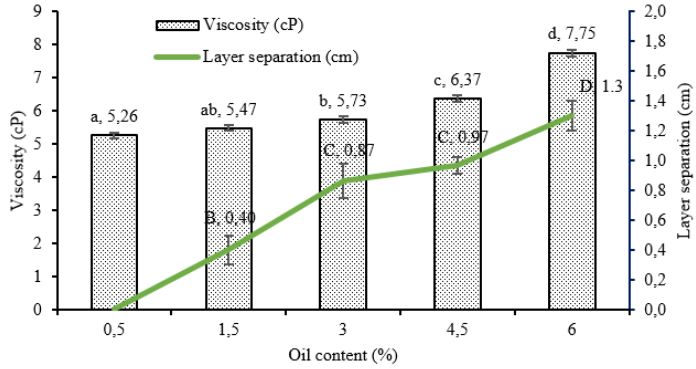

Figure 1. Viscosity and phase separation of pomegranate seed oil nanoemulsions formulated with varying oil contents
Viscosity measurement was recorded after 30 min, while phase separation was assessed after 7 days of storage. Different letters (a, b, c, d) and (A, B, C, D) indicate statistically significant differences between samples for the same response ($p < 0.05$).

As illustrated in Figure 2, the surfactant-to-oil ratio (SOR) had a statistically significant effect on the behavior of the nanoemulsions ($p < 0.05$). Viscosity increased with SOR (from 4.16 ± 0.09 to 6.01 ± 0.13 cP), and emulsions remained within the nano-size range at all tested ratios. However, both low (0.5) and high (3.0) SORs produced fewer stable emulsions, with visible phase separation occurring between 12- and 48-h post-emulsification. This instability is likely due to either insufficient emulsifier coverage at low SORs or excessive emulsifier at high SORs, which may lead to Ostwald ripening via the formation of free micelles. In summary, moderate oil concentrations (1.5–3%) and SORs (1.5–2) provided optimal stability and viscosity for nanoemulsions intended for beverage applications.

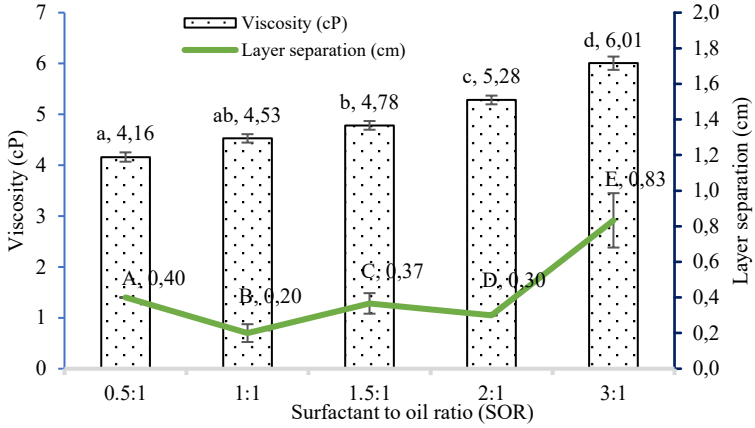

Figure 2. The viscosity and the layer separation of PSO nanoemulsion with different SOR.
Viscosity measurement was recorded after 30 min, while phase separation was assessed after 7 days of storage. Different letters A, B, C… indicate statistically significant differences between samples for the same response ($p < 0.05$).

3.1.3. Stability of nanoemulsion by emulsion phase inversion with the sonication method

The combination of EPI and sonication enhances nanoemulsion formation by improving droplet dispersion, reducing viscosity, and increasing stability while saving energy and time [6]. This study investigated the effects of oil content, SOR, ultrasonic power, and sonication time on the stability of pomegranate seed oil nanoemulsions.

