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RESEARCH ARTICLE

# LC-HRMS-MS Profiling and Partial Estrogen Agonist Evaluation of *Iresine Diffusa* Ethyl Acetate Extract in Prepubertal Rats Model

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**Abstract**

Ethnopharmacological studies on *Iresine diffusa* and prior research on its phytochemicals have identified potential estrogenic effects. This study aims to evaluate the partial estrogen agonist effects of *Iresine diffusa* ethyl acetate extract (IDEA) in a prepubertal mouse model as a candidate selective estrogen receptor modulator (SERM). SERMs are of interest because they selectively modulate estrogenic responses in specific tissues, thereby enabling therapeutic interventions with minimal side effects. The *in vivo* study involved 19-day-old prepubertal female rats, with no established hormonal production, and was divided into eight groups, each consisting of five rats. These included a normal group, an estradiol control group, three IDEA groups with estradiol to identify antiestrogenic effects, and three IDEA groups without estradiol to identify estrogenic effects. Samples were administered orally for 16 days. Observed parameters included vaginal cytology smears, wet weight of the uterus, histological analysis of the uterus and ovaries, and hormone levels (estrogen, luteinizing hormone/LH, follicle-stimulating hormone/FSH, and progesterone). All data were analyzed by one-way ANOVA using GraphPad Prism. Differences with *p*-values less than 0.05 were considered statistically significant. LC-HRMS-MS phytochemical screening of IDEA revealed the presence of Kaempferol, Bakuchiol, and Afzelin. Cytological observations in the estrogenic test group showed a high number of cornified cells (+++), whereas in the antiestrogenic group, they were present in lower numbers (++). Evaluation of partial estrogenic agonist activity in prepubertal rats revealed differences in uterine wet weight (UWW), endometrial thickness, uterine diameter, follicular maturity in the ovaries, and higher hormone levels in both the estrogenic test group (IDEA) and the antiestrogenic test group (Estradiol+IDEA) compared to the Normal group (PEN), with significant differences ($p < 0.05$). The findings regarding partial estrogen agonism were confirmed and show the illustration of the effect of partial agonism by the results of each parameter test in the antiestrogenic test group (estradiol+IDEA), which showed lower mean values compared to the estrogenic test group.

**Keywords:** *Partial* estrogen agonist, *Iresine diffusa*, Prepubertal rat, Estrogen, LH, FSH, Progesterone

## Introduction

A fundamental understanding of the estrogen signaling system and its interactions with estrogen receptors (ERs) is essential in modern biopharmaceutical research, given estrogen's central role in a wide range of physiological and pathological processes, including reproductive health, bone density, cardiovascular function, and the etiology of breast cancer and endometriosis [1,2]. Estrogenic effects, primarily mediated by ERα (alpha) and ERβ (beta), vary widely across tissues and physiological states. [3,4]. This complexity underlies the concept of Selective Estrogen Receptor Modulators (SERMs) [5]. SERMs

are a class of compounds that are uniquely capable of acting as estrogen agonists (eliciting estrogen-like responses) in specific tissues, while simultaneously functioning as estrogen antagonists (blocking estrogen responses) in others. This tissue-specific action is crucial for a favorable pharmacological profile, enabling targeted therapeutic intervention with minimal side effects. [6,7]. Research conducted by Stephen R.D. Johnston on the preclinical prerequisites for developing SERM drugs with tamoxifen-like effects identified 10 evaluation criteria for assessing a drug candidate's potential as an SERM. This study evaluated 2 key criteria: the SERMs compounds under investigation must exhibit stronger binding affinity for estrogen receptors, a critical parameter for ensuring effective receptor binding and modulation in inhibiting estrogen activity that can trigger cancer cell proliferation, such as in hormonal conditions. High affinity allows these compounds to interact selectively and effectively with both the ESR1 and ESR2 receptors, thereby offering significant therapeutic potential in the management of estrogen-dependent breast cancer [8]. Additionally, this study emphasizes the importance of reducing agonist effects in uterotrophic assays as a safety parameter. Uterotrophic testing evaluates the extent to which SERMs compounds can reduce estrogenic stimulation in uterine tissue, such as uterine wall thickening, thereby avoiding side effects like hyperplasia or the risk of uterine tumors, common challenges in conventional Tamoxifen therapy. Thus, successful compounds must demonstrate the ability to reduce these agonist effects, thereby ensuring a better safety profile for long-term clinical applications [9].

Pharmacologically, this dual mechanism of action enables SERMs to be used to treat conditions such as breast cancer (Tamoxifen) [11], to prevent postmenopausal osteoporosis (Raloxifene) [12], and to potentially reduce menopausal symptoms without increasing the risk of endometrial cancer associated with conventional estrogen hormone replacement therapy [10]. According to Johnston's 2005, one parameter among the ideal profile of a novel SERMs (in comparison with Tamoxifen) is reduced agonist effects in uterothropic assays [8]. *Iresine diffusa* has long been recognized in traditional medicine as an effective remedy for regulating the menstrual cycle and for treating aging. From an ethnopharmacological perspective, the use of this plant suggests that *Iresine diffusa* contains bioactive compounds that may act as phytoestrogens. Phytoestrogens are natural compounds with a chemical structure similar to the hormone estrogen, allowing them to interact with estrogen receptors in the human body [4]. In *Iresine diffusa*, this hypothesized phytoestrogenic activity is supported by a partial agonist mechanism against estrogen receptors, meaning the compounds present not only fully mimic the effects of natural estrogen but can also modulate receptor responses according to the body's physiological conditions. The traditional use of *Iresine diffusa* as a menstrual regulator indicates that its extract or active compounds play a role in regulating reproductive hormones, which is undoubtedly related to estrogenic action. This partial-agonist mechanism allows for safer, more controlled biological effects than full agonists, suggesting that *Iresine diffusa* may provide therapeutic benefits without causing excessive hormonal side effects. Given this potential, further research is needed to identify and characterize the phytoestrogenic compounds in *Iresine diffusa* and to determine their bioavailability and efficacy both *in vitro* and *in vivo* [11]. A combined approach involving ethnopharmacological studies, metabolomics, and molecular analyses, such as docking and bioactivity assays, will greatly support the development of *Iresine diffusa* as a natural candidate in phytoestrogen-based therapy for reproductive disorders [11].

To identify potential *SERMs* in plant extracts, such as the *Iresine diffusa* (ID) extract, this study employed a comparative approach in an important *in vivo* animal model: The prepubertal female rat. The model offers a unique hormonal context that is crucial for elucidating the agonistic and antagonistic actions of potential SERMs. The prepubertal female rat model is particularly suitable for evaluating the purely estrogenic activity of extracts. At this stage of development, the rat ovaries do not produce significant levels of endogenous estrogen, making target organs such as the uterus, vagina, and mammary glands highly responsive to estrogenic stimuli (hypoestrogenic state). Consequently, any increase in uterine weight, vaginal epithelial cell proliferation, or other morphological changes in the reproductive organs following administration of *Iresine diffusa* extract, without exogenous estrogen supplementation, would directly indicate estrogen

agonist (estrogenic) effects caused by the compounds in the extract.

In addition, this study provides a detailed description of the condition of the rats, which were specifically bred to be 19 days old and treated for 16 days, with the total duration of treatment and surgery concluding at 35 days of age. Physiologically, the rats were expected to enter puberty at 40 days of age, leading to the emergence of conditions resulting from estrogenic effects, as indicated by the WWU parameter, Uterine diameter, endometrial thickness, ovarian maturity, and hormone levels resulting from the treatment can be confirmed as due to the estrogenic effects of IDEA. Furthermore, the use of an antiestrogenic control group under estradiol+IDEA conditions also illustrates whether there is a partial agonist effect; specifically, whether the addition of a full agonist effect could potentially reduce the maximum effect observed. Under these conditions, this demonstrates IDEA's ability as a phytoestrogen to act selectively by antagonizing the effects of the full agonist estradiol administered.

Previous studies have demonstrated that, *in silico*, IDEA exhibits strong binding to estrogen receptors; however, IDEA itself is still used traditionally and has been passed down through generations in the Tawangmangu community in Solo, Indonesia, as a treatment to regulate menstruation and support breast milk production. Based on its functions and several previous studies, IDEA is a plant with phytoestrogenic properties. Therefore, this study aims to investigate whether it exhibits partial estrogenic agonist activity, with the ultimate goal of further developing it for research with greater potential, particularly for the discovery of breast cancer drugs by identifying a mechanism of Tamoxifen-like action.

## Materials and methods

### Material

Prepubertal female rats (Sprague-Dawley), *Iresine diffusa* plant (herb), Ethyl acetate extract of *Iresine diffusa* (IDEA) suspended in a suitable carrier, such as 0.5% *Carboxymethyl Cellulose Sodium* (CMC-Na) solution for oral administration. Ethinyl estradiol USPRS lot Number Q0C162 (BPOM, Indonesia). Ethanol for analysis, all other reagents and chemicals, including those used for hormone quantification (Rat/Porcine ELISA kit E2 (Estradiol) Cat log E-EL-0152 Elabscience, Rat FSH E-EL-R0391 Elabscience, Rat FSH E-EL-R00261 Elabscience, and progesterone Cat Log ER1255 V4.0 *Finetest*), 10% *Buffered Neutral Formalin* (Sigma-Aldrich, USA), absolute ethanol and graded concentrations (*Merck, Germany*), Xylene (Sigma-Aldrich, USA), and Surgipath liquid paraffin (USA). Hematoxylin (Sigma-Aldrich, USA) and Eosin Y (Merck, Germany). Object *glass* and cover slips (Marienfeld Superior, Germany), DPX mounting medium (Sigma-Aldrich, USA). Feather disposable microtome blade (Japan) and distilled water. Automated Tissue Processor (Leica Biosystems, Germany), Tissue Embedding Center (Thermo Scientific, USA), and Rotary Microtome (Leica Biosystems, Germany). Histology Water Bath (Leica Biosystems, Germany) and Hot Plate. Olympus light microscope (Japan) with digital camera. Refrigerator, freezer, fume hood, and sterile cotton buds.

### Age control prepubertal rats

Prepubertal female rats (Sprague-Dawley), aged 19 days, with an average body weight of 40 - 50 g. The dams were obtained from the Indonesian Food and Drug Administration and subsequently bred under controlled conditions, with the provision that offspring aged up to 19 days were used as experimental animals. The animals were housed in standardized polycarbonate cages under controlled environmental conditions (temperature, 22 ± 2 °C; relative humidity, 50% - 60%; light/dark cycle, 12 h) with *ad libitum* access to standard rodent feed and filtered drinking water. All experimental procedures involving animals will strictly adhere to ethical guidelines and have received prior approval from the Animal Research Ethics Committee (No. 085/KE.03/SK/04/2024), issued by the Ethics Commission of the National Research and Innovation Agency of the Republic of Indonesia.

