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Effects of *Physalis angulata* Leaf Extract on Female Reproductive Organs Following Busulfan Injection in Rats

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ABSTRACT

Physalis angulata L. (ciplukan) is traditionally used as an herbal remedy with anticancer and antioxidant properties. At the same time, busulfan, a chemotherapy alkylating agent, causes gonadotoxicity and oxidative stress that impair female reproduction. This study aimed to evaluate the effect of *P. angulata* on reproductive function after busulfan administration in female rats. Twenty-five 6-week-old female rats were randomly divided into: Group I (control), Group II (busulfan only), Group III (*P. angulata* only), Group IV (busulfan followed by *P. angulata* after 14 days), and Group V (busulfan and *P. angulata* administered for 28 days). FSHR and LHR expression in the ovaries and MDA levels in the ovaries and uterus were measured to assess reproductive changes. Busulfan showed no significant effect on FSHR and LHR, whereas *P. angulata* induced downregulation, suggesting a potential negative feedback mechanism on ovarian receptors. Ovarian MDA showed a decreasing trend with combined treatments, while uterine MDA peaked after busulfan but declined markedly with *P. angulata*, indicating its role in alleviating oxidative stress. In conclusion, *P. angulata* may modulate hormonal balance in rat ovaries and decrease oxidative stress in the uterus after busulfan treatment.

1. Introduction

Physalis angulata L., commonly known as ciplukan, has been traditionally used as an herbal remedy for various ailments. Recent studies have highlighted its potential as an anticancer agent due to its ability to inhibit metastasis and tumor angiogenesis (Khoirunnisa *et al.* 2024). As a wild plant rich in flavonoids, *P. angulata* extract demonstrates strong antioxidant activity (Ekayanti *et al.* 2022), which contributes to its role in promoting the regeneration of damaged cells (Suryani *et al.* 2022). The leaves of *P. angulata* extract contain a total flavonoid content of 2.29% (w/w) (Angela *et al*. 2023). Since flavonoids are well-known contributors to antioxidant defense (Vicente and Bosciau 2018), this relatively high content supports the strong antioxidant capacity reported in previous studies. Previous studies have shown its protective effects against tissue damage, including renal damage induced by ethylene glycol (Angela *et al.* 2023) and hypertensive conditions

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(Nugrahenny *et al.* 2022). Given the strong antioxidant potential of *P. angulata*, explore its role in mitigating the chemotherapeutic effects of agents such as busulfan, which are known to induce oxidative stress and reproductive toxicity.

Busulfan is an alkylating chemotherapeutic agent commonly employed in conditioning regimens for hematopoietic stem cell transplantation (Ciurea and Anderson 2009). Its cytotoxic effects are primarily mediated through DNA cross-linking, which disrupts DNA replication and halts cancer cell mitosis at the G1 phase (Guidolin *et al.* 2023). While effective in treating hematological malignancies and solid tumors, busulfan has well-documented gonadotoxic side effects. In male subjects, busulfan causes severe and multifaceted reproductive damage, primarily through its toxic effects on spermatogonial stem cells (SSCs), leading to spermatogenesis disruption, reduced sperm quality and quantity, hormonal imbalance, and impairment of the blood–testis barrier, which ultimately contributes to infertility (Gutierrez *et al.* 2016). In female subjects, busulfan impairs granulosa cell function, induces significant follicular atresia, and disrupts ovarian endocrine regulation (Abir *et al.* 2008; Mohamed *et al.* 2019). These effects often culminate in premature ovarian failure, characterized by diminished estrogen production and compensatory elevation of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels. Additionally, DNA double-strand breaks have been observed in oocytes from follicles exposed to busulfan metabolites (Petrillo *et al.* 2011). The resulting ovarian toxicity can significantly compromise oocyte viability and female fertility.

