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Pharmacological Potential of *Hippophae rhamnoides* L. Nano-Emulsion for Management of Polycystic Ovarian Syndrome in Animals' Model: *In Vitro* and *In Vivo* Studies

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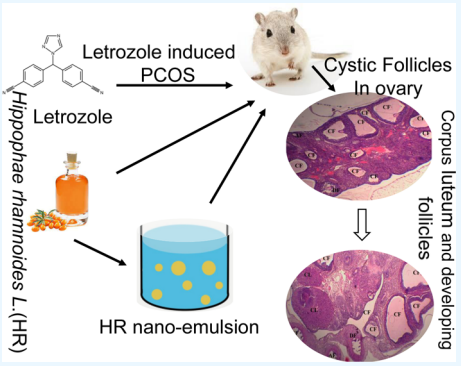
ABSTRACT: The most common female endocrinopathy, polycystic ovarian syndrome (PCOS), generally affects women of childbearing age. *Hippophae rhamnoides* L. has been traditionally used to improve menstrual cyclicity. Gas chromatography by flame ionization detection analysis showed that it contained various phytoconstituents such as omega-3 fatty acid, phytosterols, palmitic acid, oleic acid, and linoleic acid. *H. rhamnoides* L. (HR) nano-emulsion was also formulated. HR and its encapsulated nano-emulsion (HRNE) were evaluated for the treatment of PCOS. Thirty-five healthy female adult albino rats were acquired and divided into seven groups (n = 5). Letrozole (1 mg/kg) was used for 5 weeks to induce the disease. To confirm disease (PCOS) induction, the animals were weighed weekly and their vaginal smears were analyzed daily under a microscope. After PCOS induction, animals were treated with metformin, HR, and HRNE with two different doses (0.5/kg and 1 g/kg, *p.o.*) for 5 weeks. At the end of the treatment, animals were euthanized, and blood was collected for hormonal assessment, lipid profiling, and liver functioning test assessment. Both the ovaries were preserved for histopathology and liver for the purpose of assessment of antioxidant potential. The results revealed that HR and HRNE at both doses improved the hormonal imbalance; follicle-stimulating hormone, estrogen, and progesterone levels are increased, while luteinizing hormone surge and testosterone level are controlled. Insulin sensitivity is improved. Ovarian histopathology showed that normal ovarian echotexture is restored with corpus luteum and mature and developing follicles. HR and HRNE also improved the lipid profile and decreased lipid peroxidation (MDA) with improved antioxidant markers (SOD, CAT, and GSH). Results were statistically analyzed by one-way analysis of variance and were considered significant only if $p < 0.05$. In conclusion, it can be postulated that *H. rhamnoides* L. proved effective in the management of PCOS and its nano-emulsion effects were statistically more significant, which might be due to better bioavailability.



1. INTRODUCTION

PCOS (polycystic ovarian syndrome) is a common endocrine ailment that affects women during their reproductive age. PCOS may cause hyperandrogenism and oligo-anovulation, both of which have significant physiological, psychological, and social consequences.[1] Women with PCOS are prone to metabolic abnormalities, with multiple complications. There has been an increase in awareness of this illness in the general population and medical communities in recent years.[2] Because of the variability of symptoms, the concept of PCOS has been debated in various fields of medicine such as in internal medicine, gynecology, and psychiatry. The recent research explored a new diagnostic marker for PCOS blood organic solvents (VOCs) such as 4-ethylphenol and capric acid; thus, additional screening methods should be included for PCOS diagnosis.[3] As a result, elucidating the origins of the PCOS and distinguishing pathological abnormalities from secondary environmental disturbances remain persistent challenges not only for clinicians but also for researchers.[4]

PCOS is a prevalent, complicated, and diverse endocrine condition that affects 5−10% of women of reproductive age. This condition is characterized by oligomenorrhea, amenorrhea, anovulation, a large number of antral follicles, hypersecretion of the luteinizing hormone (LH) but with lesser or similar FSH levels, hirsutism, and hyperandrogenemia. Majority of patients

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also have metabolic issues, such as obesity, dyslipidemia, and insulin resistance.[5] Women with PCOS are more likely to have reproductive problems, and major proportions have metabolic dysfunction, which increases their risk of developing type II diabetes and cardiovascular disease. Insulin resistance and compensatory hyperinsulinemia are common in women with PCOS, with rates as high as 95% in obese women. Hyperinsulinemia may increase aberrant androgen production as well as alter folliculogenesis and menstrual cyclicity; all of these are hallmarks of PCOS.[6]

Hippophae rhamnoides L. (HR), a member of the family *Elaeagnaceae* commonly called sea buckthorn oil, also has a history of its traditional medicinal use to treat stomach ulcers, liver dysfunction, and various conditions of skin damage due to the presence of flavonoids, phenols vitamins, and minerals.[7] The sea buckthorn plant, also known as the Chinese medicinal herb, is indigenous to Siberia, Central Asia, China, Mongolia, and the Caucasus regions.[7,8] Currently, *H. rhamnoides* L. oil is used in skincare products because of its possible advantageous effects on the skin. The presence of flavonoids, polyphenols, polysaccharides, sterols, phenols, and tannins will mainly exert a favorable effect in alleviating PCOS-related symptoms.[9] All the plant parts possess phytoconstituents with potential antioxidant properties.[8] This is why all the tested sea buckthorn products had high antioxidant activity. In 100% sea buckthorn oil, the highest levels of total polyphenols (204.26 mg GAE/g), flavonoids (30.00 mg QE/g), and carotenoids (0.34 mg/g) were found.[10]

Because *H. rhamnoides* L. (sea buckthorn) oil has a long history of use as an anti-diabetic, antioxidant, hypolipidemic agent, anticancer, in endometriosis and vaginal atrophy, cardioprotective, anti-platelets, anti-viral, anti-bacterial, and anti-inflammatory agent, it would be worthwhile to investigate its potential in PCOS therapy. This study explores the efficacy of *H. rhamnoides* L. in the treatment of rats with letrozole-induced PCOS. *H. rhamnoides* L. is oil, and most of the time, solubility and dissolution in gastrointestinal fluids are big problems.[11] Various formulations are in practice to overcome these solubility-related problems of various drugs to enhance bioavailability. One of these types of techniques is nano-emulsion formulation, which is used to enhance the dissolution and bioavailability of various drugs. Nano-emulsion-based formulation is a good choice for oil-based herbal products.[12] *H. rhamnoides* L. nano-emulsion (HRNE) was also formulated. The aims and objectives of the study were to evaluate the efficacy of HR and its nano-emulsion (HRNE) in letrozole-induced PCOS female adult rats. Additionally, it was also expanded to assess whether nano-emulsion (HRNE) has better efficacy as compared to HR oil.

2. EXPERIMENTAL SECTION

2.1. Acquisition of *H. rhamnoides* L. *H. rhamnoides* L. oil was courteously provided by Saffron Pharmaceuticals PVT. Ltd., Faisalabad, Pakistan. It was extracted from plant berries, which were collected from the northern areas of Pakistan.

2.1.1. In Vitro Evaluation of H. rhamnoides L. (GC-FID Analysis). Fatty acid composition of the *H. rhamnoides* oil was analyzed by using GC-FID (GC-FID, Perkin Elmer). The IUPAC standard protocol was applied, which is based upon derivatization of fatty acids into FAMEs (fatty acid methyl esters) (IUPAC, 1987). FAME was analyzed on a gas chromatograph model Clarus-580 (Perkin Elmer), fitted with a SP-2330 (SUPLECO, Inc., Bellefonte, PA, USA) methyl lignoserate-coated (film thickness, 0.20 μm) polar capillary column (30 m × 0.32 mm) and a flame ionization detector. Oxygen-free nitrogen was used as a carrier gas at a flow rate of 3 mL min.

