

# New alternative herbal remedies for treatment of letrozole-induced polycystic ovary syndrome in rats

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## ABSTRACT

This study aimed to investigate and study the effectiveness as well as the efficacy of five natural products as alternatives for treating ovarian cysts and to correlate their biological activities with their active constituents. *Phyllanthus emblica* fruits, *Trigonella foenum-graecum* seeds, *Brassica oleracea* flowers, and *Persea americana* fruits were extracted and standardized using high-performance liquid chromatography (HPLC), and their phenolic constituents were determined. Meanwhile, the *Olea europaea* L. oil was tested for its purity. The extracts were evaluated for their effect in the treatment of polycystic ovary syndrome (PCOS). *Tribulus arabicus* showed the highest flavonoid content. HPLC analyses of the phenolic, flavonoid, and vitamin constituents of the different extracts were carried out. All the extracts and the *O. europaea* oil showed high antioxidant activities. The biochemical measurements showed a significant difference between rats treated with natural remedies and letrozole. Some extracts significantly reduced the elevated levels of triglycerides, cholesterol, low-density lipoprotein, and glucose compared to metformin. Progesterone and follicle-stimulating hormone levels increased, but luteinizing hormone and testosterone levels decreased in all the treated groups. Marked reduction in the elevated tumor necrosis factor-alpha and malondialdehyde levels with an increased superoxide dismutase level was observed. The histopathological data also supported the potential effect of the remedies. The results showed that *P. emblica* and *P. americana* were viable alternatives to metformin for the treatment of PCOS.

## INTRODUCTION

Polycystic ovary syndrome (PCOS) is a prevalent endocrinopathy in women during their reproductive age. Although the specific etiology of PCOS is unknown, hormonal imbalances such as hyperandrogenemia, insulin resistance (IR), and hyperinsulinemia might play a crucial role. Insulin affects the hypothala-

mus-hypophysis-ovary axis components, resulting in altered metabolic signaling in ovarian tissue. Increased androgen levels may cause IR through elevating the free fatty acid levels and changing the composition and functionality of muscle tissue, resulting in an IR-hyperinsulinemia-hyperandrogenemia cycle. Increased androgen levels are also linked to dermatological issues such as female pattern baldness, acne, and hirsutism (Rojas *et al.*, 2014). PCOS is characterized by irregular ovulation, irregular menstrual cycles, delayed pregnancy or recurrent abortions, depression, anxiety, cardiovascular disease, diabetes mellitus type II, and other health complications (Janez and Jensterle, 2017).

Not only does hormone disruption play a part in the pathophysiology of this syndrome, but so do oxidative stress and elevated inflammatory cytokines, all of which contribute to

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a variety of consequences, including cell damage. Improving insulin intolerance, lowering androgen levels, maintaining normal menstrual cycles, and fertility are all part of PCOS treatment. Nonpharmacological treatments, such as proper physical exercise, body weight control, and nutritious meals, are also beneficial (Patel *et al.*, 2020). Metformin is a drug that is used to treat diabetes mellitus type II and also used to increase insulin sensitivity in obese and PCOS patients. It has been linked to better menstrual cycles and ovulation and a decrease in testosterone levels (Elnashar, 2011). Lactic acidosis, vitamin B12 deficiency, diarrhea, nausea, vomiting, constipation, abdominal pain, and heartburn are some of the most common side effects of metformin (Aaoran and Caughey, 2018).

Oral contraceptives are also indicated in the management of PCOS, although they have side effects such as acne, hirsutism, irregular menstrual periods, increased body weight, nausea, bloating, breast tenderness, and the possibility of infertility (Rosenfield, 2015). For the prevention and treatment of some ailments, most people have used complementary medicine, which includes natural and herbal medicines, since ancient times.

*Brassica oleracea* flowers, often known as broccoli flowers, belong to the Brassicaceae family (mustard family). The blossoms are high in vitamins. It has many health benefits as the flowers contain vitamins A, E, C, K, B6, B12, niacin, riboflavin, and thiamin, as well as folic acid and minerals such as potassium, iron, zinc, selenium, and phosphorus. The plant's principal active ingredients are glucosinolates, which are organosulfur compounds. Fibers, *S*-methyl cysteine sulfoxide, and phenolic acids, in addition to flavonoids such as kaempferol, isorhamnetin, and quercetin glucosides, were detected in the plant's extract. All these constituents are responsible for the antioxidant activity of the broccoli flowers and the protection of the body cells from damage. In addition, broccoli flowers help to lower the blood glucose level, decrease weight, inhibit cancer cell development, treat osteoarthritis, prevent heart disease, and boost the immune system (Axelsson *et al.*, 2017).

The *Persea americana* fruit, also known as avocado fruit, is a tropical fruit with a pear to round shape. It belongs to the family Lauraceae, and its origin is in South-Central Mexico. Vitamins like E, C, K, riboflavin, niacin, pantothenic acid, and pyridoxine are abundant in the fruit, making it incredibly beneficial to physiological functioning. Moreover, it includes minerals like magnesium, potassium, and copper. *Persea americana* is high in oleic acid, fibers, phenolic acids like gallic acid, and bioflavonoids like quercetin, all of which are monosaturated fatty acids. Avocado is said to help with eye problems since it contains carotenoids. As a result, it can protect against age-related eye illnesses, diabetes, inflammation, and cardiac disorders. Hypertension, overweight, and hypercholesterolemia can all be treated using *P. americana*. In addition, it reduces arthritis symptoms, prevents cancer formation, and provides immune system enhancement because of its fiber content (Dreher and Davenport, 2013).

*Phyllanthus emblica* is an Indian fruit, and it is commonly known as gooseberry or amla. *Phyllanthus emblica* contains several active constituents such as phenolic acids like gallic acid, amino acids, vitamins, tannins, and flavonoids including rutin, quercetin, and apigenin. Additionally, it contains fatty acids (lauric acid). Ayurvedic medicine uses *P. emblica* to treat a variety of

ailments, including dyslipidemia, hypertension, diabetes mellitus, inflammation, and cancer. Because of its significant antioxidant activity, *P. emblica* is highly recommended in folk medicine as a therapeutic option for PCOS, which works by improving fertility and managing menstrual cycles (Variya *et al.*, 2016).

*Trigonella foenum-graecum* L. (fenugreek) is extensively cultivated in countries such as India, Egypt, Turkey, Ethiopia, and Morocco. It has antioxidant effects due to its flavonoid content, such as quercetin, apigenin, and rutin. It has been found to contain alkaloids (carpaine, trigonelline, and gentianine). *Trigonella foenum-graecum* is frequently utilized in ayurvedic medicine for the management of hypoglycemia, hypercholesterolemia, hypertension, cancer, and obesity. Moreover, it has anti-inflammatory, antibacterial, and anthelmintic effects. *Trigonella foenum-graecum* is highly recommended for PCOS treatment as it significantly reduces ovarian cyst size and controls menstrual cycles (Aher *et al.*, 2016).

Olive oil is extracted from the olive fruits which are known as *Olea europaea* L. using different physical as well as mechanical methods. *Olea europaea* is recognized to be one of the Mediterranean diets which is rich in dietary polyphenols, vitamins (vitamins K and E), monounsaturated fatty acids (e.g., oleic acid), and polyunsaturated fatty acids (such as linoleic acid). Previous studies revealed the effects of *O. europaea* in dyslipidemia and diabetes mellitus (Memon *et al.*, 2018).

*Tribulus arabicus* (family Zygophyllaceae) has been used as a traditional herb for fertility. It is adapted to grow in dry climates (deserts). It possesses significant antioxidant, antihyperuricemic, and xanthine oxidase inhibitory activities (Abu-Gharbieh *et al.*, 2018; Ksiksi *et al.*, 2017). The main active constituent that was previously isolated is ursolic acid, which showed xanthine oxidase inhibitory activity (Abu-Gharbieh *et al.*, 2018).

In this study, we aimed at investigating the effectiveness of some selected herbal remedies based on their folk medicine, e.g., *P. emblica*, *P. americana*, *B. oleracea*, *T. foenum-graecum*, the *O. europaea* oil, and the *T. arabicus* herb, as well as their published active constituents as alternative treatments of ovarian cysts to standardize the extracts of the plants and to correlate their biological activities with their active constituents.

## MATERIALS AND METHODS

### General

#### Chemicals and drugs

Methanol, ethanol, benzene, and ethyl acetate were obtained from Fisher Scientific (UK) 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, flavonoid standards, fatty acid standards, sodium carboxymethylcellulose (CMC), and the Folin-Ciocalteu reagent were purchased from Merck (Darmstadt, Germany).

Letrozole (Femara<sup>®</sup>, Cairo, Egypt) and metformin (Glucophage<sup>®</sup>, Dubai, UAE) were obtained from private retail pharmacies. Biochemical kits for triglycerides (TGs), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), and glucose were obtained from DiaSys Diagnostic Systems GmbH, Holzheim, Germany (colorimetric assay kits). Progesterone and

testosterone kits were purchased from ichroma™ and measured using the ichroma-II Immunoassay Analyzer, Boditech Med Inc. (Gan-won-do, Korea). Malondialdehyde (MDA), superoxide dismutase (SOD), and tumor necrosis factor-alpha (TNF- $\alpha$ ) kits were ordered from Novus Biologicals LLC (CO, USA), Abnova (Taipei, Taiwan), and R&D Systems (MN, USA), respectively.

### Plant material

The *P. emblica* fruits, *T. foenum-graecum* seeds, *B. oleracea* flowers, and *P. americana* fruits were obtained from local markets in Dubai, UAE, while the *O. europaea* oil (olive oil) was brought from Jordan. The *T. arabicus* plant was collected from the Muhaisnah desert, Dubai, UAE, in October 2019. The plants' identities were confirmed by the staff members of the Department of Biology, Faculty of Science, UAE. Voucher specimens were kept at the Herbarium of the Pharmaceutical Chemistry Department, Dubai Pharmacy College for Girls, UAE (#12-10-19).

### Phytochemical investigation

#### Extraction

The seeds of *T. foenum-graecum* were powdered, and the fresh plants of *P. emblica*, *B. oleracea*, *P. americana*, and *T. arabicus* were cut into small pieces.

The plants were exhaustively extracted by cold maceration in 50% alcohol (three times  $\times$  4 l). The alcoholic extract in each case was evaporated separately under reduced pressure at 50°C using a rotary evaporator. The remaining water was lyophilized using a lyophilizer (BIOBASE). The dried extracts were saved and used for both phytochemical investigation and biological evaluation. The weights of the different plants and the extractive yields are recorded in Table 1.

### Standardization of the plant extracts

#### Measurement of the phenolic contents

A colorimetric assay was used to determine the amount of the phenolic and the flavonoid constituents using a spectrophotometer. All measurements were conducted in triplicate.

The Folin-Ciocalteu reagent was used to measure the amount of phenolic content (Shehab *et al.*, 2015) (results were calculated as mg/g gallic acid dry weight of plant). Gallic acid was used to create the calibration curve. 9 ml of water and 1 ml of the Folin-Ciocalteu reagent were added to 1 ml of each sample and standard. Later, 10 ml of 7% sodium carbonate was added. All samples were kept for 90 minutes at room temperature, and then the absorbances were measured at 750 nm.