Preservation of fertility among women receiving chemotherapy remains a major clinical challenge, and antioxidant-based approaches are increasingly explored as supportive strategies. However, most studies have focused on synthetic antioxidants or other medicinal plants, while evidence on *P. angulata's* protective effects on female reproductive organs remains limited. Previous studies have reported that *P. angulata* protects male reproductive organs due to its high antioxidant content and regenerative properties. Currently, no comprehensive studies have investigated whether *P. angulata* leaf extract can attenuate busulfan-induced ovarian toxicity in females. This condition represents a critical research gap, as understanding the plant's role in mitigating oxidative damage in ovaries and uterus could contribute to the development of novel adjunct therapies for fertility preservation in cancer patients.

Therefore, *P. angulata* leaf extract is hypothesized to exert protective effects against busulfan-induced ovarian damage. This study aims to evaluate the potential of *P. angulata* leaf extract to preserve function and mitigate reproductive organ toxicity induced by busulfan administration in a female rat model.

2. Materials and Methods

2.1. Study Site and Ethical Approval

This study was conducted at the Laboratory Animal Management Unit, Division of Physiology, and Division of Anatomy, Histology and Embryology, School of Veterinary Medicine and Biomedical Sciences (SVMBS), IPB University. All experimental procedures were conducted in accordance with the OIE Principles for Animal Welfare. Our study was approved by the Animal Ethics Commission of SVMBS, IPB University, under ethical approval number 247KEH/SKE/IX/2024.

2.2. Preparation of *Physalis angulata* L. Leaf Extract

Physalis angulata L. leaves were obtained from the Balitro Medicinal and Aromatic Plants Research Center, Bogor, Indonesia. Leaves were dried at 50°C for three days and then ground into a fine powder. The extraction was performed using the maceration method with 70% ethanol at a ratio of 5:1 (solvent to powder) for 72 hr. The filtrate was concentrated using a rotary evaporator at 40°C and 50 rpm to obtain a thick ethanol extract. The extract was administered at a dose of 300 mg/kg body weight (BW) in 0.6 mL per rat per day.

2.3. Preparation of Busulfan Solution

Busulfan was first dissolved in 0.5 mL of 10% dimethyl sulfoxide (DMSO). Then, 4.5 mL of phosphate-buffered saline (PBS) preheated to 37°C was added to the solution. The mixture was vortexed at 2500 rpm for 15 min to obtain a homogeneous solution. The prepared busulfan solution was maintained at 37°C in a thermos until administration. A single dose of 40 mg/kg BW (0.1 mL/rat) was administered intraperitoneally.

2.4. Preparation of Animals and Sampling

A total of 25 female Sprague-Dawley rats, six weeks old (average body weight 130-150 g), were used in this study. The rats were divided into five groups, each group consisting of five virgin rats. The groups were I (control),

II (busulfan), III (*P. angulata* leaf extract), IV (busulfan and *P. angulata* leaf extract after 14 days of busulfan administration), and V (concurrent administration of busulfan and *P. angulata* leaf extract for 28 days). The group size (n = 5) was chosen based on precedent in similar rodent studies, ethical considerations to minimize animal use (3R principle), and logistical constraints. Rat body weight was measured weekly; the data presented were the initial body weight (D-0) and the final body weight (D-28). The body weight of the rats was measured to determine the effects of giving busulfan and *P. angulata* leaf extract.

All of the rats were euthanized for organ sampling at Day 28. The ovaries and uteri were weighed before being used for measuring other parameters. Parameters to be measured included measurement of malondialdehyde (MDA) as a stress indicator and measurement of gene expression of FSHR and LHR in ovaries using real-time PCR/qPCR.

2.5. Experimental Animals and Experimental Design

The rats were acclimatized for three weeks before treatment and given an anti-helminthic. During the experiment, rats were given standard commercial rat feed of 10% body weight and drank ad libitum. The rats' body weight and remaining feed were weighed daily to determine daily weight gain and feed consumption during treatment. Busulfan was injected on Day 0 in Groups II, IV, and V. *P. angulata* leaf extract (300 mg/kg BW) was administered in Group III for 28 days. The single dose of busulfan was 40 mg/kg BW, administered as 0.1 mL/rat. *P. angulata* leaf extract 300 mg/kg BW was given for 28 days of treatment from Day 0 till Day 28 after busulfan injection in Group V and from Day 15 until Day 28 after busulfan injection in Group IV. The dose of *P. angulata* leaf extract was 0.6 mL administration/rat/day. The research flow is shown in Figure 1.

