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Ocimum kilimandscharicum L. restores ovarian functions in letrozole - induced Polycystic Ovary Syndrome (PCOS) in rats: Comparison with metformin

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ABSTRACT

Introduction and aim: Polycystic ovary syndrome is one of the most common causes of female infertility, affecting 5–10% of the population. Women with PCOS manifest hyperandrogenism, hyperinsulinemia, low-grade systemic inflammation, and polycystic ovaries. Unfortunately, current available medications are only symptomatic without relevant reported treatment. Therefore, a pressing need for alternative safe approaches is necessitated. To this end, the present study is designed to investigate therapeutic merits of the edible plant: *Ocimum kilimandscharicum* (*Ok*), in a letrozole PCOS rat model, and compare it to metformin.

Material and methods: PCOS rats were treated with *Ok* total extract and its different fractions at 100 mg/kg orally for 10 consecutive days. Moreover, phytochemical characterization was applied using HPLC/PDA/ESI-MS to identify different secondary metabolites in the bioactive fractions.

Key findings: Results revealed that the total extract (*Ok*) and ethyl acetate (EA) fraction improved insulin sensitivity and restored normal hormonal and lipid profiles as well as normal morphological structure of the reproductive system. Furthermore, elevation of SOD and reduction of VEGF levels in comparison with metformin were recorded.

Significance: These results suggest that *Ok* extract and EA fraction halt letrozole-induced reproductive dysfunctions and restore normal morphological and physiological functions in PCOS rats, even superior to metformin.

1. Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine disorder affecting women at reproductive age, with high prevalence worldwide [1]. Stress, diet, sedentary lifestyle, in addition to genetic factors, may contribute to its pathogenesis [1]. Women are diagnosed with PCOS if two of the following hallmarks are fulfilled: hyperandrogenism, anovulation, and/or polycystic ovaries, according to the Rotterdam criteria [2]. Moreover, the prevalence and incidence of metabolic syndrome are greater in women with PCOS. Noteworthy, hyperinsulinemia results in overproduction of androgens by the ovaries [3] and hence anovulation.

Currently conventional accessible medications for PCOS are controversial due to the complex nature of this disorder. Treatment goals should focus on normalizing ovulation, reducing androgen levels and insulin resistance [2]. Metformin is an oral hypoglycemic drug initially introduced to treat T2DM [4]. Several studies advocate its use in PCOS since it enhances insulin sensitivity and restores normal hormonal profile with improved menstrual cyclicity [5]. However, accumulating body of evidence on metformin efficacy has yet been controversial [5].

New trends have been directed towards imbedding natural remedies in daily diet for PCOS management [6]. *Ocimum kilimandscharicum* (*Ok*) is an edible plant indigenous to Egyptian flora, commonly known as basil [7]. Several types of bioactive compounds have been isolated from different parts of *Ok* such as flavonoids, glycosides, and phenolic acids [7]. *Ok* is highly valued for its economic importance and traditional therapeutic potential [7] as an antioxidant, anti-inflammatory, and anticancer agent [8]. Noteworthy, letrozole is believed to recapitulate both the reproductive and metabolic phenotypes of PCOS clinically [9].

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Letrozole—a nonsteroidal aromatase inhibitor [10]—is used to induce PCOS experimentally with similar clinical reproductive and metabolic traits [11]. It blocks the conversion of testosterone to estrogen; consequently, serum and ovarian testosterone levels rise leading to abnormal follicular development and hormonal imbalance [11].

Accordingly, the present study aimed to investigate the possible therapeutic merits of *Ok* in reversing PCOS anomalies in a rat letrozole model. Furthermore, phytoconstituents in *Ok* total extract and its bioactive EA fraction have been characterized using HPLC/PDA/ESI-MS. Moreover, their potential therapeutic efficacy has been compared with the conventionally used drug: metformin.

2. Experimental

2.1. Plant material preparation

Fresh leaves of *Ok* were collected from El-Orman garden, Giza, Egypt, and authenticated by Dr. Mohamed El-Gebaly, Taxonomy Specialist, National Research Center, Egypt. Voucher specimen number 00399 was deposited at Pharmaceutical Biology Department, Faculty of Pharmacy and Biotechnology, German University in Cairo. According to Handoussa et al. (2009) [12] dried *Ok* leaves (3 Kg) were used to obtain the total extract, amounting to 370.6 g (yield 12.36% w/w). Then, solvent-solvent fractionation was applied to the total extract to obtain fractions of petroleum ether, chloroform, and ethyl acetate yielding 17 g, 10 g, and 3 g, respectively [12].

2.2. Animals

Adult virgin female Wistar Albino rats (6 weeks old, weighing 150–200 g; total $n = 54$, $n = 6$) were obtained from National Research Institute (NRI), Cairo, Egypt. They were housed in a pathogen-free facility at the German University in Cairo (GUC) under standard environmental conditions, 25 ± 2 °C temperature, 55 ± 5% humidity, and 12 h dark/light cycle and fed standard chow with water provided ad libitum. Rats were adapted to laboratory conditions for 1 week. Experimental procedures (approval no. GUC-00249) were performed in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996) [13].

2.3. Drugs and chemicals

Letrozole (4,4′-(1h-1,2,4-triazol-1-yl)methylene dibenzonitrile) purchased from Sigma-Aldrich, Saint Louis, USA, was prepared by dissolving in 0.5% Carboxymethyl Cellulose (CMC, WINLAB, UK). Metformin was purchased from CID Company, Cairo, Egypt, as metformin HCL 850 mg/tablet.

2.4. PCOS induction

To confirm PCOS induction, vaginal smears were obtained using cotton buds immersed in saline; smears were allowed to dry after rolling on a clean slide. Cells were fixed using methanol, allowed to dry, and stained using 0.76 g giemsa according to [14].

2.5. Experimental design

Animals were divided into two main groups: control groups (Gp. I) and PCOS groups (Gp. II); **Group Ia** served as negative control and received 0.5% CMC day after day for 3 weeks. **Group Ib** received metformin at a dose of 300 mg/kg for 15 days [15]. **Group Ic** received *Ok* at a dose of 100 mg/kg for 10 days (the choice of this dose is based on a comparison between different doses through preliminary histopathological investigations) (Supp. Data 1 & Supp. Data 2). **PCOS groups**: **Group IIa** received 1 mg/kg letrozole day after day for 3 weeks [16].

Following PCOS induction, animals were divided into **Gp IIb** (received metformin 300 mg/kg for 15 days); **Gp IIc** (received (*Ok*) at 100 mg/kg for 10 days); **Gp IId**, **IIe**, **and IIf** (animals received petroleum ether, methylene chloride, and ethyl acetate, respectively, at 100 mg/kg for 10 days). All groups received treatments orally. Rats were euthanized using isoflurane overdose; blood samples were collected from the heart and then centrifuged (Centurion, Scientific Ltd., USA) at 373 × *g* for 20 min. Sera were collected and stored at −80 °C until further processing. After scarification, ovaries and uteri were excised, cleaned, weighed, and then preserved in 10% formalin for histopathological investigation.

2.6. Biochemical parameters

Serum fasting blood glucose (FBG), total cholesterol (TC), and triglycerides (TG) were measured colorimetrically using their respective kits supplied from Diamond Diagnostics (kit no. 201922) and Spectrum Diagnostics®, Hannover, Germany (kit nos. 136059 & 22170), respectively. Serum VEGF was determined using ELISA diagnostic kit provided by Quantikine, Minneapolis, USA (kit no. RRV00). Serum insulin, hormonal profile [estrogen (E), progesterone (P), and testosterone (T)], and SOD were measured using their respective ELISA kits supplied from CUSABIO, Wuhan, China (kits no. CSB-E05070r, MBS703614, CSB-E07282r, CSB-E05100r, and MBS036924), respectively. HOMA index was calculated from the obtained values of fasting blood glucose (FBG) and insulin using the following equation [17]:

$$\text{HOMA} - \text{IR} = (\text{fasting glucose level} \times \text{fasting insulin level})/405$$

2.7. Histopathological analysis

Ovaries and uteri tissues were fixed in 10% buffered formalin for 24 h and were subsequently processed and embedded in paraffin wax sections for further histopathological examination using hematoxylin and eosin stain [18].

