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Effect of aqueous seed extract of *Caesalpinia bonduc* (L.) Roxb., on hormonal assay and lipid profile in induced Polycystic Ovary Syndrome albino female rats

Thirumurugan Ayyadurai[1], Senthil Kumar Thiruppathi[1], Achiraman Shanmugam[2], Ranjitha Kumari Bollipo Diana[1*]
[1] Department of Botany, Bharathidasan University, Tiruchirappalli, India
[2] Department of Environmental Biotechnology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India

Abstract
Polycystic ovarian syndrome (PCOS) is considered to be one of the most common endocrine gynaecological disorders, and the majority of female affected by PCOS is increasing daily in women. In order to investigate the effect of Caesalpinia bonduc (L.) Roxb., seed extracts on letrozole-induced PCOS were studied in female albino rats. When PCOS induced rats were administered orally with Letrozole at a concentration of 1mg/kg/day dissolved in 0.5 per cent of Carboxy Methyl Cellulose (CMC) once daily for 28 days.The present study consisted of 42 female Albino rats and equally divided into seven groups. designated as (served as normal intact animals), vehicle control which was orally administered with CMC. The negative control group animals received Letrozole (Letroz). The positive control group animals received 20 mg/kg/day Pioglitazone and other groups received 100, 200 and 300 mg/kg/day of the aqueous seed extracts. The route of Letrozole led to abnormally in Biochemical parameter and histological analysis. *C.bonduc* was able to surely exert its defensive influence by give back hormone levels, with testosterone, estrogen and progesterone. PCOS rats treated with plant extract exhibited significant reduction in Triacylglycerides (TGL), Low-density lipoprotein (LDL), Very low-density lipoprotein (VLDL) and Total cholesterol levels, with an increase in High density lipoproteins (HDL) cholesterol.

Keywords: *Caesalpinia bonduc* L. roxb, letrozole, Cysts, Uterus, Pioglitazone

Introduction
Polycystic Ovary Syndrome (PCOS) is a common heterogenous endocrinological and metabolic disorder in women of reproductive age foremost to infertility/subfertility. Thus, letrozole-induced PCOS model exhibits many histologic and biochemical findings consistent with human PCOS [1, 2]. The reproductive features of PCOS include the increased production of androgen and disordered gonadotropin secretion leading to the menstrual irregularity, hirsutism and infertility [3, 4.] PCOS is characterized by hyperandrogenism, acne, acanthosis nigricans, insulin insensitivity and chronic anovulation [5]. The risk of endometrial hyperplasia, cancer, type 2 diabetes and cardiovascular diseases increase with long-term PCOS [6]. Administration of letrozole decreased the uterus weight. The reduction in uterus weight observed in PCOS rats might be due to the reduction in oestradiol levels. Follicular atresia and unusual follicular expansion have also been observed in PCOS [7, 8]. A large number of plants are used traditionally to induce fertility in women.

Plant
Caesalpinia bonduc L. Roxb., (Syn name: *C. bonducella* L. Fleming, Syn name: *C. crista* (L.) belonging to the family: Fabaceae), a medicinally important plant. It is abundant in the tropical and subtropical regions of Asia, and the Caribbean [9-11]. The plant seeds are traditionally used in the fertility regulation in female in India. [12, 13]. Thus, reported to possess antifertility activity in plants extracts *Ficus platyphylla, Coccinia cordifolia, Ageratum conyzoides, Kaempferia parviflora and Lepidium meyenii*, [14-18].

Uses in traditional medicine
Earlier studies on *C. bonduc* L. Roxb., is reported to have several therapeutic properties a like anti-inflammatory, antipyretic, analgesic and antioxidative, antimicrobial immunomodulatory, antifilarial, anti-diabetic, Muscle contractile activity and antiestrogenic [19-26].

Previously isolated classes of constituents
The seeds of *C. bonduc* are originate to comprise several chemical elements such as hematoxylol, stereochenol A, 60 -O-acetylloganic acid, 40 -O-acetylloganic acid, and 2-O-b-D-glucosyloxy-4-methoxybenzenepropanoic acid, furanoditerpenes, phytosterinin, β-sitosterol, flavonoids, bonducellin, aspartic acid, arginine, citrulline and β – carotene and Caesalpin furanoditerpenes, [27-30].