2.6. Measurement of Malondialdehyde (MDA)

Ovarian MDA levels were assessed as an indicator of oxidative stress using the thiobarbituric acid reactive substances (TBARS) method (Messarah *et al.* 2013). Briefly, 1.5 mL of diluted ovarian homogenate in phosphate-buffered saline (PBS) was centrifuged at 3500 rpm at 4°C for 10 minutes. A 4 mL reagent mixture (15% HCl 0.25N containing 15% trichloroacetic acid (TCA), 0.38% thiobarbituric acid (TBA), and 0.5% butylated hydroxytoluene (BHT) was added to the supernatant. The mixture was incubated at 80°C for 1 hr, cooled in water, and centrifuged again at 3500 rpm for 10 minutes at 4°C. The absorbance of the supernatant was measured at 532 nm using a spectrophotometer.

2.7. RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from ovarian tissue using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, Massachusetts, USA), and complementary DNA (cDNA) was synthesized using ReverTra Ace-α (Toyobo, Tokyo, Japan), according to the manufacturer's protocols. qRT-PCR was performed using the ABI StepOne Plus Real-Time PCR System and Thunderbird SYBR qPCR Mix (Toyobo, Tokyo, Japan). Expression levels of the target genes, rat follicle-stimulating hormone (FSHR) and luteinizing hormone (LHR), were normalized against GAPDH and expressed as fold changes. Primer sequences are listed in Table 1.

2.8. Data Analysis

Data on body weight, ovarian weight, MDA levels, and gene expression (FSHR and LHR) were analyzed using one-way analysis of variance (ANOVA). When significant differences were observed, post hoc comparisons were conducted using Duncan's range test (Abuoghaba *et al.* 2021). All statistical analyses were performed using GraphPad Prism version 8.4.0. A p-value of less than 0.05 was considered statistically significant.

3. Results

Body weight was recorded at the beginning (Day 0) and end (Day 28) of the study, while ovarian and uterian weight were measured post-sacrifice, before further biochemical analyses. Table 2 summarizes the body, ovarian, and uterine weights across the experimental groups. There was no significant effect of treatment on body weight, ovarian weight, or uterine weight ($p>0.05$). Among all groups, Groups III (*P. angulata* leaf extract) and IV (busulfan and *P. angulata* leaf extract after 14 days of busulfan administration) had the highest final body, ovarian, and uterine weights. In contrast, group II (busulfan) had the lowest final body and ovarian weights among all groups.

Gene expression analysis of FSHR (Follicle Stimulating Hormone Receptor) and LHR (Luteinizing Hormone Receptor) in ovarian tissue revealed significant changes in response to busulfan and *P. angulata* extract treatments