### Plant materials and extract preparation

Plant material of *Iresine diffusa* (herb) collected from [Center for Research and Development of Medicinal Plants and Traditional Medicines, Tawangmangu, Solo, Central Java, Indonesia], authenticated by a botanist, and *voucher* specimens stored and determined with number 7906/IT1.C11.2/TA.00/2024 from a botanist at the Bandung Institute of Technology, Indonesia. The

collected plant materials were washed, dried in the shade, and then ground into a fine powder. An ethyl acetate extract of Iresine diffusa (IDEA) was prepared by maceration. Macerate, then evaporate by rotary evaporator. Crude extract of IDEA stored at –20 °C until use.

**Liquid chromatography high-resolution mass spectrophotometry (HRMS) analysis**

To perform the analysis, liquid chromatography was carried out using a Thermo Scientific™ Vanquish™ UHPLC binary pump, and a high-resolution Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer was used. The Thermo Scientific Accucore Phenyl-Hexyl analytical column has dimensions of 100×2.1 mm ID×2.6 µm. Using a gradient technique with a flow rate of 0.3 mL/min, mobile phase B was set at 5%. It is then gradually increased to 90% over 16 min, held at 90% for 4 min, and returned to the initial condition (5% B) over 25 min. The injection volume is 3 µL, and the column temperature is maintained at 40 °C. Unfocused screening is performed in full MS/dd-MS2 acquisition mode in positive polarity/ionization. At 32, 8, and 4 arbitrary units (AU), nitrogen was used as the carrier, auxiliary, and purge gases. The spray voltage was set to 3.30 kV, the capillary temperature to 320 °C, and the auxiliary gas heater temperature to 30 °C. In positive ionization mode, the resolution was 70,000 for full MS and 17,500 for ddMS2, and the scan range covered 66.7 - 1,000 m/z. XCalibur 4.4 software, developed by Thermo Scientific, based in Bremen, Germany, was used to control the system. The obtained mass spectra are used to identify potential metabolites, and the Compound Discoverer 3.2 program is used. Furthermore, these compounds are checked for peak extraction using the MzCloud and ChemSpider databases, with a minimum value of 98%. Only compounds that match both databases are selected for analysis. Tabulation and profiling were performed for each predicted compound.

**Animal treatment grouping**

Forty prepubertal female rats were divided into eight groups, with 5 rats in each group. This was arandomized, double-blinded, placebo-controlled clinical trial. The groups included a normal group, an estradiol control group, 3 doses of IDEA groups with estradiol to identify the antiestrogenic effect, and three doses ID groups without estradiol to identify the estrogenic effect, as presented in **Table 1**. Samples were administered orally via a gastric sonde at the dose determined for each group, once daily for 16 consecutive days. Body weight was measured daily to determine the dose for each test animal. Dose selection in this study, the doses of Iresine diffusa extract used were 30, 60, and 120 mg per 200 g body weight. These doses were selected because no prior studies had determined the appropriate dose for this plant extract; therefore, this study adopted an simultion-bsed pproch with dose conversion. The highest dose of 120 mg/200 g BW represents the maximum safe limit; when converted to human doses, this becomes an extremely high and impractical amount for daily use. Low and moderate doses were chosen to observe effects within a safer and more practical range. Additionally, formulation aspects such as mixing the extract with excipients in specific proportions also influenced dose selection to ensure the capsule formulation is practical and easy to consume.

**Table 1** Animal treatment grouping.

| Group | Code | Treatment | N |
|---|---|---|---|
| Control Group | PEN | CMC-Na 0.5% | 5 |
| | PEE | Estradiol 0.9 µg/200gBW | 5 |
| Estrogenic Effect | PED1 | Extract IDEA dose 1 (30mg/200gBW) | 5 |
| | PED2 | Extract IDEA dose 2 (60mg/200gBW) | 5 |
| | PED3 | Extract IDEA dose 3 (120mg/200gBW) | 5 |
| Antiestrogenic Effect | PAED1 | Extract IDEA dose 1 (30mg/200gBW)+Estradiol 0.9 µg/200gBW | 5 |

| Group | Code | Treatment | N |
|---|---|---|---|
| | PAED2 | Extract IDEA dose 2 (60mg/200gBW)+Estradiol 0.9 µg/200gBW | 5 |
| | PAED3 | Extract IDEA dose 3 (120mg/200gBW)+Estradiol 0.9 µg/200gBW | 5 |

**Vaginal smear cytological observation procedure**

This method was modified and optimized based on several methods used by [12]. Cytological examination of vaginal smears was carried out on days 13 to 16, with the duration of one estrous cycle (4 days). Sterile cutton buds are dripped with distilled water to wet the tip of the cutton bud and then apply slowly to the vagina of white rats and apply it with zig zag to the glass object, then wait about 2 min after being applied and dripped with methanol for analysis to bind the cells and not be damaged then allowed to stand for 3 min based on the best optimization time and drip it with giemsa dye and then allowed to stand for 6 min according to the best optimization time and then rinsed slowly with running water, drained, dried and then observe the cytological profile under a microscope. The cell types to be examined in this study include epithelial cells, leukocytes, and cornified cells.

**Uterine wet weight analysis**

The weight of the uterus is analysed immediately after surgery, and the uterus is placed on a tissue to be cleaned of blood and body fluids. Then, the uterus was weighed to obtain its wet weight [13].

**Histological structure analysis [12,14,15]**

After euthanasia, the uterus and ovaries were immediately excised and washed using sterile physiological saline. Both organs were then fixed in 10% Neutral-Buffered Formalin (NBF) for at least 24 h to preserve tissue structure. The dehydration was carried out gradually using alcohol of increasing concentration (70% to absolute), followed by clearing in xylene. Tissues were then infiltrated and embedded in liquid paraffin at 60 °C. Paraffin blocks were cut using a microtome with a thickness of 3 - 5 µm, and the tissue slices were then attached o a glass slide. Subsequently, Hematoxylin-Eosin (HE) staining was performed according to a standard protocol, including deparaffinization, rehydration, Hematoxylin staining, washing, Eosin staining, rehydration, clearing, and coverslip mounting with Entellan. The finished histology preparations were observed under a light microscope to assess the uterine diameter and endometrial thickness. Meanwhile, in the ovaries, the stages of follicular maturity were identified and characterized, including primordial, primary, secondary, tertiary (Graafian), and corpus luteum follicles. The number of follicles at each stage was counted across several randomly selected fields of view to assess ovarian activity [14].

**Hormonal level test**

This method was modified and optimized in accordance with the techniques described in the Elabscience manual procedure for hormone Estrogen (Catalog No: E-EL-0152), Luteinizing Hormone(E-EL-R0026), Follicle-stimulating Hormone(E-EL-R0391), and Progesterone (Pinetest No.: ER1255). 100 µL of blood plasma was added to each well of the ELISA plate, and then the plate was incubated at 37 °C for 90 min. After incubation is complete, the liquid in the wells is discarded, and 100 µL of biotinylated antibody solution is added to each well; the wells are then incubated for an additional 60 min at 37 °C. Afterward, the plate was washed 3 times to remove residual reagents. Then, 100 µL of HRP-conjugate solution was added to each well, incubated for 30 min at 37 °C, and the plate was rewashed five times. Next, 90 µL of TMB substrate reagent was added, and the mixture was incubated at 37 °C for 15 min to develop color. The reaction was then stopped by adding 50 µL of stop solution. The reaction was read immediately on a plate reader at 450 nm, and the data were analyzed to determine the target concentration in blood plasma.

**Data analysis**

Data are presented as mean ± standard deviation (SD) with 95% confidence intervals (CIs). Before

performing a one-way analysis of variance (ANOVA), normality and homogeneity of variances tests were automatically conducted using GraphPad Prism to ensure that the data met the parametric assumptions. Subsequently, a one-way ANOVA was performed using the same software, and $p$-values < 0.05 were considered significant. If the ANOVA results indicated significant differences, post hoc tests (Tukey's multiple comparisons) were conducted to determine which groups differed significantly. All significant results are marked with an asterisk (*)

## Results and discussion

### Prediction of phytochemical compounds of IDEA extract

The LC-HRMS data of the IDEA extract revealed several compounds with significant potential as phytoestrogens, notably Kaempferol, Quercetin, and Afzelin. This analytical correlation is confirmed by the chromatogram profile in Figure 1, where the main intensity peaks correspond directly to the retention times (RT) of kaempferol, quercetin and afzelin, as summarised in Table 2. Mechanistically, the polyphenolic framework and specific hydroxylation patterns of these flavonoids' chemical structures (Figure 2) are crucial in facilitating hydrogen bonding and hydrophobic interactions within the ligand-binding pockets of the oestrogen receptors alpha (ERα) and beta (ERβ). These flavonoids exhibited the highest peak areas: 99,815,495.97 for Kaempferol, 59,580,906.92 for Quercetin, and 94,421,624.52 for Afzelin, indicating their dominant presence in the extract. Structurally, these compounds belong to the flavonol and flavonoid glycoside groups, which are well-documented in literature for their partial agonist activity toward estrogen receptors ESR1 and ESR2. Their polyphenolic frameworks and specific hydroxylation patterns facilitate effective interaction with estrogen receptors, supporting estrogenic modulation without the adverse effects commonly associated with synthetic estrogens. Kaempferol and Quercetin, in particular, have been shown to induce estrogenic activity and to provide antioxidant and anti-inflammatory [15-17] benefits that protect reproductive tissues and maintain hormonal balance. Afzelin, as a flavonoid glycoside, is noted for enhanced bioavailability and stability, which can potentiate its estrogenic effects. Other compounds, such as Pinocembrin, display supportive bioactivities but have lower direct phytoestrogen potential compared to these three core flavonoids.