2.8. Quantitative analysis of bioactive compounds using HPLC/PDA/ESI-MS

Characterization of the bioactive metabolites was performed using HPLC/PDA/ESI-MS according to the method reported in Handoussa et al. (2013) on HPLC Agilent 1200 series instrument [19].

2.9. Statistical analysis

Data were expressed as means ± SD. Comparisons between different means were obtained by conducting one-way analysis of variance (ANOVA) followed by Tukey–Kramer multiple comparison test using GraphPad Prism (version 5) software (GraphPad Inc., San Diego, CA, USA); $p < 0.05$ was considered statistically significant.

3. Results

3.1. Anthropometric parameters (body, ovarian, and uterine weights)

As depicted in Table 1, letrozole group (Gp IIa) showed a significant increase in body weight by 14% and reproductive organ weight by 39% compared to CMC control group (Gp Ia). PCOS rats treated with EA fraction showed significant decrease in body weight by 3% compared to letrozole. On the other hand, PCOS rats treated with metformin, *Ok* extract and EA fraction showed significant reduction in reproductive organ weight by 31%, 36%, and 42%, respectively, compared to letrozole group.

3.2. Determination of estrous cyclicity

Vaginal smears of normal rats showed nucleated epithelial cells indicating the presence of proestrous phase of rat cycle (Fig. 1A); animals receiving letrozole showed predominant leukocytes indicating

Table 1
Effect of metformin, Ok total extract, and EA fraction on anthropometric parameters: body weight and reproductive organ weight in letrozole-induced PCOS rats.

Groups	Body weight (g)		Reproductive system weight (g)
	Baseline	After 10 days	
Control	111.29 ± 2.06	182.0 ± 15.69	0.72 ± 0.08
Metformin	108.69 ± 2.62	182.6 ± 1.77	0.70 ± 0.09
Ok extract	125.22 ± 1.20	180.6 ± 3.34	0.73 ± 0.07
Letrozole	101.18 ± 2.62	206.8[a] ± 9.93	1.00[a] ± 0.22
Letrozole + metformin	113.62 ± 0.20	216.2[a] ± 3.39	0.69[c] ± 0.12
Letrozole + *Ok* extract	116.71 ± 2.20	213.8[a] ± 8.44	0.63[c] ± 0.13
Letrozole + EA fraction	125.52 ± 2.20	200.3[a,d,e] ± 5.77	0.58[c] ± 0.09

Data are expressed as mean of 6 rats ± SD.
[a] $p < 0.05$ versus control group.
[c] $p < 0.05$ versus letrozole.
[d] $p < 0.05$ versus letrozole + metformin.
[e] $p < 0.05$ versus letrozole + *Ok* extract.

Table 2
Effect of metformin, *Ok* extract, and EA fraction on serum hormonal profile: testosterone (T), estrogen (E), and progesterone (P) in letrozole-induced PCOS rats.

Groups	(T) (ng/ml)	(E) (Pg/ml)	(P) (ng/ml)
Control	0.56 ± 0.04	21.23 ± 1.71	5.03 ± 0.17
Metformin	0.53 ± 0.04	24.53[a] ± 0.99	5.30 ± 0.28
Ok extract	0.46[a,b] ± 0.04	27.37[a,b] ± 1.3	6.30[a,b] ± 0.71
Letrozole	1.43 ± 0.08	15.47 ± 1.18	0.46 ± 0.04
Letrozole + metformin	0.86[c] ± 0.04	50.33[c] ± 3.32	1.96[c] ± 0.14
Letrozole + *Ok* extract	0.83[c] ± 0.04	105.1[c,d] ± 4.92	2.80[c,d] ± 0.18
Letrozole + EA fraction	0.39[e] ± 0.01	29.95[e] ± 0.88	4.20[e] ± 0.21

Data are expressed as mean of 6 rats ± SD
[a] $p < 0.05$ versus control group.
[b] $p < 0.05$ versus metformin group.
[c] $p < 0.05$ versus letrozole.
[d] $p < 0.05$ versus letrozole + metformin.
[e] $p < 0.05$ versus letrozole + *Ok* extract.

Table 3
Effect of metformin, *Ok* extract, and EA fraction on lipid profile: triglyceride (TG) and total cholesterol (TC) in letrozole-induced PCOS rats.

Groups	(TG) (mg/dl)	(TC) (mg/dl)
Control	182.6 ± 19.27	300.9 ± 6.59
Metformin	158.8[a]b ± 12.84	299.3 ± 28.72
Ok extract	149.6[a] ± 14.45	293.9 ± 21.11
Letrozole	216.5[a] ± 19.52	325.4 ± 15.58
Letrozole + metformin	187.7[c] ± 21.07	306.9[c] ± 5.52
Letrozole + *Ok* extract	131.0[c,d] ± 15.36	133.7[c,d] ± 11.83
Letrozole + EA fraction	131.2 ± 13.85	132.4 ± 11.89

Data are expressed as mean of 6 rats ± SD.
[a] $p < 0.05$ versus control group.
[b] $p < 0.05$ versus metformin group.
[c] $p < 0.05$ versus letrozole.
[d] $p < 0.05$ versus letrozole + metformin.

continuous diestrous phase of rat cycle (Fig. 1B). Interestingly, treatment of animals with metformin (Fig. 1C), *Ok* extract (Fig. 1D), and EA fraction (Fig. 1E) following PCOS induction restored normal estrous cycle.

3.3. Biochemical parameters

3.3.1. Effect of Ok extract and EA fraction on serum hormonal levels

Results of the present study as demonstrated in Table 2 revealed deleterious effects of letrozole on the hormonal profile, where significant elevation in T and significant reduction in E and P levels by 153%, 27%, and 9%, respectively, were reported, compared to control animals. Treatment of PCOS rats with metformin (Gp IIb), *Ok* extract (Gp IIc), and EA fraction (Gp IId) decreased testosterone levels by 40%, 42%, and 73%, significantly increased estrogen levels by 225%, 579%, and 94%, and also increased progesterone levels by 321%, 500%, and 813%, respectively, compared to PCOS untreated rats (Gp IIa).

3.3.2. Effect of Ok extract and EA fraction on lipid profiles

Table 3 confirmed significant elevation of TG levels in PCOS rats compared to control (Gp Ia) by 19%. Treatment of PCOS rats with metformin (Gp IIb), *Ok* extract (Gp IIc), and EA fraction (Gp IId) normalized lipid profile manifested as reduction in TG content by 13%, 39%, and 40%, respectively and TC levels by 6%, 59%, and 60%, respectively, compared to PCOS untreated rats (Gp IIa).

