EVALUATION OF ANTIFERTILITY ACTIVITY OF *SARACA INDICA* (ASHOKA) IN EXPERIMENTAL ANIMALS

A Thesis submitted to Gujarat Technological University

for the Award of

Doctor of Philosophy

in

Pharmacy

by

Mr. JAINIK KHAMAR, (B.Pharm, M.Pharm, ERT)

Enrollment No.: 209999901009

under supervision of

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GUJARAT TECHNOLOGICAL UNIVERSITY

AHMEDABAD

[July –2026]

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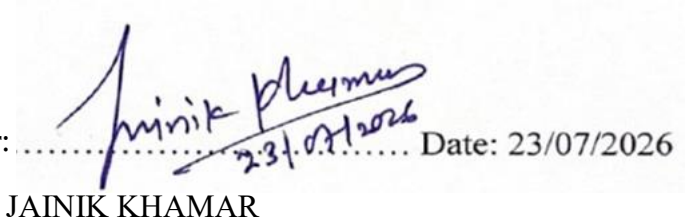

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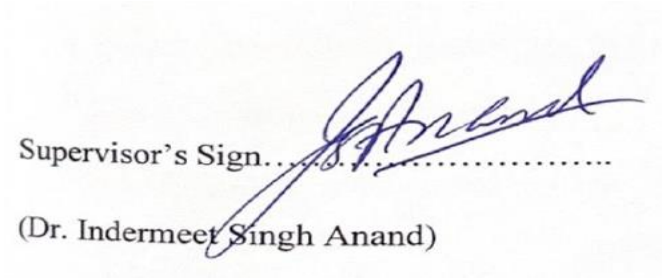

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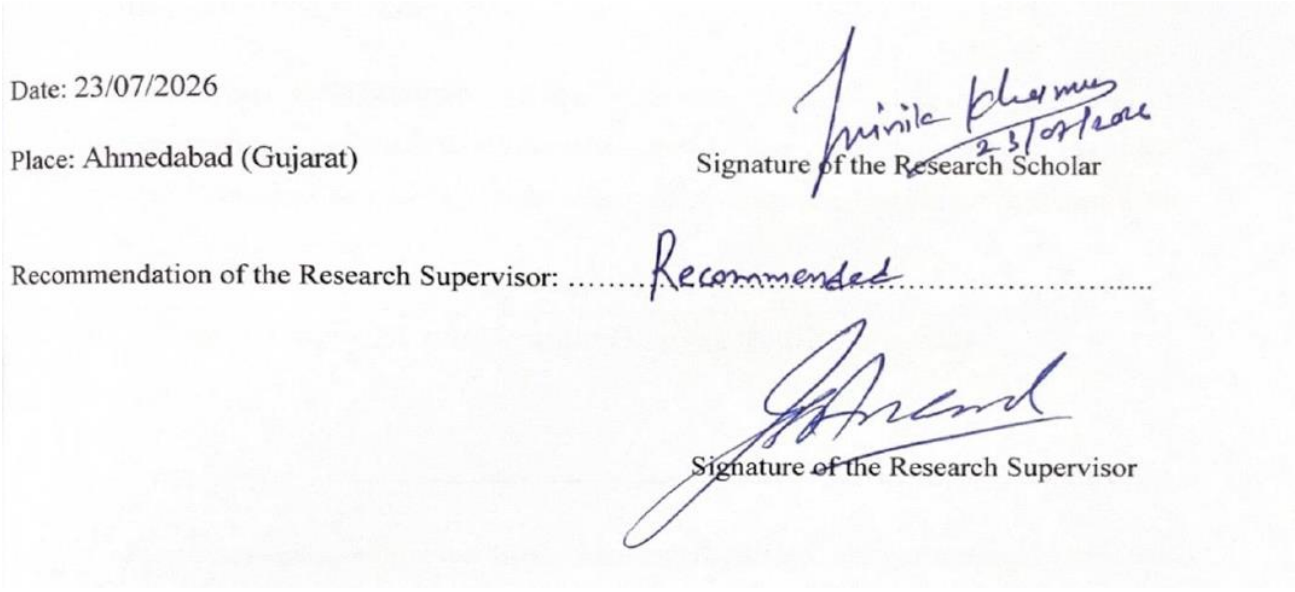

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ABSTRACT

This thesis investigates the anti-fertility potential of *Saraca indica* (Ashoka), with a specific focus on its active compound, ursolic acid, available abundance amout in the bark. The study addresses the need for safer, plant-based contraceptives by evaluating ursolic acid's effects through in vitro and in vivo experiments on experimental animals. Key methodologies include extraction and quantification of ursolic acid via maceration and HPLC (yielding 0.40% with 90% assay), in vitro oxytocic activity on rat uterine tissue, and in vivo assessments of anti-ovulatory, anti-implantation, anti-androgenic, and anti-estrogenic activities in Wistar rats and Swiss albino mice. Results demonstrate dose dependent inhibition of ovulation, implantation loss (up to 63.47% pre-implantation and 57.66% post-implantation at 250 mg/kg.bwt.), reduced sperm parameters and testicular weight in males, and decreased estrogen levels and uterine weight in females. These findings support ursolic acid's multifaceted anti-fertility mechanisms, including LH surge inhibition, cervical mucus thickening, and endometrial alteration. The original contributions highlight ursolic acid's comparable efficacy to standards like oxytocin and cyclophosphamide, validating *Saraca indica's* traditional use and suggesting its potential for developing novel, reversible contraceptives to address population control and gender equity in birth control options. Future research could explore clinical applications and molecular pathways.

