Ameliorative effects of Anacardic acid in letrozole-induced rat model of PCOS

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Abstract

The present study was designed to evaluate the Pharmacological effects of Anacardic acid in Letrozole induced polycystic ovary syndrome in female Albino wistar rats. Rats were administered with Letrozole (1mg/kg in 0.5% CMC, p.o.) for 21 days to induced PCOS characterized by hyperandrogenism, dyslipidemia and development of numerous subcapcular cysts in the ovaries. Letrozole treated animals showed significant decrease in serum progesterone, Estradiol, FSH, and LH Levels, antioxidants like GSH, Catalase, TBARS, and ovarian weights. After 21 days PCOS induced rats were administered with [Low dose (10mg/kg) and high dose (40mg/kg), p.o.] of Anacardic acid for next 15 days. Anacardic acid treatment decreased testosterone normalized anti-oxidant levels and also reduced cysts in ovaries and induced ovulation. These results suggest that Anacardic acid attenuates PCOS through maintaining sex hormones levels, antioxidant activities.

Key words: *Anacardic acid, Letrozole, PCOS and Dyslipidemia.*

1.Introduction

The polycystic ovarian syndrome is the most common heterogeneous endocrine disorder affecting 5–10% of women of reproductive age with hyperandrogenism, hyperinsulinemia, low-grade systemic inflammation, and polycystic ovaries[14]. It involves multisystem endocrinopathy with interlinked metabolic disturbances. Ovulatory dysfunction, enlarged polycystic ovaries, and hyperandrogenism characterize the syndrome. Although the exact etiology of the syndrome is still unclear, evidence suggests a clear connection between PCOS and metabolic disturbances such as insulin resistance, obesity, and type 2 diabetes [15]

Change in habits of life, surgery, and medication are the followed therapies of PCOS. The most recognized medications are Clomiphene citrate, metformin and oral contraceptives are used to induce sex hormone-binding globulin (SHBG) production and increase free

testosterone binding, consequently reducing the symptoms of high testosterone hirsutism, and inducing a return to normal menstruation[3] . In addition, Metformin is a commonly used treatment for type 2 diabetes to reduce insulin resistance and is used worldwide as an insulin resistance treatment for PCOS[6,12]. Various short term symptomatic therapies are being used to manage the syndrome but these therapies are associated with several adverse effects from mild to severe side effects such as mood swing, arthritis, breast tenderness, weight gain irregular bleeding, bloating, fatigue, sexual dysfunction, osteoporosis, hot flashes, cardiovascular and thromboembolic problems, hepatic toxicity, etc[4]. The best and most effective curative treatment is still an unmet medical need. Therefore, searching for a drug from natural sources which shows no or minimal side effects is a priority.

Anacardic acid (AA) is a bioactive phytochemical found in a nutshell of *Anacardium occidentale*. Chemically, it is a mixture of several closely related organic compounds, each consisting of salicylic acid substituted with an alkyl chain. The traditional Ayurveda depicts nutshell oil as a medicinal remedy for, amebicidal, gingivitis, malaria, and syphilitic ulcers, as a therapeutic agent in the treatment of the most serious pathophysiological disorders like cancer, oxidative damage, inflammation, and obesity. Furthermore, AA was found to be a common inhibitor of several clinically targeted enzymes such as NFkB kinase, histone acetyltransferase (HATs), lipoxygenase (LOX-1), xanthine oxidase, tyrosinase, and ureases[10,16].

2. Materials and methods

2.1. Animals and experimental procedure

Female Albino Wistar adult rats weighing between 150-200 g were used for the study. Animals were procured from Tina laboratories, Kukatpally, Hyderabad and were maintained under standard laboratory i.e. temperature of 22 ± 3°c, humidity 55±5% and a 12 light/dark cycle. They were fed with standard diet and water provides ad labium. Experiments were carried out with strict compliance to ethical principles and guidelines provided by committee for the purpose of control and supervision of Experiments on Animals (CPCSEA). Approval was taken from institutional Animal Ethical committee in school of pharmacy, Anurag Group of Institutions, Venkatapur, Ghatkesar Hyderabad, prior to the experiments[9,11,19] (I/IAEC/LCP/04/2018/WR-30).