Other conditions were as follows: initial oven temperature, 180 °C; ramp rate, 5 °C/min; final temperature, 220 °C; injector temperature, 230 °C; detector temperature, 240 °C; FAMEs were identified by comparing their relative and absolute retention times with those of authentic standards of FAMEs (Sigma Chemical Co., St Louis, MO, USA). The quantification was done using the external standard method by plotting the concentration against the peak areas of different standards concentrations. The fatty acid composition was reported as a relative percentage of the total peak area.[13]

Gas chromatography by flame ionization (GC-FID) is a widely used method for the identification of lipids and fatty acids in oils. GC-FID of *H. rhamnoides* L. oil was performed by forming its methyl ester using a Restek (RTX-1) (30 m × 0.25 mm i.d.) 100% dimethyl polysiloyane column, with an FID detector 220 temperature. An HP 7673 autosampler and a split−split less injector (split ratio of 1:15) were used to inject the samples (1 μL). The oven's temperature was set at 120 °C; ABS nitrogen, oxygen, and hydrogen gases were utilized as the carrier gas and an inlet pressure of 175 kPa. The temperatures of the injector and detector were operated at 120 and 220 °C, respectively, and a column flow of 1 mL/min was achieved. HP 3365 Series II ChemStation software (version A.03.34) was used to collect the data. The samples were all tested in triplicate. It has been observed that palmitic, docosahexaenoic acid (DHA), oleic acids, and linoleic acids were prevalent in the fatty acids quantified using myristinic acid as a standard.[14]

At room temperature, methanol and KOH (potassium hydroxide) were combined to create methyl ester. After 1 h of stirring, the mixture was allowed to settle, and the product of the reaction was simply decanted into two parts. The fatty acid methyl ester (FAME) upper layer was removed to further purify it by washing with water (10 times). The volatile part was then removed under reduced pressure, while the FAME was kept standing over magnesium sulfate after being dissolved in dichloromethane. Methanol, oil, and KOH were used in the transesterification process in a total molar ratio of 7:1:0.01.[15,16]

2.2. Preparation of *H. rhamnoides* L. Nano-Emulsion Formulation. *H. rhamnoides* L. nano-emulsion was formulated by using various concentrations of the surfactant (Tween 60 and 80) and co-surfactant (PEG 400) by adding these into HR and cinnamon oil in 3:2:1:1, and then, its effect in treatment of PCOS was evaluated by administering to letrozole-induced PCOS rats.

2.2.1. Evaluation/Characterization of Nano-Emulsion Formulation. The prepared nano-emulsion was evaluated by FTIR, DSC, and TGA. Thermal stability of the HR oil in nano-emulsion was evaluated by DSC weight loss of nano-emulsion, and the formulation under different temperature conditions was also evaluated. The interaction of HR oil with other used oils and excipients was checked by FTIR.

2.3. Protocols for Experimental Animals. Thirty-five healthy female Wistar albino rats, weighing 100−130 g, were acquired and housed in the animal facility of the Government College University Faisalabad. Standard laboratory conditions were maintained (10/14 h light/dark cycle, 25−30 °C, 45−55% relative humidity). Rats were given a standard laboratory diet and water ad libitum. Animals were handled according to ARRIVE guidelines. Study protocols were approved by the

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Ethical Review Committee of Government College University Faisalabad, and ref no. (GCUF/ERC/ 52.1) was obtained.

2.4. Disease Induction in Rats. Animals were allowed for a 1 week adaptation period. Letrozole (1 mg/kg) was used to induce the PCOS. Letrozole suspension was prepared by dissolving letrozole in 0.5% of CMC carboxymethyl cellulose solution. Letrozole suspension was administered to rats for 5 weeks for the induction of PCOS. To confirm the disease induction, estrous cycle monitoring and weight changes were recorded weekly. To determine the stages of the estrous cycle, vaginal cytology was performed daily, which was indicated by the ratio of cornified, leukocyte, and epithelial cells under light microscopy.[17]

2.5. Experimental Protocols. *2.5.1. Research Methodology for H. rhamnoides L.* A complete randomized design was used for animals' division into groups. There were seven groups, and each group contained five animals. Groups were labeled as normal control (N.C.), diseased (PCOS), standard (metformin), HR (0.5 g/kg), HR (1 g/kg), HRNE (0.5 g/kg), and HRNE (1 g/kg). All the drugs were administered orally through gavage. A vaginal smear was observed daily, and weight changes were recorded weekly.

2.6. Dosage Schedule. The dosage schedule for the experimental designs of *H. rhamnoides* L. is explained in Table 1.

Table 1. Dosage Plan for *H. rhamnoides* L. and Its Formulation

group name	formulation	dose/route/duration
Disease induction phase (5 weeks)		
N.C. (normal control)	0.5% w/v CMC	1 mg/kg/day (*p.o.*) 10 weeks
PCOS (disease-induced)	letrozole in 0.5% w/v CMC	1 mg/kg/day (*p.o.*) 10 weeks
Treatment phase (5 weeks)		
standard (metformin) group	metformin in 0.5% w/v CMC	20 mg/kg/day (*p.o.*)
HR (0.5 g/kg)	*H. rhamnoides* L. pure oil	0.5 g/kg/day (*p.o.*)
HR (1 g/kg)	*H. rhamnoides* L. pure oil	1 g/kg/day (*p.o.*)
HRNE (0.5 g/kg)	*H. rhamnoides* L. nano-emulsion	0.5 g/kg/day (*p.o.*)
HRNE (1 g/kg)	*H. rhamnoides* L. nano-emulsion	1 g/kg/day (*p.o.*)

2.7. Experimental Procedures. Estrous cycle stages were determined by analyzing the vaginal smear.[18] After completion of the treatment duration, rats were euthanized and blood was taken by cardiac puncture. Serum was separated by centrifugation to identify hormonal evaluation such as luteinizing hormone (LH), follicle-stimulating hormone (FSH), serum insulin, progesterone, estrogen, and testosterone. The liver functioning test (LFT) and lipid profile were also examined. Histopathology of rat ovaries was also performed to visualize the effect on follicular cysts. Oxidative stress markers were also tested by antioxidant assays on liver homogenate.

2.8. Estrous Cycle Monitoring and Vaginal Smear Cytology. To prepare vaginal smears of rats, each animal was removed from the cage and the vaginal hole was opened carefully. A damp cotton gauze moistened with distilled water was placed into the vagina of the rat. A cotton swab was rotated softly for a while and removed. A smear was created on a cleaned grease-free glass slide with the help of this cotton sab. The slide was then air-dried, washed with 10% methanol solution, and stained with a dye such as methylene blue. It should be cleaned with distilled water, allowed to air-dry, and then examined using a binocular microscope (accuscope) to recognize the various stages of the estrous cycle. The estrous cycle comprises four phases, such as proestrus, estrus, metestrus, and diestrus.[18]

2.9. Ovary Collection and Histology. Animals were euthanized after completion of the blood drawing procedure. Female rats were dissected, and ovaries were removed by following the standard procedure, according to ARRIVE guidelines. Ovaries from each female rat were fixed in 4% paraformaldehyde (PFA) and further in 20% sucrose solution. Later, tissue embedding was done in Tissue-Tek (O.C.T. compound; Sakura) and frozen at − 80 °C overnight, and tissues were cut into sections (6 μm thickness). Hematoxylin and eosin (H&E) stain was used to stain the tissues, according to the standard histological procedures. Ovarian morphology was examined in a way that the glass slide was mounted with every 12th section. All the antral follicles and corpora lutea were counted. The follicle diameter was calculated as the mean distance between opposite basal membrane portions, while the wall thickness was calculated as the sum of theca internal and granulosa cell layers. All large fluid-filled cysts with an attenuated granulosa cell layer and thickened theca cell layer were considered as cystic follicles.[19]

2.10. Serum Hormonal Estimation. For the hormone analysis radioimmunoassay (RIA) and enzyme-linked immune-sorbent assay (ELISA), kit methods were used on the blood serum of rats. Blood samples were collected by cardiac puncture, and then, the serum was separated by cold centrifugation at 3000 rpm for 5 mins. Serum follicle-stimulating hormone (FSH; mIU/mL), progesterone (ng/dL), and testosterone (ng/dL) levels were measured by the radioimmunoassay (RIA; Gamma counter) utilizing the kit of Beckman Coulter, Inc. USA, while serum luteinizing hormone (LH; mIU/mL) concentration was determined by the enzyme-linked immune-sorbent assay (ELISA) method using the kit of Pointe Scientific Inc. USA. Serum estrogen (pg/mL) was assessed by using the kit of ALPCO, USA, and serum insulin (ulU/mL) was determined by using the kit of Calbiotech Inc., USA.

2.11. Lipid Profile and Liver Functioning Tests (LFTs). Analysis of total serum cholesterol and triglycerides was done spectrometrically. Serum HDL and LDL levels were analyzed enzymatically (DiaSys Diagnostic System GmbH, Germany). Likewise, different markers of liver function tests, i.e., levels of ALT, AST, and ALP, along with the total and direct bilirubin levels were also evaluated. The liver functioning test (LFT) was also performed spectrophotometrically on a semi-automated chemistry analyzer (microlab-300).

