Development and evaluation of Novel krill oil based Clomiphene Microemulsion as a therapeutic strategy for PCOS treatment

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Abstract

Polycystic ovary syndrome (PCOS) is frequently diagnosed hormonal disorder with reproductive and metabolic complications. The most common symptoms include cyst in ovaries, anovulation, insulin resistance, and obesity. Clomiphene citrate, an ovulating agent, is the first-line drug used to treat PCOS. We hypothesized that clomiphene citrate, by stimulating ovarian function, with krill oil used as an oil phase to improve solubility, by addressing PCOS-associated symptoms might be effective in PCOS. Hence, our goal was to target hormonal imbalance along with PCOS-associated symptoms using single formulation. The concentration of water (X1), oil (X2) and Smix(surfactant-cosurfactant mixture) (X3) were selected as independent variables, in a simplex lattice design, from microemulsion area derived from pseudoternary phase diagram while the globule size (Y1) was selected as dependent parameter. The optimized microemulsion showed good sphericity having 41 nm globule size, 0.32 Poly dispersibility index and + 31mV zeta potential. The optimized microemulsion was further evaluated *in-vivo* using letrozole induced PCOS rats. Formulation treated group reversed the effect of letrozole on body weight and oestrus cycle in comparison to disease control group ($p < 0.001$). The formulation was also effective in reducing insulin resistance, cholesterol and serum testosterone level ($p < 0.001$). The *in-vivo* results were supported by histopathological studies where formulation treated group showed a marked decrease in the number of cystic follicles and a remarkable increase in the number of growing follicles at variable stages, similar to the

normal control group. Thus, the results confirmed that novel krill oil based clomiphene microemulsion may become promising therapeutic choice for treatment of PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder, associated with hormonal imbalance and metabolic anomalies, having a global prevalence of 6–16% [1]. The clinical features include hyperandrogenism, anovulation, insulin resistance, obesity, dyslipidemia, cysts in the ovaries, hirtusis, and seborrhea. Along with obesity, PCOS may also lead to psychological impairments, including depression and other mood disorders [1, 2]. It is the leading cause of anovulatory infertility in reproductive-aged women globally [3].

Clomiphene citrate (CC), a nonsteroidal selective oestrogen receptor modulator, is approved as first line treatment for oligo- or anovulation, primarily in patients with polycystic ovaries. It has an oestrogen agonist-antagonist properties. As an anti-oestrogen, clomiphene citrate displaces endogenous oestrogen form hypothalamic and pituitary oestrogen receptor sites alleviating negative feedback leading to alter the pulsatile gonadotropin-releasing hormone secretion. As a result, the pituitary gland releases FSH and LH which stimulates ovarian function. Despite the fact that clomiphene is particularly efficient at stimulating ovulation, long-term CC therapy leads to thinning of the endometrium linked to a decrease in endometrium receptivity and resistance to CC therapy [4, 5]. To overcome the stated constraint and improve the solubility of CC, alternative delivery systems such as lipid-based nanoformulation, cyclodextrin, and liquisolid tablets have been proposed [6–8].

Microemulsion (ME) is a multiphase, transparent, thermodynamically stable nanosystem composed of water, oil, surfactant, and co-surfactant. ME is the most exhaustively studied drug carrier due to its distinguishing characteristics like the ease of preparation, increased permeation, high solubilization capacity, high bioavailability, less intra- and inter-individual variation in pharmacokinetics, and the ability to carry hydrophilic and lipophilic drugs simultaneously [9–12].

MEs development using bioactive natural oils has piqued the interest of researchers and demonstrated significant commercial potential [13–16]. The combination of the intrinsic benefits of ME may be able to work in synergy with natural compounds [17, 18]. Krill oil is a marine source obtained from a species of Antarctic krill, Euphausia Superba, and is included in the list of foods generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA). It is a rich source of omega-3 fatty acids and has EPA and DHA fatty acids that are 1.6 times more absorbable than the EPA and DHA in fish oil [19, 20]. It has been used to treat high blood pressure, stroke, cancer, osteoarthritis, depression, and premenstrual syndrome (PMS) Krill oil activates AMP-activated protein kinase (AMPK), a therapeutic target for obesity and type 2 diabetes [20–25]. According to various research papers, krill oil can help reduce insulin resistance, hyperandrogenism, acne, and triglyceride levels, as well as symptoms of

cardiovascular disease and non-alcoholic fatty liver disease (NAFLD). It was hypothesized that preparing novel clomiphene citrate microemulsion was effective in stimulating ovarian function along with addressing other symptoms of PCOS like obesity and insulin resistance simultaneously.

Materials And Methods

1.1 Materials

Clomiphene Citrate (CC) was a gift sample obtained from Palam Pharma Pvt. Ltd, Ahmedabad, India. Krill oil was purchased from Inlife Health Care supplements, Hyderabad, India. Labrasol (Caprylocaproyl Polyoxyl-8 glycerides) and Transcutol HP (Diethylene glycol monoethylether) were received as gift samples from Gattefosse Pvt. Ltd. (Mumbai, India). Tween 80, Tween 20, Span 80, Polyethylene glycol 400, polyethylene glycol 600, and propylene glycol were purchased from S.D. Fine chemicals (Mumbai, India). All of the other ingredients were procured commercially and were of analytical quality. Throughout the experiment, double distilled water was utilized.

Selection of animals

Virgin, adult female Wistar Albino rats weighing 180–200 g were employed for the study. Animals were acquired from Torrent Research Centre, Ahmedabad, India and housed in our institute animal house for three weeks prior to study. During the study, animals were maintained at 25 ± 2°C temperature, 55 ± 5% humidity and a 12 h light/dark cycle. Animals were fed with standard diet and water ad libitum. Only rats with at least three consecutive regular oestrus cycle were used in the study. The study was approved by Institutional Animal Ethics Committee of L. J. Institute of Pharmacy with protocol number LJIP/IAEC/22–23/02 and animal care was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA).

1.2 Saturation Solubility studies:

For the optimization of the composition of CC ME, the saturation solubility of CC was assessed in various oils (oleic acid, coconut oil, spearmint oil, castor oil and krill oil), surfactants (Tween 80, Span 80, and Tween 20), and co-surfactants (ethanol, PEG 400, PEG 600, Transcutol HP, and propylene glycol). In the method, an excess of CC was added to each cap vial containing 5 ml of the selected vehicles. The resulting suspension was heated at 40 ± 2°C in a water bath, followed by shaking on a magnetic stirrer for 24 hours at room temperature to attain equilibrium. The resultant suspension was then centrifuged (R2, REMI) at 10, 000 rpm for 15 min, and the resultant supernatant was filtered through a Millipore membrane filter (0.22 µm) to eliminate any particles. The filtrate was diluted with methanol, and absorbance was measured against its corresponding blank at 260 nm by a UV spectrophotometer (UV 1800, Shimadzu, Japan). The test was done in triplicate [10, 24].

1.3 Selection of surfactant:

Surfactant screening is as important in microemulsion formulation as selection of an appropriate oil. The surfactant and co-surfactants were selected based on their ability to form a microemulsion. Tween 80, Span 80, Tween 20, PEG 400, PEG 600, PG and ratios of Tween 80: Span 80 (1:1), Tween 80: Span 80 (1:2), Tween 80: Span 80 (1:3), Tween 80: Span 80 (2:1), Tween 80: Span 80 (3:1) were taken for surfactant selection. For this, a 15% v/v solution of surfactant was prepared by dissolving it in water. From this solution, 2.5 ml was taken and 4 µg of oil was added each time slowly with constant stirring on a magnetic stirrer till a separate layer of oil was seen on the surface [26]. The produced emulsions were maintained for 1 hour before being tested for transmittance using UV spectrometry at 630 nm.

1.4 Development of Pseudoternary phase diagram:

The area of ME formation representing the suitable concentration of each component could be identified by the construction of a pseudoternary phase diagram. Three phase diagrams were constructed using Krill oil as an oil, Tween 80: Span 80 (3:1) as surfactant and Transcutol HP: Ethanol (1:1) as co-surfactant. The surfactant: cosurfactant (Smix) weight ratio (Km) used were 1:1, 2:1 and 3:1. The oil and Smix were mixed in the weight ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2 and 9:1 in separate glass vials and small amount of distilled water (10 µl) was added with continuous stirring and after attainment of equilibrium the mixture was visually checked for transparency and phase separation. The point at which the mixture exhibits turbidity or phase separation is considered as an end point [11–13].

1.5 Preparation of Clomiphene loaded microemulsions using simplex lattice design:

The data from preliminary batches was used for further optimization of microemulsion. The batch showing maximum oil incorporation in preliminary trials was selected for optimization by simplex lattice design. The factors like Water concentration (X1), Oil (Krill oil) concentration (X2) and surfactant (Tween 80: Span 80): co-surfactant (Transcutol HP:Ethanol) (**Smix)** concentration (X3) which were found to have significant effect on globule size (Y1) during preliminary study were selected as independent variables **(**Table 1**)**. Simplex lattice design using design of experiment software (Version 11.0, M/s Stat-Ease, Minneapolis, USA) [27] was used to find optimum concentration of water, oil and Smix in order to achieve globule size less than 70nm. Globule size was measured by using Malvern zetasizer (Nano ZS, Malvern instruments, UK) which is based on dynamic light scattering principle [28]. Each sample was suitably diluted 50 times with filtered distilled water to avoid multiple scattering effects and placed in a disposable zeta cell. To confirm the efficacy of the polynomial equation and generated contour plots in the manufacture of microemulsion check point tests were run.