2.2. Drugs and reagents

All the drugs and chemicals used were of analytical grade. Letrozole was obtained from Natco pharma Limited, Hyderabad. Clomiphene citrate was obtained from fertile super, Ar-Ex Laboratories Pvt.Ltd. Rat testosterone ELISA kit, estrogen kit, FSH kit, LH kit and triglyceride kit were obtained from sincere biotech, Beijing, China.

2.3 . PCOS induction

All the experimental animals except control group, were orally administered with Letrozole at a dose of 1 mg/kg dissolved in 0.5% Carboxy Methyl Cellulose (CMC) once daily for 21 days[9]. Control group received vehicle only (0.5% CMC). Vaginal Smears were collected daily and evaluated microscopically using Giemsa stain to confirm the induction of PCOS.

2.4 Study design

The study consisted of 30 female Albino Wistar rats equally divided into five groups designated as group 1 (served as control group), group 2 (served as PCOS induced group), group 3 (served as standard group), groups 4 and 5 served as treatment groups. Following Letrozole administration, standard group was administered with Clomiphene Citrate at a dose of 1 mg/kg in 0.5% CMC per oral and treatment groups 4 and 5 were administered Anacardic acid with the dose of 10 mg/kg (Low dose) and 40 mg/kg (High dose) per body weight respectively in 0.5% CMC per oral for 15 days i.e., from day 22 to day 36.

2.5 Blood sampling

After 21 days, PCOS control group and after 36 days, animals from other groups were fasted overnight and anaesthetized with diethyl ether. Blood was collected by retro orbital puncture then serum was separated by centrifugation and was used for estimation of hormones, glucose and lipid parameters.

2.6 Tissue collection and ovarian morphometry

Uteri and ovaries were dissected out from all the animals and freed from extra fats. Weight of ovaries, their diameter and ovarian organ index were recorded. Ovarian tissues were serially dehydrated in graded ethanol and xylene. Specimens were embedded in paraffin block and sections of approximately 5 µm thick were cut and stained with hematoxylin and eosin stain and visualized under light microscope. One ovary from each animal was fixed in 10% formalin for histological study.

2.7 Serum hormone analysis

Serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), Serum testosterone (TET) levels 17β-estradiol (EST) levels were measured using ELISA Kit. All hormone levels were measured according to the supplier's instructions.

2.8 Lipid profile

Triglycerides (TG), low density lipid cholesterol (LDL) and high density lipid-cholesterol (HDL) were estimated using enzymatic kits procured from Sincere Biotech Co., Ltd, China.

2.9 Determination of superoxide dismutase activity

Superoxide dismutase (SOD) was measured by the procedure as described earlier[13]. Cocktail containing sodium carbonate (1.0 mL, 50 mM), nitro blue tetrazolium (0.4 mL, 25 µm) and hydroxylamine hydrochloride (0.1 mM of 0.2 mL) that was freshly prepared. The reaction mixtures were mixed by turning the tubes upside down several times followed by the addition serum sample (0.1 mL, 1:10 v/v). The change in absorbance of samples was monitored at an optical density 560 nm.

2.10 Estimation of catalase

Catalase (CAT) was measured by the procedure as described elsewhere[17]. Reaction mixture (1.5 mL) containing 0.01 M pH 7.0 phosphate buffer (1.0 mL), serum sample (0.1 mL) and hydrogen peroxide (H2O2; 2 M, 0.4 mL). The reaction was arrested by the adding

dichromate-acetic acid reagent (2.0 mL, 5% potassium dichromate and glacial acid mixed in the ratio of 1:3). The optical density was measured at 620 nm and the activity was expressed as moles of H2O2- consumed/min/mg protein.

2.11 Statistical analysis

The results among different groups were analyzed by using one-way ANOVA, with Tukey multiple comparison tests. Statistical difference was considered significant at $p < 0.05$.

3. Results

3.1 Body, ovary and uterine weight in the experimental groups

All rats in all groups completed the experimental procedure. At first, the body weights of all rats were measured on the first and last day of the experiment because the increase in body weight is considered one of the most important clinical characteristics of PCOS. Our results showed that letrozole administration resulted in an increase in the final body weight in the PCOS group ($P < 0.001$ Fig. 1a). Rats which received anacardic acid (AA) showed reduced body weight compared to the PCOS group. However, they were not statistically significant.

