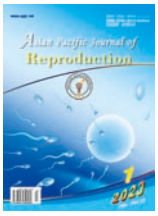

Original Article

Asian Pacific Journal of Reproduction

Journal homepage: www.apjr.net

doi: 10.4103/2305-0500.365229

A novel herbal combination ameliorates ovarian dysfunction and regulates altered biochemical parameters in rats with letrozole-induced polycystic ovary syndrome

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ABSTRACT

Objective: To investigate the potential activity of novel herbal combination and novel herbal formulation (syrup) in female Sprague Dawley rats with letrozole-induced polycystic ovary syndrome (PCOS).

Methods: Forty-two rats were randomly divided into seven groups with six rats in each group. Group 1 received 0.5% carboxy methylcellulose orally for 37 days and served as the normal control group. Group 2 was orally administered with letrozole of 1 mg/kg for 21 days and served as the PCOS induction group without treatment. Group 3 to 7 were administered with letrozole of 1 mg/kg for 21 days orally to induce PCOS, and then respectively received clomiphene citrate at 1 mg/kg, 100 and 200 mg/kg of novel herbal combination, 200 mg/kg of novel herbal formulation (syrup), and 400 mg/kg of marketed formulation of *Pushyanuga churna*, once daily for 15 days. Effects of the novel herbal combination and its syrup formulation were evaluated on the hormonal profile, the levels of antioxidants, the lipid profile and on the ovarian morphology, using letrozole-induced PCOS model in rats.

Results: Letrozole caused alterations in hormonal levels and lipid levels similar to PCOS and ovarian histology showed presence of ovarian cysts confirming the induction of PCOS in rats. On treatment with the novel herbal combination and its syrup formulation in PCOS-induced rats, the altered hormonal and lipid profiles showed significant recovery to normal levels. Ovarian histology confirmed the restoration of folliculogenesis in the PCOS-induced rats. The treatment with the syrup formulation of novel herbal combination was found to be more effective than novel herbal combination and showed better recovery in various parameters evaluated. The results of the study, however, suggested that treatment with novel herbal combination and its syrup formulation provided minimal protection against oxidative stress caused due to the induction of PCOS.

Conclusions: The integrated approach for management of PCOS is to counterbalance the limitations associated with modern therapy. Both the novel herbal combination and the syrup formulation of novel herbal combination show efficacy in the management of PCOS in rats and restore folliculogenesis in the ovary. The syrup formulation of novel herbal combination is most effective in the management of PCOS and shows potential to be developed as an adjuvant therapeutic agent.

KEYWORDS: Polycystic ovarian syndrome; Polyherbal combination; Letrozole; *Pushyanuga churna*; Clomiphene citrate; Histopathology; Biochemical; Herbal

Significance

Modern drugs are reported to cause a wide range of adverse side effects in the treatment of polycystic ovary syndrome (PCOS) which target the symptoms in isolation rather than targeting the underlying interlinked physiological causes of PCOS. The study reveals that the novel herbal combination and the novel herbal formulation (syrup), which have been validated phytopharmacologically and evaluated experimentally for their efficacy, show potential to be used as alternative treatment regimens in the management of PCOS. The syrup formulation of the novel herbal combination could be a patient compliant adjuvant therapy in the management of PCOS.

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How to cite this article: Shailajan S, Menon S, Singh S, Patil Y. A novel herbal combination ameliorates ovarian dysfunction and regulates altered biochemical parameters in rats with letrozole-induced polycystic ovary syndrome. *Asian Pac J Reprod* 2023; 12(1): 23-34.

Article history: Received: 13 June 2022; Revision: 9 October 2022; Accepted: 16 November 2022; Available online: 6 January 2023

1. Introduction

Polycystic ovary syndrome (PCOS) is a common heterogeneous, endocrinological and metabolic disorder that is becoming an epidemic around the world due to its prevalence in women of reproductive age especially in the adolescent girls[1]. Studies on the prevalence of PCOS reported that on an average, 8% to 13% women are affected by PCOS[2,3]. PCOS is a multifaceted clinical condition, with physiological and biochemical effects ranging from amenorrhea, irregular menstrual cycles, androgenic features and even infertility. Other complications associated with PCOS include hyperlipidemia, insulin resistance and obesity[4]. In its long-term consequences, PCOS may also increase the risk of endometrial cancer, type 2 diabetes mellitus, hypertension and cardiovascular disorders. The origin of PCOS is uncertain, but genetic factors, life style factors along with insulin resistance, lipid imbalance and oxidative stress have been reported to be important contributors[5,6]. Due to the combined effect of sociological and psychological factors, every woman suffering from PCOS presents a different combination of symptoms with varied intensities[7].

Clinically, PCOS normally manifests with the presence of enlarged ovaries and deranged neuroendocrine regulatory system. Increase in levels of ovarian steroids causes fluctuation in gonadotropin-releasing hormone (GnRH) which lead to hypersecretion of luteinizing hormone (LH) and decrease in levels of follicle-stimulating hormone (FSH). This eventually leads to anovulation and hyperandrogenism[8-11]. Due to this manifold presentation of PCOS, the treatment needs have to be individualized. Current treatment regimen for PCOS is essentially dictated by the symptoms associated with PCOS and depends upon either infertility being the primary grievance of the patient or other associated aesthetic or physiological concerns[4,12]. The original treatment reported by Freiler Stein and Michael Leventhal included surgical mediation. However, modern pharmacological interventions are now preferred to avoid post-operative side effects. Modern main line treatments by clomiphene citrate or use of oral contraceptives, anti-androgens with metformin, have been reported to cause a wide range of adverse side effects[4,7]. These treatment regimens are targeted for symptomatic relief at best, as there is no single pharmacological therapy reported so far as a direct and comprehensive treatment of PCOS[13]. Thus, research on alternative and complementary medicine approaches to manage PCOS has gained prominence. Recent studies suggest that such an alternative approach may possess the potential to alleviate all symptoms associated with PCOS as a single unifying therapy.