Table 1
Simplex lattice design for CC microemulsion

Formulation components(%)/coded values			
Sample No	**Oil(Actual/coded)**	**Smix(2:1)(Actual/coded)**	**Water(Actual/coded)**
1	16.66/0.167	16.66/0.167	66.66/0.667
2	30/0.500	30/0.500	40/0.00
3	10/0.00	50/1.000	40/0.00
4	36.66/0.667	16.66/0.167	46.66/0.167
5	16.66/0.167	36.66/0.667	46.660.167
6	10/0.00	10/0.00	80/1.000
7	10/0.00	10/0.00	80/1.000
8	23.33/0.333	23.33/0.333	53.33/0.333
9	30/0.500	10/0.00	60/0.500
10	30/0.500	30/0.500	40/0.00
11	10/0.000	50/1.000	40/0.000
12	50/1.000	10/0.000	40/0.000
13	10/0.000	30/0.500	60/0.500
14	50/1.000	10/0.000	40/0.000

1.6 Physicochemical characterization of optimized clomiphene citrate microemulsion:

The viscosity of the optimized ME was measured using Brookfield Viscometer (Brookfield HADV III, Brook — field Engineering Laboratory, USA) using 61 number spindle rotating at 100 rpm at room temperature. Electrical conductivity of microemulsion was measured using a conductivity meter (CM 180 ELICO, India) at room temperature and the conductivity results were used to determine phase system. To confirm phase system results the dilution test and dye solubility test was performed. The pH of microemulsion was measured using a pH meter at room temperature (Systronic 335, India). Experiments were performed in triplicate for each sample. Percentage transmittance of prepared formulations was measured at 630 nm using distilled water as reference by UV spectrophotometer (UV 1800, Shimadzu, Japan) to check transparency of the ME. Zeta potential was measured using Malvern zetasizer (Nano ZS, Malvern instruments, UK). Globule size and polydispersibility index was measured by method described previously

in section 2.5. Zeta potential of undiluted ME was determined by using electrophoretic light scattering using Malvern zetasizer (Nano ZS, Malvern instruments, UK). Zeta potential data was collected over 30 continuous readings and all measurements were performed in triplicate. Transmission electron microscopy (TEM) (Talos F200i S/TEM (HRTEM-200KV) was used to study the ME's appearance. Copper nets were mounted onto a stencil plate to prepare samples for negative staining. MEs were gently placed onto the film after diluting it, which was then allowed to dry for approximately 20 minutes. The film was then treated with a drop of 2% w/v phosphotungstic acid and allowed to dry for 10 minutes before being examined under a TEM. Stability was also major quality parameter to be studied for ME. Thus, the optimized ME was studied by subjecting it to centrifugation test (3000 rpm for 15 min), and freeze thaw cycle [24, 26].

1.7 *In vitro* drug release study:

The *In vitro* drug release study was performed by using a regenerated seamless cellulose dialysis membrane of molecular weight cutoff of 12–14 KDa (D9402, HiMedia Laboratories Pvt. Ltd., India). The membrane was activated prior to use by washing the tubing with running tap water for 3–4 hrs. followed by treating it with a 0.3% w/v solution of sodium sulfide at 80°C for 1 minute. Then it was washed with hot water (60°C) for 2 minutes, followed by acidification with a 0.2% v/v solution of sulfuric acid and rinsing with hot water. The sac was prepared by using activated dialysis membrane and ME equivalent to 10 mg drug was placed into it. The prepared dialysis sac was immersed into 100 ml of 0.1 N HCl containing 20% PEG 400 v/v in order to maintain sink condition. The mixture was uniformly stirred at 50 rpm and the temperature was maintained at 37 ± 1℃ using water bath. The drug release study was performed for 2 hrs. and at every 15 min the aliquots from receptor media were withdrawn and replaced with equal volume of fresh media. The collected samples were filtered using 0.45 µm nylon filter paper, and the drug content was measured using a UV spectrophotometer. Similarly the study was performed for pure drug suspended in distilled water and marketed tablet preparation. The release data obtained were fitted in to the power law to determine release mechanism [9].

$Mt / M \infty = kt^{n}$

Where Mt/M ∞ is the fraction of drug release time t and k is the rate constant and n is the perfusion coefficient related to the mechanism of the drug release. The value of n between 0.5 and 1.0 indicates non-Fickian of anomalous perfusion, while n = 0.5 indicates Fickian release and n = 1 indicates zero-order release

1.8 *In vivo* studies:

1.8.1 PCOS Induction and Study Design: Females with regular oestrus cycle were selected for the study. The study consisted of 30 females divided into five groups of 6 each. Group 1 was treated as a control group, received 0.5% Carboxy Methyl Cellulose (CMC) for 21 days. All the experimental animals except

control group, were administered with Letrozole (1 mg/kg/day, per oral) dissolved in 0.5% w/v CMC solution for 21 days to induce PCOS [29]. Following PCOS induction, Group 3 received Clomiphene Citrate (2 mg/kg/day, per oral) in 0.5% w/v CMC solution and group 4 and 5 received microemulsion of Clomiphene Citrate (2 mg/kg/day) and krill oil (500 mg/kg/day) for 14 days (from day 22 to day 36) respectively[30, 31]. After completion of dosing period (21 days for Group 2, 36 days for group 3, and 4, and 5) animals were fasted overnight and anaesthetized with isoflurane. Blood was collected by retro orbital puncture and used for serum estimation of estradiol, testosterone, and cholesterol. Animals were sacrificed and ovaries excised, cleaned and weighed. The experimental design for in vivo studies is depicted in Fig. 1.

1.8.2 Oestrus Cycle Monitoring: Vaginal smears were collected daily by vaginal lavage using phosphate buffered saline and stained with Toluidine blue O to determine the reproductive cycle of each animal. As described previously [32] predominance of nucleated epithelial cells of relatively uniform appearance and size was classified as proestrus (First stage), Estrus stage (second stage) was characterized by anucleated keratinized epithelial cells occurring in clusters. Metaestrus (third stage) was characterized by a combination of neutrophils and anucleated keratinized epithelial cells. Lastly, diestrus stage (fourth stage), was characterized by moderate to low cellularity with a combination of neutrophils, large and small nucleated epithelial cells, and low numbers of anucleated keratinized cells.

1.8.3 Oral Glucose Tolerance Test: The test was done after induction of PCOS (21st day), and following completion of treatment (36th day). Rats were fasted overnight and glucose levels were determined in serum using glucose oxidase-peroxidase (GOD-POD)based kit before (time 0) a single oral administration of glucose (2 g/kg body weight) and after 30, 60, and 120 minutes of glucose administration. [33]

1.8.4 Sacrifice and Sample Collection: After completion of protocol, blood was collected by retro orbital puncture and used for cholesterol, estradiol and testosterone evaluation. Rats were sacrificed by cervical dislocation under diazepam (10mg/kg) and ketamine (50 mg/kg) anesthesia. Ovaries were removed, weighed and histological study was done.

1.8.4.1 Biochemical Parameters: Serum total cholesterol was estimated using standard colorimetric kits (Span Diagnostic Ltd., Gujarat, India). Serum estradiol and testosterone were assayed by immunosorbent Sandwich ELISA colorimetric method in ELISA reader (Multiskan™ GO, Thermo Fischer scientific) using GenXbio kit.

1.8.4.2 Histopathological Evaluation: Ovaries were fixed in 10% v/v neutral buffered formalin for 24 hours, following paraffin embedding. They were longitudinally and serially sectioned at 5 µm with microtome. The samples were stained with hematoxylin and eosin and assessed microscopically.

1.8.5 Statistical Analysis: The data for animal study is presented as mean ± S.E.M (Standard Error of the Mean). The significance of the difference in means between control and treated animals for different parameters was determined by One-way Analysis of Variance (ANOVA). Post-hoc comparison was carried out using Tukey's test. The analyses was performed using the software GraphPad Prism, Version 8.0.2 (Graphpad software, Inc.,California, USA). In all tests, the criteria for statistically significance was $p < 0.05$.

Result And Discussion

2.1 Solubility studies

The solubility of clomiphene citrate in various oils, surfactants, co-surfactants were determined and represented in Fig. 2. Highest solubility was observed in Krill oil that is also considered as Generally Recognized As safe (GRAS) by United States food and drug department (FDA). Previous studies reported reduction in synthesis of prostaglandins through inhibition of cyclooxygenase 2 (COX-2) along with reduction in cholesterol absorption and low-density lipoprotein-cholesterol synthesis, improves LDL receptor activity. It is also reported that omega-3 fatty acid present in krill oil has a beneficial effect on some cardiometabolic risk factors in women with PCOS. The selected oil phase must also increase absorption and bioavailability. Most of the fatty acids present in krill oil are in the form of phospholipids, which helps in increase in absorption of simultaneously administered drug [34]. According to various research papers shows beneficial effect of Krill oil in reducing insulin resistance, hyperandrogenism, acne, triglyceride levels that will also help in cardiovascular disease symptom [35]. Thus Krill oil was selected as an oil phase with the hypothesis of getting synergistic effect.