The AlCl<sub>3</sub> method, described by Dewanto *et al.* (2002), was used to measure total flavonoid content. Quercetin, a flavonoid

aglycone, was used to create the calibration curve. 0.1 ml from each extract was added to 0.3 ml of distilled water and 0.03 ml of sodium nitrite (5%). After 5 minutes, 0.03 ml of aluminum chloride (10%) was added; after another 5 minutes, 0.2 ml of 1 mM sodium hydroxide and 1 ml of distilled water were added to the tested samples. The absorbances of the yellow color produced were measured at 510 nm. The total flavonoid and phenolic percentage values are recorded in Table 2.

### High-performance liquid chromatography (HPLC) analysis of the phenolic and flavonoid constituents

An Agilent 1100 HPLC apparatus, with a quaternary pump and a UV detector (280 nm), was used for the phenolic acid constituents. Both an Alltima C18 Column (particle size 5  $\mu$ m, 150  $\times$  4.6 mm) and an Alltima C18 Guard Column (5 mm) were used (Alltech, USA), while a Hypersil ODS C18 Column (particle size 5  $\mu$ m, 4.6  $\times$  250 mm) with a UV detector (325 nm) was used for the flavonoid constituents. Two solvents, A (methanol) and B [acetic acid in water (1:25 v/v)], were applied to the column to separate the phenolic acid constituents.

A methanol/water (50:50 v/v, pH 2.8) mixture was used (isocratic flow rate of 1.0 ml/minute) for the separation of the flavonoids. The HPLC analysis of the flavonoids and phenolic constituents is recorded in Tables 3 and 4 and Figures 1 and 2, respectively (Kuntic *et al.*, 2007; Lin *et al.*, 1996).

#### HPLC analysis of water-soluble vitamins

The Agilent 1100 HPLC (UV 220 nm) system was used for the identification of the vitamins. The Titan C18 Column (5 cm  $\times$  2.1 mm D) and a mixture of 20 mM potassium phosphate (pH 3.0) (A) and methanol (B) were used for the separation. Gradient elution was carried out according to Rokayya *et al.* (2014). The results of the water-soluble vitamins are recorded in Table 5 and Figure 3.

#### TLC analysis

All the extracts were analyzed for their triterpenoid contents using TLC and different solvent systems (benzene: ethyl acetate 86:14 v/v; chloroform: methanol 9:1 v/v). The spots were visualized under UV lamps 365 (with or without ammonia vapor). The chromatograms were sprayed with *p*-anisaldehyde (spray reagent) and then heated at 110°C.

### Antioxidant activity

#### DPPH radical scavenging assay

*In vitro*, the antioxidant activities of the extracts and *O. europaea* oil were evaluated through hydrogen donating (radical

**Table 1.** The weight of the plants and the extractive yield.

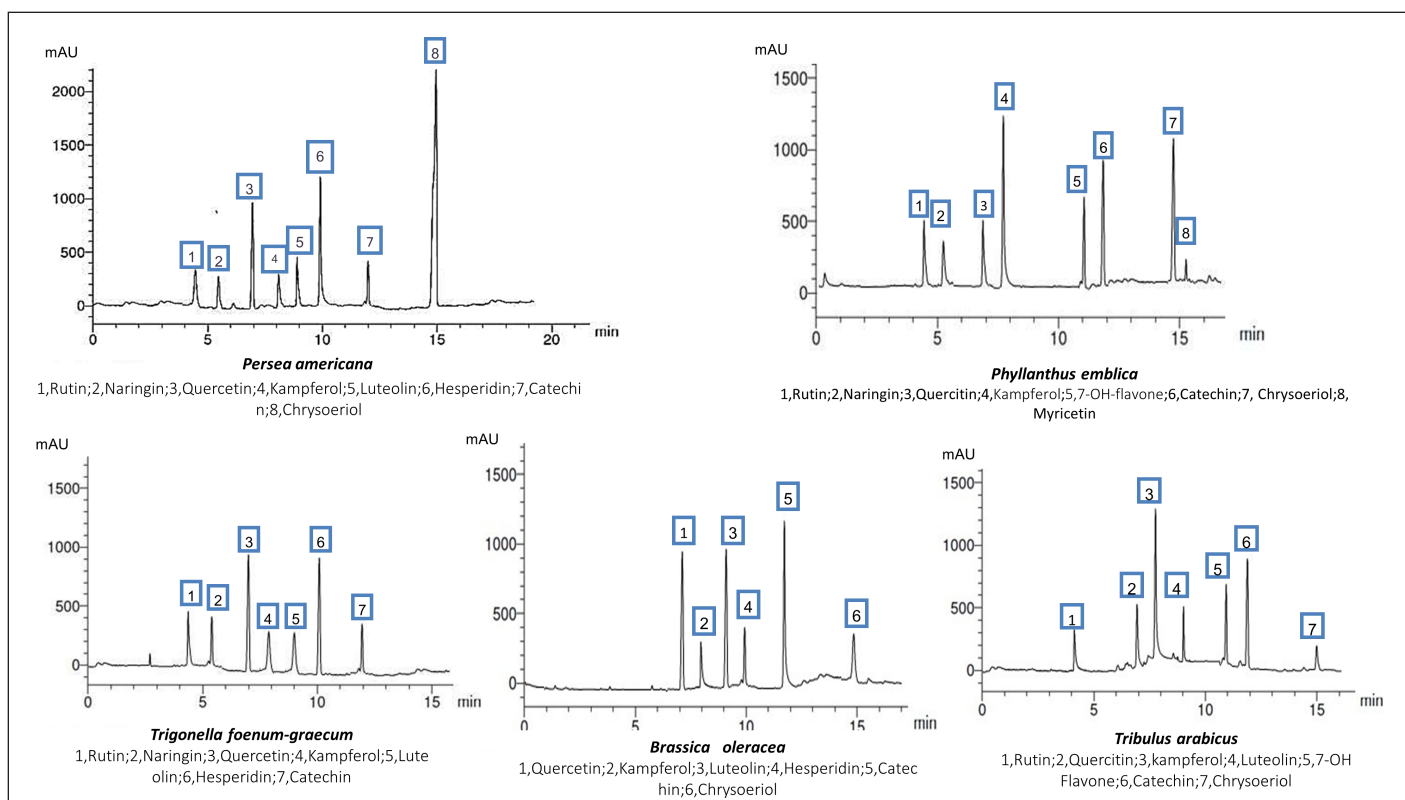
Plants	W. of plant (g)	Wt. of residue(g)	Percentage yields
<i>T. foenum-graecum</i>	1,500	309.86	20.6
<i>P. emblica</i>	2,988	144.0	4.8
<i>B. oleracea</i>	605	484	80
<i>P. americana</i>	521	375	72
<i>T. arabicus</i>	900	41.96	4.7

**Table 2.** Percentages of the flavonoid and phenolic contents in different plant extracts.

Plant name	Percentage of the phenolic content	Percentage of the flavonoid content
<i>T. foenum-graecum</i>	1.60	2.40
<i>P. emblica</i>	4.34	0.39
<i>B. oleracea</i>	0.24	0.04
<i>P. americana</i>	0.39	1.15
<i>T. arabicus</i>	1.45	1.32

**Table 3.** HPLC analysis of flavonoid content of the different plant extracts at wavelength 325 nm.

Flavonoid	RT (minute)	Relative area %				
		<i>P. americana</i>	<i>P. emblica</i>	<i>T. foenum-graecum</i>	<i>B. oleracea</i>	<i>T. arabicus</i>
Rutin	4.6	0.523	0.614	0.814	—	0.814
Naringin	5.2	0.412	0.504	0.704	—	—
Quercetin	7.0	0.921	0.731	1.004	1.145	0.923
Kaempferol	8.0	0.405	1.947	0.517	0.412	0.817
Luteolin	9.0	0.609	—	0.588	1.215	2.213
Hesperidin	10.0	1.245	—	1.156	0.632	—
7-OH-Flavone	11.0	—	1.116	—	—	1.102
Catechin	12.0	0.714	1.078	0.652	1.856	1.511
Chrysoeriol	14.9	2.208	1.544	—	0.521	0.566
Myricetin	15.2	—	0.125	—	—	—

**Figure 1.** HPLC analysis of the flavonoid constituents of the different plant extracts at wavelength 325 nm.

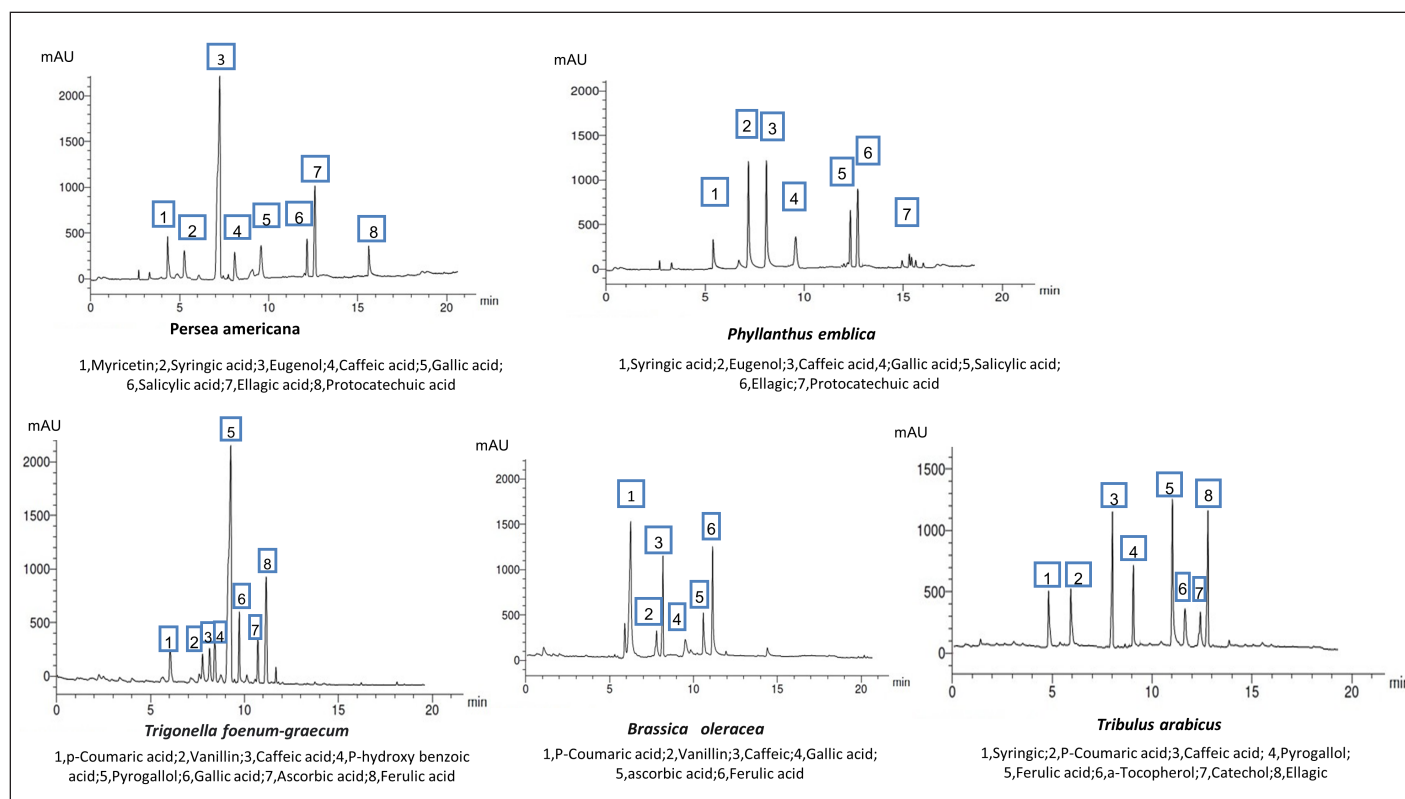


Figure 2. HPLC analysis of the phenolic constituents of different plant extracts at wavelength 280 nm.