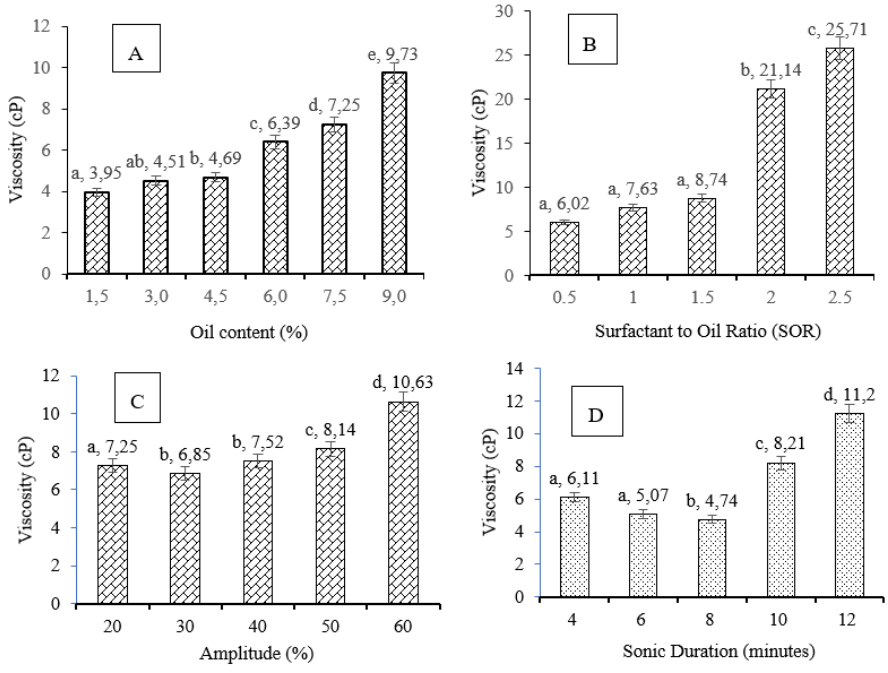

Figure 3. The viscosity of the pomegranate seed oil nanoemulsion with different oil content (A), SOR (B), amplitude (C) and sonic duration (D)

As shown in Fig 3A, increasing oil content (from 1.5% to 9%) raised the emulsion's viscosity and reduced its stability after 7 days, as shown by centrifugation. However, stable nanoemulsions were still formed at high oil levels, suggesting feasibility for applications requiring higher oil content. At a fixed oil concentration of 3%, increasing SOR from 0.5 to 1.5 raised viscosity from 6.02 ± 0.09 cP to 8.74 ± 0.09 cP. formed nanoemulsions, while SOR 2 and 2.5 were out of the ideal viscosity range and SOR 3 resulted in gelation and water separation within 24h, indicating poor stability (Fig 3B).

From Figure 3D, viscosity decreased with increasing sonication time from 4 to 8 min but increased at 12 minutes due to possible emulsifier degradation and gel formation. An 8-minute duration provided the lowest viscosity, while durations of 12 min exceeded the ideal viscosity range (>10 cP), compromising emulsion stability. The nanoemulsion system is consistent with research that when the ultrasound time is long, the process of decomposition of the emulsifier will occur, increasing the viscosity [23]. Results from Figure 3C show that when increasing ultrasonic power, the viscosity of the nanoemulsion system will increase. Based on ANOVA results, it shows differences at ultrasound levels of 20%, 30%, 40% and 50% compared to 60%. The increase in viscosity at ultrasonic power from 20% to 60% is explained by the increase in ultrasonic power by reducing the particle size and increasing the viscosity of the emulsion system [24].

