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Biological investigation of supersaturated self-nanoemulsifying drug delivery system of *Piper cubeba* essential oil

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Abstract

Piper cubeba essential oil (PCEO) is used in many ailments but its mechanism is not very well reported especially in case of pain and inflammation. Therefore, in this work, the mechanism of inflammation of PCEO alone and its supersaturated self-nanoemulsifying drug delivery system (S-SNEDDS) was evaluated. S-SNEDDS formulations of PCEO were developed by aqueous phase titration method. Thermodynamically stable S-SNEDDS were characterized for droplet size, polydispersity index, zeta potential, viscosity, refractive index, % transmittance and surface morphology. Based on the best physicochemical parameters, S-SNEDDS F1 was selected for biological investigations in rats. The dose of pure PCEO was 400 mg/kg body weight while the S-SNEDDS F1 was administered in two different doses i.e. 40 mg/kg and 80 mg/kg body weight. The results of this work indicated that the pretreatment with PCEO and S-SNEDDS F1 reduced the exudate volume and polymorphonuclear cells significantly. Moreover, the levels of MPO, NO and proinflammatory cytokine (TNFα and IL-β) were also reduced by PCEO and S-SNEDDS F1 and this observation was also supported by histological observation. The results of S-SNEDDS F1 were superior over PCEO alone even at significantly lower doses. These results indicated the potential of developed S-SNEDDS in enhancing therapeutic efficacy of PCEO.

Keywords: Anti-inflammatory; *Piper cubeba*; Pleurisy; Proinflammatory cytokine; S-SNEDDS.

1. Introduction

Essential oils are commonly used flavoring agents in cosmetics and perfumes in food, beverages and pharmaceutical industries[1]. They have been investigated with variety of medicinal properties such as antimicrobial, antibacterial, antifungal, antibiotic, anti-leukemic, analgesics, anti-inflammatory, antiviral, anticancer and antioxidant[2-4]. *Piper cubeba* plant belongs to family Piperaceae which is commonly known as 'Kababa' in Arab countries. Literature survey revealed gastroprotective activity of this plant[5, 6]. However, no scientific data available to validate the traditional claim of anti-inflammatory effects of *P. cubeba*. Neutrophils play an important role in inflammatory response[7]. However, polymorphonuclear (PMN) cells have an important role in lung damage[8]. Analgesic and anti-inflammatory effects of methanolic and hydroalcoholic extracts of *P. cubeba* have been reported in literature[9, 10].

In the last decade, various lipid based nanosized formulations such as nanoemulsions, microemulsions, self-microemulsifying drug delivery systems (SMEDDS) and self-nanoemulsifying drug delivery system (SNEDDS) have been investigated successfully to enhance therapeutic efficacy of various anti-inflammatory drugs[11-17]. SNEDDS are transparent mixtures of drug, oil/lipid, surfactant and cosurfactant which upon agitation with water or gastrointestinal (GI) fluids produced very fine nanoemulsions (droplet size < 100 nm)[18, 19]. If these SNEDDS could produced nanoemulsions whose size is too fine or ultrafine (< 20 nm), these systems can be considered as supersaturated-SNEDDS (S-SNEDDS)[20]. As a drug delivery carriers, S-SNEDDS offer several advantages such as thermodynamic stability, self-nanoemulsification efficiency, ease of preparation (spontaneous emulsification), minimization of adverse effects and nanosized droplets (less than 50 nm) which could results in rapid absorption of PCEO and finally enhanced anti-inflammatory effects over unstable dispersions[18-20]. Because

of ultra low size of these systems, they are known to enhance therapeutic efficacy and bioavailability of several poorly water-soluble drugs[15-20]. Because, no scientific data are available on anti-inflammatory mechanism of *Piper cubeba* essential oil (PCEO), attempts were made to develop S-SNEDDS formulations of PCEO and to evaluate its anti-inflammatory effects in comparison with pure PCEO and standard indomethacin in Carrageenan-induced pleurisy in rat model. Various S-SNEDDS formulations of PCEO were developed by aqueous phase titration method using Sefsol-218, Triton-X100, Transcutol-HP and water as oil phase, surfactant, cosurfactant and aqueous phase, respectively. SNEDDS are the systems which are able to self-emulsify with GI fluids when administered orally. Due to their potential for self-emulsification, these systems can only be administered by oral route. In order to provide similar conditions for statistical comparisons, PCEO was also administered orally. Moreover, mice were not used as animal model in this work because mice are small animals and they do not have sufficient amount of GI fluids for self-nanoemulsification of SNEDDS. All the components of S-SNEDDS are nontoxic and fall under generally regarded as safe category of excipients.

2. Materials and methods

2.1. Materials

Piper cubeba (*P. cubeba*) plant was purchased from local market in Riyadh, Saudi Arabia. The plant was authenticated by a taxonomist Dr. Mohammed Y. Yaqoob at the College of Pharmacy, King Saud University, Riyadh, Saudi Arabia.

PCEO was extracted and characterized by gas chromatography-mass spectrometry technique in the laboratory. Propylene glycol monocaprylic ester (Sefsol-218) was procured from Nikko Chemicals (Tokyo, Japan). Highly purified diethylene glycol monoethyl ether (Transcutol-HP) was obtained as a kind gift sample from Gattefossé (Lyon, France). Iso-

octylphenoxypolyethoxyethanol (Triton-X100), dimethyl sulfoxide (DMSO) and ethanol were procured from Sigma Aldrich (St. Louis, MO). Ultra-pure chromatographic grade water (deionized water) was collected from Milli-Q water purification system (Berlin, Germany). All other materials and reagents used were of analytical grade and used without any further purification.

2.2. Extraction of PCEO essential oil from *Piper cubeba*

The dried fruits of *P. cubeba* were coarsely pulverized and hydro-distilled for 3 h by using clevenger-type apparatus according to the European Pharmacopoeia. The obtained oil was dried over anhydrous sodium sulfate, filtered and stored at 4 °C till further use. The detailed procedure about its extraction and characterization is given in our previous article[21].

2.3. Selection of components for S-SNEDDS preparation of PCEO

For the selection of components for the preparation of SNEDDS, the solubility of biological molecule in different components is one of the most important criteria[20]. Nevertheless, solubility studies were not performed in this work because biological molecule in this work was PCEO which is easily miscible in oils and surfactants. Therefore, the safety and nontoxicity were main criteria for selection of oil phase, surfactant and cosurfactant in this work. Based on above criteria, Sefsol-218, Triton-X100 and Tarnscutol-HP were selected as oil phase, surfactant and cosurfactant, respectively for the development of S-SNEDDS of PCEO. Deionized water was selected as aqueous phase because of its frequent use in literature[16].

2.4. Construction of pseudo-ternary phase diagrams

For the preparation of S-SNEDDS of PCEO, pseudo-ternary phase diagrams were constructed by aqueous phase titration method as reported in literature[11, 12]. Briefly, surfactant (Triton-X100) and cosurfactant (Transcutol-HP) were mixed in the mass ratios of 1:0, 1:2, 1:1, 2:1, 3:1 and 4:1.

The total stock of the mixture of surfactant and cosurfactant (S_{mix}) was 20 g. Sefsol-218 and a particular S_{mix} were then mixed at mass ratios of 1:9 to 9:1. Pseudo-ternary phase diagrams were constructed by aqueous phase titration method. In this method, the mixture of oil phase (Sefsol-218) and specific S_{mix} was titrated by slow addition of deionized water (aqueous phase) and visual observations were made depending upon their clarity[12, 22]. The clear, transparent and easily flowable SNEDDS zones were marked on a phase diagram with one axis representing the aqueous phase, second oil phase (Sefsol-218) and third representing the specific mass ratio of surfactant (Triton-X100) to cosurfactant (Transcutol-HP).