While considering alternative treatment regimen, there are numerous medicinal plants, which have been reported useful both, individually and also as part of a formulation for the management of female reproductive disorders[14]. Hence, a combination of therapeutically active medicinal plants has been suggested, which have been reported to possess the ability to alleviate the symptoms associated with PCOS as a single treatment. This combination thus has been hypothesized to be a potent alternative treatment regimen in the management of PCOS. The plant ingredients that have been proposed in novel herbal combination like *Myrica esculenta*, *Symplocos racemosa*, *Mimosa pudica*, *Cyperus rotundus*, *Aegle marmelos*, *Saraca asoca* and *Berberis aristata* have already been reported for their potential therapeutic effects in PCOS conditions[15-21].

In the current research work, based on available database and prior studies, a combination of ten medicinal plants has been proposed. The combination includes *Myrica esculenta*[22], *Symplocos racemosa*[16], *Mimosa pudica*[17], *Cyperus rotundus*[23], *Asteracantha longifolia*[24], *Saraca asoca*[25], *Aegle marmelos*[26], *Berberis aristata*[27], *Woodfordia fruiticosa*[28] and *Terminalia arjuna*[29]. The efficacy of the novel herbal combination and syrup formulation of the novel herbal combination have been evaluated using an experimental study using letrozole-induced PCOS model in rats.

2. Materials and methods

In this study, the novel herbal combination prepared using ten medicinal plant ingredients was standardized using validated scientific methods and chromatographic techniques. The herbal combination has been hypothesized to work synergistically in the management of PCOS. The efficacy of the novel herbal combination and syrup formulation of the novel herbal combination was evaluated using letrozole-induced PCOS model in Sprague Dawley rats. Biochemical and histopathological parameters were evaluated and the results were compared with those after treatment with the modern drug - clomiphene citrate in the study. The results were also compared with the known Ayurvedic formulation - *Pushyanuga churna*, which has been prescribed by Ayurvedic practitioners in the management of female reproductive disorders such as menorrhagia, leucorrhoea, menstrual disorders etc.

2.1. Reagents and marketed formulations

Letrozole was obtained as Femara® (Novartis Pharma Stein AG, Stein, Switzerland). Clomiphene citrate (Fertomid-50 Manufactured by Cipla Ltd., Kumrek, Rangpo Sikkim, 737132, India) was purchased from the market. Ayurvedic polyherbal formulation -*Pushyanuga churna* was used as a traditional control in the study. The marketed formulation of *Pushyanuga churna* (Dhootpapeshwar Batch no: DB042001) was purchased from the Ayurvedic pharmacy, Mumbai. For blood withdrawal, heparin injection IP- Hep 25, (power: 25 000 IU) (Gland Pharma Ltd., Hyderabad, India; B. No. OK503) and heparinized capillaries (Top Tech Lab Equipment Pvt. Ltd., Mumbai, India) were used.

2.2. Plant materials

In order to prevent errors of collection, each of the plants were individually collected from their natural habitats. In order to reduce phenological deviation, the plants were collected during their individual flowering/ fruiting seasons. The plant parts collected were washed on site to remove all foreign matter and soil from it, dried thoroughly and transported to the lab for further processing. A twig of each plant with leaves and flowers/fruits was also collected and pressed in order to prepare a herbaria which was further used for authentication purposes. The collection locations and plant authentication (specimen voucher number) are described in Table 1.

2.3. Preparation of novel herbal combination

Plant materials were air-dried and ground. Each plant powder was mixed with ethanol and water in 1:1 ratio, kept overnight on rotary shaker. The solvent was evaporated at 40 ℃ to prepare hydroalcoholic extracts. Three separate combinations (combination of hormonal supplement-50%), combination for diabetes control -25% and combination for fertility enhancement-25%) were prepared with equal ratios of each plant extracts. Combination of hormonal supplement-50% contains *Myrica esculenta*, *Symplocos racemosa*, *Mimosa pudica*, *Cyperus rotundus*, *Asteracantha longifolia*, and *Saraca asoca*. Combination for diabetes control-25% includes *Aegle marmelos, Berberis aristata* whereas *Woodfordia fruiticosa* and *Terminalia arjuna* are present in combination for fertility enhancement-25%. The three combinations were then taken and mixed together to obtain the novel herbal combination.

2.4. Evaluation of stability of the plant extracts using high performance thin layer chromatography (HPTLC)

A standardized HPTLC method was employed on three individual extracts corresponding to hormonal supplements, diabetes control, and fertility enhancement along with the final novel herbal combination in order to evaluate extract stability. Chromatographic separation was achieved on silicagel $60F_{254}$ precoated HPTLC plates using toluene: ethyl acetate: formic acid (8:2:1 v/v/v) as mobile phase. The plates were derivatized in 10% methanolic sulfuric acid and photodocumented.

2.5. Preparation of syrup formulation from novel herbal combination

The sugar base for the syrup was prepared by adding 660 g of sugar in 1 000 mL of de-ionized water heated upto 90 ℃ followed by mixing with a mechanical stirrer. Sugar base was filtered through nylon cloth and mixed with 0.1% w/v sodium benzoate as preservative. Accurately weighed herbal extracts were added in a measured quantity as described in section 2.3 and mixed with a mechanical stirrer until stable consistency was achieved. The syrup was then filtered through nylon cloth[30]. The final syrup solution was transferred to amber colour bottle, labeled and used further for efficacy study.

2.6. Animals

Forty-two healthy adult (8-12 week old) nulliparous and nonpregnant Sprague Dawley rats, weighing 180-200 g were procured from Bharat Serum and Vaccines Pvt. Ltd., Thane, Mumbai, India. Animals were kept in the Animal House Facility (CPCSEA No.: 315/PO/Re/S/2000/CPCSEA) at Ramnarain Ruia Autonomous College, Matunga, Mumbai, India and quarantined for at least two weeks. The animals were housed in clean and dry polypropylene cages (B. I. K Industries, Mumbai, India) provided with sterilized, clean and dry rice husk bedding and acclimatized at a temperature of (22±3) ℃, relative humidity of 50%-55% and 12 hours light-dark cycle (artificial light, 6.00 a.m. to 6.00 p.m.). Animals were fed with commercially available standard pellet diet (supplied by Amrut Feed, India). Water was provided *ad libitum*. The experimental procedures were performed in accordance with guidelines provided by the Institutional Animal Ethics Committee's (IAEC) rules and regulation of this institute and the experiments

Table 1. Plant details.