Methods and materials
Plant materials
The mature dried seeds of *C.bonduc* L. Roxb., were collected from Kollidam river bank, Tiruchirappalli District, Tamil Nadu, India. The plant was authenticated with the type specimen available in the Rapinat Herbarium. St. Joseph's College (Autonomous), Tiruchirappalli, Tamil Nadu, India.

Preparation of the extract
The appearance-dehydrated seeds of 50 g were powder and then extracted with (500 ml) of aqueous by exhausting soxhlet apparatus. The filtrates evaporated in the oven at 65°C. The final product was a dry powder of 4.84% w/w. The plant seed crude extract was dissolved in water and used for the valuation of PCOS.

Experimental design

Adult female albino Wistar rats weighing 150 – 230g were purchased from Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy, Kerala and used throughout the study. They were housed in polypropylene cages in a controlled environment (Temperature 25±2°C) with 12h light: 12 h dark cycle. The animals were fed with standard laboratory diet (Sai Durga feeds and foods, Chennai) and water ad libitum. The study was duly approved by Institutions Animal Ethics Committee for the use of animals and care of the animals was carried out as per the guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA) with protocol number. BDU/IAEC/Re 05/2019/AWR- 42.

Experimental procedure

The study consisted of 42 female Albino Wistar rats equally divided into seven groups. Designated as Group I (served as normal Intact animals), Group II animals served as vehicle control which was orally administered with 0.5 % CMC (CMC). Group III served as PCOS Induced group. The animals received 1mg/kg body weight Letrozole (Letroz) dissolved in 0.5% CMC as vehicle for 28 days. In Group IV the animals received 20 mg/kg/day Pioglitazone. Group V animals were administered with 100 mg/kg seed extract (PCOS + C. bonduc) for 28 days after PCOS Induction. Group VI animals were administered with 200 mg/kg Seed extract (PCOS + C. bonduc) for 28 days after PCOS Induction. Group VII animals were administered with 300 mg/kg Seed extract (PCOS + C. bonduc) for 28 days after PCOS Induction.

Blood Collection and Tissue Processing

The rats were anesthetized with an overdose of Pentobarbital 45 mg/kg B.W and then blood samples were obtained directly by cardiac puncture. The blood samples were centrifuged 3000 rpm for 15 minutes at 4 °C and the serum was stored at -20 °C until further analysis. After blood sampling, the animals were sacrificed, ovaries and uterus were excised, cleaned and weighed. Randomly, one ovary from each group was fixed in 10 % buffered formalin. Fixed tissues were processed routinely for paraffin embedding, and 4 µm sections were prepared and dyed with haematoxylin-eosin; stained areas were viewed using an optical microscope (Olympus, Tokyo, Japan).

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 28 days. Control group received vehicle only (0.5% CMC). Vaginal Libels were together daily and calculated microscopically using Giemsa stain to ratify the induction of PCOS [31].

Biochemical estimations

Hormonal assay

The serum Estradiol and Testosterone levels were analyzed using Chemiluminescent Microparticle Immunoassay (CMIA) using ARCHITECT Reagent Kit and progesterone using ADVIA Centaur PRGE kit.

Assessment of lipid profile

Total Cholesterol

The total cholesterol level in the serum of the experimental animals was measured using (BioSystems Kit, Spain, Product code: COD11505). The standard protocol mentioned by the manufacturer was followed. The concentration of total Cholesterol was measured directly using BioSystems Semi analyzer (BTS350). 1ml of working reagent (35 mmol/L PIPES, 0.5 mmol/L Sodium cholate, 28 mmol/L Phenol, Cholesterol esterase > 0.2 U/ml, Cholesterol oxidase > 0.1 U/L, peroxidase > 0.8 U/ml, 0.5 mmol/L 4 - aminoantipyrine) was added to 10µl of serum. The tubes were mixed well and incubated at room temperature for 10 minutes and the absorbance was read at 500nm against blank.