The stability of nanoemulsion was assessed by centrifugation using the Ke value, which quantifies the turbidity difference before and after centrifugation. A lower Ke indicates higher stability [25], [26]. As shown in Figure 4A, Ke values increased over 7 days, indicating reduced stability. The 4.5% oil concentration exhibited the highest stability (Ke: 11.54 ± 0.14% on day 1 to 24.74 ± 12.88% on day 7), while 9% showed the lowest. ANOVA results confirmed that 4.5% oil yielded the most stable system with no phase separation after 7 days. Figure 4B shows that the increasing SOR raised Ke values. SOR 0.5 showed the least stability due to the lack of an emulsifier, which led to layer separation. On the other hand, SOR 1 had the lowest Ke (3.34 ± 1.75% to 13.98 ± 4.41%) due to electrostatic repulsion between droplets [27]. Higher SORs (e.g., 2 and 2.5) increased viscosity and Ke, indicating reduced stability, consistent with increased Zeta potential. Figure 4C indicates that higher ultrasonic power (20–60%) increased viscosity due to smaller droplet sizes.

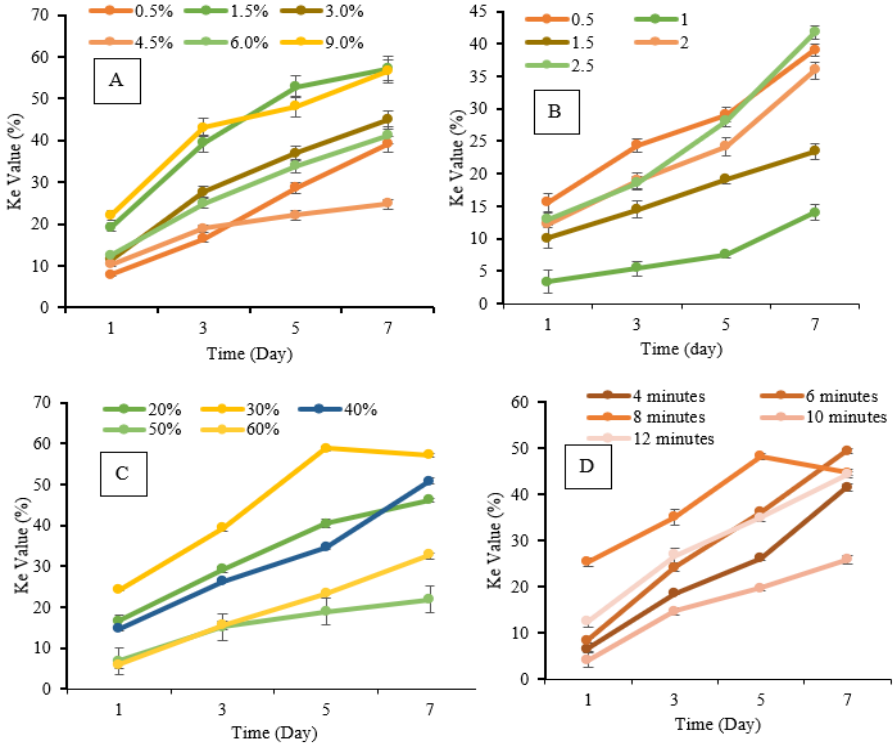

Figure 4. The Ke Value of the pomegranate seed oil nanoemulsion with different oil content (A), SOR (B), amplitude (C) and sonic duration (D).

The ANOVA results indicated that 50% sonication amplitude provided the highest stability on day 7, whereas 30% amplitude resulted in the lowest stability. At the initial stage of sonication, lower amplitudes primarily facilitate dispersion of oil droplets, but are insufficient to induce effective cavitation. In contrast, higher amplitudes enhance cavitation intensity, leading to more efficient disruption and size reduction of oil droplets within the same sonication duration, thereby improving emulsion stability [28], [29]. An amplitude range of 20–50% was found to maintain optimal viscosity and emulsion stability. From Figure 4D, centrifugation tests demonstrated that 10 min of sonication produced the most stable emulsions after 7 days of storage. However, extending the sonication time to 12 min led to an increase in the instability index (Ke value), likely due to degradation of the emulsifier, which compromised the system's stability. In contrast, emulsions subjected to 8 min of sonication exhibited inconsistent stability outcomes. These findings highlight the importance of optimizing sonication duration to achieve a balance between effective droplet size reduction and long-term stability. Overall, the stability of nanoemulsions prepared via the EPI -ultrasonic is predominantly influenced by key formulation and processing parameters, including oil concentration, surfactant-to-oil ratio (SOR), ultrasonic amplitude, and sonication time.