Name of the plants	Plant part	Place of collection	Authenticated by	Plant authentication (specimen voucher number)
Myrica esculenta[22]	Bark	Khirsu, Panghot, Uttarakhand	National Botanical Research Institute, Lucknow	NBRI-LWG103078
Mimosa pudica[17]	Whole plant	Rajapur,Thane, Maharashtra	Agharkar Research Institute, Pune	ARI-Auth 10-75
Symplocos racemosa[16]	Bark	Mahabaleshwar, Maharashtra	Agharkar Research Institute, Pune	ARI-10-173
Cyperus rotundus[23]	Root	Thane, Maharashtra	National Institute of Science Communication and information Resources, Delhi	NISCAIR 2309
Asteracantha longifolia[24]	Whole plant	Thane, Maharashtra	Agharkar Research Institute, Pune	Auth 11-122
Saraca asoca[25]	Flowers	Mumbai, Maharashtra	Agharkar Research Institute, Pune	Auth 13-015
Aegle marmelos[26]	Bark	Girnar hills, Gujrat; Thane, Maharashtra	Agharkar Research Institute, Pune	Auth 11-196
Berberis aristata[27]	Root	Pippalkoti, Kathgodam Khirsu, Uttarakhand	Alaknanda Ghaati Shilpi Federation, Uttarakhand	AAGAAS-04/19
Woodfordia fruticosa[28]	Flowers	Khandala Ghat, Maharashtra	Agharkar Research Institute, Pune	Auth 14-005
Terminalia arjuna[29]	Bark	Boisar and Thane, Maharashtra	Herbal Research Lab, Ramnarain Ruia Autonomous College, Mumbai	HRL/2015/04

were carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The animals were acclimatized for one week prior to study initiation.

2.7. Experimental design

Forty-two rats were randomly divided into seven groups with six rats in each group. Group 1 was the normal control group, receiving 0.5% carboxy methylcellulose orally for 37 consecutive days. Group 2 was orally administered with letrozole of 1 mg/kg for 21 days and served as PCOS-induced group without treatment (the PCOS induction group). The PCOS induction group was sacrificed on 22nd day after letrozole treatment. Group 3 was orally administered with letrozole of 1 mg/kg for 21 days and daily oral dose of clomiphene citrate (a well-known modern PCOS treatment drug) at 1 mg/kg for 15 days in 0.5% carboxy methylcellulose per oral and served as the clomiphene citrate group. Groups 4 and 5 were administered with letrozole of 1 mg/kg for 21 days and daily oral dose of 100 mg/kg and 200 mg/kg of novel herbal combination each, once daily for 15 days. Group 6 was administered with letrozole of 1 mg/kg for 21 days and daily oral dose of 200 mg/kg of novel herbal formulation (syrup) once daily for 15 days. Lastly, the group 7 was administered with letrozole of 1 mg/kg for 21 days and daily oral dose of 400 mg/kg of marketed formulation of *Pushyanuga churna* aqueous slurry once daily for 15 days[31–33].

2.8. Sacrifice and sample collection

At the end of experimental period for each group, blood samples (500 μL per animal) were collected from the retro orbital plexus of the treated rats under anesthesia. Serum was separated and kept at -20℃ in freezer.

2.8.1. Biochemical assay

Hormonal, lipid and antioxidant profile were evaluated using standard assay kits. Hormonal profile includes estimation of testosterone, progesterone, estrogen, LH, FSH using standard kit by CLIA, Abnova. Antioxidant profile was evaluated in terms of catalase, superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione (GSH) and lipid peroxidation (LPO) using standard kit procured by Canvax Biotech, Spain. Lipid profile includes estimation of triglycerides, cholesterol, random using standard kits, obtained from Spinreact, Spain. After blood collection, rats were euthanized by CO_2 and the ovaries and uterus were excised.

2.8.2. Histopathological evaluation

Histological studies were evaluated after processing them using a standard procedure. The small piece of the ovarian tissue was fixed in neutral formalin for 24 h. The tissue after fixation was washed in water to remove excess of fixative. Washed tissues were then dehydrated through graded series of ethyl alcohol cleared with xylene and embedded in paraffin. Sections of 5 to 7 μm thickness were mounted on glass slides. The sections were stained routinely with hematoxylin and eosin technique. The stained slides were observed and photographed using light microscope with 4×, 10× and 40× objective lenses and 10× eyepiece. The collage was prepared using Autostitch™ automatic panorama stitching software; Copyright (2005), University of British Columbia.

The rat ovary samples were cut into pieces (1-2 mm^2) and fixed in 3% glutaraldehyde (pH=7. 4) for 6-8 h at 4℃. They were washed 0.1 M sodium cacodylate buffer for 4-6 h and post fixed in 2% osmium tetroxide (O_sO_4) for 2 h, at 4℃. The tissue was dehydrated in a graded series of ethanol baths over a two-hour period embedded in araldite resins. Semi-thin sections (1 μm) were cut using LEICA ULTRACUT R microtome and stained with 1% toluidine blue in dilute borax, mounted and studied under a light microscope. Sections showing required ovarian features were selected and ultra-thin sections (silver interference) were cut using ultra-microtome with a glass knife. The ultra-thin sections were stained with uranyl acetate and alkaline lead citrate. The sections were viewed using a JEOL (Japanese Electron Optics Limited) -JEM 1010 electron microscope and changes in their cellular and subcellular structures were analysed and compared.

2.9. Statistical analysis

The statistical analysis were performed by using GraphPad Prism 5 software version 5.03 (GraphPad Software, Inc., California, USA). Statistically analysis was done by using one-way analysis of variance followed by Tukey's test multiple comparison tests and data were expressed as mean±standard deviation (mean±SD). $P<0.05$ was considered to be statistically significant. Values which were more than 4SD in deviation from mean were considered as outliers and were not included in statistical comparisons.

2.10. Ethical statement

The Institutional Animal Ethics Committee of Ramnarain Ruia Autonomous College (CPCSEA No.: 315/PO/Re/S/2000/CPCSEA), Matunga, Mumbai, India reviewed and approved the experimental protocol (RRC/IAEC/09/2017) used in this study. An approval letter was issued by the Institutional Animal Ethics Committee (Date of approval letter: 11th June, 2018).