Acknowledgement and / or Dedication

First and foremost, I reverentially “dedicate this work to my beloved Father and my esteemed Grandparents, whose blessings, values, and inspiration have been the guiding light of my life” and have shaped every step of this academic journey. Their unwavering faith, moral teachings, and constant encouragement have instilled in me the perseverance, discipline, and integrity required to pursue and complete this research work.

This endeavour “would not have been possible without the unconditional love, patience, and support” of my Wife, Mother, Sister, and Brother-in-law. Their constant motivation, understanding during long working hours, and sacrifices during my moments of absence have been a continuous source of strength and emotional stability throughout every challenge of this study. In times of doubt and exhaustion, their kind words, prayers, and belief in my potential renewed my determination to move forward and give my best.

I express “my deepest and most sincere gratitude to my respected Supervisor for his invaluable guidance, continuous encouragement, and insightful advice throughout the entire course of my research. His scholarly expertise, critical feedback, and patient mentoring have” not only enriched the quality of this work but have also significantly contributed to my professional growth as an emerging researcher. I am truly indebted to him for the time, effort, and trust he has invested in me, and for always challenging me to think critically, work diligently, and maintain high scientific and ethical standards.

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My profound appreciation goes to the Management Team of Accuprec Research Labs Pvt. Ltd. for their steadfast support, for “providing all the necessary facilities and resources, and for being the true backbone of my professional development and research endeavors. The state-of-the-art infrastructure, well-equipped laboratories, and highly disciplined research environment at Accuprec created an enabling ecosystem that allowed this work to be executed with precision and reliability. I am sincerely grateful for the opportunities, trust, and encouragement extended to me” by the management throughout this journey.

I sincerely acknowledge the dedicated efforts of my Animal House Team, whose technical expertise, cooperation, and commitment “played a vital role in the successful execution of the experimental studies. Their meticulous animal care, adherence to ethical practices, and timely assistance during experimental procedures ensured the smooth progress and scientific integrity of this research.

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With deepest gratitude,
Mr. Jainik Khamar

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List of Abbreviation

B. wt.	Body Weight
CDSCO	Central Drugs Standard Control Organisation
CCSEA	Committee for Control and Supervision of Experiments on Animals
ºC	Degree Centigrade
g	gram
h	Hour
ID	Identification
IAEC	Institutional Animal Ethics Committee
IV	Intravenous
IP	Intraperitoneal
mL	Milliliter
Kg	Kilogram
UA	Ursolic Acid

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CHAPTER – 1 INTRODUCTION

TITLE: Evaluation of Anti fertility Activity of *Saraca Indica* (Ashoka) in Experimental Animals

1. INTRODUCTION

Human being is known to be the first and perfect creation of god on this planet. God has created such a beautiful hierarchy of species according to their evolution to bring about a perfect equilibrium in the cycle of life, but the starting number of humans and their disproportionately increasing number pose a great harm towards the ecological balance on earth. With an alarming rise in the number of people, the competition for basic resources such as food, water, cloth, and living space is increasing, resulting in the **Charle Darwin's** theory of the survival of the fittest [1]. As a result, our own counterparts living all around the planet are being deprived of the very basic amenities of life, not to mention the standards of living. If ever increasing population growth is not checked, the earth is going to be populated by 1 billion people in 14 years' time. According to the **UNITED NATIONS** statistics, world population will be **8.9 billion** around **2150** [2].

India, our motherland, is better known as the land of spirituality, prosperous kings, and rich cultural heritage. There are various demons, such as poverty, illiteracy, etc., which, if not checked, could really hamper our growth as per global scenario. The root cause of all these problems lies in the devastatingly rising number of Indians.

India's population is going to reach **1.545 billion** by **2035** A.D., making it the most populous country in the world. Therefore, population controls are undoubtedly one of the major socio-economic issues that need to be dealt with seriously. Recent population data shows that the population in India reached the billion mark on 11th May 2000, and expected projection for the population by 2027 is around 1,441.2 million at the current growth rate, which is 1.9% each year (**WHO report**) [3]. With a population of 1027,015,247 and an area of 3.28 million square kilometers, as reported by the 2001 Census, it constitutes more than 1.6% of the world's population but only 2.4% of its total area. Due to improved medical facilities, the number of diseases and epidemics has been controlled, thus life expectancy has increased from 20 years in 1920 to 65 years now, which means the death rate has decreased significantly. However, the birthrate has not been lowered, hence the increase in growth rate.