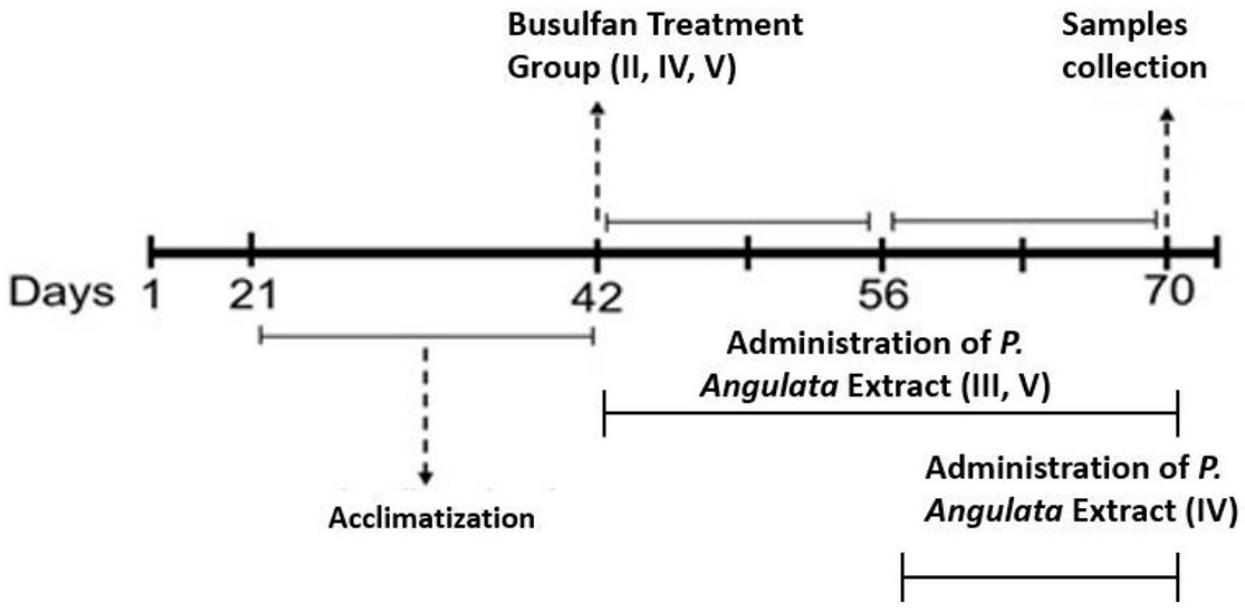

Figure 1. Research flow

Table 1. primer used for qRT-PCR

Gene	Sequences (5'-3')	Size (bp)
FSHR forward	CAT CAC TGT GTC CAA GGC CA	101
FSHR reverse	TGC GGA AGT TCT TGG TGA AAA	
LHR forward	CGG GCT GGA GTC CAT TCA	70
LHR reverse	TTC TTT GGA GGG CAG TGT TTT C	
GAPDH forward	TGC CAA GTA TGA TGA CAT CAA GAA G	71
GAPDH reverse	AGC CCA GGA TGC CCT TTA GT	

Table 2. The body, ovarian, and uterine weights of rats during the treatment

Parameters	Groups				
	I	II	III	IV	V
Initial body weight	139.11±9.89	138.40±7.12	145±15.83	151.7±11.33	139.7±14.70
Final body weight	204.17±12.22	201.86±20.73	211±23.35	225.43±16.24	200.29±19.40
Δ Body weight	22.72	22.56	30.40	42.73	26.29
Ovarian weight	0.065±0.010	0.062±0.012	0.082±0.017	0.089±0.010	0.068±0.012
Uterine weight	0.304±0.107	0.391±0.166	0.397±0.226	0.448±0.226	0.333±0.115

(Figures 2A and B). A slight downregulation of FSHR and LHR gene expression was observed in the busulfan-only group (II) compared to the control (I), as indicated by a 2.3x fold change. Intriguingly, a significant down-regulation of FSHR and LHR genes ($p<0.05$) was observed in group III that had been treated by *P. angulata* leaf extract, suggesting *P. angulata* impairs follicular development and FSH-LH signaling pathways. Furthermore, a slight downregulation of FSHR and LHR gene expression was observed in groups IV and V, which were administered both substances (busulfan and *P. angulata* leaf extract).