HDL – Cholesterol

The HDL cholesterol level in the serum of the experimental animals was measured using (BioSystems Kit, Spain, Product code: COD11648). The standard protocol mentioned by the manufacturer was followed. The concentration of HDL Cholesterol was measured directly using BioSystems Semi analyzer (BTS350). Initially the HDL cholesterol was precipitated by adding 0.5ml of HDL cholesterol precipitating reagent (0.4 mmol/L Phosphotungstate and 20 mmol/L Magnesium chloride) to 0.1ml of serum and mixed well. The tubes were incubated at room temperature for 10 mins and further centrifuged at 4000 rpm for 10 mins. 100µl of supernatant was collected in fresh tubes and 1ml of cholesterol reagent (COD 11505) containing 35 mmol/L PIPES, 0.5 mmol/L Sodium cholate, 28 mmol/L Phenol, Cholesterol esterase > 0.2 U/ml, Cholesterol oxidase > 0.1 U/L, peroxidase > 0.8 U/ml, 0.5 mmol/L 4 - aminoantipyrine was added to it. The tubes were mixed well and incubated at room temperature for 15 minutes and the absorbance was read at 500 nm against blank.

Triacylglycerides (TAG)

The concentration of Triacylglycerides in the serum of the experimental animals was measured using commercially available kit (BioSystems Kit, Spain, Product code: COD11828). The concentration of TAGs was measured directly using BioSystems Semi analyzer (BTS350).1ml of working reagent containing 45 mmol/L Pipes, 5 mmol/L Magnesium Chloride, 6 mmol/L 4 – chlorophenol, Lipase > 100 U/ml, Glycerol kinase > 1.5 U/ml, glycerol – 3-phosphate oxidase > 4 U/ml, peroxidase > 0.8 U/ml, 0.75 mmol/L 4 – Aminoantipyrine and 0.9 mmol/L ATP was added to 10µl serum. The tubes were mixed thoroughly and incubated at room temperature for 15 mins. The absorbance (A) of the sample and standard (Glycerol equivalent to 200 mg/dL) were read at 500 nm against blank.

VLDL – Cholesterol

The level of VLDL-cholesterol was calculated by following the Friedewald's equation

VLDL= Concentration of Triglycerides/ 5.

Statistical analyses

Statistical analyses were performed using SPSS version 16.0 Values were expressed as mean ± SD (N=6). Bar with different alphabets are significantly different from each

other and with same alphabets have insignificant changes ($p<0.05$).

Results

Organ weights

In the present study, we aimed to study the organ weight upon treatment with different concentrations of plant extract in comparison with controls. Among the treatment tested, In letrozole treated animals, the organs (Uterus and Ovary) weight significantly decreased in comparison with vehicle (CMS) and control (Fig. 1 and 2). However, In letrozole induced rat after 28 days of treatment with plant extract with different dosages (Low dose, Medium dose and High dose), medium dosage (200 mg/kg) showed similar response in comparison with control and vehicle treatments. We also observed that, with increasing the concentration of dosage the organs weight is gradually decreasing.

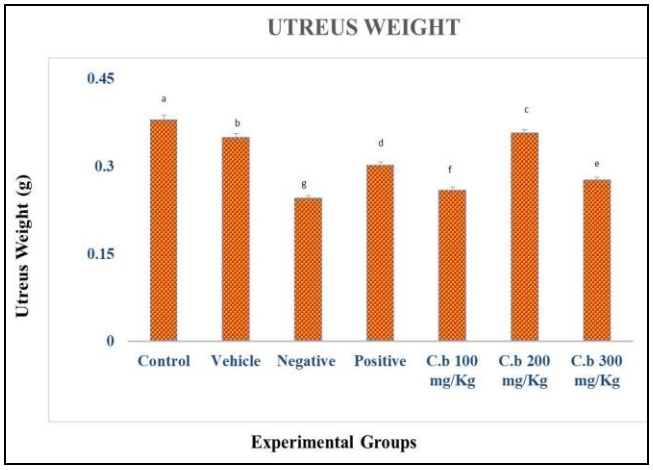

Fig 1: Effect of aqueous Seeds extract of *C. bonduc* on Uterus weight in PCOS induced rats. Values were expressed as mean ± SD (N=6). Bar with different alphabets are significantly different from each other and with same alphabets have insignificant changes ($p<0.05$).

