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Research Article

# *PIPER LONGUM* HEXANE FRACTION INDUCES INFERTILITY BY MODULATION OF INFLAMMATORY MEDIATORS AND GONADOTROPIN INSUFFICIENCY IN FEMALE RATS

**ABU HASNATH MD GOLAM SARWAR[a], SHIKHA SHARMA[a], MOHAMMED ARIF[a], SARJEET SINGH THAKUR[b], FAREEDA ATHAR[a], BEENA KHILLARE[c], SONU CHAND THAKUR[a]***

**[a]Centre for Interdisciplinary Research in Basic Sciences, Jamia Millia Islamia, New Delhi 110025, India, [b]National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi 110067, India, [c]Department of Reproductive Biomedicine, National Institute of Health and Family Welfare, Munirka, New Delhi 110067, India. Email: sonuchandthakur@gmail.com**

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**ABSTRACT**

Objective: The present study was designed to explore the antifertility efficacy of fruits of *Piper longum* hexane fraction (PLHF) and to study the parameters related to female reproduction.

Methods: Mature female rats were orally administered with two different doses (150 and 250 mg/kg) of PLHF for thirty days. All reproductive parameters were evaluated like estrous cycle, histological changes in uterus and ovary, hormone levels in the serum, antioxidant enzymes and NO concentration, measurement of cytokines, COX-2 expression, antifertility and anti-implantation activity.

Results: Treatment with PLHF significantly ($p < 0.001$) prolonged the length of estrous cycle at both doses. At the dose of 250 mg/kg there was drastic reduction ($p < 0.001$) in the number of implantation sites. Histopathology of the uterus revealed degeneration of uterine glands and endometrial epithelial cells. The graffian follicle in the ovary showed lack of cumulus oocyte complex. The serum levels of LH and FSH reduced significantly ($p < 0.001$) and the level of estradiol increased significantly ($p < 0.001$). The ovarian cytokines, nitric oxide and COX-2 level was decreased two fold after PLHF treatment. Furthermore, antioxidant enzymes level was also significantly ($p < 0.001$) reduced at 250 mg/kg dose.

Conclusion: All the above observations reveal, PLHF disturbed normal progression of reproductive senescence and induces infertility via gonadotropin insufficiency and modulation of inflammatory mediators.

**Keywords:** *Piper longum*; Gonadotropin; Cytokines; Cyclooxygenase-2; Antioxidant enzymes; Infertility.

## INTRODUCTION

*Piper longum* Linn. (Piperaceae) is a tropical climbing shrub grown throughout India. It is cultivated for its fruit, which is usually dried and used as a spice and seasoning. Aqueous extract of the roots of *P. longum* are used as food in the western part of India. Different species of *Piper* (Piperaceae) such as *P. nigrum* Linn., *P. longum* Linn. and *P. retrofractum* Vahl. have been widely used as spice all over the world.

The major constituents of *P. longum* are piperine (most abundant and active), piperlongumine, piperlonguminine, sesamin, fargesin, tridecyl-dihydro-*p*-coumarate, eicosanyl-(E)-*p*-coumarate, caryophyllene, bisaboline, etc. [1].

Varied pharmacological activities possessed by the fruits of *P. longum* include thermogenic, aphrodisiac, carminative, expectorant, anti-giardiasis, anti-amoebic, and antiseptic activity [2]. Dried fruits of *P. longum* have been shown to be useful in the prevention of recurrence of asthma [3]. Extract of this plant has been reported to have hepatoprotective action against carbon tetrachloride induced liver damage [4]. Earlier, immunomodulatory and anti-inflammatory activities of *P. longum* has been demonstrated [5,6]. Other studies have indicated antifertility property of *P. longum* [7,8]. However, the detailed study of effect of plant on different reproductive parameters has not been carried out. Hence, the present study has been undertaken to evaluate the antifertility efficacy of *P. longum* and to study various parameters related to female reproduction in order to understand the mechanism involved in it.