2.2 Selection of surfactant:

The selection of surfactant is also a critical step for effective production of ME as it imparts to the decrement of interfacial tension by establishing a film at the oil-water interface. Tween 80 was selected as surfactant from solubility study results but it has been observed that single surfactant was able to emulsify only small quantity of krill oil and hence emulsifying capacity of single surfacatnts and surfactant blends were determined for krill oil. The capacity of surfactant and surfactant mixtures to emulsify maximum amount of krill oil was determined by incorporating 4 µl of oil gradually till oil gets separated from the phase and results were represented in Fig. 3. It has been observed that higher oil can be emulsify by using combination of surfactants that achieves more surfactant partitioning to the interface in comparison to partitioning to the bulk phase that will allow stabilization of larger interfacial area and thus a high level of solubilization. Tween80: Span 80 (3:1) with HLB of 12.2 showed maximum emulsification for oil hence selected as surfactant for further studies. Similar results for drug solubility and optimum HLB were recorded by other investigators [36, 37].

2.3 Development of Pseudoteranry phase diagrams:

The components and their ratio selected from solubility study and emulsifying capacity study were further optimized by plotting the pseudoternary phase diagram (Fig. 4). In preliminary study it has been observed that addition of Transcutol as cosurfactant increase drug solubility but simultaneously reduces water incorporation and hence produced ME with very high viscosity exhibiting gelling behavior. Thus Ethanol has been added along with Transcutol as it has been reported that addition of short chain alcohol like ethanol leads to formation of a large one-phase single microemulsion region by increased disorder in the interfacial film [38]. Ethanol places itself in between surfactant heads results in higher dielectric constant and decrement in mixture viscosity making it easier for Tween 80 to reach to the

interface rapidly (Ibuprofen ME). Three pseudoternary phase diagrams were prepared by using oil (Krill oil), surfactant (Tween 80: Span 80 (3:1)) and cosurfactant (Transcutol: Ethanol (1:1)) as cosurfactant. Three pseudoternary phase diagrams were developed by employing three Km ratios (surfactant:cosurfactant) (1:1, 2:1and 3:1). The results indicated highest microemulsion region obtained with the Km ratio of 2:1. Thus Km ratio 2:1 showed increase in solubility as well as increases water incorporation and decrease viscosity of ME thus, it was chosen for further research.

2.4 Preparation of Clomiphene loaded microemulsions using simplex lattice design: The levels for independent variables were selected based on pseudo ternary phase diagrams and are as below:
$10\% \leq X1 \leq 50\%$

$10\% \leq X2 \leq 50\%$

$40\% \leq X3 \leq 80\%$

To make the computations easier, the real levels of oil, Smix, and water were converted to a simpler level using a simplex lattice design, with the minimum level equaling zero and the highest level equaling 100 [39]. On the basis of pseudo ternary phase diagrams, the levels of three independent variables were chosen. The overall concentration of water, surfactant, and oil phase in the formulation remained fixed at one while the ratio of the three was altered. Fourteen batches were created as follows: three vertexes (A, B, C), three one-third points between vertices (AB, BC, AC), three two-third points between vertices (AB, BC, AC), and the centre point (ABC). The globule size is the defining hallmark for the stability of microemulsion [40] and hence the identification of the combination that yield smallest globule size was essential step for the process of optimization.

The ANOVA for globule size (Table 2) was performed suggesting a Quadratic model with an F value of 12.82. The lack of fit was non-significant, which is good as we want the model to fit. The polynomial equation generated for globule size is as below:

Equation for Globule Size Y = + 47.68 water + 286.71 oil + 18.07 smix + 305.37 water.oil – 143.86 water. smix – 319.93 oil. smix

As seen from the polynomial equation, with increase in water concentration, oil and Smix concentration, the globule size increased whereas water and smix and oil and smix were found to have negative effect on globule size. As the quantity of oil along with Smix in the microemulsion globules is increased, the globules tend to constrict [41]. The contour plot and 3D response surface graphs for globule size are depicted in Fig. 5. The overlay plot showing design space as indicated by yellow region is shown in Fig. 6.

The checkpoint batches to validate model were prepared, and responses were determined. The observed and predicted responses obtained are shown in Table 3. It was discovered that the predicted and observed responses did not differ by more than 5%, indicating the validity of applied mathematical model.

Table 2
ANOVA for globule size

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	12412.38	5	2482.48	12.82	0.0012	Significant
Linear Mixture	11970.04	2	5985.02	30.91	0.0002	
AB	221.41	1	221.41	1.14	0.3161	
AC	34.20	1	34.20	0.1766	0.6853	
BC	169.16	1	169.16	0.8736	0.3773	
Residual	1549.03	8	193.63			
Lack of Fit	1317.28	4	329.32	5.68	0.0605	not significant
Pure Error	231.75	4	57.94			
Cor Total	13961.41	13				

Table 3
Checkpoint Batches

Sample No	Globule size(nm)	
	Actual	Predicted
Checkpoint Batch 1	59.9	60.00
Checkpoint Batch 2	41.1	43.16
Checkpoint Batch 3	50.1	51.0
Checkpoint Batch 4	87.6	89.26

2.5 Physicochemical characterization of optimized clomiphene citrate microemulsion: The conductivity and pH of the 40optimized ME was 49 ± 4 µS/cm and 6.4 ± 0.2 respectively. ME with low conductivity (less than 10 µS/cm) was considered as w/o ME and those with conductivity value higher than 10 µS/cm was considered as o/w ME [39] and thus the conductivity results confirms the prepared ME was o/w. The results were further confirmed by the dilution test, it was discovered that the optimized CC ME could be easily diluted with water, implying that the generated MEs were of the O/W type. A staining test revealed that water soluble dye, methylene blue, was easily miscible with ME, indicating that O/W ME had formed. The pH value obtained were near to physiological pH range. The globule size was found to be 41.1 ± 0.047 nm with the PDI value of 0.323 (Fig. 7a) which indicates globule size were in nanometer range with even distribution in formulation [40, 41]. The zeta potential was 31 ± 0.068 mV (Fig. 7b), the positive zeta potential value obtained might be due to traces of mineral impurities present in Krill oil [42]. As nonionic

surfactant stabilize the microemulsion by stearic hindrance zeta potential does not play any significant role in terms of stability of optimized microemulsion. Viscosity of the optimized ME was 142 ± 5 cps. TEM images depict that the particles present on optimized MEB16 have uniform and spherical particle distribution (Fig. 7c). Particles are seen under 100x and 50x magnifications and particle size was observed between 35-49.5 nm which is found to be similar to the results obtained of globule size. There was no phase separation in the produced CC ME after the centrifugation and freeze thaw cycle, indicating that the ME was stable. The globule size obtained after centrifugation cycle and freeze thaw cycle was 42.54 nm and 43.78 nm respectively suggesting formation of stable CC ME.

2.6 *In vitro* drug release study:

The *In vitro* drug release study was executed for optimized CC ME, pure drug and marketed preparation using activated cellulose dialysis membrane to understand possible effect of formulation components such as oil, surfactants and cosurfactants on drug release. The comparative release profile of CC from different formulations was depicted in Fig. 8 and it has been observed that CC ME showed faster drug release in comparison to pure drug and marketed preparation and might be due to presence of surfactant and smaller globule size. The biphasic release profile obtained can be explained by the initial higher permeation due to miceller solubilization of drug while the slower release rate later might be due to drug release from the oil droplets to the receiver chamber through the continuous phase in the donor chamber [12]. The drug release profile were further evaluated by fitting the *In vitro* release data to equations describing different kinetic orders & linear regression analysis were made. The results indicated the drug release can be best explained by Higuchi model as obtained R^2 was 0.9099. Higuchi's mathematical model suggest a pure diffusion release mechanism of drug from ME. The release mechanism was further verified by applying Korsemeyar peppas model as a decision parameter. As n value obtained after data fitting into power law was 0.5345 suggested the release mechanism was non-Fickian or anomalous diffusion. The results conformed to the release kinetics obtained for ME by previous scientists [13].

3.7 *In vivo* studies:

3.7.1 Effect of different treatments on Body weight and relative weight of Ovaries

As showed in Fig.9, body weight prior to PCOS induction was comparable across all groups. Following 21-day letrozole administration, body weight increased by at least 17% ($p<0.001$) as compared with control group. In comparison to the disease control group, 14 days of treatment with the standard, microemulsion, and krill oil reversed the effects of letrozole on body weight by at least 13.83% ($p<0.001$), 14.19% ($p<0.001$), and 3.93% ($p<0.001$), respectively. After letrozole administration, a significant weight gain was observed, which may be attributed to hyperandrogenism and hyperinsulinemia. The antiestrogenic actions of the CC may have contributed to the reversal effect on body weight after therapy (groups 3 and 4) [43, 44]. We also observed significant weight loss in the krill oil-treated group. Krill oil-dependent AMPK activation suppresses fatty acid synthesis genes, including ACC, FAS, and SERBP-1

[45]. Consequently, CC and krill oil may both lower body weight through several mechanisms involving, respectively, anti-estrogenic characteristic45s and AMPK activation.

In the disease control group compared to the control, there was a significant rise in mean ovarian weight ($p < 0.001$), as shown in Fig. 10. After 14 days of treatment with standard ($p < 0.01$), microemulsion ($p < 0.01$), and krill oil ($p < 0.05$), ovarian weight reverted to normal.

The anabolic effect of letrozole on lipid metabolism and glucose intolerance may have contributed to the rise in relative weight of the ovaries following treatment. In a histological investigation, the formation of multiple cysts was also noted [46, 47]. Treatment with CC (groups 3 and 4) induced a significant reduction in relative ovarian weight, which might account for the restoration of normal follicular formation and function as confirmed by histopathology. We also observed a decrease in ovarian weight in the krill oil-treated group compared to the disease control group, even though the difference was not statistically significant. This might be because krill oil contains n-3 PUFAs, which are known to improve intracellular catabolism and lipid regulation [48].