3.1.4. Pseudo-ternary phase diagram construction

Pseudo-ternary phase diagrams were constructed to evaluate the phase behavior of pomegranate seed oil nanoemulsions prepared via both EPI and EPI-sonication methods. The diagrams were based on varying oil concentrations and surfactant-to-oil ratios (SOR), with Tween 80 as the emulsifier. The diagrams classify 20 formulations (Figure 5A) and 17 formulations (Figure 5B) into three distinct phases: gel phase, oil-in-water (O/W) emulsion (>200 nm), and O/W nanoemulsion (≤200 nm, viscosity 1–10 cP). As shown in Fig 5A, gel phase formed when water content was <75% (v/v), especially at high oil concentrations (≥9%) and SORs of 2.0, 2.5, and 3.0. O/W emulsions were formed at SOR 0.5–1.5 and oil content 6–10%, but viscosity exceeded 10 cP. Stable nanoemulsions formed when water content >85%, oil content 0.5–6%, and Tween 80 content <10%, with optimal SORs of 1.0–1.5. Similar results were obtained using EPI with sonication method, shown in Fig. 5B. Gel phase formed at water content <75%, especially with SORs ≥2.0 and oil content >9%. Nanoemulsions formed at oil content 1.5–10%, water content >80%, Tween 80 <15%, and SORs of 1.0–1.5, exhibiting stable systems (no separation) with ideal viscosity (1–10 cP). In the study of Syed and Peh, the gel formation area is between 25% and 60% of the water content [30]. In our study, the gel system will be formed when the water content is below 75% (v/v). Along with that, when SOR 2, 2.5, and 3 with an oil content of over 9% formed a gel system. When the water content continues to be added to the gel phase, the system will form an oil-in-water emulsion system (milky solution) at an oil concentration of 15%.

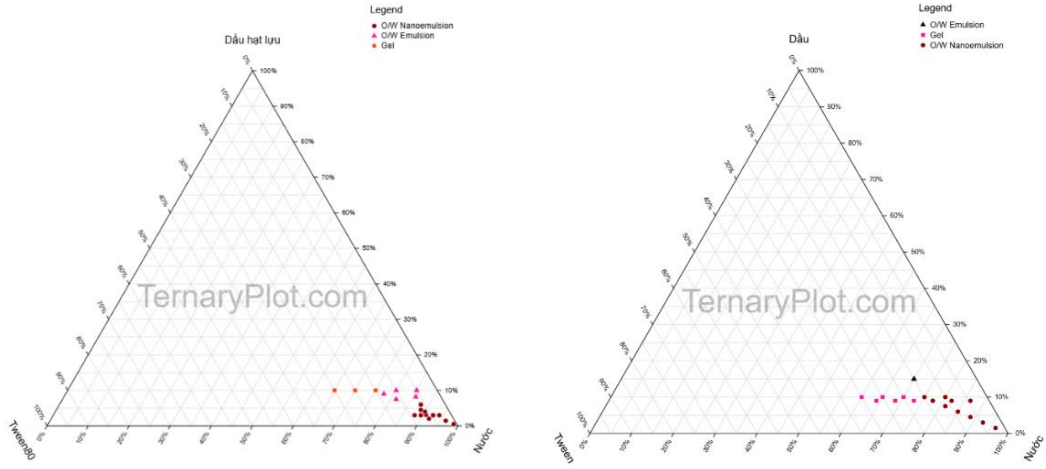

Figure 5. Pseudo-ternary phase diagram of three components by EPI method (A) and EPI with sonication method (B).

The typical drop size of microemulsion and nanoemulsion, fabricated by EPI and EPI with sonication method was represented in Figure 6.