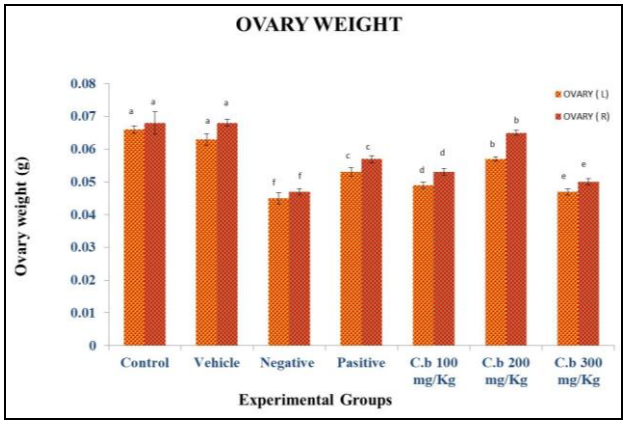

Fig 2: Effect of aqueous extracts of Seeds of *C. bonduc* on Weights of Ovaries in PCOS induced rats. Values were expressed as mean ± SD (N=6). Bar with different alphabets are significantly different from each other and with same alphabets have insignificant changes ($p<0.05$).

Histomorphological changes Ovarian and Uterus

The ovaries in the control and vehicle group showed healthy follicles and usual presence with small and average sized corpora luteum (Fig. 3*a* and *b*). The ovaries in letrozole induced PCOS assembly showed typical PCOS -like changes including presence of upper quantities of animal showing several cysts follicles related with the intact and vehicle group (Fig. 3*c*). In letrozole induced rat after 28 days of treatment with Pioglitazone preserved rats exhibited lesser quantities of cystic follicles, upper quantities of undeveloped corpora luteum compared with PCOS group (Fig. 3*d*). However, In letrozole induced rat after 28 days of treatment with plant seed extract with different dosages (Low dose, Medium dose and High dose), medium dosage (200 mg/kg) showed similar healthy follicles at different developmental stages and Corpus luteum (Fig. 3*f* and *g*). Response in comparison with control and vehicle treatments. We also observed that, with increasing the concentration of dosage the organ ovary showed follicles larger in size and few corpora luteum.

A histological examination of uterus upon treatment with different concentrations of plant extract in comparison with controls and Vehicle. The Uterus in letrozole induced PCOS animal showing Damage cells indicated with (arrows), stroma, glandular epithelium, luminal epithelium and few numbers of glands. Compared with the control and vehicle group (Fig. *4c*). However, In letrozole induced rat after 28 days of treatment with plant extract with different dosages (Low dose, Medium dose and High dose), medium dosage (200 mg/kg) showed similar healthy luminal epithelial cells, endometrial glands and healthy stroma filled with blood vessels (Fig.4*f* and *g*).

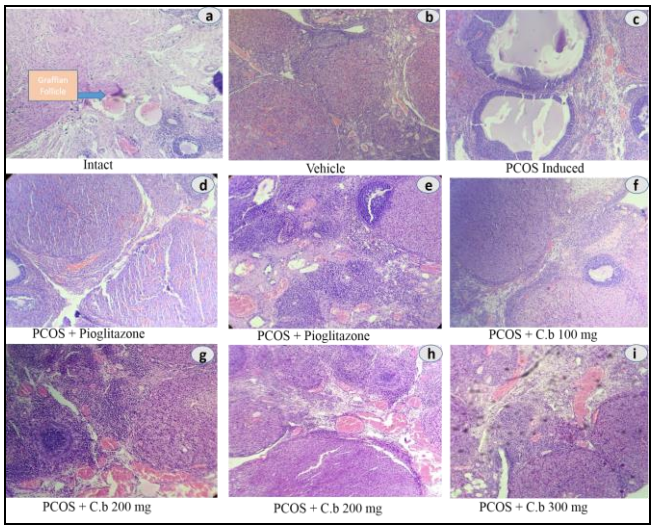

Fig 3: Photomicrograph of transverse sections of Ovary from rats different experiments groups. (a). Control group showing the presence of healthy follicles and corpus luteum (b). Vehicle group showing normal arrangement-like control (c). Section of ovary from Letrozole induced PCOS animal showing Multiple cysts (d and e). Letrozole in combination with Pioglitazone group showing healthy follicles in various stages of development (f). Section of ovary from *C.bonduc* treated (100 mg/kg) animal showing large follicles (g and h). Section of ovary from *C. bonduc* treated (200 mg/kg) animal showing follicles at different developmental stages and Corpus luteum (i). Section of ovary from PCOS rat treated with *C. bonduc* 300 mg/kg showing the presence of normal developing follicle.