2.12. Determination of Antioxidant Activity. *2.12.1. Evaluation of Oxidative Stress Markers.* Tissue homogenate was prepared by using 0.1 M phosphate buffer (pH 7.4) and 1 g of tissue from the liver and grinded it with a tissue homogenizer. The phosphate buffer contained 10 mM potassium chloride, 1 mmol of ethylene diamine tetra-acetic acid (EDTA), 0.25 M sucrose, and 1 mM phenylmethylsulfonyl-fluoride. Then, the mixture was centrifuged at 800 rpm at 4 °C for 30 min to obtain the supernatant. The supernatant solution was separated and used for the assessment of antioxidant parameters (CAT, MDA, SOD, and GSH).

2.12.2. DPPH (2,2-Diphenylpicrylhydrazyl) Analysis. DPPH analysis was done according to established protocols and already published in our previous study.[14,20,21] The capacity to scavenge the DPPH radical was calculated by the following equation:

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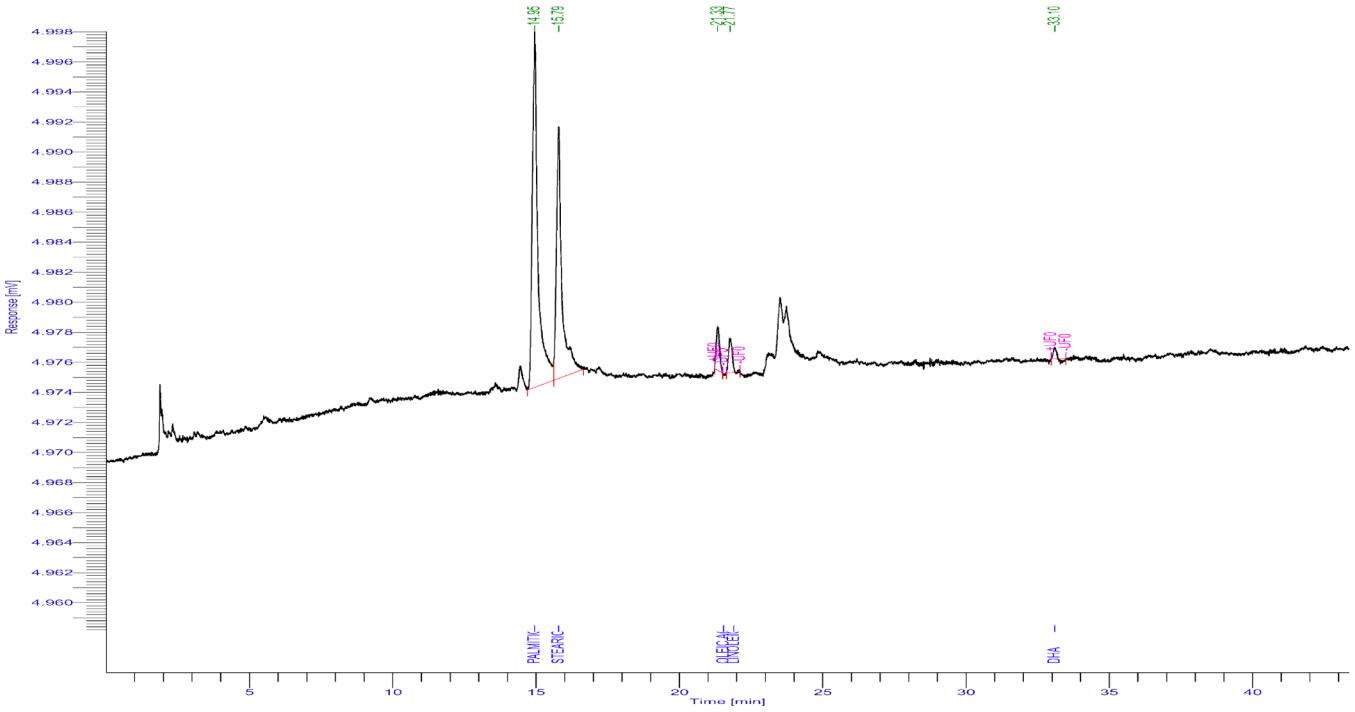

Figure 1. GC-FID analysis of *H. rhamnoides* L. by GC-FID.

$$\text{DPPH scavenged (\%)} = \text{AB} - \text{AA/AB} \times 100$$

where AB is the absorbance of blank at 0 min and AA is the absorbance of the sample to be tested at 30 min.

2.12.3. Total Phenolic Content (TPC) Estimation. The total phenolic content of HR was measured by using the method described by Shukla et al.[22] and the already published methods in our previous study.[23] The TPC of all the samples was determined using the following formula:

$$C = cV/m$$

where V is the volume of the extract in mL, m is the mass of the extract in grams, C is the total phenolic content mg GAE/g dry extract, and c is the gallic acid concentration calculated from the calibration curve (mg/mL).

2.12.4. Total Flavonoid Content (TFC) Estimation. The total flavonoid content was measured as quercetin equivalent by making its standard curve (10−130 ppm). The TFC was determined according to the standard protocol and already published studies.[21,24]

2.13. Statistical Analysis. Data was presented as mean ± SEM. Statistical analyses were performed with a one-way analysis of variance (ANOVA), and Tukey's multiple comparison test was used to evaluate the hormonal differences among the groups. GraphPad Prism version 8.02 was used for all the statistical analyses. A two-way analysis of variance with repeated measures for a time was used to test the significance of various groups. The data was considered statistically significant if $p < 0.05$.

3. RESULTS

3.1. Characterizations of *H. rhamnoides* L. *H. rhamnoides* L. has a very rich source of fatty acids and omegas. Fatty acids/omegas present in HR oil are palmitic acid (13.97%), stearic acid (14.76%), oleic acid (19.94%), linoleic acid (20.357), DHA (31.10%), and traces of other saturated fatty acids (Figures 1 and 2 and Table 2).

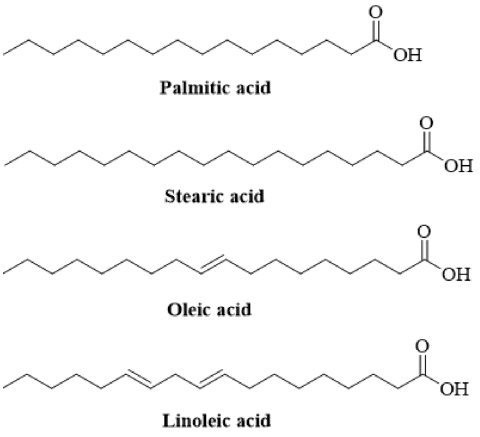

Figure 2. Structures of phytoconstituents present in *H. rhamnoides* L.

3.1.1. GC-FID Analysis of H. rhamnoides L. GC-FID analysis of the *H. rhamnoides* L. chromatogram is given in Figure 1.

3.1.2. TPC Analysis. The total phenolic content of *H. rhamnoides* L. was 1713 ± 0.124 μg/100 g and is mentioned in Table 3. The TPC is given as gallic acid equivalent (GAE).

Table 2. GC-FID Analysis of Fatty Acids/Sterol Contents of *H. rhamnoides* L.

fatty acids	concentration (mg/g)
palmitic acid	13.97%
stearic acid	14.76%
oleic acid	19.94%
linoleic acid	20.357
DHA	31.10%

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Table 3. Determination of the Total TPC and TFC in *H. rhamnoides* L.

analysis	TPC as GAE (μg/100 g)	TFC as QE (μg/100 g)
mean	1713 ± 0.124	310 ± 0.08

3.1.3. TFC Analysis. The total flavonoid content of the *H. rhamnoides* L. was 310 ± 0.08 μg/100 g and is demonstrated in Table 3. The TFC is given as quercetin equivalent (QE).

3.1.4. DPPH Assay. DPPH scavenging % by *H. rhamnoides* L. is mentioned in Table 4. The sample is obtained in parts per million. Ascorbic acid was used as a reference to compare the results. The % age inhibition of *H. rhamnoides* L. is (67.27 μg/mL) in comparison to ascorbic acid (75.34 μg/mL).