Table 4. HPLC analysis of the phenolic constituents of the different plant extracts at wavelength 280 nm.

Phenolic	RT (minute)	Relative area %				
		<i>P. americana</i>	<i>P. emblica</i>	<i>T. foenum-graecum</i>	<i>B. oleracea</i>	<i>T. arabicus</i>
Myricetin	4.50	1.20	—	—	—	—
Syringic acid	5.10	0.91	0.712	—	—	0.922
<i>p</i> -Coumaric acid	6.00	—	—	0.823	2.251	0.968
Eugenol	7.00	3.11	1.605	—	—	—
Vanillin	7.80	—	—	0.755	0.742	—
Caffeic acid	8.10	0.433	1.526	1.526	1.387	1.963
<i>p</i> -Hydroxybenzoic	8.50	—	—	0.712	—	—
Pyrogallol	9.02	—	—	—	—	1.451
Gallic acid	9.70	0.612	0.614	1.266	0.412	—
AA	10.50	—	—	0.823	0.961	—
Ferulic acid	11.00	—	—	1.809	2.011	2.112
Alpha-tocopherol	11.50	—	—	—	—	0.745
Salicylic acid	12.00	0.76	0.856	—	—	—
Ellagic acid	13.00	1.46	0.947	—	—	1.833
Protocatechuic	15.50	0.96	0.125	—	—	—
Total identified compounds		8	7	7	6	7

scavenging ability) using the stable DPPH radical. The procedure was carried out according to Cheng *et al.* (2006). Moreover, the samples were shaken after the addition of the reagent and kept in the dark at 37°C for 30 minutes. The absorbance was measured at 517 nm using a UV microplate reader. The inhibition percentage of the DPPH radical (antioxidant activity) was calculated using the following equation:

$$\% \text{ inhibition} = [A_0 - (A_1 - A_2)] / A_0 \times 100\%$$

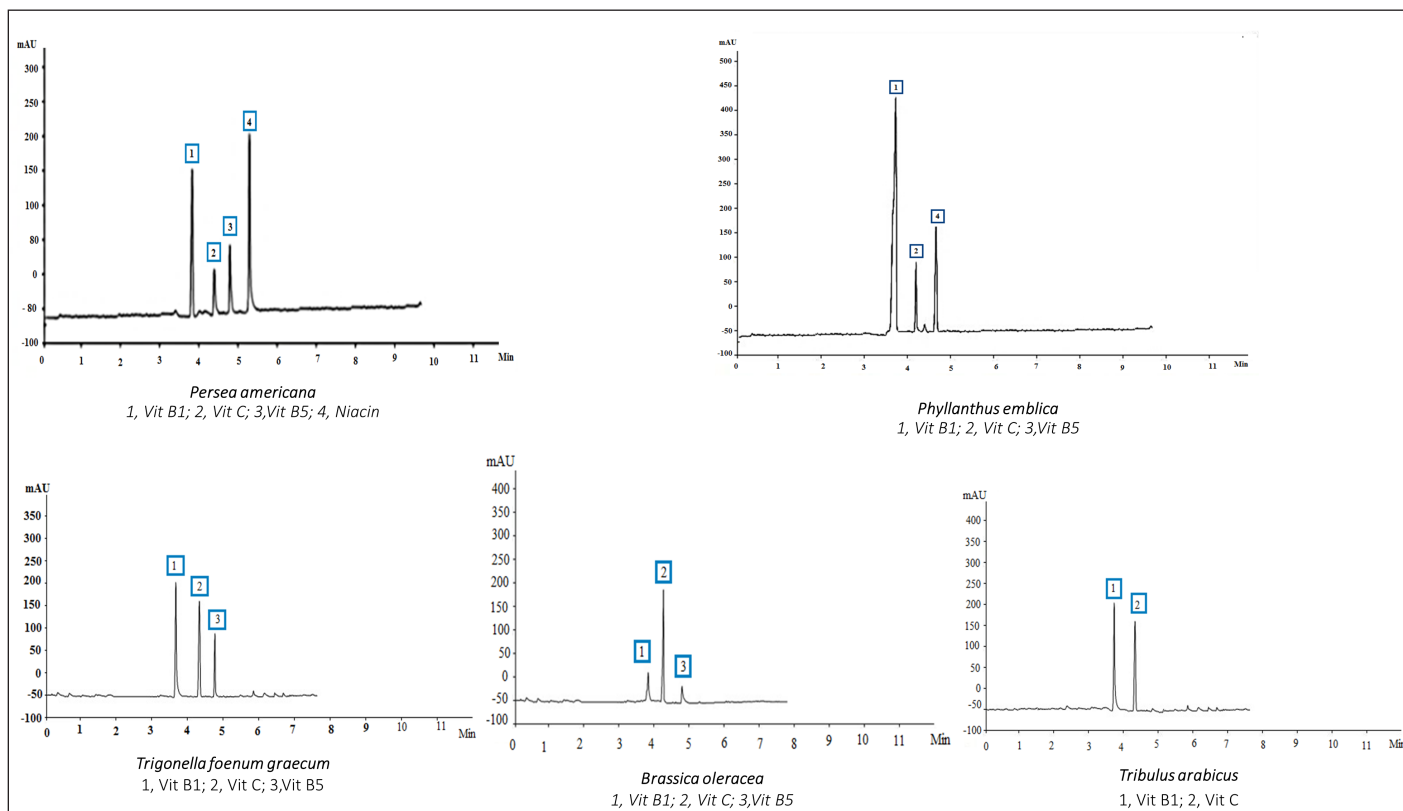
where,  $A_0$  is the absorbance of the control,  $A_1$  is the absorbance of the sample + DPPH, and  $A_2$  is the absorbance of the sample without DPPH.

Ascorbic acid (AA) was used as a standard. The samples were analyzed in triplicate (Fig. 4).



**Table 5.** HPLC analysis of the vitamins of the different plant extracts at 220 nm.

Vitamins	RT (minute)	Relative area %				
		<i>P. americana</i>	<i>P. emblica</i>	<i>T. foenum-graecum</i>	<i>B. oleracea</i>	<i>T. arabicus</i>
Vitamin B1	3.8	1.316	1.76	1.02	0.414	0.866
Vitamin C	4.3	0.560	0.49	0.91	1.120	0.754
Vitamin B5	4.9	0.790	0.78	0.67	0.162	—
Niacin	5.3	1.780	—	—	—	—
Total percentages	—	4.446	3.03	2.60	1.696	1.62

**Figure 3.** HPLC analysis of the vitamins of the different plant extracts at 220 nm.

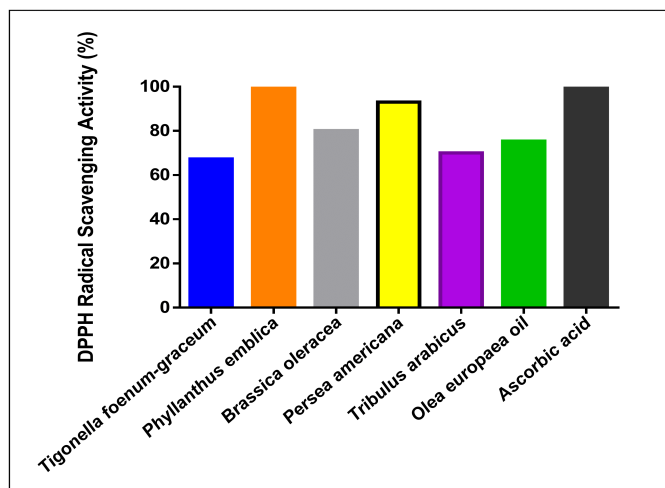
## Biological study

### Animals

Fifty-four mature female Wistar rats weighing 170–190 g, with 4–5 days of regular estrus cycles, were selected for this study and maintained in a controlled environment at a temperature of 25°C and a 12 hours light/dark cycle with free access to food and water. All the animal investigations were performed according to the ethical standards and upon approval of the Research and Ethical Committee of Dubai Pharmacy College for Girls, Dubai, UAE (REC-FD-2020-05).

### Experimental design

All the animals except the control group were given letrozole (Femara®, Cairo, Egypt) orally at a dose of 1 mg/kg dissolved in 1% of CMC for 35 days to induce PCOS. This dose was selected according to previous studies on inducing cystic follicle formation (Karateke *et al.*, 2018).

**Figure 4.** DPPH radical scavenging inhibition activity of the plant extracts and AA.

During the study, the estrus cycle was evaluated microscopically by taking a vaginal smear and analyzing the relative proportion of epithelial cells, cornified cells, and leukocytes to confirm the PCOS model by the existence of the irregular estrus cycle (Fig. 5) (Zhou *et al.*, 2019). After that, the animals were divided into nine groups of six rats per group: Group 1: control group received CMC only; Group 2: negative control group was administered letrozole only; Group 3: positive control group received letrozole and then metformin (70 mg/kg); and Groups 4–9: each group received letrozole and one of these plants' extracts (500 mg/kg): *T. arabicus*, *P. americana*, *P. emblica*, *T. foenum-graecum*, *B. oleracea*, and *O. europaea* oil, respectively.

#### Termination of the procedure

On the 56th day, after 24 hours of receiving the last dose of the treatment, the rats were fasted overnight, and blood samples were collected from the retro-orbital plexus. Plasma was obtained by centrifugation for measurement of the biochemical markers, e.g., serum glucose, cholesterol, TGs, and LDL, in addition to progesterone and testosterone. The animals were weighed before dissection. The two ovaries were separated from each rat, detached from any surrounding tissue or fat, and weighed for the measurement of morphological changes in the ovaries of rats. The two ovaries were weighed, and the average ovary weight to the body weight for each animal in the group was calculated (Kafali *et al.*, 2003; Mihanfar *et al.*, 2021). The left ovaries were kept in 10% formalin for the histopathological measurements, and the right ones were used for the preparation of tissue homogenates using Wise instrument model HG-15D for the oxidative stress determinations, e.g., MDA, SOD, and TNF- $\alpha$ .

#### Measurement of circulating levels of progesterone and testosterone

Progesterone and testosterone levels were analyzed by fluorescence immunoassay using the ichroma™ II instrument for

the quantitative determination of sex hormones in human plasma. Plasma (75  $\mu$ l) was mixed with 30  $\mu$ l of the displacing reagent; the mixture was shaken several times and then incubated at room temperature for 3 minutes. An aliquot of 75  $\mu$ l of the prepared sample mixture was added to the sample well on the cartridge and was incubated for 15 minutes at room temperature, then scanned, and recorded in Table 6.