2.5. Formulation development of PCEO

From pseudo-ternary phase diagrams, it was observed that the highest SNEDDS zones were exposed by 1:1 mass ratio of Triton-X100 and Transcutol-HP, hence 1:1 mass ratio was selected for the preparation of SNEDDS of PCEO. From the phase diagram, different SNEDDS with formulation codes of F1-F9 were precisely selected. Almost the entire region of SNEDDS zones were taken into account. In formulations F1-F5, the concentration of oil phase (Sefsol-218) was kept constant at 5 % w/w and the concentration of S_{mix} was varied from 10-50 % w/w. However, in formulations F6-F9, the concentration of S_{mix} was kept constant at 40 % w/w and the concentration of oil phase was varied from 10-25 % w/w in order to cover the entire SNEDDS zones in the phase diagram. After selection of blank SNEDDS from phase diagram, 5 % w/w of PCEO was incorporated in each SNEDDS by vortexing at 1000 rpm and 25 $^{\circ}$C for about 5 min. The composition of PCEO loaded SNEDDS is listed in Table 1.

2.6. Thermodynamic stability and self-nanoemulsification tests

Thermodynamic stability tests on developed PCEO loaded SNEDDS (F1-F9) were performed to remove any unstable or metastable formulation. These tests were performed viz. centrifugation,

heating & cooling cycles and freeze-pump-thaw cycles. The detailed procedure of these tests is given in our previously published articles[20, 22]. The purpose of self-nanoemulsification test was to investigate any phase separation or precipitation upon dilution with water, acid buffer (0.1 N HCl) and phosphate buffer (pH 7.4). This test was carried out by diluting 1 ml of each PCEO loaded SNEDDS (F1-F9) with water, 0.1 N HCl and phosphate buffer (pH 7.4) in the dilution ratio of 1:500. The self-nanoemulsification efficiency of each formulation was assessed visually with the help of following grading systems[20, 22]:

Grade A: Rapid/spontaneously forming clear nanoemulsion

Grade B: Rapid/spontaneously forming bluish slightly less clear nanoemulsion

Grade C: Slowly forming turbid emulsions

Grade D: Dull, grayish slowly forming turbid emulsions

Grade E: Turbid emulsions with the presence of oil globules at the surface

2.7. Physicochemical characterization of PCEO-S-SNEDDS

Developed S-SNEDDS of PCEO were physicochemically characterized for droplet size distribution, polydispersity index (PI), zeta potential (ZP), viscosity, refractive index (RI), percentage of transmittance (% T) and surface morphology using transmission electron microscopy (TEM). The mean droplet size, PI and ZP of PCEO-S-SNEDDS (F1-F9) were measured using Malvern Particle Size Analyzer (Malvern Instruments Ltd., Holtsville, NY) at 25 ± 1 °C at a scattering angle of 90°. The detailed procedure for the measurement of droplet size, PI and ZP is presented in our previous article[20]. The viscosity of PCEO-S-SNEDDS (F1-F9) was measured using Brookfield Viscometer (Brookfield Engineering Laboratories, Middleboro, MA) at 25 ± 1 °C as reported previously[22]. However, the RIs of PCEO-S-SNEDDS (F1-F9) were measured using Abbes type Refractometer (Precision Testing Instruments Laboratory, Germany)

at 25 ± 1 °C as reported previously[20]. The % T of PCEO-S-SNEDDS (F1-F9) was determined spectrophotometrically at 550 nm as reported in literature[20].

The surface morphology and structure of droplets of optimized PCEO-S-SNEDDS formulation F1 was evaluated using JEOL TEM technique (JEOL JEM-2100 F, USA). TEM analysis was carried out under light microscopy operating at 100 KV. The detailed procedure for TEM analysis is presented in our previous article[12].

2.8. Animals and study protocol

Male Albino Wistar rats (weighing in the range of 180-220 g) were collected from the Experimental Animal Care Center of the Institute. The animals were provided controlled environmental conditions. All the rats were receiving free access to standard pellet diet and tap water. The study protocol was approved by Research Ethics Committee of the Institute (Clearance No. 021656-0621). The institutional guidelines were strictly followed for these studies. Based on the best physicochemical properties of S-SNEDDS of PCEO, formulation F1 was selected as an optimized formulation for in vivo investigations in rats. The rats were divided into six groups with six rats per group: Group-I: control, Group-II: caragenan only, Group-III PCEO, Group-IV: F1 (80 mg/kg), Group-V: F1 (40 mg/kg) and Group-VI: indomethacin (standard).

Formulation F1 at two different doses (40 and 80 mg/kg body weight) were administered orally once a time for the period of 4 days. However, control and carrageenan groups were given an equivalent volume of 0.5 % suspension of sodium carboxymethyl cellulose. Approximately, 0.3 ml of saline or saline containing 1% carrageenan was injected directly into the pleural cavity of animals under anesthesia on the last day i.e. fourth day. After 4 h of carrageenan injection, the animals were sacrificed, the chest was opened and the pleural cavity was rinsed with

approximately 2 ml of sterile saline solution with heparin (5 U/ml). The exudate was taken out by aspiration and the total volume was determined. The samples were centrifuged and cell pellet was resuspended in the solution of phosphate buffer saline. The number of neutrophils in the exudates were counted with the help of optical microscope after Giemsa staining.

2.9. Determination of nitric oxide (NO)

Production of NO was carried out by the quantification of its related end products such as nitrite/nitrate using Griess reaction reported previously[23, 24]. The measurement was carried out using the 540 nm filter in a titrated Biotek ELISA reader.

2.10. Myeloperoxidase (MPO) level of pleural exudates

MPO assay was performed for the measurement of neutrophil recruitment which was measured indirectly by means of MPO activity. MPO activity was determined by adopting the procedure reported by Campos et al. (2002)[25]. The measurements were performed at 690 as reported in literature[25].

2.11. Cytokine determination

The levels of cytokines such as TNF-α, IL-6 and IL-1β in lung tissues were determined colorimetrically using commercial ELISA kit (R&D systems, USA).

2.12. Total RNA isolation and cDNA synthesis

After 4 h of carrageenan administration, the lung samples were obtained and being used for total RNA isolation. Total RNA isolation was performed using manufacturer's instructions of the Trizol reagent (Life Technologies, Inc., NY, USA). Following total RNA isolation, the reverse transcription from total RNA to cDNA was processed by high capacity cDNA reverse transcription kit of Applied Biosystems according to the manufacturer's instructions[26].

2.13. Expression of iNOS-2 and COX-2 mRNA in lung tissue

The analysis of specific mRNA expression was carried out by RT-PCR by subjecting the resulting cDNA to PCR amplification using 96-well optical reaction plates in the ABI Prism 7500 System (Applied-Biosystems). The 25 µl reaction mixture containing 0.1 µl of 10 µM forward primer and 0.1µl of 10 µM reverse primer (40 nM final concentration of each primer), 12.5 µl of SYBR green universal master mix, 11.05 µl of nuclease-free water and 1.25 µl of cDNA sample. Rat primers iNOS-2, COX-2 and β-ACTIN gene were procured from Integrated DNA Technologies (IDT, Coralville, IA) (Table 1). The fold change in the level of mRNA between treated and untreated groups was corrected by the levels of β-ACTIN. The RT-PCR data were analyzed using the relative gene expression, that is (DD CT) method, as described and explained previously[27, 28].

2.14. Western blot analysis

This analysis was carried out according to method proposed by Towbin et al. [29, 30]. The proteins were electrophoretically transferred to PVDF membranes, blocked in 5 % skim milk in Tris buffer saline (TBS) containing 1 % Tween-20 for the period of 2 h at room temperature and probed with the polyclonal iNos (sc-651), COX-2 (sc- sc-1746) and β-actin (sc-47778) polyclonal rabbit-anti rat antibodies as solutions in PBS containing 1 % Tween-20 on a shaker for 2 h at room temperature followed by horseradish peroxidase-(HRP-)conjugated secondary antibodies (1: 3000) for 1 h and visualization with the enhanced chemiluminescence system (Santa Cruz Biotechnology, USA). β-actin was used as the loading control for total proteins densitometric analysis of immunoblots was performed with the Image J software (NIH).

