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# Effect of Combined Treatment of Modern and Herbal Supplement in the Management of Letrozole Induced Polycystic Ovary Syndrome

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## 1 Abstract

Polycystic Ovary Syndrome (PCOS) has a prevalence of developing obesity, Type II diabetes, and cardiovascular disorder. These metabolic disorders and PCOS pathogenesis involve Oxidative Stress (OS) and low-grade inflammation. There are many modern drugs available for the treatment of PCOS, such as Clomifene Citrate (CC) but these drugs not work with above-mentioned etiologies of PCOS. Hence, treatment of PCOS required an herbal supplement to manage the OS and low-grade inflammation. Exudates of *Commiphora Mukul* (CM) having anti-inflammatory and antioxidant properties was used as an herbal supplement. The purpose of the study was to evaluate the protective effect of CM alone and in combination with CC in Letrozole (LTZ) induced PCOS rats. Letrozole induced PCOS rats showed a significant increase in testosterone level, High Sensitive C-Reactive Protein (HSCRP), interleukin-6 (IL-6), Lipid Peroxidation (LPO), significant decrease in the level of Superoxide Dismutase (SOD) and catalase. Combined treatment of CM and CC significantly decreases testosterone, HSCRP, IL-6, LPO and increases SOD and catalase. The combined treatment also induces ovulation and decreases number of cystic follicles. This study indicates the supplement of CM with CC have higher therapeutic value as compared to modern drug CC.

## 2 Keywords

*Oxidative stress, inflammation, polycystic ovary syndrome, Commiphoramukul, clomifene citrate, hyperandrogen*

## 3 Introduction

The Polycystic ovary syndrome (PCOS) is a hormonal disorder in women of reproductive age. Their clinical symptoms include menstrual abnormalities, polycystic ovary, obesity, infertility, hairy, acne, and hyperandrogenism. It is often accompanied by hyperlipidemia and insulin resistance [1]. Chronic low-grade inflammation and oxidative stress (OS) have emerged as a key contributor to the pathogenesis of PCOS [2]. A positive correlation has been reported between high sensitive C-reactive protein (hsCRP), insulin resistance (IR) and body weight in PCOS patients [3].

PCOS affected by many factors, but IR play important role in the manifestation of pathogenesis of this disease. A risk factor for developing diabetes, cardiovascular diseases, obesity, dyslipidemia are increasing in this syndrome [4].

Reactive oxygen species (ROS) and inflammation are important in the regulation of many physiological functions of female reproduction, such as development of oocyte, folliculogenesis, ovulation, and steroidogenesis [5].

There is a vice-versa relationship occurring in between inflammation and ovarian steroid hormone [6]. In PCOS chronic low-grade inflammation markers such as hsCRP, interleukin-18 (IL-18), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6) are elevated. Systemic inflammation occurs in obesity, but the level of circulating pro-inflammatory cytokines are also elevated in PCOS independent of obesity [7].

Glucose induced OS is found in mononuclear cell in polycystic ovary that leads to stimulation of the local inflammatory response which could induce more production of androgens by ovarian theca cells [8]. In some clinical study it was shown that ROS formations have a positive correlation with hyperandrogen production [9]. Insulin resistance and free fatty acid are also involved in production of osin body [10,11].

There are many modern drugs available for induction of ovulation and treatment of infertility in PCOS, such as Clomifene Citrate (CC). It is an anti-androgenic, non-steroidal estrogen agonist [12]. However, these modern medicines have some limitation

such as long-term treatment and side effects so there is a need of using herbal supplement with no side effects for management of PCOS. Commiphora mukul (CM) belongs to family Burseraceae. Its gumresin contains steroid, sterol, alcohols and esters. The main active compounds found in CM are guggulsterones Z and E [13]. C.mukul useful in the treatment of obesity, liver dysfunction, leucoderma etc. Its gum has anti-inflammatory, anti-arthritis activity and also used in the treatment of menstrual dysfunction and ovulation disorders [14,15]. Here the present study based on to find positive effect of gum resin of CM on the management of PCOS in isolation and in combination with CC.