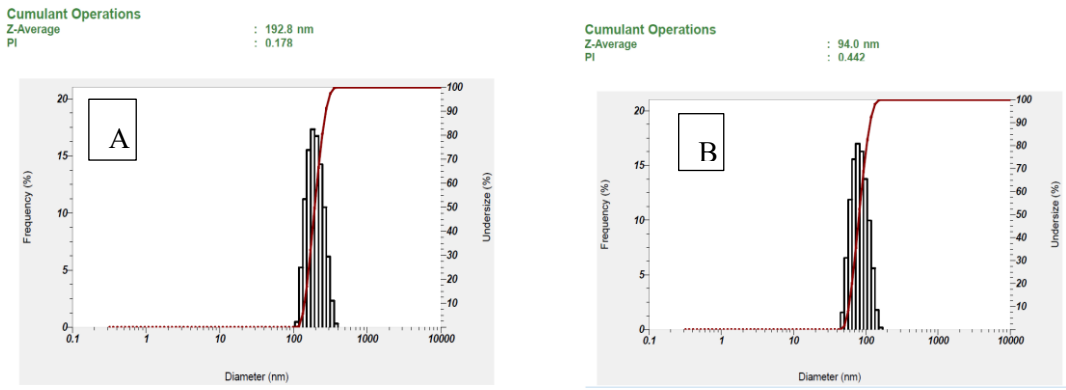

Figure 6. Droplet size of emulsion system formed by EPI and EPI with sonication method
A- nanoemulsion via EPI method; B - nanoemulsion via EPI with sonication method

In addition, the stability of the obtained nanoemulsions was evaluated using zeta potential (ZP) measurements, a key parameter for assessing and predicting colloidal stability, as highlighted by Behl et al. and Honary and Zahir [31]. Zeta potential was measured at 3h and after 7 days of storage for nanoemulsions prepared using both the phase inversion (EPI) method and the EPI method combined with ultrasonication. At the initial time point (3h), the nanoemulsion prepared via EPI with sonication exhibited a mean ZP of –45.6 ± 0.8 mV, while the EPI-only sample showed a ZP of –41.1 ± 1.5 mV. These values suggest strong electrostatic repulsion between droplets, indicative of excellent colloidal stability and reduced likelihood of coalescence or phase separation, thereby maintaining consistent droplet size over time [32]. After 7 days of storage, the ZP decreased significantly to –22.3 ± 0.5 mV for the EPI with sonication method and –8.4 ± 1.1 mV for the EPI-only method. These findings align with the physical observations of stability: the sonicated nanoemulsion remained homogeneous with no visible phase separation, whereas the non-sonicated sample exhibited marked phase separation. These results underscore the effectiveness of ultrasonication in enhancing the long-term stability of nanoemulsions.

Table 2 summarizes the physicochemical characteristics of typical nanoemulsion (NE) systems prepared using the EPI method and the EPI with sonication method.

Table 2. Comparison of characteristics of typical nanoemulsion systems

Parameters	NE via EPI method [a]	NE via EPI with sonication method [b]
Droplet size (nm)	197.57 ± 0.86^{A}	93.00 ± 1.11^{B}
Polydispersity index (PDI)	0.15 ± 0.03^{B}	0.43 ± 0.01^{A}
Zeta potential (mV)	-41.1 ± 1.49^{A}	-45.6 ± 0.83^{A}
Viscosity (cP)	5.73 ± 0.27^{B}	9.00 ± 0.12^{A}
pH	4.7 ± 0.1^{A}	4.5 ± 0.2^{A}

[a]Emulsion phase inversion method (3% pomegranate seed oil, SOR 1.5:1, flow rate 2 mL/min); [b]Emulsion phase inversion combined with sonication (3% pomegranate seed oil, SOR 1.5:1, flow rate 2 mL/min, amplitude 50%, sonic duration 10 minutes) Different letters A, B in a row indicate statistically significant differences between samples for the same response ($p < 0.05$).