Growth Rate = Birth rate – Death rate

If is estimated that the increase in India's population every year is equivalent to Australia's total population. The time has arrived for this scenario to change and for the universal problem of population explosion be rectified [4].

Therefore, it is imperative to discover the perfect contraceptive agent—one that is 100% reversible, has no adverse effects, is simple to administer, and has no negative effects on libido or the gonads. Although steroidal estrogen and progesterone-containing contraceptives are available, their lack of 100% antifertility action and the risk of side effects like coronary thrombosis, which can lead to myocardial infarction or stroke, an increase in B.P., genital carcinoma, gallstones, etc., indicate the need for research into more dependable contraceptive agents.

In this context, medicinal plants used in traditional systems such as Ayurveda and Unani have attracted considerable attention as potential sources of safer fertility-regulating agents. Systematic surveys by Kamboj and Dhawan [5] and others have documented several hundred plant species with reported antifertility activity in different experimental models, and the World Health Organization (WHO) and the Indian Council of Medical Research (ICMR) have proposed guidelines for their pharmacological evaluation. These observations provide a scientific basis for selecting and screening ethnomedicinal plants that are traditionally indicated for reproductive and gynaecological disorders.

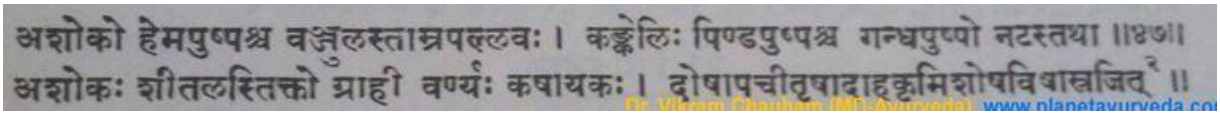
Ancient Indian literature mentions extensive use of plants and plant products as a reliable means of curing various ailments. The "**Ayurvedic** as well as **Unani**" system of medicine mentions a list of various plants that have shown potent antifertility activity. Mother Nature has blessed us with a vast amount of herbal resources, which, if utilized judiciously and carefully, would definitely serve as a boon to usher in an ideal contraceptive agent [6].

Various plants have been randomly screened for their fertility regulation activity. Plants can be broadly categorized as those used in female contraception and make contraception. Under the plants screened as female contraceptives, they have been categorized as antiovulatory plants,

interceptive plants, abortifacient plants, and contraceptive plants [5]. Fertility regulators in males are categorized as spermicidal plants, antispermicidal plants, and fertility-inhibiting plants.

In order to produce repeatable results, **WHO** and **ICMR** offer systematic standards for the assessment of fertility-regulating plants, such as appropriate authentication and systematic screening techniques. A list of 298 plants with possible antifertility has been listed in literature [7].

Saraca indica, known in Hindi as Asoka and in English as Asok tree, has been listed in ancient Indian literature as a plant possessing varied pharmacological actions. [8]


अशोको हेमपुष्पश्च वञ्जुलस्ताम्रपल्लवः। कङ्केलिः पिण्डपुष्पश्च गन्धपुष्पो नटस्तथा ॥४७॥
अशोकः शीतलस्तिक्तो ग्राही वर्ण्यः कषायकः। दोषापचीतृषादाहकृमिशोषविषास्रजित् ॥

Meaning: As described in the mentioned shloka, the herb is known by several synonymous names, encompassing Ashok, Hempushpa, Tamarpallav, Vajjul, Kankeli, Ghandpushp, Pindpushpa, and Nut. The drug is characterized by an astringent and bitter taste, cold potency, and absorbent properties, and is traditionally regarded as effective in the management of wounds and ulcers. It is employed for pacifying Vata dosha and is indicated in the treatment of tumors, excessive thirst, burning sensations, helminthic infestations, emaciation, poisoning, and various bleeding disorders. According to Rajnighantu, this herb is also employed in the management of tumors (Apachi), cardiac disorders (Hridyaroga), and abdominal pain (Udarshoola).

The Asoka tree is considered sacred in India and is well-known for its application in the treatment of gynecological conditions, particularly menorrhagia. The bark of *Saraca indica* serves as a significant raw material in Asokarishta, a fermented formulation, as well as in various other medicinal products. Modern pharmacological and clinical observations support the traditional claim that *Saraca indica* (syn. *Saraca asoca*) bark exerts prominent actions on the female reproductive system. Hot-water or hydroalcoholic extracts of the bark have been

reported to act as uterine stimulants with an ergot-like effect, to reduce excessive uterine bleeding and to regularize menstrual cycles without major adverse effects. The herb is widely used in Ayurvedic formulations such as Asokarishta for the management of menorrhagia, dysmenorrhoea, leucorrhoea and other uterine disorders, indicating that it can influence endometrial function, ovarian activity and hormonal balance in women.