**Table 2** Annotated compound of ethyl acetate extract from *I. diffusa* through LC-HRMS.

| Rt (minute) | Area ($10^8$) | MS (m/z) BMC | CMW | Molecule formula | Annotated compound | Groups |
|---|---|---|---|---|---|---|
| 14.90 | 34,250,398.89 | 256 | 256.18 | C18 H24 O | Bakuchiol | (Terpenoid) |
| 14.26 | 11,591,275.13 | 146 | 145.05 | C9 H7NO | 4-Indolecarbaldehyde | (Alkaloid) |
| 12.62 | 44,265,471.34 | 431 | 430.38 | C29 H50 O2 | D-α-Tocopherol | Tocopherol (Vitamin E) |
| 12.84 | 59,580,906.92 | 279 | 278.22 | C15 H10 O7 | Quercetin | Flavonol |
| 10.70 | 99,815,495.97 | 279 | 278.22 | C18 H10 O6 | Kaempferol | Flavonol |
| 10.55 | 18,296,041.11 | 257 | 256.07 | C15 H12 O4 | Pinocembrin | Flavanone |
| 9.79 | 94,421,624.52 | 431 | 432.10 | C21 H20 O10 | Afzelin | Flavonoid glycoside |

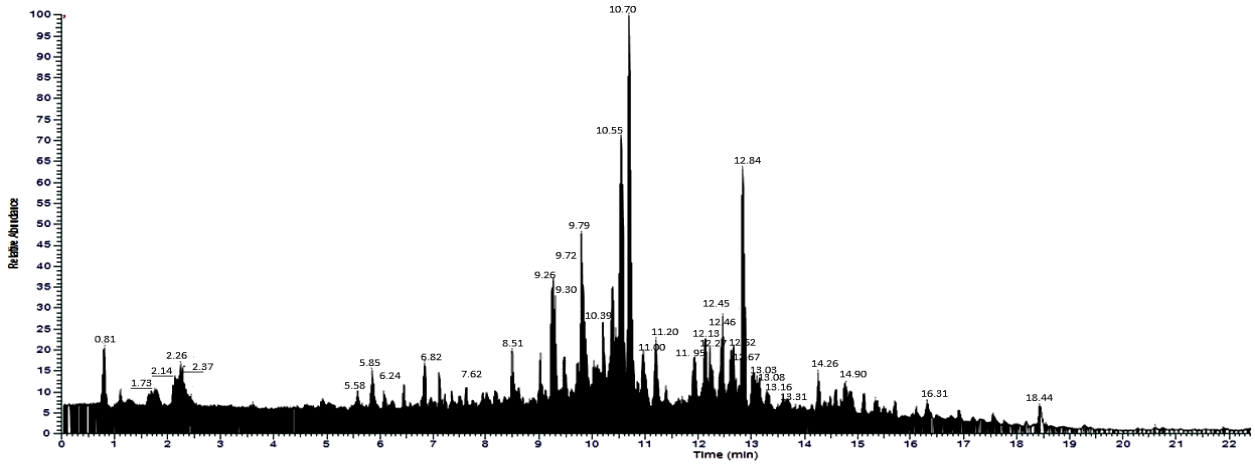



**Figure 1** The chromatogram in positive ion mode of the extract of IDEA.

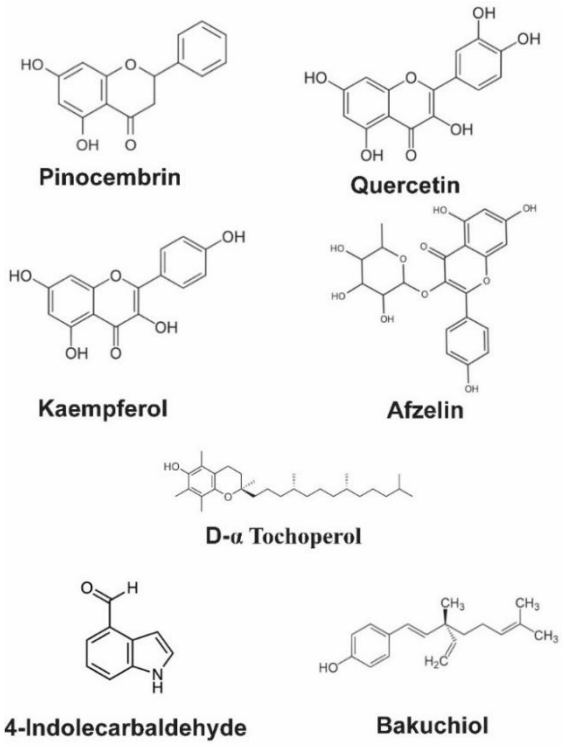



**Figure 2** Chemical structures of secondary metabolites identified in IDEA extract.

**Partial agonist estrogenic evaluation of IDEA in prepubertal rats**

This study used 19-day-old rats with a treatment duration of 16 days. This was to prevent the rats from reaching puberty and to ensure that any observed estrogenic or antiestrogenic effects were attributable to the *Iresine diffusa* extract. Several test parameters were used to support these effects, such as cytology smears, wet uterine weight, endometrial thickness, uterine diameter, ovarian follicle maturity, and hormone levels.

**Effect of IDEA on vaginal smear cytology**

Cytological observations were conducted to determine whether administration of IDEA extract could elicit partial agonist effects, as indicated by the types of cells formed. The formation of the estrous cycle is evident in cytological observations, which reveal the presence of various cell types, including epithelial cells, leukocytes, and keratinized cells. The cytological observation results for determining partial agonist status are presented in **Table 3**, and the microscopic observation results are shown in **Figure 3**.

**Table 3** Effect of IDEA on vaginal smear cytology.

| Effect | Groups | Cornified epithelial cells | Cycle estrous |
|---|---|---|---|
| Control Group | PEN | - | - |
| | PEE | ++ | ✓ |
| Estrogenic Effect | PED1 | ++ | ✓ |
| | PED2 | ++ | ✓ |
| | PED3 | +++ | ✓ |
| Antiestrogenic Effect | PAED1 | + | ✓ |
| | PAED2 | + | ✓ |
| | PAED3 | ++ | ✓ |

**Description:** ✓ (There is an estrus cycle); - (No estrus cycle); (+) Less; (++) moderate; (+++) many.
**PEN:** CMC 0.5%, **PEE:** Estradiol 0.9µg/200gBW, **PED1:** IDEA extract Dose 1 (30mg/200gBW), **PED2:** IDEA extract Dose 2 (60mg/200gBW), **PED3:** IDEA extract Dose 3 (120mg/200gBW) **PAED1:** IDEA extract Dose 1 (30 mg/200gBW)+Estradiol 0.9µg/200gBW, **PAED2:** IDEA extract Dose 2 (60mg/200gBW)+Estradiol 0.9µg/200gBW, **PAED3:** IDEA extract Dose 3 (120mg/200gBW)+Estradiol 0.9µg/200gBW.

The estrous cycle in rats is a short, recurring reproductive cycle, generally lasting 4 - 5 days, and is strictly regulated by hormonal interactions between the hypothalamus, pituitary gland, and ovaries; observation of the estrous cycle can be done through cytological examination of vaginal smears [18]. Vaginal cytology analysis focuses on the relative proportions and morphology of vaginal epithelial cells, particularly nucleated epithelial cells, cornified epithelial cells, and leukocytes, whose presence and numbers reflect the stage of the estrous cycle, as shown in **Figure 3**.

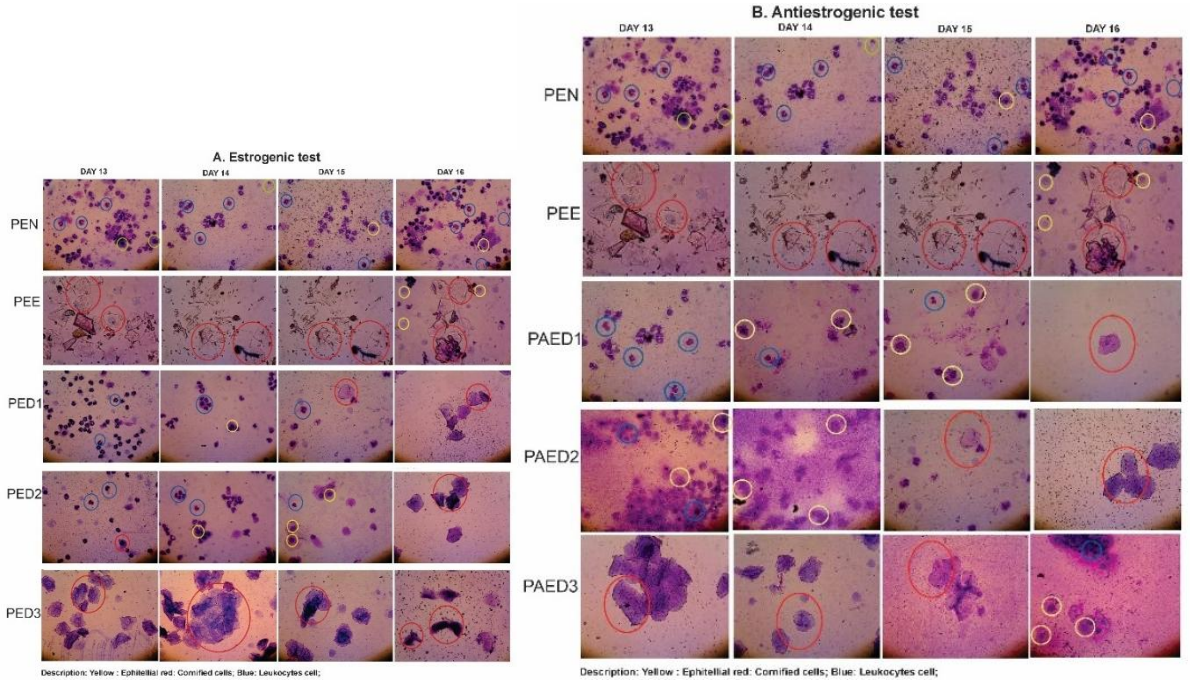



**Figure 3** Differences in cytological observations of vaginal smears from prepubertal female rats in (A) estrogenic and (B) antiestrogenic tests (40x magnification). **PEN:** CMC 0.5%, **PEE:** Estradiol 0.9µg/200gBW, **PED1:** IDEA extract Dose 1 (30mg/200gBW), **PED2:** IDEA extract Dose 2 (60mg/200gBW), **PED3:** IDEA extract Dose 3 (120mg/200gBW) **PAED1:** IDEA extract Dose 1 (30 mg/200gBW)+Estradiol 0.9µg/200gBW, **PAED2:** IDEA extract Dose 2 (60mg/200gBW)+Estradiol 0.9µg/200gBW, **PAED3:** IDEA extract Dose 3 (120mg/200gBW)+Estradiol 0.9µg/200gBW.

During the Proestrus phase, driven by increased estrogen, vaginal smears are dominated by large nucleated epithelial cells, sometimes accompanied by cornified cells. The peak of the cycle, Estrus, is characterized by the dominance of flat, non-nucleated cornified epithelial cells in response to the highest estrogen levels, indicating a receptive period for mating; in this phase, nucleated epithelial cells and leukocytes are scarce.

This study investigates the partial agonist potential of the tested extract in prepubertal rats by examining estrous cycle cytology. In the antiestrogenic test, treatment with doses of 120 and 60 mg/200gBW of the extract showed lower estrous cycle results, as seen from the reduction in cornified cells compared to the control group that only received estrogenic effects (PEE) or pure estrogenic testing, and also compared to the estrogenic model with the same dose. This finding is highly relevant because it is consistent with prior literature indicating that partial agonists elicit a lower microscopic response than full agonists or compounds with pure estrogenic effects. This occurs because partial agonists can compete with full agonists (in this case, estradiol) for binding to estrogen receptors or act as weak antagonists when full agonists are present, resulting in submaximal receptor activation and thereby moderating estrogenic effects [19]. Microscopic observation of Figure 3 visually confirms the semi-quantitative data presented in Table 3 (note: the previous reference in the text to '**Table 2**' should be corrected to 'Table 3'). In **Figure 3A** (PED3 group), there is a clear predominance of flattened, polygonal, anucleate keratinised epithelial cells (score +++), indicating strong oestrogenic agonist activity of the extract. Conversely, Figure 3B for the anti-oestrogenic group (PAED) shows the reappearance of nucleated epithelial cells and leukocytes alongside a reduction in the density of keratinised cells, visually demonstrating IDEA's ability to fully antagonise the proliferative effects of oestradiol. Therefore, lower cytological parameter values, such as the number of keratinized cells at a given dose in the antiestrogenic assay, indicate that the extract may act as a partial agonist, capable of modifying the existing estrogenic response based on the data showing the presence of keratinized cells in **Table 2** [20].