3. Results

3.1. Evaluation of stability of the novel herbal combination using HPTLC

In the phytochemical fingerprint developed by HPTLC, the identity and stability of active substances were evaluated in form of the bands resolved on the HPTLC plate. In the different combinations of medicinal plants and different batches of novel herbal combination, the separated bands were found to be consistent and stable (Figure 1). After ascertaining the stability, the novel herbal combination was formulated into a syrup.

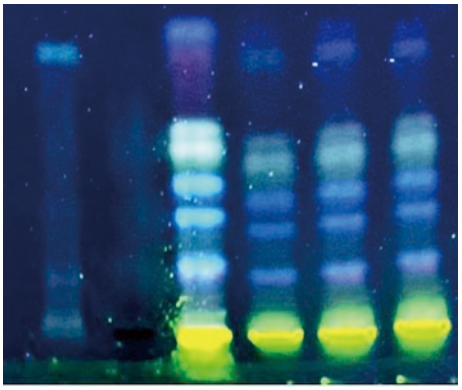

Figure 1. High performance thin layer chromatography (HPTLC) plate photo at 366 nm to check the consistency of the novel herbal combination different batches as a quality control tool. Track 1: Combination for hormonal supplements; Track 2: Combination for fertility enhancement; Track 3: Combination for diabetes control; Track 4: novel herbal combination Batch 1; Track 5: novel herbal combination Batch 2; Track 6: novel herbal combination Batch 3.

3.2. Effect of treatment with novel herbal combination and its syrup formulation on hormonal profile

3.2.1. Testosterone

The mean concentration of testosterone in the PCOS induction group was significantly increased after letrozole administration of 21 days compared to the normal control group (P<0.001). The treatment of 100 mg/kg and 200 mg/kg novel herbal combination and 200 mg/kg syrup formulation from novel herbal combination for 15 days significantly decreased the testosterone levels compared to the PCOS induction group (all P<0.001). The clomiphene citrate and *Pushyanuga churna* treatment groups also showed significant decrease in testosterone levels compared to the PCOS induction group (all P<0.001). There was no significant difference in the level of testosterone among treated rats from all other treatment groups (Table 2).

3.2.2. Progesterone

PCOS-induced rats showed decrease in progesterone level compared to the normal control group, though the decrease was statistically not significant. Progesterone level, which decreased in PCOS induced rats from (8.39±3.60) ng/mL of the normal control group to (1.77±0.30) ng/mL of the the PCOS induction group, increased to (8.50±2.22) ng/mL in the syrup formulation group and it was on par with those of the clomiphene citrate treatment group. The mean concentration of progesterone hormone in the groups of 100 mg/kg and 200 mg/kg novel herbal combination was found to

Table 2. Effects of treatment on hormonal profile in rats.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Testosterone, ng/mL	19.14±1.56	245.76±31.61^{a***}	19.64±4.78^{b***}	41.30±4.44^{b***}	19.99±5.38^{b***}	52.02±5.57^{b***}	36.01±19.13^{b***}
Progesterone, ng/mL	8.39±3.60	1.77±0.30	7.55±1.03	4.09±0.91	6.40±2.22	8.50±2.22	10.93±3.12^{b*}
Estradiol, pg/mL	24.29±8.80	26.18±1.11	23.93±2.36	21.78±7.75	20.52±7.92	25.46±1.75	18.04±6.55
Luteinizing hormone, mIU/mL	0.12±0.02	0.30±0.08^{a***}	0.15±0.06^{b**}	0.15±0.07^{b**}	0.13±0.05^{b**}	0.15±0.08^{b**}	0.18±0.02
Follicle stimulating hormone, mIU/mL	1.11±0.39	1.65±0.44	0.358±0.08^{b***}	0.51±0.16^{b**}	0.93±0.87	0.34±0.06^{b***}	0.77±0.40^{b*}

Data are expressed as mean±SD; n=6 in each group. a: compared with the normal control group, $^{***}P$<0.001; b: compared with the induction group, $^{*}P$<0.05; $^{**}P$<0.01; $^{***}P$<0.001. Group 1 receives 0.5% carboxy methylcellulose orally for 37 days and serves as the normal control group. Group 2 is orally administered with letrozole of 1 mg/kg for 21 days and serves as the PCOS induction group. Group 3 is orally administered with letrozole of 1 mg/kg for 21 days and daily oral dose of clomiphene citrate (a well-known modern PCOS treatment drug) at 1 mg/kg for 15 days in 0.5% carboxy methylcellulose per oral and serves as the clomiphene citrate group. Groups 4 and 5 are administered with letrozole of 1 mg/kg for 21 days and daily oral dose of 100 mg/kg and 200 mg/kg of novel herbal combination each, once daily for 15 days. Group 6 is administered with letrozole of 1 mg/kg for 21 days and daily oral dose of 200 mg/kg of novel herbal formulation (syrup) once daily for 15 days. Group 7 is administered with letrozole of 1 mg/kg for 21 days and daily oral dose of 400 mg/kg of marketed formulation of *Pushyanuga churna* aqueous slurry once daily for 15 days.

be (4.09±0.91) ng/mL and (6.40±2.22) ng/mL, respectively; there were no significant differences compared to the the PCOS induction group. Progesterone concentration of *Pushyanuga churna* treatment group significantly increased compared to the the PCOS induction group (P<0.05). In addition, the increase in progesterone was greater in rats of *Pushyanuga churna* treatment group than rats from other treatment groups (Table 2).

3.2.3. Estradiol

In rats treated with letrozole, a mild increase in mean estradiol levels was noticed, but the increase was not statistically significant. The estrogen levels in the rats form all treatment groups did not show any significant changes as compared to the PCOS induction group. In rats treated with clomiphene citrate, the estradiol level showed inter-animal variabilities within the animals of the treatment group. The treatment with novel herbal combination at doses of 100 mg/kg and 200 mg/kg and the syrup formulation at 200 mg/kg did not lead to significant changes in the estradiol levels as compared to the PCOS induction group (Table 2).

3.2.4. LH and FSH

The mean concentration of LH in the PCOS induction group increased significantly as compared to the normal control group (P<0.001). After the treatment for 15-days with clomiphene citrate, 100 mg/kg and 200 mg/kg novel herbal combination, and 200 mg/kg syrup formulation, there was significant decrease in LH levels compared to the PCOS induction group (all P<0.01), whereas the changes in LH levels after treatment with *Pushyanuga churna* was not significant, compared to the PCOS induction group (Table 2).