These traditional and experimental findings suggest that *Saraca indica* may possess properties relevant to fertility regulation, including modulation of uterine contractility, endometrial receptivity and ovarian hormones. However, despite its long history of use in gynaecological conditions, its possible antifertility potential and the contribution of its individual phytoconstituents have not been systematically evaluated in validated *in vitro* and *in vivo* models. This gap justified the selection of *Saraca indica* as a candidate plant for detailed antifertility investigations in the present work.The tannins present in bark have been shown to exert an astringent effect that mitigates hemorrhaging hemorrhoids, excessive menstrual bleeding, bleeding ulcers, and hemorrhagic diarrhea. The bark is soluble in water. Consequently, the aqueous extract of bark has been employed for assessing pharmacological activity [9].

Phytochemical studies have shown that the bark of *Saraca indica* contains tannins, flavonoids and pentacyclic triterpenoids, among which ursolic acid has been identified as a major constituent. Ursolic acid is a widely distributed triterpenoid with diverse biological activities, including anti-inflammatory, antioxidant and endocrine-modulatory effects, and several recent animal studies indicate that it can influence reproductive parameters and gonadal function. On this basis, ursolic acid was considered a rational lead compound and chemical marker for exploring the antifertility potential associated with *Saraca indica* bark.

Although *Saraca indica* bark is traditionally used in gynaecological formulations, its crude extracts contain multiple phytoconstituents that complicate mechanistic interpretation in experimental studies. Ursolic acid was therefore selected as a defined chemical marker and lead compound representing the major triterpenoid fraction potentially responsible for reproductive effects. Phytochemical analysis of the bark (Objective 2) first confirmed ursolic acid presence

(TLC identification) and quantified its content (HPLC assay: 90% w/w in enriched fraction, 0.40% w/w bark yield), establishing the phytochemical link between the plant source and the test compound. Pure ursolic acid was then employed in all subsequent pharmacological experiments to ensure precise dosing, excellent reproducibility and unambiguous attribution of antifertility effects to this specific bioactive constituent, while maintaining scientific relevance to Saraca indica bark activity.

Saraca indica is prime ingredient in ***Ashokarishta***, a well-known Ayurvedic preparation for gynaecological ailments. In order to link the traditional use of *Saraca indica* with a defined bioactive marker, the present study first confirms the presence and quantifies the level of ursolic acid in the bark and then evaluates the antifertility profile of pure ursolic acid in appropriate in vitro and in vivo models. However, there is no evidence of its pharmacological effect on rodents as well as in mammals. So current research has been conducted to find out the evidence for this drug, and it has been screened as an antifertility agent [9].

Thus, integrating the ethnomedicinal use of *Saraca indica* in female reproductive disorders with its documented phytochemical profile and the emerging evidence on the reproductive effects of ursolic acid, the present study was designed to test the hypothesis that ursolic acid, as a major triterpenoid constituent of *Saraca indica* bark, can exert multifaceted antifertility actions through anti-implantation, anti-estrogenic, anti-ovulatory, oxytocic and anti-androgenic mechanisms in validated rodent models.

CHAPTER – 2 REVIEW OF LITERATURE

TITLE: Evaluation of Anti fertility Activity of *Saraca Indica* (Ashoka) in Experimental Animals

2. REVIEW OF LITERATURE

Rapid growth is the cause of grave concern as it forms a major obstacle for the socio-economical development of the country. By 2035 AD, India's population is projected to reach 1.545 billion, establishing it as the most populous nation globally. Consequently, human fertility is a significant domain of biological research. Despite the availability of numerous contraceptive medications in the market, the prevalence of severe side effects associated with their usage renders them less acceptable to the general population. Significant adverse effects encompass uterine, cervical, and ovarian cancer, increased risk of coronary and cerebral thrombosis leading to myocardial infarction or stroke, genital carcinoma, and gallstones, among others. Consequently, rigorous study is necessary to create safe and effective contraceptive drugs. An ideal contraceptive is one that possesses 100% efficacy, reversibility of activity, is free from any side effect and does not have any damaging effect on the gonads and libido.

Effects have been put in the direction of developing a safer contraceptive agent from plant sources. A constant emphasis has been laid on the regulation of fertility through plants and their products. The use of plants as emmenagogues enhances menstrual flow in a non-gravid uterus and has been widely employed to induce abortion. A review indicates that 120 plant species from **103** taxa and **54** families have been identified as emmenagogues and/or abortifacients in ancient Indian literature. Nearly 75 years ago, the Central Drug Research Institute started systematic integrated methodologies using authenticated plant samples and suitable animal models. These efforts were augmented by the collaboration task force program of **WHO in 1982** [10], with one of its centers established in India, based on computer rating of antifertility plants.

2.1 Hormonal Control of Reproduction:-

The fertilized ovum's transition into the blastocyst, which implants itself in the endometrium at the embryonic pole, is a consistent characteristic of the ovum once it enters the uterus. The following are the main implementation episodes:-

1. Development of the decidual reaction of the endometrium.

2. Orientation of the blastocyst, so that its trophoblast is able to penetrate the endometrium and make firm attachments.

3. A real invasive process, in which the trophoblast is either passively absorbed by the nidus or actively enters the endometrium [11-14].

The following hormonal parameters are concerned on preparing the uterus for implantation in the mouse.