These findings reinforce the partial agonist effect of the extract, whereby it modulates estrogen activity with a submaximal or controlled response. Previous studies on various extracts, including those from *Pueraria mirifica* and *Glycyrrhiza glabra*, have also reported similar patterns in which bioactive compounds provide moderate estrogenic stimulation, as reflected in uterine cytological changes, including a decrease in the number of cornified cells and leukocyte infiltration, compared with full estrogen [21]. This suggests that the partial agonist mechanism is a common feature of several herbal extracts, enabling selective and safe estrogenic effects without overstimulating target tissues [22].

The presence of cornified cells, epithelium, and leukocytes in cytological observations is an essential indicator of the estrous cycle in female rats. Cornified cells are indicative of the proestrus and estrus phases, which are characterized by estrogen-induced thickening and desquamation of the vaginal epithelium [22]. The dominance of epithelial cells indicates estrogen activity that prepares the reproductive tract for possible reproduction. At the same time, leukocyte counts typically increase during the metestrus or diestrus phases as part of an immune response to tissue changes. Therefore, the presence of these three cell types in the group receiving the IDEA extract indicates estrogenic stimulation that triggers the estrous cycle, suggesting that the extract has a genuine estrogenic effect on the reproductive cycle of these female rats [23].

**Analysis of partial agonist effects of IDEA based on uterine analyses**

To evaluate the effect of IDEA as a partial agonist phytoestrogen candidate on reproductive organs, analyses uterine were conducted, with parameters wet weight uterine and histological analysis of uterine (measurement of endometrial thickness and diameter uterine). The measurement data are presented in **Table 3**, which provides quantitative information on changes in uterine mass as an indicator of the estrogenic activity of the AS herb, supported by significant differences between groups, as shown in **Figure 2**.

Observation of the wet weight uterine in prepubertal female rats treated with the extract at various doses, along with estrogenic and antiestrogenic

controls, revealed significant differences in response related to partial estrogen agonist activity. The estrogenic control group treated with estradiol (PEE) exhibited the most pronounced increase in uterine weight, indicating maximal stimulation by endogenous and exogenous estrogen, consistent with the characteristic uterine tissue sensitivity to estrogen [24]. The estrogenic extract treatment groups (PED1, PED2, PED3) showed uterine weights lower than PEE but still higher than the normal control (PEN), suggesting that the extract could be have estrogenic activity, though with a more moderate efficacy compared to PEE. Conversely, the extract treatment groups combined with estradiol at antiestrogenic doses (PAED1, PAED2, PAED3) showed significantly lower uterine weights, approaching those of PEN, indicating antagonistic potential against exogenous estrogen stimulation (**Figure 4**).

**Table 4** Histological analysis of uterine.

| **Effect** | **Groups** | **Wet weight uterine (mg)** ***p*-value 0.001*** | **Endometrial Thickness (µm)** ***p*-value 0.001*** | **Diameter Uterine (µm)** ***p*-value 0.001*** |
|---|---|---|---|---|
| Control Groups | PEN | $0.128 \pm 0.02^{a}$ | $1.43 \pm 0.17^{a}$ | $3.48 \pm 0.11^{a}$ |
| | PEE | $0.320 \pm 0.02^{b}$ | $3.81 \pm 013^{b}$ | $12.55 \pm 0.52^{b}$ |
| Estrogenic Effect | PED1 | $0.112 \pm 0.04^{b}$ | $1.15 \pm 0.13^{b}$ | $2.95 \pm 0.31^{b}$ |
| | PED2 | $0.178 \pm 0.06^{a,b}$ | $2.56 \pm 0.20^{a,b}$ | $5.58 \pm 0.52^{a,b}$ |
| | PED3 | $0.206 \pm 0.06^{a,b}$ | $3.23 \pm 0.17^{a,b}$ | $10.53 \pm 0.20^{a,b}$ |
| Antiestrogenic Effect | PAED1 | $0.101 \pm 0.02^{b}$ | $1.08 \pm 0.03^{b}$ | $2.40 \pm 0.02^{b}$ |
| | PAED2 | $0.126 \pm 0.02^{b}$ | $2.18 \pm 0.07^{a,b}$ | $4.37 \pm 0.02^{b}$ |
| | PAED3 | $0.191 \pm 0.01^{a,b}$ | $3.10 \pm 0.04^{a,b}$ | $9.19 \pm 0.01^{a,b}$ |

**PEN:** CMC 0.5%, **PEE:** Estradiol 0.9µg/200gBW, **PED1:** IDEA extract Dose 1 (30mg/200gBW), **PED2:** IDEA extract Dose 2 (60mg/200gBW), **PED3:** IDEA extract Dose 3 (120mg/200gBW) **PAED1:** IDEA extract Dose 1 (30 mg/200gBW)+Estradiol 0.9µg/200gBW, **PAED2:** IDEA extract Dose 2 (60mg/200gBW)+Estradiol 0.9µg/200gBW, **PAED3:** IDEA extract Dose 3 (120mg/200gBW)+Estradiol 0.9µg/200gBW.

This phenomenon aligns with the mechanism of selective estrogen receptor modulators (SERMs), which can interact with estrogen receptors to elicit partial agonist or antagonist effects depending on tissue type and hormonal context. SERMs, such as Tamoxifen, act by competing with endogenous ligands for binding to the ligand-binding domain of the estrogen receptor, thereby altering receptor conformation and selectively modulating the transcriptional activity of estrogen target genes [25].

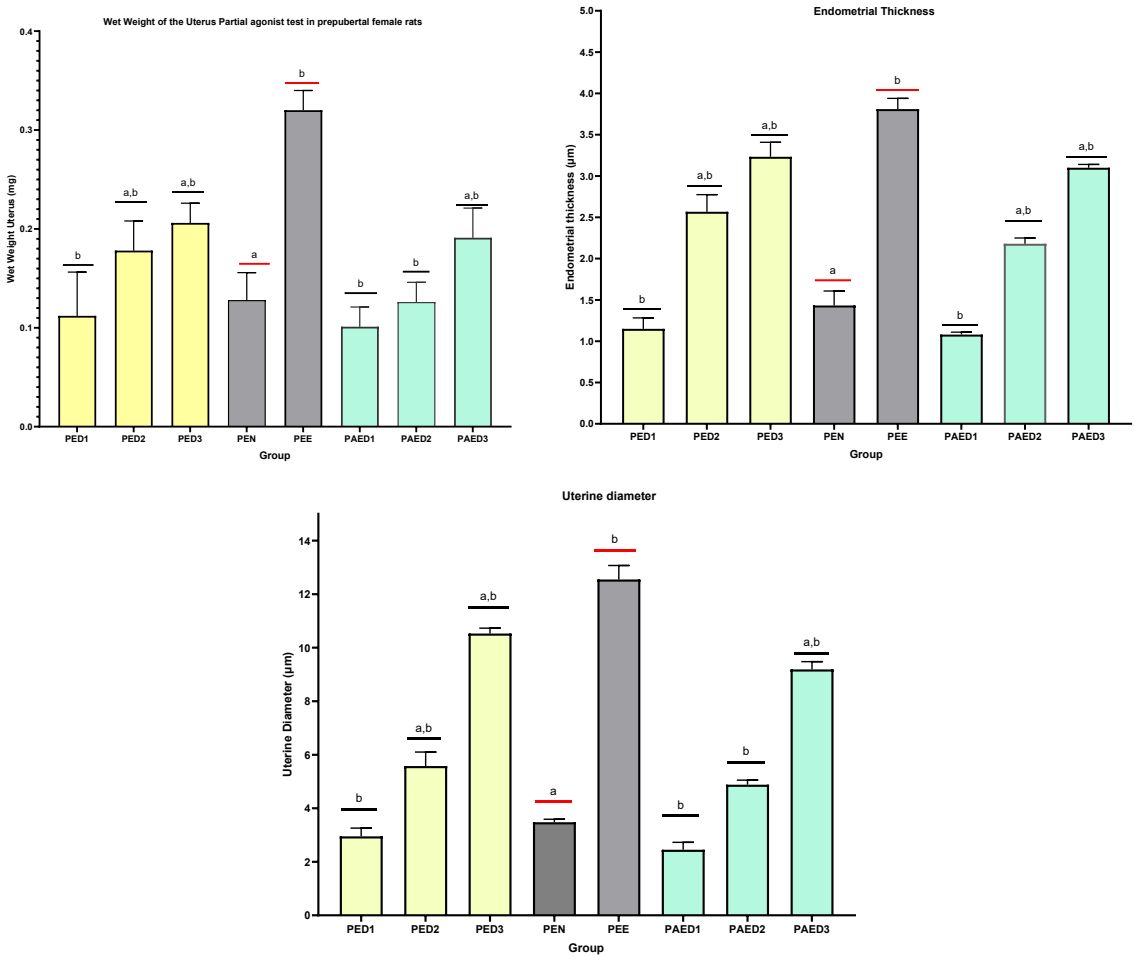



**Figure 4** Analyses of the uterus in the partial agonist estrogenic effect of IDEA in prepubertal rats. **PEN:** CMC 0.5%, **PEE:** Estradiol 0.9µg/200gBW, **PED1:** IDEA extract Dose 1 (30mg/200gBW), **PED2:** IDEA extract Dose 2 (60mg/200gBW), **PED3:** IDEA extract Dose 3 (120mg/200gBW) **PAED1:** IDEA extract Dose 1 (30 mg/200gBW)+Estradiol 0.9µg/200gBW, **PAED2:** IDEA extract Dose 2 (60mg/200gBW)+Estradiol 0.9µg/200gBW, **PAED3:** IDEA extract Dose 3 (120mg/200gBW)+Estradiol 0.9µg/200gBW; The same symbol indicates significant differences ($p < 0.05$).