#### Measurement of circulating levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH)

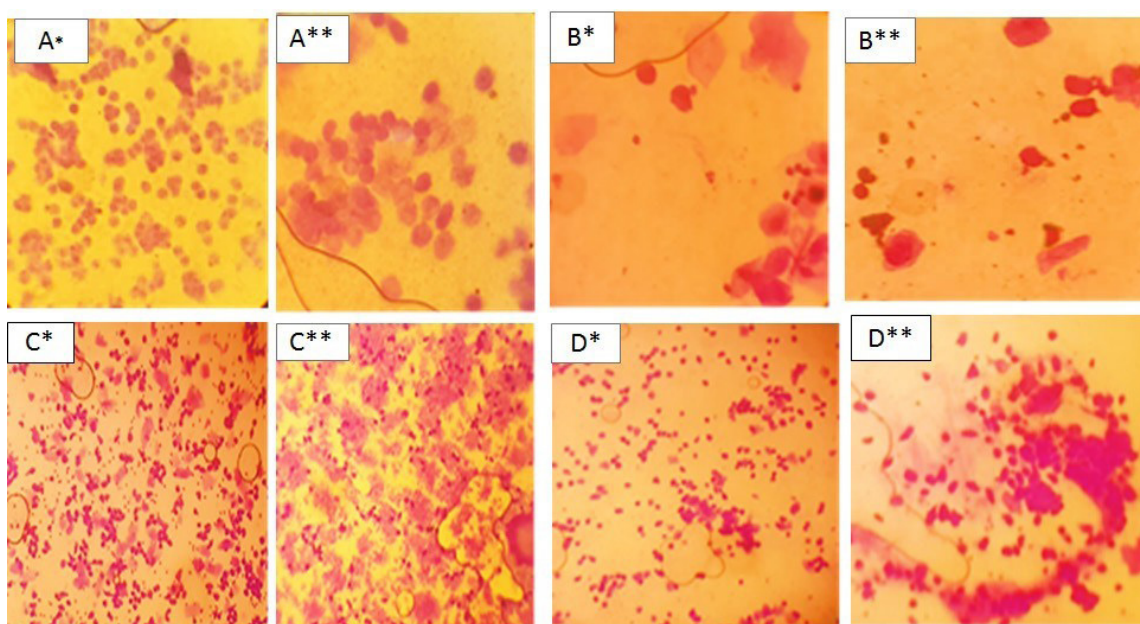
Samples (50  $\mu$ l) were added to the wells, and 50  $\mu$ l of an horseradish peroxidase (HRP) conjugate was added to all wells except the blank. The plate was mixed and incubated for 1 hour at 37°C after the addition of a 50  $\mu$ l antibody to all the wells. The washing step was performed three times. 50  $\mu$ l of substrate A and 50  $\mu$ l of substrate B were also introduced and mixed into all wells. The plate was incubated in a dark place for 15 minutes at 37°C, and then 50  $\mu$ l of a stop solution was used to stop the reaction, and the optical density was determined at 450 nm within 10 minutes. The results are recorded in Figure 7.

#### Measurement of blood lipids and glucose levels

The levels of cholesterol, LDL, TGs, and serum glucose were determined using spectrophotometric techniques from the various blood samples.

**Determination of serum cholesterol.** Using commercial diagnostic kits from CHOD-PAP, the level of serum cholesterol was determined. 10  $\mu$ l of the sample and 1 ml of the reagent (Good's buffer phenol, 4-aminoantipyrine, cholesterol esterase, cholesterol oxidase, and peroxidase) were combined in the two vessels. After 10 minutes of incubation, the absorbance was measured at 546 nm against the reagent blank (Artiss *et al.*, 1997).

**Determination of serum LDL.** According to Bairaktari *et al.* (2005), serum LDL-cholesterol was determined by a color-producing enzymatic reaction.



**Figure 5.** Crystal violet-stained vaginal smear from normal\* and PCOS group\*\* showing proestrus (predominance of nucleated epithelial cells) A\* and A\*\*, estrus (consists of anucleated cornified cells) B\* and B\*\*, metestrus (equal proportion among leukocytes, cornified, and nucleated epithelial cells) C\* and C\*\*, and diestrus (leukocytes mainly) D\* and D\*\*.

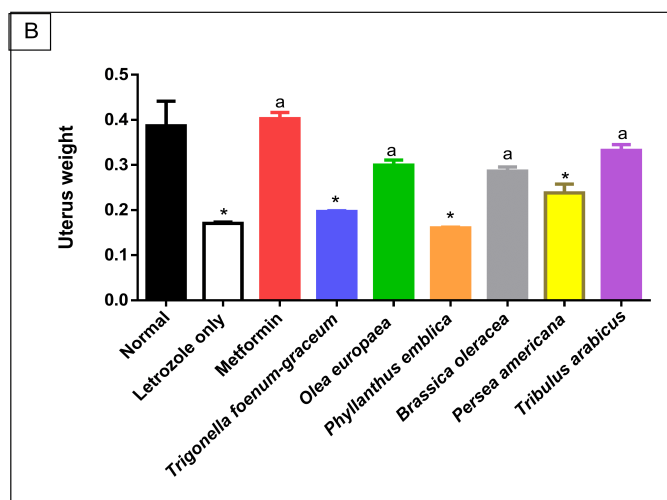
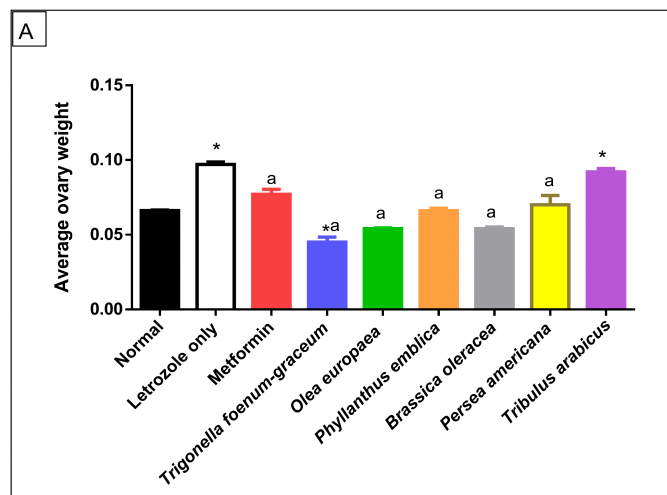
**Table 6.** Effects of letrozole, metformin, and the plants' extracts on progesterone and testosterone levels.

Groups	Progesterone (nmol/l)	Testosterone (ng/ml)
Normal	61.560 ± 6.790 <sup>b</sup>	0.343 ± 0.007 <sup>b</sup>
Letrozole	20.224 ± 1.512	8.847 ± 0.964
Metformin	44.897 ± 4.305 <sup>a</sup>	0.416 ± 0.076 <sup>a</sup>
<i>T. foenum-graecum</i>	56.587 ± 9.969 <sup>a</sup>	0.770 ± 0.225 <sup>a</sup>
<i>O. europaea</i> oil	32.210 ± 1.165 <sup>b</sup>	0.546 ± 0.027 <sup>a</sup>
<i>P. emblica</i>	60.333 ± 8.033 <sup>a</sup>	0.50 ± 0.100 <sup>a</sup>
<i>B. oleracea</i>	40.187 ± 5.065	0.583 ± 0.243 <sup>a</sup>
<i>P. americana</i>	52.107 ± 1.868 <sup>a</sup>	0.393 ± 0.053 <sup>a</sup>
<i>T. arabicus</i>	61.475 ± 4.877 <sup>a</sup>	0.50 ± 0.050 <sup>a</sup>

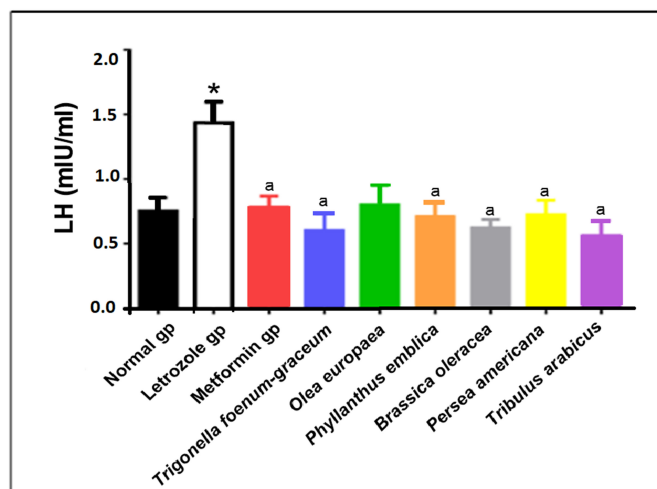
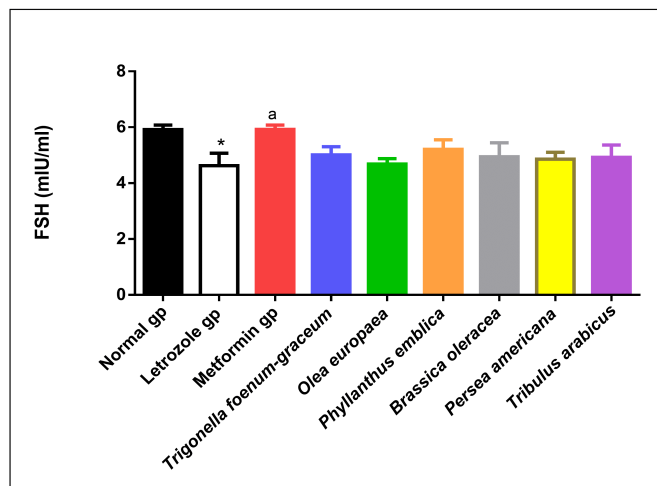
<sup>a</sup>Significant difference when compared with letrozole-only-treated animals.

<sup>b</sup>Significant difference when compared with normal control.

Data are presented as statistical mean ± SEM.



**Figure 6.** Morphological changes in ovary (A) and uterus (B) weight of normal, letrozole, and different treatment groups of rats. \* indicates significant difference when compared with normal control. <sup>a</sup> indicates significant difference when compared with letrozole-only-treated animals. Data are presented as statistical mean ± standard error of mean (SEM).



**Figure 7.** LH and FSH levels for normal, letrozole, and different treatment groups of rats. \* indicates significant difference when compared with normal control. <sup>a</sup> indicates significant difference when compared with letrozole-only-treated animals.

**Determination of serum TGs.** Glycerol-3-phosphate oxidase was used in the colorimetric enzymatic testing procedure to measure the amounts of TGs. As a blank, 10 µl of distilled water was used, and 1,000 µl of the reagent—buffer Good's phenol, 4-aminoantipyrine, cholesterol esterase, cholesterol oxidase, and peroxidase—was added to the blank and sample solutions. For 10 minutes, the mixtures were incubated at 37°C. At 546 nm, the absorbance was measured in comparison to the reagent blank (Rifai *et al.*, 1999).

**Determination of serum glucose.** Using the “GOD-PAP” enzymatic photometric test, the serum glucose level was determined. The reagent used in this assay was composed of phosphate buffer (250 mM), phenol (5 mM), 4-aminoantipyrine (0.5 mM), glucose oxidase (GOD, ≥10 kU/l), and peroxidase (≥1 kU/l). 10 µl of distilled water was used as a blank, and 1,000 µl of the reagent was added and mixed with the sample. After 20 minutes incubation at 20°C–25°C, absorbance against the blank within 60 minutes was obtained (Trinder, 1969). The results are recorded in Figure 8.