## MATERIALS AND METHODS

### Chemical reagents

Citric acid, 5,5'-dithiobis (2-nitro-benzoic acid) (DTNB), 1-chloro-2,4-dinitro-benzene (CDNB), L-glutathione reduced (GSH), L-glutathione oxidized (GSSG), nicotinamide adenine dinucleotide phosphate (NADPH), *o*-phenylenediamine dihyrochloride (OPD), griess reagent and protease inhibitor cocktail were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Recombinant proteins and antibodies of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were purchased from Peprotech Inc., USA and secondary antibodies from Santa Cruz Biotechnology Inc, USA. All other chemicals and solvents used were of analytical grade and purchased from Merck Ltd., India.

### Animals

Experiments were performed on female holtzman rats of 6-7 weeks of age (weighing 150-180 g). These animals were procured from the central animal house facility, National Institute of Health and Family Welfare (NIHFW), Munirka, New Delhi, India and housed separately in cages under standard conditions of 12 hours day/night cycle, fed with standard rodent chow and water *ad libitum*. The environmental temperature and humidity maintained at 25°C ± 2 and 42% ± 5 respectively. The approval for the animal experimentations was taken from Institutional Animals Ethics Committee of NIHFW (IAEC No. 168/1999).

### Collection of plant material and preparation of dose

The dried fruits of *P. longum* were purchased from a local dealer, Delhi and authenticated by Dr MP Sharma, Department of Botany, Jamia Hamdard, Delhi, India. The fruits were ground into powder and exhaustively extracted thrice with absolute ethanol (10 times of the initial sample weight) for 36 hours in an orbital shaker. The extract was filtered and concentrated *in vacuo* to get the gummy mass. This ethanol extract was further partitioned with hexane. The resultant fraction was collected, concentrated on rotary evaporator, and stored at -20°C till further use and designated as *Piper longum* hexane fraction (PLHF).

### Animal treatment and experimental design

The final doses (150 and 250 mg/kg body weight) of PLHF were prepared by suspending the desired amount in 2% gum acacia and orally administered for 30 days. To investigate the effects of PLHF, animals were divided into three groups of 9 rats each. Animals that had typical four day estrous cycle were included in the study. The first group was kept as vehicle control and given 2% gum acacia. The other

two groups were treated with two different doses of PLHF (150 and 250 mg/kg body weight) for 30 days. Vaginal smears were checked twice daily to characterize the estrous cycle of the animals. Air-dried smears were stained with haematoxylin/eosin. After last oral dose, as soon as the animals came into estrus phase the control and treated animals were sacrificed by cervical dislocation after mild anesthesia. For hormone analysis blood was collected from the dorsal aorta using a heparinized syringe attached with 21 gauge syringe needle. Ovaries and uteri from control and treated animals were processed for enzymatic activity, cytokine levels, nitric oxide (NO) production, cyclooxygenase-2 (COX-2) protein expression and histology.

**Assessment of estrous cycle, antifertility and anti-implantation activity**

During the treatment period of 30 days, rats from control and treated groups were daily assessed for the phases of estrous cycle (proestrus, estrus, metestrus and diestrus) as well as the for the length of each phase. Total number of cycles per animal was also analyzed. For fertility and implantation studies, animals were treated with two different doses of PLHF (150 and 250 mg/kg body weight) for 30 days and caged with fertility proven males. The presence of copulatory plug was taken as evidence of fertile mating and was counted as day 1 of pregnancy. The mated females were lapratomized on the day 11 of pregnancy and uteri were examined to determine the number of implantation sites in the control and treated rats. Mating that did not lead to successful pregnancy was considered as infertile mating.

**Histological evaluation:** For histological observations, ovaries were fixed in buffered formaldehyde solution, embedded in paraffin and serially sectioned at 5 µm thickness. Sections were stained with hematoxylin and eosin for microscopic observation.

**Hormone analysis:** The circulating serum levels of estradiol, luteinizing hormone (LH) and follicle stimulating hormone (FSH) were determined by ELISA kit (Uscn Life Science Inc., Wuhan) as per the instructions laid by the manufacturer.