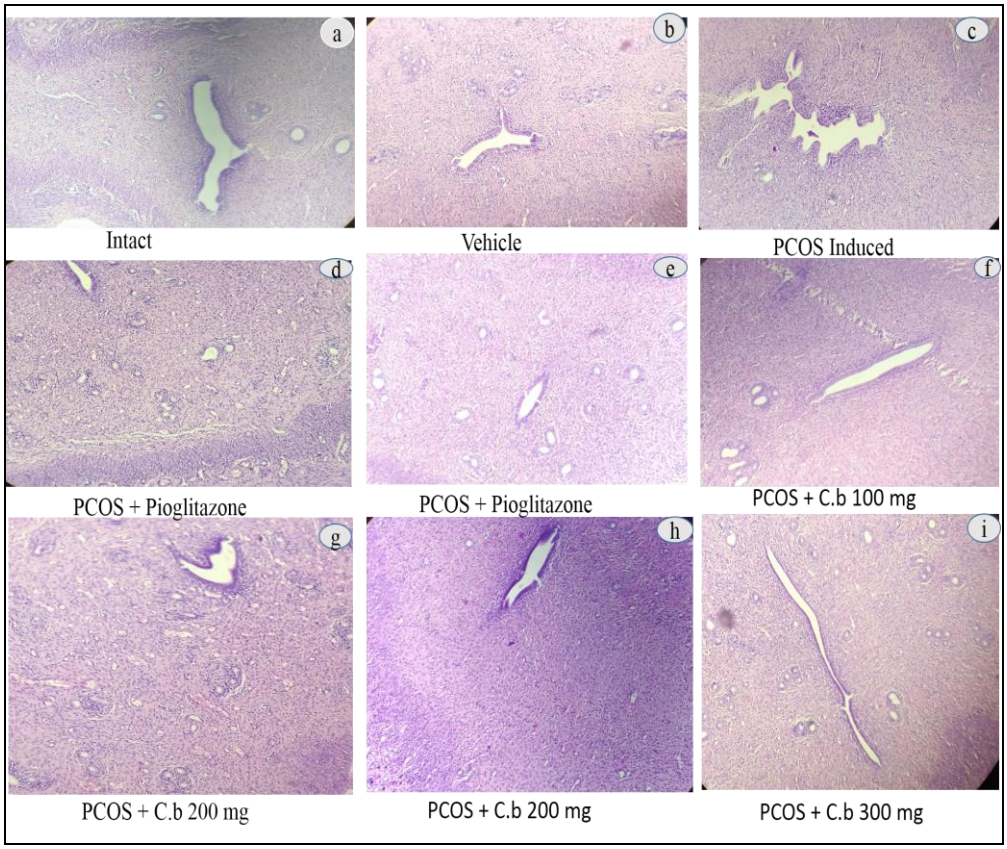

Fig 4: Photomicrograph of transverse sections of Uterus from rats different experiments groups: (a and b). Control & vehicle groups showing normal histological appearance of the uterine healthy stroma of endometrium, glandular epithelium and single layer of luminal epithelium with normal distribution of collagenous fibers (c). Section of Uterus from Letrozole induced PCOS animal showing Damage cells (arrows), stroma, glandular epithelium, luminal epithelium and few numbers of glands (d and e). Letrozole in combination with Pioglitazone group showing healthy luminal epithelium cells and number of glands (f). Letrozole in combination with Uterus from *C. bonduc* treated (100 mg/kg) group showing normal luminal epithelium but also exposed condensed stroma (g and h). Section of Uterus from *C. bonduc* treated (200 mg/kg) animal showing healthy luminal epithelial cells, glands and healthy stroma (i). Section of Uterus from *C. bonduc* treated (300 mg/kg) animal showing normal luminal epithelium with normal distribution of collagenous fibers.