Table 4. DPPH Scavenging % of *H. rhamnoides* L.

ascorbic acid standard		*H. rhamnoides* L. oil	
sample in ppm	percent inhibition	sample in ppm	percent inhibition
20	44.31	20	40.02
40	57.26	40	49.71
60	64.43	60	56.82
80	69.70	80	62.44
100	75.34	100	67.27

3.2. Preparation and Characterization of Nano-Emulsion. Nano-emulsion was prepared successfully and was evaluated by DSC, TGA, and FTIR. HR oil showed a broad endothermic peak at 160 °C and a second endothermic peak at 424 °C, as shown in Figure 3A. PEG 400 showed a sharp endothermic peak at 112 °C. The cinnamon oil and Tween 80 showed endothermic peaks at 203 and 199 °C, respectively. The nano-emulsion showed an endothermic peak at 150 C, and no other peak was observed, indicating the thermal stability of the oil in formulation. The weight loss of HR oil started at 190 °C and continued till 500 °C. A rapid weight loss of PEG 400 was observed between 55 and 95 °C. The weight loss of cinnamon oil started at 31 °C, and 99% weight loss occurred at 211 °C. The TGA curve of Tween 80 showed that weight loss started at 32 °C and continued until 500 °C. The weight loss of nano-emulsion was only 0.1% at 33 °C, and 50% weight loss was observed at 32 °C, indicating that the formulation was stable under accelerated temperature conditions (Figure 3B). HR oil showed peaks at 2750, 1680, 1200, and 950 cm^{-1} due to the presence of the hydroxyl −OH group and alkane −CH group and the presence of aromatic rings. FTIR of PEG 400 displayed peaks at 3000, 2700, and 1700 cm^{-1} due to the CH stretching, CH bending, and C−O−H stretching vibrations, respectively. The C═O ester group's stretching band at 1512 cm^{-1} and the hydroxyl stretching vibrations' strong band at 3300 cm^{-1} were both visible in the FTIR spectra of Tween 80. The spectrum displays distinctive bands at 1500−1400 cm^{-1} owing to alkane deformation, signals at 1650 cm^{-1} corresponding to C═C stretching, and stretching vibrations of C═O groups at 1660−1680 cm^{-1}. One of the strongest IR absorptions, the carbonyl stretching absorption is highly helpful in determining the structure since it allows one to count the amount of carbonyl groups. The FTIR spectra of SNEDDS showed peaks of the hydroxyl group of HR oil, C═C stretching of cinnamon oil, C−H bending of PEG 400, and C═O stretching of Tween 80, indicating the chemical stability of formulation (Figure 3C).

3.3. Body Weight Changes of Rats. *3.3.1. Body Weight Changes during the Disease Induction Phase.* After continuous administration of letrozole for 5 weeks in the disease group, a significant increase ($p < 0.05$) was noticed in the body weight of the rats as compared to the normal control

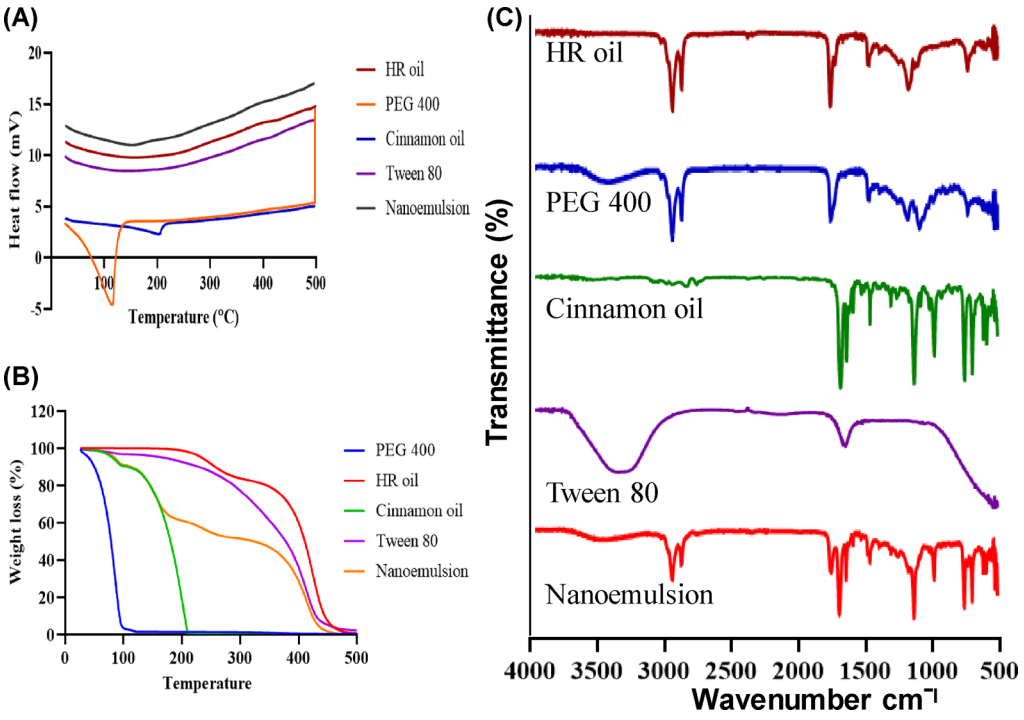

Figure 3. Characterization of *H. rhamnoides* L. Nano-emulsion (A) DSC, (B) TGA, and (C) FTIR of HR oil, PEG400, cinnamon oil, Tween 80, and HR nano-emulsion. DSC of nano-emulsion showed no endothermic peak, indicating the uniform distribution of HR oil, and TGA of the final formulation showed a two-step degradation. Characteristic peaks of HR oil also shown in the FTIR spectra of nano-emulsion indicated the chemical stability of formulation.

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group. The increase in weight of diseased rats was observed at 20%, and contrarily, control rats' weight increased by 13.27% of their starting weight (Table 5).

Table 5. Body Weight Changes during Disease (PCOS) Induction

weeks	normal control group (weight/g)	diseased (PCOS) group (weight/g)
first	113.6 ± 1.8	105.0 ± 4.2
second	117.4 ± 1.9	110.4 ± 4.4
third	119.4 ± 2.2	115.6 ± 4.8
fourth	124.0 ± 2.09	121.2 ± 4.6
fifth	128.0 ± 2.168	126.2 ± 4.4
increase in body weight	13.27%	20%

3.3.2. Body Weight Changes during the Treatment Period. At the end of the trial period, rats in the diseased group weighed more than the normal control group. However, the weight gain of the metformin group was increased in a much similar way as that of the PCOS group. However, rats from the HR 0.5 g/kg group showed a rise in body weight as that of the normal control group. The weight of rats from HR 1 g/kg increased in a parallel manner that is almost one-third of the PCOS groups. There was also a similar increase in body weight at both doses of HRNE. Changes in body weights of rats for the period of treatment are given in Figure 4.

3.4. Histopathology of the Ovary and Vaginal Smear Analysis for the Identification of the Estrous Cycle. One of the main hallmarks of PCOS is the delayed estrous cycle. To confirm the estrous cycle regulation, vaginal cytology was done throughout the study regularly. A change in the estrous cycle of the normal control group was recorded at a fixed time interval, which confirms a regular estrous cycle. All the estrous stages of normal healthy female rats are shown in Figure 5. In the proestrus phase, cornified nucleated epithelial cells were predominant, whereas in the estrus phase, cells were non-nucleated cornified epithelial. The diestrus phase mostly contained leucocytes, while in the metestrus phase, both the cornified epithelial and nucleated cornified cells were found.

Rats with PCOS show delays in their normal estrous cycle as they remain in the diestrus phase for a prolonged time. Results are shown in Figure 6.

3.5. Effect of *H. rhamnoides* L. on the Histopathology of Rat Ovaries. The ovaries in the normal control group's histopathological slides had normal architecture, tiny to middle-sized follicles, and many corpus luteum, granulosa cells, and follicles in numerous phases of development (Figure 7A). The PCOS group's ovary slides (Figure 7B) showed atretic antral follicles' typical cystic follicles and disorganized granulosa cell section with variable granulosa cell thickness. Ovaries treated with letrozole have a high quantity of cystic cavities and lack the corpus luteum. In the letrozole-treated group, these alterations can be attributed to lower FSH and elevated testosterone levels. However, a prominent decrease in the number and size of cystic follicles occurs after the administration of metformin (Figure 7C). Group (HR 0.5 g/kg) substantially restored the normal anatomy of the ovary (Figure 7D), while group (HR 1 g/kg) showed even better results with the reduction of cystic follicles, increasing corpus luteum and developing follicles (Figure 7E). Both doses of formulation also have comparable effects like oil with betterment of follicle development and reduction in the number of cystic follicles (Figure 7F,G).

3.6. *H. rhamnoides* L. (HR) Effect on Hormonal Status. The results of the study clearly showed that there was fluctuation in the hormonal status after disease induction, and significant improvement was seen with treatment. PCOS induction significantly ($p > 0.01$) lowered the follicle-stimulating hormone (FSH) level as compared to the control group. Meanwhile, all the treatment groups improved the serum hormone level. The most significant ($p > 0.001$) was seen with the HR 1 g/kg nano-emulsion (HRNE) formulation group (Figure 8A). PCOS also significantly lowered the luteinizing hormone (LH) level. HRNE at both doses significantly ($p > 0.05$) abridged the LH as compared to PCOS and similarly better effects compared to HR both groups. Results are shown in Figure 8B. Figure 8C depicts that animals with PCOS has a lower level of estrogen as compared to the normal ones, while all the treatment groups improved the estrogen level. The most betterment ($p > 0.01$) was observed with nano-emulsion formulation as compared to the HR group.