## 4 Material and Methods

### 4.1 Animal and Experimental design

Institutional Animal Ethical Committee (letter no.-Dean/12-13/CAEC/15) approved the protocols of animal experiments. The animals purchased from the central animal facility of our institute. The 30 adult female albino rats (9 week old) of CF strain, weighing 100-110gm. With normal estrous cycle, randomly divided into 5 groups, each with 6 rats. All animals housed under controlled temperature (22 °C to 25 °C) with a relative humidity of 40% to 55% and 12 hours light and dark cycle with unlimited access to water and dry rat food. Group-1 was control animal receive 1% aqueous solution of Carboxy methyl cellulose (CMC) p.o. Once daily. Group-2 was received letrozole (LTZ) (dissolved in 1% aqueous solution of the carboxymethyl cellulose solution) at a concentration of 3mg/kg. The LTZ dose was selected based on a preliminary experiment in which rats were treated with different doses of LTZ ranging from 1-5mg/kg body weight. Significant changes were observed in rats treated with a minimum dose of 3 mg/kg body weight, so that dose was selected for further study. Group-3 was received LTZ and cm (40mg/100gm body weight), Drug dose was decided on the basis of separate toxicity experiment. This dose was safe for the animals and no toxicity found in biochemical parameters of liver and kidney (Table-1). Group-4 was treated with LTZ and cc (1mg/kg body weight) and group-5 was treated with LTZ (3mg/kg body weight), CC (1mg/kg body weight) and CM (40mg/100gm body weight). All doses were given p.o. Once daily.

The estrous cycle of each rat was monitored by daily examination of vaginal smear. The body weight of each rat was recorded at the start of the experiment and subsequently at 3 days interval till autopsy. Twenty-four hours after the last treatment, animals were euthanized and the serum was kept at -20°C for hscrp and test oster one assays. One ovary of each rat was fixed in bouin's fluid for histological study, whereas the other was stored at -40°C until used for biochemical and immune blot analysis.

Preparation of extract of C.mukul: Dried exudates of the bark of CM are known as "guggulu" in India. Gum-guggulu obtained from an ayurvedic pharmacy of BHU was air-dried. As guggul used as, an ayurvedic medicine from ancient time and its gum resin also have many clinical applications so we prepared water decoction of its gum resin. Plant doses were decided on the basis of toxicity experiment.

**Table 1** Effect of aqueous extract of Commiphora mukul on biochemical blood parameters of rats after 20-day oral administration

Parameters	Normal	Drug vector	C.mukul treatment (mg/100gm body weight)		
			20	40	80
Glucose (mg %)	63.8± 2.9	60± 4.0	61± 7.3	68± 4.5	65± 5.2
Aspartate aminotransferase (AST) (IU/L)	47.3± 2.6	45± 3.3	42± 3.8	35± 2.3	44± 4.1
Alanine aminotransferase (ALT) (IU/L)	50± 2.5	58± 3.2	53.7± 3.6	59.2± 4.5	61± 4.3
Triglyceride (mg%)	74± 3.8	72± 3.6	70± 4.5	65± 3.8	69± 6.1
Urea (mg%)	43.8± 2.2	40± 2.6	45.5± 1.8	39± 3.2	43.7± 2.9
Creatinine (mg%)	0.63± 0.03	0.44± 0.013	0.37± 0.02	0.40± 0.03	0.49± 0.02

The values are the mean ± S.D. of parameters measured.

### 4.2 Screening of vaginal smears

A cotton swab dipped in normal saline was inserted gently into the vaginal opening of the female rats. It was rolled on a clean grease-free glass-slide to make a smear and observed under a light microscope to assess the changes occurred in the regulation of the estrous cycle.

Histomorphometry of ovary: the ovaries fixed in bouin's fix at five were subsequently processed for histological study. Thin serial paraffin sections (5µm) of the ovary were stained with hematoxylin-eosin. Nikon microscope (Eclipse 50i, Japan) and Nikon imaging software examined follicular diameter, thickness of the granulosa and theca layers and changes in follicles. Different types (healthy antral, cystic, and atretic) of follicles were counted in every fifth serial sections of each ovary. The numbers of healthy antral, atretic, and cystic follicle at different duration of pcos development were compared with those in the control. Follicle showing features, such as hyper trophied granulosa cells, pyknotic cell nuclei in the granulosa cells and an abnormal oocyte were categorized as abnormal follicles. Histological changes were categorized and scored according to the severity of changes in the ovary: No changes = 0; mild = +; moderate = ++; and intense = +++.