A. Nidation estrogen, i.e., estrogen secreted just prior to implantation [15-18]
B. Progesterone
C. Estrogen: secreted by the ovary during diestrus and proestrus

2.2 Decidualisation Concepts:-

Decidualisation occurs in a sensitized endometrium during a certain timeframe, approximately on the 25th day of the menstrual cycle in humans and on the 4th day in rats. It is a distinctive structural stromal cell type characterized by multinucleate cells containing numerous cytoplasmic filaments.

It has been proposed that the process is related to a particular change in nucleic acid protein synthesis that is progesterone-dependent. In the cyclic rat, estrogen initiates a cascade of processes that result in the proliferation of stem cells, whose offspring act as precursor cells for decidualization, hence determining the uterine capacity for decidualization. which, while not dividing, mature into capable precidual cells for DNA synthesis in response to the estrogen action phase during progestation [12]. "Histamine," which is either produced by experimental stimulation or released when the blastocyst interacts with uterine components, serves as the stimulus for differentiation into primary decidual cells. The predecidual cells have the capacity to change temporarily, but they degenerate after a day or two if they are not activated. The blastocyst-uterus interaction exhibits characteristics of a delayed hypersensitivity-type immune response.

Histamine, which is released by the ovary's surge of estrogen, acts on hormonally primed progravid endothelium to promote decidualisation and the development of decidual tissues into

which the blastocyst can implant [14,15]. The findings have been validated by the uterine freezing methods of [19] and [20], which selectively eliminate mast cells. Consequently, histamine cannot be mobilized, and decidualization cannot occur.

2.3 Nidation:-

Nidation is characterized by a sequence of overlapping periods with dominating occurrences in each. Priming, consisting of proestrus and estrus, is controlled by estrogen, which encourages uterine constituent turnover and new element creation, allowing the uterus to respond to decidual induction but also making it vulnerable to decidualization inhibition. Priming transitions into a second stage known as **"sensitization."** Histamine accumulation and coitally enhanced leukocyte infiltration of the uterus are the hallmarks of this period. The uterine sensitivity is now formed once this phase is finished by an estrogen surge that results in the period of sensitization. Decidualization starts at this stage when the blastocyst releases histamine and sheds its zona pellucida. The blastocyst starts to penetrate the uterine epithelium and embed itself in the nidus once decidualisation is complete. After implantation and nidation are finished, trophoblastic and embryonic tissue proliferation start.

The synergistic actions of progesterone and estrogen appear to be important for synchronization of shedding and dissolution of the zona so that they occur together at the specific time. Shedding of the zona pellucida precedes the attachment of the blastocyst to the uterus in the rat and mouse. The treatment of pregnant rats with progesterone and estrogen influences the time of zona shedding [21, 22, 23]. The maximum sensitivity of the mouse endometrium to a decidual stimulus is brought about by both the ovarian hormones acting on the uterus in an orderly pattern with regard to time and dosage. Martin and Finn's 1968 study found that progesterone and nidatory estrogen production begin 48 hours after mating and lead to blastocyst implantation two days later [24]. High levels of estrogen secreted during proestrus initiate the endometrial changes, which are completed by progesterone and low doses of estrogen. It appears that estrogen just prior to decidual stimulus, called estrogenic surges, is essential to induce sensitivity of the epithelial to induces sensitivity of the epithelial cells, which remains and transduces the decidual stimulus, while the earlier estrogen is necessary for maximum

decidualisation of the stromal cells [17].

The mesometrial side of the implantation sites initially showed extravasation in deep endometrial stoma venous components. In Hoffman et al. discovered macromolecular leakage at rabbit implantation sites. They found that systemic administration of indomethacin, an anti-inflammatory drug, prevented vascular leakage caused by the formation of endothelial gaps but had little to no effect on trophoblast knob penetration of vessels [25]. They also found that anti-inflammatory drug treatment decreased, but was unable to totally prevent, changes in vascular permeability at implantation sites. In the early stages of pregnancy, estrogen levels are comparatively low. During proestrus, estrogen secretion was found to be high, and it quickly decreased before ovulation. Before implantation, the amount of estrogen rises, according to a previous study that used a bioassay of total estrogen in ovarian blood flow [26-28]. The level of the pre-implantation of pre-implantation estrogen, however, is much less than that observed on the day of proestrous.

In pregnant rats, nursing a large litter delayed implantation. This is also true in mouse and the Mongolian gerbil. Estrogen abolishes the delay in implantation in pregnant, lactating rats [29]. A small dose of estradiol (2-5mg) injected locally into mesometrial adipose tissue exerted an action on the limited portion of the uterus, close to injection site, and reduced implantation of blastocyst, which happened to be within that portion of the uterus [27]. To enable a blastocyst to implant into the endometrium and for a pregnancy to continue to term, precise hormonal regulation of cellular functions in the endometrium is essential. Lobe clearly laid down basic biological features of decidualisation [30].

- It is necessary to prime the uterus by exposing it to ovarian hormones for a specific amount of time.
- A condition of sensitivity is attained following priming and lasts for a brief period of time.
- The application of a suitable stimulus during the induction phase leads to the formation of a deciduoma.