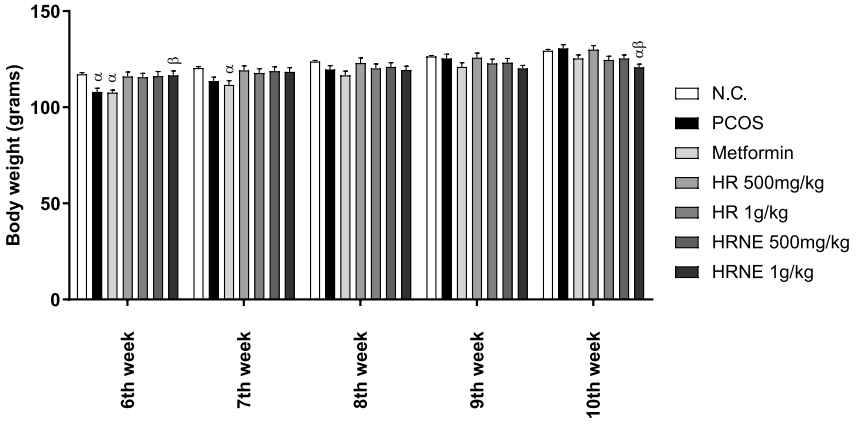

Figure 4. Effect of *H. rhamnoides* L. on body weight changes after disease induction. Values are expressed as mean ± SEM ($n = 5$). α designated a significant difference from N.C., while β designated a significant difference from the PCOS group (disease control). One-way analysis of variance (ANOVA) was used to evaluate the statistical significance between the groups, and multiple comparison was done by Tukey's post-hoc test. N.C.: normal control group, HR: *H. rhamnoides* L., HRNE: *H. rhamnoides* L. nano-emulsion formulation.

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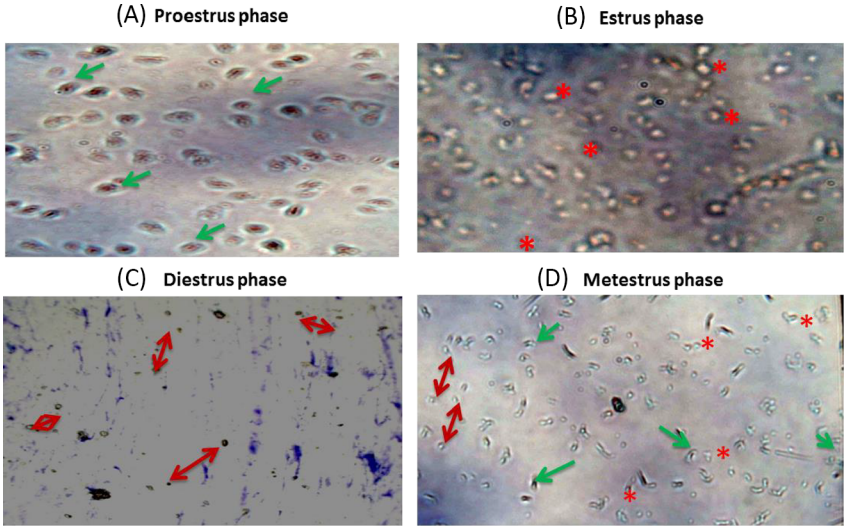

Figure 5. Vaginal cytology of a normal control rat demonstrating the different estrous cycle phases. Vaginal smears of rats' estrous cycle presenting different estrous phases. (A) In the proestrus phase, cornified epithelial cells were predominately nucleated (green arrows). (B) Cornified epithelial cells are classed as the estrus phase (*). (C) The diestrus phase primarily consists of leukocytes (red arrows). (D) Cornified epithelial cells and nucleated cornified cells are all existing in the metestrus stage.

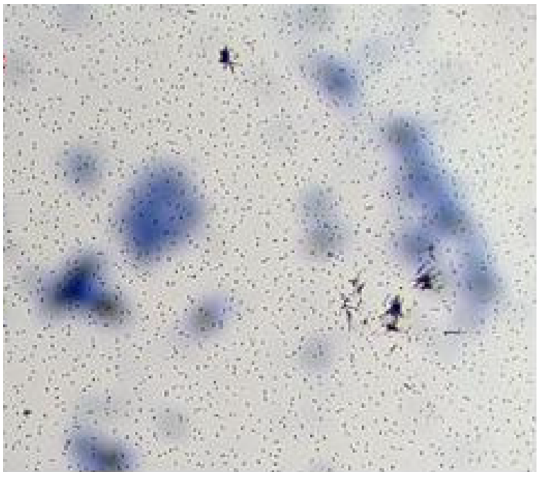

Figure 6. Vaginal cytology of diseased rats (PCOS group with the diestrus phase).

Our results exhibited that PCOS significantly ($p > 0.001$) decreased the progesterone level, and metformin has no effect. All the treatment groups improved the progesterone level, and there was no significant difference between lower doses of HR and HRNE, but 1 g/kg HRNE has comparable better effects in incrementing the progesterone level (Figure 8D). The progesterone level in the serum of the disease group was enhanced. Metformin was the most efficacious ($p > 0.001$) drug in lowering the testosterone level, while all the treatment groups also have lowered its level (Figure 8E). As we know, PCOS has a strong link with insulin resistance. The similar findings were observed in our results where the serum insulin level was markedly ($p > 0.001$) elevated in the disease group. All the treatment groups lowered the serum insulin. Results are shown in (Figure 8F).

3.7. *H. rhamnoides* L. Effect on the Lipid Profile. PCOS is a metabolic abnormality, and it always disturbs the lipid profile. In this study, the total cholesterol (TC), high density lipoprotein (HDL), low density lipoprotein (LDL), and triglyceride (TG) levels were determined. Disease induction significantly elevated TC, LDL, and TG and lowered HDL. After treatment with metformin and HR oil and HR nano-emulsion formulation, there was a significant improvement in the HDL level and decrease in the elevated level of TC, LDL, and TG. Additionally, HR nano-emulsion has better effects compared with HR. Results are shown in Figure 9.

3.8. *H. rhamnoides* L. Effect on Oxidative Stress and Lipid Peroxidation Status. Oxidative stress markers such as catalase (CAT), superoxide dismutase (SOD), and glutathione (GSH) were assessed in the liver homogenate of letrozole-induced female rats to check the effects of treatment with *H. rhamnoides* L. The levels of all these antioxidant enzymes were reduced by 4.67 ± 0.008 μg/mg, 66.5 ± 0.29 units/mg, and 1.85 ± 0.015 units/mg, respectively, in the PCOS group (letrozole-treated) (Figure 10A−C). This clearly showed that letrozole lowered the antioxidant enzyme level and caused oxidative stress. Data indicated that all the treatment groups significantly ($p < 0.05$) improved the level of these parameters. Letrozole administration (1 mg/kg) in rats of PCOS groups caused a significant rise ($p < 0.05$) in the level of lipid peroxidation markers, such as malondialdehyde (MDA) in rat livers (15.94 ± 0.01 mmol/mg). Rats in the normal control group showed a normal level of MDA (14.352 ± 0.08 mmol/mg) (Figure 10D). Meanwhile, MDA levels were improved in all the treatment groups including metformin. The most significant effects were seen in HR nano-emulsion formulation that was comparable with the metformin-treated group.

3.9. *H. rhamnoides* L. Effect on the Liver Functioning Test (LFT). Liver aminotransferase was also determined from the liver tissues. There was a significant ($p < 0.05$) modulation in the aspartate aminotransferase (AST) level in the PCOS group as compared to the control group, but all the treatments could not produce any significant change in the ALT level after a whole treatment period. When the alanine transaminase (ALT) and alkaline transferase levels were assessed, the PCOS group contained significant elevated levels but treatment with plant oil and nano-emulsion formulation significantly lowered the elevated levels of liver enzyme markers. Results are shown in Figure 11.