**Measurement of NO and cytokine concentration:** Concentration of ovarian cytokines was evaluated using ELISA kits for rat TNF-α and IL-1β (Peprotech Inc., USA). NO was measured using Griess reagent as per the manufacturer's protocol.

**Western blot analysis:** For immunodetection, lysate of ovaries were prepared in Laemmli buffer, ovarian proteins were resolved by SDS-PAGE and were electroblotted onto a nitrocellulose membrane and incubated with primary antibodies. After incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase (AP), the immunoreactive bands were detected.

**Estimation of antioxidant activity**

To prepare tissue homogenate, ovaries were dissected out, rinsed quickly in cold saline solution and homogenized in 0.05 M Tris-HCl buffer (pH 7.4) to yield a 10% (w/v) homogenate. An aliquot of the homogenate (0.5 ml) was used for assaying soluble sulfhydryl group (-SH) while the remainder was centrifuged at 1000 × g for 10 min. The resultant supernatant was transferred into pre-cooled centrifugation tubes and centrifuged at 12,000 × g for 30 min. The supernatant (cytosolic fraction) was used for assaying the antioxidant enzyme activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST). The fraction was stored at -80ºC.

**Estimation of acid soluble sulfhydryl (–SH) group**

The level of acid soluble sulfhydryl group was estimated as total non-protein sulfhydryl group as reported by Singh et al. (2000) [9]. Reduced glutathione was used as a standard to calculate µM of –SH content/g tissue.

**Determination of SOD, CAT, GPx, GR and GST activity**

The cytosolic SOD, CAT, GPx, GR and GST activities were determined spectrophotometrically at 37°C [9]. Enzyme activity was expressed as units/mg protein.

**Statistical analysis**

Results are expressed as mean ± standard deviation of n = 9 animals per experimental group. Statistical analysis was performed using GraphPad Prism software version 6.00. The significance of differences between groups was assessed by Student's *t*-test. Values of $p < 0.05$ were considered to be statistically significant.

**RESULTS**

**Estrous cycle**

Treatment with PLHF significantly ($p < 0.001$) prolonged the length of estrous cycle at both doses. Treatment with 150 mg/kg dose significantly increased metestrus phase ($p < 0.01$) while decreased the proestrus ($p < 0.01$) and diestrus phase ($p < 0.05$). The higher dose (250 mg/kg) significantly increased metestrus phase ($p < 0.001$) while decreased the proestrus ($p < 0.001$) and estrus phase ($p < 0.05$). The total number of cycles per animal was also reduced significantly after the administration of PLHF at 150 mg/kg ($p < 0.001$) and 250 mg/kg dose ($p < 0.001$) as compared to vehicle control rats (Table 1).

**Table1: Effect of PLHF treatment on the estrous cycle of rats.**

| Treatment | Cycle per animal | Length of the cycle | Total duration in each phase (in days) | | | |
|---|---|---|---|---|---|---|
| | | | Metestrus | Diestrus | Proestrus | Estrus |
| Control | 5.17 ± 0.21 | 4.03 ± 0.18 | 7.33 ± 0.35 | 6.67 ± 0.48 | 4.33 ± 0.60 | 3.67 ± 0.65 |
| PLHF (150 mg/kg) | 3.50 ± 0.22*** | 5.08 ± 0.21*** | 8.33 ± 0.43** | 6.00 ± 0.33* | 2.67 ± 0.76** | 3.00 ± 0.47$^{ns}$ |
| PLHF (250 mg/kg) | 3.33 ± 0.13*** | 5.33 ± 0.10*** | 10.00 ± 0.45*** | 5.67 ± 0.52* | 2.00 ± 0.58*** | 2.67 ± 0.58* |

Data are mean ± S.E.M. of 9 replicates

* Significant difference at $p < 0.05$ level, when compared with control group.