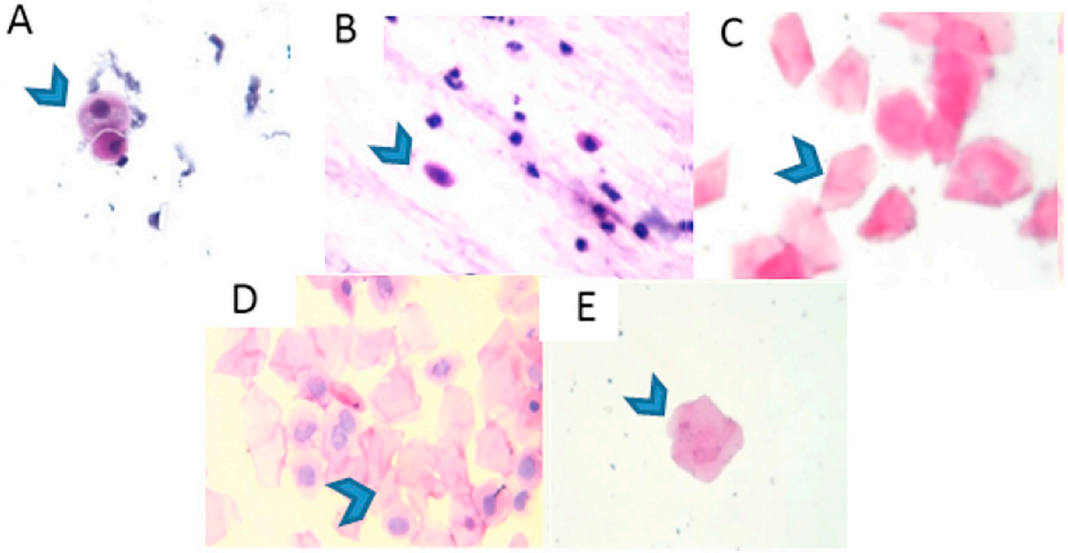

Fig. 1. Representative photomicrographs of vaginal smears from each experimental group (H&E, magnificent scale x 80). (A) Control group showing proestrous phase (nucleated epithelial cells). (B) Letrozole group showing cells of diestrous phase (leukocytes). (C) Letrozole + metformin group showing estrous phase (anucleated cornified cells). (D) Letrozole + *Ok* extract group showing estrous phase (anucleated cornified cells). (E) Letrozole + EA fraction showing estrous phase (anucleated cornified cells).

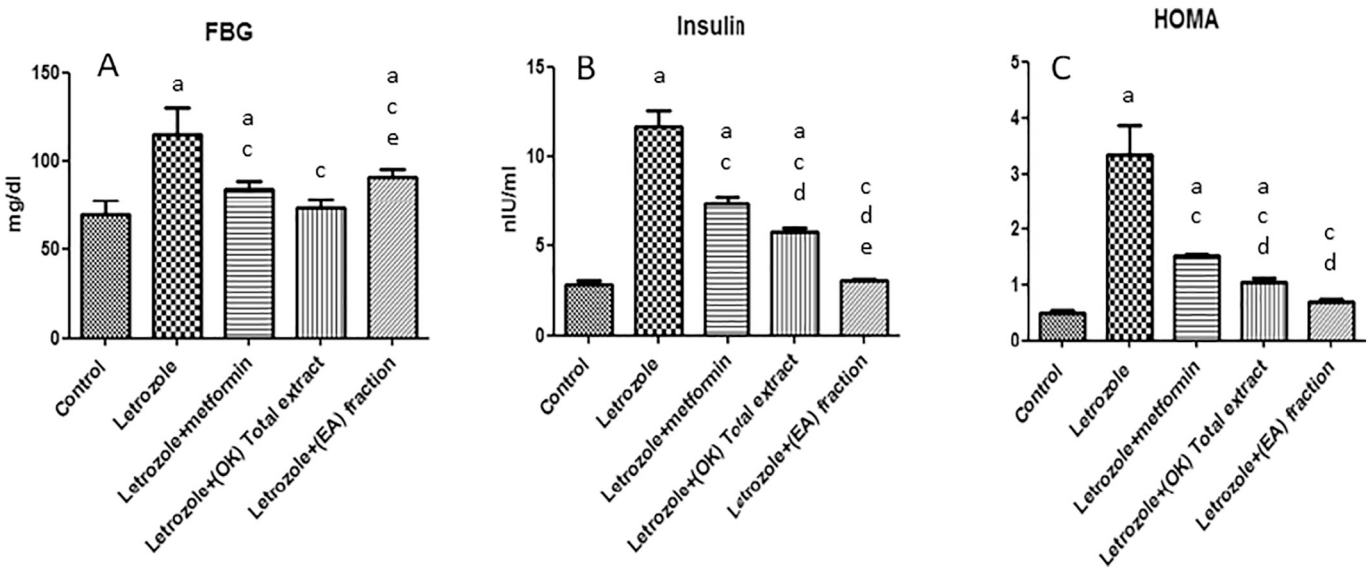

Fig. 2. Effect of different experimental groups on (A) FBG; (B) insulin; (C) HOMA index. Data are expressed as mean of 6 rats ± SD. [a]$p < 0.05$ versus control group, [b]$p < 0.05$ versus metformin group, [c]$p < 0.05$ versus letrozole, [d]$p < 0.05$ versus letrozole + metformin, and [e]$p < 0.05$ versus letrozole + *Ok* total extract.

3.3.3. Effect of Ok extract and EA fraction on insulin, fasting blood glucose, and HOMA index

PCOS induced rats (Gp IIa) showed marked elevation in INS, FBG, and HOMA index compared to control rats by 318%, 66%, and 529%, respectively. Treatment of PCOS rats with metformin (Gp IIb), *Ok* extract (Gp IIc), and EA fraction (Gp IId) significantly reduced FBG levels by 27%, 23%, and 19% (Fig. 2A), INS levels by 37%, 51%, and 74% (Fig. 2B), and HOMA levels by 55%, 69%, and 79%, respectively, compared to PCOS untreated rats (Gp IIa) (Fig. 2C).

3.3.4. Effect of (Ok) extract and EA fraction on antioxidants and cytokines activity

Results in Table 4 showed marked reduction in SOD enzyme and elevation in the mitogenic cytokine VEGF levels in letrozole group compared to its control counterpart by 67% and 490%, respectively. Metformin (Gp IIb), *Ok* extract, (Gp IIc), and EA fraction (Gp IId) increased SOD activity level by 101%, 198%, and 208%. Moreover, these experimental groups significantly reduced VEGF level by 39%, 63%, and 89%, respectively, compared to PCOS untreated rats (Gp IIa).

3.4. Histopathological analysis

PCOS rats (Gp IIa) revealed multiple follicular cysts (FC) and necrosis (N) in the central portion of some corpus luteum (C) (Fig. 3B) with mild uteri hyperplasia and polyps formation in the mucosal lining epithelium (E) besides hypertrophy in the muscular layer (M) as well as atrophy in uterine glands (U) (Fig. 4B). On the other hand, animals that received metformin (Figs. 3C & 4C), *Ok* extract (Figs. 3D & 4D), and EA fraction (Figs. 3E & 4E) after PCOS induction showed normal reproductive organs architecture. These groups showed multiple mature Graafian follicles (F) with corpus luteum (C) at the cortical portion of the ovaries with active proliferative phase of glandular structure, similar to the control group (Figs. 3A & 4A). Raw data of histopathological studies are represented in Supp. Data 3.

Table 4
Effect of metformin, *Ok* extract, and EA fraction on SOD antioxidant and VEGF cytokine levels in letrozole-induced PCOS rats.

Groups	(SOD) (U/ml)	(VEGF) (Pg/ml)
Control	32.23 ± 1.25	26.50 ± 1.34
Metformin	40.87[a] ± 3.15	25.97 ± 0.91
Ok extract	63.90[a,b] ± 4.20	21.13[a,b] ± 1.28
Letrozole	10.73 ± 1.00	124.8 ± 6.24
Letrozole + metformin	21.60[c] ± 1.27	76.03[c] ± 7.24
Letrozole + *Ok* extract	32.00[c,d] ± 1.52	46.27[c,d] ± 3.20
Letrozole + EA fraction	36.17[e] ± 1.51	13.55[e] ± 0.67

Data are expressed as mean of 6 rats ± SD.
[a] $p < 0.05$ versus control group.
[b] $p < 0.05$ versus metformin group.
[c] $p < 0.05$ versus letrozole.
[d] $p < 0.05$ versus letrozole + metformin.
[e] $p < 0.05$ versus letrozole + *Ok* extract.

3.5. Quantitative analysis of bioactive compounds using HPLC/PDA/ESI-MS

Characterization of metabolites in the bioactive total extract (Table 5A) and EA fraction (Table 5B) was performed using HPLC/PDA/ESI-MS. Several phytochemical classes were identified as phenolic acids, flavonoids, and their corresponding glycosides.

