



The pharmacological evaluation of antifertility role of *Plumeria acuminata* on the reproduction of female Wistar rats

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ABSTRACT

Treatment-induced imbalance in female reproductive hormones can lead to difficulty in ovulation and preparing the endometrium for efficient reception can cumulatively suggest the antifertility role of the drug. Traditionally, ancient herbal medicines are proficiently used to rule out fertility and its control-related issues. This research aims to explore a fertility regulatory profile of leaves and roots ethanolic extract from *Plumeria acuminata* of the *Apocynaceae* family. *Plumeria acuminata* leaves and roots ethanolic extracts were prepared by cold maceration process, subjected to qualitative phytochemical analysis, and acute toxicity test. Based on the LD50 values, 100, 200, and 400 mg/kg doses of both extracts were determined for antifertility activity in adult Wistar female rats. Test-item treatments were given orally from day 1 to 28. Morphological, hematological, hormonal, and histological examinations were performed on day 29 after euthanizing animals. Administration of extracts expressively altered the hormonal intensities up to ~7%–99%, i.e., a decrease in estrogen, progesterone, and luteinizing hormone level as well as an increased in follicle-stimulating hormone level. Anatomical alterations in reproductive organs were confirmed by observing cystic follicles and atrophied squamous cells during histopathological evaluation. Vaginal smear evaluation confirmed the disturbance of the estrous cycle with an increase in estrous cycle length up to ~8%–64% as well as a decrease in the number of the estrous cycle and each phase up to ~0%–118% except for diestrus phase. Achieved antifertility results can be attributed to hormonal imbalance, mainly caused by the stigmaterol, saponins, flavonoids, and alkaloids groups of phytochemicals in leaves and roots ethanolic extracts.

INTRODUCTION

Advanced medical technologies have increased life expectancy and decreased mortality rate exploding the human population in the modern era, a significant global burning issue, adversely affecting the economic, social, and technological developments (Amit and Pankaj, 2018; Jain *et al.*, 2015). Unwanted pregnancies raised due to inappropriate contraception or negligence sometimes become life-threatening for women (Mziray *et al.*, 2020; Thanamool, 2013). Thus, conception and fertility control become critical issues and make the population

control tops on the priority list through the use of contraceptives and family planning to improve the quality of life (Asuquo, 2012; Bandyopadhyay, 2010; Shah *et al.*, 2016b; Sundar *et al.*, 2013; Thanamool, 2013).

Great advances in modern medicine in recent decades have enriched today's market with highly potent synthetic contraceptives. These drugs prevent or inhibit conception and/or modify the cyclicity of endogenous hormonal production resulting in menstrual irregularity. This cumulatively leverages the physical and mental stress and put the patient at risk of developing a variety of disorders, such as breast cancer, endometriosis, gastrointestinal disturbance, massive painful uterine contraction, etc., to name a few. This explains a need for exploring the hidden wealth of medicinal plants with better effectiveness and lesser side effects (Fajriaty *et al.*, 2017). In Indian local communities, several plants and their parts have become crucial due to their action on the

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ovarian-uterine axis, biological effectiveness, factorial role in the regulation of fertility, eco-friendliness, cheapness, affordable for all segments of society, and capability to provoke changes in the configuration of the reproductive cycle (Daud *et al.*, 2015; Mansouri *et al.*, 2016; Singh *et al.*, 2017).

So far, a small number of plants have been reported to have antifertility potential and out of them, only a few have been reached at clinical evaluation. Thus, hunting for herbal drugs with the capacity to disturb the endocrine function of the hypothalamic-pituitary-gonadal axis that can replace the synthetic steroidal/nonsteroidal medications as birth-control regulation strategies is in demand (Chen *et al.*, 2021; Goyal, 2014; Udiwal *et al.*, 2014).

Plumeria acuminata (L.) commonly known as Frangipani (Eng.) belongs to the *Apocynaceae* family exhibited antifertility, antipyretic, antinociceptive, antibacterial, antidiabetic, antitumor, and immune-modulatory activity (Devi *et al.*, 2015; Farooque *et al.*, 2012; Gupta, 2016; Gomathi *et al.*, 2013, 2016; Singh *et al.*, 2017; Sunitha and Mohan, 2017; Sunitha and Naga, 2018; Taid *et al.*, 2016; Yadav *et al.*, 2016). Active ingredients present in leaves and roots, such as stigmasterol, saponins, flavonoids, and alkaloids, are believed to be having antifertility and abortive activities (Devi *et al.*, 2015; Gupta, 2016). Stigmasterol possesses both estrogenic and antiestrogenic effects due to its estrogen-like structure (Thanamool, 2013). Saponin, a lipid derivative triterpenoid, plays an important role in steroid biosynthesis and caused disturbances of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) release affecting the estrous cycle. Saponins possess antizygotic, blastotoxic, and anti-implantation activity in rodents (Fajriaty *et al.*, 2017; Mziray *et al.*, 2020). Flavonoids, which are nonsteroidal phytoestrogens, exhibit antifertility potential in male rats by decreasing the production of spermatids (Choudhary *et al.*, 2017; Mukhram *et al.*, 2012). It is reported that flavonoids block the cyclooxygenase-I and II which are believed to be essential for ovulation (Nayaka *et al.*, 2014). Alkaloid is thought to possess oxytocic-like effects that affect the environment of the uterus in rodents (Kayode *et al.*, 2007).

As there are scanty reports on the antifertility effects of the *P. acuminata* leaves (PAL) and *P. acuminata* roots (PAR) extracts, none of these have documented their possible mode of action for these extracts. The mechanism behind the extracts' action that brings out changes in the estrous cyclicity and its length, the hormonal alterations during these changes, and histological modifications in the female reproductive organ system, are focused on in the present study. In the represented work, by emphasizing the possible antifertility role of *P. acuminata* leaves and roots extract in female Wistar rats, we try to explain how these extracts bring changes in the histology of female reproductive organs, hormonal levels and alter their regular estrous cyclicity.

MATERIALS AND METHODS

Identification of plant and collection of plant materials

Plant *P. acuminata* (L.), has been identified and authenticated by a resource person, and fully grown healthy leaves, as well as roots, were collected from the herbal botanical garden of Shree Dhanvantary Pharmaceutical Analysis and Research Centre, Surat, Gujarat (India).

Preparation of plant materials

Plumeria acuminata leaves and roots were washed thoroughly with deionized water to remove the adherent impurities like soil debris, sliced into small pieces followed by proper air-drying under shade at room temperature (25°C–30°C) for a week. The dried material was then ground to a uniform powder with the help of a suitable grinder and the powdered plant parts were then stored in an airtight container and kept in a cool dry place for further analysis.