Serum hormonal profile

In the present study, we aimed to study the Hormones were tested upon treatment with different concentrations of plant extract in comparison with controls. The serum levels of Testosterone were abnormally increased in PCOS induced In letrozole group. While those of Progesterone and Estradiol decreased in comparison to the control and along with vehicle (CMC) group. Whereas testosterone levels was observed in standard, low dose, medium dose and high dose groups. Progesterone levels also increased considerably in all the treatment groups i.e., in groups 4, 5, 6 and 7. Only standard group and high dose group showed significant increase in estradiol levels when compared to PCOS induced group. (Table. 1).

Table 1: Effect of aqueous s eed extracts of *Caesalpinia bonduc* L. Roxb., on the level of testosterone, progesterone and estradiol in the serum of pcos induced rats.

Groups	Testosterone (ng/dL)	Progesterone (ng/mL)	Estradiol (pg/mL)
Control	32.01 ± 2.94	38.21 ± 3.88	36.6 ± 10.15
Vehicle	26.5 ± 7.91	28 ± 4.76	26.8 ±9.06
PCOS control	156.2 ±20.95	13.26 ± 1.13	16.7 ±1.13
Standard	13.18 ±5.57	36.7 ± 13.63	40.0 ± 8.37
Low dose	45.92 ±4.58	26.96 ±3.24	18.51 ± 5.76
Medium dose	35.5 ± 3.83	27.5 ±5.16	23.9 ±7.48
High dose	71.4 ±12.29	46.7 ± 9.17	41.6 ± 1.25

Vehicle: CMC; PCOS control: Letrozole; Standard: Pioglitazone; Low dose: *C. bonduc* 100 mg/kg; medium dose: *C. bonduc* 200 mg/kg High dose: *C. bonduc* 300 mg/kg; a,b PCOS control vs. control and Vehicle; c Standard vs. PCOS control; d *C. bonduc* low dose vs. PCOS control; e *C. bonduc* medium dose; f *C. bonduc* high dose.

The values were expressed as mean ± SD for six animals.Means in each column followed by the same superscript letters are not significantly different according to DMRT at $P < 0.05$

Serum lipid profile

In the present study, we also aimed to study the lipid profile were tested upon treatment with different concentrations of plant extract in comparison with control and vehicle group. Letrozole treatment caused significant changes in serum lipid as compared to control and vehicle. TC, TGL, LDL

and VLDL were greatly increased as respectively while HDL levels were notably decreased in PCOS induced letrozole group. (Figs. 5,6,7,8 & 9) portrays effect of *C. bonduc* (L.) Robx plant seed extract with different dosages (Low dose, Medium dose and High dose), on lipid profile. Pioglitazone treatment significantly decreased TGL, LDL and VLDL levels when compared to PCOS induced letrozole group. Whereas HDL levels were particularly increased in Pioglitazone treatment group. Different dosages of *C.bonduc* (L.) Robx., decreased levels of TGL, LDL and VLDL significantly but HDL levels significantly increased in comparison to PCOS induced group. However, In letrozole induced rat after 28 days of treatment with plant extract with different dosages (Low dose, Medium dose and High dose) We also observed that, (Fig. 5, 6, 7, 8 & 9). With increasing the concentration of dosage the serum lipids level of TGL, LDL and VLDL is gradually decreasing. as respectively while HDL levels were notably increased treatment with plant extract with different dosages groups.

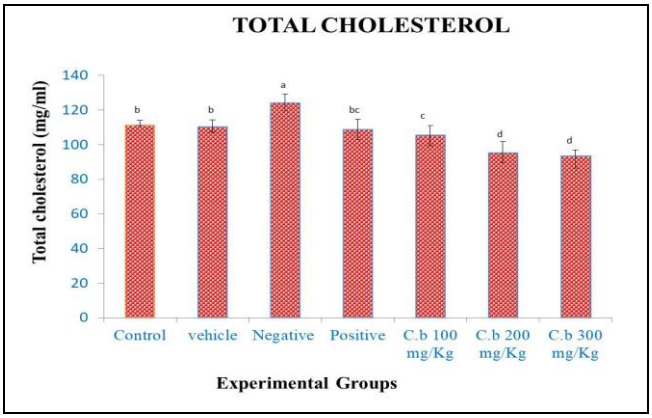

Fig 5: Effect of aqueous seed extracts of *Caesalpinia bonduc* on level of Total Cholesterol in the serum of PCOS induced rats. Values were expressed as mean ± SD (N=6). Bar with different alphabets are significantly different from each other and with same alphabets have insignificant changes ($p<0.05$)