As shown in Table 2, both methods successfully produced nanoemulsions with droplet sizes below 200 nm. The droplet size of the NE prepared via the EPI method (197.57 ± 0.86 nm) (Figure 6A), is consistent with results reported by Ahmad et al. [33], who achieved a similar size (199.44 ± 2.1 nm) using a comparable method and a higher surfactant concentration. When combined with Sonication (Figure 6B), the droplet size of nanoemulsion has significantly decreased to 93.00 ± 1.11 nm, which leads to the confirmation that combining sonication will help reduce particle size better. Applying the sonication helps to reduce the droplet size more effectively than using high energy method to form a nanoemulsion compared with the results presented by Dimitra et al [34]. The polydispersity index (PDI), a measure of particle size distribution, is a critical parameter reflecting formulation, uniformity and stability. Lower PDI values, such as 0.15 observed in the EPI sample, indicate a narrow size distribution

and better physical stability. In contrast, the higher PDI (0.43) in the sonicated sample may reflect broader size distribution due to high-energy input during sonication, although it still remains within acceptable limits for nanoemulsions. Zeta potential values for both samples exceeded –30 mV, suggesting strong electrostatic repulsion and high colloidal stability. The sample prepared with sonication had a slightly higher absolute ZP (–45.6 mV), reinforcing its enhanced stability. Viscosity was also affected by the preparation method, with the sonicated nanoemulsion exhibiting higher viscosity (9.00 cP) compared to the non-sonicated counterpart (5.73 cP), potentially due to increased droplet interaction at reduced particle sizes. Overall, the combination of EPI and ultrasonication effectively reduced droplet size while maintaining system stability, which may help better preserve the bioactive compounds present in pomegranate seed oil. Moreover, the incorporation of sonication significantly enhanced the emulsification process by introducing high shear forces that reduce droplet size and improve uniformity. This combination yielded stable nanoemulsions with smaller droplet sizes and reduced surfactant concentrations, making the system more suitable for food applications.

3.2. Bioactivity screening

Pomegranate seed oil is known for its valuable biological activities, particularly its antioxidant and antibacterial properties [24]. However, its practical application is limited due to poor water solubility, low dispersibility, and limited bioavailability. To address these challenges, nanoemulsion technology has emerged as an effective strategy to enhance the solubility and bioavailability of the oil's active components. In this study, the antioxidant and antibacterial activities of pomegranate seed oil nanoemulsions were evaluated against both Gram-positive and Gram-negative bacterial strains. The biological activities of nanoemulsions produced by two different methods were assessed and compared with those of the original pomegranate seed oil.

3.2.1. Antioxidant capacity

The antioxidant properties of pomegranate seed oil and the nanoemulsions prepared using the EPI method, with and without sonication, were evaluated by measuring their free radical scavenging activity using DPPH and ABTS assays, as well as their total polyphenol content (TPC). The results are summarized in Table 3.

The antioxidant activity of pomegranate seed oil (PSO) and its nanoemulsion formulations was assessed using DPPH and ABTS radical scavenging assays. The IC_{50} value for PSO was found to be 52.48 mg/mL for the DPPH assay and 118.43 mg/mL for the ABTS assay. Notably, nanoemulsions prepared using both the emulsion phase inversion (EPI) method and the EPI method combined with ultrasonication exhibited significantly lower IC_{50} values, indicating enhanced antioxidant activity compared to the raw oil. These results agree with the findings of Ahmad et al. [33], who also reported improved antioxidant performance in nanoemulsified systems.