Preparation of extracts

The ethanol extracts were prepared by soaking 1,000 g each of dry powdered plant parts in 2,000 ml of ethanol in an airtight container for about 14 days. Residual particulates and extracts were separated through filtration using Whatman Filter Paper No.1. The filtrate was concentrated to semisolid paste using a rotary evaporator with the water bath set at 50°C. The resulting paste in the form of crude ethanolic extract of *P. acuminata* leaves and roots, respectively, referred to as PAL and PAR, was weighed and stored in an airtight container till further used. The percentage of yield was calculated using Equation (1) (Hyacinth and Nwocha, 2011; Marin *et al.*, 2020; Taid *et al.*, 2016; Shah *et al.*, 2016b).

$$\text{Extract yield (\%)} = \frac{\text{Net weight of extract (g)}}{\text{Total weight of powder used for extraction (g)}} \times 100 \quad (1)$$

Phytochemical analysis

Individual PAL and PAR extracts were qualitatively analyzed to identify the presence of various phytoconstituents, such as carbohydrates, alkaloids, glycosides, amino acids and proteins, phenols, tannins, flavonoids, sterols, saponins, steroids, and terpenoids by using reported standard tests (Ahirwar *et al.*, 2010; Edwin *et al.*, 2008; Jaber and Jasim, 2014; Jain *et al.*, 2013).

Chemicals

Analytical grade reagents and chemicals were consumed for this analysis.

Animal studies

Wistar rats were used as an animal model for the antifertility model.

Age, sex, and housing condition of animals

The antifertility activity of the crude ethanolic extract was studied using adult female rats. The animals were housed under standard environmental conditions of temperature $22 \pm 3^\circ\text{C}$, at humidity of $50 \pm 20\%$, and a 12-hour light-dark cycle. Rats were given a standard rodent pellet chow diet and water *ad libitum*.

Dose selection for PAL and PAR extracts

The safe dose of the PAL and PAR extracts were determined by performing acute toxicity studies as per the OECD 423. Starting dose of 2,000 mg/kg was given to three adult female Wistar rats in the first step. Postdosing the animals were kept under special observation and screened every 30 minutes till 4 hours for any toxic response. Further till day 14, every day, the animals

were monitored for the same. The absence of any mortality was confirmed by a repetition of the same dose in the second step and the LD₅₀ value was determined.

Experimental procedure and study design

An experimental study was designed to explore the antifertility characteristics of PAL and PAR extracts. Three different doses of 100, 200, and 400 mg/kg of PAL and PAR extracts were determined and henceforth, the doses were referred to as PAL-100, PAL-200, PAL-400, PAR-100, PAR-200, and PAR-400 for the study.

Antifertility model

The effect of PAL and PAR extract on the estrous cycle was determined using nulliparous and non-pregnant adult female Wistar rats. Vaginal smears from female rats were collected through flush techniques for about 12 days (~3–4 estrous cycles) to monitor normal cyclicity. A total number of 42 rats with normal cyclicity were selected for the present model and randomized into seven different groups comprising six animals each. The normal control (Group I) group received normal saline from day 1 to day 28 (D1–D28). Test item treatment for Group II–VII was given from D1 to D28. During these 28 days, i.e., ~6–7 estrous cycles, the vaginal smear was taken on the everyday morning between 8 and 10 am from each animal and subjected to microscopic examination for distinguishing between the four-phase, i.e., estrus (E), metestrus (M), diestrus (D), and proestrus (P). On D29, after collecting blood from retro-orbital sinus, animals were euthanized by CO₂ asphyxiation. White blood cells (WBC), Red blood cells (RBC), Hemoglobin (Hb), Hematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), platelet, and neutrophil counts were evaluated from the blood sample using a hematology analyzer (Siemens, Advia 2120i). FSH, LH, estrogen, and progesterone levels were quantified from serum samples using ELISA. Ovaries, uterus, vagina, oviduct, and cervix were collected, surrounding tissue was removed, blotted on filter paper, and weighed quickly. Collected organs were fixed in 10% formalin buffer, dehydrated in alcohol, and embedded in paraffin for sectioning. 6 μ thick sections were fixed on slides and subjected to the hematoxylin-eosin (H & E) staining process. The stained sections were observed for histological changes in the organs using a digital light microscope (ZEISS, Axio Lab A1) (An *et al.*, 2020; Bhaskar *et al.*, 2009; Byers *et al.*, 2012; Daud *et al.*, 2015; Mittal, 2020; Mziray *et al.*, 2020).

Cyclicity calculations

During 28 days of dosing, different phases of estrous cycles were determined using the flush technique. Days spent in different phases of each cycle were calculated individually throughout the study and at the end of 28 days, the relative time spent in each phase was cumulatively calculated for 28 days in percentage as mentioned in the Equation (2).

Relative time spent in one particular phase (in %)

$$= \frac{\text{total no of days detected in that phase during evaluation}}{\text{total no of days for estrous cycle evaluation}} \times 100 \quad (2)$$

Statistical analysis

All the data are expressed as mean ± standard error of mean for $n = 6$. Statistical analysis was performed with one-way analysis of variance followed by Dunnett's post-test at a confidence level of 0.05 (95% confidence interval) using Graph Pad Prism Version 5.03. For comparison with normal control, differences were considered to be statistically significant when $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. With decreasing the p values, the difference between the two groups is more significant and there is a lesser chance that this difference is due to an error or noise.

RESULTS

Extract yield

The percentage yield from different parts of the *P. acuminata* was calculated from the formula given in Equation (1), and the values for leaves and roots are 15.3% and 13.5%, respectively.

Qualitative phytochemical screening

Qualitative analysis of the phytochemical compounds from the PAL and PAR extracts is represented in Table 1. The presence of carbohydrates, alkaloids, phenolic, sterols, flavonoids, saponins, and terpenoids was detected in both extracts. Proteins and amino acids were absent in both extracts, whereas tannins were detected from the root but absent from the leaves extract.

Acute toxicity test

In the acute toxicity test, a limit dose of 2,000 mg/kg was tested on six female rats. Three rats were used at each step. All rats were observed individually for a sign of toxicity after 30 minutes of dosing, with given special attention during the first 4 hours and then daily thereafter up to 14 days. No signs of toxicity like changes in skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic, central nervous systems, somatomotor activity, behavior patterns, and deaths were observed during 14 days period at the doses tested. The median lethal dose (LD50) was determined to be higher than the highest dose tested, i.e., 2,000 mg/kg.