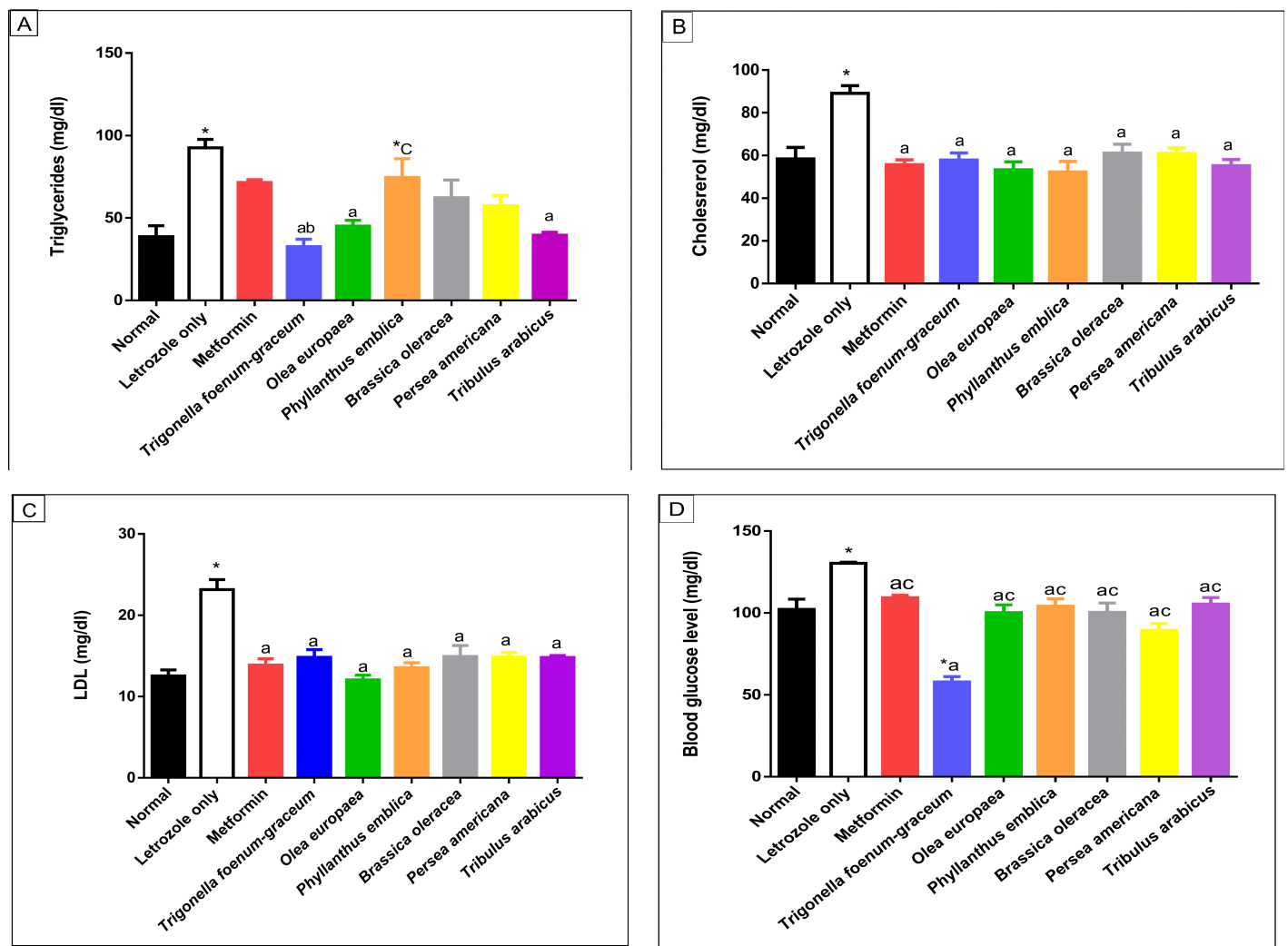
### Measurement of oxidative stress markers

**Measurement of MDA level.** MDA level was investigated using the competitive ELISA method. A precoated microplate with MDA was used to compete with the sample or standard MDA for sites on the biotinylated detection Ab specific to MDA. After washing out the excess conjugate and unbound sample or standard, avidin conjugated to HRP was added to all the wells and incubated for 45 minutes. A TMB substrate solution was then added to all the wells, and then the stop solution (2 M H<sub>2</sub>SO<sub>4</sub>) was added to terminate the reaction. To measure the color intensity at a wavelength of 450 nm, the ROBONIK Readwell TOUCH ELISA Plate Analyzer (Ambarnath, India) was used, and MDA concentrations in the samples were measured ([https://](https://www.novusbio.com/support/support-by-application/elisa/direct-sandwich-protocol)

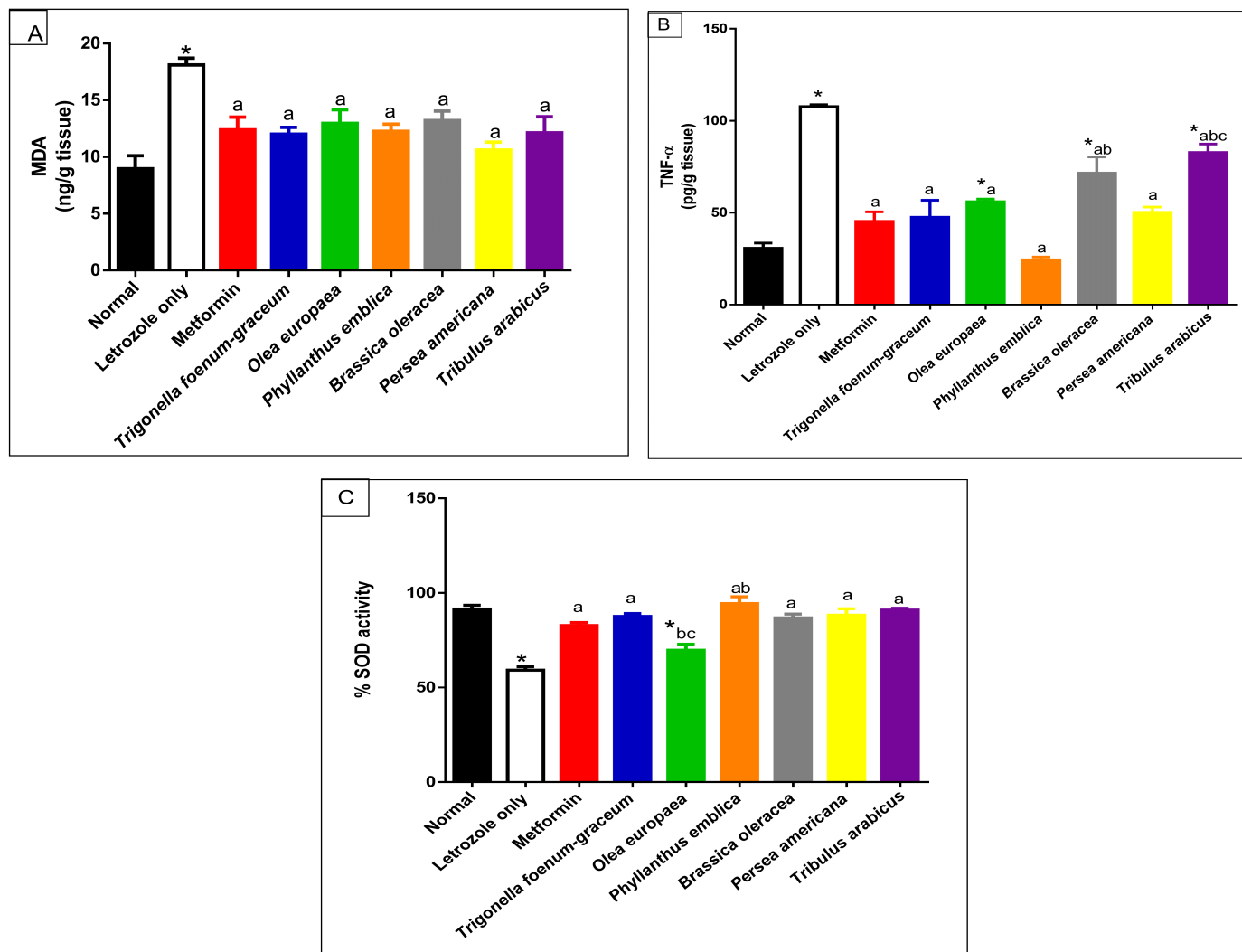
[www.novusbio.com/support/support-by-application/elisa/direct-sandwich-protocol](https://www.novusbio.com/support/support-by-application/elisa/direct-sandwich-protocol)).

**Measurement of SOD activity.** To measure SOD activity, the colorimetric assay was employed. The WST-1 reagent was used as it produces a formazan dye upon reduction with superoxide anion. The rate of the reduction with a superoxide anion was linearly correlated with xanthine oxidase activity and was hampered by SOD. The results are recorded in Figure 9.

**Measurement of TNF- $\alpha$  level.** This was done utilizing the sandwich ELISA technique. Standards, control, and samples were added to wells precoated with monoclonal antibodies specific for rat TNF- $\alpha$ . TNF- $\alpha$  is bound to the immobilized antibody. After removal of the unbound substances by washing, the enzyme-linked polyclonal antibody specific for rat TNF- $\alpha$  was added and incubated for 2 hours. A substrate solution was added following the washing



**Figure 8.** Lipid profile including TGs (A), cholesterol (B), LDL (C), and blood glucose (D) levels, respectively, for normal, letrozole, and different treatment groups of rats. \* indicates significant difference when compared with normal control. <sup>a</sup> indicates significant difference when compared with letrozole-only-treated animals. <sup>b</sup> indicates significant difference when compared with metformin. <sup>c</sup> indicates significant difference when compared with *T. foenum-graecum*.



**Figure 9.** Oxidative stress markers including MDA (A), TNF- $\alpha$  (B), and SOD (C) activity for normal, letrozole, and different treatment groups of rats. \* indicates significant difference when compared with normal control. <sup>a</sup> indicates significant difference when compared with letrozole-only-treated animals. <sup>b</sup> indicates significant difference when compared with metformin. <sup>c</sup> indicates significant difference when compared with *T. foenum-graecum*.

step producing a blue color after 30 minutes incubation. The termination step was done using the stop solution giving a yellow color whose intensity is proportional to the amount of TNF- $\alpha$  bound.

### Histopathological investigation

The left ovaries of the rats were excised and weighed before preparation. After cutting, the tissues were fixed in 10% formal saline for 24 hours. After that, all specimens were cleaned, gradually dehydrated using ascending grades of ethanol, and then embedded in paraffin at 56°C. Sections of 4–5  $\mu$  thickness were prepared using a microtome (Shandon Cat. No. 0525, England). On glass slides, the sections were collected, deparaffinized, and stained using hematoxylin and eosin. All slides were evaluated by a light microscope (Leica ICC50 E, MD 500, Germany). The follicles containing oocytes with nuclei were counted as healthy ones. The follicles were classified as follows: a follicle that is still growing, a preantral follicle with a single layer of cuboidal granulosa cells and an intact, expanded oocyte with a visible nucleus. Regardless of whether the cavity was visible or not, the antral follicle had two or more layers of granulosa cells. Degenerating oocytes or pyknotic granulosa cells are present in

atretic follicles. The theca interna is thickened, and the granulosa cell layer of the cystic follicle is attenuated.