In addition to body weight, ovary and uterine weight were also monitored in all groups. In the PCOS group, the ovary weight significantly increased as compared to control ($P < 0.001$). AC administration decreased ovary weight (Fig. 1b). These differences were not significant statistically. Besides, by measuring uterine weight, we found that rats with letrozole-induced PCOS had significantly reduced uterine weight ($P < 0.05$; Fig. 1c). On the other hand, AA led to an increase in the uterine weight up to normal values ($P < 0.05$). In brief, the AA as a therapeutic option was effective in reversing the letrozole induced adverse alterations in uterine weight in rats with PCOS.

3.2 Effects of AA on Plasma Hormonal Changes

The hormone profile of experimental groups was assessed by measuring serum levels of testosterone, estrogen, progesterone LH, FSH and LH/FSH ratio. The PCOS induction by letrozole resulted in a significant increase in serum levels of testosterone LH, FSH and a significant decrease in estrogen and progesterone serum levels in the rats which received letrozole compared to the controls (tab 1; $P < 0.001$). On the other hand, the treatment with AA attenuated the letrozole-induced changes in hormonal levels, including reducing testosterone LH, FSH levels and elevating estrogen and progesterone in the AA group when compared to the PCOS group ($P < 0.001$).

3.3 Effects of AA on Lipid profile

The serum levels of TG, LDL and HDL as main lipid profile parameters, were measured in all groups. Our results showed that in rats with letrozole-induced PCOS, the serum concentrations of TG, and LDL were significantly higher when compared to controls, which approved disturbed lipid profile in PCOS ($P < 0.001$; tab 2). The administration of AA effectively decreased these two parameters compared to the PCOS group ($P < 0.001$). Besides, letrozole also led to a significant decrease in HDL serum levels in rats with PCOS, and treatment with AA restored HDL levels to normal control values.

3.4 Effects of AA on antioxidans

The serum levels of antioxidant parameters SOD and Catalase, were measured in all groups. Our results showed that in rats with letrozole-induced PCOS, the serum concentrations of SOD and Catalase were significantly declined when compared to controls ($P < 0.001$; tab 2). The administration of AA effectively increased these two parameters compared to the PCOS group ($P < 0.001$).

3.5 Effects of AA on Histopathology

The histological analysis of the ovarian section from control group showed normal cortex and medulla. Cortex shows numerous healthy growing follicles and a follicle with oocyte, healthy granulosa and theca layer, well developed corpus luteum with uniform round cells and eosinophilic cytoplasm. Negative control group shows multiple follicular cysts, diminished granulosa layer, vacuolated granulosa and stroma (fig 2). Histopathological changes after treatment with AA shows developing healthy follicles, Healthy stroma, normal granulosa and theca were observed. Thickened numerous blood vessels with congestion was observed. Standard control group also shows formation of oocyte, healthy stroma, granulosa and theca, well developed corpus luteum along with some cystic follicles.

4. Discussion

The present study was undertaken to evaluate the effect of Acridic acid (AA) against letrozole induced PCOS in female Wistar rats. The efficacy of letrozole (an aromatase inhibitor) in establishing PCOS in rats is well documented. Letrozole-received animals showed the characteristics phenotype of PCOS similar to human phenotype, including increased body and ovarian weight, decreased uterine weight, disrupted estrous cycle, increased cystic follicles and decreased corpus luteum, dysregulated lipid profile, elevated testosterone levels, and reduced estradiol and progesterone levels. In contrast with normal control rats, letrozole administration expanded the diestrous condition, indicating that the model imitated anovulation, which is typical of PCOS.