In uterine tissue, SERMs typically function as antagonists or partial agonists, inhibiting tissue proliferation despite high estradiol levels. This is reflected in the PAED groups, where reduced uterine weight despite estradiol administration indicates antiestrogenic activity via receptor binding that blocks full estrogenic stimulation by endogenous ligands. [26]. These data corroborate previous studies reporting that compounds with SERM activity reduce uterine weight and epithelial proliferation even under estrogen hyperstimulation conditions. Such studies demonstrate that SERMs not only inhibit estrogen action in target tissues such as the uterus but can also preserve or enhance agonist effects in other tissues, such as bone and the cardiovascular system, thereby offering more selective and targeted therapies. Accordingly, extract administration in the PAED groups exhibits partial agonist and estrogen antagonist activities, similar to SERMs, with potential utility as bioactive agents in the development of selective hormonal therapy [27]. The wet uterine weight observations thus reinforce the initial hypothesis that the test extract may act as an estrogen receptor modulator with partial agonist and antiestrogenic effects consistent with SERM pharmacology. These findings bear important implications for the development of phytochemical agents capable of safer and more selective estrogen receptor modulation, particularly relevant for breast cancer therapy and other hormonal disorders [28]. In this way, the extract helps reduce excessive cell

proliferation and uterine swelling while maintaining sufficient estrogenic function to maintain hormonal balance and reproductive health [29]. This phenomenon is consistent with the mechanism of action of (SERMs), which provide controlled estrogenic effects without causing side effects such as uterine tissue hyperplasia [30].

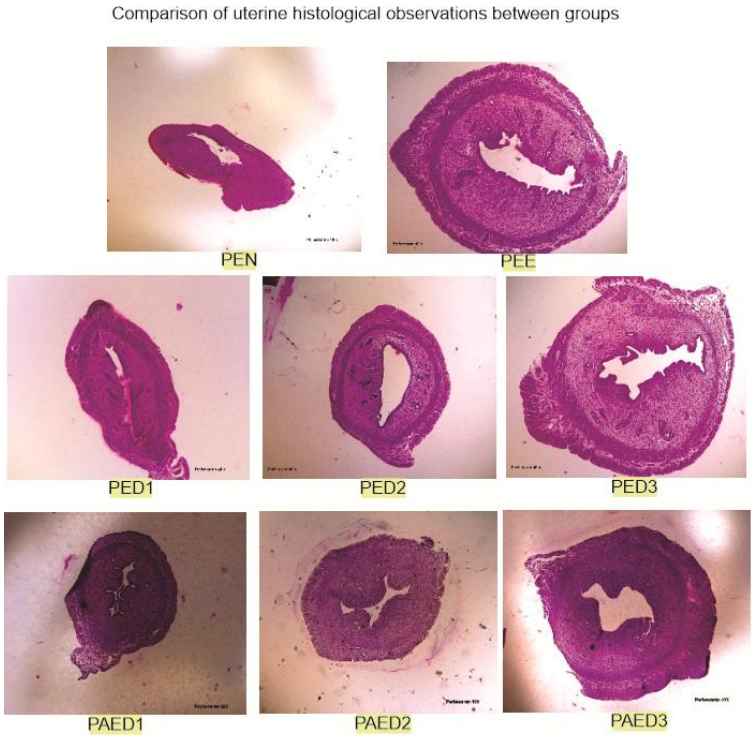



**Figure 5** Comparison of uterine histological observations between groups. **PEN:** Normal with CMC 0.5%, **PEE:** Estradiol 0.9µg**, PED1:** Prepubertal Estrogen Dose 30 mg/200gBW, **PED2:** Prepubertal Estrogenic Dose 60 mg/200gBW, **PED3:** Prepubertal Estrogenic Dose 120 mg/200gBW **PAED1:** Prepubertal Antiestrogenic Dose 30 mg/200gBW, **PAED2:** Prepubertal Antiestrogenic Dose 60 mg/200gBW, **PAED3:** Prepubertal Antiestrogenic Dose 120 mg/200gBW.

The results of uterine diameter and endometrial thickness measurements showed (**Table 4**) a quantitative pattern consistent with BBU measurements in the treatment group receiving estradiol (PEE), which exhibited higher mean values for uterine diameter and endometrial thickness compared to the treatment groups, whether those receiving IDEA or Estradiol+IDEA. This quantitative evidence is strongly supported by the histological findings shown in Figure 5. Uterine sections from the positive control group (PEE) revealed highly active proliferation of luminal epithelial cells, stromal oedema, and significant hypertrophy of the myometrial layer. Administration of IDEA at doses PED2 and PED3 resulted in moderate endometrial hypertrophy without extreme hyperplasia. Interestingly, in the PAED2 and PAED3 combination group, there was a marked reduction in the height of the luminal epithelial cells and the thickness of the uterine stroma compared with the PEE group, which visually confirms the protective effect of IDEA as a selective oestrogen receptor modulator (SERM) in preventing uterine hyperstimulation. The mean values indicate an increase in uterine diameter and endometrial thickness as the dose increases, or a dose-dependent effect. IDEA's ability to modulate uterine proliferation is characterized by an increase in endometrial thickness and uterine diameter, demonstrating IDEA's potential for such modulation based on the mean values and statistical significance obtained. Endometrial thickness showed higher values and was significantly different in the estrogenic test groups (PED2 and PED3) at ($p$ = 0.022) and ($p$ = 0.015), respectively, compared to the

PEN group, and higher mean endometrial thickness values were also observed in the antiestrogenic test groups (PAED2 and PAED3) compared to the PEN group, with statistically significant differences at *p*-values of 0.038 and 0.022, respectively (**Figure 4**). Uterine diameter also showed higher mean values compared to the PEN group and was statistically significantly different in the estrogenic test groups (PED2 and PED3) compared to the PEN group, with p-values of 0.028 and 0.001, respectively; higher mean uterine diameter values in the antiestrogenic test groups (PAED2 and PAED3) compared to the PEN group, and were statistically significantly different with significance values of ($p$ = 0.042) and ($p$ = 0.020), respectively. The mean values of uterine diameter and endometrial thickness obtained in the antiestrogenic treatment groups were lower compared to the estrogenic groups. This pattern indicates a difference in the mechanism and effects of IDEA as a partial agonist, as evidenced by the lower mean values observed in the group administered estradiol + IDEA.

The difference in follicular maturity levels between the test group and the control group demonstrates the strong potential of IDEA as a natural SERMs in stimulating follicular maturation; the average pattern observed with the administration of estradiol + IDEA showed quantitatively lower maturity values compared to the group administered IDEA alone (**Table 5**). The differences shown were statistically significant between PED1, PED2, and PED3, respectively ($p$ = 0.037), ($p$ = 0.022), and ($p$ = 0.012). The statistical significance regarding follicular maturity in the antiestrogenic groups (PAED1, PAED2, and PAED3) compared to the normal group was, respectively, ($p$ = 0.040), ($p$ = 0.032), and ($p$ = 0.020). Significant differences were also observed between the estrogenic and antiestrogenic test groups and the control group administered estradiol (PEE) (**Figures 6** and **7**). This indicates that although IDEA possesses potential as a partial estrogenic agonist, it has not yet been able to match the effects of the estrogen estradiol.

The pattern of these research results is consistent with the mechanism of action of SERMs, which are capable of interacting with estrogen receptors and producing partial agonist or antagonist effects depending on the tissue and hormonal conditions [31]. Theoretically, SERMs such as tamoxifen have been shown to work through competitive binding to the estrogen receptor domain, altering receptor conformation and selectively modulating the transcriptional activity of estrogen target genes. In uterine tissue, SERMs typically act as antagonists or partial agonists that inhibit tissue proliferation even when estradiol is present at high concentrations [32]. This is reflected in the PAED group, which showed a reduction in uterine weight, uterine diameter, and endometrial thickness despite administration of estradiol + IDEA; this finding supports previous research demonstrating antiestrogenic activity that binds to estrogen receptors, displaces them with beta-estrogen receptors, and blocks full stimulation by estrogen ligands.

The data from this study are also consistent with previous research demonstrating that genistein, a phytoestrogen with SERM activity, can reduce uterine weight and uterine epithelial proliferation even under conditions of estrogen hyperstimulation. The study also demonstrated that SERMs not only inhibit estrogen action in specific target tissues such as the uterus but can also maintain or enhance the efficacy of agonists in other tissues, such as bone or the cardiovascular system, thereby offering a more selective and targeted therapy. Thus, the results obtained in the estradiol+IDEA (PAED) group represent partial agonist and estrogenic antagonist activity similar to SERMs, which holds potential as a bioactive agent in the development of selective hormonal therapy [33].

Measurements of BBU, uterine diameter, endometrial thickness, and follicular maturity demonstrate IDEA's potential as a natural SERM based on the "Tamoxifen-like effect" approach by (Stephen R.D. Johnston, 2011), which states that natural SERMs can reduce agonist effects in uterotrophic assays; under conditions where a full agonist is added, they can produce a submaximal reduction in agonist effects. These measurements reflect the proliferation and hypertrophy of uterine smooth muscle due to estrogenic stimulation, while the increase in endometrial thickness and follicular maturity in the ovaries indicates increased proliferation and differentiation of the mucosal layer as a direct response to estrogen binding to its receptors. Variations in both are used to confirm the partial agonist or antagonist effects of IDEA as a test sample in this study.

**Mature ovarian follicles**

Mature ovarian follicles exhibit a physiological response to estrogenic stimulation, so changes in this level of maturity may reflect the extract's ability to activate or modulate estrogen receptors partially. In other words, the results of the IDEA extract in **Table 5** were able to increase the number of follicles, indicating that the extract has partial estrogenic activity, as shown in the antiestrogenic test, where the level of follicle maturity in prepubertal female rats was lower (**Figures 6** and **7**).

**Table 5** Analyses of mature ovarian follicles.

| Effect | Group | Number of follicle at each stage (*p*-value 0.591) | | | |
|---|---|---|---|---|---|
| | | **F1** | **F2** | **F3** | **F4** |
| Control Groups | PEN | 3.6 ± 1.01 | 1.8 ± 0.4 | 1.2 ± 0.7 | 0 |
| | PEE | 0 | 2.0 ± 0.6 | 2.8 ± 0.7 | 4 ± 0.4 |
| Estrogenic Effect | PED1 | 1.6 ± 0.4 | 2.4 ± 0.4 | 2.4 ± 0.4 | 0.8 ± 0.4 |
| | PED2 | 0.6 ± 0.4 | 2.6 ± 0.8 | 1.8 ± 0.9 | 1.8 ± 0.7 |
| | PED3 | 0 | 0.8 ± 0.4 | 1.6 ± 0.4 | 3.6 ± 0.3 |
| Antiestrogenic Effect | PAED1 | 1.2 ± 0.2 | 2.0 ± 0.2 | 1.9 ± 0.2 | 0.6 ± 0.2 |
| | PAED2 | 0.2 ± 0.2 | 2.0 ± 0.2 | 1.3 ± 0.4 | 1.4 ± 0.3 |
| | PAED3 | 0 | 0.4 ± 0.2 | 1.4 ± 0.4 | 3.0 ± 0.3 |

***p* > 0.05 (One-Way ANOVA)** F1: Primordial follicel; F2: Primary follicle; F3: Secondary follicle; F4: Mature follicle ***PEN:*** CMC 0.5%, **PEE:** Estradiol 0.9 µg/200gBW, **PED1:** IDEA extract Dose 1 (30 mg/200gBW), **PED2:** IDEA extract Dose 2 (60 mg/200gBW), **PED3:** IDEA extract Dose 3 (120 mg/200gBW) **PAED1:** IDEA extract Dose 1 (30 mg/200 gBW)+Estradiol 0.9 µg/200gBW, **PAED2:** IDEA extract Dose 2 (60 mg/200gBW) + Estradiol 0.9 µg/200gBW, **PAED3:** IDEA extract Dose 3 (120 mg/200gBW) + Estradiol 0.9 µg/200gBW.