The numbers of developing atretic, cystic, and corpora lutea (CL) follicles in each group's ovaries were counted. In the control group and PCOS groups, the thickness of the "tunica albuginea" capsule, hyperplasia of the theca interna, reduction in the number of CL, and subcapsular follicular cysts were examined.

### Statistical analysis

Data were statistically analyzed using one-way analysis of variance followed by Tukey-Kramer's multiple comparisons test. Statistical analyses were conducted using GraphPad Prism software (GraphPad Software Inc., V 4.03, San Diego, CA). The significance of the biochemical parameters was assumed at  $p < 0.05$ .

## RESULTS

### Phytochemical investigation

#### Extractive yields

As shown in Table 1, the *B. oleracea* extract yield showed the highest percentage (80%), followed by the yield of

the *P. americana* extract (72%) among the other herbal remedies' extracts.

### Standardization of the remedies' extracts

#### Measurement of the phenolic and flavonoid contents

Table 2 reveals that *T. foenum-graecum* seeds showed the highest flavonoid content (2.4%), followed by the *T. arabicus* herb (1.32%) and *P. americana* fruit (1.15%), while the least amount of flavonoids appeared in *B. oleracea* flowers (0.04%). On the other hand, the *P. emblica* fruit showed the highest phenolic content (4.34%), calculated as gallic acid, followed by the *T. foenum-graecum* seeds (1.6%) and *T. arabicus* herb (1.45%).

#### HPLC analysis of the flavonoid and phenolic acids

The HPLC analysis of the flavonoid constituents of the different plant extracts showed that quercetin aglycone, catechin, and kaempferol exist in all the plants under investigation at different concentrations. Luteolin was detected in all the plant extracts under investigation except *P. emblica*. *Phyllanthus emblica* showed the highest content of kaempferol (1.947) and 7-OH-flavone (1.116) among all the remedies. Meanwhile, *P. americana* showed the highest content of chrysoeriol (2.208) and hesperidin (1.245). Catechin was a predominant component in *B. oleracea* (1.856), while the highest content of rutin existed in *T. arabicus* and *T. foenum-graecum* (0.814). Myricetin was detected only in *P. emblica* (0.125). Hesperidin and quercetin were the predominant flavonoid constituents in *T. foenum-graecum* (1.156 and 1.004, respectively). Naringin was detected in all the plants except *B. oleracea* and *T. arabicus*.

As represented in Table 4, the HPLC analysis of the remedies' extracts under investigation allowed the identification of various phenolic compounds. Seven components were identified in all extracts except *P. americana* and *B. oleracea* (eight and six, respectively). The percentages of the total identification were 9.445%, 6.385%, 7.714%, 7.764%, and 10.064% for *P. americana*, *P. emblica*, *T. foenum-graecum*, *B. oleracea*, and *T. arabicus*, respectively. The highest phenolic constituents were detected in *T. arabicus*, followed by *P. americana*, as shown in Table 4. Caffeic acid was detected in all the plant extracts under investigation, while gallic acid was detected in all the plant extracts except *T. arabicus*. Eugenol, a phenolic compound, was a predominant compound in *P. americana*, followed by ellagic acid and myricetin aglycone (3.11%, 1.46%, and 1.02%, respectively). However, AA was detected only in *T. foenum-graecum* seeds and *B. oleracea* flowers. Ferulic acid is a major phenolic acid in both *T. foenum-graecum* and *T. arabicus*. *Brassica oleracea* showed the highest content of *p*-coumaric acid (2.251%) among the other plants' extracts.

Table 5 reveals that setting the detector at 220 nm and using another type of column allowed the detection of vitamin C and vitamin B1 in all the plant extracts at a different concentration, while niacin, a form of vitamin B, was only detected in *P. americana*. Furthermore, vitamin B5 was detected in all the plant extracts except *T. arabicus*. The highest vitamin content was explored in *P. americana* and *P. emblica* (4.446% and 3.03%, respectively). Moreover, *P. emblica* showed the highest amount of Vitamin B1 (1.76%), followed by *P. americana* (1.316%) and *T. foenum-graecum* (1.02%). Vitamin C was a prevalent component

in *B. oleracea*, followed by *T. arabicus* (1.12% and 0.754%, respectively).

#### TLC screening of fatty acids and triterpenes of the different extracts

Preliminary screening of fatty acids and triterpenoidal constituents showed that *O. europaea* had palmitic, oleic, arachidonic, lauric, and stearic acids and alpha-amyrin. In addition, alpha-amyrin was detected in all the plants under investigation.

#### In vitro antioxidant activities of different plants

Figure 4 reveals that all the plants as well as the *O. europaea* oil showed high antioxidant activities. The highest DPPH free radical inhibition was shown in *P. emblica* (100%) and *P. americana* (93%) as compared to AA (100%).

#### Vaginal cytology

The types and number of cells from the vaginal smear of each rat were examined under the microscope daily to recognize the estrous cycle phases (Marcondes *et al.*, 2002). It was noticed that the animals persisted in the diestrus stage in the letrozole-treated animals more than in the normal control group, and the vaginal smears were composed of cornified cells in all the phases of the estrous cycle in the PCOS group. This indicates the irregular cycle of the PCOS model if compared with the regular cycle of the normal group (Zhou *et al.*, 2019).

#### Morphological changes in ovaries and uteri of rats

The average ovary weight increased significantly in the PCOS-negative control group compared to the normal group ( $p < 0.001$ ). However, a marked reduction in uterus weight was recorded in the PCOS-negative control group compared to the normal group ( $p < 0.001$ ). All the treated plant extracts were like metformin, except *T. arabicus* ( $p < 0.001$ ), in decreasing the elevated ovaries' weight to near-normal values and were superior to the effect of *T. foenum-graecum* ( $p < 0.001$ ). On the other hand, *T. arabicus* ( $p < 0.001$ ), the *O. europaea* oil ( $p < 0.01$ ), and *B. oleracea* ( $p < 0.05$ ) were superior to *P. americana* ( $p > 0.05$ ), *P. emblica* ( $p > 0.05$ ), and *T. foenum-graecum* ( $p > 0.05$ ) in restoring uterus weight compared to the PCOS group, as shown in Figure 6.

#### Measurement of LH and FSH levels

A significant elevation in LH levels accompanied by a significant reduction in FSH levels was observed in rats with PCOS compared to the normal control group ( $p < 0.05$ ). PCOS rats treated with metformin as well as the plants' extracts showed significant improvement in LH and FSH levels compared to the PCOS untreated group (Fig. 7).

#### Measurement of circulating levels of progesterone and testosterone

As represented in Table 6, progesterone levels significantly decreased in the letrozole-treated groups compared to the normal control group ( $p < 0.001$ ). *Phyllanthus emblica* ( $p < 0.001$ ), *P. americana* ( $p < 0.01$ ), and *T. arabicus* ( $p < 0.001$ ), but not the *O. europaea* oil ( $p > 0.05$ ), were superior to metformin ( $p > 0.05$ ) in the elevation of progesterone levels compared to the letrozole-only group with no significant difference with the control group ( $p > 0.05$ ) or *T. foenum-graecum* group ( $p > 0.05$ ). The PCOS model

without treatment (letrozole only) showed a remarkable increase in the level of the testosterone hormone compared to the control ( $p < 0.001$ ) or the plants' extracts ( $p < 0.001$ ). All the tested extracts reduced the level to near the normal value ( $p > 0.05$ ).

### Measurement of lipid profile and glucose levels

The data of lipid profiles in the normal, letrozole-only, and different plant groups are provided in Table 7 and Figure 8. Significant elevation in TGs ( $p < 0.001$ ), cholesterol ( $p < 0.001$ ), and LDL levels was observed in the group treated with letrozole only in comparison to the normal group. The *O. europaea* and *T. arabicus* groups showed significant reduction of the TG levels to near the normal value ( $p > 0.05$ ) compared to the letrozole group ( $p < 0.01$ ) and superior to the metformin group ( $p > 0.05$  with letrozole). *Brassica oleracea* and *P. americana* showed similar results to those of the metformin group compared to the normal and letrozole groups ( $p > 0.05$ ), as shown in Table 7 and Figure 8A.

The natural plants used successfully decreased the elevated LDL and cholesterol levels to near the normal value ( $p > 0.05$ ) compared to the letrozole group [all,  $p < 0.001$ , *B. oleracea* in cholesterol ( $p < 0.01$ )], as represented in Figure 8B and C.

The blood glucose level increased significantly in the PCOS group ( $p < 0.01$ ) compared to the normal group. *Trigonella foenum-graecum* demonstrated the most significant effect in lowering the blood glucose level compared to metformin ( $p < 0.001$ ), the plants' extracts ( $p < 0.001$ ), and even the normal control group ( $p < 0.001$ ). The *O. europaea* oil ( $p < 0.001$ ), *B. oleracea* ( $p < 0.01$ ), *P. americana* ( $p < 0.001$ ), *P. emblica* ( $p < 0.01$ ), and *T. arabicus* ( $p < 0.01$ ) significantly decreased the blood glucose level compared to rats with PCOS and near the normal value ( $p > 0.05$ ), as represented in Table 7 and Figure 8D.

### Measurement of oxidative stress markers

The letrozole-only-treated group showed a marked increase in MDA level compared to the normal group ( $p < 0.001$ ). Treatment with *P. emblica*, *P. americana*, and *T. arabicus* significantly reduced the elevated lipid peroxidation level compared to the letrozole group ( $p < 0.01$ ). The *O. europaea* oil-

and *B. oleracea*-treated groups ( $p < 0.05$ ) showed comparable results, as represented in Figure 9A.

The letrozole-treated groups showed a significant elevation of the TNF- $\alpha$  cytokine level. All the plant extracts used in the current study reduced the elevated TNF- $\alpha$  level compared to the group that received letrozole only. *Persea americana* and *P. emblica* succeeded in decreasing the level to be close to the normal group ( $p > 0.05$ ), as represented in Figure 9B.

On the other hand, SOD activity was significantly decreased in the letrozole group compared to the normal rats ( $p < 0.001$ ). *Trigonella foenum-graecum*, *P. emblica*, *B. oleracea*, *P. americana*, and *T. arabicus* increased the SOD activity to near the normal ratio, as illustrated in Figure 9C.

### Histopathological examination

The ovaries of the negative control animals showed a thin covering ovarian capsule and follicles (F) in various stages of development, as well as fresh CL and corpus albicans (CA). Some of the follicles were antral while others were preantral. The Graafian follicle (GF) was clear, as well as the thin covering capsule (C) (Fig. 10A and A\*). The ovarian sections of the animals treated with letrozole showed thickening of the ovarian capsule, the appearance of many large subcapsular cysts (Cy), and very few CL, but a greater number of degenerated ovarian follicles (F) were noticed. The subcapsular cysts revealed follicular debris and were lined with a thin layer of granulosa cells (GC) with hyperplasia of the theca interna cells. The ovarian medulla showed dilated congested blood vessels (BVs) (Fig. 10B and B\*).