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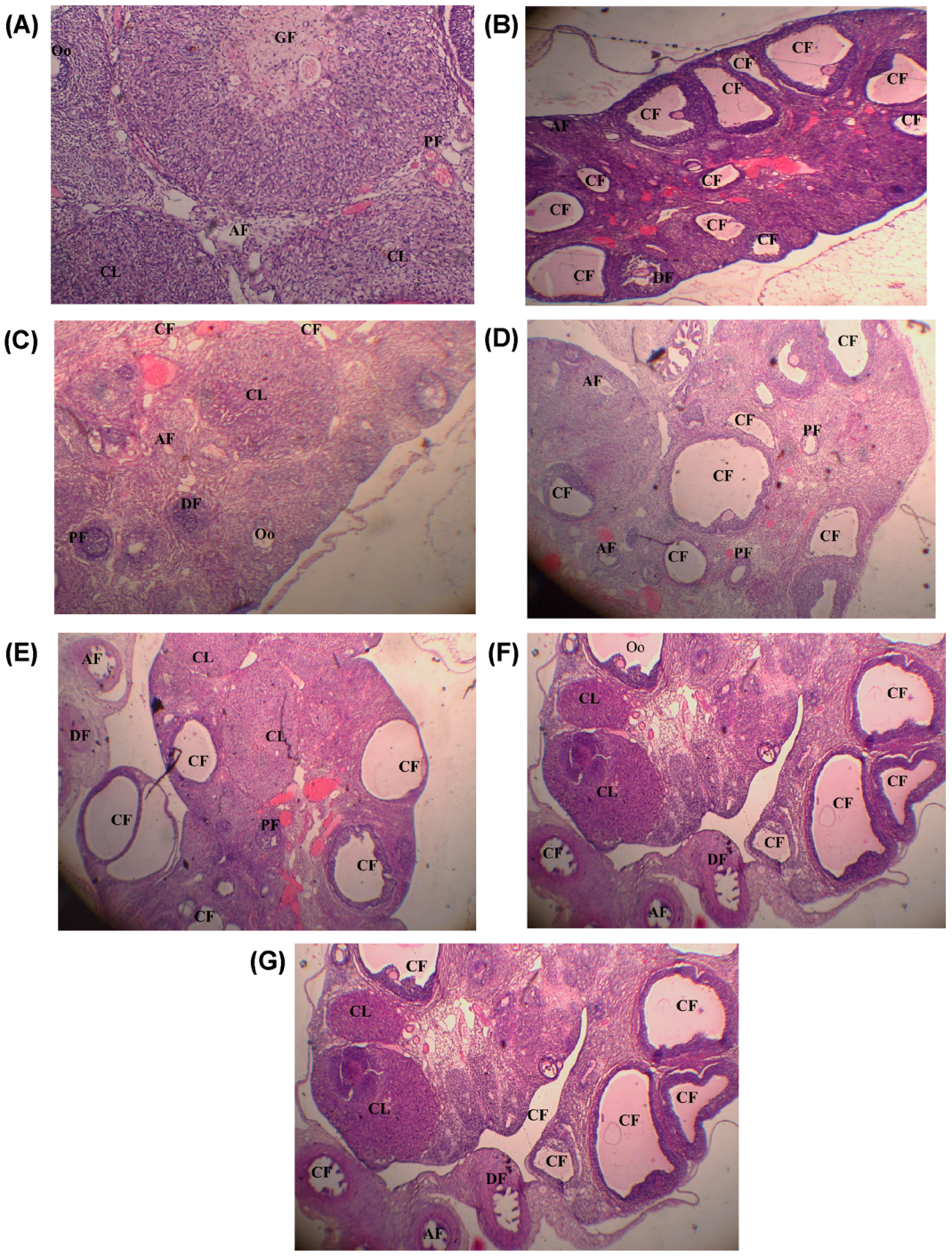

Figure 7. Microscopic examination of a cross section of the ovary of a rat stained by hematoxylin−eosin. (A) Ovarian section of control group rats exhibiting normal morphology of the rat ovary with different stages of ovarian follicles showed atretic follicles, developing follicles, and corpus luteum. (B) PCOS group showing typical cystic follicles and disorganized granulosa cells. (C) Ovarian section of the metformin-treated rat showed corpus luteum and less number of cystic follicles with developing follicles. (D) Ovarian section of rats treated with (*H. rhamnoides* L.) HR (0.5 g/kg) showed reduction in the number of cystic follicles and more developing follicles, primary follicles, and atretic follicles. (E) Ovarian section of HR (1 g/kg) treated rats showed the presence of developing follicles, primary follicles, and atretic follicles. (F) Ovarian section of HR 0.5 g/kg, nano-emulsion showed a good number of developing, primary, and atretic follicles. (G) Section of nano-emulsion (1 g/kg) showed corpus luteum, primary and developing follicles, and oocyte with a lesser number of cystic follicles. CF: cystic follicles, AF: atretic follicles, DF: developing follicles, CL: corpus luteum, PF: primary follicles, GF: growing follicles, Oo: oocyte.

4. DISCUSSION

In this project, the effect of *H. rhamnoides* L. (HR), also called sea buckthorn, is evaluated for its potential to be used for the management of PCOS in the adult female rat model. *H. rhamnoides* L. nano-emulsion (HRNE) was also formulated, and its efficacy was also assessed in the same animal model. *H. rhamnoides* L. is a member of the family *Elaeagnaceae* and has a fertile history due to its traditional medicinal uses. It is used to treat stomach ulcers, liver dysfunction, and various conditions of skin damage. *H. rhamnoides* L. also possesses anti-diabetic, anti-

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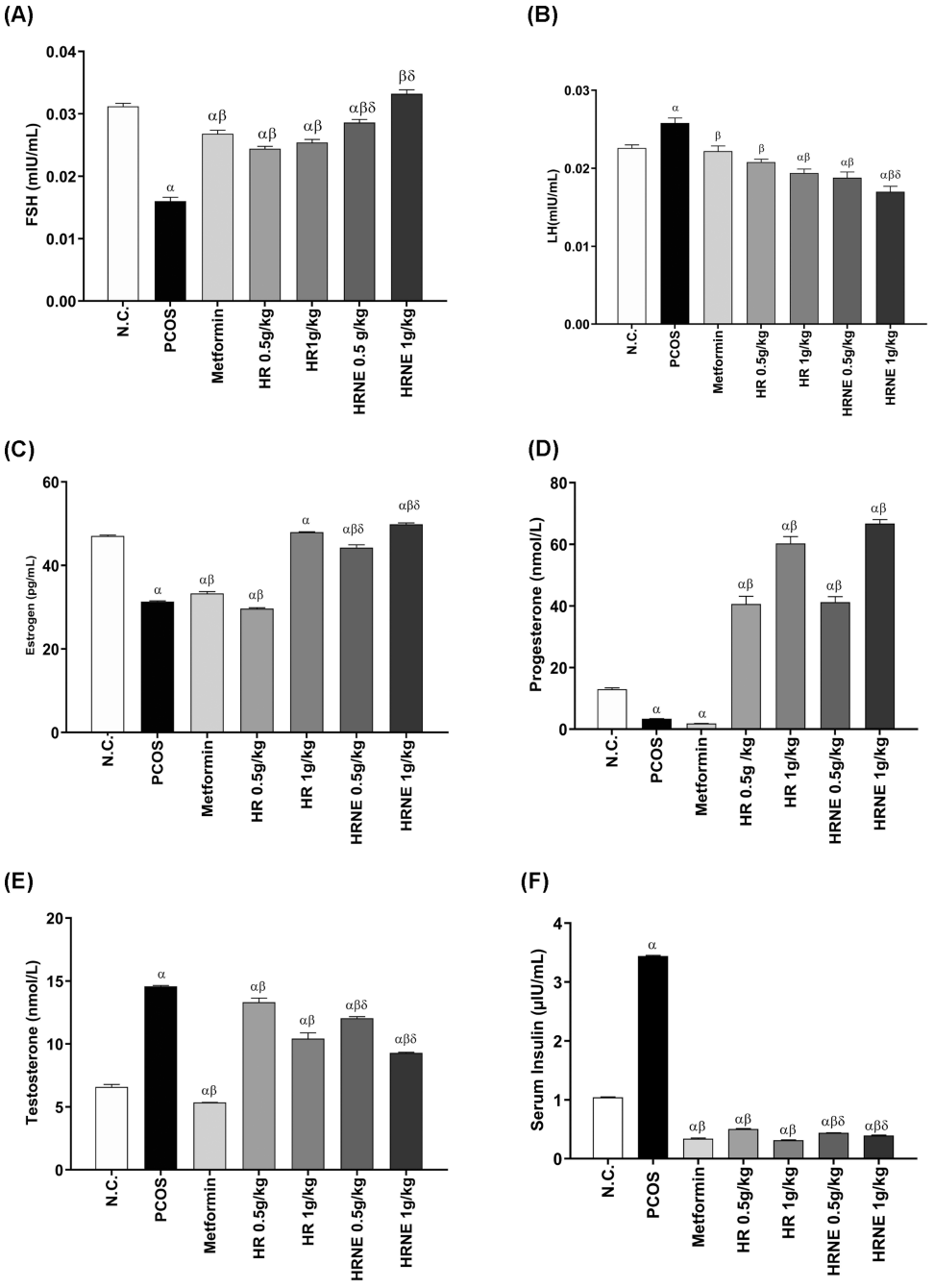