Upon induction, a deciduoma proliferates rapidly, and the stromal cells undergo differentiation into decidual cells. In most of the mammalian species so far investigated, progesterone seems

essential to prime the uterus for decidualisation. Although in some, such as "hamster [31, 32], progesterone" alone appears to be sufficient to prepare the uterus for the formation of the decidual tissues. It is strongly believed that in many other species including rats and mice, the combined actions of progesterone and estrogen are essential to prime the uterus for decidualisation [33, 13]. Much important evidence in this regard comes from works on delayed implant, e.g., in rats [34]. Shelesynak and his colleagues [13, 15] concluded from the biological evidence available that the estrogen during the prenidatory period was secreted as a surge immediately before the onset of ovum implantation.

The data published by Nimrod et al failed to analyse the presence of a peak of estrogen during the prenidatory period, although the pre-implantation rise was confirmed [35]. A distinctive cytological aspect of the uterus during the pre-treatment phase of ovum implantation is the transition of mitotic activity from the epithelial stromal components of the endometrium. The initial phenomenon delineated by [36], subsequently by Finn and Martin in mice [18], was later extrapolated to rats by Shelenyak and Marcus in [14], and by Tachie et al. in [37]. The observed shift of the mitotic activity was shown to result from the combined action of progesterone and estrogen [24, 37].

Nimrod et al. demonstrated that estradiol secretion started increasing in the afternoon of the second day post-coitum, reaching a plateau by the afternoon of day three, which continued much after implantation, lasting at least until day eight [35]. The exact time of peak uterine sensitivity for the induction of decidualization, occurring in the afternoon of day 5, is likely dependent upon an early and sustained elevation in ovarian estrogen secretion.

The findings suggest that LH is responsible for the pre-implantation rise of estrogen in the mouse. In rats, FSH is considered to facilitate implantation, with the efficacy of both endogenous and exogenous FSH reliant on low serum prolactin levels. The function of FSH is facilitated by the ovarian follicles, perhaps due to increased estrogen production [38]. This notion aligns with the findings that FSH promotes estrogen synthesis in ovarian cultures and granulosa cell incubation systems [39, 40, 41]. Research utilizing well-characterized antisera for LH and PRL demonstrated that LH is more significant than PRL in pregnant rats [42].

Dickmann showed that this is not essential since priming with progesterone 24 hours prior to the experimental estrogen administration is compatible with implantation [21].

Deansely showed that a small amount of progesterone is required to induce implantation of ova in the guinea pig ovariectomised after mating [43]. Recent histopathological observation has shown that progesterone alone induced negligible stromal proliferation; the addition of a small quantity of estrogen to progesterone induced pronounced mitotic activity in the stroma. In typical pregnant guinea pigs, many epithelial cells undergo mitosis immediately before and after ovulation, with peak mitotic numbers in the glandular epithelium occurring approximately four days post-ovulation. Cell division in the stroma reached its zenith on the sixth day of the cycle, immediately preceding the anticipated time of implantation. This transition of uterine mitotic activity from the luminal epithelium to the glandular epithelium and then to the stroma is noted in the mouse. The above observation shows that both progesterone and estrogen are secreted prior to ovum implantation in the guinea pigs [44].

The hormonal control of uterine reactivity for ovum implantation in the rats is similar to that in the mouse. In mouse models, estrus estrogen plays a crucial function in regulating the uterine response to following hormones [18]. Where as in the rats, estrus estrogen does not appear to influence the subsequent uterine responses to hormones because progesterone alone renders the uterus "**pre receptive**", a condition which can be activated with estrogen [27].

Pseudopregnancy is not usually induced by synthetic progestins alone. The uterus of ovariectomised rats receiving daily injections of progesterone is maximally sensitized for decidual reaction with a single injection of concomitant estrogen. The duration of receptive stage is about 12 hours [45], and the uterus becomes refractory to stimulation for decidual reaction. The uterus refractory state can be sustained for a lengthy duration, provided that daily progesterone therapy and a four-day estrogen injection schedule are followed. The refractory state can be abolished if progesterone treatment is discontinued for 2 days [27].

2.4 Trends in Contraception:-

Intraceptives are the agents acting after fertilization. Contraceptives are agents that prevent ovulation and act before fertilization.

Contraception methods available for birth control can be grouped as follows:-

1. Natural methods
2. Barrier methods
3. Mechanical sterilization
4. Immunological methods
5. Hormonal methods [46]

Among these, oral contraceptives are the most widely used method for contraception.

2.4.1 Natural methods:-

Natural methods of contraception utilize the barriers in space and time or use the natural antifertility effect of breastfeeding. There prevent pregnancy without the use of chemical agents or physical devices. These methods include:-

- Abstinence
- Coitus interrupts
- Safe periods
- Temperature methods

These natural methods have a high rate of failure this is the biggest limitation of these natural methods.

Natural, physical contraceptive approaches rely on the understanding of specific changes in the body that occur throughout the menstrual cycle. The lactational amenorrhea method (LAM) relies on the physiological impact of suckling to inhibit ovulation, resulting in the absence of ovum [47].