3.6. Phenolic acids and their derivatives

Rosmarinic acid which was previously detected in *Ok* [7] was also identified as peak *Ok7* in the total extract (Fig. 5A) based on its characteristic fragments' ions at *m/z* 123, *m/z* 135, *m/z* 161, *m/z* 179, and *m/z* 197 [20]. Rosmarinyl glucoside (*Ok24*) appeared as $[M - H]^-$ at *m/z* 521.28 showed fragments' ions at *m/z* 359 $[M - H] - C_6H_{10}O_5]^-$ due to loss of glucose and *m/z* 197 $[M - H] - C_9H_6O_3]^-$ as a result of caffeoyl group loss [21]; that compound was firstly isolated from *Eryngium alpinum* according to Le Claire (2005) [22]. Furthermore, peak *Ok25* which was originally isolated from *Blepharis ciliaris* [23] was identified in EA fraction (Fig. 5B) as decarboxyrosmarinic acid-galactosyl rhamnoside. Moreover, peak *Ok16* with $[M - H]^-$ at *m/z* 529.13 yielded fragments' ions of feruloylquinic acid at *m/z* 367 $[M - H -$ caffeic acid$]^-$, *m/z* 353 $[M - H -$ caffeoyl quinic acid$]^-$, and *m/z* 191 of quinic acid moiety; thus, it was identified as feruloyl-caffeoylquinic acid derivative [24]. In addition to presence of ursolic acid (*Ok*17) and sinapic acid (*Ok*18), both peaks were identified according to their fragmentation pattern as reported in [25,26], respectively. It is noteworthy that both compounds were previously isolated from aerial parts of *Ok* [7].

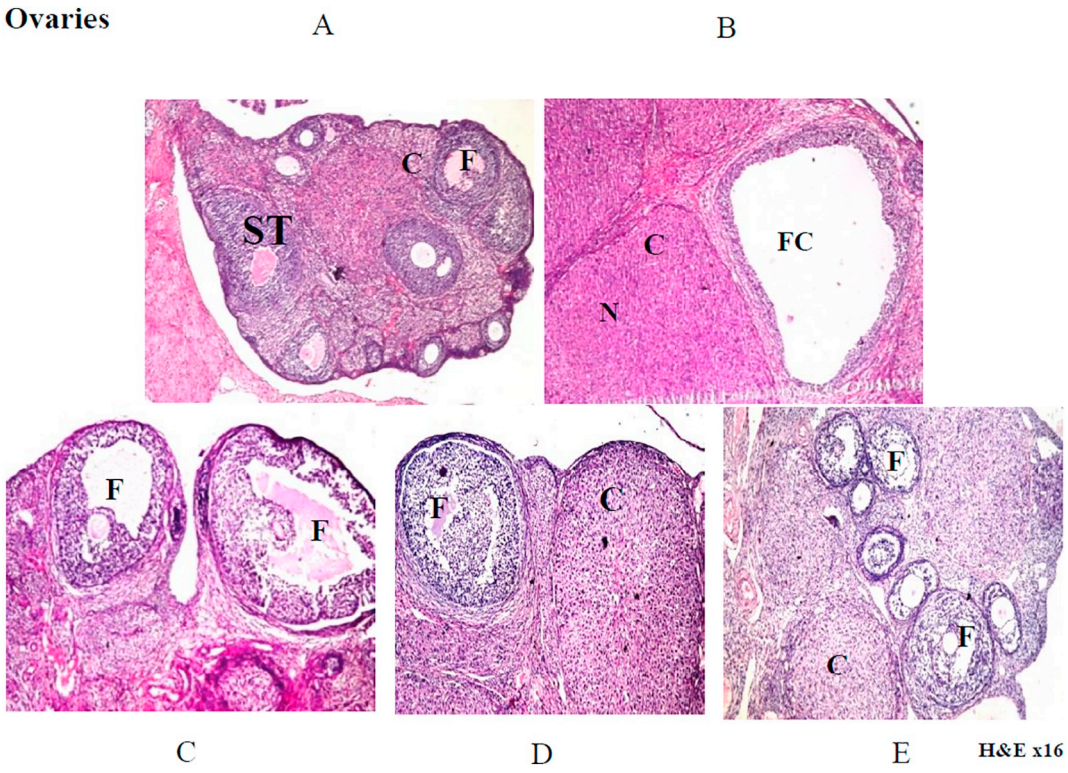

Fig. 3. Representative histopathological photomicrographs of ovaries from each experimental group (H&E, magnificent x16): (A) control, (B) letrozole, (C) letrozole + metformin, (D) letrozole + Ok extract, and (E) letrozole + EA fraction groups. (F) graafian follicle, (FC) follicular cysts, (N) necrosis, (ST) stroma and (C) corpus luteum.

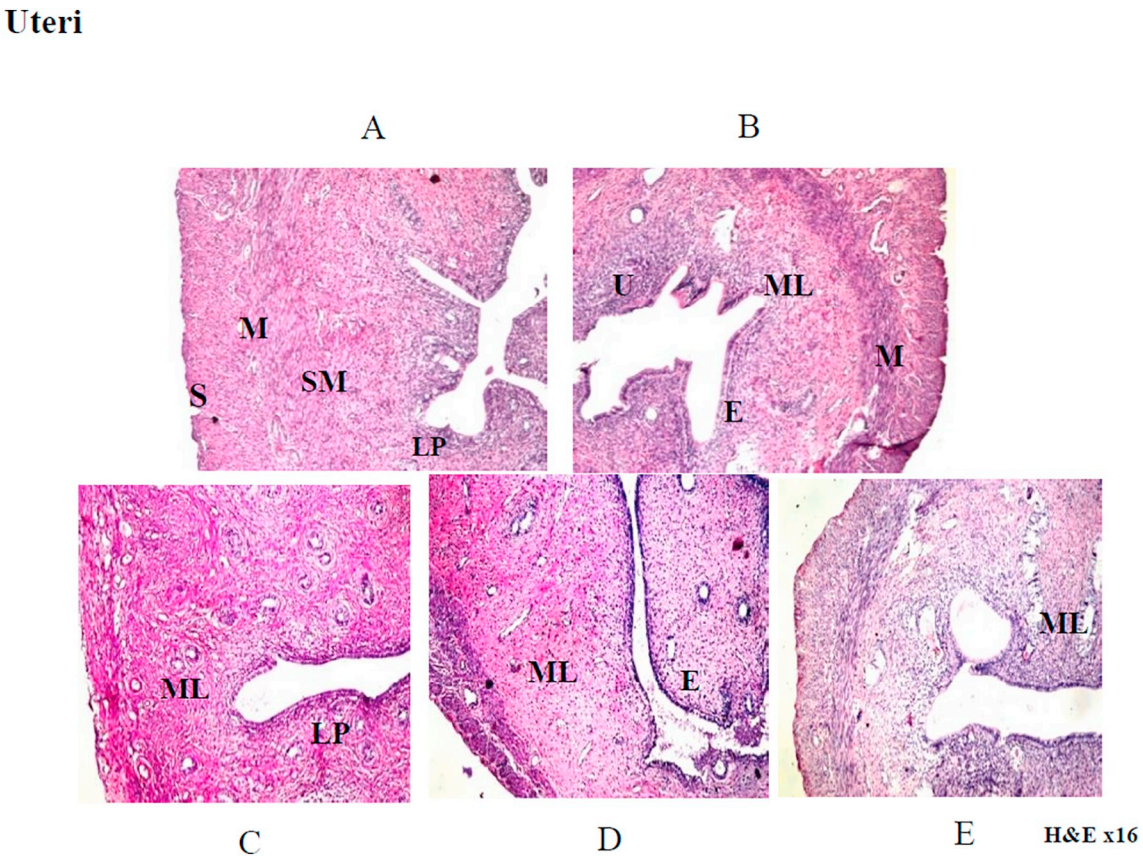

Fig. 4. Representative histopathological photomicrographs of uteri from each experimental group (H&E, magnificent x16): (A) control, (B) letrozole, (C) letrozole + metformin, (D) letrozole + Ok extract, (E) letrozole + EA fraction. (M) muscular layer, (ML) mucosal layer,(SM) submucosa, (LP) lamina propria, (E) Epithelium, (S) serosa and (U) uterine glands.