Antifertility model

Effect on body weight gain

The effect of PAL and PAR extract administration on body weight change is given in Table 2. Measurement and comparison of body weight on every seventh day unveiled the slower body weight gain by animals after treatment in comparison with normal control. Although the body weight gain measured on these interim as well as terminal days was statistically significant for the treatment groups when compared with the normal control group, there was a dose-dependent decrease in this body weight gain after PAL and PAR treatment. When terminally compared with normal control, the PAL and PAR treatment resulted in lesser bodyweight gain and this decrease in body weight gain was statistically significant for all treatments.

Effect on each phase of the estrous cycle

As shown in Figure 1, a comparison of relative time span in individual phases throughout 28 days for test-item

Table 1 Results of qualitative phytochemical screening of ethanolic extract of PAL and PAR.

Test	Reference Result	Observed Result	PAL	PAR
Test for detection of carbohydrates				
Molisch's test	The reddish violet ring at the junction	Dark Reddish Violet ring present at the junction	+	+
Benedict's test	Red precipitate	Light right precipitate	+	+
Fehling's test	Brick red precipitate	Red precipitate	+	+
Test for detection alkaloids				
Mayer's test	Dull white precipitate	Light white precipitate	+	+
Hager's test	Yellow precipitate	Yellow precipitate	+	+
Wagner's test	Reddish-brown precipitate	Brown precipitate	+	+
Test for detection of glycosides				
Killer-Kilani test	The reddish-brown colour at the junction	Reddish colour present at the junction	+	+
Salkowski's test	Development of reddish- brown colour	Reddish-brown colour	+	+
Libermann's test	violet to blue to green colour	Green colour	+	+
Test for detection of proteins and amino acids				
Xanthoproteic test	Orange colour	No colour development	-	-
Millon's test	The appearance of white precipitate which turns red upon gentle heating	No white precipitate appears	-	-
Ninhydrin test	Blue to purple colour	No colour	-	-
Test for detection of Phenol				
Ferric chloride test	Formation of bluish-black colour	The appearance of dark blue colour	+	+
Test for detection of Tannin				
Lead acetate test	Development of a white precipitate	White precipitate	-	+
Test for detection of steroids				
Libermann's test-Burchard test	Formation of dark pink colour	Pink colour appeared	+	+
Salkoeski test	The red colour obtained in the upper chloroform layer	The light red colour appeared in the chloroform layer	+	+
Test for detection of flavonoids				
Alkaline reagent test	Intense yellow colour upon addition of base and turns colourless on the addition of acid	Yellow colourisation on the addition of NaOH and become colourless on the addition of HCl	+	+
Shinoda test	Red colour	Red colour appears	+	+
Lead acetate test	The appearance of yellow colour precipitate	Yellow colour appears	+	+
Test for detection of saponins and terpenoids				
Froth test	Formation of froth	1-2 cm height froth formed	+	+

+: Present was detected for a component in the test; -: Present was not detected for a component in the test.

Table 2 Effect of PAL and PAR extract on body weight gain during anti-fertility activity.

Group and treatment	Mean Body weight gain (g)				
	Day (1-7)	Day (7-14)	Day (14-21)	Day (21-28)	Day (0-28)
Normal Control	6.72 ± 0.87	7.57 ± 0.87	7.43 ± 0.80	6.97 ± 0.56	28.68 ± 2.17
PAL_100	6.50 ± 1.01	7.02 ± 1.24	6.30 ± 0.90	6.27 ± 0.99	26.08 ± 1.44
PAL_200	6.13 ± 0.90	6.63 ± 0.82	5.90 ± 1.62	5.73 ± 1.34	24.40 ± 2.20**
PAL_400	5.90 ± 0.76	6.10 ± 1.84	5.53 ± 1.68	5.40 ± 1.13*	22.93 ± 1.55***
PAR_100	6.00 ± 1.21	6.92 ± 1.02	6.00 ± 0.96	5.83 ± 0.58	24.75 ± 1.97*
PAR_200	5.37 ± 1.20	5.90 ± 0.85	5.30 ± 1.39*	5.10 ± 0.88*	21.67 ± 2.27***
PAR_400	4.92 ± 1.58	5.50 ± 0.55*	4.90 ± 0.83*	4.75 ± 0.44**	20.07 ± 1.91***

Values are expressed in Mean ± SEM for n=6, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Normal control

treated groups and normal control groups, revealed a statistically significant dose-dependent decrease in duration of estrus and proestrus phase after PAL and PAR treatments. Even though no dose-dependency was observed in the decreased relative time span of the metestrus phase, the decrease was statistically significant for the 400mg/kg dose of PAL and PAR extracts. On contrary to this, the earmarked observation significantly escalated the relative time span of the diestrus phase in a dose-dependent manner after PAL and PAR treatments. These disturbances in the individual phase can alter the overall cyclicity and its length as shown below. These disturbances in each phase of the estrous cycle might be due to hormonal disturbances caused by both extracts.

Effect on length and number of estrous cycles

The effect of administration of different extracts on the length and number of estrous cycles is given in Table 3. When compared to normal control, increased length of the estrous cycle has resulted in the reduced cycle numbers suggesting the reciprocal relation between cycle length and number. This dose-dependent increase in cycle length and the resulting decrease in cycle number were found statistically significant for groups treated with 200 and 400 mg/kg doses of PAL and PAR extract.

Effect on hematological parameters

As shown in Table 4, hematological parameters such as WBC, RBC, Hb, HCT, MCV, MCH, platelet, and neutrophil did not have any specific effects of PAL and PAR extract treatments when compared with the normal control group. Percentage changes in WBC, RBC, Hb, HCT, MCV, MCH, platelet, and neutrophil count were in the range of ~1%–3%, ~2%–3%, ~2%–3%, ~0%–3%, ~4%–4%, ~0%–3%, ~2%–5%, and ~4%–8%, respectively. When we compared with the normal control group, they did not exhibit any significant changes.

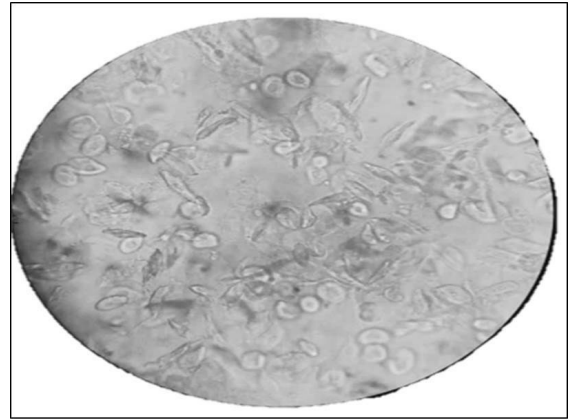


Figure 1. (A)-(ii). Microscopic image of estrus phase observed during evaluation of estrous cycle during anti-fertility model.