2.15. Histological examination of the lungs

The lung tissues were taken out from rats, fixed in 10 % formalin for the period of 7 days at room temperature, dehydrated and finally embedded in paraffin blocks. Sections were deparaffinized using xylene and stained using hematoxylin-eosin. The sections of lung tissues were examined under light microscopy.

2.16. Data analysis

All values of biological investigations are expressed as mean ± standard error of mean (SEM). The statistical significance of differences between the groups was analyzed by ANOVA. A value of $P<0.05$ was considered statistically significant.

3. Results and discussion

3.1. Selection of components for S-SNEDDS preparation of PCEO

As stated previously that the solubility of therapeutic drug molecules in different components is the main criteria for their selection. In this work, we have not used any solid crystalline drug molecule, solubility studies were not performed. Moreover, PCEO used in this study is easily miscible in oils and surfactants. Nevertheless, different components were selected on the basis of safety and nontoxicity of oil phase, surfactant and cosurfactant in this work. Based on these criteria, Sefsol-218, Triton-X100, Tarnscutol-HP and deionized water were selected as oil phase, surfactant and cosurfactant and aqueous phase, respectively for the development of PCEO-S-SNEDDS.

3.2. Construction of pseudo-ternary phase diagrams and formulation development

For the development of PCEO-S-SNEDDS, pseudo-ternary phase diagrams were constructed using Sefsol-218, Triton-X100, Transcutol-HP and water. The results of this study are presented in Figure 1. However, the brief summary of results is listed in Table 2. From Figure 1, it was clear that S_{mix} ratio of 1:0 (surfactant alone) showed poor zones of SNEDDS (Figure 1A). The

maximum amount of Sefsol-218 (oil phase) that was solubilized by 1:0 ratio was observed as 9 % w/w with respect to 82 % w/w of S_{mix}. But when the concentration of surfactant Transcutol-HP (cosurfactant) was increased with respect to Triton-X100 (surfactant) [S_{mix} ratio of 1:2), the SNEDDS zones were increased as compared to 1:0 ratio (Figure 1B). The maximum amount of Sefsol-218 that was solubilized by 1:2 ratio was higher than Figure 1A i.e. 14 % w/w with respect to 55 % w/w of S_{mix} (Table 2). When the concentration of surfactant and cosurfactant was kept equal (S_{mix} ratio of 1:1), the SNEDDS zones were increased significantly in comparison with previous S_{mix} ratios (Figure 1C). The maximum amount of Sefsol-218 that was solubilized by 1:1 mass ratio was observed as 28 % w/w with respect to 41 % w/w of S_{mix} (Table 2). However, when the S_{mix} ratio of 2:1 was studied, the SNEDDS regions were found to be reduced in comparison with 1:1 S_{mix} ratio (Figure 1D). The maximum amount of Sefsol-218 that was solubilized by 2:1 mass ratio was observed as 22 % w/w with respect to 52 % w/w of S_{mix} (Table 2). When the concentration of Triton-X100 was further increased with respect to Transcutol-HP (S_{mix} ratio of 3:1), the SNEDDS zones were found to be decreased further as compared to 1:1 and 2:1 ratios (Figure 1E). The maximum amount of Sefsol-218 that was solubilized by 3:1 ratio was recorded as 16 % w/w by utilizing 67 % w/w of S_{mix} (Table 2). When the concentration of Triton-X100 was further increased with respect to Transcutol-HP (S_{mix} ratio of 4:1), the SNEDDS zones were decreased further as compared to 1:1, 2:1 and 3:1 ratios (Figure 1F). The maximum amount of Sefsol-218 that was solubilized by 4:1 ratio was recorded as 15 % w/w by incorporating around 59 % w/w of S_{mix} (Table 2).

Aqueous phase titration studies indicated that highest SNEDDS zones were exposed by S_{mix} ratio of 1:1 (Figure 1C). Therefore, different SNEDDS formulations for PCEO were precisely selected from Figure 1 C. Almost the entire region of SNEDDS zones were taken into account in Figure

1C. In first five formulations (F1-F5), the concentration of Sefsol-218 was kept constant at 5 % w/w and the concentration of S_{mix} was varied from 10-50 % w/w. However, in next four formulations (F6-F9), the S_{mix} concentration was kept fixed (i.e. 40 % w/w) and Sefsol-218 concentration was changed from 10-25 % w/w in order to cover the entire SNEDDS zones in Figure 1C. After selection of blank SNEDDS, 5 % w/w of PCEO was incorporated in each SNEDDS by vortexing at 1000 rpm and 25 °C for about 5 min (Table 1).

3.3. Thermodynamic stability and self-nanoemulsification tests

The primary objective of thermodynamic stability tests was to remove any metastable/unstable SNEDDS because observations during phase titration studies were made visually. Hence, selected SNEDDS were subjected to different thermodynamic stability tests. The qualitative results of these studies are presented in Table 3. From Table 3, it can be seen that all developed SNEDDS survived at all steps of thermodynamic stability tests. Developed PCEO-SNEDDS (F1-F9) were further investigated for self-nanoemulsification efficiency test because it is mandatory for oral emulsifying formulations[22]. The primary objective of self-nanoemulsification test was to investigate any phase separation or precipitation upon dilution with aqueous media (deionized water), acid buffer (0.1N HCl) and phosphate buffer (pH 6.8) and qualitative results are presented in Table 3[20, 22]. It was observed that all the SNEDDS of PCEO survived this test with grade A in the presence of all three diluents. Because, all the formulations remained transparent (passed the test with grade A), these were considered as supersaturated SNEDDS (S-SNEDDS). Overall, these results indicated that PCEO was maintained in solubilized form at molecular state in developed SNEDDS and its self-nanoemulsification behavior of all formulations was independent of pH[31].

3.4. Physicochemical characterization of PCEO-S-SNEDDS

The results of physicochemical characterization of developed PCEO-S-SNEDDS (F1-F9) are presented in Table 4. The droplet size of SNEDDS is one of the most important characterization parameter. From Table 4, it can be seen that the droplet size of PCEO-S-SNEDDS (F1-F9) was observed in the range of 7.53-52.45 nm. When the concentration of oil phase (Sefsol-218) was kept constant at 5 % w/w and the concentration of S_{mix} was varied from 10-50 % w/w (F1-F5), the droplet size of S-SNEDDS was found to be changed slightly. The droplet size of formulations was decreased slightly with increasing the concentration of S_{mix} (Table 4). However, when the concentration of S_{mix} was kept constant at 40 % w/w and the concentration of Sefsol-218 was varied from 10-25 % w/w (F6-F9), the droplet size of S-SNEDDS was found to be changed significantly. The droplet size of these formulations was found to be enhanced rapidly with increase in Sefsol-218 concentration in S-SNEDDS (Table 4). These results indicated that S_{mix} had little impact on droplet size of PCEO-S-SNEDDS while Sefsol-218 had greater impact on droplet size of PCEO-S-SNEDDS. Overall, the largest droplet size was observed in formulation F9 (52.45 ± 4.17 nm) which was probably due to the presence of highest amount of Sefsol-218 (25 % w/w) in F9. However, the droplet size of PCEO-S-SNEDDS F1 was observed as lowest (7.53 ± 0.56 nm) that was probably due to the presence of lowest amount of Sefsol-218 (5 % w/w) in F1.

The measurement of PI is useful in evaluation of uniformity of droplets in developed SNEDDS. The PIs of PCEO-S-SNEDDS (F1-F9) were recorded as 0.119-0.247 as shown in Table 4. The least PI was observed in PCEO-S-SNEDDS F1 (0.119), indicating higher uniformity of droplet size distribution as compared to other PCEO-S-SNEDDS. However, the highest PI value was observed in PCEO-S-SNEDDS F9 (0.247). Overall, the PIs were less than 0.3 in all formulations, indicating good uniformity of droplet size distribution in all formulations.