Table 5A
Peak assignments using UPLC/PDA/ESI-MS/MS of metabolites detected in total extract of *Ocimum kilimandscharicum* L. from negative mode.

Peak# (compound)	Identified compounds	Retention time (min)	UV–Vis (λ_{max})	$[M-H]^-$ (*m/z*)	Fragment ions (*m/z*)	Molecular formula	Reference
*Ok*1	Rutin	1.01	254, 354	609.28	301,179,151	$C_{27}H_{30}O_{16}$	[19]
*Ok*2	Dihydroxy dimethoxy flavone	1.10	275, 334,	328.06	313	$C_{18}H_{17}O_6$	[43]
*Ok*3	Isorhamnetin-3-*O*-hexoside	3.46	250, 342	477.36	315	$C_{22}H_{22}O_{12}$	[28]
*Ok*4	(Iso)rhamnetin 3-*O*-gentiobioside	5.85	249, 362	639.19	315	$C_{28}H_{32}O_{17}$	[44]
*Ok*5	Genistein (trihydroxyisoflavone)	8.85	226, 278	269.02	225	$C_{15}H_{10}O_5$	[45]
*Ok*6	Hydroxy-trimethoxy-flavone	9.18	275, 334,	327.34	312	$C_{17}H_{12}O_7$	[24]
*Ok*7	Rosmarinic acid	9.77	254,330	359.06	179,161	$C_{18}H_{16}O_8$	[20]
*Ok*8	Diosmetin di-C-hexoside	10.2	250, 268, 342	623.30	503,383,312	$C_{32}H_{44}O_{12}$	[28]
*Ok*9	Dihydroxy-trimethoxy-methyl homoisoflavanone	10.7	296	373.25	358,355	$C_{18}H_{17}O_6$	[46]
*Ok*10	Caffeoyl-hexose-deoxyhexoside	11.14	226, 318	487.38	308	$C_{21}H_{29}O_{14}$	[28]
*Ok*11	Caffeoyl hexose-deoxyhexoside	11.83	275	293.12	308,179	$C_{30}O_3H_{50}$	[28]
*Ok*12	Strictinin ellagitannin	12.07	270	633.41	463,275	$C_{27}H_{22}O_{18}$	[47]
*Ok*13	Methylepigallocatechin gallate	12.17	268	471.36	407	$C_{25}H_{32}O_{12}$	[47]
*Ok*14	Apigenin-C-[6-deoxy-2-O-rhamnosyl]-xylo-hexos-3-uloside	12.56	270, 340	559.35	395,321,269	$C_{28}H_{32}O_{12}$	[48]

3.7. Glycosides

Quercetin derivatives were assigned to several peaks in both bioactive fractions. For example, *Ok1* which showed deprotonated molecule at *m/z* 609.28 and was identified as rutin (quercetin-3-*O*-rutinose) that was confirmed by comparison to its reported pattern as molecular ion appeared at *m/z* 463 due to quercetin hexoside and at *m/z* 301 due to quercetin aglycone [19]. Also, hydroxy-trimethoxy-flavone (*Ok6*) with its $[M-H]^-$ at *m/z* 327.34 showed fragments at *m/z* 312 and *m/z* 297, which evidenced successive loss of two methyl groups from corresponding parent ion [24]. Additionally, quercetin glucoside (*Ok15*) has its deprotonated ion $[M-H]^-$ at *m/z* 463.08 with its fragments at *m/z* 301 $[M-162-H]^-$ and *m/z* 271 $[M-H-CO]^-$, which are typical to flavon-3-*O*-monoglycoside [26]. Likewise, quercetin arabinopyranoside was assigned for peak *Ok20* and identified based on its $[M-H]^-$ at *m/z* 433.07 and the detected pentose moiety at *m/z* 132 [26], besides another pentoside derivative that was assigned for peak *Ok22* that was recognized as quercetin-glucosyl-pentoside [27].

The identity of isorhamnetin derivatives was confirmed with its distinguished fragment ion at *m/z* 315 [24]. *Ok28* was identified as methyl-isorhamnetin-*O*-glucoside with $[M-H]^-$ ion at *m/z* 491.22 and peak *Ok3* recognized as isorhamnetin-3-*O*-hexoside; the occurrence of hexose moiety was confirmed by the loss of 162 amu [28].

Furthermore, apigenin-7-*O*-glycuronyl (*Ok29*) was observed as $[M-H]^-$ at *m/z* 445.22 due to the presence of fragment ion peak at *m/z* 269, corresponded to glucuronyl unit loss [29]. In addition, a molecular ion peak appeared at *m/z* 431.13, which experienced a hexoside loss to produce an apigenin ion at *m/z* 269 and, thus, *Ok30* was identified as glucosyl-apigenin [24].

4. Discussion

Polycystic ovary syndrome is a complex and polygenic disorder imposing high economic burden both personal and societal [30]. Clinical features associated with PCOS such as abdominal adiposity, acne, scalp bolding, and hirsutism associated with high androgens predispose women to anxiety and depression [1], in addition to other complications such as infertility, cardiovascular problems, and insulin resistance. Having said that, new pharmacological approaches with minimal side effects should be addressed.

The current study highlights a potential role of *Ok* total extract and EA fraction in restoring PCOS induced hormonal and lipid profile alterations and histological abnormalities as well as enhancing insulin sensitivity and normalizing FBG. Moreover, structural diversity of secondary metabolites in the bioactive total extract of *Ok* (Fig. 5A, Table 5A) and EA fraction - the most bioactive fraction (Fig. 5B, Table 5B) - were identified using HPLC-MS based method which was developed to detect the presence of several glycosides and phenolic acids; most of these identified compounds are characteristic for family Lamiaceae [31]. Interestingly, this study is the first ethanopharmacological study to characterize the secondary metabolites of *Ok*.

PCOS induction was confirmed in the present investigation through vaginal smears [14] that displayed predominant leukocytes in letrozole

Table 5B
Peak assignments using UPLC/PDA/ESI-MS^n of metabolites detected in ethyl acetate (EA) fraction of *Ocimum kilimandscharicum* L. from negative mode.

Peak #	Identified compounds	Retention time [min]	UV–Vis [λ_{max}]	$[M-H^-]$ [*m/z*]	Fragment ions [*m/z*]	Molecular formula	References
*Ok*15	Quercetin-*O*-hexoside	1.09	254, 362	463.0878	301,273, 179, 151	$C_{21}H_{19}O_{12}$	[26]
*Ok*16	Feruloyl-caffeoyl-quinic acid	1.34	246, 362	529.1349	367,193,179	$C_{26}H_{26}O_{12}$	[24]
*Ok*17	Ursolic acid	1.45	255, 363	455.6754	439,411,203, 191	$C_{30}H_{48}O_3$	[25]
*Ok*18	Sinapic acid	1.52	256, 328	223.212	2018,178	$C_{11}H_{12}O_5$	[26]
*Ok*19	Quercetin-*O*-hexoside	2.373	254, 362	463.0878	301,273,179,151	$C_{21}H_{19}O_{12}$	[26]
*Ok*20	Quercetin arabinopyranoside	3.95	275, 550	433.0774	398,329	$C_{20}H_{18}O_{11}$	[27]
*Ok*21	Rutin	8.67	254, 354	609.2813	301,179,151	$C_{27}H_{30}O_{16}$	[19]
*Ok*22	Quercetin-glucosyl-pentoside	9.04	254,350	595.73	463,301,179	$C_{26}H_{28}O_{16}$	[27]
*Ok*23	Quercetin-*O*-deoxyhexoside	9.39	254, 350	447.1239	301,271,255,179	$C_{21}H_{20}O_{11}$	[26]
*Ok*24	Glucopyranosyl rosmarinic acid [Rosmarinyl glucosid]	10.2	226, 333	521.28	359,197,161	$C_{24}H_{26}O_{13}$	[21]
*Ok*25	Decarboxyrosmarinic acid-galactosyl rhamnoside	11.09	290,323	623.43	359,197,161	$C_{32}H_{32}O_{13}$	[23]
*Ok*26	Rosmarinic acid derivative	11.53	265, 330	671.33	359,161	$C_{36}H_{32}O_{13}$	[21]
*Ok*27	Dimethyl quercetin	12.05	254,350	329.19	301,179	$C_{17}H_{14}O_7$	[24]
*Ok*28	Methyl-isorhamnetin-hexoside	13.5	250,342	491.2236	315, 301	$C_{23}H_{25}O_{12}$	[28]
*Ok*29	Apigenin-7-*O*-glycuronyl	13.89	270, 340	445.4132	269	$C_{21}H_{18}O_{11}$	[29]
*Ok*30	Apigenin-7-hexoside	15.2	272,345	431.1311	269	$C_{21}H_{20}O_{10}$	[49]