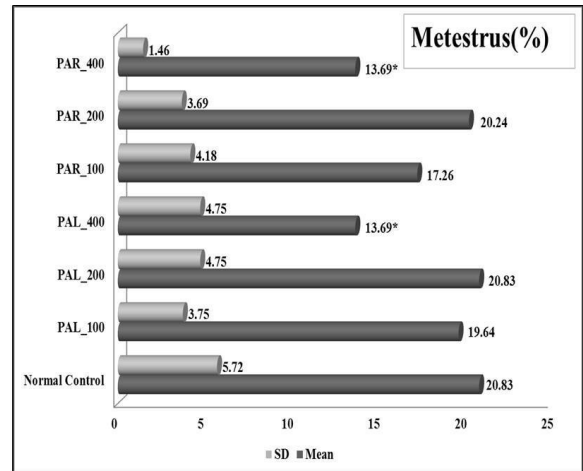


Figure 1. (B)-(i). Effect of PAL and PAR extract on metestrus phase of estrous cycle during anti-fertility model. Values are expressed as Mean ± SEM for n=6, *p<0.05, **p<0.01, ***p<0.001 vs Normal control

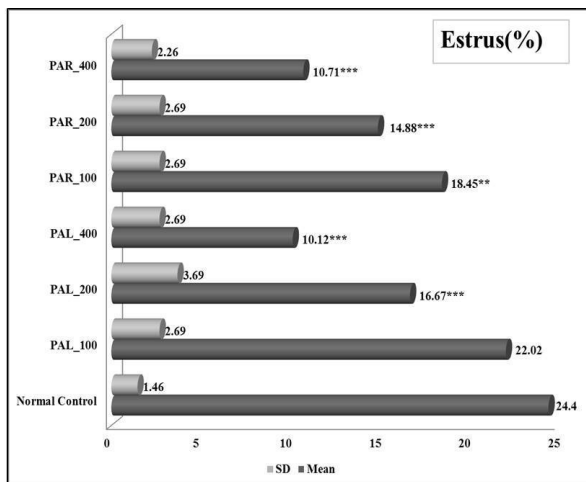


Figure 1. (A)-(i). Effect of PAL and PAR extract on estrus phase of estrous cycle during anti-fertility model. Values are expressed as Mean ± SEM for n=6, *p<0.05, **p<0.01, ***p<0.001 vs Normal control

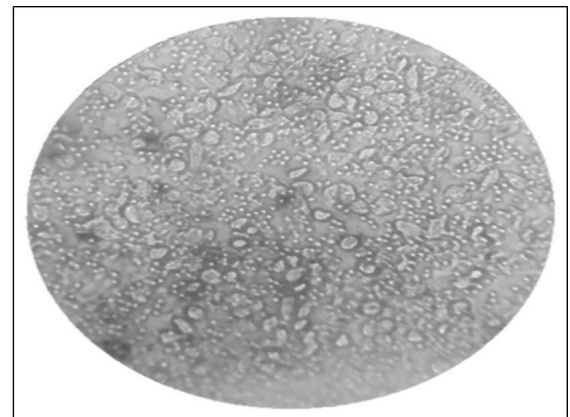


Figure 1. (B)-(ii). Microscopic image of metestrus phase observed during evaluation of estrous cycle during anti-fertility model.

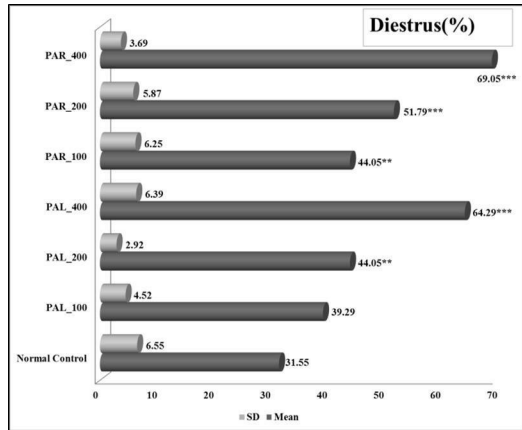


Figure 1. (C)-(i). Effect of PAL and PAR extract on diestrus phase of estrous cycle during anti-fertility model. Values are expressed as Mean \pm SEM for n=6, *p<0.05, **p<0.01, ***p<0.001 vs Normal control

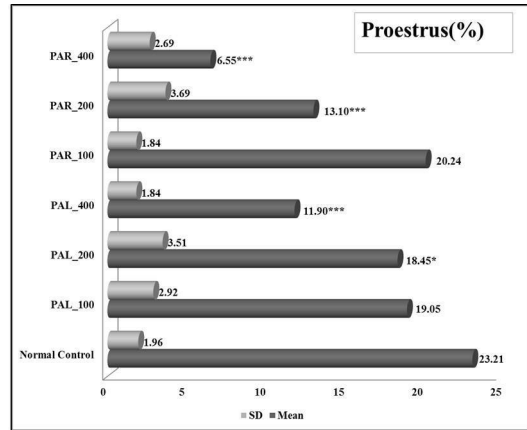


Figure 1. (D)-(i). Effect of PAL and PAR extract on prooestrus phase of estrous cycle during anti-fertility model. Values are expressed as Mean \pm SEM for n=6, *p<0.05, **p<0.01, ***p<0.001 vs Normal control

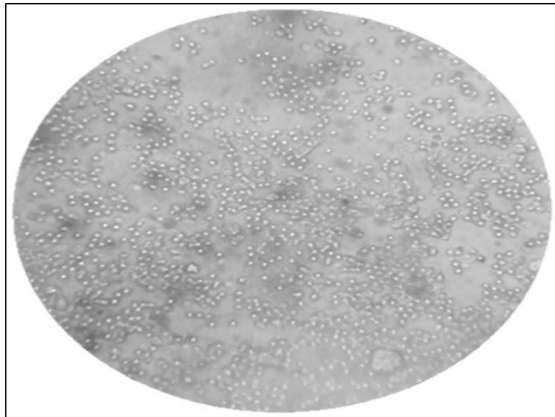


Figure 1. (C)-(ii). Microscopic image of diestrus phase observed during evaluation of estrous cycle during anti-fertility model.