The increase in FSH level in the PCOS induction group was not statistically significant when compared with the normal control group. The clomiphene citrate and syrup formulation groups showed significant reduction in FSH levels, compared to the PCOS induction group (both P<0.001). The 100 mg/kg novel herbal combination (P<0.01) and *Pushyanuga churna* (P<0.05) showed significant reduction in FSH levels compared to the PCOS induction group. But 200 mg/kg novel herbal combination did not show significant reduction compared to the PCOS induction group (Table 2).

3.3. Effect of novel herbal combination and its syrup formulation treatment on antioxidant profile

3.3.1. Catalase

Rats of the PCOS induction group showed a reduction in catalase activity compared to the normal control group, but there was no significant difference. Catalase activity was found to be significantly increased after treatment of PCOS induced rats with clomiphene citrate (P<0.05). The treatment with syrup formulation, however, did not increase the catalase activity significantly. Similarly, no significant differences in catalase levels were observed after treatment with novel herbal combination at doses of 100 mg/kg and 200 mg/kg as compared to clomiphene citrate and *Pushyanuga churna* treatment groups (Table 3).

3.3.2. SOD

Rats of the PCOS induction group showed a reduction in the activity of SOD compared to the normal control group, but there was no significant difference. However, in all treatment groups including the clomiphene citrate group, there was no significant change in the levels of SOD in the treated rats, compared to the the PCOS induction group (Table 3).

3.3.3. LPO

The LPO level increased significantly after letrozole-induced PCOS in rats compared to the normal control group (P<0.001). The level of

Table 3. Effects of treatment on antioxidant profile in rats.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Catalase, units/mg protein	18.46±1.66	15.47±0.28	19.84±0.65^{b*}	17.23±2.14	15.77±2.42	19.23±3.08	16.13±2.83
SOD, units/mg protein	21.96±1.19	18.37±0.31	26.68±11.92	18.79±2.37	19.20±2.63	18.48±4.19	20.83±1.86
LPO, nmole/g Hb	95.44±12.0	115.47±0.38^{a***}	97.34±4.57^{b**}	123.33±6.09	117.90±5.80	107.51±10.95	116.79±3.98
GPx, μg GSH utilized/mg protein/min	3.28±0.51	3.71±0.17	3.55±0.35	3.33±0.56	3.23±0.27	3.50±0.40	3.26±0.49
GST, microgram/L	26.56±3.44	28.03±0.51	32.61±1.99	30.22±4.94	29.17±3.99	24.32±2.92	27.03±7.46

Data are expressed as mean±SD; n=6 in each group. a: compared with the normal control group, $^{***}P$<0.001; b: compared with the induction group, $^{*}P$<0.05; $^{**}P$<0.01. SOD: super oxide dismutase; LPO: lipid peroxidation; GPx: glutathione peroxidase; GST: glutathione s-transferase.

Table 4. Effects of treatment on lipid profile in rats.

Parameters	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
Triglycerides, mg/dL	180.87±26.77	157.33±3.45	111.04±40.17	86.50±25.81^{b***}	93.17±16.70^{b**}	123.17±45.81	76.17±8.11^{b***}
Cholesterol, mg/dL	41.48±2.12	39.33±3.62	33.69±5.03	44.33±5.13	46.67±4.50	47.00±14.35	36.00±6.81
Random blood sugar, mg/dL	82.88±8.61	110.67±13.41^{a***}	94.48±6.92	84.00±7.32^{b***}	82.50±8.55^{b***}	89.67±11.48^{b**}	88.33±10.65^{b**}

Data are expressed as mean±SD; n=6 in each group. a: compared with the normal control group, $^{***}P$<0.001; b: compared with the induction group, $^{***}P$<0.01, $^{***}P$<0.001.

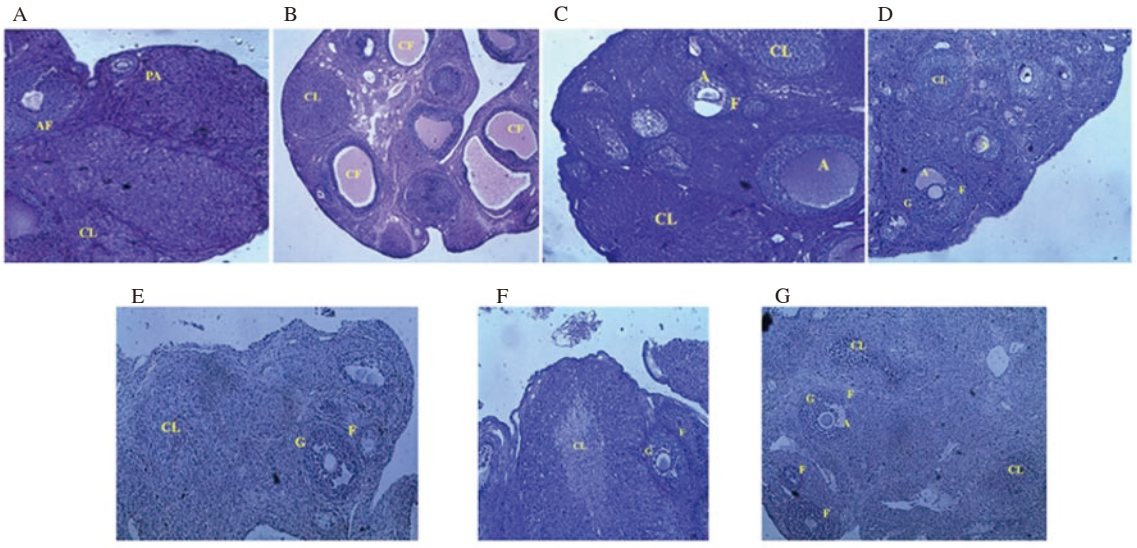

Figure 2. Histological section of the ovarian tissue in rats (hematoxylin and eosin staining; magnification: ×40). A: The normal control group shows primary follicle (PA), antral follicle (AF), and corpus luteum (CL) in the sections from ovarian tissue. B: The PCOS induction group shows multiple cystic follicles (CF) with thin granulosa layer and less number of corpus luteum (CL) in section. C: The clomiphene citrate group shows primary follicle with oocyte and antral follicle and follicles (F) in various stages of development. D: The 400 mg/kg *Pushyanuga churna* group shows primary follicle with no abnormalities, better tissue arrangements, and corpus luteum (CL). E: The 100 mg/kg novel herbal combination group shows follicles (F) with oocyte along with corpus luteum (CL), and granulosa layer (G). F: The 200 mg/kg novel herbal combination group shows developing follicle with oocyte and corpus luteum (CL), and better tissue arrangements in section. G: The 200 mg/kg syrup formulation of novel herbal combination group shows multiple growing follicle with no abnormalities and corpus luteum (CL). PA: primary follicle; AF: antral follicle; CL: corpus luteum; F: follicle; A: antral cavity; G: granulosa layer; CF: cystic follicle.