2.4.2 Barrier methods:-

The goal of these techniques is to keep live sperm from coming into contact with the ovum. They have the benefit of not having the negative effects of IUDs and pills [48].

a. *Chemical methods:-*

These include spermicides, which comprise

- Soluble films
- Suppositories
- Foam tablets and aerosol
- Creams, Jellies, and Pastes [49]

Surface-active compounds, which bind to spermatozoa and prevent oxygen intake, are commonly used spermicides.

b. Physical methods:-

- Vaginal sponge:-

Forms a barrier that prevents sperm from entering the cervix and either kills or immobilizes them. Olive oil or vinegar is used to soak the sponge. It is a little sponge made of polyurethane foam that has been saturated with spermicidal monoxynol-9. Protected is the only sponge in the market, but recently Allendale Pharmaceuticals bought the rights to manufacture Today sponge [50].

- Diaphragm:-

It is a vaginal barrier. It is a side-effect-free, simple, and efficient form of birth control. However, it is not a perfect and foolproof kind of birth control [51].

Condom:-

Around the world, it is the most often utilized barrier device. The advantages and disadvantages are identical to those of the diaphragm [52].

2.4.3 Mechanical sterilization:-

Mechanical methods of contraception include "intrauterine devices (IUDs)". IUDs could be copper-releasing, inert, or progestin-releasing. The most likely explanation for IUD actions is the migration of WBCs in the uterine cavity due to the presence of a foreign body. The cells probably capture the ascending sperms during intercourse and probably alter and destroy the blastocyst in case they are formed. TCu – 200 is the IUD releasing copper ion which is being used in the national program in India. **Progestasert** [53] is another medicated IUD, which releases 65 microgram of natural progesterone for about a year. LNG-20 IUD, a norgestrel-

releasing device, has been found to remain effective for 5-7 years. The use of IUDs in certain females has been associated with potential risks, encompassing excessive menstrual bleeding, infertility, pelvic inflammatory disease, and pain [53].

2.4.4 Immunological methods:-

For the development of vaccines for contraception, four different molecules have been identified. They are FSH, HCG, sperm surface antigen, and Zona Pellucida antigen. An injectable vaccine, TALSUR (Talwar and Suri) [54], has been evaluated in non-human male animals, and it has been reported that after weeks of single administration in Cauda epidydmis, sperm becomes free of sperm and complete azoospermia is obtained.

Modugal et al 1998 developed a vaccine against FSH for males, employing bovine FSH as an immunogen. Immunization with FSH vaccines leads to oligospermia [55]. HCG-based immunization is an advanced stage of development. Talwar vaccine consists of HCG linked to LH and conjugated with diphtheria toxoid to form a hapten carrier complex and claims to show promise in phase 2 clinical trials. Active immunization with IDH-C4 (Lactate dehydrogenase C4 sperm antigen) suppressed fertility in mice, rabbits, and several other vaccines have been developed against sperm antigens.

2.4.5 Hormonal methods:-

These methods act via the following mechanisms

- By suppressing ovulation
- By changing endometrium
- By thickening of cervical mucus, thus preventing sperm penetration
- By decreasing sperm movement in upper genital tract (Fallopian tubes) or decreasing likelihood of implantation

I) Hormonal contraceptives *may be classified as follows.*

A) Oral pills:-

- Progesterone-only pills
- Long-acting pills

- Combined pills
- Male pill

B) Depot formulations:-

- Subcutaneous implant
- Injectables
- Vaginal rings

II) ORAL CONTRACEPTIVES:-

II a. Combined pill:-

Both progestin and estrogen are present. With experience, the amount of estrogen has decreased: 30µg of ethylestradiol per day is regarded as the threshold. The estrogen is either ethinylestradiol or its methyl ester, mestranol, which is 1.6 times weaker. Since they have strong antiovulatory effects when taken alone, the progestin is a 19-nortestosterone molecule. The ovulation inhibitory dose (per day) of the progestins now in use is estimated to be 400 mg of norethindrone, 60 mg of levonorgestrel, 60 µg of desogestrel, 200 µg of norgestimate, and 40 µg of gestodene. Progestin ensures on-time bleeding at the end of a cycle and lowers the risk of endometrial cancer caused by estrogen, even though progestins and estrogen work together to prevent ovulation. On the fifteenth day of menstruation, one progestin pill should be taken every day for 20–22 days. The following treatment is started six to eight days after the bleeding has stopped. Consequently, a 28-day cycle is maintained. There are calendar packs available. This is the most widely used and effective technique [56].

II b. Sequential pill:-

The first 15 tablets contained only estrogen, and the next 6 had a progestin added. The dose of estrogen in this region had to be relatively larger, and failure rates were slightly higher.

Further, estrogen alone could increase the incidence of endometrial carcinoma. Therefore, these have been withdrawn [57].

II c. Phased regimens:-

These have been developed to allow for a decrease in the overall dosage of steroids without

compromising effectiveness. These can be either triphasic or biphasic. In the second and third phases, there is little progestin and a steady estrogen dosage (or a minor variation between 30 and 40 µg). Women over 35 have been advised to use phasic tablets [58].