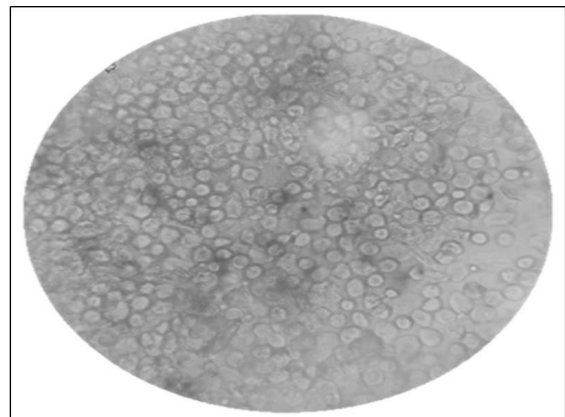


Figure 1. (D)-(ii). Microscopic image of prooestrus phase observed during evaluation of estrous cycle during anti-fertility model.

Table 3 Effect of PAL and PAR extract on length and number of the estrous cycle during anti-fertility activity.

Group and treatment	Length of Estrous Cycle (days)	Number of Estrous Cycle
Normal Control	4.29 \pm 0.14	5.83 \pm 0.41
PAL_100	4.67 \pm 0.23	5.17 \pm 0.75
PAL_200	5.20 \pm 0.51*	4.67 \pm 1.03*
PAL_400	6.19 \pm 0.81***	2.83 \pm 0.41***
PAR_100	5.05 \pm 0.41	4.83 \pm 0.41
PAR_200	6.13 \pm 0.56***	3.67 \pm 0.82***
PAR_400	7.06 \pm 0.95***	2.50 \pm 0.55***

Values are expressed in Mean \pm SEM for n=6, *p<0.05, **p<0.01, ***p<0.001 vs Normal control

Effect on reproductive organ weight

Table 5 summarizes day 29 weight data of reproductive organs, i.e., ovaries, oviducts, and uterus. In comparison to the normal control group, a statistically significant decrease in the weight of ovaries and uterus was found in both PAL and PAR

extract-treated groups in a dose-dependent manner. On the other hand, no drastic change in the oviduct weight was noted.

Effect on hormone analysis

Effects of PAL and PAR extract treatments on estrogen, FSH, LH, and progesterone levels are shown in Figure 2. When

Table 4 Effect of PAL and PAR extract on haematological parameters during anti-fertility activity.

Group and treatment	Haematological Analysis							
	WBC (10 ⁹ /L)	RBC (10 ¹² /L)	Hb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	Platelet (10 ⁹ /L)	Neutrophil (%)
Normal Control	5.04 ± 0.18	12.25 ± 1.40	12.42 ± 0.70	46.62 ± 4.35	53.07 ± 4.56	12.23 ± 0.48	1231.34 ± 104.23	18.83 ± 3.37
PAL_100	5.06 ± 0.12	12.61 ± 1.15	12.67 ± 0.64	45.70 ± 2.93	54.96 ± 3.57	12.27 ± 0.55	1244.82 ± 94.10	18.26 ± 3.03
PAL_200	5.07 ± 0.08	12.41 ± 0.96	12.11 ± 0.76	45.59 ± 3.27	55.44 ± 3.48	12.47 ± 0.48	1252.37 ± 77.86	17.66 ± 3.05
PAL_400	5.01 ± 0.10	12.07 ± 1.29	12.12 ± 1.02	47.08 ± 3.53	54.11 ± 3.21	12.32 ± 0.56	1270.97 ± 93.22	17.71 ± 3.84
PAR_100	5.10 ± 0.06	12.19 ± 1.35	12.07 ± 0.89	46.25 ± 3.90	51.43 ± 3.71	12.40 ± 0.49	1212.68 ± 108.62	18.65 ± 3.38
PAR_200	5.04 ± 0.08	12.07 ± 1.41	12.32 ± 1.08	46.41 ± 3.51	54.24 ± 4.67	12.29 ± 0.60	1239.90 ± 69.73	19.72 ± 2.89
PAR_400	5.05 ± 0.08	12.05 ± 0.99	12.35 ± 0.70	46.36 ± 4.55	51.99 ± 3.41	12.53 ± 0.50	1293.73 ± 81.45	17.37 ± 4.09

Values are expressed in Mean ± SEM for n=6

Table 5 Effect of PAL and PAR extract on organs weight during anti-fertility activity.

Group and treatment	Organ weight (mg)		
	Ovary (L+R)	Oviduct (L+R)	Uterus
Normal Control	98.06 ± 2.79	15.81 ± 0.46	288.95 ± 7.42
PAL_100	94.84 ± 2.17	15.60 ± 0.71	279.52 ± 7.76
PAL_200	92.84 ± 1.84***	15.44 ± 0.64	272.10 ± 5.69***
PAL_400	89.47 ± 2.08***	15.20 ± 0.72	265.90 ± 6.40***
PAR_100	93.20 ± 1.37**	15.30 ± 0.69	275.85 ± 6.27*
PAR_200	89.96 ± 1.25***	14.80 ± 0.50	265.85 ± 4.56***
PAR_400	85.68 ± 1.68***	14.00 ± 0.90***	258.03 ± 5.29***

Values are expressed in Mean ± SEM for n=6, **p*<0.05, ***p*<0.01, ****p*<0.001 vs Normal control

compared with the normal control, a dose-dependent decrease in estrogen, progesterone, and LH levels was observed after treatment with PAL and PAR extracts. On contrary to this, a dose-dependent increase in FSH levels was noted. In most cases, this difference was statistically significant for 200 and 400 mg/kg doses of treatments. With an increased dose of PAL, there was a ~7%–43% drop in estrogen, ~13%–49% drop in progesterone, and ~6%–22% drop in LH levels. Similarly in the case of PAR with increased dose, 14%–46% drop in estrogen, ~24%–57% drop in progesterone, and 14%–35% drop in LH levels. However, the FSH level was increased by about 19%–99% in the case of PAL and around 48%–140% in the case of PAR extracts-treated groups.

Effect on histopathology

In histopathological studies (Fig. 3, Row 1), the ovarian section from the normal control group exhibited normal cellular morphology with plenty of primary and secondary follicles, graafian follicles, or corpora lutea. The treatment of PAL and PAR extracts has slowed down the maturation of follicles and delayed the ovulation that resulted in a follicular cyst or vacuolization, i.e., the formation of fluid-filled bubble-like structure. During the observation of oviduct sections (Fig. 3, Row 2), ciliated simple columnar cells and nonciliated peg cells of the mucosal portion from the ampulla region have been observed for the following alterations. The thickness of the mucosal layer, which was higher in the case of the normal control group due to a large number