3.7.2 Effect of different treatments on oestrus cycle

Vaginal smears showed nucleated epithelial cells, anucleated keratinized epithelial cells, and neutophiles. Proestrus phase indicated a high number of nucleated epithelial cells. Estrus phase revealed anuleated keratinized epithelial cells, metaestrus was confirmed by the presence of nutrophiles, and dieastrus revealed nucleated cells together with small and large nucleated epithelial cells.

Estrus cycle in control group lasted for 5 days with following successive phases: proestrus, estrus, metaestrus and diestrus. In other groups, Letrozole blocked the cycle in the diestrus phase. The cyclic appearance resumed in standard and microemulsion treated group post 10 days and 7 days respectively. Krill oil-treated group showed proestrus phase after 9 days and resumed normal after 13 days of krill oil administration (**Fig. 11**).
The oestrus cycle was negatively impacted in PCOS rats, mostly as a result of changes to the steroidal hormones that control ovarian function [49]. In contrast to control rats, letrozole-treated rats displayed an irregular oestrus cycle (75.5% of days were spent in the diestrus phase). The percentage of days spent in the diestrus phase after treatment with conventional and microemulsion decreased to 56%. As previously stated, CC stimulates FSH and LH release through a negative feedback mechanism, and the CC treated groups showed a normal oestrus cycle. In addition, the krill oil-treated group spent 67% fewer days than the disease control group. By lowering testosterone levels, omega-3 fatty acids are thought to normalise the menstrual cycle [50]. As a result, Krill oil, which contains Omega-3 fatty acids, may aid in the improvement of the oestrus cycle.

3.7.3 Effect of different treatments Oral Glucose Tolerance Test

An insulin-resistance phenotype was confirmed by an oral glucose tolerance test where Letrozole disturbed glucose homeostasis. Following glucose loading, the levels in control animals peaked after 60

minutes and returned to normal after 120 minutes on day 21. However, not every group experienced the same reversal of glucose. The area under the curve (AUC) differed significantly from the control, demonstrating the presence of insulin resistance (Fig. 12)

In comparison to the control group, the groups that received standard and microemulsion treatments had lower insulin resistance (with statistical significance, $P < 0.0001$). The AUC of the microemulsion-treated group significantly differed from the disease control group, as shown in **Fig. 13a and 13b**, demonstrating an improvement in insulin resistance. We also observed a significant difference between the groups that received conventional and formulation treatments.

Insulin resistance has been discovered in 75% of obese and 30% of lean PCOS patients, and its severity is more than would be expected given the age and weight [48, 51]. Compared to fasting glucose test, the Androgen Excess Society advises an oral glucose tolerance test (OGTT) for PCOS [52, 53]. Following the delivery of letrozole for 21 days and the 14-day treatment period, we assessed the OGT. By preventing the conversion of testosterone to oestrogen, letrozole increases endogenous testosterone levels [54]. High endogenous testosterone attenuates insulin sensitivity and impairs oral glucose tolerance [55]. After administering letrozole for 21 days, we noticed comparable results. We observed improved glucose tolerance after 14 days of therapy with standard and microemulsion. Additionally, the krill oil-treated group showed improved glucose tolerance in comparison to the disease control group, however the difference was not statistically significant. While raising plasma levels of IGFBP-I, clomiphene considerably reduces the IGF I: IGFBP I ratio [56]. According to reports, krill oil reduces insulin resistance; the bioavailability and structure of the − 3 fatty acids in krill oil may be responsible for this effect. Therefore, in our investigation, the presence of ω-3 fatty acids and a lower IGF: IGFBP I ratio may have enhanced insulin tolerance.

3.7.4 Effect of different treatments on serum cholesterol

The serum cholesterol test was performed both after disease induction and at the end of treatment. We noticed that letrozole-treated groups had higher serum cholesterol levels than control groups, which supported the induction of PCOS. When compared to the disease control group, the changes were dramatically recovered to normal levels in the standard ($p < 0.01$) and microemulsion treated groups at the end of treatment. (Fig. 14)

PCOS has been linked to dyslipidaemia. Following letrozole treatment, we observed a significant rise in cholesterol, which is linked to hyperandrogenism [57]. Androgen receptors found on adipocytes and testosterone block catecholamine-induced lipolysis in abdominal subcutaneous preadipocytes [57, 58]. Because Letrozole inhibits aromatase, it causes hyperandrogenism, which raises cholesterol levels due to its antilipolytic action. However, the results of clomiphene therapy differ. Numerous studies [59, 60] found that clomiphene citrate treatment increased plasma triglyceride and cholesterol levels, while other studies [61, 62] found improvements in lipid profiles, notably cholesterol levels. We found improved plasma cholesterol after the administration of clomiphene citrate in both standard and microemulsion forms. The result could be attributed to clomiphene's estrogenic action [63]. Oestrogen lowers cholesterol levels in

rats by stimulating LDL-receptors [64]. We also noticed decreased cholesterol levels in the krill oil-treated group, even though the difference was not statistically significant. The expression of mitochondrial citrate carrier (CiC), which prevents the buildup of cholesterol in hepatocytes, is downregulated by krill oil, which limits the production of fatty acids [48]. As a result, clomiphene, which has estrogenic activity, and kill oil, which inhibits fatty acid synthesis, may have improved cholesterol levels in the microemulsion-treated group.

3.7.5 Effect of different treatment on Testosterone and Estradiol

When compared to the control group, letrozole treatment reduced the serum level of estradiol by 60.73% ($p < 0.001$). As compared to the disease control group, treatment with the standard, formulation, and krill oil increased estradiol levels by 50.92% ($p < 0.01$), 92.06% ($p < 0.001$), and 33.52% ($p < 0.05$), respectively **(**Fig. 15**).**

Serum testosterone levels increased by 40.22% ($p < 0.001$) more in the letrozole-treated group compared to the control group. Treatment with standard and microemulsion reduced this by 16.60% ($p < 0.01$) and 13.86% ($p < 0.05$) compared to disease control, respectively.

The amount of oestrogen produced by the dominant follicle has an effect on follicular vitality and ovulation success [65]. LH induces theca cells to create androgen in a healthy ovary, while FSH prompts granulosa cells to aromatize this androgen into estradiol. Together, these two hormones aid in the maturation and growth of the follicles [66]. After 21 days of Letrozole treatment, inhibition of androgen aromatization to oestrogen results in low serum estradiol concentrations and high levels of androgen compared to disease control levels. The results are consistent with previous studies [65]. Clomiphene citrate treatment increased oestrogen levels while decreasing testosterone levels in the hypothalamus by promoting follicular growth and inducing ovulation. Omeg-3 fatty acids in krill oil, according to reports, reduce the availability of arachidonic acid by preventing LH-stimulated steroidogenesis [67, 68]. Arachidonic acid is essential for the regulation of the steroidogenic acute regulatory gene and the synthesis of steroids [69]. Therefore, by having an anti-estrogenic action and preventing steroidogenesis, a microemulsion may improve estradiol and testosterone levels.

3.7.6 Histopathology: Histopathological results were assessed based on the visibility of corpora lutea, cystic and, graafin follicles. In the control group, the growing follicles (at variable stages) with several fresh corpora lutea were visible. A high number of follicles with cysts and a minimal number of corpora lutea was reported in the disease control animal. CC-treated animals (Groups 3 & 4) showed a less number of cystic follicles and an increase in the numbers of both corpora lutea and growing follicles. Additionally, group 4 (microemulsion treated) showed a marked decrease in the number of cystic follicles and a remarkable increase in the number of follicles (at variable stages), similar to the normal control group. Krill oil treated group also showed beneficial results with less number of cystic follicles (Fig. XVI).

Conclusion

PCOS is a frequently diagnosed hormonal disorder with reproductive and metabolic complications. Excess androgens and LH levels are the main anomalies under reproductive complications, and clinical symptoms are typically seen at a reproductive age. The novel CC ME in krill oil was developed using a simplex centroid design. The ME was stable with globule sizes in the nm range and showed better diffusion in comparison to the marketed preparation. The ME was able to reduce insulin resistance, cholesterol, and testosterone and improve ovulation, as confirmed by a histopathology study. Thus, the prepared formulation is a good candidate for PCOS treatment as it treats various symptoms simultaneously.