Table 3. Antioxidant capacity of pomegranate seed oil (PSO) and nanoemulsion formed by EPI and EPI with sonication method

Parameters	*PSO*	*NE via EPI method* [b]	*NE via EPI with sonication method* [c]
Total polyphenol content (mg gallic acid/g)	1.36 ± 0.03^{C}	10.29 ± 0.38^{B}	12.40 ± 0.19^{A}
IC50 for the DPPH method (mgOE/mL)[a]	52.48 ± 1.4^{A}	1.01 ± 0.13^{B}	0.88 ± 0.04^{C}
IC50 for the ABTS method (mgOE/mL)	118.43 ± 2.1^{A}	0.62 ± 0.01^{B}	0.47 ± 0.03^{C}

[a] *mgOE/mL* - mg Oil Equivalent/ mL*;* [b]*Emulsion phase inversion method (3% pomegranate seed oil, SOR 1.5:1, flow rate 2 mL/min);* [c]*Emulsion phase inversion combined with sonication (3% pomegranate seed oil, SOR 1.5:1, flow rate 2 mL/min, amplitude 50%, sonic duration 10 min).*

Different letters A, B, C in a row indicate statistically significant differences between samples for the same response ($p < 0.05$).

Specifically, from Table 3, nanoemulsions prepared via the EPI with sonication method showed greater antioxidant activity than those prepared by the EPI method. For the DPPH assay, the IC_{50} decreased from 1.01 mg EO/mL (EPI) to 0.88 mg EO/mL (EPI with sonication), and for the ABTS assay, from 0.62 mg EO/mL to 0.47 mg EO/mL. This enhancement can be attributed to the more efficient dispersion of oil droplets in the aqueous phase facilitated by the cavitation effect during

sonication, as described by Wirawati et al. Sonication induces localized high pressure and temperature, reducing droplet size and yielding a more homogeneous emulsion. This not only improves emulsion stability but also increases the mass transfer of bioactive compounds, thereby enhancing free radical scavenging capabilities [35].

As illustrated in Table 2, the total phenolic content (TPC) of nanoemulsions formed via both EPI and EPI with sonication was significantly higher than that of PSO. This suggests that the nanoemulsion system enhances the functionality of PSO by improving the dispersion and availability of its bioactive constituents. Literature reports [1][2] confirm that pomegranate seed oil is rich in bioactive compounds, particularly polyunsaturated fatty acids (notably punicic acid) and polyphenols, which contribute to its potent antioxidant, anticancer, anti-inflammatory, and antibacterial properties. In the present study, both antioxidant assays confirmed that nanoemulsification significantly improved the antioxidant potential of PSO. The nanoemulsion prepared via EPI combined with sonication showed an approximately 1.8-fold increase in DPPH activity and a 7.5-fold increase in ABTS activity compared to raw PSO. These findings are consistent with the study by Ahmad et al., which reported a 1.47-fold enhancement in antioxidant activity upon nanoemulsification of PSO, as measured by DPPH assay [33].

3.2.2. Antibacterial activity

The antibacterial test results demonstrated that PSO nanoemulsion, prepared via the EPI with sonication, exhibited inhibitory activity against *Escherichia coli* but not against other Gram-negative bacteria *Pseudomonas aeruginosa*, nor against the tested Gram-positive strains (*Staphylococcus aureus* and *Bacillus subtilis*.

The pomegranate seed oil (PSO) nanoemulsion exhibited inhibitory activity against *E. coli* at concentrations of 50 mg/mL and 12.5 mg/mL, with inhibition rates of 39% and 30%, respectively. The IC_{50} value for *E. coli* inhibition was determined to be 95.8 mg/mL. Although PSO is rich in polyphenolic compounds such as punicalagin and punicic acid, which are known for their antimicrobial properties [28], the antibacterial efficacy of the nanoemulsion depends on both the concentration of these bioactives and their ability to penetrate bacterial cell membranes. The observed selective antibacterial activity may be attributed to differences in bacterial cell wall structures. *E. coli*, a Gram-negative bacterium, possesses an outer membrane containing lipopolysaccharides that typically act as a permeability barrier. However, these structures may also interact specifically with certain bioactive components of the PSO nanoemulsion, enhancing membrane permeability and facilitating antimicrobial action [36]. In contrast, the lack of antibacterial activity against Gram-positive bacteria may be due to their thick peptidoglycan layer and absence of an outer membrane, which can hinder the penetration of hydrophobic bioactive compounds. Additionally, the limited interaction between these compounds and the cell wall components of Gram-positive bacteria may reduce their susceptibility to PSO nanoemulsion treatment [36].