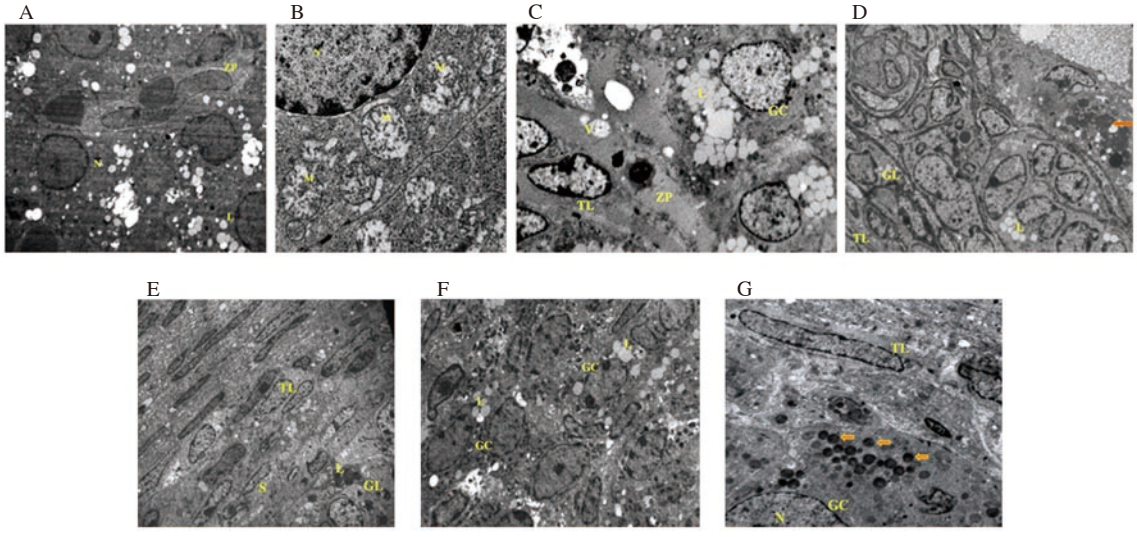

Figure 3. Electron micrograph of the ovary in rats. A: The normal control group shows nucleus of granulosa cells (N), zona pellucida (ZP), and lipid droplets (L), (3 000×). B: The PCOS induction group shows nucleus (N) and disrupted cristae in mitochondria (M) in granulosa cell, (15 000×). C: The clomiphene citrate group shows lipid (L) laden granulosa cells (GC) and thecal layer, vesicle (V) in zona pellucida (ZP), (15 000×). D: The 400 mg/kg *Pushyanuga churna* group shows distinctive thecal layer (TL) and granulosal layer (GL). The inner layer of the granulosa cells shows presence of autophagosomes (arrow), (2 000×). E: The 100 mg/kg novel herbal combination group shows distinctive thecal layer (TL), secretory cells (S) and lipid droplets (L). The cellular and nuclear morphology of cells in both these layers are normal, (2 000×). F: The 200 mg/kg novel herbal combination group shows normal granulosa cells (GC) with lipid droplets (L), (3 000×). G: The 200 mg/kg syrup formulation of novel herbal combination group shows active granulosa cell (GC) with nucleus (N) and electron dense secretory vesicles and normal thecal layer and electron dense secretory vesicles (arrows) and normal thecal layer, (6 000×).

LPO did not show any significant change after treatment with novel herbal combination at doses of 100 mg/kg and 200 mg/kg, syrup formulation and *Pushyanuga churna,* compared to the the PCOS induction group. However, the clomiphene citrate group showed a significant decrease in LPO level as compared to the PCOS induction group (P<0.01) (Table 3).

3.3.4. GPx

The mean GPx activity was found to be increased in the PCOS induction group as compared to the normal control group, but there was no significant difference. After the treatment period of 15 days, a decrease in GPx activity was observed in all treatment groups compared to the PCOS induction group, but the decrease was not statistically significant (Table 3).

3.3.5. GST

The mean GST activity was found to be increased after PCOS-induction with letrozole administration, but there was no significant difference. However, after all the treatment regimen including clomiphene citrate, the GST levels in treated rats did not show any significant changes compared to the PCOS induction group (Table 3).

3.4. Effect of novel herbal combination and its syrup formulation treatment on lipid profile

3.4.1. Triglycerides

The triglyceride level was found to be decreased in the PCOS induction group as compared to the normal control group, but there was no significant difference. The mean triglyceride levels decreased after treatment with syrup formulation and clomiphene citrate but the decrease was not statistically significant. The triglyceride level significantly decreased after treatment with novel herbal combination at doses of 100 mg/kg (P<0.001) and 20 mg/kg (P<0.01) and *Pushyanuga churna* (P<0.001) compared to the PCOS induction group (Table 4).

3.4.2. Cholesterol

The cholesterol level was found to be decreased in the PCOS induction group as compared to the normal control group, but there was no significant difference. The levels of cholesterol in treated rats from all treatment groups did not show any significant changes after treatment, compared to the PCOS induction group. The range of the cholesterol values was very wide in the rats treated with the novel herbal combination and syrup formulation and was more evident after treatment with syrup formulation (Table 4).