of ciliated simple columnar cells and nonciliated peg cells, was found to decrease after treatment with PAL and PAR extracts as the treatment resulted in a reduced number of ciliated simple columnar cells and nonciliated peg cells. The uterine section (Fig. 3, Row 3) from the endometrium portion was majorly focused for histological evaluation of the luminal endothelial comprising three-layer, i.e., *stratum compactum*, *stratum spongiosum*, and *stratum basalis*. Treatment with PAL and PAR extracts yielded notable changes in the endometrium (uterine gland and myometrium layer) as compared to the normal control group. Treatment with extracts resulted in the dilation of uterine glands, absence of pits and folds, and caused degeneration of the luminal epithelium compared to the normal control group. Cumulatively, all these effects must have controlled the enlargement of the endometrium layer making embryo implantation and growth difficult. From the cervical sections (Fig. 3, Row 4), it was observed treatment with PAL and PAR extracts has decreased the thickness of metaplastic squamous cells, columnar epithelium lining, and endo-cervical glands in a comparison with a normal control group and these changes have made the cervical region more hostile passage for sperms. Vaginal sections (Fig. 3, Row 5) of the normal control group, exhibited thick layers of nonkeratinized stratified squamous epithelium layer, which is consist of three layers, i.e., *stratum corneum*, *stratum granulosum*, and *stratum germinativum*, along with the elastic lamina propria as well as fibromuscular layer exhibited alterations in the same region from the groups which

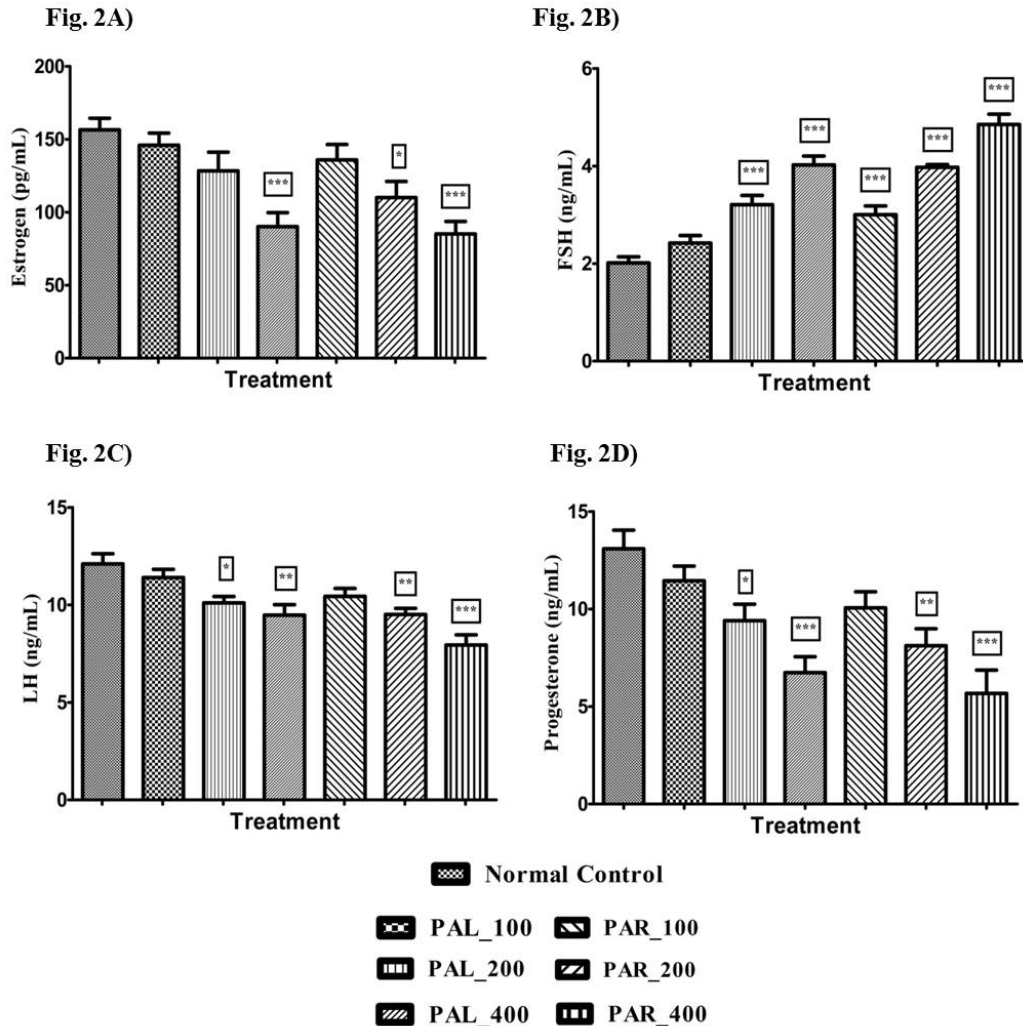


Figure 2. Effect of PAL and PAR extracts on hormonal levels during anti-fertility activity.

Fig. 2A) Estrogen, Fig. 2B) FSH, Fig. 2C) LH, and Fig. 2D) Progesterone. Values are expressed as Mean \pm SEM for n=6, *p<0.05, **p<0.01, ***p<0.001 vs Normal control

received test item treatments. Decreased thickness of all these layers has been seen after treatment probably due to decreased proliferation rate.

DISCUSSION

The present study focuses on the investigating antifertility efficacy of PAL and PAR extracts in laboratory animals. Drugs with antifertility potential have diverse modes of action and might target one or more organ systems in females, such as the hypothalamus, anterior pituitary, ovary-oviduct, uterus, cervix, and vagina, to disrupt the normal preovulatory functions (Amit and Pankaj, 2018). Such disruption may be attributed to the failure of hormonal balance and normal estrous cyclicity, leading to the inability of releasing mature ovum as well as failure to provide a healthy passage to ova and sperms. These can individually or cumulatively lead to fertilization failure suggesting an antifertility activity.

Alteration of the estrous cyclicity is one such method that regulates female fertility. Traditionally accurate determination of

four estrous cycle stages and their durations, i.e., E, M, D, and P in the same order, is being done in rats by studying vaginal cytology. The observed mean duration of 4–5 days for a cycle length in normal control rats has been supported by the literature. Usually, the E phase lasts for 12–14 hours, the M phase for 24 hours, the D phase for 55–57 hours, and the P phase for 12–14 hours. The types of cells present in the smear can help in determining the estrous cycle phase for individual females. A large number of cornified epithelial cells are present during the E phase. A few numbers of cornified epithelial cells and polymorphonuclear leukocytes are present during the M phase. D phase represents the longest phase, lasting more than 2 days with leukocytes and a small number of non-nucleated epithelial cells. The next phase is the P phase which is having mostly nucleated epithelial cells and at the end of the P phase, some cornified epithelial cells can be seen. At the end of the P phase or onset of the E phase, the female rat becomes sexually receptive to males during the dark period of time. Any interruption by pregnancy, pseudopregnancy, hormonal imbalance, or anestrus