Malondialdehyde (MDA) levels in ovarian tissue were assessed on day 28 as an indicator of lipid peroxidation and oxidative stress (Figure 3A). The control group (I) showed the highest average MDA level, representing the baseline oxidative status. In the busulfan-treated group (II), MDA concentration decreased slightly, which is unusual, as busulfan is expected to induce oxidative stress. This result might suggest a physiological compensatory antioxidant response that reflects the cytotoxic effects of busulfan, leading to suppressed cellular metabolism or structural damage. Administration of *P. angulata* leaf

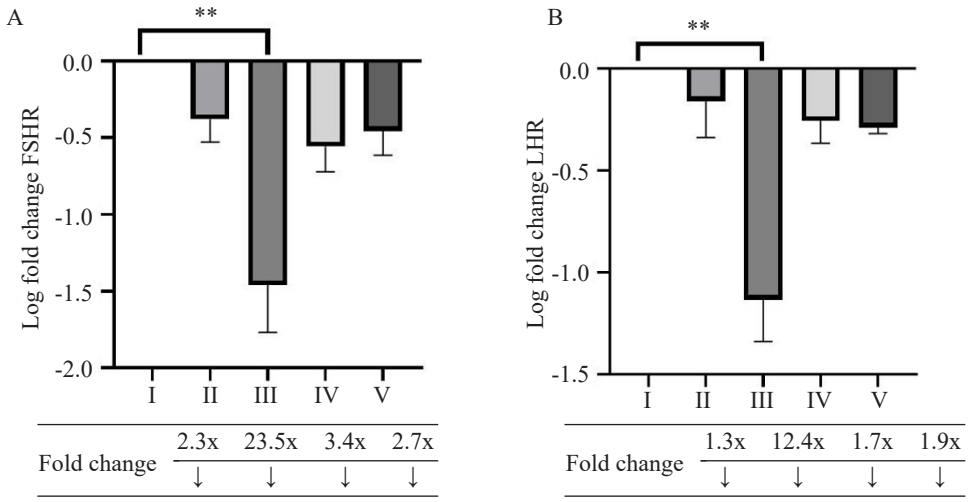

Figure 2. Gene expression changes of (A) FSHR and (B) LHR in rat ovaries. Relative expression is expressed as fold change of control (I), normalized to GAPDH values. The Kruskal-Wallis test determined statistical significance–Wallis test: *p<0.05, **p<0.01. ↓: down-regulation, ↑: up-regulation

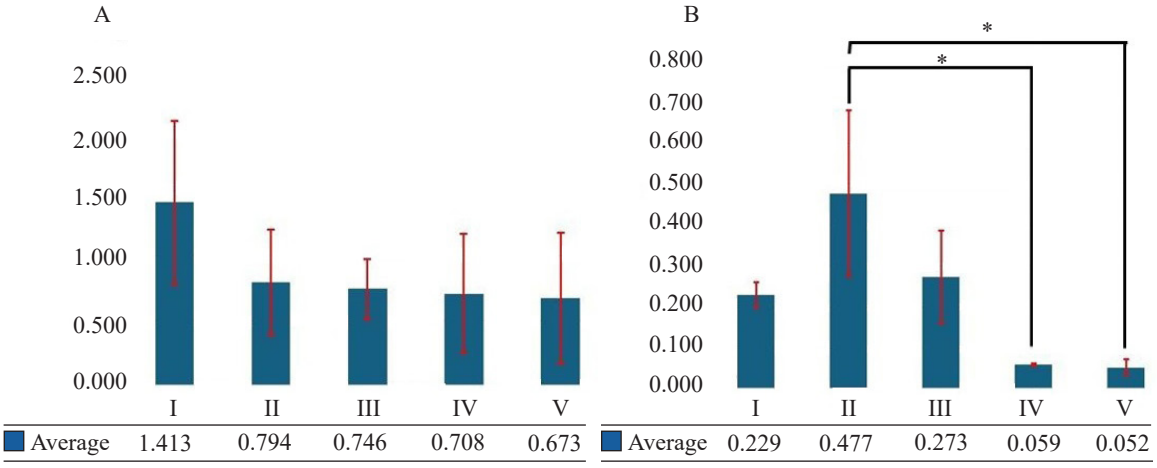

Figure 3. MDA level (A) in the ovarium and (B) in the uterus

extract (group III) resulted in an MDA level similar to that of group II, suggesting mild antioxidant activity under normal physiological conditions. Further reductions in MDA levels were observed in groups III, IV, and V, which showed slightly lower levels than in the busulfan-administered group (Group II). These trends suggest that *P. angulata* may confer beneficial effects against oxidative damage in the ovary, particularly when continuously administered alongside the oxidative insult. However, statistical analysis indicated that these differences were not significant ($p>0.05$). Despite the lack of significance, the consistent trend of decreasing MDA in the *P. angulata*-treated groups supports the hypothesis of its potential antioxidant role in ovarian tissue.