The ovaries of the animals treated with metformin were covered with a thin capsule. They revealed a suitable number of CL and several ovarian follicles in different stages of development. Some of the CL showed vacuolated (V) granulosa lutein cells. Other corpora revealed many BVs in the surrounding theca cells layer. There were no observed subcapsular cysts. The medulla showed dilated congested BVs (Fig. 10C and C\*).

When the rats with PCOS were treated with *T. foenum-graecum*, their ovarian sections revealed developing ovarian follicles (F), a GF, and a degenerated follicle (DF). Many CL with vacuoles were seen, and the capsule appeared thin. The medulla (M) had dilated congested BVs (Fig. 10D and D\*).

**Table 7.** Effect of letrozole, metformin, and the plants' extracts on lipid profile as well as blood glucose levels.

Groups	TGs (mg/dl)	Cholesterol (mg/dl)	LDL (mg/dl)	Blood glucose level (mg/dl)
Normal	38.68 ± 6.62	58.40 ± 5.38	12.53 ± 0.75	102.23 ± 6.23
Letrozole	92.57 ± 5.15 <sup>d</sup>	89.17 ± 3.53 <sup>d</sup>	23.15 ± 1.23 <sup>d</sup>	130.30 ± 0.69 <sup>d</sup>
Metformin	71.50 ± 1.78	55.70 ± 2.23 <sup>a</sup>	13.88 ± 0.76 <sup>a</sup>	109.30 ± 1.56 <sup>c</sup>
<i>T. foenum-graecum</i>	32.60 ± 4.59 <sup>ab</sup>	57.90 ± 3.27 <sup>a</sup>	14.83 ± 0.95 <sup>a</sup>	57.90 ± 3.27 <sup>da</sup>
<i>O. europaea</i>	45.13 ± 3.47 <sup>a</sup>	53.30 ± 3.76 <sup>a</sup>	12.05 ± 0.58 <sup>a</sup>	100.23 ± 4.76 <sup>ac</sup>
<i>P. emblica</i> oil	74.52 ± 11.36 <sup>dc</sup>	52.30 ± 4.91 <sup>a</sup>	13.55 ± 0.61 <sup>a</sup>	104.15 ± 4.50 <sup>ac</sup>
<i>B. oleracea</i>	62.33 ± 10.73	61.10 ± 4.19 <sup>a</sup>	14.93 ± 1.34 <sup>a</sup>	100.37 ± 5.68 <sup>ac</sup>
<i>P. americana</i>	57.48 ± 6.24	60.97 ± 2.64 <sup>a</sup>	14.85 ± 0.58 <sup>a</sup>	89.40 ± 4.21 <sup>ac</sup>
<i>T. arabicus</i>	39.53 ± 1.85 <sup>a</sup>	55.20 ± 2.90 <sup>a</sup>	14.80 ± 0.25 <sup>a</sup>	105.48 ± 3.96 <sup>ac</sup>

<sup>a</sup>Significant difference when compared with letrozole-only-treated animals.

<sup>b</sup>Significant difference when compared with metformin.

<sup>c</sup>Significant difference when compared with *T. foenum-graecum*.

<sup>d</sup>Significant difference when compared with normal control.

Data are presented as statistical mean ± SEM.



**Table 8.** Histopathological examination of the extracts-, letrozole-, and metformin-treated groups.

Drug/herbal	Ovarian capsule	Cysts	Follicles	CL
Letrozole	Thick	Present	Many DFs	Very small number
Metformin	Thin	Absent	Moderate number	Suitable/moderate
<i>T. arabicus</i>	Moderately thickened	Absent	Moderate number	Small number
<i>P. americana</i>	Mildly thick	Absent	Moderate number	Big number
<i>P. emblica</i>	Thin	Absent	Moderate	Big number
<i>B. oleracea</i>	Thin	Absent	Moderate	Many
<i>O. europaea</i> oil	Thin	Absent	Many	Many
<i>T. foenum-graecum</i>	Thin	Absent	Many	Many

The treatment of PCOS induced in the animals with *T. arabicus* showed moderately thickened covering ovarian capsules (C). There were ovarian follicles (F) in different stages of development, but there were no subcapsular cysts. The number of CL was smaller and showed vacuolation of the granulosa lutein cells. The medulla (M) revealed dilated congested BVs (Fig. 10E and E\*).

When PCOS rats were treated with *P. americana*, their ovarian sections revealed some ovarian follicles (F) in different stages of development, some DFs, and numerous CL with vacuolations (V). The capsule (C) was mildly thick with no subcapsular cysts. The medulla (M) revealed dilated congested BVs (Fig. 10F and F\*).

The ovarian sections of the PCOS animals treated with *P. emblica* showed that the ovaries were full of CL and some developing ovarian follicles (F) while no subcapsular cysts were seen. The covering capsule (C) was thin. The medulla (M) revealed dilated and congested BVs (Fig. 10G and G\*).

The treatment of PCOS animals with *B. oleracea* revealed some developing ovarian follicles (F) and many CL with vacuoles (V). The capsule appeared thin with no subcapsular cysts (Fig. 10H and H\*).

The ovarian sections of the animals treated with the *O. europaea* oil showed many ovarian follicles (F) in different stages of development as well as many CL. The corpora showed BVs in the center (corpora hemorrhagicum), and the capsule (C) was thin. The medulla (M) revealed dilated and congested BVs. There were no subcapsular ovarian cysts seen (Fig. 10I and I\*).

## DISCUSSION

This study investigated the effect of some plant extracts in the PCOS model. A woman with PCOS needs long-term treatment because PCOS is a lifelong endocrinopathy. PCOS is a hormonal and metabolic disorder that may lead to infertility in many women during their reproductive age (Salehpour *et al.*, 2016). Many synthetic drugs such as oral contraceptives and insulin sensitizers, e.g., metformin, are used to control PCOS (Dehghan, 2012). However, the adverse effects of such drugs shift the research interest toward the use of plants as safer alternatives for women with PCOS (Kurzthaler *et al.*, 2014).

Letrozole was used in some studies to induce the PCOS model in experimental animals to resemble that of humans (Demirel *et al.*, 2016). As evidence of the induction of PCOS, a marked elevation in testosterone and LH levels with reduction of the progesterone and FSH levels was observed. In addition, an irregular estrus cycle including elongation of both the diestrus and estrus phases and persistent cornified cells in the vaginal smears

was observed (Marcondes *et al.*, 2002). The blood glucose level increased in the rats with PCOS, which indicated IR (Glintborg and Andersen, 2010). The etiology of IR in PCOS is still unclear to date. The elevated blood glucose level may be due to a defect in insulin binding, glucose transporters, or glucose metabolism. The lipid profile was elevated compared to the normal group; this dyslipidemia with prominent levels of TGs, TC, and LDL along with the reduction in progesterone level and increase of testosterone may be due to hyperandrogenemia, which is characteristic of the PCOS model (Andersson *et al.*, 2002).

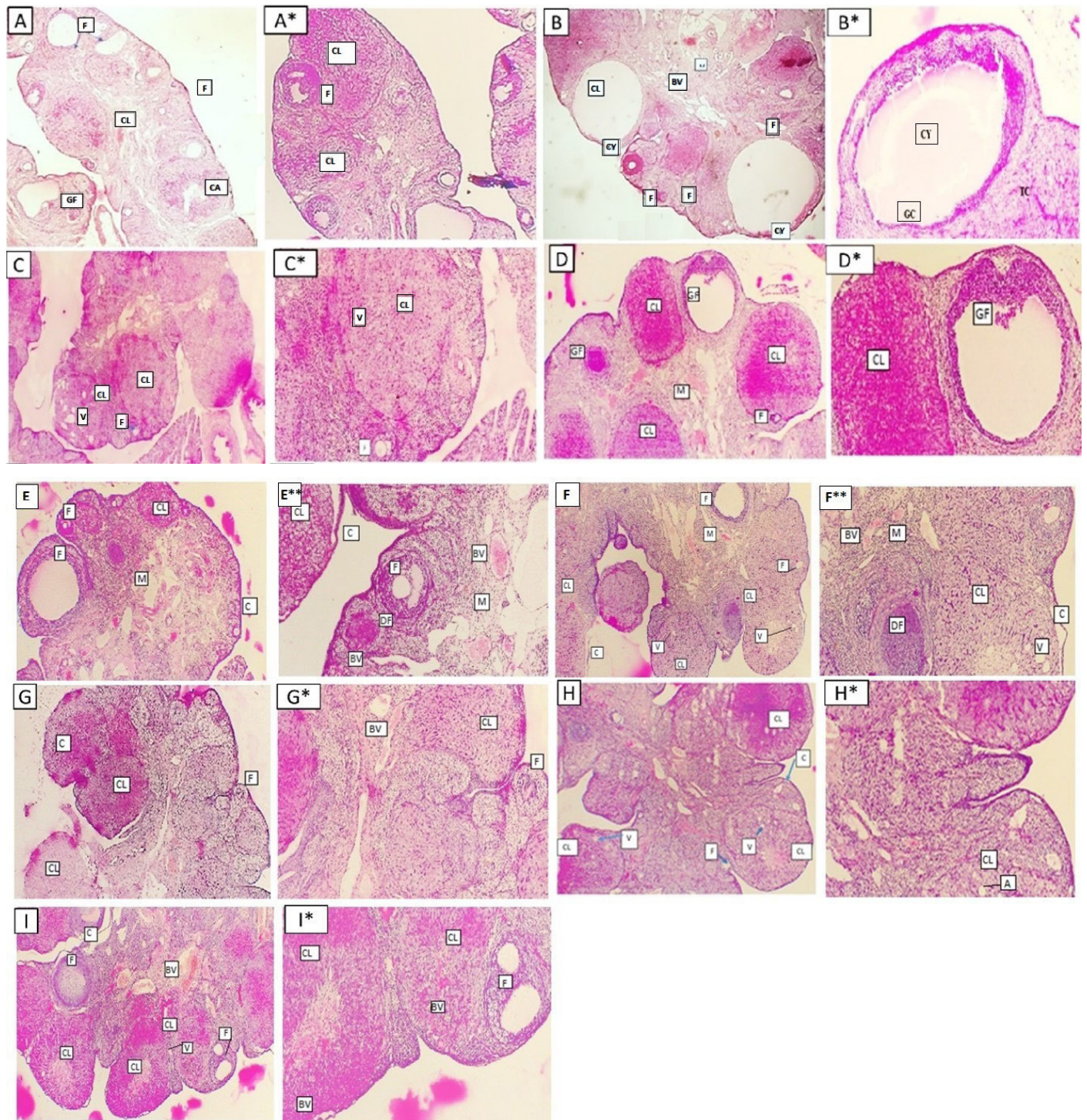
Oxidative stress is the imbalance between the overproduction of reactive oxygen species (ROS) and the limited antioxidant defenses. In PCOS, there is an increase in free radical damage causing an elevated MDA level in ovary tissue with the reduction in the SOD activity, which protects the tissues from the harmful effects of superoxide radicals (Papalou *et al.*, 2016). TNF- $\alpha$  is markedly increased in our study in accordance with other reports (Mohammadi *et al.*, 2017).

In this study, we selected plants based on their folk medicine used for the treatment of PCOS, their reported active constituents, and their antioxidant activities. The study compared the activities of the selected plant extracts for PCOS treatment by both synthetic and natural drugs. Currently, metformin and fenugreek are present as products in the market for the treatment of PCOS (metformin and fenugreek, respectively).