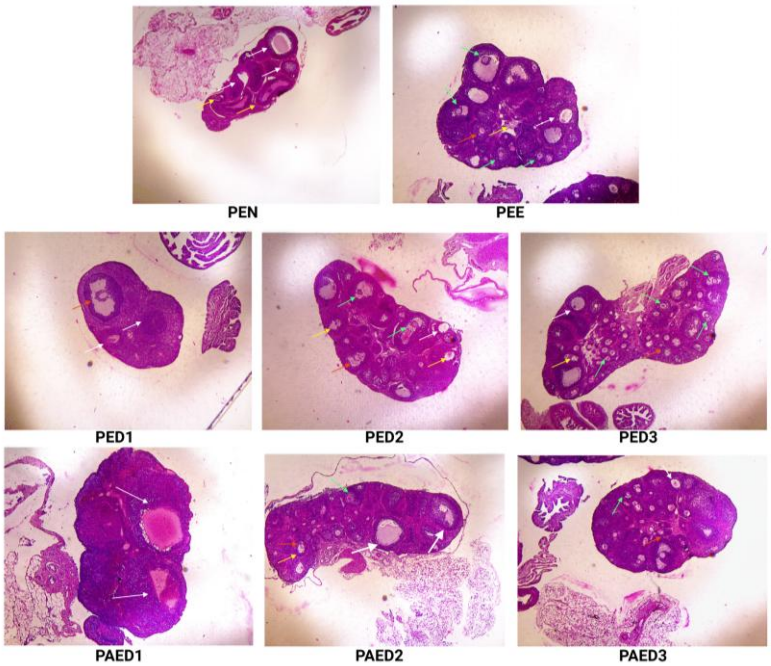



**Figure 6** Mature Ovarian Follicle. **PEN:** CMC 0,5%**, PEE:** Estradiol 0.9 µg/200gBW**, PED1:** IDEA extract Dose 1 (30 mg/200gBW), **PED2:** IDEA extract Dose 2 (60 mg/200gBW), **PED3:** IDEA extract Dose 3 (120 mg/200gBW) **PAED1:** IDEA extract Dose 1 (30 mg/200gBW)+Estradiol 0.9 µg/200gBW, **PAED2:** IDEA extract Dose 2 (60mg/200gBW)+Estradiol 0.9 µg/200gBW, **PAED3:** IDEA extract Dose 3 (120 mg/200gBW)+Estradiol 0.9 µg/200gBW; (→) Primordial Folicle (→) Primary Folicle (→) Secondary Folicle (→) Mature Folicle.

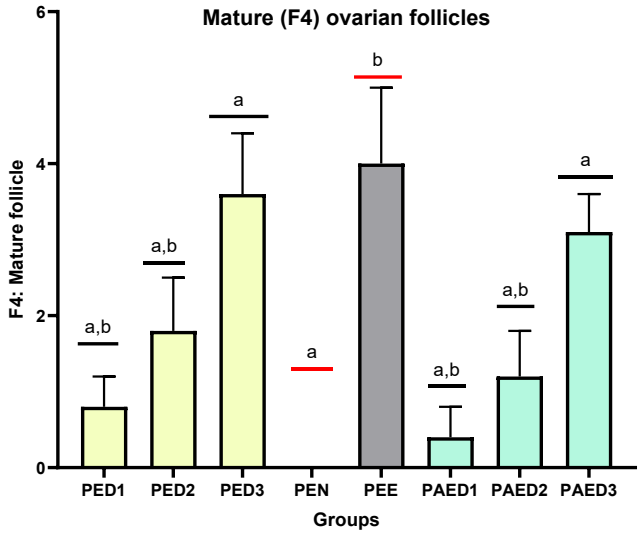



**Figure 7** Number of Mature Folicle (F4) in each groups. **PEN:** CMC 0.5%, **PEE:** Estradiol 0.9 µg/200gBW, **PED1:** IDEA extract Dose 1 (30 mg/200gBW), **PED2:** IDEA extract Dose 2 (60 mg/200gBW), **PED3:** IDEA extract Dose 3 (120 mg/200gBW) **PAED1:** IDEA extract Dose 1 (30 mg/200gBW) + Estradiol 0.9 µg/200gBW, **PAED2:** IDEA extract Dose 2 (60 mg/200gBW)+Estradiol 0.9 µg/200gBW, **PAED3:** IDEA extract Dose 3 (120 mg/200gBW)+Estradiol 0.9 µg/200gBW; The same symbol indicates significant differences ($p < 0.05$).

Observation of ovarian follicle maturation levels in prepubertal rats revealed significant differences between treatment groups, particularly evident in the number of mature follicles. Quantitative data showed that the group receiving estradiol, the positive control, had the highest mean number of mature follicles (4.0 ± 0.4). In contrast, the group treated with the ethyl acetate extract of sambang colok at a dose of 120 mg/200 g body weight showed the second-highest significant increase relative to the negative control group. These findings indicate that compounds present in the sambang colok extract possess estrogenic potential that can induce ovarian follicle maturation through mechanisms resembling those of endogenous estrogen hormones. Theoretically, estrogen is a key hormone in regulating follicle maturation, produced by granulosa cells and triggering cell proliferation and differentiation toward secondary and mature follicle stages [34]. Interaction of phytoestrogens with estrogen receptors (ERα and ERβ) can activate similar signaling pathways, resulting in partial agonist effects that support follicle maturation without hormonal overstimulation. Similar studies using phytoestrogenic compounds, such as soybean isoflavones (*Glycine max*) or kaempferol from other plants, have reported comparable mechanisms. These compounds increase the number of follicles from the primary to the mature stage by modulating estrogen receptor activity in prepubertal rat ovaries [35]. Several investigations also show that administration of phytoestrogen-rich plant extracts significantly increases the number of mature follicles, reinforcing evidence that these plant compounds stimulate ovarian follicle development through humoral and local effects on ovarian tissue. Thus, these observations align with the concept that phytoestrogens act as reproductive modulators with estrogenic effects, which can be harnessed both in alternative therapy development and pharmacological research focused on women's reproductive health [36].

Moreover, histological profiles indicating follicle maturation, as assessed macroscopically, further corroborate the quantitative data, lending scientific validity to these findings as genuine biological responses to phytoestrogenic treatment. Further examination of molecular expression of estrogen receptors and associated hormones is a key focus to deepen understanding of the mechanisms of IDEA phytoestrogens in the reproductive biology context of prepubertal rats, These results are consistent with the existing literature, confirming the role of partial agonists in inducing a controlled estrogenic response without overstimulation, which is crucial in maintaining

hormonal balance and reducing the risk of side effects associated with full agonists [18,19,37]. The SERMs concept supports these findings, whereby the substance can provide sufficient receptor activation to stimulate follicular maturation and ovarian function, while avoiding excessive stimulation that can cause reproductive dysfunction [38].

**Hormone levels analysis**

Measurements of estradiol, FSH, progesterone, and LH hormones provide a biological picture of how the tested ID extract affects hormonal regulation and estrogen receptor activity. By monitoring changes in these hormone levels, it is possible to see the extent to which the extract mimics or alters the estrogenic response, as well as its modulatory capacity under normal conditions and in the presence of estrogen inhibition. The hormonal measurement results are presented in **Table 6**.

**Table 6** Hormonal levels of estrogen, Luteinizing hormone, Progesterone, and Follicle-stimulating hormone.

| Effect | Groups | Estrogen(pg/mL) *p*-value 0.001* | LH(mIu/mL) *p*-value 0.001* | Progesteron(ng/mL) *p*-value 0.001* | FSH(ng/mL) *p*-value 0.001* |
|---|---|---|---|---|---|
| Control Groups | PEN | $9.01 \pm 0.25^{a}$ | $1.87 \pm 0.02^{a}$ | $1.51 \pm 0.35^{a}$ | $5.81 \pm 0.42^{a}$ |
| | PEE | $43.26 \pm 2.64^{b}$ | $23.66 \pm 0.26^{b}$ | $1.29 \pm 0.23^{b}$ | $6.47 \pm 0.35^{b}$ |
| Estrogenic Effect | PED1 | $16.63 \pm 1.48^{a,b}$ | $5.98 \pm 0.11^{a,b}$ | $3.97 \pm 0.52^{a,b}$ | $11.09 \pm 0.29^{b}$ |
| | PED2 | $21.29 \pm 1.35^{a,b}$ | $10.19 \pm 0.19^{a,b}$ | $5.72 \pm 0.88^{a,b}$ | $8.85 \pm 0.43^{b}$ |
| | PED3 | $34.52 \pm 1.62^{a,b}$ | $13.87 \pm 0.60^{a,b}$ | $8.67 \pm 0.27^{a,b}$ | $6.79 \pm 0.54^{a,b}$ |
| Antiestrogenic Effect | PAED1 | $13.28 \pm 0.21^{b}$ | $4.74 \pm 0.25^{b}$ | $1.59 \pm 0.07^{ns}$ | $10.39 \pm 0.69^{b}$ |
| | PAED2 | $19.37 \pm 0.51^{a,b}$ | $8.42 \pm 0.37^{a,b}$ | $2.56 \pm 0.28^{ns}$ | $7.91 \pm 0.85^{b}$ |
| | PAED3 | $32.28 \pm 1.34^{a,b}$ | $10.36 \pm 0.33^{a,b}$ | $4.46 \pm 0.16^{a,b}$ | $6.60 \pm 0.30^{b}$ |

**PEN:** CMC 0.5%, **PEE:** Estradiol 0.9 µg/200gBW, **PED1:** IDEA extract Dose 1 (30 mg/200gBW), **PED2:** IDEA extract Dose 2 (60 mg/200gBW), **PED3:** IDEA extract Dose 3 (120 mg/200gBW) **PAED1:** IDEA extract Dose 1 (30 mg/200gBW)+Estradiol 0.9 µg/200gBW, **PAED2:** IDEA extract Dose 2 (60 mg/200gBW)+Estradiol 0.9 µg/200gBW, **PAED3:** IDEA extract Dose 3 (120 mg/200gBW)+Estradiol 0.9 µg/200gBW; The same symbol indicates significant differences ($p < 0.05$).