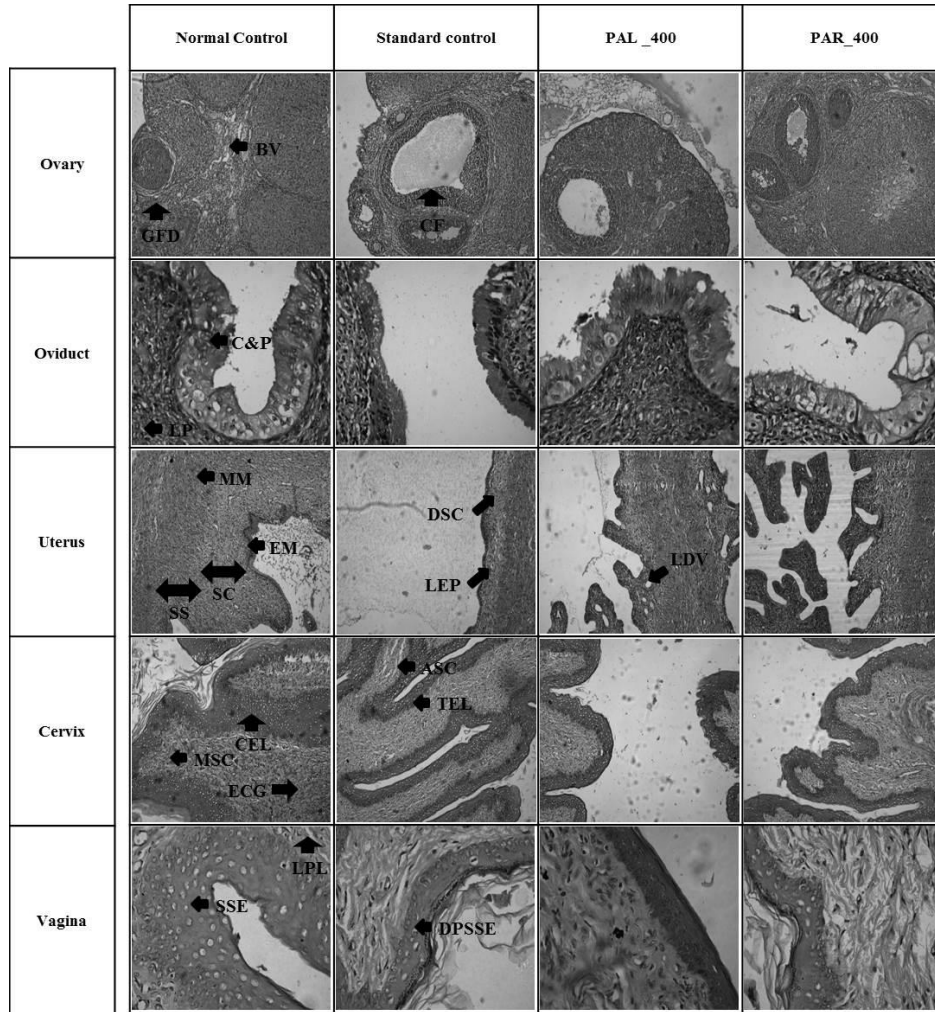


Figure 3. Effect of PAL and PAR extracts on histology of reproductive organs during anti-fertility activity. Representative H & E stained photomicrographs of the (Row 1-5) ovary, oviduct, uterus, cervix and vagina respectively from diverse treatment groups. Respective treatment given to animal is mentioned on the top of each column. Ovary: Graafian follicle degeneration (GFD), Secondary follicle (SF), Primordial follicle (PF), Zona pellucida (ZP), Ovarian stroma (OS), Vacuolisation (VSL), Blood vessels (BV), Cystic follicle (CF) and antrum (ATM) visualized at 10x; Oviduct: Ciliated and peg cell (C&P), Lamina propria (LP) visualized at 40x; Uterus: Stratum compactum (SC), Stratum spongiosum layer (SS), Endometrium (EM) and Myometrium (MM), Uterine gland (UG), Dilatation of uterine gland (DUG), Absence of pits and folds (APF) and Luminal epithelium degeneration (LED) visualized at 10x; Cervix: Metaplastic squamous cells (MSC), Columnar epithelium lining (CEL), Endo-cervical glands (ECG) and Decrease epithelium lining (DEL) visualized at 10x; Vagina: Stratum corneum (SC), Stratum granulosum (SG), Stratum germinativum (SGM), lamina propria layer (LPL), Fibromuscular layer (FML) and decreased proliferation of stratified squamous epithelium (DPSSE) visualized at 40x.

can alter the length of one or more phases and in turn lengthen the cycle (Byers *et al.*, 2012; Ngadjui *et al.*, 2015; Paccola *et al.*, 2013; Sanabria *et al.*, 2019).

The reproductive function in mammals is mainly regulated by the decapeptide called gonadotropin-releasing hormone (GnRH) which is secreted by the hypothalamo-hypophyseal axis and particularly acts on the anterior pituitary gland. Stimulation of the GnRH receptor causes the anterior pituitary to release gonadotropins, i.e., FSH and LH which are essential hormones for cyclicity, steroidogenesis, and gametogenesis. FSH initiates follicular growth and LH stimulates ovarian follicles development. LH stimulates the theca cells to produce androgens, which are

taken up by granulosa under FSH influence and converted into estrogens. LH also triggers ovulation and promotes corpus luteum development, which produces estrogen and progesterone which cumulatively prepare and maintain the endometrium for implantation (Mittal, 2020; Oyewopo *et al.*, 2012; Paccola *et al.*, 2013; Sanabria *et al.*, 2019; Sari *et al.*, 2016).

For healthy female rats with normal cyclicity, FSH and LH which are at lower levels during M and D phases, begin to increase in the P phase. LH reaches a peak and decreases as the cycle transits from P to E and becomes low, whereas FSH gradually increases and achieves a peak at the beginning of E and then decreases by end of the E phase. Estrogen is considered as

a primary hormone as its level begins to rise at the start of the M phase, achieves a peak during the mid P phase, stimulates the gonadotropin release, triggers ovulation at the late P/early E phase, and returns to point of departure at the end of E phase. Progesterone level gradually rises and sets during both M and D phases, and by the end of the P phase, it again increases to the peak followed by a decrease in the E phase. Decreased level of progesterone follows a positive feedback mechanism which increases FSH and LH production from the anterior part of the pituitary gland. LH plays the central character in ovulation and is responsible for oocyte maturation, luteinization, and rupturing of pre-ovulatory follicles (Ganaie and Shrivastava, 2010; Marcondes *et al.*, 2002; Van De Lagemaat *et al.*, 2009).