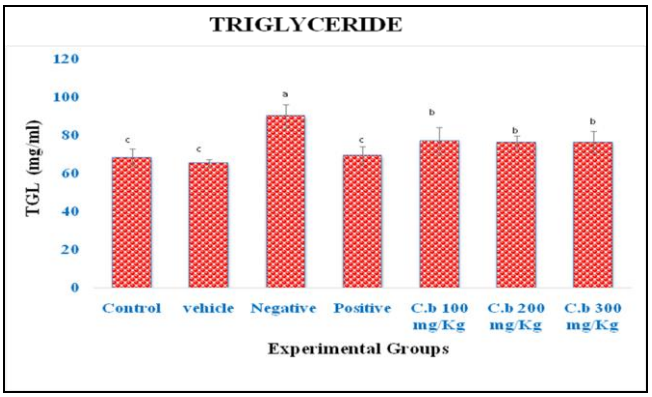

Fig 6: Effect of aqueous seed extracts of *Caesalpinia bonduc* on level of Triglyceride in the serum of PCOS induced rats. Values were expressed as mean ± SD (N=6). Bar with different alphabets are significantly different from each other and with same alphabets have insignificant changes ($p<0.05$).

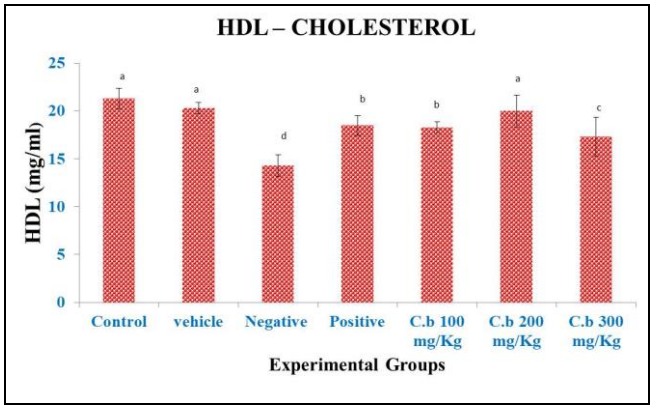

Fig 7: Effect of aqueous seed extracts of *Caesalpinia bonduc* on level of HDL in the serum of PCOS induced rats. Values were expressed as mean ± SD (N=6). Bar with different alphabets are significantly different from each other and with same alphabets have insignificant changes ($p<0.05$).

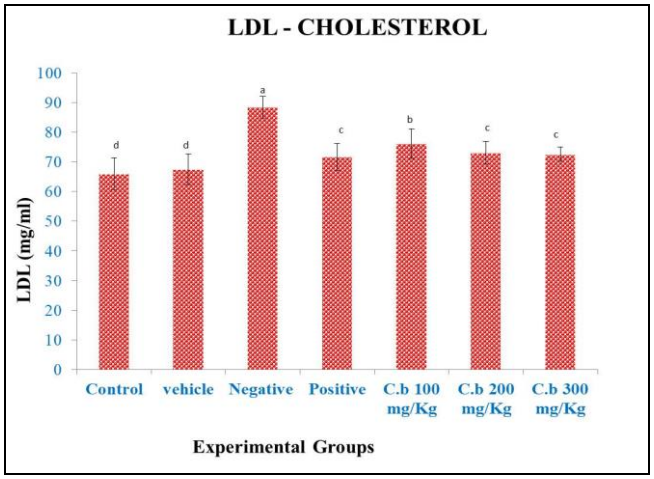

Fig 8: Effect of aqueous seed extracts of *Caesalpinia bonduc* on level of LDL in the serum of PCOS induced rats. Values were expressed as mean ± SD (N=6). Bar with different alphabets are significantly different from each other and with same alphabets have insignificant changes ($p<0.05$).