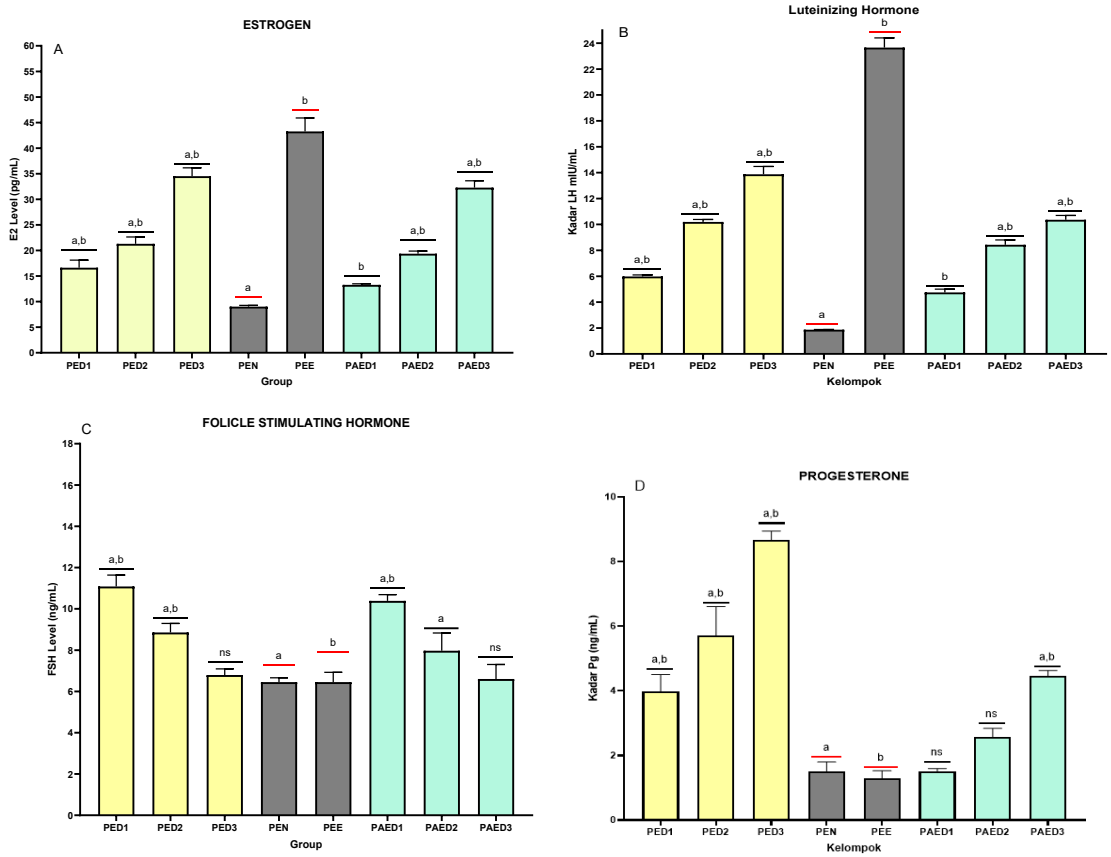



**Figure 8** Hormone levels after treatment in the estrogenic test of prepubertal female white rats included: (A) Estradiol hormone; (B) Luteinizing hormone; (C) Progesterone hormone; and (D) FSH hormone. The treatment groups were as follows: ***PEN:*** *CMC 0.5%,* **PEE:** Estradiol 0.9 µg/200gBW, **PED1:** IDEA extract Dose 1 (30 mg/200gBW), **PED2:** IDEA extract Dose 2 (60 mg/200gBW), **PED3:** IDEA extract Dose 3 (120 mg/200gBW) **PAED1:** IDEA extract Dose 1 (30 mg/200gBW)+Estradiol 0.9 µg/200gBW, **PAED2:** IDEA extract Dose 2 (60 mg/200gBW)+Estradiol 0.9 µg/200gBW, **PAED3:** IDEA extract Dose 3 (120 mg/200gBW)+Estradiol 0.9 µg/200gBW; The same symbol indicates significant differences ($p < 0.05$); ns: Not significance difference.

Measurements of hormone levels, including estrogen, luteinizing hormone (LH), follicle-stimulating hormone (FSH), and progesterone, in a group of prepubertal rats treated with estrogenic and antiestrogenic agents revealed significantly different hormonal profiles between the normal group and the group administered estradiol alone as a control. **Table 6** shows that estrogen levels in the positive control group (PEE) administered estradiol consistently exhibited the highest increase in estrogen levels (43.26 pg/mL) and were the highest compared to all other groups. Estrogen levels in the estrogenic treatment group showed higher mean estrogen levels compared to the PEN group overall across all three doses (PED1, PED2, and PED3) administered and were statistically significantly different with *p*-values of ($p = 0.021$), ($p = 0.019$), and ($p = 0.014$), respectively. The mean LH hormone level in the PEE group was also the highest at 23.66 ± 0.26 mIU/mL, indicating that the effect of estradiol administration aligns with the HPA axis mechanism, where high estrogen levels are followed by high LH levels [39]; LH levels in the PEE group were used as a reference for comparison to determine the effects resulting from IDEA administration, whether in estrogenic or antiestrogenic testing. Overall, all three doses administered PED1, PED2, PED3, and PAED1, PAED2, PAED3 all showed an increase in LH levels compared to the PEN group and were statistically significant with a p-value ($p < 0.05$) for PED1, PED2, PED3, PAED2, and PAED3; however, there was no statistically significant difference for the PAED1 group ($p < 0.068$).

A similar pattern for FSH levels showed a lower mean value in the PEE group (6.47 ± 0.35), which was

close to the mean value in the normal control group (PEN) and was not higher than in the other treatment groups. The mean results for FSH hormone levels in the estrogenic and antiestrogenic test groups compared to PEN were statistically significant in the PED1 and PAED1 groups, respectively ($p = 0.033$) and ($p = 0.40$), and were not statistically significantly different ($p > 0.05$) from the PED2, PED3, and PAED2 groups. PAED3. These results indicate a suppression of FSH levels in a dose-dependent manner. The low FSH levels in the PEE and treatment groups suggest the activation of the HPA axis's negative feedback mechanism, whereby high estrogen levels suppress FSH levels in the body. PG hormone levels showed low mean values in the group administered estradiol (PEE) when compared to the treatment groups, including both the estrogenic and antiestrogenic test groups.

This indicates an increased effect of reduced progesterone levels resulting from hormonal fluctuation mechanisms; when estrogen levels rise, progesterone levels decrease. This is supported by the mean values: The PEE group showed a level of only $1.29 \pm 0.23$, the lowest value, alongside the PEN group at $1.51 \pm 0.35$. Statistically, the differences in levels between the estrogenic test groups PED1, PED2, and PED3 showed higher mean values compared to the control groups (PEN and PEE), and these differences were statistically significant with *p*-values for all ($p < 0.05$) however, conversely, the antiestrogenic test groups showed mean values not significantly different from the PEN and PEE groups and were also not statistically significantly different from the PAED1 and PAED2 groups; meanwhile, PAED3 itself showed an increase in the mean PG level compared to PAED1 and PAED2 and was statistically significant ($p = 0.029$).

Testing of E2, LH, FSH, and PG hormone levels in prepubertal female rats following IDEA treatment revealed differences in the mean values of each hormone level, which tended to be lower in the antiestrogenic treatment group (estradiol + IDEA) compared to the estrogenic treatment group given IDEA alone. The pattern of decreased levels in the estradiol + IDEA treatment provides an explanation that the presence of estrogen derived from estradiol, combined with the addition of IDEA, simultaneously exerts a suppressing effect on estradiol, as evidenced by the decreased hormone levels. Overall, the results of this study align with the theory demonstrating that when prepubertal conditions are exposed to estrogen, estrogen concentrations in the bloodstream increase significantly, mimicking or enhancing the function of natural estrogen. This rise in estrogen levels sends a strong signal to the hypothalamus to modulate the function of the pituitary gland, which plays a role in producing gonadotropin hormones such as FSH and LH. Under normal conditions, the hypothalamus and pituitary gland operate through a strict hormonal feedback system; however, during prepuberty with the addition of exogenous estrogen, stimulation occurs that triggers the release of FSH and LH. The rise in these 2 hormones is crucial because they stimulate the growth and maturation of ovarian follicles, which is the initial step in reproductive readiness [40].

The surge in LH reflects a positive feedback mechanism for ovulation, while conversely, FSH levels are suppressed as a negative feedback response to prevent excessive stimulation of mature follicles; progesterone levels typically decrease when exogenous estrogen is administered to prepubertal rats. This is because progesterone is primarily synthesized by the corpus luteum after ovulation, whereas during the prepubertal period, ovulation and corpus luteum formation have not yet occurred actively. Additionally, high estrogen levels can inhibit progesterone production through a negative feedback mechanism, making the decrease in progesterone levels more pronounced. This decrease also reflects that the reproductive cycle has not yet entered the characteristic luteal phase, in which progesterone is dominant.

**Conclusions**

This study demonstrates that all observed parameters such as vaginal cytology, wet weight, and uterine histological findings (including endometrial thickness and uterine diameter), ovarian follicular maturity, and hormone profiles (E2, LH, progesterone, and FSH) consistently support the hypothesis that the IDEA extract exhibits partial agonist activity at estrogen receptors. This extract induces moderate, dose-dependent estrogenic effects in prepubertal rats, although these are less potent than those of pure estradiol. Furthermore, under estradiol stimulation, the IDEA extract modulates the response, reduces excessive effects, and maintains hormonal and physiological

balance in a controlled manner. Findings regarding partial estrogen agonism were confirmed by the results of each parameter test in the antiestrogenic test group (estradiol+IDEA), which showed lower mean values compared to the estrogenic test group; this illustrates the effect of partial agonism, where the addition of a full agonist actually reduces the maximum effect of that full agonist.

**Limitations of the study and directions for future research**

This study was conducted based on the drug discovery of Selective Estrogen Receptor Modulators (SERMs), which was developed following the achievement of the preclinical study criteria proposed by Stephen R.D. Johnston in the discovery of the "Tamoxifen-like effect" drug, based on the existing 10 criteria, This study evaluates two main criteria: The SERM compounds under investigation, specifically conducting an initial screening to identify potential phytochemical compounds present in IDEA that belong to the phytoestrogenic group, and subsequently demonstrating a reduction in agonist effects in an *in vivo* uterotrophic assay in prepubertal rats. This research warrants continuation to address the 8 follow-up points in the future to support the potential demonstrated in this study, thereby supporting more detailed and selective follow-up testing and proving the potential of IDEA as a candidate for natural SERMs in the future

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**CRediT author statement**

**Kurnia Agustini**: Conceptualization; Methodology; Software. **Armansyah Maulana Harahap**: Data curation; Writing - Original draft preparation. **Sri Ningsih**: Visualization; Investigation. **Anton Bahtiar**: Supervision. **Yuli Widyastuti**: Software; Validation. **Kurnia Agustini** and **Armansyah Maulana Harahap**: Writing - Reviewing and Editing.