Before performing the biological study, standardization of the plant extracts was carried out by the investigation of their constituents via HPLC analysis as well as determination of their phenolic and flavonoid contents and evaluation of their *in vitro* antioxidant activities. The highest flavonoid content was detected in the *T. arabicus* herb and *P. americana* fruit, while the *P. emblica* fruit showed the highest phenolic content compared to the standard used which is *T. foenum-graecum* seeds. All the plant extracts under investigation showed variable concentrations from the flavonoid, phenolic, and vitamin constituents. The highest content of rutin existed in *T. arabicus*, while *P. americana* showed the highest content of chrysoeriol and hesperidin. On the other hand, *P. emblica* showed the highest content of kaempferol. In addition, the highest phenolic constituents were detected in *T. arabicus* followed by *P. americana*. AA and vitamin B1 were detected in all plant extracts at different concentrations while niacin, a form of vitamin B, was only detected in *P. americana*.

In the current study, treatment with metformin improved most of the studied parameters in accordance with other studies (Weerakiet *et al.*, 2004). This can be attributed to its insulin-sensitizing and steroidogenic effects. Metformin is a synthetic drug whose use is accompanied by many side effects, such as





**Figure 10.** Ovarian sections of different groups of rats (A and A\*: normal group, B and B\*: letrozole group, C and C\*: letrozole and metformin, D and D\*: letrozole and *T. foenum-graecum*, E and E\*: letrozole and *T. arabicus*, F and F\*: letrozole and *P. americana*, G and G\*: letrozole and *P. emblica*, H and H\*: letrozole and *B. oleracea*, and I and I\*: letrozole and *O. europaea* oil treated groups). Some of them showing ovarian follicles (F), corpora lutea (CL), vacuoles (V), corpus albicans (CA), Graafian follicle (GF), capsule (C), subcapsular ovarian cysts (Cy), the medulla (M), and blood vessels (BV) (A: I, H&E  $\times 40$ ) (A\*: I\*, H&E  $\times 100$ ).

lactic acidosis, dizziness, GI pain, diarrhea, nausea, or vomiting. In addition, some females complain of drowsiness, muscle pain, breathing difficulty, and irregular heartbeats (Nasri *et al.*, 2014). Therefore, herbal alternatives with fewer side effects are superior to metformin and oral contraceptives.

*Trigonella foenum-graecum* (fenugreek) was reported as an alternative to synthetic drugs for PCOS management due to its

antidiabetogenic as well as antiandrogenic outcomes (Swaroo *et al.*, 2015). However, *T. foenum-graecum* should be averted in females with peanut and chickpeas hypersensitive reactions as it might lead to cross-reactivity and asthma. It has been considered a potentially emerging allergen and as a herb is not favorable by all people (Dutau *et al.*, 2013).



The doses of herbal remedies, as well as olive oil, were chosen according to their LD<sub>50</sub> that were mentioned in previously published articles. The plant extracts used in our studies showed relevant contents of flavonoids known for their antioxidant and anti-inflammatory activities. This explained the reduction of lipid peroxidation and TNF- $\alpha$  activity with the increase in the antioxidant effect by increasing the SOD level due to the free radical scavenging activities of the plants' extracts. Quercetin, kaempferol, myricetin, and rutin, which were detected in most of the plants under investigation, are known to possess antioxidant activities and exhibit anti-inflammatory effects (Narayana *et al.*, 2001).

The scavenging activity of the flavonoids is in the order myricetin > quercetin > naringenin > catechin > kaempferol > flavone, which may explain the slight difference between the plant extracts used in the reduction of oxidative stress. *Phyllanthus emblica* showed a high concentration of kaempferol, and this may explain the normalization of the oxidative stress markers (MDA and TNF- $\alpha$ ) and the elevation of the antioxidant and SOD levels, as shown in the present study. In addition, rutin was reported to decrease the size of polycystic ovaries (Ratty and Das, 1988; Sarwat *et al.*, 2016), which explains the current finding related to the effect of *T. arabicus* to improve PCOS in the animal model as it showed the highest content of rutin.

Flavonoid contents of the tested plant extracts have also been reported in Noro *et al.* (1983), to have hormone-regulatory activity by binding to both 17 $\beta$ -hydroxysteroid dehydrogenases and 3 $\beta$ -hydroxysteroid dehydrogenase. Binding to these enzymes regulates estrogen and androgen in addition to progesterin and androgen levels in humans, respectively. *Tribulus arabicus* in addition to *P. americana* revealed the highest content of flavonoids that led to a significant improvement in the testosterone and progesterone levels in our study model. The reduction in testosterone level can clarify the restoration of the lipid profile as it was mentioned in another report that testosterone has an antilipolytic activity by its selective inhibition of catecholamine-induced lipolysis (Noro *et al.*, 1983), which

might be the explanation for the improvement in the lipid profile by *T. arabicus*. The flavanone, naringenin, which had the highest concentration in *P. emblica* modifies steroidogenic enzyme activity in rats with letrozole-induced PCOS (Hong *et al.*, 2019). Moreover, quercetin that was detected in most of the plant extracts promotes the proliferation of pancreatic  $\beta$ -cells and enhances glucose metabolism and insulin secretion (Tabrizi *et al.*, 2020). This may clarify the reduction in the blood glucose level shown in this research. Furthermore, hesperidin can improve the follicular development of the isolated ovarian follicles of mice (Shoorei *et al.*, 2019), which was proved by our histopathological study, where ovarian sections of *P. americana* showed ovarian follicles in various stages of development compared to PCOS.

Nonflavonoid phenolic compounds such as caffeic acid that was detected in all plant extracts with the highest concentration in *T. arabicus* were also documented in several studies to possess antioxidant effects through inactivation of ROS and inhibiting lipid peroxidation (Patil and Masand, 2018), while gallic acid was reported to modify the sex hormones' level in PCOS rats due to its antioxidant activity. Both *P. americana* and *P. emblica* contain high concentrations of gallic acid, which gives more interpretation of the current findings. Most of the tested plant extracts contain vitamins B1, B5, and C. Vitamin C is known as an antioxidant and potentiates the antioxidant effects of the plant extracts (Mazloom *et al.*, 2017). Niacin was detected in *P. americana*, which is known for its effect in the treatment of lipid disorders (Kamanna *et al.*, 2013). This provides more clarification of the improvement in the lipid profile in the group treated with *P. americana* in addition to the previously mentioned effect due to testosterone reduction. All of that adds to its usefulness in women with PCOS to be not only a treatment but also a source rich in vitamins.

Many reports encourage people to increase the consumption of olive oil because of both health benefits and its effect on cardiovascular disorders, diabetes, many types of cancer, inflammation, and metabolic disorders. The antioxidant activity of olive oil is due to its constituents such as phenolic compounds, tocopherols, and sterols (Gamze *et al.*, 2021).

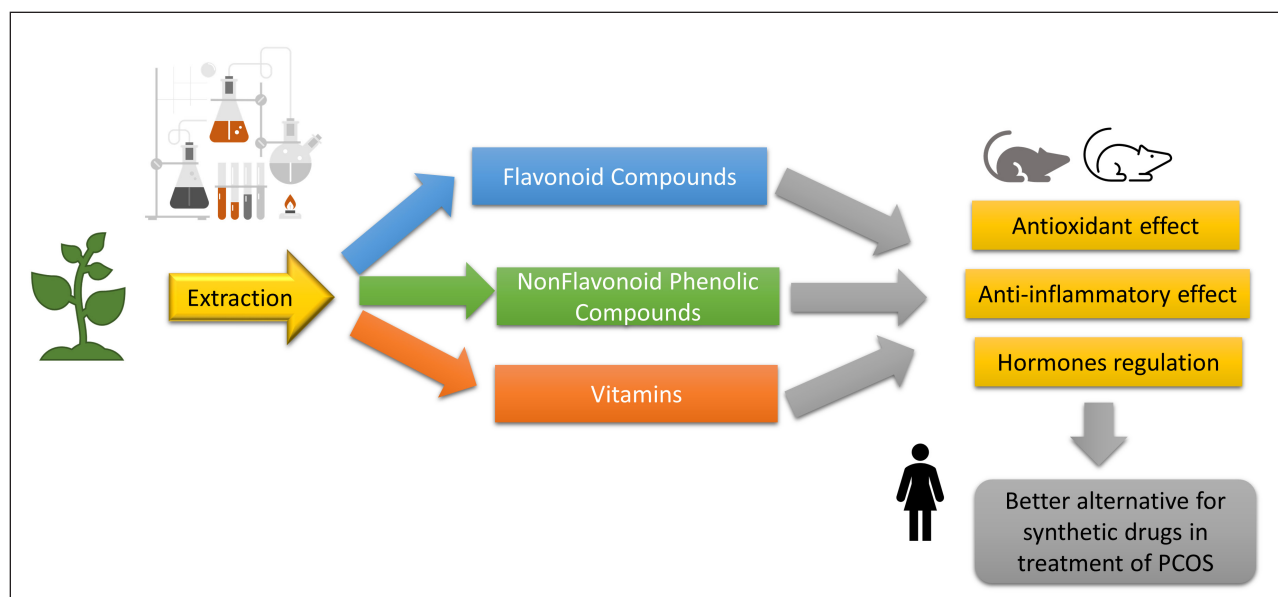


Figure 11. Summary of plant extract constituents and their effects on PCOS model.

These explanations of our results together with the histopathological findings confirmed the potential, useful effects of the tested natural extracts in the treatment of PCOS. **Figure 11** summarizes the constituents of the tested plant extracts and their potential effects on the PCOS model.

## CONCLUSION

The extracts of *T. arabicus*, *P. americana*, and *P. emblica* were investigated as potential alternatives to metformin for the treatment of PCOS. These natural extracts were found to be rich in flavonoids, phenols, and vitamins. They decreased the elevated lipid profile, reduced oxidative stress, regulated hormonal levels, and improved the histopathological findings of ovaries in the *in vivo* model of letrozole-induced PCOS. These plant extracts can be promising candidates for the treatment of PCOS.

## LIST OF ABBREVIATIONS

CMC: Carboxymethylcellulose; CL: Corpora lutea; DF: Degenerated follicle; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; FSH: Follicle-stimulating hormone; GF: Graafian follicle; HPLC: High-performance liquid chromatography; PCOS: Polycystic ovary syndrome; LDL: Low-density lipoproteins; LH: Luteinizing hormone; MDA: Malondialdehyde; ROS: Reactive oxygen species; SOD: Superoxide dismutase; TNF- $\alpha$ : Tumor necrosis factor-alpha; TGs: Triglycerides.

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## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

## AUTHORS' CONTRIBUTIONS

**Naglaa Shehab** contributed to conceptualization, methodology, formal analysis, original draft, project administration, and review and editing. **Hanan S. Anbar** contributed to methodology, formal analysis, original draft, and review and editing. **Nadia Mahmoud Alrouby** contributed to methodology and original draft. **Aya Abouelalamin** contributed to investigation and original draft. **Lama Lutfi** contributed to investigation and original draft. **Israa Tyseer Allo** contributed to investigation and original draft. **Salma Mohamed Elayoty** contributed to investigation and original draft.

## ETHICAL APPROVALS

The approval of the Research and Ethical Committee of Dubai Pharmacy College for Girls, Dubai, UAE (REC-FD-2020-05).

## DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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