The measurement of ZP is important in evaluation of net surface charge and stability of developed SNEDDS. The values of ZP for formulations (F1-F9) were recorded as -28.98 to -25.14 mV (Table 4). The least value of ZP was recorded in PCEO-S-SNEDDS F1 (-28.98 mV). However, the highest value of ZP was recorded in PCEO-S-SNEDDS F9 (-25.14 mV). The negative values of ZP in all PCEO-S-SNEDDS were possibly due to the presence of negatively charged fatty acid esters in Sefsol-218[32]. The experimental ZP values in the magnitude of ± 30 mV indicated the stable formation of PCEO-S-SNEDDS[20].

The measurement of viscosity is important in evaluation of flow behavior of developed SNEDDS. The viscosity of all formulations (F1-F9) was recorded as 12.80-61.24 cp (Table 4). The viscosity results were correlated with formulation compositions and droplet size. It was observed that when the Sefsol-218 concentration was kept fixed (5 % w/w) and S_{mix} concentration was varied from 10-50 % w/w (F1-F5), the viscosity of PCEO-S-SNEDDS was found to be changed slightly. The viscosity of these formulations (F1-F5) was found to be decreased slightly with respect to S_{mix} concentration (Table 4). However, when the S_{mix} concentration was kept constant (40 % w/w) and Sefsol-218 concentration was varied from 10-25 % w/w (F6-F9), the viscosity of PCEO-S-SNEDDS was found to be changed significantly. The viscosity of these formulations (F6-F9) was found to be increased significantly with increasing the concentration of Sefsol-218 (Table 4). Moreover, the viscosity of all PCEO-S-SNEDDS was also found to be reduced with decrease in droplet size of formulations. These results indicated that S_{mix} had little impact on viscosity of PCEO-S-SNEDDS. However, Sefsol-218 and droplet size had greater impact on viscosity of PCEO-S-SNEDDS. Overall, the highest viscosity was also observed in PCEO-S-SNEDDS F9 (61.24 ± 5.21 nm) while the lowest one

was observed in PCEO-S-SNEDDS F1 (12.80 ± 1.09 cp). Overall, the lower values of viscosity in all formulations indicated the free flowing behavior of developed PCEO-S-SNEDDS.

The measurement of RI is useful in evaluation of transparent behavior and type of nanoemulsions. The RIs of PCEO-S-SNEDDS (F1-F9) were observed in the range of 1.337-1.347 (Table 4). The highest value of RI was observed in PCEO-S-SNEDDS F9 (1.347 ± 0.11). However, the lowest value of RI was recorded in PCEO-S-SNEDDS F1 (1.337 ± 0.06). The RIs of all formulations were very close to RI of water (1.333), indicating transparent nature and oil-in-water type behavior of all PCEO-S-SNEDDS.

The measurement of % T is also useful in evaluation of transparent behavior of developed SNEDDS. The % T of developed formulations (F1-F9) was recorded as 96.0-98.7 % (Table 4). Formulation PCEO-S-SNEDDS F1 showed the highest value of % T (98.7 ± 0.3 %). While, the formulation PCEO-S-SNEDDS F9 showed the lowest value of % T (96.0 ± 0.5 %). These results indicated transparent behavior of all PCEO-S-SNEDDS.

TEM evaluation was conducted to investigate the morphological characters of optimized PCEO-S-SNEDDS F1. TEM images of formulation F1 were taken and evaluated for surface morphology and droplet size (Figure 2). The size of all droplets of F1 was observed within nanometer range (Figure 2). The shape of droplets of F1 was observed as spherical. The spherical shape of formulation F1 could be due to the presence of Sefsol-218 and Triton-X100[22].

3.5. Effect of PCEO and its S-SNEDDS F1 on carrageenan-induced pleurisy

As shown in Figure 3, the exudate volume was found to be increased from 0.373 ± 0.012 to 1.27 ± 0.383 ml/rat at 4 h in pleural cavity of rats induced by carrageenan ($P<0.01$). However, the number of neutrophils were found to be increased from 2.71 ± 0.098 to 62.41 ± 3.35 × 10^6 cells/rat ($P<0.01$). Pretreatment with PCEO and formulation F1 showed the significant reduction

in exudate volume in dose dependent manner which indicated that PCEO has anti-inflammatory effect while formulation F1 was highly comparable with standard indomethacin (Figure 3). Moreover, the number of polymorphonuclears (PMNs) were also reduced to significantly by formulation F1 in dose dependent manner ($P < 0.01$).

In this study, the results showed that PCEO and its S-SNEDDS formulation could mitigate carrageenan-induced lung inflammation response. Further studies showed PCEO and its S-SNEDDS formulation could decrease the degree of lung inflammation in dose dependent manners in rats. This observation was probably due to a reduction of PMNs infiltration and release of inflammatory factors. Carrageenan in plural cavity elicited an acute inflammatory reaction characterized by accumulation of plural exudate with large amount of PMNs infiltration. Neutrophils were recruited to the site of aggregations and can be highly activated by a vide arrays of ligands. The results showed that PCEO and S-SNEDDS formulation could attenuate the number of polymorph nuclear cells and exudate volume. These results suggested that PCEO and its S-SNEDDS formulation could inhibit carrageenan-induced pleurisy in rats[33, 34]. Neutrophils are known to adhere to the endothelial layer which could be activated by mediators released locally[33]. The pathological changes in rat lung were also examined. In comparison with rats treated with vehicle, the rats treated with carrageenan showed the pathological changes of the lungs with inflammatory cells infiltration and local edema.

3.6. MPO level of pleural exudates

We further evaluated the MPO activity in this work. MPO is an indicator of polymorphonuclear leukocyte accumulation in pleural exudates. MPO level was measured with the help of colorimetric, commercial kit. As shown in Figure 4, the level of MPO was found to be increased from 36.71 ± 1.0 to 260.08 ± 9.13 U/l in pleural exudates of carrageenan-induced rats ($P < 0.01$).

The result showed that PCEO and its S-SNEDDS formulation could decrease the MPO level to 210.45 ± 5.55, 196.88 ±3.96 and 176.80 ± 3.39 in dose dependent manner. The indomethacin group showed potent inhibition of MPO level. These investigations further confirm the anti-inflammatory effect of PCEO and its S-SNEDDS formulation. The MPO, the principal enzyme released from PMNs stimulation is used as biomarker for measuring acute inflammation[35]. MPO may lead to tissue damage during inflammation[36]. The results showed that PCEO and its S-SNEDDS formulation inhibit the MPO levels in pleural exudate of the carrageenan treated rats in dose dependent manners. Acute inflammation in rats results in a significant infiltration of inflammatory cells in lung tissue, the results demonstrated that treatment with PCEO and its S-SNEDDS formulation along with standard drug indomethacin reduced infiltration of these inflammatory cells.

3.7. Cytokine levels of the lungs

As shown in Figure 5, the levels of TNF-α and IL-1β were found to be increased in the lungs of carrageenan-induced rats ($P < 0.01$). PCEO and its S-SNEDDS formulation could significantly decrease the release of TNF-α and IL-1β in a dose-dependent manner ($P < 0.05$, $P < 0.01$). Inflammation is mediated by a variety of soluble factors, including a group of secreted polypeptides known as cytokines[37]. Cytokines activation has been reported as the key process of inflammatory reaction[38]. The release of cytokines can induce chemotaxis to attract migrating granulocytes or leukocytes which further release cytokines and inflammatory mediators[37]. TNF-α, Ilβ and IL-6 are associated with lung inflammation in animal models[38-41]. The activated neutrophils in lung are known to release pro-inflammatory cytokines on endotoxin administration[34]. This study demonstrated that PCEO and its S-SNEDDS formulation significantly attenuated the levels of TNF-α IL 6 and IL-1β in the lungs of carrageenan-injected

rats leading to anti-inflammatory effect. Therefore, anti-inflammatory action of PCEO may be due to attenuation of chemotaxis and inhibition of TNF-α and IL-1β in dose dependent manner.