Body weight gain has been reported as an important parameter that significantly regulates gonadotropin secretions from the pituitary gland and the balance of these ovarian and extra-ovarian hormones, as mentioned above, contributes to maintaining steady cyclicality in females (Yinusa *et al.*, 2010). Therefore, a significant decrease in the body weight gain after both, PAL and PAR treatments (as observed in Table 2), might have actively contributed to altering gonadotropin levels, particularly estrogen, affecting the normal functionality of the ovary and uterus resulting in the disturbing cyclicality. Reproductive organs like the ovary, uterus, and vagina are the target organs that manifest differential sensibility to hormones as their structural and functional maintenance is completely dependent on hormones. Any changes in hormonal circulatory levels affect the internal environment of reproductive organs and thereby lead to alteration in histology and weight of organs. Thus, complex relationships within and between, these self-balanced ovarian and extra-ovarian hormones (i.e., estrogen, progesterone, FSH, and LH), as well as various events and phases of ovarian and estrous cycles (i.e., maturation of pre-ovulatory follicles and ovulation), determine the histological, physiological, morphological and biochemical changes within the reproductive organs during these phases (Kafali *et al.*, 2004; Karateke *et al.*, 2019; Renczes *et al.*, 2020; Shah *et al.*, 2016b).

In the present antifertility model, reduced estrogen levels (Fig. 2) must have disrupted the gonadotropins' balance, which might have disturbed cyclicality by extending the duration of the mean length of the estrous cycle and reducing the number of the estrous cycles (Table 3). This reduction in estrous cycle counts can be attributed to decreased LH, progesterone, and estrogen levels which leads to as follows: (i) a prolonged percentage of D phase where follicle matures but do not ovulate, causing a failure of luteal regression at the normal time in non-pregnant females (Mair and Watson, 2019) and (ii) shortened percentage of P and E phases, where the endometrium and vaginal epithelium do not get sufficient time to prepare themselves for successful fertilization, implantation, and sustaining a pregnancy. Thus, a significant reduction in the number of the estrous cycle in females, after treatments with PAL and PAR extracts, can certainly be correlated with the fertility decrement. A shorter number of estrous cycling can cause systemic pathology characterized by profound hormonal deregulation of reproductive physiology impairing the successful ovulation, fertilization of an ovum, and/or implantation of a zygote

or sometimes premature spontaneous abortion or miscarriage (Jaini *et al.*, 2015).

After PAL and PAR treatment, estrogen, LH, and progesterone levels decreased dose-dependently, but, FSH levels increased instead. It has been documented that estrogen, produced by maturing ovarian follicles, is the primary trigger for pre-ovulatory gonadotropin surge in rodents as well as non-rodents. Estrogen induces both positive and negative feedback on gonadotropin secretion. Negative feedback on estrogen can raise the level of FSH (Mahesh and Muldoon, 1987). Decreased levels of progesterone (Fig. 2) from corpora lutea might also have affected uterine, vaginal, and cervical histology (Fig. 3). Previous studies showed that decreased levels of progesterone produced by corpora lutea affect the implantation as well as maintenance of gestation (Goyeneche *et al.*, 2003).

Estrogen and progesterone have been reported to modulate the growth of ciliated and nonciliated cells in the oviduct (Barton *et al.*, 2020). A decrease in the numbers of ciliated and non-ciliated cells, from the section of oviduct ampulla, after PAL and PAR treatments, might have retarded movement of sperms, ovum, and/or zygote making them unable to reach the destination causing either failure of fertilization or failure of implantation. A major noteworthy change was an alteration in the thickness of the endometrial wall of the uterus. Sloughing off of decidualized endometrial wall due to significantly decreased glandular and luminal epithelium proliferation and increased apoptosis of cells can be attributed to the low levels of progesterone as similar effects have been reported in the literature (Al-Qudsi and Linjawi, 2012). This resulted in a reduction of endometrial wall thickness making uterine conditions favorable for preimplantation loss. In the cervix, reduced proliferation of different epithelial layers can be attributed to decreased levels of progesterone. As per the studies conducted in the past, estrogen regulates the differentiation of vaginal epithelial cells, increased weight, thickening, keratinization in the squamous epithelium layer as well as in increasing density of lamina propria and fibromuscular layer (Li *et al.*, 2017, 2018). Similarly in the vaginal region, due to hormonal imbalance, decreased proliferation of stratified squamous epithelium layer, the height of epithelium and thickness of the epithelium, and increased squamous epithelium hypoplasia were observed. All these changes in the cervix and vagina cumulatively resulted in a hostile environment for sperm transport. Thus, the above discussed histological several changes in different reproductive organs can be linked with the hormonal imbalance in the antifertility model. Studies have also reported that low levels of estrogen cause degeneration of follicles and mineralization of follicles in the ovary (Gilbreath *et al.*, 2014). The hormonal imbalance by both extracts resulted in reduced numbers of mature follicles as well as corpora lutea, observed from the ovarian section.

CONCLUSION

Prompt quest for an innovative constituent entity with improved salutary action and the lesser side effects is always on demand. Phyto-constituents acquired from herbal preparations are essential because of their fewer or no side effects. The outcomes of the present exploration indicate that PAL and PAR extract

retains antifertility properties, which are facilitated via disturbing hormonal balance, changing endometrium receptiveness, and altering the histological behavior of organs. Future investigation can be carried out to find out the exact molecular mechanism for these phyto-constituent's modes of action through the use of advanced medical technologies. This provides a guidance corridor and opportunities for the application of such phytochemicals acting as fertility inhibitors by using up-to-date medical tools that work through various mechanisms to support healthy birth control practices (contraception).

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ETHICAL APPROVAL

All authors hereby declare that the "Committee for the Purpose of Control and Supervision of Experiments on Animals" (Compendium of CPCSEA, 2018) was followed. Authorization and ethical approvals were acquired proceeding to commencement of the research work on animals, and that the experiments were accomplished in harmony with the above guidelines. The entire animal research was executed in the department of pharmacology, VNS College of Pharmacy, Bhopal (India) with due permission from the Institutional Animal Ethics Committee (IAEC) (Reg. No. PH/IAEC/VNS/2K21/05).

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DECLARATION OF COMPETING INTEREST

This investigation research was hypothesized and implemented by the first author under the supervision of the second author. Data exploration and manuscript preparation were through by the first and second authors together. There is no conflict of interest.

AUTHORS' CONTRIBUTIONS STATEMENT

First author: Conceptualization, Investigation, Methodology, Software. Second author: Supervision, Validation. Third author: Writing, draft preparation, reviewing, editing

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the first author upon reasonable request.

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