**References**

[1] M Ghanavati, Y Khorshidi, M Shadnoush, ME Akbari, SH Ardehali, Y Chavarri-Guerra, A Akbari, R Barragan-Carrillo, MA Amlashi, Z Javid and J Rahmani. Tamoxifen use and risk of endometrial cancer in breast cancer patients: A systematic review and dose-response meta-analysis. *Cancer Reports* 2023; **6(4)**, e1806.

[2] C Dipankar, S Murugan and PU Devi. Review on medicinal and pharmacological properties of *Iresine herbstii*, *Chrozophora rottleri* and *Ecbolium linneanum*. *African Journal of Traditional, Complementary and Alternative Medicines* 2011; **8(5)**, 124-129.

[3] D Mouttet, M Laé, M Caly, D Gentien, S Carpentier, H Peyro-Saint-Paul, A Vincent-Salomon, R Rouzier, B Sigal-Zafrani, X Sastre-Garau and F Reyal. Estrogen-receptor, progesterone-receptor and *HER2* status determination in invasive breast cancer. concordance between immuno-histochemistry and MapQuant™ microarray based assay. *PLoS One* 2016; **11(2)**, e0146474.

[4] NY Paniagua-Zambrana, RW Bussmann and C Romero. *Iresine diffusa Humb. & Bonpl. ex Willd. Iresine herbstii Hook Amaranthaceae*. Springer Nature, Switzerland, 2020, p. 991-995.

[5] R Matsushima-Nishiwaki, N Yamada, Y Hattori, Y Hosokawa, J Tachi, T Hori and O Kozawa. SERMs (selective estrogen receptor modulator), acting as estrogen receptor β agonists in hepatocellular carcinoma cells, inhibit the transforming growth factor-α-induced migration via specific inhibition of AKT signaling pathway. *PLoS One* 2022; **17(1)**, e0262485.

[6] CT Stork, M Bocek, MP Crossley, J Sollier, LA Sanz, F Chédin, T Swigut and KA Cimprich. Co-transcriptional R-loops are the main cause of estrogen-induced DNA damage. *eLife* 2016; **5**, e17548.

[7] C Nencini, F Cavallo, G Bruni, A Capasso, V de Feo, L de Martino, Giorgio and L Micheli. Affinity of *Iresine herbstii* and *Brugmansia arborea* extracts on different cerebral receptors. *Journal of Ethnopharmacology* 2006; **105(3)**, 352-357.

[8] SRD Johnston. Endocrinology and hormone therapy in breast cancer: Selective oestrogen

receptor modulators and downregulators for breast cancer - have they lost their way? *Breast Cancer Research* 2005; **7**, 119.

[9] S Martinkovich, D Shah, SL Planey and JA Arnott. Selective estrogen receptor modulators: Tissue specificity and clinical utility. *Clinical Interventions in Aging* 2014; **9**, 1437-1452.

[10] HY Ma, S Chen, LL Lu, W Gong and AH Zhang. Raloxifene in the treatment of osteoporosis in postmenopausal women with end-stage renal disease: A systematic review and meta-analysis. *Hormone and Metabolic Research* 2021; **53(11)**, 730-737.

[11] AI Suárez. New biflavonoids from *Iresine diffusa*. *Revista de la Facultad de Farmacia* 2022; **85(1y2)**, 91-99.

[12] M Srinivasan. A comparative study on staining techniques for vaginal exfoliative cytology of rat. *Journal of Pharmacology & Clinical Research* 2017; **3(3)**, 555615.

[13] OECD, Terms & Conditions, Available at: http://www.oecd.org/termsandconditions, accessed July 2025.

[14] Y Li, H Zeng and J Fu. Preovulatory progesterone levels are the top indicator for ovulation prediction based on machine learning model evaluation: A retrospective study. *Journal of Ovarian Research* 2024; **17**, 169.

[15] S Yani, T Opik, R Siti, K Ida and P Epa. *In silico* analysis of kaempferol as a competitor of estrogen on estrogen receptor alpha of endometrial cancer. *Journal of Physics: Conference Series* 2019; **1402**, 066109.

[16] Fadilah, BC Edina, RA Rahmawati, L Wiyono, L Erlina, RI Paramita and A Tedjo. Molecular dynamic of pinostrobin and pinocembrin from *Kaempferia pandurata* Roxb. towards Estrogen Receptor Positive (ESR) and Estrogen Receptor Negative (VEGFR) of Breast Cancer. *Asian Journal of Pharmaceutics Supplementary Issue* 2018; **12(4)**, 1473-1480.

[17] S Özsoy, G Soykut and E Becer. Quercetin: A phytochemical with pro-apoptotic effects in colon cancer cells. *Cyprus Journal of Medical Sciences* 2022; **7(5)**, 587-592.

[18] I Setyawati, NI Wiratmini and I Narayani. Estrogen hormone level of prepubertal female rat treated with *Calliandra calothyrsus* ethanolic leaf extract. *Journal of Physics: Conference Series* 2018; **983**, 012190.

[19] J Łuczak, M Bagłaj, R Ciaputa, A Szymerowski and M Nowak. Does open ovarian biopsy in prepubertal age affect ovarian reserve in a rat model? *Journal of Pediatric Surgery* 2021; **56(2)**, 360-367.

[20] JM Goldman, SC Laws, SK Balchak, RL Cooper and RJ Kavlock. Endocrine-disrupting chemicals: Prepubertal exposures and effects on sexual maturation and thyroid activity in the female rat. A focus on the EDSTAC recommendations. *Critical Reviews in Toxicology* 2000; **30(2)**, 135-196.

[21] JM Oliveira, IM Oliveira, HK Sleiman, GOD Forno, MA Romano and RM Romano. Consumption of soy isoflavones during the prepubertal phase delays puberty and causes hypergonadotropic hypogonadism with disruption of hypothalamic-pituitary gonadotropins regulation in male rats. *Toxicology Letters* 2022; **369**, 1-11.

[22] R Pelayo, H Marina, A Suárez-Vega, G Hervás, C Esteban-Blanco, B Gausseres, G Foucras, JJ Arranz and B Gutiérrez-Gil. Influence of a temporary restriction of dietary protein in prepubertal ewe lambs on first lactation milk traits and response to a mammary gland inflammatory challenge. *Research in Veterinary Science* 2023; **159**, 57-65.

[23] L Zhou, KY Wong, W Yu, CCW Poon, H Xiao, CO Chan, DKW Mok, Y Zhang and MS Wong. Selective estrogen receptor modulator-like activities of *Herba epimedii* extract and its interactions with tamoxifen and raloxifene in bone cells and tissues. *Frontiers in Pharmacology* 2020; **11**, 571598.

[24] GS Bustani and HFK Alghetaa. Aryl hydrocarbon receptor signaling in male fertility: Protective role of resveratrol and disruptive effects of CH223191 in adult male rats. *Veterinary World* 2025; **18(5)**, 1274-1287.

[25] S Huang, B Qi, L Yang, X Wang, J Huang, Y Zhao, Y Hu and W Xiao. Phytoestrogens, novel dietary supplements for breast cancer.

*Biomedicine & Pharmacotherapy* 2023; **160**, 114341.

[26] S Goel, R Sharma, A Hamilton and J Beith. LHRH agonists for adjuvant therapy of early breast cancer in premenopausal women. *Cochrane Database of Systematic reviews* 2009; **2009(4)**, CD004562.

[27] W Wang, C Zhang, Y Fan, S Yue, Y Yang, R Liu, L Zhang, T Wang and F Fu. Dopamine receptor agonist rotigotine-loaded microspheres ameliorates sexual function deteriorated by fluoxetine in depression rats. *ASN Neuro* 2021; **13(1)**, 17590914211052862.

[28] TM Borjeson, J Pang, JG Fox and A García. Administration of luteinizing hormone releasing hormone agonist for synchronization of estrus and generation of pseudopregnancy for embryo transfer in rats. *Journal of the American Association for Laboratory Animal Science* 2014; **53(3)**, 232-237.

[29] K El-Sayed, DA Ali, SA Maher, D Ghareeb, S Selim, S Albogami, E Fayad and E Kolieb. Prophylactic and ameliorative effects of PPAR-γ agonist pioglitazone in improving oxidative stress, germ cell apoptosis and inflammation in gentamycin-induced testicular damage in adult male albino rats. *Antioxidants* 2022; **11(2)**, 191.

[30] JM Bazioli, JH Costa, L Shiozawa, ALTG Ruiz, MA Foglio and JE de Carvalho. Anti-estrogenic activity of guajadial fraction, from guava leaves (*Psidium guajava* L.). *Molecules* 2020; **25(7)**, 1525.

[31] J Gorzkiewicz, G Bartosz and I Sadowska-Bartosz. The potential effects of phytoestrogens: The role in neuroprotection. 2021; **26(10)**, 2954.

[32] VP Chavda, AZ Chaudhari, PC Balar, A Gholap and LK Vora. Phytoestrogens: Chemistry, potential health benefits, and their medicinal importance. *Phytotherapy Research* 2024; **38(6)**, 3060-3079.

[33] NN Suhaimi, MN Waras and NM Ansor. Association between phytoestrogen consumption and female reproductive health: A systematic review of experimental models. *Ibnosina Journal of Medicine and Biomedical Sciences* 2023; **15**, 152-160.

[34] HK Patel and T Bihani. Selective estrogen receptor modulators (SERMs) and selective estrogen receptor degraders (SERDs) in cancer treatment. *Pharmacology & Therapeutics* 2018; **186**, 1-24.

[35] HS Chung, HS Lee and K Park. Estrogen modulates epithelial progenitor cells in rat vagina. *Investigative and Clinical Urology* 2021; **62(3)**, 349-353.

[36] I Falsetti, G Palmini, C Aurilia, S Donati, T Iantomasi and ML Brandi. Selective estrogen receptor modulators in post-menopausal osteoporosis. *International Journal of Bone Fragility* 2022; **2(3)**, 93-96.

[37] QN Khusmitha, EHW Putri, Nurdiana and NP Sari. Potential effects of tempeh extract on ovarian folliculogenesis in female wistar rats. *Journal of Research in Midwifery and Healthcare* 2025; **1(1)**, 14-19.

[38] O Karlsson, K Kultima, H Wadensten, A Nilsson, E Roman, PE Andrén and EB Brittebo. Neurotoxin-induced neuropeptide perturbations in striatum of neonatal rats. *Journal of Proteome Research* 2013; **12(4)**, 1678-1690.

[39] M Levi, R Shalgi and I Ben-Aharon. Pretreatment with gonadotropin-releasing hormone antagonist protects against chemotherapy-induced testicular damage in mice. *Therapeutic Advances in Medical Oncology* 2022; **14**, 17588359221113274.

[40] HQ Wang, WD Zhang, B Yuan and JB Zhang. Advances in the regulation of mammalian follicle-stimulating hormone secretion. *Animals* 2021; **11(4)**, 1134.