In women with PCOS, higher levels of LH and an increased LH/FSH ratio increase the production of androgens and lower the FSH, resulting in endocrine disruption of intra follicles due to the inefficient aromatization of estrogens[8].As a high LH/FSH ratio severely impairs follicle development and results in anovulation, the production of follicular cysts, and irregular estrous cycle and infertility[8] , it is necessary to control the concentration of LH and the level of LH/FSH ratio when designing therapeutics for PCOS[8,11,19]. Letrozole is a nonsteroidal drug that interfered with aromatase, an enzyme necessary for the synthesis of estrogen that converts androgens to estrogen[9]. Therefore, we used Letrozole-induced PCOS rats as an animal models with reproducible and metabolizing properties similar to women's PCOS.The present study examined the level(s) of LH and FSH, and the LH/FSH ratio in the serum of Letrozole-induced rats. Elevated level(s) of LH and LH/FSH significantly decreased following treatment with AA. In addition, a decrease in estrogen level(s) following treatment with Letrozole was observed, and its reduction was restored by AA treatment in Letrozole-induced rats. In contrast to estrogen, the serum level of testosterone increased in Letrozole-induced rats, and this was changed by AA treatment. As shown in another study, plasma

hormonal levels of letrozole induced rats showed similar tendencies, including increased LH and testosterone levels and decreased estrogen levels, except for FSH.

Dyslipidemia is common in PCOS characterized by elevated TGs and LDL. Dyslipidemia in PCOS occurs independent of BMI. However, obesity and insulin resistance in PCOS has synergistic harmful effect that is equivalent to that seen in type-2 diabetes mellitus. In PCOS, dyslipidemia has multiple causes. Letrozole administration increased the circulating lipid parameters including TG and LDL in PCOS and lowered the HDL levels. The AA treatment provided strong evidence of its anti-hyperlipidemic effects by reversing the dyslipidemic status in the group of letrozole induced PCOS rats. In the current study AA effectively reduced the lipid profile in rodents with hyperlipidemia complications[7].

It has been reported that the antioxidant enzymes decline in patients with PCOS[1,2]. In several studies, oxidative stress has been established as one of the major PCOS pathologic factors[18]. Enhanced oxidant levels can alter the stereo diagnosis in ovaries, that ultimately leads to increased production of androgen and polycystic ovaries. In the current study, the letrozole induced PCOS rats showed higher oxidative stress markers. Letrozole administration to rats significantly declined the SOD, CAT activity in the group of PCOS rats and subsequent treatment with AA regained their activities. Lipid peroxidation is probably considered one of the biggest markers for oxidative tissue damage, since it provokes free radical damage to the cell membrane components resulting in cell necrosis and inflammation.

In this study, we evaluated the effects of AA in the ovarian histology of Letrozole-induced rats. In the Letrozole-induced rats, the number of cystic follicles significantly decreased following AA treatment in the rats with PCOS. The administration of AA resulted in the effective improvement of PCOS-like symptoms, i.e., a thinned granulosa cell layer, thickened theca cell layer, and decreased number of antral follicles. The cell–cell interactions, granulosa, and theca, are essential for the development of regular follicular structures and maintenance of their functions. Furthermore, the hyperproliferation of the internal and/or external theca is a major cause of ovarian dysfunction conditions including PCOS[5,6].

5. Conclusion

In conclusion, AA showed many beneficial effects similar to Clomiphene citrate in treating PCOS conditions and inducing ovulation. It restored the hormone and lipid profile, antioxidant and glycemic status as well as ovarian morphology in Letrozole induced PCOS animals. These effects may be ascribed to its multiple pharmacological activities like estrogenic, antihyperlipidemic, antioxidant, and hypoglycemic effects which could be useful in managing PCOS conditions and preventing ovarian cell dysfunction, ovulation and thereby improving fertility. Together broad-spectrum biological effects of anacardic acid make it a promising drug for treating clinical and pathological abnormalities in PCOS conditions.