Conversely, MDA levels in the uterus on day 28 demonstrated a different trend (Figure 3B). The highest MDA concentration was observed in the group receiving busulfan (Group II), as expected, suggesting oxidative stress in the uterus. The MDA level in group III was similar to that of group I. Interestingly, administration of both substances (Groups IV and V) results in a significant reduction in MDA levels compared to Group II, which received only busulfan, suggesting a stronger antioxidant effect of *P. angulata* in uterine tissue, especially when

administered alongside or following an oxidative challenge.

4. Discussion

In this study, busulfan administration did not significantly alter overall body weight gain, with Group II (busulfan only) showing a profile similar to that of the control group (Group I). Although statistical significance was not observed, the lowest weight gain in Group II suggests a subtle negative impact on general metabolism, likely reflecting the cytotoxic and gonadotoxic properties of busulfan. Busulfan is well known to disrupt cellular replication through DNA cross-linking, which can reduce cellular proliferation in various organs, leading to decreased organ and body weight (Ahar *et al.* 2014; Ali *et al.* 2023). Interestingly, the highest body weight was recorded in Group IV, which received busulfan followed by *P. angulata* leaf extract treatment, followed by Groups III and V. This pattern suggests a trend toward enhanced recovery or growth-promoting effects of *P. angulata* leaf extract, which may be associated with its antioxidant and regenerative properties. However, these observations did not reach statistical significance. Previous studies have reported that *P. angulata* leaf extract contains flavonoids, polyphenols, and other phytochemicals that support cellular repair and mitigate oxidative stress (Ahar *et al.* 2014; Abdollahifar *et al.* 2020).

Uterine weight among the experimental groups did not differ significantly. However, the highest uterine weight was observed in Group IV, suggesting a trend toward tissue maintenance or partial recovery following *P. angulata* administration. Regarding ovarian weight, no significant differences were observed among Groups I, II, and V, although a slight reduction was noted in the busulfan-only group. This subtle reduction may indicate early or mild ovarian toxicity induced by busulfan, which is consistent with previous findings that busulfan impairs granulosa cell function and induces follicular atresia (Abir *et al.* 2008; Mohammed *et al.* 2019). Notably, the highest ovarian and uterine weights were observed in Group IV, suggesting that extended *P. angulata* treatment may confer a potentially beneficial effect on reproductive tissues, possibly through its antioxidant properties. These findings emphasize the importance of integrating tissue weight data with biochemical and molecular assessments, as organ mass alone may not fully capture functional or structural impairment.

MDA levels were measured in ovarian and uterine tissues to evaluate lipid peroxidation and oxidative damage. Group II (busulfan-treated) showed elevated uterine MDA levels compared with other groups, indicating oxidative damage, consistent with previously reported effects of busulfan in promoting ROS formation and lipid peroxidation (Fang and Zhong 2020). Elevated MDA levels reflect peroxidative damage to membrane polyunsaturated fatty acids, which compromises cellular homeostasis and may contribute to tissue dysfunction (Powers and Jackson 2008; Ayala *et al.* 2014). Groups III, IV, and V, which received *P. angulata* either after or concurrently with busulfan, showed lower ovarian and uterine MDA levels than both the control and busulfan-only groups. While reductions in ovarian MDA were not statistically significant, uterine MDA levels were significantly decreased in these groups, suggesting a tissue-specific response to *P. angulata* treatment. The lack of a significant reduction in ovarian MDA may reflect intrinsic tissue variability, compensatory antioxidant mechanisms, or the relatively small sample size, underscoring the need for cautious interpretation of trends.