** Significant difference at $p < 0.01$ level, when compared with control group.

*** Significant difference at $p < 0.001$ level, when compared with control group, ns Non-significant

**Antifertility and anti-implantation activity:** Administration of PLHF exhibited a significant effect in the fertility performance of female rats. The effect was dose dependent. The anti-implantation activity was not observed in the control group as this group showed a large number of implantation sites in all the rats.

There was a reduction in the number of the implants per animal, when a dose of 150 mg/kg was administered. At the dose of 250 mg/kg there was a drastic reduction ($p < 0.001$) in the number of implantation sites as compared to vehicle treated control animals (Table 2).

**Table 2: Effect of PLHF treatment on implantation of rats**

| Treatment | No. of implantation sites |
|---|---|
| Control | 8.33 ± 0.45 |
| PLHF (150 mg/kg) | 6.33 ± 0.50 |
| PLHF (250 mg/kg) | 0.67 ± 0.78*** |

Data are mean ± S.E.M. of 9 replicates.

*** Significant difference at $p < 0.001$ level, when compared with control group

**Histopathological changes**

The histology of the ovary obtained from the vehicle treated control group showed normal structure of the graffian follicle along with the developing ovum inside (Fig. 1A). The dose 150 mg/kg of PLHF exerted mild effect on the histoarchitecture of the ovary. Some of the antric follicles showed degenerative changes as compared to vehicle-treated control. The follicular growth was inhibited when the dose of PLHF was increased to 250 mg/kg and most of the graffian follicles showed lack of cumulus oocyte complex (COC) (Fig. 1B).

Treatment with higher dose of PLHF (250 mg/kg) for 30 days developed vacuolization of the endometrial epithelial cells (Fig. 1D) when compared to the vehicle control (Fig. 1C). The lower dose of PLHF (150 mg/kg) showed normal endometrial epithelial cells and normal architecture of the uterine glands were comparable to vehicle control. However at 250 mg/kg dose of PLHF the uterine glands were dilated and degenerated with reduced lumen (Fig. 1F) as compared to vehicle control (Fig. 1E).

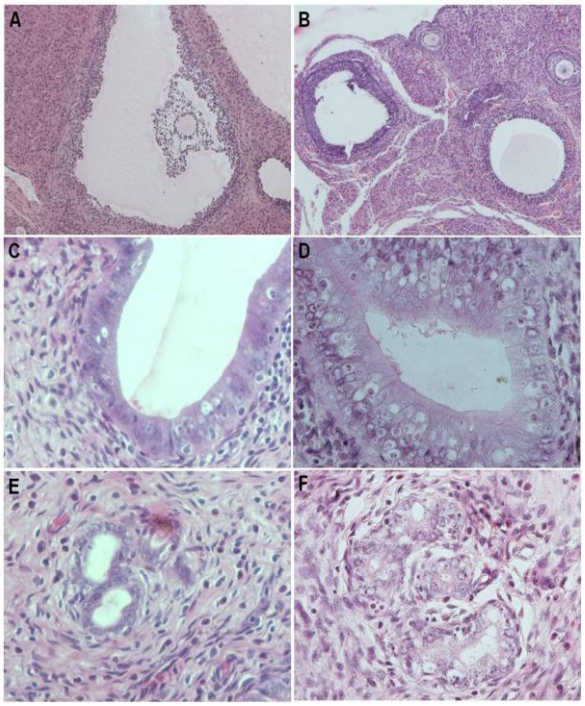


**Fig. 1: Representative photomicrographs of rat ovary and uterus sections.**

A. Section of ovary from control rat showing normal graffian follicle along with the developing ovum inside B. Ovary section from PLHF treated rat (250 mg/kg) showing lack of ovum in the graffian follicle (100X magnification). C. Uterus section from control rat showing normal endometrial epithelial cells D. Section of uterus from PLHF (250 mg/kg) treated rat showing vacuolated endometrial epithelial cells E. Section of uterus from control rat showing normal compact cells of endometrial glands F. Section of uterus from PLHF (250 mg/kg) treated rat showing degenerated cells of the endometrial glands (400X magnification).