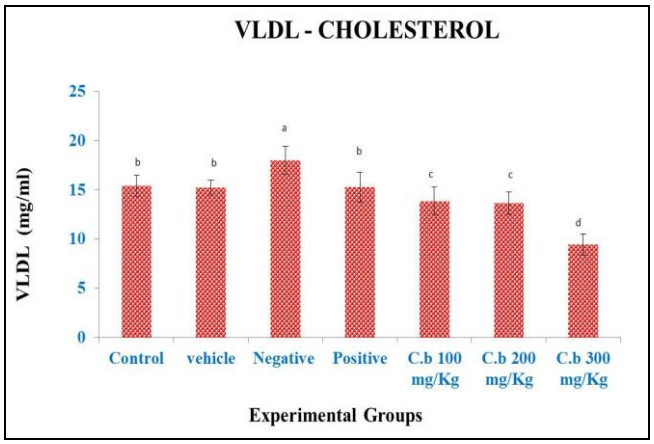

Fig 9: Effect of aqueous seed extracts of *Caesalpinia bonduc* on level of VLDL in the serum of PCOS induced rats. Values were expressed as mean ± SD (N=6). Bar with different alphabets are significantly different from each other and with same alphabets have insignificant changes ($p<0.05$).

Discussion

In the present study, demonstrated that an imbalance gonadotropin hormone as a negative effect of PCOS could affect follicular development. Letrozole-aromatase inhibitor, was used to induce Polycystic Ovary Syndrome in female albino rats. Previous reports suggest that Letrozole induced PCOS condition depicts human PCOS in many ways [14, 31, 32-34]. The related outcomes were obtained in the current research work. When the rats were induced with PCOS by letrozole, the testosterone levels were found to be expressively improved when compared with intact and vehicle albino rats. PCOS induced rats treated with *C. bonduc* (200 mg/kg) seed extracts showed reduced levels of testosterone. While those of Progesterone and Estradiol increased in comparison to the PCOS Induced group.

Literature revealed that *C. bonduc* in albino rats mediated through direct effect on the reproductive organs possibly by suppressing follicular growth in the ovary and trouble of the hormonal balance in the hypothalamo-hypophysial ovarian and uterine axis, in terms of Ovarian weights, there were no significant changes among the groups. However, advanced to a significant decrease in Ovarian weights, uterine weight were reduced due to Letrozole treatment [31, 35-37]. *C. bonduc* treatment (200 mg/kg) seed extracts showed Ovarian and uterine weight similar response which matched to those in control and vehicle treatments. Histological examination of ovaries from letrozole treated rats revealed an increase in the number of atretic secondary follicles were randomly and large cystic follicles [35, 37-40] *C. bonduc* treatment (200 mg/kg) showed similar healthy follicles at different developmental stages and Corpus luteum response in comparison with control and vehicle along with pioglitazone treatments. A repetitive administration was reported in *Foeniculum vulgare*, Curcumin, *Mentha piperita*, *Symplocos racemosa, Aloe barbadensis*, and *S. racemose* [37, 39, 41-44]. Decreased cholesterol levels significantly. Present study exhibited similar results in lipid profile. Letrozole induced treatment caused significant changes in serum lipid as compared to control and vehicle. TC, TGL, LDL and VLDL were greatly increased as respectively while HDL levels were notably decreased in PCOS induced letrozole group. The similar outcome was seen in the present research work later the induction of PCOS. The letrozole induced PCOS rats showed raised lipid Profile TC,TGL, LDL and VLDL which decreased significantly While HDL levels greatly increased when the PCOS induced rats were treated with medium dose of 200mg/kg *C. bonduc* and our study concurred with these findings. However, further studies are required to determine the PCOS syndrome of this extract and other possible contrivance through which the extract may act. Furthermore, the bioactive component of this extract should be characterised.

Conclusion

The aqueous extract of *C. bonduc* showed similar effect is Comparision with pioglitazone in treating PCOS and inducing ovulation. Phytotherapy is frequently measured to be not as much of toxic and free from side effects Than artificial drugs. The *C. bonduc* seed extract also formed an increase in serum estrogen levels and number of corpus luteum, when administered to healthy female albino rats. Further studies are needed to decipher the mechanism of action of this plant and the component responsible for these actions.

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Conflict of interest

Authors declared that there is no conflict of Interest

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