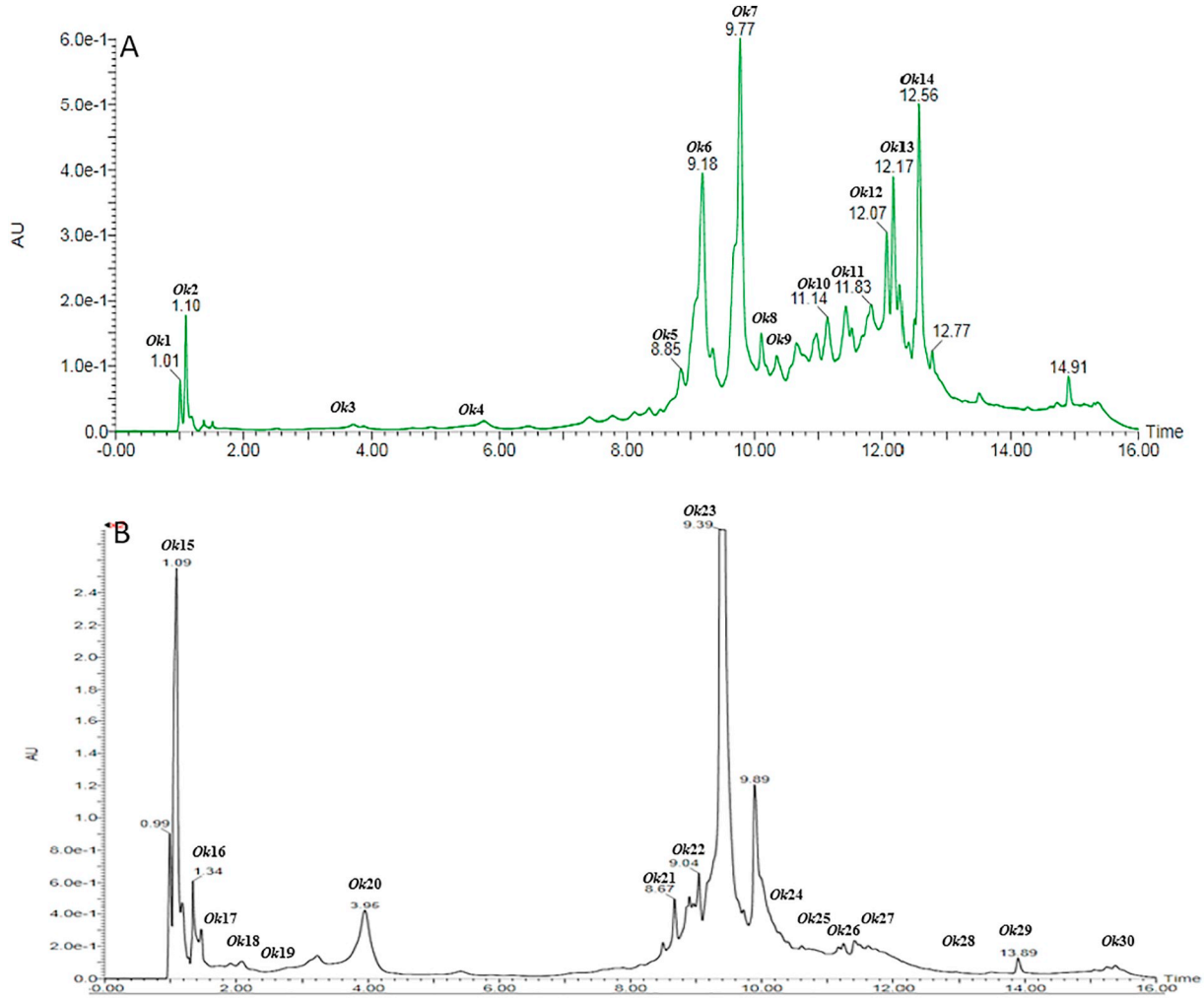

Fig. 5. Negative HPLC/PDA/ESI/mass chromatogram of metabolites from (A) total extract and (B) ethyl acetate fraction obtained from *Ocimum kilimandscharicum* leaves.

groups indicating continuous diestrous phase (Fig. 1B). Noteworthy, letrozole blocks the conversion of androgens into estrogens and results in hyperandrogenism [9]. This leads to estrous cycle dysregulation and elevated body and reproductive organ weight [32]. Furthermore, thickened ovarian capsule, hyperplasia in the mucosa, uterine muscular hypertrophism, and eventually follicle depletion take place [32]. Administration of *Ok* extract and EA fraction showed regular estrous cycle restoration and normal reproductive organ weight accompanied with reduction in testosterone and enhancement of estrogen and progesterone levels. This potential effect could be attributed to the presence of specific phytoconstituents such as rutin [33] and quercetin [34] in the *Ok* total extract and EA fraction. Noteworthy, these phytoconstituents are reported to attain antiandrogenic effects by improving aromatase activity, hence reducing testosterone and enhancing estrogen and progesterone levels [33].

Consistent with Di Pietro et al. (2015) [15], hormonal profile alterations provoked insulin resistance and increased HOMA index in the letrozole group of the present study [15]. Interestingly, PCOS rats receiving *Ok* and EA fraction improved insulin sensitivity evidenced by reducing FBG and insulin levels with remarkable decline in HOMA index. This finding could be correlated to the insulin sensitization effect of rutin thereby increasing glucose uptake by target cells [34]. In addition to the presence of the antihyperglycemic flavone apigenin [35] and isorhamnetin glucoside [36].

Indeed, hyperinsulinemia and hyperandrogenism in PCOS stimulate adipocytes to increase catecholamine-induced lipolysis resulting in high serum free fatty acids and hence dyslipidemia [37]. Consequently, increased free fatty acids synthesis in the liver results in elevated levels of VLDL and TG in blood [38]. Rats receiving letrozole displayed high levels of TC and TG compared to the control naïve animals. The extract and EA fraction demonstrated a significant reduction in TG and TC levels compared to letrozole nontreated and letrozole metformin-treated groups. Similar effect of *Ocimum sanctum* was reported in Ahmad et al. (2018) [25].

The role of inflammation and oxidative stress in PCOS pathogenesis has been tracked in the current investigation. VEGF is an angiogenic factor [38] playing a key role in developmental, physiological, and pathological angiogenesis. In a feed forward loop, oxidative stress causes a proinflammatory state that induces insulin resistance and hyperandrogenism [30]. Consequently increased VEGF secretion in PCOS women is reported [39]. Mechanistically, androgen receptors (AR) binding sites are present on the VEGF promoter region. Binding of androgens to these sites causes the activation of VEGF gene expression [40]. Moreover, as suggested by Artini et al. (2009) [39] soluble VEGF receptors decrease in the serum of PCOS women; consequently, the bioavailability of VEGF increases. Such findings coincide with the observed increase in VEGF levels in the letrozole group. Indeed, several phytoconstituents present in *Ok* extract and EA fraction such as phenolic acids and polyphenolics possess antioxidant activity, thereby illustrating the reason behind elevated SOD [19] and reduced VEGF levels [41] observed in the present research compared to the letrozole group. Besides, the insulin sensitization effect of rutin [42] and the

presence of the antihyperglycemic flavone apigenin improve insulin sensitivity and reduce VEGF [35].