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Figures & Tables

Figure 1a

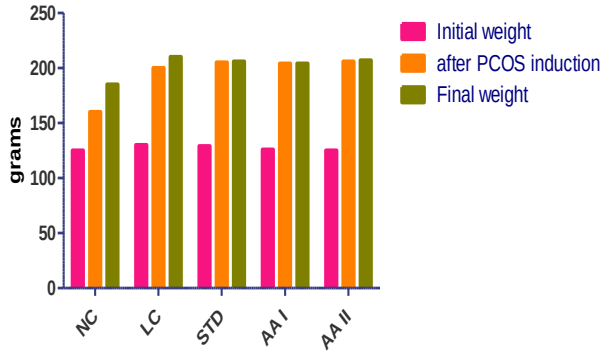


Figure 1b

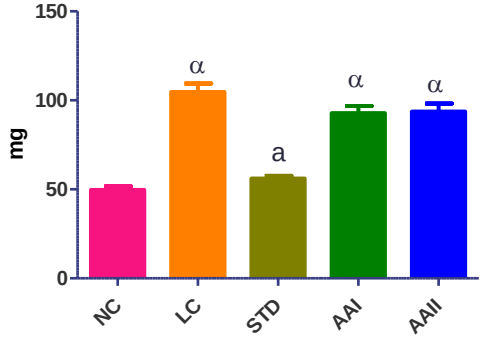
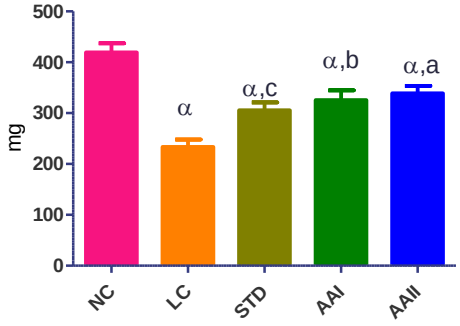


Figure 1c



Effect of anacardic acid on a. body weight, b. ovaries and c. uterus. NC: CMC; LC: Letrozole; STD: Clomiphene citrate; AAI: Anacardic acid 10 mg/kg; AA II: C Anacardic acid 40 mg/kg; Data presented as Mean ± SEM (n=6). $^{\alpha}P<0.001$ compared with NC ; $^{a}P<0.001$ compared with LC.