Figure 8. Effect of *H. rhamnoides* L. on the serum hormone level: (A) FSH, (B) LH, (C) estrogen, (D) progesterone, (E) testosterone, and (F) insulin. Values are expressed as mean ± SEM ($n = 5$). α designated a significant difference from N.C., while β designated a significant difference from the PCOS group (disease control). δ designated a significant difference from the HR group. One-way analysis of variance (ANOVA) was used to evaluate the statistical significance between the groups, and multiple comparison was done by Tukey's post-hoc test. N.C.: normal control group, HR: *H. rhamnoides* L., HRNE: *H. rhamnoides* L. nano-emulsion formulation.

cancer, antioxidant, hypolipidemic, and cardio-protective activities. It is also effective in treating endometriosis and improving the integrity of the endometrium.[25]

The fruit and seed oil of *H. rhamnoides* L. (HR) are rich in omega-3, omega-6, omega-7, phytosterols, antioxidants, vitamins E and K, and carotenoids.[26] Fatty acids and omegas are abundant in HR like palmitic acid (13.97%), stearic acid (14.76%), oleic acid (19.94%), linoleic acid (20.357), dihydroxy acetic acid (DHA), and omega-3 (31.10%) (Figures 1 and 2 and Table 2). Total phenolic (TPC) and flavonoid contents (TPC) of HR oil were 1713 ± 0.124 and 310 ± 0.08 µg/100 g, respectively (Table 3). Additionally, DPPH radical scavenging potential of HR oil was assessed, which had a good radical scavenging potential with a % inhibition of 67.27 µg/mL as compared to ascorbic acid (75.34 µg/mL) (Table 4). It has a good rationale with PCOS because in PCOS, there is background inflammation and ROS production that might have a role in disease progression.[27] Thus, the HR oil has good DPPH activity-depicted HR potential to reduce the disease progression and might be involved in hormonal imbalance restoration.[2,27−29] Due to the presence of all the above phytoconstituents and omega-3 fatty acids, HR showed good

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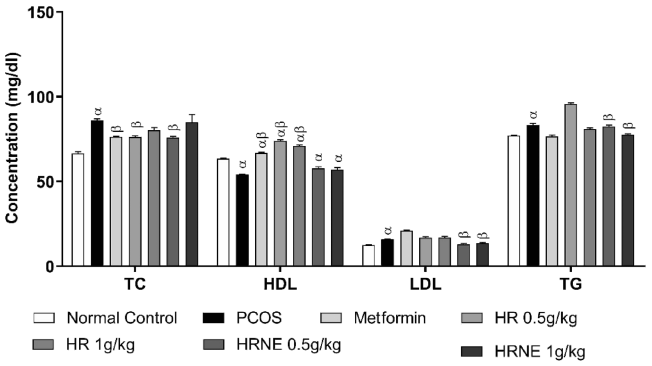

Figure 9. Effect of *H. rhamnoides* L. on the lipid profile. Values are expressed as mean ± SEM ($n = 5$). α designated a significant difference from N.C., while β designated a significant difference from the PCOS group (disease control). δ designated a significant difference from the HR group. One-way analysis of variance (ANOVA) was used to evaluate the statistical significance between the groups, and multiple comparison was done by Tukey's post-hoc test. N.C.: normal control group, HR: *H. rhamnoides* L., HRNE: *H. rhamnoides* L. nano-emulsion formulation.

antioxidant potential. Antioxidant potential is depicted with good DPPH values. These substances work together to safeguard cell membranes and improve cell regeneration. These antioxidant substances also reduce reactive oxygen species (ROS) production that play a role in inflammatory episodes. These inflammatory episodes play a role in worsening of the PCOS symptoms.[2,19]

HR nano-emulsion was prepared, and its purpose was to enhance its bioavailability and solubility. DSC of the nano-emulsion showed no endothermic peak, indicating the uniform distribution of HR oil, and TGA of the final formulation showed two-step degradation. Characteristic peaks of HR oil also shown in the FTIR spectra of nano-emulsion indicated the chemical stability of formulation (Figure 3A−C). All the results of HR oil and HR nano-emulsion are compared and discussed in the upcoming sections. The disease was induced by the administration of letrozole (1 mg/kg) for 5 weeks.[28] Disease induction was confirmed by vaginal smear analysis (Figure 5), and weight variations were recorded (Table 4 and Figure 4). Vaginal smear analysis revealed the confirmation of the disease in the PCOS group as the number of leukocytes increased plentifully presenting the diestrus stage of the estrous cycle (Figure 5). Rats with PCOS stayed in their diestrus stage for a persistent period.[19,28] Meanwhile, the treatment with *H. rhamnoides* L. obliterates the abnormality of the estrous cycle and regulates the cyclicity (Figure 6).

Moreover, histopathology of the ovaries of the normal control group revealed the presence of normal architecture, tiny to middle-sized follicles, and many corpus luteum, granulosa cells, and developing follicles (Figure 7A). The PCOS group's ovary slides (Figure 7B) showed atretic antral follicles and are cystic in nature. However, a prominent decrease in the number and size of cystic follicles occurs after the administration of metformin (Figure 7C). Group (HR 0.5 g/kg) substantially restored the normal anatomy of the ovary (Figure 7D), while group (HR 1 g/kg) showed even better results with the reduction of cystic follicles, increasing corpus luteum and developing follicles (Figure 7E). Both doses of formulation (HR nano-emulsion) also have comparable effects like oil with maturation of cystic follicles into developing follicles (Figure 7F,G).

The hypothalamic gonadotropin-releasing hormone (GnRH) is in charge of pituitary hormone control, including FSH and LH. Unfortunately, in PCOS, normal control is disrupted, and an LH surge occurs, depleting FSH and altering the LH/FSH ratio.[2] These gonadotrophin irregularities are aggravated by interrupting the HPA feedback mechanism. The ovarian cytochrome P450 $17A_1$ ($CYP45017A_1$) gene was over-expressed, and it ultimately enhanced the level of the 17-hyroxylase enzyme, which might be responsible for the over-production of androgens from progesterone. All of these abnormalities are due to the abnormal triggering of the P13k/Akt pathway. More testosterone is created as a result of this spike, increasing anti-Müllerian hormone levels (AMH). Many follicles are present, but their development is halted, and they are unable to mature sufficiently to cause ovulation, as shown in Figure 7B. The overall impact on the hormone level is reduced FSH and increased level of LH (Figure 8B,C). This LH surge deteriorated the ovarian architecture with more cystic and less mature follicles. Estrogen and progesterone levels also reduced, while testosterone and insulin levels increased (Figure 8D−F).

Hyperinsulinemia has multiple reasons such as impairment of beta cells in the pancreas and peripheral insulin resistance. This type of impairment has a strong family tendency, but the actual appearance of this syndrome must have a strong impact from various environmental factors. Metformin is one of the most used drugs to overcome insulin resistance. In our study, we also observed similar findings; metformin lowered the serum insulin level significantly (Figure 8F). Thus, improving insulin tolerance may play a role in management of PCOS. Both doses of HR oil and HRNE have a significant effect in the improvement of the FSH level, and they also played a pivotal role in normalizing the LH surge that is necessary for the rectification of LH/FSH surge. Thus, cumulation will improve the follicle maturing ability of ovaries (Figure 8A,B). Moreover, plant oil and its formulation also have beneficial effects on normalizing the progesterone, estrogen, and testosterone levels, and in this way, they improved ovarian functionality. Thus, there were more mature follicle corpus luteum and lesser number of immature cystic follicles (Figure 8C−E).