3.8. mRNA expression and protein expression of iNOS and COX-2 in lungs

A significant increase in iNOS and COX-2 mRNA and protein expression 4 h after carrageenan injection was detected in lungs obtained from rats subjected to carrageenan-induced pleurisy as shown in (Figure 6). The pretreatment with PCEO and its S-SNEDDS formulation showed dose dependent inhibition of iNOS and COX-2 expression in carrageenan induced pleurisy. This further confirms the anti-inflammatory potential of PCEO and its S-SNEDDS formulation. NO is very important in the regulation of vascular permeability and cell migration induced by pro-inflammatory agents such as carrageenan. To further elucidate the mechanism underlying the preventive action of PCEO and its S-SNEDDS formulation on lung tissue, we examined the mRNA and protein expression levels of inflammatory mediators in lung tissue using RT-PCR and western blot analysis. The mRNA levels of iNOS and COX-2 were significantly increased in the carrageenan injected rats, whereas the treatment with PCEO and its S-SNEDDS formulation attenuated the mRNA and protein expression of these pro-inflammatory mediators. The activation of iNOS catalyzes the formation of a large amount of NO, which plays a key role in the pathogenesis of a variety of inflammatory diseases[42].

The inhibitors of iNOS activity reduce the increase of carrageenan induced inflammation[43]. Induction of COX-2 gene is responsible for the increase of PGE2, a key mediator of inflammation and inhibitors of COX-2 may attenuate the inflammation in lungs. We further demonstrate here that the increase in expression level of iNOS and COX-2 caused by carrageenan into lung tissue is attenuated by PCEO and its S-SNEDDS formulation deregulated the PGE2 and no synthesis leading to attenuation in classical sign of inflammation redness,

swelling and pain. The results of NO level clearly showed that there is up regulation of NO in carrageenan induced rats while PCEO and S-SNEDDS formulation attenuate NO synthesis. These results clearly indicate that PCEO and its S-SNEDDS formulations has anti-inflammatory activity.

3.9. Histological analysis of lung tissues

As compared to vehicle group (Figure 7A), the lung tissues treated with carrageenan showed pathological changes with inflammatory cells infiltration and local edema (Figure 7B). The results showed that PCEO and its S-SNEDDS formulation could decrease the inflammation in lung of rats treated with carrageenan (Figure 7C-F). The histological observation further substantiates our finding. Therefore, PCEO and S-SNEDDS formulation were able to mitigate the acute inflammation in dose dependent manner in carrageenan injected rats. There are reports indicating that some monoterpenes from essential oils are strong inhibitors of certain inflammatory mediators such as prostaglandins and other arachidonic acid metabolites. The present GC-MS investigation revealed that monoterpenes e.g. sabinene, 4-terpineol, γ-terpinene and α-thujene were the major components in PCEO which could be responsible for the observed activities[44, 45].

4. Conclusions

In this work, various S-SNEDDS formulations of PCEO were developed, characterized, evaluated for anti-inflammatory effects as compared to pure PCEO and standard indomethacin. Based on lowest droplet size (7.53 nm), least PI (0.119), lowest viscosity (12.80 cp), optimal values of ZP (-28.98 mV) & RI (1.337) and highest % T (98.7 %) S-SNEDDS F1 containing 5 % w/w of PCEO, 5 % w/w of Sefsol-218, 25 % w/w of Triton-X100, 25 % w/w of Transcutol-HP and 40 % w/w of water was selected for biological

investigation in rat model. The results indicated that the pretreatment with PCEO and S-SNEDDS F1 reduced the exudate volume and PMN cells significantly. Moreover, the levels of MPO, nitric oxide and proinflammatory cytokine (TNFα and IL-β) were also reduced by PCEO and S-SNEDDS F1 and this observation was also supported by histological observation. The results of S-SNEDDS F1 were superior over PCEO alone even at significantly lower doses. These results indicated the developed S-SNEDDS could be successfully used for enhanced therapeutic efficacy of PCEO.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure captions

Figure 1 Phase diagrams developed aby aqueous phase titration method for SNEDDS zones of PCEO (dotted area) for Sefsol-218, water, Triton-X100 and Transcutol-HP at S_{mix} ratios of A. 1:0, B. 1:2, C. 1:1, D. 2:1, E. 3:1 and F. 4:1

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Figure 3 Effect of PCEO and optimized PCEO-S-SNEDDS (F1) on carrageenan-induced pleurisy in rats; values are mean ± SEM of 6 rats in each group; P values: * < 0.05, ** <0.01, * <0.001 compared with toxic control (carrageenan)**

Figure 4 Effect of PCEO and optimized PCEO-S-SNEDDS (F1) on nitrous oxide and MPO level in rats; values are mean ± SEM of 6 rats in each group; P values: * < 0.05, ** <0.01, * <0.001 compared with toxic control (carrageenan)**

Figure 5 Effect of PCEO and optimized PCEO-S-SNEDDS (F1) on cytokines levels in carrageenan-induced pleurisy in rats; values are mean ± SEM of 6 rats in each group; P values: * < 0.05, ** <0.01, * <0.001 compared with toxic control (carrageenan)**

Figure 6 mRNA expression and protein expression of i-NOS and COX-2 by PCEO and optimized PCEO-S-SNEDDS (F1) in lungs; values are mean ± SEM of 6 rats in each group; P values: * < 0.05, ** <0.01, * <0.001 compared with toxic control (carrageenan)**

Figure 7 Effect of PCEO and PCEO-S-SNEDDS (F1) on lung inflammation (HE × 100); lung section of the rats treated with normal saline (A); lung section of the rats treated with carrageenan (B); lung section of the rats treated with carrageenan and PCEO (C, D) and lung section treated with carrageenan and S-SNEDDS (F1) (E, F)

Table 1 Formulation components of S-SNEDDS formulations of PCEO (F1-F9) prepared using Sefsol-218, Triton-X100, Transcutol-HP and water

Code	Formulation composition (% w/w)					S_{mix} ratio
	PCEO	Sefsol-218	Triton-X100	Transcutol-HP	Water	
F1	5	5	25	25	40	1:1
F2	5	5	20	20	50	1:1
F3	5	5	15	15	60	1:1
F4	5	5	10	10	70	1:1
F5	5	5	5	5	80	1:1
F6	5	10	20	20	45	1:1
F7	5	15	20	20	40	1:1
F8	5	20	20	20	35	1:1
F9	5	25	20	20	30	1:1

Table 2 Summary of observations made during aqueous phase titration of nanoemulsions

Figure	S_{mix} ratio	Surfactant	Cosurfactant	Nanoemulsion zones	Oil phase solubilized (% w/w)[a]	S_{mix} solubililized (% w/w)[b]
1A	1:0	Triton-X100	Transcutol-HP	Low	9	82
1B	1:2	Triton-X100	Transcutol-HP	Higher than 1A	14	55
1C	1:1	Triton-X100	Transcutol-HP	Highest	28	41
1D	2:1	Triton-X100	Transcutol-HP	Lower than 1C	22	52
1E	3:1	Triton-X100	Transcutol-HP	Lower than 1C & 1D	16	67
1F	4:1	Triton-X100	Transcutol-HP	Lower than 1C, 1D & 1E	15	59

[a]The maximum amount of Sefsol-218 (oil phase) that was solubilized

[b]The maximum amount of Smix (Triton-X100:Transcutol-HP) phase that was solubilized with respect to maximum amount of oil phase

Table 3 Results of thermodynamic stability and self-nanoemulsification test in the presence of deionized water, 0.1 N HCl and phosphate buffer (pH 6.8)