Histopathological results of the present study confirmed that letrozole induced ovarian dysfunction, where ovaries of PCOS rats showed follicular cysts (Fig. 3B) and necrosis in the central portion of some corpus luteum (Fig. 4B). This result could be attributed to high luteinizing hormone (LH) level triggering the ovary to secrete androgens [30]. Moreover, the observed necrosis in the corpus luteum illustrates anovulation and irregular estrous cycle [30]. Obviously, treatment with metformin, *Ok*, and EA fraction reversed anomalies induced in the reproductive organs of rats following letrozole administration. Such results are evidenced by the presence of different stages of mature follicles with corpus luteum indicating ovulation and regular estrous cycle [16] following treatment with metformin (Fig. 3C), *Ok* extract (Fig. 3D), and (EA) fraction (Fig. 3E). Worth mentioning, earlier studies reported that rutin decreases the number of ovarian cysts by upregulating the expression of aromatase enzyme, thus reducing androgen levels [34] and restoring normal ovarian architecture [42].

Taken all together, results of the present study advocate the use of *Ok* extract and EA fraction in PCOS experimentally induced in rats. *Ocimum kilimandscharicum* amends the biochemical, vascular, and histological alterations associated with this syndrome. Results revealed the superior effects achieved by *Ok* extract and EA compared to metformin.

5. Conclusion

The current study is the first to introduce *Ocimum kilimandscharicum* as a potential treatment rather than just a symptomatic approach for PCOS. Improving the quality of life of PCOS sufferers would recuperate women self-image, mental health, and social status. Further research to enhance the feasibility of nutraceuticals-based treatment of PCOS is highly urged.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116640.

References

[1] R.J. Rodgers, L. Suturina, D. Lizneva, M.J. Davies, K. Hummitzsch, H.F. Irving-Rodgers, et al., Is polycystic ovary syndrome a 20th century phenomenon, Med. Hypotheses 124 (2019) 31–34 Mar.
[2] U.A. Ndefo, A. Eaton, M.R. Green, Polycystic ovary syndrome: a review of treatment options with a focus on pharmacological approaches, P T 38 (6) (2013) 336–355.
[3] R.L. Barbieri, M.D. Hornstein, Hyperinsulinemia and ovarian hyperandrogenism: cause and effect, Endocrinol. Metab. Clin. N. Am. 17 (4) (1988) 685–703 Dec.
[4] M.K. Moon, K.Y. Hur, S.H. Ko, S.O. Park, B.W. Lee, J.H. Kim, et al., Combination therapy of oral hypoglycemic agents in patients with type 2 diabetes mellitus, Korean J. Intern. Med. 32 (6) (2017) 974–983.
[5] H. Lashen, Role of metformin in the management of polycystic ovary syndrome, Ther. Adv. Endocrinol. Metab. 1 (3) (2010) 117–128.
[6] M. Kolivand, A. Keramat, A. Khosravi, The effect of herbal teas on Management of Polycystic Ovary Syndrome: a systematic review, J. Midwifery Reprod. Heal. 5 (4) (2017) 1098–1106.
[7] V. Singh, P. Krishan, R. Shri, *Ocimum kilimand scharicum* Guerke: phytochemical and pharmacological aspects: a review, Res. Rev. J. Pharmacogn. Phytochem. 2 (3) (2014) 1–11.
[8] V.T. de Lima, M.C. Vieira, C.A.L. Kassuya, C.A.L. Cardoso, J.M. Alves, M.A. Foglio, et al., Chemical composition and free radical-scavenging, anticancer and anti-inflammatory activities of the essential oil from *Ocimum kilimandscharicum*, Phytomedicine 21 (11) (2014) 1298–1302 Sep.
[9] A.S. Kauffman, V.G. Thackray, G.E. Ryan, K.P. Tolson, C.A. Glidewell-Kenney, S.J. Semaan, et al., A novel letrozole model recapitulates both the reproductive and metabolic phenotypes of polycystic ovary syndrome in female Mice1, Biol. Reprod. 93 (3) (2015).
[10] G.E. Ryan, S. Malik, P.L. Mellon, Antiandrogen treatment ameliorates reproductive and metabolic phenotypes in the letrozole-induced mouse model of PCOS, Endocrinology 159 (4) (2018) 1734–1747. Apr 1.
[11] B. Usluogullari, C.Z. Duvan, C.A. Usluogullari, Use of aromatase inhibitors in practice of gynecology, J. Ovarian Res. 8 (1) (2015) 4 Dec 25.
[12] H. Handoussa, N. Osmanova, N. Ayoub, L. Mahran, Spicatic acid: a 4-carboxygentisic acid from Gentiana spicata extract with potential hepatoprotective activity, Drug Discov. Ther. 3 (6) (2009) 278–286.
[13] U. Albus, Guide for the care and use of laboratory animals (8th edn), Lab. Anim. 46 (3) (2012) 267–268.
[14] V. Soumya, Muzib Yi, P. Venkatesh, A novel method of extraction of bamboo seed oil (*Bambusa bambos* Druce) and its promising effect on metabolic symptoms of experimentally induced polycystic ovarian disease, Indian J. Pharmacol. 48 (2) (2016) 162.
[15] M. Di Pietro, F. Parborell, G. Irusta, N. Pascuali, D. Bas, M.S. Bianchi, et al., Metformin regulates ovarian angiogenesis and follicular development in a female polycystic ovary syndrome rat model, Endocrinology 156 (4) (2015) 1453–1463.
[16] P.S. Reddy, N. Begum, S. Mutha, V. Bakshi, Beneficial effect of curcumin in letrozole induced polycystic ovary syndrome, Asian Pacific J. Reprod. 5 (2) (2016) 116–122. Apr.
[17] M.T. Michalczuk, C.R. Kappel, O. Birkhan, A.C. Bragança, M.R. Álvares-da-Silva, HOMA-AD in assessing insulin resistance in lean noncirrhotic HCV outpatients, Int. J. Hepatol. 2012 (2012) 1–7.
[18] Banchroft JD, SA and TD. Theory and Practice of Histological Techniques. Fourth Edition, Churchill Livingstone. New York, London, San Fr Tokyo. 1996.
[19] H. Handoussa, R. Hanafi, I. Eddiasty, M. El-Gendy, A. El Khatib, M. Linscheid, et al., Anti-inflammatory and cytotoxic activities of dietary phenolics isolated from *Corchorus olitorius* and *Vitis vinifera*, J. Funct. Foods 5 (3) (2013) 1204–1216 Jul.
[20] R.S. Sundaram, M. Ramanathan, R. Rajesh, B. Satheesh, D. Saravanan, LC-MS quantification of rosmarinic acid and ursolic acid in the *Ocimum sanctum* Linn. leaf extract (Holy basil, Tulsi), J. Liq. Chromatogr. Relat. Technol. 35 (5) (2012) 634–650 Mar.
[21] I. Chkhikvishvili, T. Sanikidze, N. Gogia, T. McHedlishvili, M. Enukidze, M. Machavariani, Rosmarinic acid-rich extracts of summer savory (Satureja hortensis L.) protect jurkat T cells against oxidative stress, Oxid Med Cell Longev. (2013) 9 https://www.hindawi.com/journals/omcl/2013/456253/.
[22] Claire E. Le, S. Schwaiger, B. Banaigs, H. Stuppner, F. Gafner, Distribution of a new rosmarinic acid derivative in *Eryngium alpinum* L. and other Apiaceae, J. Agric. Food Chem. 53 (11) (2005) 4367–4372.
[23] M.S. Afifi, A novel 4′-Odiglycoside of decarboxylosmarinic acid from *Blepharis ciliaris*, Pharm. Biol. 41 (7) (2003) 487–490.
[24] M.J. Simirgiotis, J. Benites, C. Areche, B. Sepu, Antioxidant capacities and analysis of phenolic compounds in three endemic nolana species by HPLC-PDA-ESI-MS, Molecules 20 (6) (2015) 11490–11507.
[25] A. Ahmad, M.F. Abuzinadah, H.M. Alkreathy, B. Banaganapalli, M. Mujeeb, Ursolic acid rich ocimum sanctum L leaf extract loaded nanostructured lipid carriers ameliorate adjuvant induced arthritis in rats by inhibition of COX-1, COX-2, TNF-α and IL-1: pharmacological and docking studies, PLoS One 13 (3) (2018).
[26] L.L. Saldanha, W. Vilegas, A.L. Dokkedal, Characterization of flavonoids and phenolic acids in *Myrcia bella* Cambess. using FIA-ESI-IT-MS(n) and HPLC-PAD-ESI-IT-MS combined with NMR, Molecules 18 (7) (2013) 8402–8416 Jul 16.
[27] H.E. Khoo, A. Azlan, A. Ismail, F. Abas, Antioxidative properties of defatted dabai pulp and peel prepared by solid phase extraction, Molecules 17 (8) (2012) 9757–9773.
[28] A. Brito, J.E. Ramirez, C. Areche, B. Sepúlveda, M.J. Simirgiotis, HPLC-UV-MS profiles of phenolic compounds and antioxidant activity of fruits from three citrus species consumed in Northern Chile, Molecules 19 (11) (2014) 17400–17421.
[29] M.E.P. Martucci, R.C.H. De Vos, C.A. Carollo, L. Gobbo-Neto, Metabolomics as a potential chemotaxonomical tool: application in the genus *Vernonia* Schreb, PLoS One 9 (4) (2014).
[30] A.A.Z. El-bahya, R.A. Radwanb, M.Z. Gadc, S.M. Abdel, A Closer Insight into the Role of Vitamin D in Polycystic Ovary Syndrome (Pcos), vol. 6(4), (2018).
[31] C.M. Uritu, C.T. Mihai, G.-D. Stanciu, G. Dodi, T. Alexa-Stratulat, A. Luca, et al., Medicinal plants of the family Lamiaceae in pain therapy: a review, Pain Res. Manag. 2018 (2018) 1–44 May 8.
[32] R.L. Rosenfield, D.A. Ehrmann, The pathogenesis of polycystic ovary syndrome (PCOS): the hypothesis of PCOS as functional ovarian hyperandrogenism revisited, Endocr. Rev. 37 (5) (2016) 467–520.
[33] R.K. Rajan, M.S.S. Kumar, B. Balaji, Soy isoflavones exert beneficial effects on letrozole-induced rat polycystic ovary syndrome (PCOS) model through anti-androgenic mechanism, Pharm. Biol. 55 (1) (2017) 242–251.
[34] H. Ullah, A. Almajwal, A. Mehboob, S. Razak, F. Munir, Q.U. Ain, et al., Ameliorative effects of rutin against metabolic, biochemical and hormonal disturbances in polycystic ovary syndrome in rats, J. Ovarian Res. 9 (1) (2016).
[35] U.J. Jung, Y.Y. Cho, M.S. Choi, Apigenin ameliorates dyslipidemia, hepatic steatosis and insulin resistance by modulating metabolic and transcriptional profiles in the liver of high-fat diet-induced obese mice, Nutrients 8 (5) (2016).
[36] Yokozawa T, Kim HY, Cho EJ, Choi JS, Chung and HY. Antioxidant effects of Isorhamnetin 3, 7-Di- O - D -glucopyranoside isolated from mustard leaf (*Brassica juncea*) in rats with streptozotocin-induced diabetes. J. Agric. Food Chem. 2002;50(19):5490–5.
[37] E. Diamanti-Kandarakis, A.G. Papavassiliou, S.A. Kandarakis, G.P. Chrousos, Pathophysiology and types of dyslipidemia in PCOS, Trends Endocrinol. Metab. 18 (7) (2007) 280–285.