Declarations

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Author contribution: Darshita Panchal: methodology; validation; **Tosha Pandya:** writing original draft, editing; **Vijay Kevlani:** Animal study, editing; **Dr.Shreeraj Shah:** funding resources; **Dr.Sheetal Acharya:** conceptualization, resources, supervision, project administration, funding acquisition,

Availability of data and materials All data generated or analyzed during this study are reported in this article (and its Supplementary information files) and the datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate The experiments reported herein did not involve any human or animal subjects. The experiments performed herein comply with the current laws of the country.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests

Author contribution: Darshita Panchal: methodology; validation; **Tosha Pandya:** writing original draft, editing; **Vijay Kevlani:** Animal study, editing; **Dr.Shreeraj Shah:** funding resources; **Dr.Sheetal Acharya:** conceptualization, resources, supervision, project administration, funding acquisition,

Availability of data and materials All data generated or analyzed during this study are reported in this article (and its Supplementary information files) and the datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

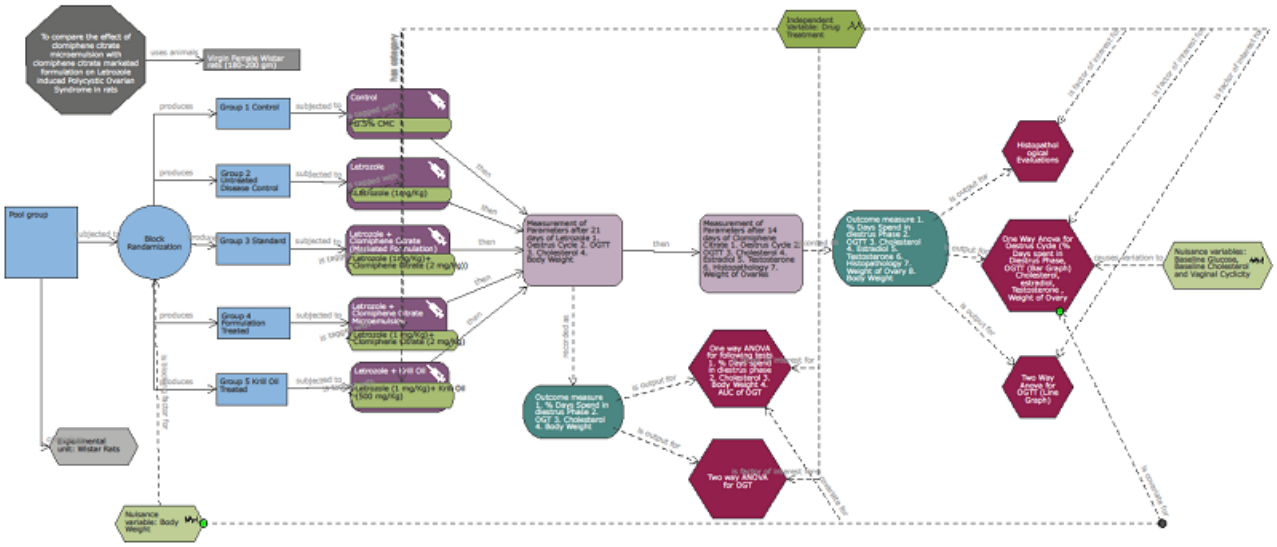


Figure 1

Experimental Design for *in vivo* study (Prepared using Experimental Design Assistant, NC3Rs)

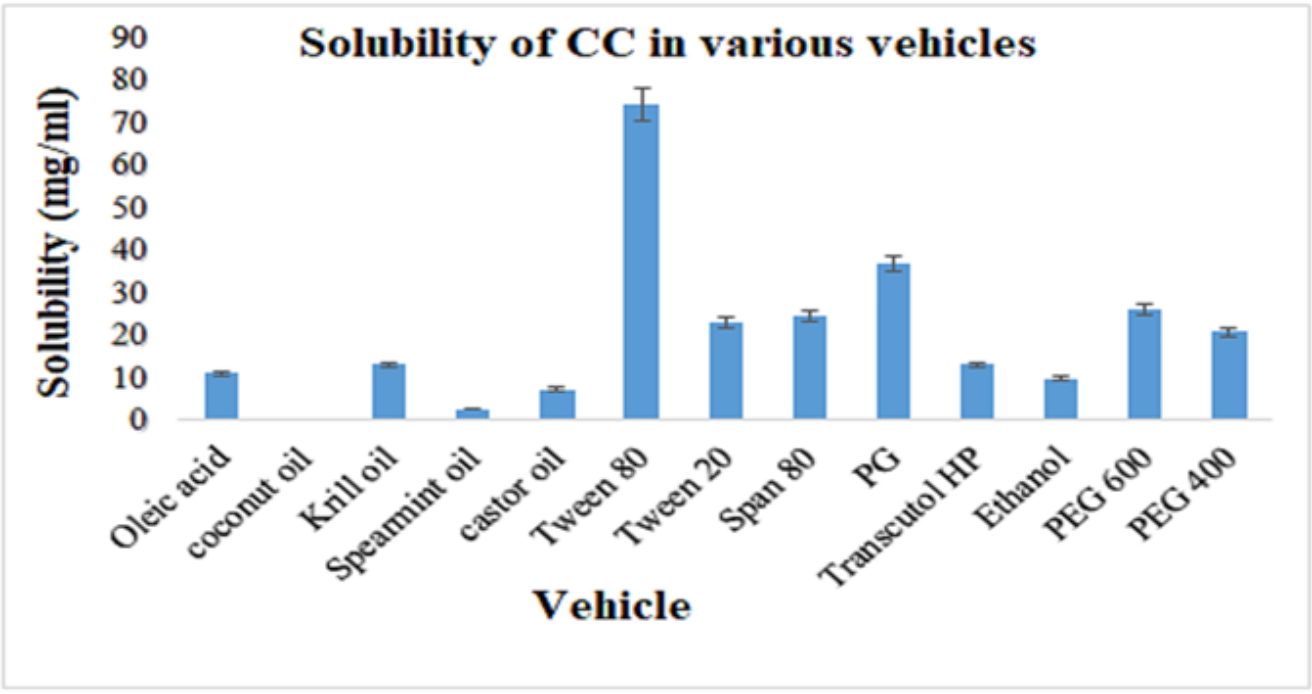


Figure 2

Solubility of clomiphene citrate in various oils

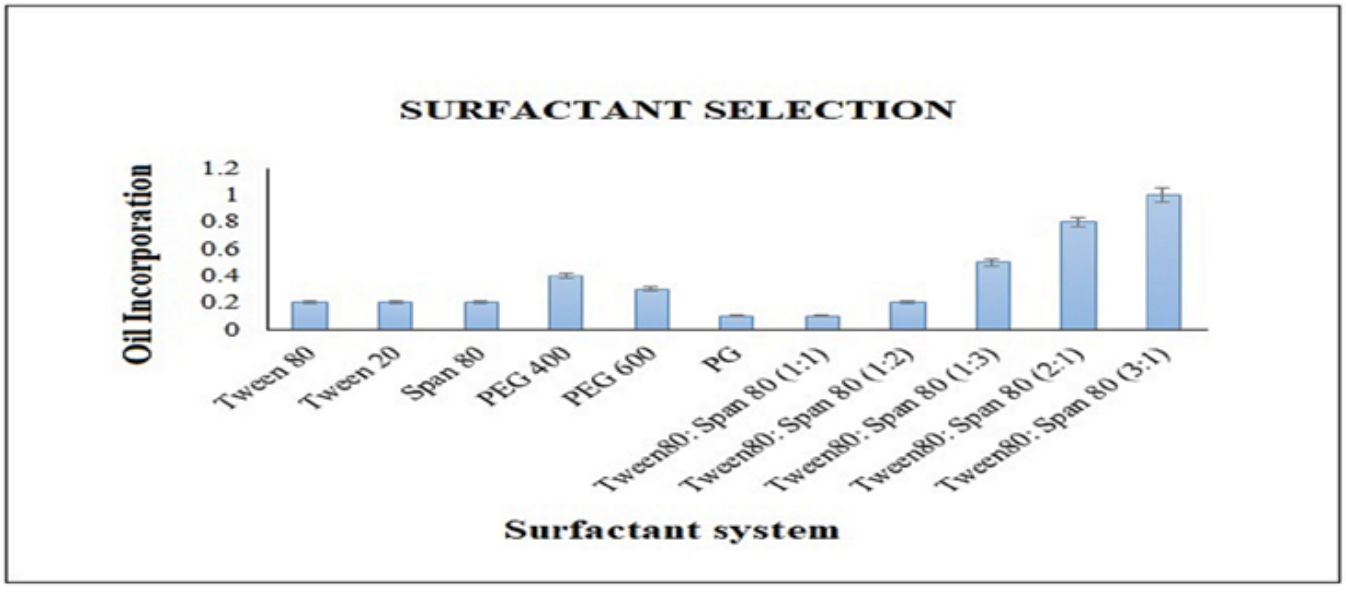


Figure 3

Surfactant and surfactant mixtures effect to emulsify maximum amount of Krill oil