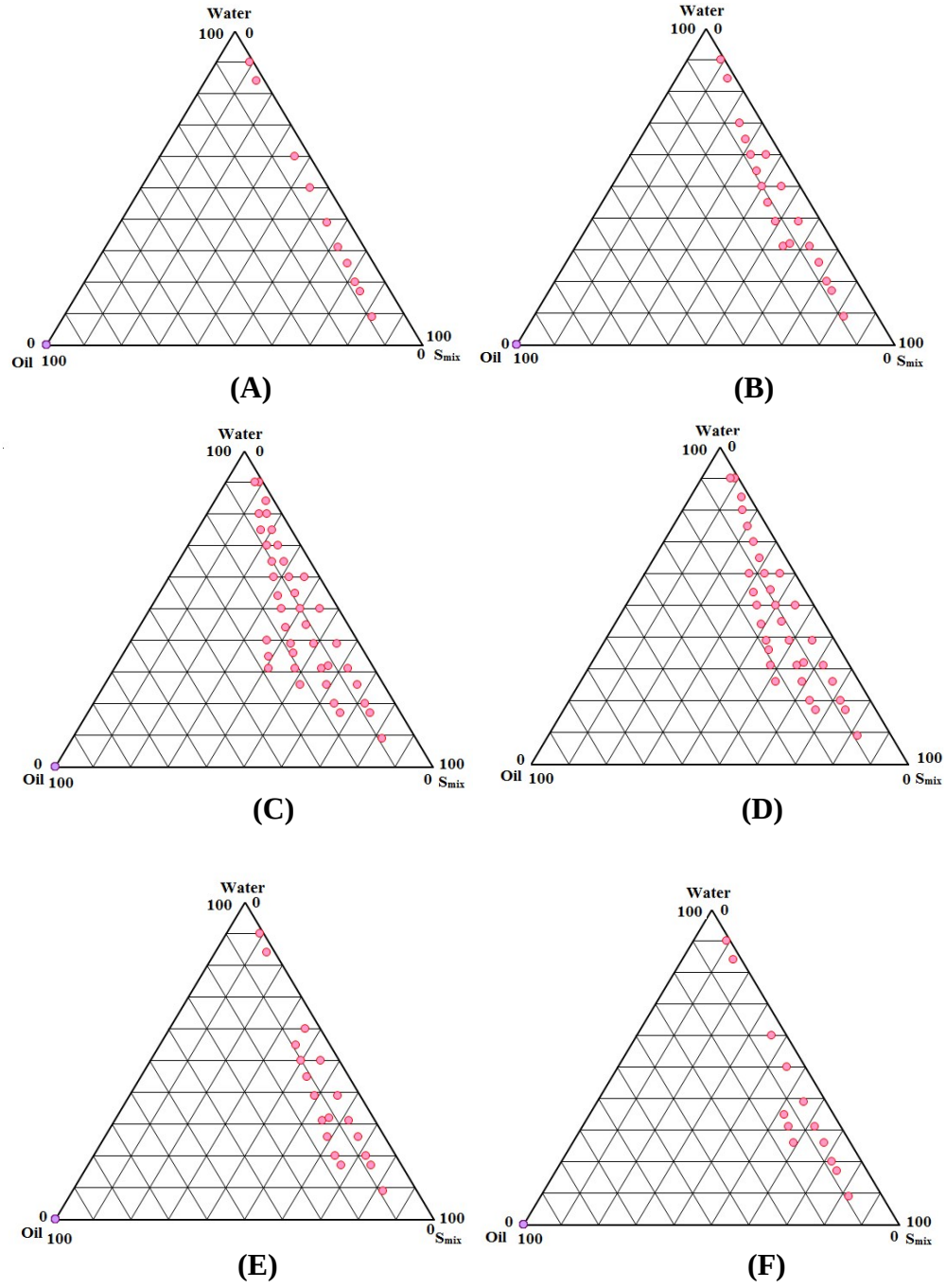
Code	*Self-nanoemulsification test grade	Thermodynamic stability tests		
		Cent.	H&T	FPT
F1	A	√	√	√
F2	A	√	√	√
F3	A	√	√	√
F4	A	√	√	√
F5	A	√	√	√
F6	A	√	√	√
F7	A	√	√	√
F8	A	√	√	√
F9	A	√	√	√

√(Passed the respective test), cent. (centrifugation), H&T (heating and cooling cycles), FPT (freeze-pump-thaw cycles), * (all the formulations passed this test with grade A in the presence of deionized water, 0.1 N HCl and phosphate buffer)

Table 4 Various physicochemical parameters of PCEO-S-SNEDDS (F1-F9)

Code	Characterization parameters					
	Δ_{dm} ± SD (nm)	**PI**	**ZP (mV)**	**η ± SD (cp)**	**RI ± SD**	**% T ± SD**
F1	7.53 ± 0.56	0.119	-28.98	12.80 ± 1.09	1.337 ± 0.06	98.7 ± 0.3
F2	8.81 ± 0.84	0.187	-27.56	13.42 ± 1.21	1.338 ± 0.05	98.4 ± 0.2
F3	9.35 ± 0.90	0.198	-27.12	14.34 ± 1.41	1.339 ± 0.08	97.5 ± 0.1
F4	10.21 ± 0.95	0.202	-26.38	15.41 ± 1.53	1.341 ± 0.10	97.3 ± 0.4
F5	11.13 ± 0.98	0.210	-26.16	16.32 ± 1.74	1.342 ± 0.12	97.1 ± 0.3
F6	21.62 ± 1.21	0.209	-25.80	26.4 ± 2.12	1.343 ± 0.09	96.7 ± 0.5
F7	33.45 ± 2.42	0.234	-25.64	38.62 ± 3.13	1.344 ± 0.07	96.3 ± 0.2
F8	41.32 ± 3.45	0.242	-25.32	48.76 ± 4.61	1.345 ± 0.08	96.2 ± 0.3
F9	52.45 ± 4.17	0.247	-25.14	61.24 ± 5.21	1.347 ± 0.11	96.0 ± 0.5

Mean droplet diameter (Δd_m), polydispersity index (PI), viscosity (η), % transmittance (% T), zeta potential (ZP), refractive index (RI), standard deviation (SD)