[38] P. Peitsidis, R. Agrawal, Role of vascular endothelial growth factor in women with PCO and PCOS: a systematic review, Reprod. BioMed. Online 20 (4) (2010) 444–452.
[39] P.G. Artini, M. Ruggiero, M.R. Parisen Toldin, P. Monteleone, M. Monti, V. Cela, et al., Vascular endothelial growth factor and its soluble receptor in patients with polycystic ovary syndrome undergoing IVF, Hum. Fertil. 12 (1) (2009) 40–44.
[40] K. Eisermann, C.J. Broderick, A. Bazarov, M.M. Moazam, G.C. Fraizer, Androgen up-regulates vascular endothelial growth factor expression in prostate cancer cells via an Sp1 binding site, Mol. Cancer 12 (1) (2013).
[41] Y. Rojanasakul, Z. He, Y.C. Chen, B. Li, G.O. Rankin, Selecting bioactive phenolic compounds as potential agents to inhibit proliferation and VEGF expression in human ovarian cancer cells, Oncol. Lett. 9 (3) (2014) 1444–1450.
[42] T. Hu, X. Yuan, R. Ye, H. Zhou, J. Lin, C. Zhang, et al., Brown adipose tissue activation by rutin ameliorates polycystic ovary syndrome in rat, J. Nutr. Biochem. 47 (2017) 21–28.
[43] E. Salih, P. Fyhrquist, A. Abdalla, A. Abdelgadir, M. Kanninen, M. Sipi, et al., LC-MS/MS tandem mass spectrometry for analysis of phenolic compounds and pentacyclic triterpenes in antifungal extracts of *Terminalia brownii* (Fresen), Antibiotics 6 (4) (2017) 37.
[44] K. Ablajan, Z. Abliz, X.Y. Shang, J.M. He, R.P. Zhang, J.G. Shi, Structural characterization of flavonol 3,7-di-O-glycosides and determination of the glycosylation position by using negative ion electrospray ionization tandem mass spectrometry, J. Mass Spectrom. 41 (3) (2006) 352–360.
[45] M. Ye, W.Z. Yang, Liu K. Di, X. Qiao, B.J. Li, J. Cheng, et al., Characterization of flavonoids in *Millettia nitida* var. hirsutissima by HPLC/DAD/ESI-MSn, J. Pharm. Anal. 2 (1) (2012) 35–42.
[46] M. Ye, D. Guo, G. Ye, C. Huang, Analysis of homoisoflavonoids in *Ophiopogon japonicus* by HPLC-DAD-ESI-MSn, J. Am. Soc. Mass Spectrom. 16 (2) (2005) 234–243.
[47] R. Guimarães, L. Barros, M. Dueñas, A.M. Carvalho, M.J.R.P. Queiroz, C. Santos-Buelga, et al., Characterisation of phenolic compounds in wild fruits from Northeastern Portugal, Food Chem. 141 (4) (2013) 3721–3730 Dec.
[48] A.G. Bezerra, G. Negri, J.M. Duarte-Almeida, S.S. Smaili, E.A. Carlini, Phytochemical analysis of hydroethanolic extract of *Turnera diffusa* Willd and evaluation of its effects on astrocyte cell death, Einstein (São Paulo) 14 (1) (2016) 56–63.
[49] M.J. Simirgiotis, G. Schmeda-Hirschmann, J. Brquez, E.J. Kennelly, The *Passiflora tripartita* (banana passion) fruit: a source of bioactive flavonoid C-glycosides isolated by HSCCC and characterized by HPLC-DAD-ESI/MS/MS, Molecules 18 (2) (2013) 1672–1692.