### 4.3 Western blot analysis of IL-6 in ovarian tissue

The ovarian tissue was washed with ice cold PBS, and 10% homogenate was prepared in ice cold lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 1 mM sodium orthovanadate, protease inhibitor cocktail and 1 mM PMSF). The tissue lysate was centrifuged at 13,000 RPM (4°C) and supernatant were saved. The protein was estimated by Bradford method and the sample volume containing 60 µg proteins was loaded in each well along with tracking dye on SDS PAGE (sodium dodecyl sulphate polyacrylamide gel). In one lane, protein molecular weight marker was loaded. The electrophoresis

was run in the cold room. Finally, the separated proteins were electro-transferred from gel to PVDF membranes as described earlier [16].

The membrane was blocked with 3% BSA (Bovine Serum Albumin) in TBST (Tris buffer saline tween-20, for 4-5 hours at room temperature (RT). The blot was then incubated overnight at 4°C with primary antibody IL-6 (1:1000) in TBST. Next day, this blot was extensively washed with TBST (5 washes, each of 5 min) and incubated for 2 hrs with secondary antibody at dilutions (1:1000) in TBST at RT. Proteins were detected by the enhanced chemiluminescence (ECL) system (Millipore India Pvt. LTD) in LAS500- imagequant (GE healthcare, hongkong) and the immune blot was quantified by Alpha imager. The blot was stripped and re-probed with housekeeping gene  $\beta$ -actin (1:1000) in a similar manner.

#### 4.4 High sensitive C-reactive protein and testosterone determination

Serum hscrp, was estimated by ELISA kit (Diagnostics Biochem Canada) based on method [17]. Control, standard and blank tubes were run in parallel with the serum sample. Intra-assay coefficient of variation was found to be less than 12%. Testosterone, was quantitatively determined by chemiluminescent micro particle immunoassay (Architect Testosterone Reagent kit, Abbott Ireland) [18].

#### 4.5 Superoxide dismutase activity

Total (Cu-Zn and Mn) superoxide dismutase (SOD) activity was determined as per method mccord with slight modification [19]. One unit of SOD is defined as the enzyme activity causing 50% inhibition in formazone formation. The SOD activity was expressed as units/mg protein.

#### 4.6 Catalase activity

Catalase activity was determined according to Aebi's method with slight modifications [20,21]. It was determined in terms of rate of H<sub>2</sub>O<sub>2</sub> decomposition, measured at 240 nm. The catalase activity was expressed as U/mg protein.

#### 4.7 LPO product level

The products of LPO were determined in terms of Thiobarbituric acid reactive substances (TBARS), with slight modification [22,19]. The lipid peroxides and Thiobarbituric acid react, when kept for 40 min in boiling water bath. It produces pink pigment with absorption maximum of 532 nm. The results were expressed as nmol/mg protein of according to standard graphics, which were prepared with serial dilutions of standard 1,1,3,3 tetramet hoxyp propane.

#### 4.8 Estimation of biochemical parameters

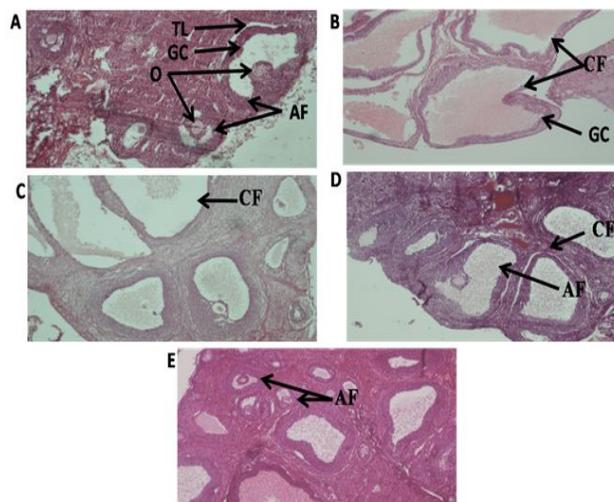
Blood glucose, Aspartate aminotransferase (AST), Alanine Aminotransferase (ALT), Triglyceride, Urea and Creatinine were estimated by using biochemical kit (Accurex biomedical Pvt. Ltd, Mumbai).