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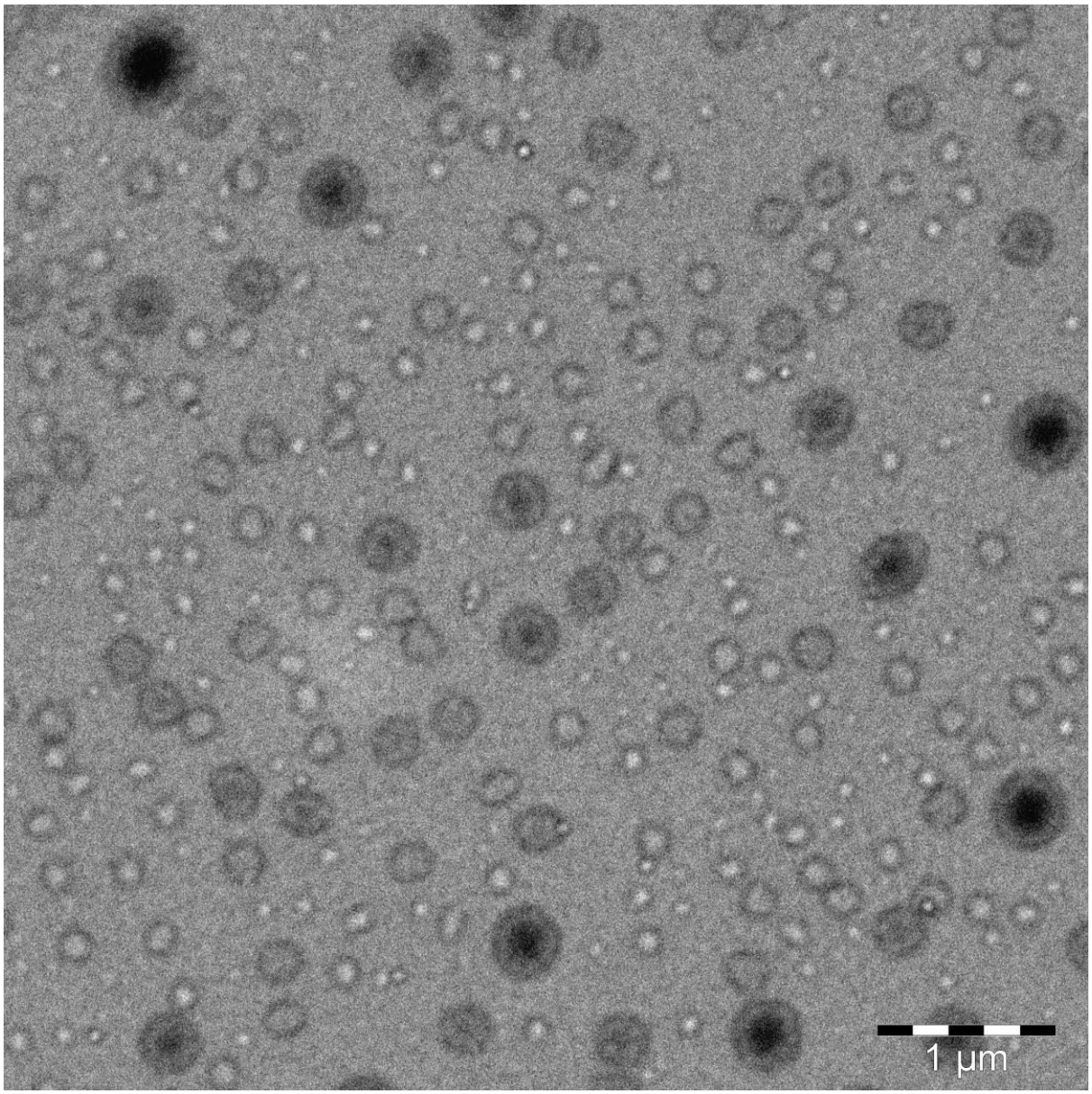
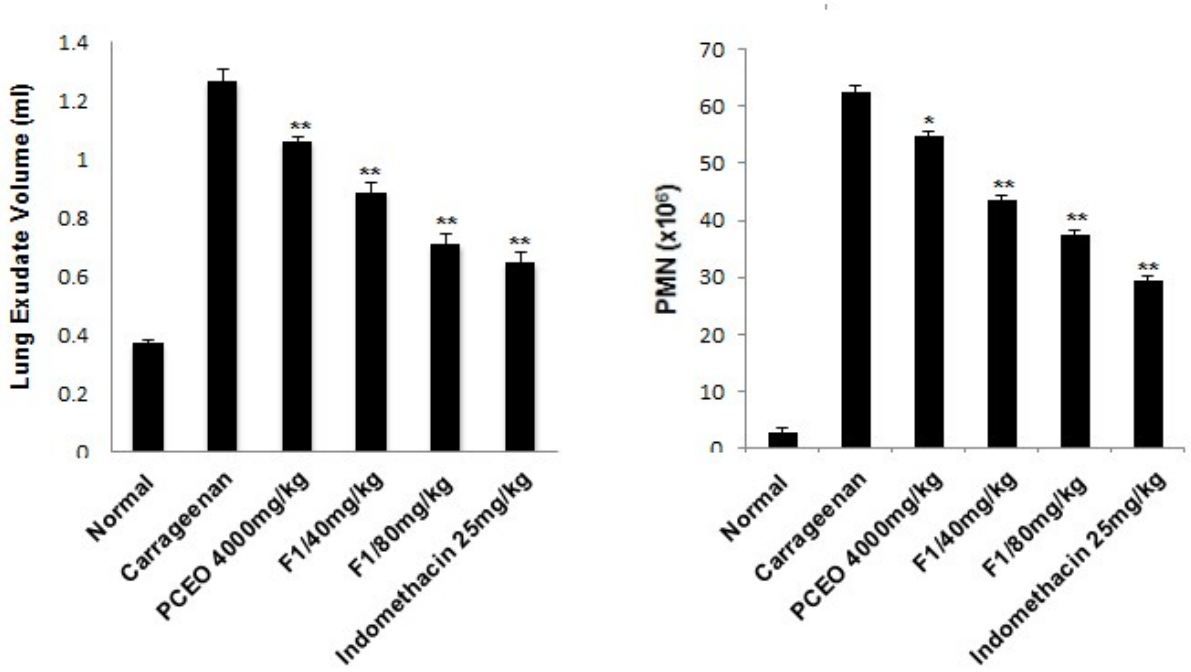


Figure 2 TEM images of optimized PCEO-S-SNEDDS (F1) having spherical shaped droplets in nanometer range



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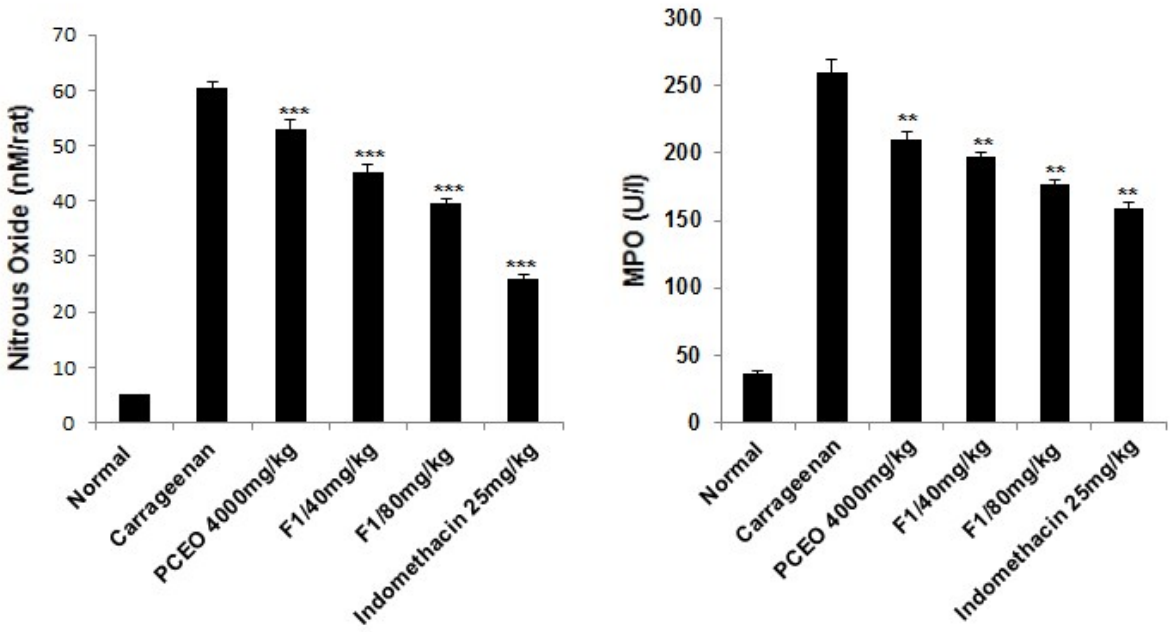