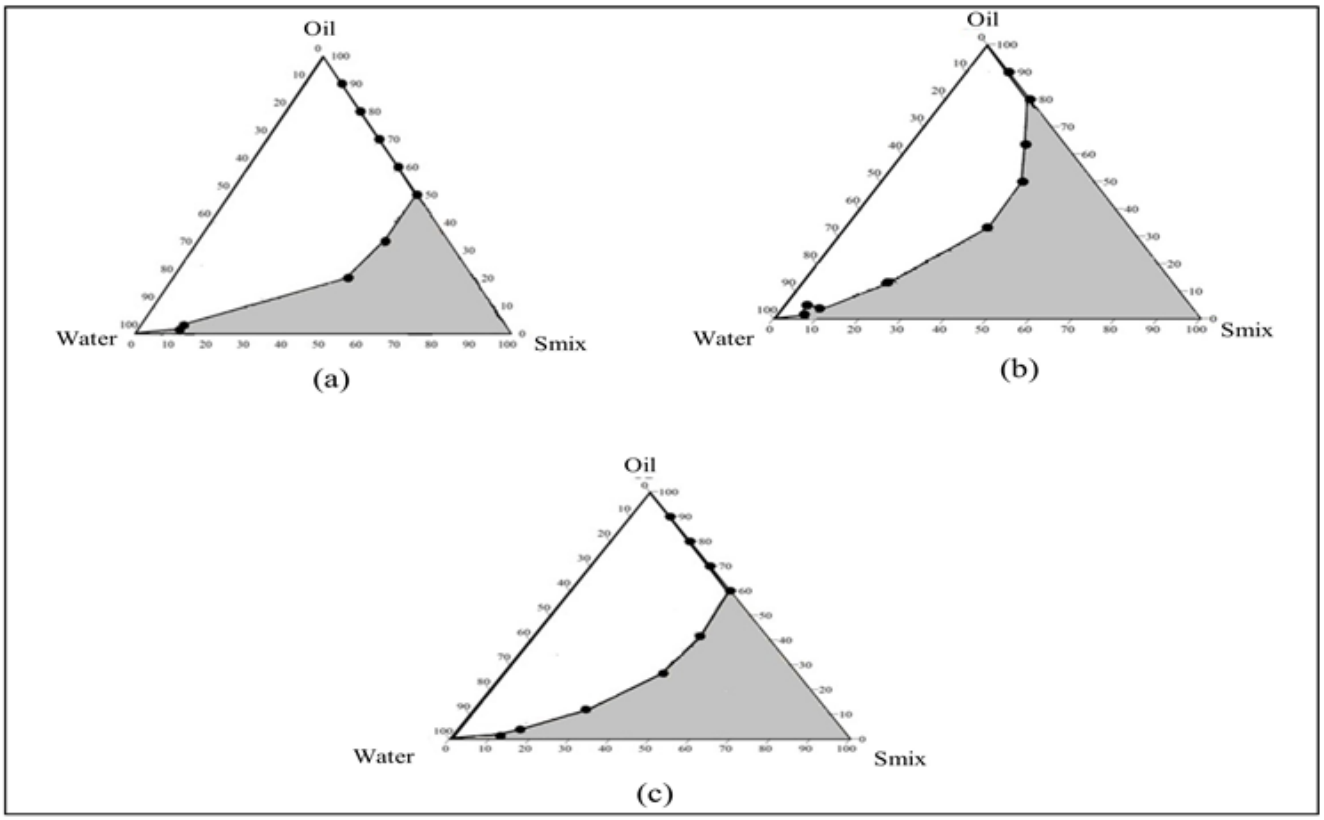


Figure 4

Pseudoternary phase diagram to select different components and their ratios for microemulsion

Figure 5

Contour plot and 3D surface plots for globule size

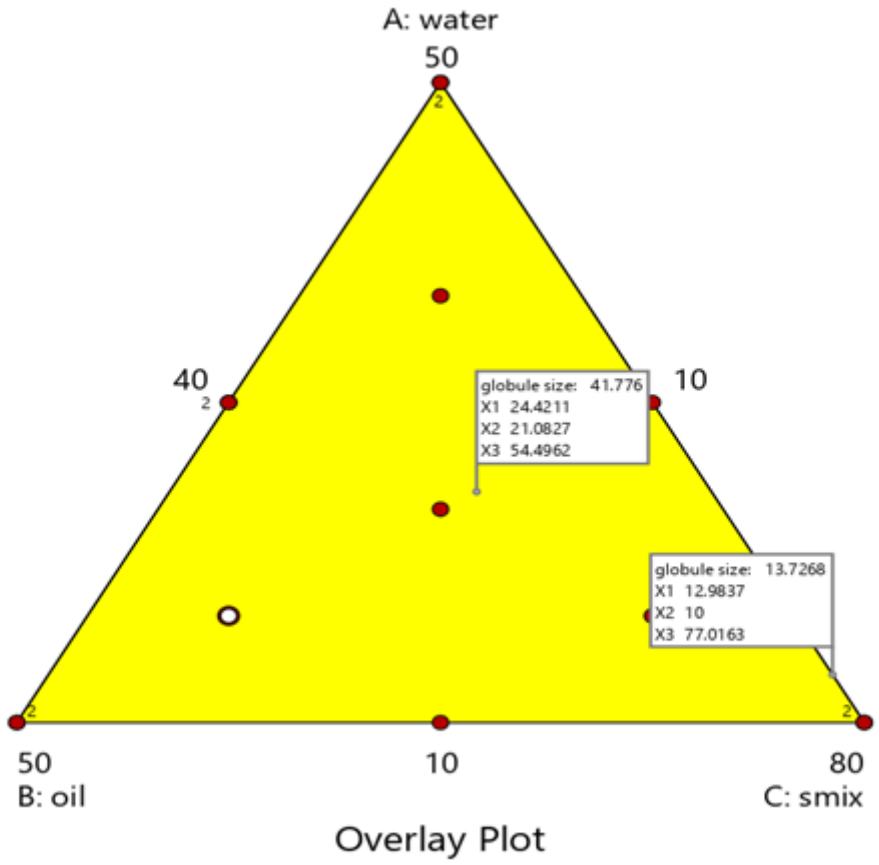
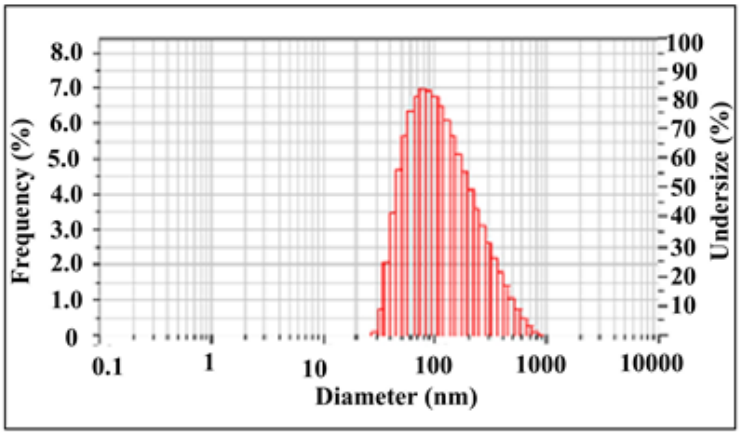
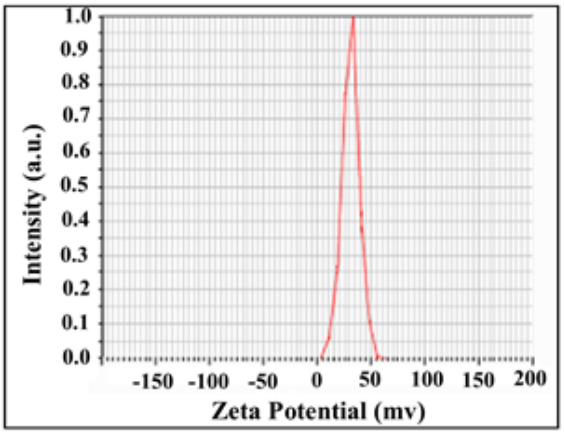


Figure 6

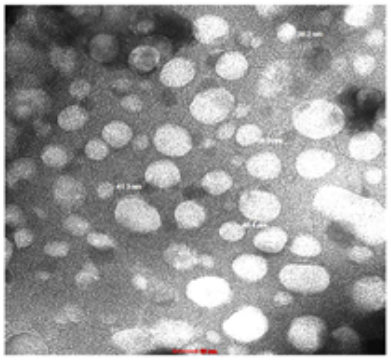
Overlay Plot



(a)



(b)



(c)

Figure 7

(a) Particle size distribution (b) Zeta potential analyis (c) TEM image of optimized microemulsion