#### 4.9 Statistical analysis

All data expressed as mean  $\pm$  standard error mean. Data were analyzed by one way annova analysis of variance followed by tukey hsdposthoc test. Correlation studies were performed to compare the data from different groups. SPSS software 20 for windows (SPSS, Chicago, IL) was used for statistical analysis. The data were considered significant if  $p < 0.05$ .

### 5 Results

Effect of cm treatment on body weight and ovarian weight: Letrozole and LTZ+CC treated rats gained more weight as compared to control group, but animals treated with LTZ+CM, LTZ+CC+CM didn't gain significant ( $p < 0.001$ ) weight as compared to control animals (Table-2). The group treated with LTZ gained higher ovarian weight but other groups also gained more ovarian weight as compared to control animals. LTZ+CC+CM treated animal having minimum ovarian weight as compared to other groups (Table-3). Effect of cm treatment on Histo-



**Figure 1:** Effect of Combine drug treatment on ovarian histology of Rats (n=6/group):

(A) Transverse section of ovary of control rat showing mature follicle with oocyte.

(B) Transverse section of ovary from rat treated with LTZ showing cystic follicles.

(C) Transverse section of ovary from rat treated with LTZ+CM showing cystic follicles.

(D) Transverse section of ovary from rat treated with LTZ+CC showing cystic follicles and Antral follicles.

(E) Transverse section of ovary from rat treated with LTZ+CC+CM showing Antral follicles. (Magnification  $\times 10$ )

AF- Antral follicles, CF- Cystic follicles, GC- Granulosa cell, TL- Theca cell layer, O-Oocyte

LTZ- letrozole, CM- Commiphora mukul, CC- Clomifene citrate

morphometry of the ovary: Ovaries of LTZ treated rats had increased numbers of atretic and cystic follicles. Follicles having a thin layer of granulosa cells and filled with follicular fluid were characterized as cystic follicles. In LTZ treated ovary, there was a clear absence of mature follicles and corpus luteum. In CM

**Table 2** Changes in body mass, follicular diameter and thickness of granulosa and thecal layers in the ovary of control and treated rats (n=6/group).

Parameters	Control	Only LTZ	LTZ+ CM	LTZ+ CC	LTZ+ CC+ CM
Body weight (g)	107.9 ± 5	153.4 ± 12.90 ***	120.5 ± 4.78 ***	133.41 ± 6.45 ***	124.90 ± 7.68 ***
Ovary weight (g)	0.055 ± 0.0034	0.082 ± 0.0035 ***	0.072 ± 0.0049 ***	0.075 ± 0.0037 ***	0.068 ± 0.005 ***
Antral follicles	6.75 ± 1.1	0 ***	1.5 ± 0.10 ***	3.43 ± 0.98 ***	4.75 ± 1.07 ***
Cystic follicles	0	8.5 ± 1.67 ***	5.1 ± 1.28 ***	4.25 ± 1.12 ***	4.18 ± 1.89 ***
Follicular diameters (µm)	267.54 ± 36.56	397.35 ± 53.28	312.17 ± 41.76 ***	298.11 ± 21.66	274.11 ± 36.25
Granulosa thickness (µm)	63.18 ± 3.98	47.35 ± 7.47 *	55.17 ± 8.11*	59.46 ± 5.97	61.66 ± 6.28
Thecalayer thickness (µm)	37.14 ± 3.35	28.71 ± 4.25 *	35.16 ± 5.17	34.73 ± 3.15	36.19 ± 2.98
Vascularisation of follicular wall	-	+++	++	++	++
Disintegration and dispersion of granulosa cell	-	+++	++	+	+

The values are the mean ± S.D. of parameters measured. Differences between control and treated groups: \*p<0.05, \*\*\*p<0.001, - No changes= 0 ; mild = + ; moderate = ++ ; and intense = +++

**Table 3** Correlation coefficient (r-value) for various parameters of control and Letrozole treated rats. Serum hsCRP, testosterone, SOD, catalase and LPO correlated with SOD, catalase, LPO, IL-6 and cystic follicles of ovary.