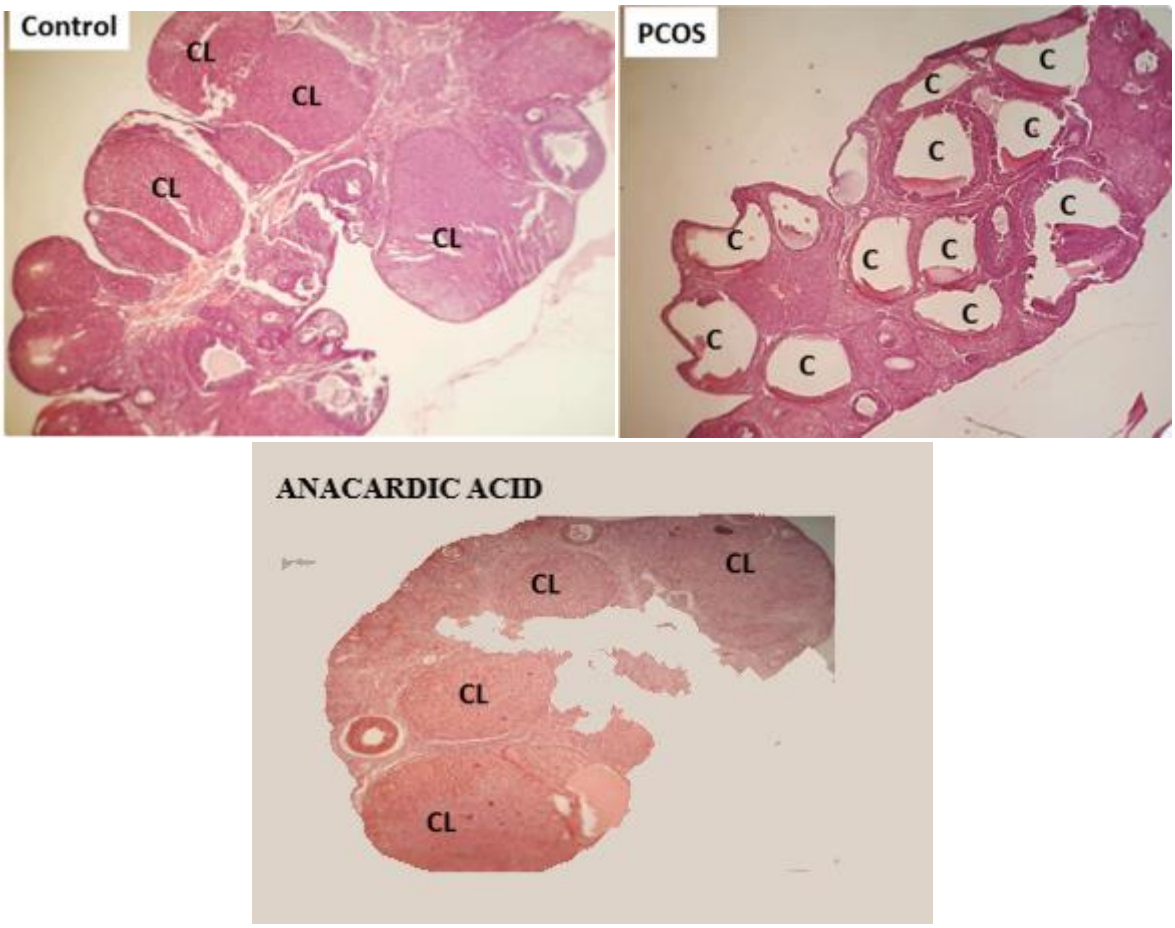


Fig 2. Effect of Anacardic acid on histomorphological alterations in ovarian tissue of the rats with PCOS (H&E staining). C, cystic follicle; CL, corpora lutea.

Tab.1. Effect of various treatments on serum sex Harmones

Groups	Testosterone (ng/dl)	Estrogen (pg/dl)	Progesterone (ng/dl)	FSH (ng/dl)	LH (ng/dl)	LH/FSH
NC	54.67±2.8	53.17±3.4	27.67±2.7	9.25±2.1	13.67±2.7	1.49±0.2
LC	209±5.4$^{\alpha}$	28±2.5$^{\alpha}$	5.983±0.6$^{\alpha}$	20.17±2.0$^{\alpha}$	41.17±5.9$^{\alpha}$	2.05±0.3^{ns}
STD	100.2±10.8$^{\alpha,a}$	42.67±3.5$^{\alpha,a}$	24.17±1.9$^{\gamma,a}$	11.67±1.7^{a}	17.17±2.7^{a}	1.50±0.3^{ns}
AA I	98.67±15.4$^{\alpha,a}$	34.33±4.1$^{\alpha.c}$	18.28±1.0$^{\beta,a}$	13.67±1.3$^{\beta,a}$	23.5±3.3$^{\beta,a}$	1.74±0.3^{ns}
AA II	70.67±16.2^{a}	40.63±1.0$^{\alpha,a}$	23.53±1.3$^{\alpha,a}$	9.5±1.0^{a}	16.67±2.8$^{\alpha,a}$	1.77±0.3^{ns}

NC: CMC; LC: Letrozole; STD: Clomiphene citrate; AAI: Anacardic acid 10 mg/kg; AA II: C Anacardic acid 10 mg/kg; Data presented as Mean ± SEM (n=6). $^{\alpha}P<0.001$, $^{\beta}P<0.01$ compared with NC; $^{a}P<0.001$, $^{C}P<0.05$ compared with LC

Tab.2. Effect of various treatments on serum Antioxidants and lipid profile

Groups	TG mg/dl	LDL mg/dl	HDL mg/dl	SOD U/mg protein	Catalase U/mg protein
NC	102.8±3.1	44.4±2.7	43.83±4.1	44.17±3.4	132.8±3.7
LC	162.7±7.9 $^{\alpha}$	69.83±4.6 $^{\alpha}$	25.17±2.8 $^{\alpha}$	24.67±2.2 $^{\alpha}$	81.67±5.1 $^{\alpha}$
STD	108±3.1 ^{a}	48.83±3.6 ^{a}	40.67±2.9 ^{a}	41.17±2.9 ^{a}	128.2±2.8 ^{a}
AA I	125.2±6.3 $^{\alpha,a}$	53.67±3.9 $^{\alpha,a}$	32.83±2.6 $^{\alpha,b}$	33.83±2.3 $^{\alpha.a}$	102±3.5 $^{\alpha,a}$
AA II	110.3±4.6 ^{a}	46.33±1.6 ^{a}	43.5±2.8 ^{a}	40.83±3.5 ^{a}	120.8±7.6 $^{\beta,a}$

NC: CMC; LC: Letrozole; STD: Clomiphene citrate; AAI: Anacardic acid 10 mg/kg; AA II: C Anacardic acid 40 mg/kg; Data presented as Mean ± SEM (n=6). $^{\alpha}P<0.001$, $^{\beta}P<0.01$ compared with NC ; $^{a}P<0.001$, $^{b}P<0.01$ compared with LC.