4. CONCLUSION

This study successfully developed and characterized pomegranate seed oil (PSO) nanoemulsions using both the emulsion phase inversion (EPI) method and a combination of EPI with ultrasonication. Among the evaluated emulsifiers, Tween 80 demonstrated the highest emulsifying efficiency and stability, producing nanoemulsions with droplet sizes below 200 nm and maintaining homogeneity for extended periods. The stability of nanoemulsions was significantly influenced by oil concentration, surfactant-to-oil ratio (SOR), sonication amplitude, and duration. Optimal stability was achieved at moderate oil concentrations (1.5–3%) and SORs (1.0–1.5), particularly when sonication was applied at 50% amplitude for 8–10 min. Phase behavior analysis via pseudo-ternary diagrams further confirmed the formation of stable nanoemulsions under these conditions. The incorporation of ultrasonication notably enhanced emulsion uniformity and long-term colloidal stability, as evidenced by smaller droplet sizes, improved zeta potential, and reduced viscosity. Nanoemulsions exhibited significantly greater antioxidant capacity and total phenolic content than raw PSO, particularly those prepared with sonication. Moreover, PSO nanoemulsions showed selective antibacterial activity against *E. coli*, likely due to enhanced dispersion and bioactive compound accessibility. Overall, nanoemulsification—especially via EPI combined with sonication—presents a promising approach to improve the solubility, stability, and bioavailability of PSO for functional food or nutraceutical applications.

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TÓM TẮT

NGHIÊN CỨU HỆ NHŨ TƯƠNG NANO TỪ DẦU HẠT LỰU: QUÁ TRÌNH TỔNG HỢP, ĐẶC TÍNH VÀ SÀNG LỌC HOẠT TÍNH SINH HỌC

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Axit punicic, một axit béo chính của dầu hạt lựu (PSO), thể hiện nhiều hoạt tính sinh học có lợi cho sức khỏe, bao gồm tác dụng chống oxy hóa, chống viêm, chống ung thư và bảo vệ tim. Nghiên cứu này nhằm mục đích xây dựng công thức nhũ tương nano PSO bằng hai phương pháp năng lượng thấp: điểm đảo pha (EPI) và điểm đảo pha kết hợp với siêu âm (EPI-siêu âm). Biểu đồ giản pha ba thành phần được xây dựng để xác định các vùng ổn định. Công thức EPI tối ưu (3% PSO, SOR 1,5) tạo ra các giọt có kích thước nano (197,57 ± 0,86 nm) với độ ổn định lên đến 7 ngày. Việc kết hợp siêu âm làm giảm thêm kích thước giọt (93,00 ± 1,11 nm) và tăng cường độ ổn định ở SOR thấp hơn (1,0). Các thí nghiệm chống oxy hóa (DPPH và ABTS) cho thấy hoạt động vượt trội trong công thức EPI-siêu âm (IC_{50} = 0,88 và 0,47 mgOE/mL), so với phương pháp EPI (IC_{50} = 1,01 và 0,62 mgOE/mL). Đánh giá kháng khuẩn cho thấy sự ức chế chọn lọc đối với *Escherichia coli*. Những phát hiện này làm nổi bật tiềm năng của nhũ tương nano dầu hạt lựu như chất mang ổn định để phân phối hoạt tính sinh học trong thực phẩm chức năng hoặc các ứng dụng dược phẩm chức năng.

Từ khóa: Nanoemulsion, axit punicic, điểm đảo pha (EPI), siêu âm, chống oxi hóa, ức chế *E. coli*.