Parameters	SOD in ovary	CAT in ovary	LPO in ovary	IL-6	Cystic follicle
hsCRP	-0.229	-.450*	.774**	.822**	.934**
Testosterone	-.575**	-.600**	.697**	.839**	.716**
SOD in blood	.551*	.702**	-.714**	-.873**	-.794**
Catalase in blood	.583**	.735**	-.729**	-.878**	-.777**
LPO in blood	-0.339	-.489*	.937**	.856**	.857**

\*\*Correlation is significant at the 0.01 level (2-tailed).

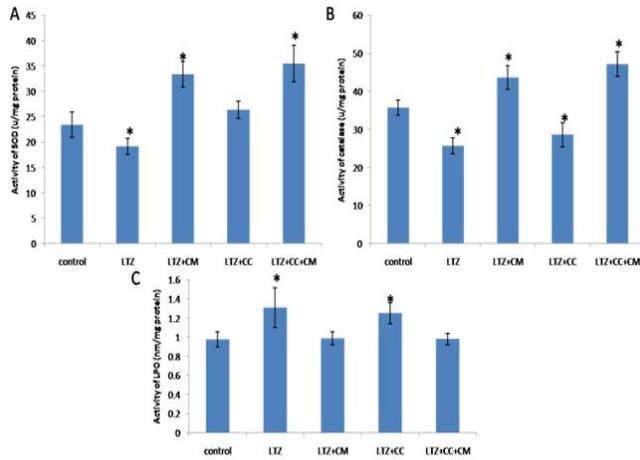
\*Correlation is significant at the 0.05 level (2-tailed).

treated rats, there was a less number of ovarian atretic and cystic follicles as compared to LTZ treated group. The group treated with clomiphene citrate also developed cystic follicles, but in this group, more number of healthy follicles found as compared to LTZ+CM group. Treatment with LTZ+CC+CM improve all the histological features of ovary and developed more antral follicles as compared to CM and CC alone treatment (Figure-1). Effect of cm treatment on superoxide dismutase and catalase activity and lipid peroxide product level: The activity of LPO was higher in both ovarian tissue and in blood plasma of LTZ treated group as compared to control and other treated group. LTZ+CC+CM and LTZ+CM treated groups showed significant (p < 0.001) decrease in level of LPO product as compare to LTZ+CC group. SOD and catalase enzymes were decreases in LTZ treated group as compared to control. LTZ+CC+CM treatment and LTZ+CM treatment groups were not shown significant (p<0.001) difference in SOD, catalase and LPO activity as compared to control (Figure-2,3).

Effect of CM treatment on ovarian expression of interleukin-6 (IL-6) protein and high sensitive C-reactive protein (hsCRP): The LTZtreated rats had increased serum level of hsCRP as compared to control, whereas LTZ+CC+CM and LTZ+CM treatment significantly (p < 0.001) reduced level of hsCRP in serum as compared to LTZ+CC treatment (Figure-4). Ovarian expression of IL-6 was increased in LTZ treated group whereas it decreases both in LTZ+CM and LTZ+CC+CM treated groups (Figure-5). Effect of cm treatment on serum testosterone level: Serum testosterone level was significantly (p<0.001) high in LTZ treated group as compared to control where as it became normal in LTZ+CC, LTZ+CM and LTZ+CC+CM treated groups (Figure-4).

## 6 Discussion

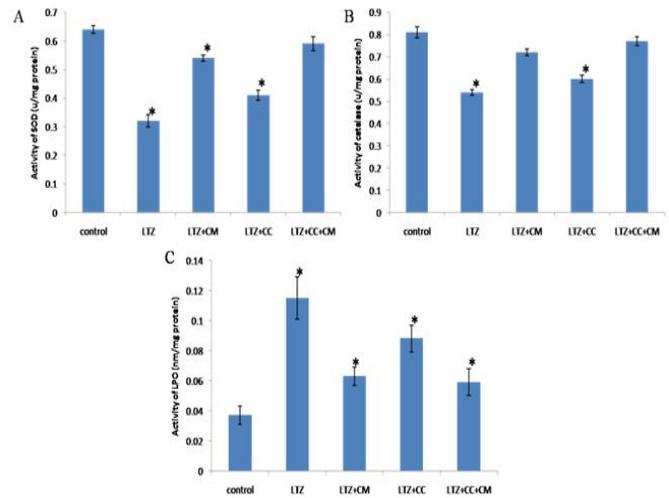
In this study, we showed the therapeutic approach of combined treatment of herbal supplement and modern medicine in