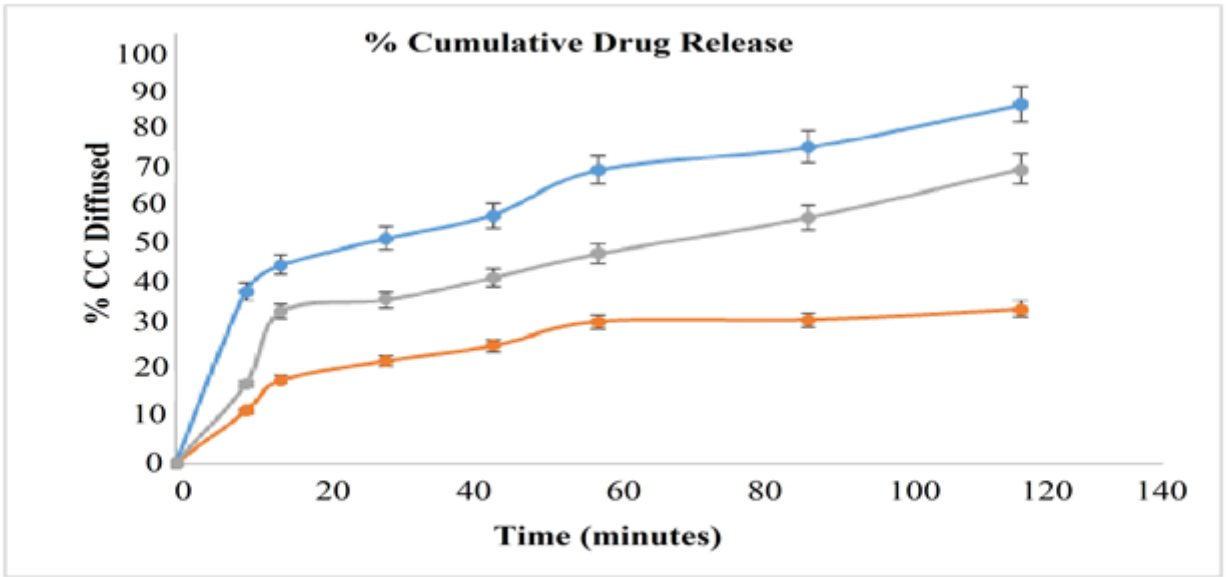


Figure 8

In vitro drug release study

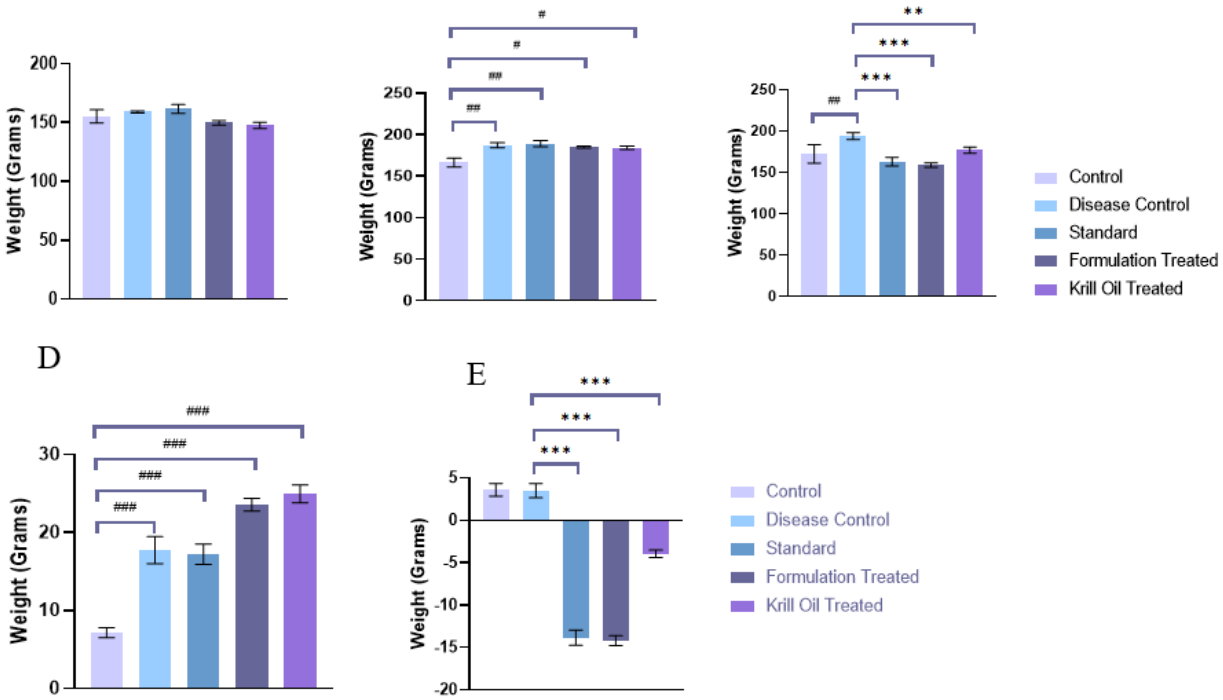


Figure 9

Body Weight. A: Body weight before Letrozole, B: Body Weight following 21 days Letrozole treatment, C: Body weight following 14 days treatment, D: % Body Weight Change following Letrozole Administration, E: % Body Weight Change following 14 days Treatment (Results are presented as mean±SEM, n= 6, ### p<0.001 vs Control and *p<0.05, **p<0.01 vs Disease Control.)

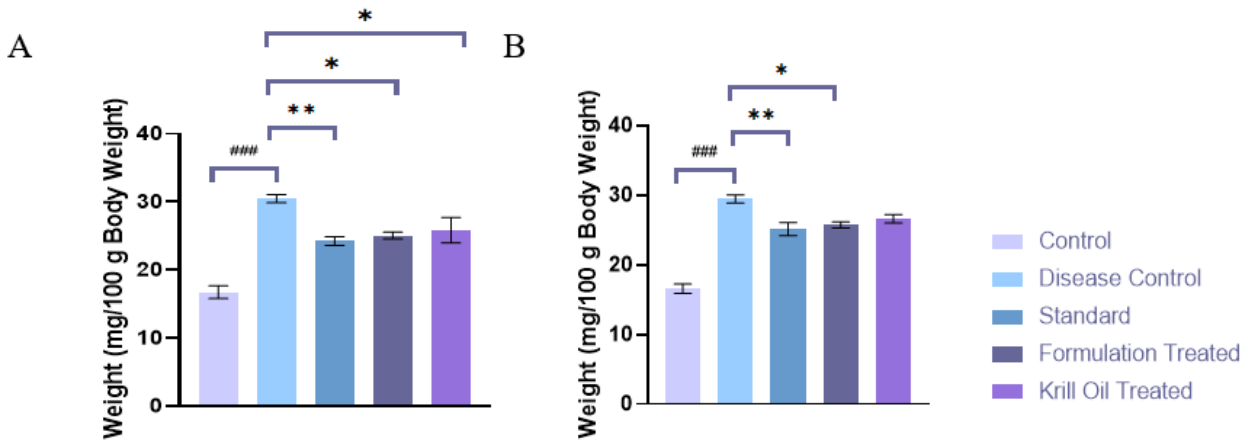
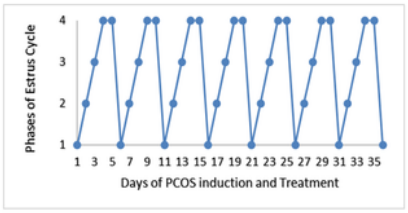
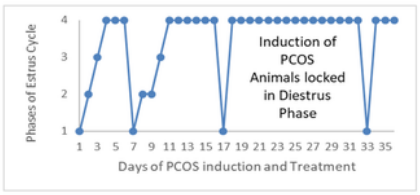


Figure 10

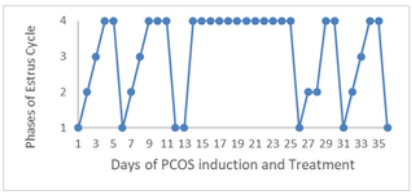
Weight of ovary after treatment. A: Relative weight of right ovary, B: Relative weight of left ovary



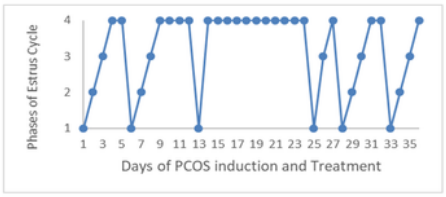
A



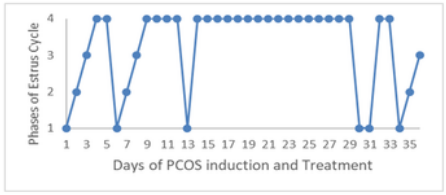
B



C



D



E

Figure 11

Effect of letrozole and treatment on estrus cycle. A: Control, B: Disease Control, C: Standard, D. Formulation Treated, E: Krill Oil Treated, 1: Prophase, 2: Estrus Phase, 3: Metaestrus Phase, 4: Diestrus Phase

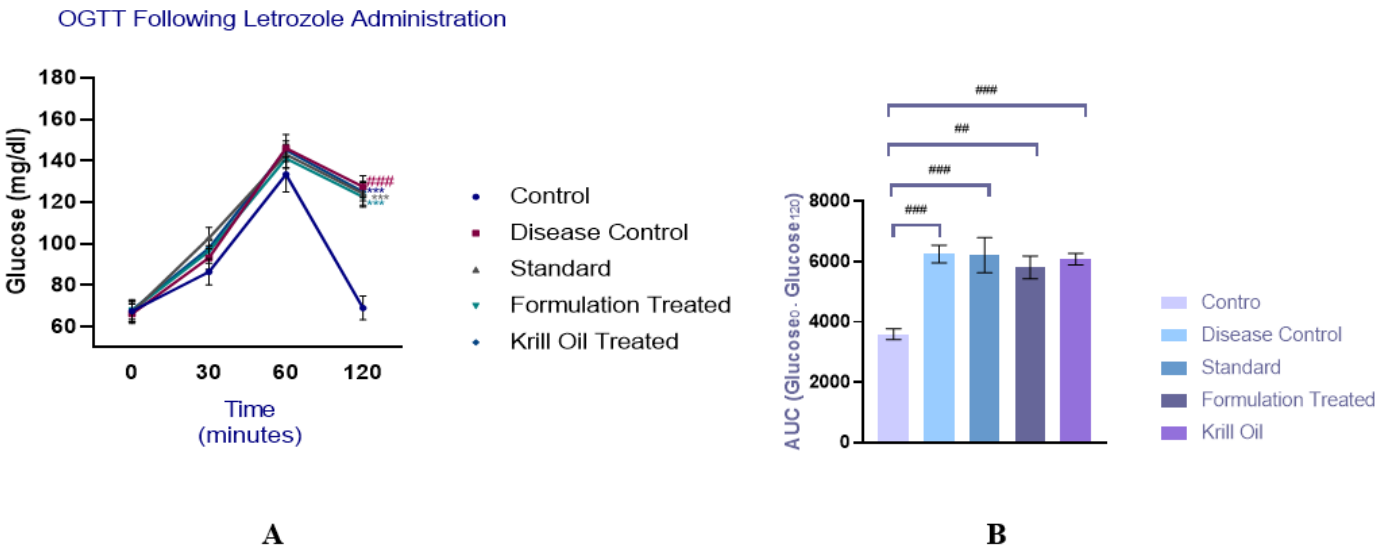


Figure 12

Effect of letrozole on OGT day 21. A: OGTT following Letrozole Administration, B: AUC following Letrozole administration