**FSH, LH and estradiol level**

The serum levels of FSH ($p < 0.05$) and LH ($p < 0.001$) were reduced significantly with 150 mg/kg dose. Similarly, administration of 250 mg/kg of PLHF significantly reduced mean levels of FSH ($p < 0.001$) and LH ($p < 0.001$) as compared to the vehicle treated control group (Fig. 2A, B). PLHF caused a significant increase ($p < 0.001$) in the level of estradiol in a dose dependent manner when compared to vehicle-treated control rats (Fig. 2C).

**Cytokines level**

Ovarian concentrations of TNF-α were highest during ovulation in control rats. After treatment with 250 mg/kg dose, ovarian concentrations of bioactive TNF-α were markedly reduced i.e., 2 fold approximately. Ovarian concentrations of TNF-α at 150 mg/kg dose also remained significantly low as compared to control (Fig. 2D).

Ovarian concentrations of IL-1β decreased significantly ($p < 0.001$) after PLHF treatment at 150 and 250 mg/kg dose. At 250 mg/kg dose the concentration of IL-1β decreased approximately 2 fold as compared to control rats (Fig. 2E).

**NO accumulation**

Nitrite levels as a measure of NO accumulation were measured in the ovaries after PLHF treatment in estrus phase. Approximately, 1.6 fold decrease in nitrite level occurred at 150 mg/kg while there was 2.0 fold decrease at 250 mg/kg dose group (Fig. 2F).

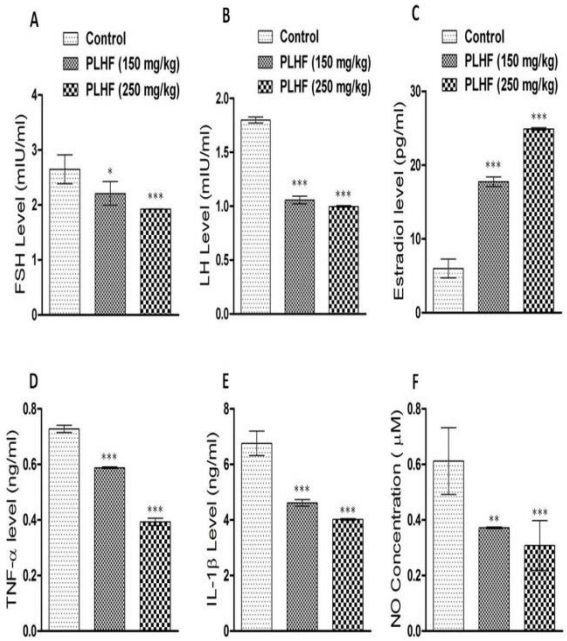


**Fig. 2: Effect of PLHF treatment on the serum levels of, A. FSH B. LH C. Estradiol and ovarian concentrations of, D. TNF-α E. IL-1β F. NO**

Data are mean ± S.E.M. of 9 replicates. Significant difference at * $p <$ 0.05 level, ** $p < 0.01$, *** $p < 0.001$ level, when compared with control group.

**COX-2 expression**

We determined the expression level of COX-2 in the ovary during ovulation as shown in Fig. 3. PLHF caused a decrease in COX-2 expression at 150 mg/kg dose (lane 2). At 250 mg/kg dose (lane 3), there was more than 2 fold decrease in the level of COX-2 expression as compared to control (lane 1).

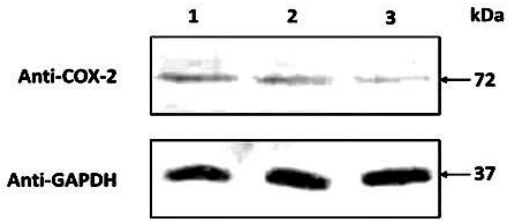


**Fig. 3: Immunoblot analysis of COX-2 expression in ovarian lysates of control (Lane 1), 150 mg/kg dose (Lane 2) and 250 mg/kg dose (Lane 3). Another set of samples was probed with anti-GAPDH antibody.**

**Enzymic antioxidant levels**

Fig. 4A summarizes the content of reduced glutathione (GSH) in the ovarian homogenate measured as acid soluble sulfhydryl group (-

SH) in all the experimental rats. The ovaries of rats treated with 150 mg/kg of PLHF showed a decrease of almost 5.24 fold whereas, treatment with 250 mg/kg dose suppressed the antioxidant enzymes to a greater extent by 6.08 fold when compared to the vehicle-treated control rats during ovulation.