**Figure 2:** Change in the activity of superoxide dismutase (SOD) (A), catalase (B) and lipid peroxidation (LPO) (C), in ovarian homogenate of control and treated rats (n=6/group). The values are the mean  $\pm$  SEM of parameters measured. Difference between control and treated groups is significant :(\*p<0.001.)  
LTZ- letrozole, CM- Commiphora mukul, CC- Clomifene citrate

the PCOS rat model. Letrozole animal model of PCOS was characterized by an increase in androgen biosynthesis. It increases serum testosterone concentration due to inhibition of aromatase enzyme activity. Ovaries treated with letrozole shows large follicular cyst, less or no corpus luteum, thin granulosa cells and thickened theca cell layers [23]. Due to blocking of aromatase enzyme activity, secretion of ovarian androgen increases, which resulted into high levels of testosterone, LH and FSH. Letrozole treatment also showed some metabolic features resemble to human PCOS, such increased body weight, body fat, cholesterol and triglycerides.

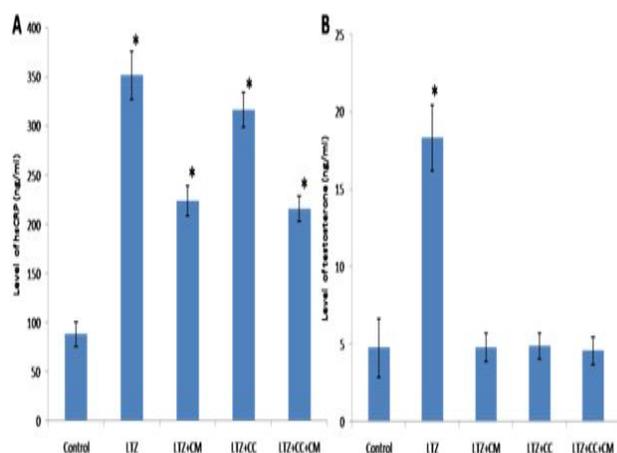
In case of PCOS, number of atretic primordial follicles, atretic growing follicles, atretic graafian follicles, cystic follicles increases as compared to normal ovary so proliferation of granulosa cells increases but disintegration and dispersion of granulosa cell, decreases granulosa cell layer thickness around cystic follicles [24,25]. This is a characteristic feature of PCOS. As granulosa cells convert androgen to estradiol with the help of the aromatase enzyme. As already discussed that letrozole is a aromatase inhibitor, thus administration of letrozole, increased the concentration of androgen, which may be the cause of increased number of atretic follicles in ovary [26]. Detachment of granulosa cells is one of the morphological characteristic features of follicular atresia. It also showed complete an ovulation and significant increase in weight of the ovary. Formation of cystic follicles is positively correlated with hyperandrogenism, low-grade inflammatory marker and OS markers. Letrozole treated group showed elevated level of inflammatory marker in both serum (hsCRP) and ovary (IL-6). There is a positive relationship between inflammatory markers (IL-6, hsCRP) and hyperandrogenism. Our result is consistent with other studies in which level of TNF- $\alpha$  correlated with hyperandrogenism [25].



**Figure 3:** Change in the activity of superoxide dismutase (SOD) (A), catalase (B) and lipid peroxidation (LPO) (C), in blood of control and treated rats (n=6/group). The values are the mean  $\pm$  SEM of parameters measured. Difference between control and treated groups is significant :(\*p<0.001.)  
LTZ- letrozole, CM- Commiphora mukul, CC- Clomifene citrate

Oxidative stress also increased in LTZ treated group and it was evidenced by significantly increased level of LPO product. Some earlier reports suggest that LTZ increases testosterone by inhibiting estradiol, which protect granulosa cell of ovary by oxidative stress mediated flaws [27]. Antioxidant enzymes (SOD, catalase) activity also decreases in LTZ treated rats.