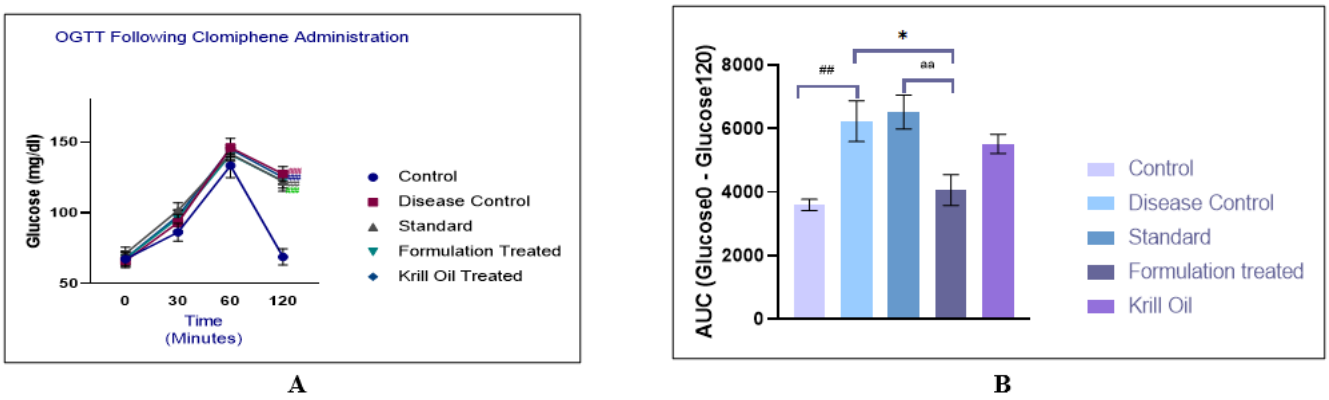


Figure 13

Effect of Treatment on OGT day 36. A: OGTT following Treatment, B: AUC following Treatment

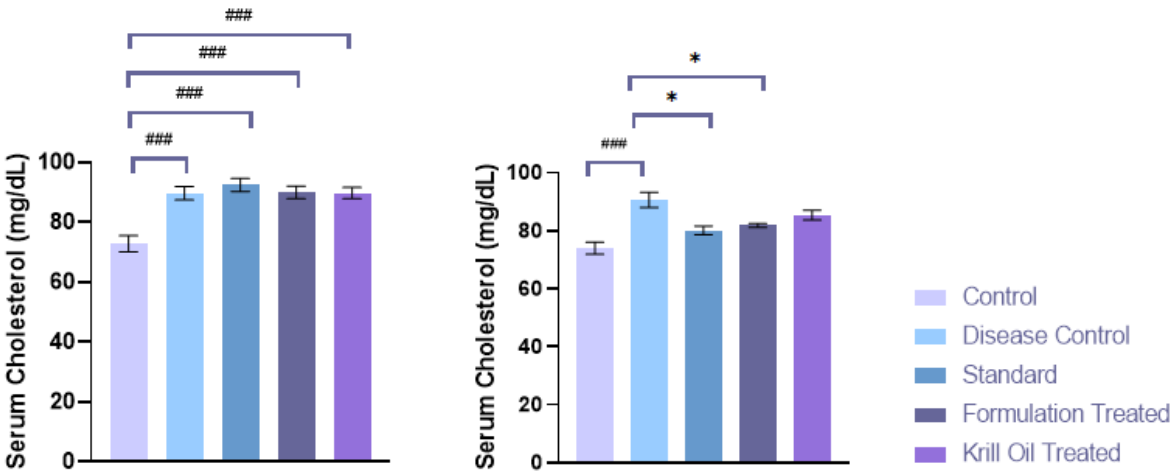


Figure 14

Serum cholesterol. A: Cholesterol following 21 days Letrozole administration, B: Cholesterol following 14 days treatment

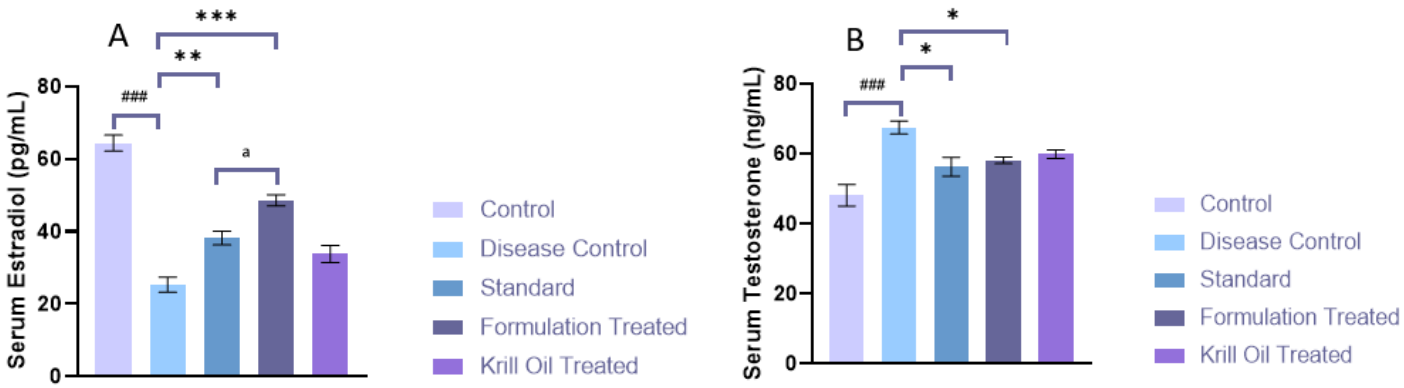


Figure 15

Hormonal Levels following treatment A: Estradiol, B: Testosterone

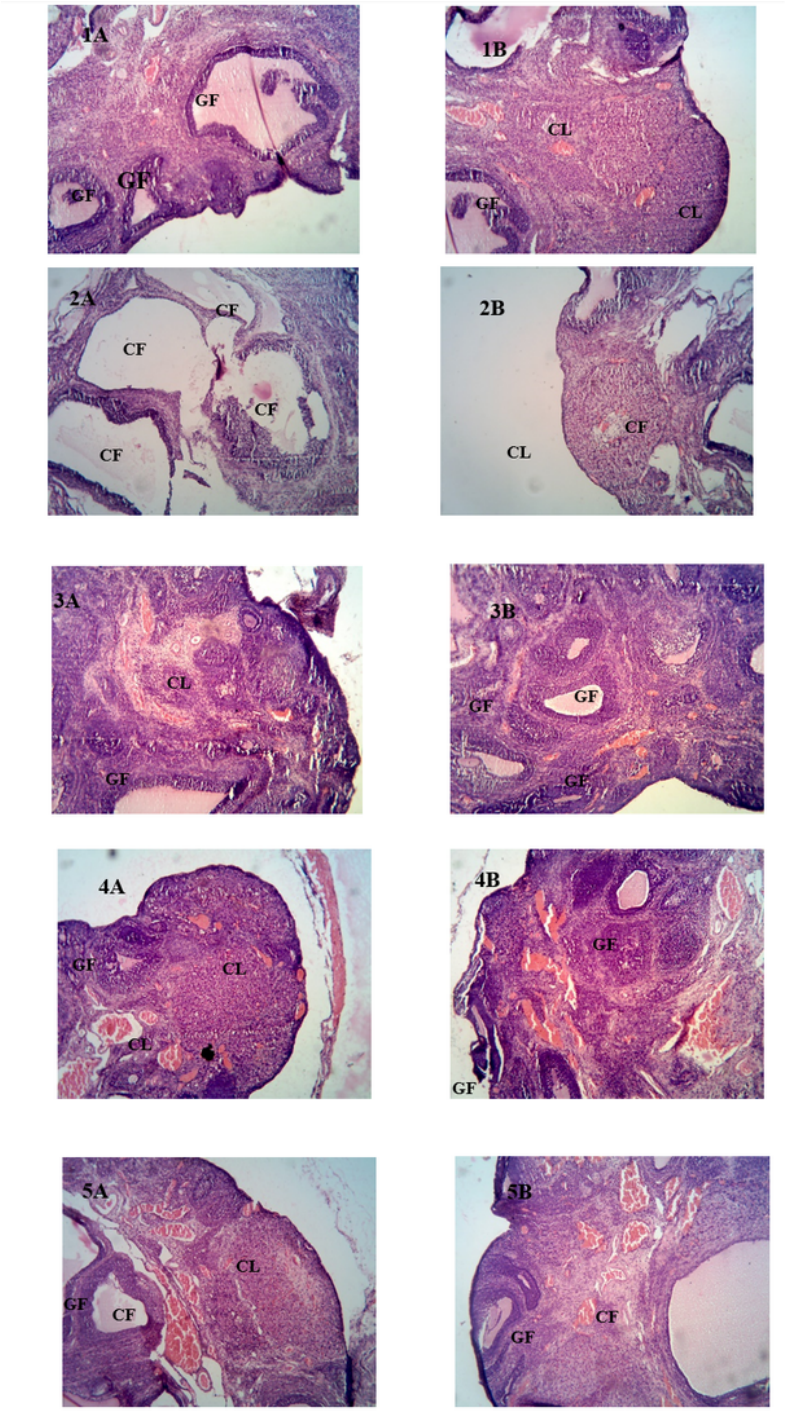


Figure 16

Histopathology of Ovary. Control: 1A & 1B; Disease Control: 2A & 2B; Standard: 3A & 3B; Microemulsion-treated; 4A & 4B; Krill oil-treated; 5A & 5B. All images are H&E stained and observed under 100X. CF: cystic follicle, GF: growing follicle/Graafian follicle, CL: corpus luteum

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- GraphicalAbstract.png