The antioxidant enzyme activities (SOD, CAT, GPx, GR and GST) were also determined in the ovarian homogenate of rats during ovulation. The results are presented in Fig. 4 (B-F). The treatment of rats with both the dosages of PLHF (150 and 250 mg/kg) induced a significant decrease in all these enzymatic antioxidants. At 150 mg/kg the enzymatic activities of SOD, CAT, GPx, GR and GST decreased by 1.06, 1.29, 1.44, 1.72 and 4.91 fold respectively, however at 250 mg/kg dose there was a further decline by 1.26, 1.64, 1.99, 2.01, 6.81 fold respectively, as compared to the control group.

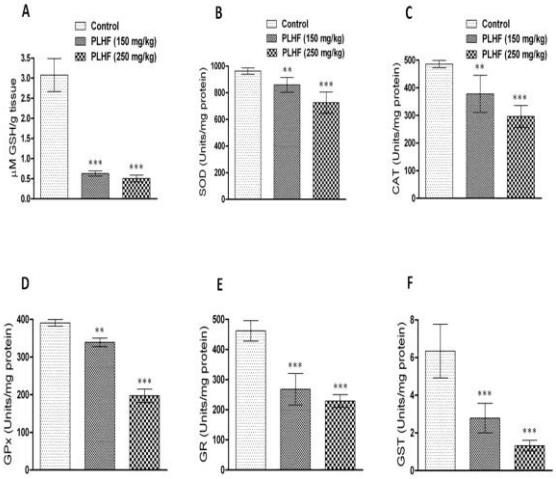


**Fig. 4: Effects of PLHF treatment on the levels of different non-enzymic and enzymic antioxidants measured in the ovary of rats from each group. Results of (A) GSH expressed as µM GSH/g tissue, (B-F) antioxidant enzymes (SOD, CAT, GPx, GR, GST) are expressed in units/mg protein.**

Each value represents the mean ± S.D. of n = 9 animals per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ versus control + vehicle group.

## DISCUSSION

Plant extracts and their constituents are being extensively studied because of their potent activities and low toxicity. For health promoting effects their consumption is on the rise throughout the world.

Our findings indicate that PLHF disturbed the normal process of reproductive senescence. PLHF treatment suppresses gonadotropin hormone level, thus disturbing the estrous cycle. Since gonadotropin hormones are associated with the estrous cycle and also responsible for the growth and development of ovary and uterus, it is quite possible that gonadotropin insufficiency might have disturbed the estrous cycle and caused histopathological changes in the uterus and affected cumulus matrix synthesis in the ovary [2]. As previously reported, FSH and LH sequentially stimulate cumulus matrix synthesis during ovulation [10]. In addition, the level of estradiol increased significantly after PLHF treatment. It is probable that without sufficient LH, ovulation does not occur and estrogen levels remain high.

Inflammation accompanying the process of ovulation is characterized by an increase in the cytokine production [11]. There is an interrelationship between the endocrine and the cytokine systems. Cytokines directly stimulate active endocrine tissues and on the other hand are produced by them in notable concentrations [12]. In the present study, we have compared the levels of cytokines (TNF-α and IL-1β) during ovulation in normal and PLHF treated ovaries. We found that the level of TNF-α and IL-1β decreased after PLHF treatment that might be due to the suppression of gonadotropins (FSH and LH). It is widely known that gonadotropins activate production of both TNF-α and IL-1β during ovulation [13]. Furthermore, TNF-α and IL-1β are involved in two different pathways leading to the follicular rupture which involve prostaglandin and NO [13].