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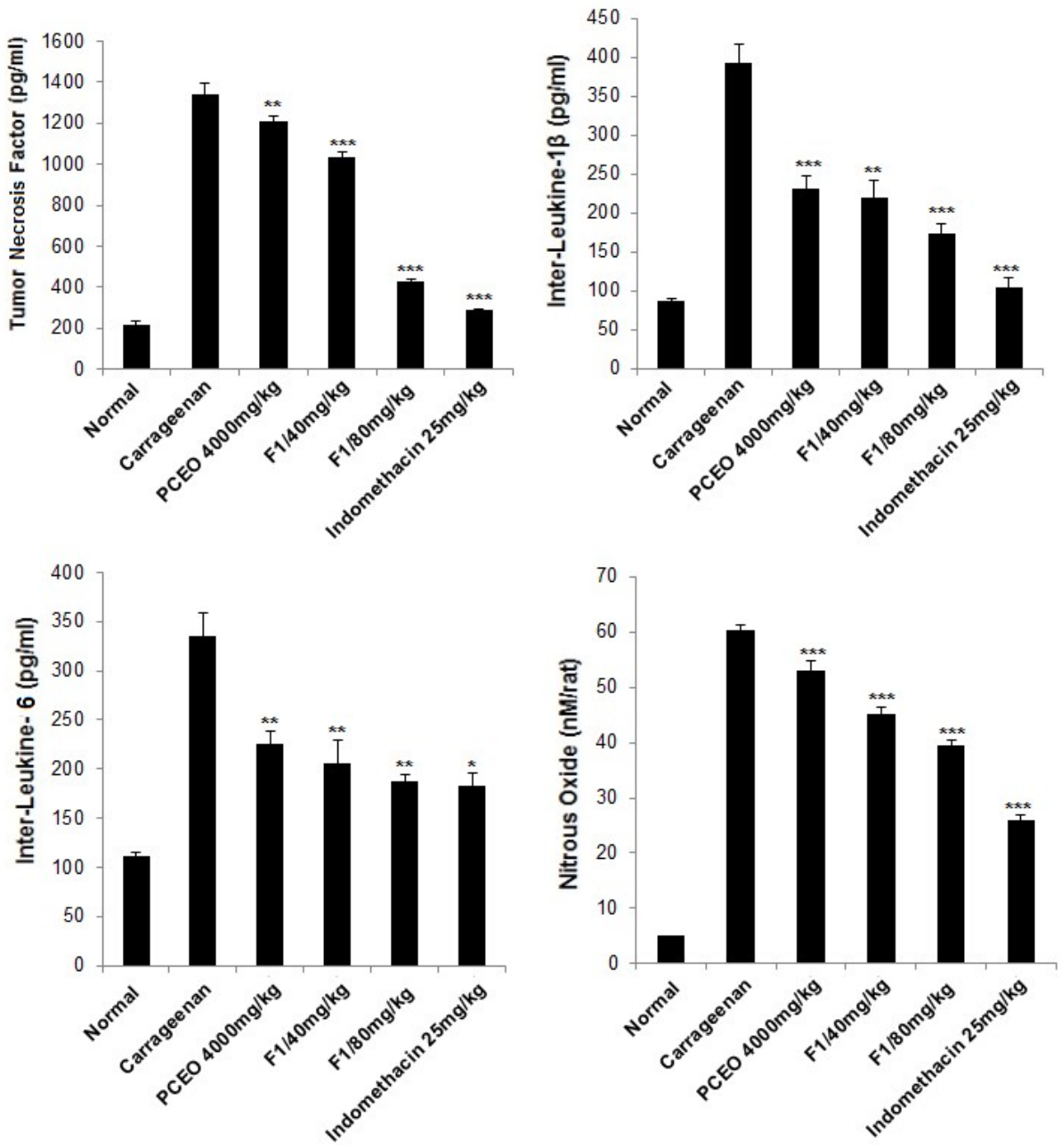

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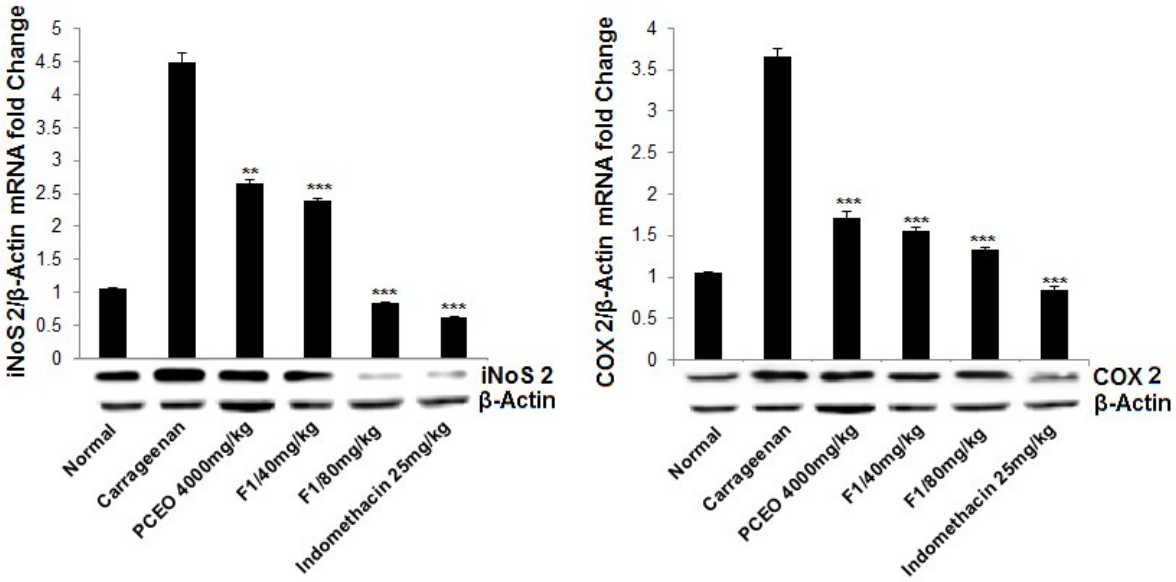

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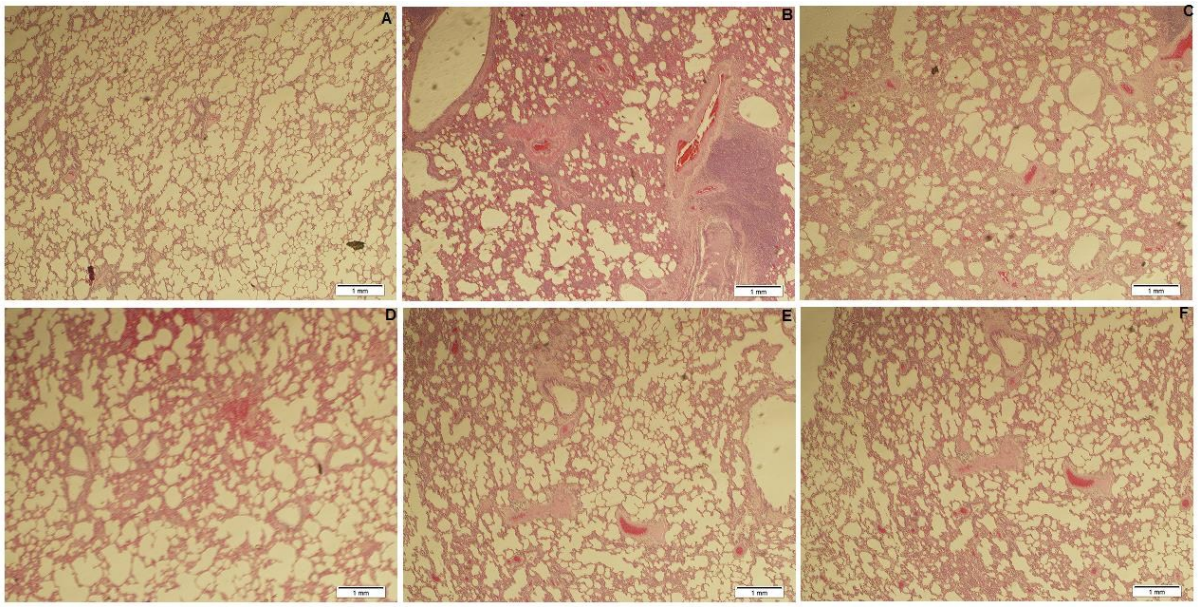

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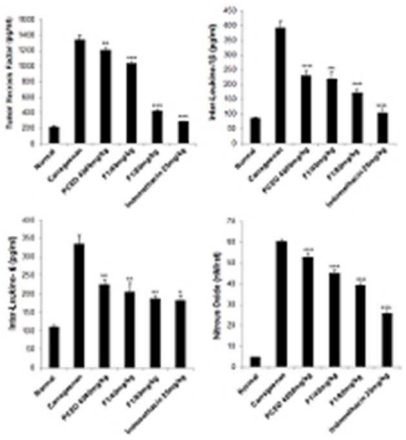

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