These results are in line with previous studies showing that oxidative stress can impair folliculogenesis and steroidogenesis, which in turn compromises ovarian function (Paine *et al.* 2013). The observed decrease in MDA levels following *P. angulata* leaf extract administration suggests a potentially beneficial mechanism, likely due to its high levels of flavonoids, polyphenols, and vitamin C. These compounds are known to scavenge free radicals, upregulate endogenous antioxidant defense systems, and potentially reduce lipid peroxidation in reproductive tissues (Speisky *et al.* 2022).

Busulfan is well documented to disrupt the balance between pro-oxidant and antioxidant mechanisms, leading to excessive generation of reactive oxygen species and oxidative byproducts such as MDA (Jalili *et al*. 2018; Li *et al.* 2018). Oxidative stress plays a key role in female reproductive dysfunction by compromising membrane integrity, inducing cellular apoptosis, and reducing tissue viability (Agarwal and Mellado 2012). Lipid peroxidation primarily targets polyunsaturated fatty acids in cell membranes, resulting in structural damage, altered membrane fluidity, and impaired signal transduction. Thus, MDA serves as a reliable biomarker for evaluating oxidative damage within tissues, though non-significant trends, such as those observed in ovarian MDA levels, require careful interpretation (Ayala *et al.* 2014).

FSHR and LHR gene expression in the ovaries revealed notable variations across treatment groups.

FSHR is primarily expressed in granulosa cells of developing follicles, while LHR is expressed in theca cells of mature follicles and the corpus luteum (Khalil *et al.* 2022). In the control group, normal expression levels reflect physiological baseline gonadotropin receptor status. Busulfan administration slightly reduced FSHR and LHR expression, which may reflect decreased granulosa and theca cell viability, consistent with its cytotoxic and gonadotoxic effects (Gabrielsen and Tanrikut 2016). Groups treated with *P. angulata* leaf extract also showed reduced FSHR and LHR expression compared with controls, with LHR exhibiting a more pronounced decrease. Given that *P. angulata* contains phytoestrogens, such as withanolides, flavonoids, and polyphenols (Rodrigues *et al.* 2018), these reductions may reflect negative feedback via the hypothalamic-pituitary-gonadal axis rather than direct cytotoxicity. Phytoestrogens could suppress GnRH secretion, thereby reducing FSH and LH release and downregulating receptor expression in ovarian tissues (Marques *et al.* 2016; Bosch *et al.* 2018).

During normal pubertal development, pulsatile GnRH release regulates FSH and LH secretion, with low-frequency GnRH pulses increasing FSH mRNA levels. At the same time, estrogen provides negative feedback to both GnRH and gonadotropin release (Oduwole *et al.* 2011; Sharma *et al.* 2021). Any modulation of the HPG axis by exogenous phytoestrogens, such as those found in *P. angulata*, could thus influence gonadotropin receptor expression, follicular maturation, and ovarian function.

Overall, the data indicate that busulfan potentially increases oxidative stress in reproductive tissues, while *P. angulata* leaf extract may exert moderately mitigating effects through antioxidant activity and phytoestrogenic modulation. Although many observed changes did not reach statistical significance, the trends observed in MDA levels, gonadotropin receptor expression, and organ weights are consistent with previously reported mechanisms of oxidative stress mitigation and hormonal modulation by plant-derived antioxidants. These results provide a basis for further investigation of *P. angulata* leaf extract as a candidate for supporting reproductive health under chemotherapeutic stress. Future studies should include larger sample sizes, functional fertility assessments, and detailed histological quantification to validate these preliminary findings.

In Conclusion, Busulfan-induced receptor suppression may be attributed to cytotoxic effects and oxidative damage, whereas *P. angulata* extract estrogens-like compounds may trigger a negative feedback mechanism, reducing gonadotropin receptor expression. Future studies with optimized dosing, extended treatment duration, and molecular profiling are recommended to further explore its therapeutic potential for preserving female reproductive health.

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