The NO pathway plays an important role in the cytokine mediated regulation of ovulation and contributes to the processes of tissue destruction. Studies inhibiting nitric oxide synthase (NOS) *in vivo* or *ex vivo* in rodents also demonstrate that NO is required for ovulation [14]. It has been demonstrated that administration of the inducible NOS inhibitors significantly reduces the ovulation rate in rats and this effect could be reversed with the NO generator (sodium nitroprusside) [14]. To investigate the effect of PLHF in NO production, we compared the level of NO during ovulation in control and treated ovaries. Our results showed a decrease in the level of NO after PLHF treatment which indicates that PLHF inhibited NO production thus affecting the ovulation rate.

Estradiol regulates NO production and it has previously been shown that inhibitors of the NOS enzyme significantly increase secretion of estradiol from human granulosa luteal cells [15]. Further it is known that high estradiol concentrations inhibit IL-1β production. Estrogens also inhibit IL-6 and TNF-α production [16]. Our results are in consistence with these reports. Increased level of estradiol might have inhibited the production of NO and the synthesis of IL-1β and TNF- α.

TNF-α has been reported to play multiple roles in ovarian function in a variety of species. TNF-α caused the stimulation of luteal prostaglandins (PGs) [17]. TNF-α accumulates in the expanded COC during ovulation suggesting that this cytokine plays an active role in preventing the degeneration of oocytes until fertilization [18]. IL-1β also stimulates COC expansion [19] and serum levels correlated with successful fertilization [20] or implantation in assisted reproductive cycles [21]. So decrease in the level of IL-1β and TNF-α might have affected formation of COC and leads to infertility and anti-implantation activity. Earlier, *P. longum* extract has also been reported to inhibit TNF-α induced expression of intercellular adhesion molecule-1 [22].

Prostaglandin production via COX-2 enzyme is crucial to ovulation and infertility has been reported in COX-2 knockout mice as a result of defects in ovulation [23]. Likewise, ovulation can be blocked in mice and rat with the treatment of COX-2 inhibitors such as indomethacin [23]. In the present study, reduced level of COX-2 may have affected ovulation. Earlier, an inhibitory activity of piperine and piper extracts on prostaglandin and COX-1 has been reported which exhibits their anti-inflammatory activity [24].

Cells under aerobic conditions have a defense system against reactive oxygen species (ROS) which are enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) etc. [25]. During ovulation, ROS is produced which has an important physiological role [26]. ROS are important mediators of the inflammatory reactions involved in the follicle rupture, hormone signaling, ovarian steroidogenesis and luteolysis [27]. Likewise ovulation may be inhibited by anti-inflammatory agents [28]. The source of ROS appears to be inflammatory cells, such as macrophages and neutrophils as they are notably present in ovary at the time of ovulation [29] and produce tremendous amount of free radicals. ROS has been proposed to initiate cellular damage via a number of independent mechanisms such as lipid peroxidation, protein oxidation and DNA damage.

A certain threshold level of ROS has been reported as a potential marker for predicting success rate of *in vitro* fertilization [27]. The overproduction of oxygen free radicals during ovulation leads to altered activities of SOD, CAT, GPx and GR enzymes. These results suggest that decrease in the level of antioxidant enzymes can be one of the factors that lead to infertility in female rats. This is in agreement with the study [30] which reports that increase in the level of ROS is associated with successful ovulation.

## CONCLUSION

Taken together these results imply that *Piper longum* hexane fraction (PLHF) impaired reproduction and induce infertility in

female rats. These effects appear to be mediated by gonadotropin insufficiency and modulation of inflammatory mediators like cytokines, nitric oxide, COX-2 and reactive oxygen species that ultimately affected fertility. The antifertility activity of the plant is due to the synergistic effects of the compounds present in the extract. Further efforts should be made to identify and isolate the compounds present in the hexane extract which may be responsible for fertility regulation.

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