3.4.3. Random blood sugar

In the PCOS induction group, a significant increase in glucose level was noticed as compared to the normal control group (P<0.001). After the treatment of 15 days with novel herbal combination at doses of 100 mg/kg and 200 mg/kg, there was significant reduction in blood sugar level as compared to the PCOS induction group (both P<0.001). After the treatment with syrup formulation and *Pushyanuga churna,* the random blood sugar showed a significant reduction as compared to the PCOS induction group (both P<0.01). The reduction in random blood sugar was more significant with novel herbal combination treatment than that with syrup formulation treatment. The treatment with clomiphene citrate did not significantly reduce the blood sugar levels, compared to the PCOS induction group (Table 4).

3.5. Ovarian morphology

3.5.1. Light microscopy

Multiple cystic follicles with thin granulosa layer and randomly interspersed atretic cyst were observed in ovaries of rats from the letrozole-induced PCOS group. The ovaries in these rats had lesser number of corpus luteum, whereas the normal control group rats showed presence of healthy growing follicles and corpus luteum. Histological studies of the ovarian section of clomiphene citrate and *Pushyanuga churna* treatment groups showed primary follicle with oocyte and antral follicles. The ovary showed many follicles in various stages of development along with corpus luteum. Histological studies of ovaries of rats treated with novel herbal combination at doses of 100 and 200 mg/kg and syrup formulation at 200 mg/kg showed many growing follicles along with corpus luteum. The ovary showed better cytoarchitecture with antral follicles and lesser number of cysts as compared to the PCOS induction group (Figure 2).

3.5.2. Electron microscopy

Electron microscopic examination of the ovarian stroma of normal control rats showed presence of electron dense cytoplasmic granules, spindle shaped granular cells, Balbiani's vitelline body, closely packed vesicles and peripheral dense fibers. Letrozole-induced PCOS rats in the PCOS induction group showed signs of cell damage in granulosa cells, with reduction in secretory granules, altered mitochondrial structure, poorly developed endoplasmic reticulum. The nuclear chromatin material appeared condensed. Electron microscopic examination of the ovarian stroma of clomiphene citrate group showed presence of granulosa cells, thecal layer and vesicle in zona pellucida, whereas low power electron micrograph of ovary of *Pushyanuga churna* treatment group showed distinctive thecal layer and granulosal layer. The inner layer of the granulosa cells showed presence of autophagosomes.

Electron microscopic observation of ovaries of rats from the treatment groups such as novel herbal combination at doses of 100

and 200 mg/kg and syrup formulation at 200 mg/kg showed normal cytoarchitecture of granulosa cells, secretory cells and thecal layer. The granulosa cells showed large nucleus with numerous dense chromatin aggregates. Lipid droplets were present in the cytoplasm along with significant amounts of secretory granules. Prominent mitochondria and other cell organelles like endoplasmic reticulum and golgi complex were also observed (Figure 3).

4. Discussion

PCOS is a multifactorial disease with physiological, endocrinological biochemical changes that lead to a variety of clinical manifestations that could be distinctive to individual women. In an attempt towards integrated approach to treat PCOS, ten medicinal plants were shortlisted as per their reported pharmacological properties and also, based on our previous research work on these individual plants. The current study involved phytopharmacological evaluation of novel herbal combination and its syrup formulation containing these ten medicinal plants in an attempt to develop a syrup formulation as a potential alternative therapeutic agent for PCOS.

Both novel herbal combination and its syrup formulation contain medicinal plants combined in specific ratio, based on their reported therapeutic effects like hormonal supplementation (50%), diabetes control (25%) and fertility enhancement (25%). The combination attempts to address three major areas of concern in the treatment of PCOS. Both novel herbal combination and its syrup formulation have been hypothesized to be effective in reducing insulin resistance, managing hyperandrogenism; regulating menstrual cycle, facilitating ovulation thus promoting fertility and improving liver and adrenal functions.

To evaluate and validate the hypothesis of multipronged approach in the management of PCOS through the novel phytotherapy the current research study used letrozole-induced PCOS model in rats[34,35].

Decrease in the levels of progesterone and estrogen, with significant increase in testosterone concentration, observed in the study, are the consistent hormonal features reported in PCOS-women[36]. In PCOS, estradiol levels have been reported to be increased due to the compensatory response of the ovary. Impaired LH: FSH ratio in PCOS condition plays an important role in the causation of abnormal ovarian physiology. According to results of the current study, treatment with novel herbal combination and its syrup formulation caused recovery of the altered hormonal profile in PCOS-induced rats. The effects are predominantly observed in the levels of testosterone, progesterone, LH and FSH. The treatment, however, had minimal effect on estrogen levels. This indicates that the treatment with novel herbal combination and syrup formulation of novel herbal combination possibly lead to resolution of changes in estrogen levels. The physiological changes induced by letrozole on ovary and changes seen in various blood biochemical parameters were normalized after treatment with novel herbal combination and its syrup formulation. The treatment with syrup formulation was found to be more effective in ovarian stimulation and restoration of follculogensis as compared to all other treatment regimens evaluated in the current work[34,37].

PCOS is closely associated with metabolic dysfunction such as dyslipidemia, insulin resistance, obesity and inflammation[38]. The treatment with novel herbal combination at doses of 100 and 200 mg/kg, and its syrup formulation at 200 mg/kg were able to effectively normalize the elevated levels of triglycerides and cholesterol. Elevated levels of random blood sugar were also controlled with treatment of novel herbal combination and its syrup formulation in PCOS induced rats.

PCOS is also recognized as a state of increased oxidative stress due to decreased concentration of antioxidants and is considered one of the important factors involved in PCOS pathogenesis[39]. A decrease in catalase activity was seen in the rats induced with PCOS, which was in consonance with earlier reports indicating the accumulation of reactive oxygen species and consequent oxidative stress after letrozole administration[31]. The apparent elevation in GPx activity in rats after induction of PCOS signifies accumulation of free radicals due to inactive peroxides[31,39]. Concomitant treatment with novel herbal combination and its syrup formulation resulted in the restoration of GPx activities, thus reducing the causative agents of oxidative stress[31,40]. SOD activity has been reported to decrease in PCOS due to the increased consumption of SOD to counterbalance ROS produced by hyperglycemia and excessive fatty acids[40]. Elevated levels of LPO and GST in PCOS indicate increased oxidative stress[41,42]. It was found that in all treatment groups, including clomiphene citrate and *Pushyanuga churna* treatment groups, there was reduced LPO, SOD and GST activities suggesting some control being effective on the oxidative stress. These changes were not statistically significant, suggesting that for better control on oxidative stress, increased treatment period or increased dosages may be required. The oxidative stress protection provided by treatments with novel herbal combination and its syrup formulation was evident from the levels of antioxidant enzymes such as catalase and GPx. The physiological effects of these treatments were, however, minimal with respect to the antioxidant enzymes SOD, GST and LPO. This also suggests that there is a need for a different treatment regimen for novel herbal combination and its syrup formulation like increasing treatment period or using a different dosage regimen to get better control of antioxidant enzymes.