Omega-3 DHA (docosahexaenoic acid) is classified as polyunsaturated fatty acids (PUFAs). DHA omega-3 has the potential to be an antioxidant, anti-obesity, and insulin sensitizer.[30] DHA is also helpful in balancing the hormonal profile of PCOS patients.[31] Omega-3 DHA boosts the secretion of the anti-inflammatory adiponectin and improves insulin sensitivity while decreasing the production of pro-inflammatory cytokines like tumor necrosis factor (TNF) and IL-6.[32] PCOS is characterized by insulin resistance, obesity, and cardiometabolic changes like dyslipidemia, diabetes, and hypertension that typically appear in PCOS individuals after the age of 40.[33]

PCOS is a metabolic abnormality, and it has a bad impact on the weight and lipid profile. PCOS increases total cholesterol (TC), LDL, and TG, while it decreases the HDL level significantly.[6] PCOS may lead to obesity, which also plays a role in hypertriglyceridemia.[34] All the doses of HR oil and formulation have a significant effect in improvement of HDL and lowered the levels of TC, LDL, and TG. The most significant improvement was seen with the highest dose of formulation (HRNE 1 g/kg). *H. rhamnoides* L. oil and formulation HRNE show a positive effect in the regulation of the lipid profile (Figure 9). The most prominent effects were observed with HRNE 1 g/kg. Likewise, metformin, a well-known sensitizing insulin, also has a similar type of lipid profile

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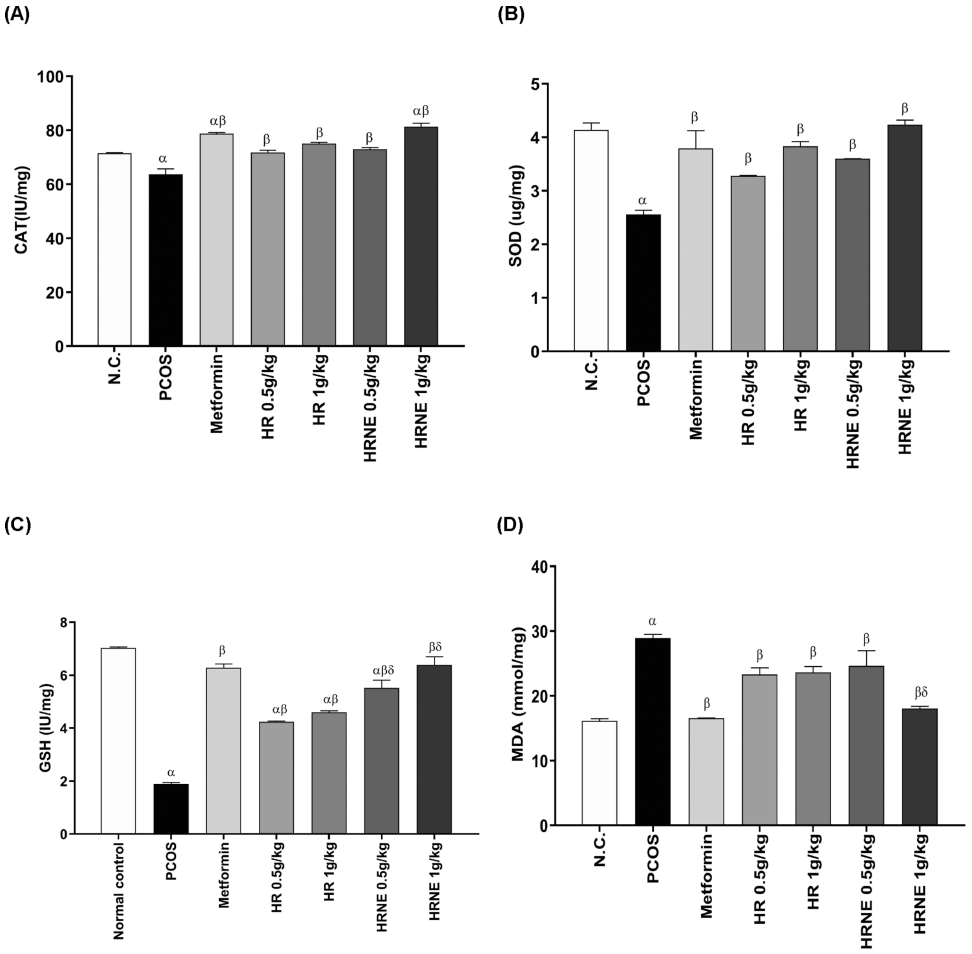

Figure 10. (A–D) Effect of *H. rhamnoides* L. on oxidative stress and lipid peroxidation markers. Values are expressed as mean ± SEM (n = 5). α designated a significant difference from N.C., while β designated a significant difference from the PCOS group (disease control). δ designated a significant difference from the HR group. One-way analysis of variance (ANOVA) was used to evaluate the statistical significance between the groups, and multiple comparison was done by Tukey's post-hoc test. N.C.: normal control group, HR: *H. rhamnoides* L., HRNE: *H. rhamnoides* L. nano-emulsion formulation. CAT: catalase, GSH: glutathione, MDA: malondialdehyde, SOD: superoxide dismutase.

improvement. These findings are persistent with previous reports that HR is effective in reducing weight gain as well as lowering the TC and TG.[6,35]

PCOS patients have hyperinsulinemia that may also cause hyperglycemia, possibly due to insulin resistance. In PCOS patient's, the proposed mechanism behind hyperglycemia, dyslipidemia, and insulin resistance comorbidities is an increase in the visceral adipose tissue. An excess of macronutrients in the visceral adipose tissues stimulates the adipose tissues to release inflammatory mediators such as IL-6 and TNF-α, disposing oxidative stress and pro-inflammatory state.[36] Omega-3 (DHA) inhibits the release of TNF-α and other inflammatory mediators, so the anti-inflammatory and anti-diabetic effects of *H. rhamnoides* L. are justified by the presence of these phytoconstituents.[37]

H. rhamnoides L. oil in part avoids UV-induced ROS generation and improves the level of antioxidant enzymes such as glutathione (GSH), thioredoxin (Trx), and vitamins A and E. Oxidative stress is also involved in the etiology of PCOS, and the level of antioxidant decrease is frequent in PCOS females. Oxidative stress is caused by an imbalance of oxidants and antioxidants. PCOS is associated with an oxidative condition. Letrozole is used for PCOS induction, and it has a bad impact on antioxidant markers such as catalase (CAT), superoxide

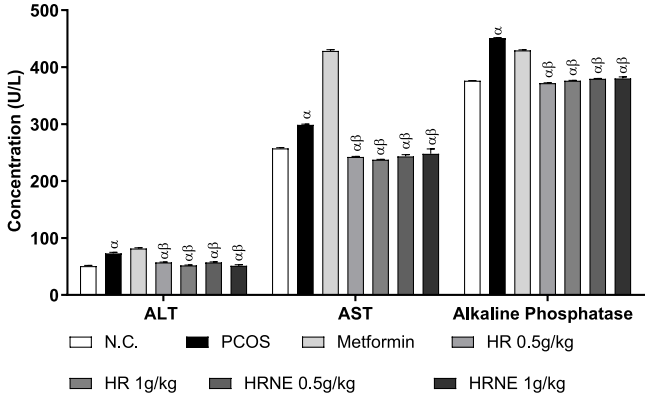

Figure 11. Effect of *H. rhamnoides* L. on liver functioning tests (LFTs). Values are expressed as mean ± SEM (n = 5). α designated a significant difference from N.C., while β designated a significant difference from the PCOS group (disease control). δ designated a significant difference from the HR group. One-way analysis of variance (ANOVA) was used to evaluate the statistical significance between the groups, and multiple comparison was done by Tukey's post-hoc test. N.C.: normal control group, HR: *H. rhamnoides* L., HRNE: *H. rhamnoides* L. nano-emulsion formulation, AST: aspartate aminotransferase, ALT: alanine transaminase.

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dismutase (SOD), and glutathione (GSH) in the body. Similar findings were seen in our study, where levels of SOD, CAT, and GSH were perturbed (Figure 10). Additionally, the lipid peroxidation marker MDA was increased.[38] HR oil along with its formulation (HRNE) improved SOD, CAT, and GSH and lowered the level of MDA (Figure 10A−D). The most significant improvement was observed with formulation. All the phytochemicals present in HR such as omega-3, palmitic acid, stearic acid, linoleic acid, and oleic acid[39] have an excellent antioxidant potential.[40] Thus, the antioxidant potential of oil HR might be due to the presence of these phytoconstituents. One more experiment was included to assess the effects of HR and HRNE on liver functioning tests (LFTs). PCOS disturbed the level of LFTs, but HR and HRNE improved these parameters. However, we did not observe any significant toxic effects of HR and HRNE (HR nano-emulsion) on ALT, AST, and alkaline phosphatase (Figure 11).

5. CONCLUSIONS

Herein, it can be concluded that the results of this study revealed that *H. rhamnoides* L. oil (HR) and its formulation (nano-emulsion) at both doses effectively restored the hormonal imbalance in the letrozole-induced PCOS rat model. Additionally, HR and HRNE improved ovarian health with mature and developing follicles. This was possible due to the presence of various phytochemicals that reduced lipid peroxidation and ROS production, reduced underground inflammation and weight control, and improved insulin sensitivity. HRNE efficacy was more significant as compared to HR, which might be due to its better bioavailability.

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Notes

The authors declare no competing financial interest.

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