Clomifene citrate (CC) is a modern drug used for ovulation induction. It is a non-steroidal compound and similar to estrogen. By blocking the estrogen receptor of hypothalamus this triggers the release of follicle steroid hormone (FSH) and luteinising hormone (LH) from the pituitary gland. Rats treated with CC shown reverse morphological features induced by LTZ. Its treatment significantly decreases the numbers of cystic follicles in ovary and increase numbers of healthy antral follicles in ovary. These rats also showed increases in thickness of granulosa and theca cell layer. Treatment of CC also reverses the serum testosterone level as compare to the LTZ treated group. But CC was not improved the low-grade inflammation and oxidative stress induced by LTZ administration. The level of serum hsCRP and expression of IL-6 in the ovary was not showing significant difference in CC treated and LTZ treated group. Level of LPO product and activity of SOD and catalase were also not significantly differing in between CC treatment and LTZ treatment. Some studies also suggest that CC induces generation of ROS. Its treatment increases hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) formation and reduced catalase activity in the ovary [28]. Thus treatment of CC was not showing any effect on biochemical parameters. Herbal supplement (C.mukul) showed a positive effect on all above discussed parameters. C.mukul treatment reduces the adverse effect induced by LTZ and improved histological parameters, but ovarian histological features had much better improve in modern drug treated group. Its treatment also normalize the level of serum testosterone as obtained in the CC



**Figure 4:** Changes in testosterone (A) and hsCRP (B), in serum of control and treated rats at different time intervals (n=6/group). The values are the mean  $\pm$  SEM of parameters measured. Difference between control and treated groups is significant: (\* $p < 0.001$ ).

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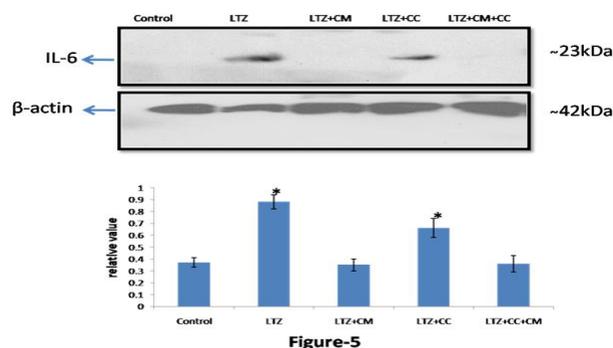
treated group and significantly decreases in the level of serum hscrp and ovarian IL-6 protein expression. Rats treated with CM also showed low level of LPO product and increase in activity of SOD and catalase hence decreases the low-grade inflammation and oxidative stress comparatively better as found in the CC treated group.

C.mukul has anti-inflammatory effect and also has antioxidant potential [13]. The active compound found in the gum resin of CM is z-guggulsterone. It regulates the expression of different transcription factors such as NF- $\kappa$ B, STAT-3 and also regulates androgen receptor and glucocorticoid receptor [29]. The pathway of low-grade inflammation and oxidative stress are also linked by NF- $\kappa$ B. Z-guggulsterone down-regulated the NF- $\kappa$ B which decreases the expression of Bcl-2, MMP-9, COX-2 and VEGF [30]. It inhibits NF- $\kappa$ B signalling pathway and release of proinflammatory cytokines (TNF- $\alpha$ , IL-6) [31]. Z-guggulsterone also suppresses lipid peroxidation and the formation of free radicals [32]. Some previous studies also showed that Z-guggulsterone decreases lipid peroxidation and increases the superoxide dismutase activity [33].

On giving combined treatment of CM and CC showed better histological features. Serum testosterone level was also found lowest in combined treatment of both herbal supplement and modern medicine. Markers of low grade inflammation and OS were also at the lowest level as compared to individual treatment of CC and CM.

## 7 Conclusion

There was a link between hyperandrogenism, local and systemic inflammation, and oxidative stress. C.mukul had a positive effect on the management of oxidative stress, inflammation, and hyperandrogenism in PCOS. Combined therapy of the herbal supplement with modern allopathic medicine showed great potential



**Figure 5:** Western blot analyses of interleukin-6 (IL-6) in ovary of control and treated rats (n = 6/group).

The values are the mean  $\pm$  SEM of parameters measured. Difference between control and treated groups is significant: (\* $p < 0.001$ ).

LTZ- letrozole, CM- Commiphora mukul, CC- Clomifene citrate

to treat the PCOS manifestation.

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