The characteristic histological feature under light microscope, of an ovary in PCOS-induced rat is the presence of multiple cysts in the ovarian tissue. After letrozole-induced PCOS in rats, numerous

cystic follicles with thin granulosa layer were visible indicating successful induction of PCOS[28]. PCOS induction also reduced the number of corpus luteum. After treatment for 15 days, with novel herbal combination and its syrup formulation, the PCOS-induced rats showed reduction in cystic follicle and increase in antral follicle along with corpus luteum. Ovarian section of rats from novel herbal combination and syrup formulation groups also showed follicles in various stages of development and a significant presence of corpus luteum[17]. The number of corpus luteum and tissue arrangements in ovarian section was, however, better after treatment with syrup formulation at the dose of 200 mg/kg as compared to novel herbal combination at 100 and 200 mg/kg[43]. Histomorphological improvement seen in the ovary after treatment with syrup formulation of novel herbal combination is comparable with those after treatment with modern drug-clomiphene citrate and the Ayurvedic formulation-*Pushyanuga churna*. The findings in the study are in agreement with the findings reported previously and the data reported in support of the restorative effects of quercetin on ovarian cytoarchitecture in letrozole-induced PCOS rat model[34].

Electron microscopic study of ovary from the rats of the control group showed granulosa cells with a prominent nucleus, dense cytoplasmic granules, closely packed vesicles and peripheral dense fibers. Letrozole-induced PCOS rats showed presence of altered mitochondrial structure, poorly developed endoplasmic reticulum and desmosomes with condensed nuclear chromatin material, indicating cell damage in granulosa cells induced by letrozole administration. After the PCOS rats were treated with novel herbal combination, its syrup formulation and clomiphene citrate, the ovarian stroma showed signs of recovery when compared with the letrozole administration group. The stroma showed the presence of Balbiani's vitelline bodies associated with the follicle. Prominent desmosomes are seen in between the cells. Stroma also showed the presence of many prominent lipid droplets. The ovarian cells showed presence of finger-like microvilli and accumulation of collagen fibers. Similar findings were reported by Çelik *et al* on the restorative effects of vitamin D on ovary in dehydroepiandrosterone-treated PCOS rat model[44]. Thus, the observations of electron microscopic evaluation supports and confirms the biochemical as well as light microscopic findings.

As multiple factors are involved in pathogenesis of PCOS, treatment with a single agent will not be able to counterbalance the damage associated with the syndrome. Comparative analysis of various treatment groups suggests that syrup formulation of novel herbal combination (200 mg/kg) show better efficacy in the management of PCOS in terms of hormonal, lipid and histopathological profiles. The oxidative protection after treatment with syrup formulation of novel herbal combination is relatively less, suggesting the need for a modified treatment regimen. Interestingly, results obtained after treatment with syrup formulation of novel herbal combination were comparable with those after treatment with the modern drug-clomiphene citrate and the Ayurvedic marketed formulation-*Pushyanuga churna*. This is an important finding that supports the conclusion that syrup formulation of novel herbal combination is potentially an effective alternative therapeutic agent for PCOS.

The therapeutic efficacies of novel herbal combination and its syrup formulation in the present study are reflected by the positive responses in terms of correcting hormonal, lipid, and histopathological profiles by bringing them close to normalcy. The results of hormonal levels, lipid assays and histopathology support the hypothesis of making a novel herbal combination with ten medicinal plants in which standardized extract from each plant is expected to target different clinical symptoms of PCOS.

The limitation of the study probably was inability to obtain significant improvements in oxidative enzymes. This calls for longer treatment period or a different dosage regimen that could lead to better management of oxidative stress related to PCOS.

In conclusion, the result of the current study shows that oral administration of syrup formulation of novel herbal combination at the dose of 200 mg/kg for 15 days significantly restores the ovarian folliculogenesis and normalizes the hormonal levels and lipid profile in PCOS induced rat model. The treatment with novel herbal combination and its syrup formulation also provided protection against oxidative stress occurring in PCOS. In the current study, however, the reduction in oxidative stress is not significant suggesting the need for either a longer treatment period or a different dosage regimen for novel herbal combination and its syrup formulation. Since PCOS is a long-term metabolic syndrome associated with multiple clinical factors, a longer treatment duration of 30 days seems desirable. Since the syrup formulation of novel herbal combination is a polyherbal formulation containing ten plants having their own specific therapeutic efficacy towards various symptoms of PCOS, the cumulative effect of the ingredient medicinal plants provides the multidimensional approach to PCOS management. This study suggests that syrup formulation of novel herbal combination (200 mg/kg) can be a potentially effective agent for clinical use in PCOS management, especially as an adjutant therapeutic agent with a modern medicine like clomiphene citrate, whereby the dose of the modern drug could be reduced. This could also effectively reduce the PCOS-related risk of diabetes and cardiovascular problems. The syrup formulation of novel herbal combination has potentials to be developed into an alternative therapy for PCOS patients, especially those with clomiphene citrate resistance.

Conflict of interest statement

The authors declare no conflict of interest.

Funding

This study was supported by DST-SERB, Government of India *via* sanction No. EMR/2017/001106.

Authors' contributions

Sunita Shailajan is Principal Investigator and Sasikumar Kumar is Co-Principal Investigator in the project.Yugandhara Patil and Swati Singh are Project Fellows. Yugandhara Patil and Swati Singh performed the experiment and submitted the data for analysis. Sasikumar Menon was involved in the designing and interpretation of animal studies while Sunita Shailajan was involved in designing the formulation composition, chromatography and interpretation of phytochemical studies. All authors were involved in the preparation of manuscript.

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