II d. Mini pill (luteal supplement):-

Since estrogen has been linked to a number of long-term hazards, it has been developed to remove it. Every day, without fail, a low-dose progestin-only pill is taken. Twenty to thirty percent of women ovulate, and menstruation becomes irregular. When amenorrhea lasts longer than two months, efficacy decreases to 96–98% from 98–99% with a combination pill; look for pregnancy. This method is uncommon [59].

II e. Post coital (Morning after) pill:-

High dose of on estrogen (stilboestrol 25mg or ethylestradiol 2.5mg twice daily for 5 days) or ethylestradiol 0.1mg + levonorgesteral 1.0mg twice 12hours apart starting within 3 days of intercourse. It is less effective (90-95%). Nausea and other side effects are marketed, and if pregnancy occurs, the offspring has high risk [60].

III Injectable:-

These were developed to remove the requirement for everyday pill consumption. Given through the muscle as an oily solution, they are quite effective and have been used by over 40 million women. However, they have several drawbacks.

a) Animal studies show carcinogenic risk. After 30 years, human research has produced no results. The risk of cervical, ovarian, and liver cancer has not increased. Younger women (under 35) may have a higher breast cancer risk.

b) Menstrual irregularities and amenorrhea are prevalent; fertility may resume after 6 to 30 months post-discontinuation; permanent infertility may develop in certain women [61].

III a. Two types of preparation have been tested:-

Inject long-acting progestin once every few months, depending on steroid dose. Marketing two compounds:

a) After i.m. injection of 150-400mg DMPA at 3-6 month intervals, peak blood levels are

reached in 3weeks and fall with t1/2 of about 50days.

b) Norethindrone enanthate 200mg at 2-3 months intervals. The most important undesirable property is complete disruption of menstrual bleeding pattern and total amenorrhea in many cases.

c) Long-acting progestin combined with long-acting estrogen administered monthly.

These have undergone limited testing in only a few countries and are not being further pursued. The primary advantage is that they facilitate a normal menstrual bleeding pattern in the majority of cases. Their evident disadvantage is the presence of a long-acting estrogen, which poses possible risks [62].

IV Implants:-

These are under-the-skin implanted drug delivery devices that release steroids gradually over a one to five-year period. Norplant: For subcutaneous implantation, a package of six capsules comprising 36 mg of levonorgestrel (a total of 216 mg) has been sold in the USA and other nations. It lasts for five years.

In certain nations, an intrauterine insert that is impregnated with progesterone **(PROGESTASERT)** [53] has been introduced. It has less progestin, which mostly affects the endometrium locally. The efficacy rating is lower, and the device must be updated annually (**K.D. Tripathi 2025**) [64] Voluntary sterilization includes female tubal occlusion and male vasectomy. Two of the substances, which have been reported as promising vasoccluding agents, are carbolic acid cynoacrylate glue, and polyurethane elastomer. The term "induced abortion" describes the early removal of the products of conception from the uterus, typically prior to the twentieth week of pregnancy. Abortion, sometimes known as non-surgical abortion, can be induced by some medications, most notably the French medication **RU 486**. Mifepristone, often known as RU 486, is an antiprogestin that binds to progesterone receptor sites in the endometrium. Prevents endometrial progesterone action. Within 12 and 72h, the endometrium weakens and loses. After conception, RU 486 can be used for five weeks. It is followed by prostaglandin, which increases uterine contents and helps expel the endometrium. Uterine hemorrhage is one of the medication's adverse effects. In the United States, RU 486 is undergoing clinical testing. One example of an estrogen antagonist is **ENTCHROMAN**

(Saheli), a non-steroidal substance that was created as a weekly pill that works by stopping implantation in the uterus [63].

2.5 Antifertility agents from plant sources:-

Due to the rise in the human population, there is an extensive search for a suitable contraceptive that has no or minimal side effects. A constant emphasis has been laid on the regulation of fertility through plants and their products. India is a country that has a rich medicinal plant history. According to Indian ayurvedic belief, almost every plant possesses some medicinal property which is yet to be searched out. Furthermore, the efficacies of these plants have not yet been confirmed in repeated investigations. Hence the efforts are still being made in this field to find a plant with potent antifertility activity [65].

2.5.1 Antifertility plants can broadly be classified into:-

A. Plants active in females:-

1. Anti-ovulatory plants
2. Anti-estrogenic plants
3. Anti-implantation plants

B. Plants active in males:-

1. Spermicidal plants
2. Anti-spermatogenic plants

1. Spermicidal plants:-

Over 2200 plant extracts have been tested at CDRI (LUCKNOW), out of which 16 showed spermicidal activities [42, 43]. Of these, the potential plants in order of activity are *Sapindus mukorossi* fruits pericarp, *Albizzia lebbeck* pod and root, *Madhuca butyraceae* seed, and *Trigonella foenum-graecum* seed. Another group found that Azardiachta indica neem oil kills monkey and human spermatozoa [44]. In rhesus monkeys, intravaginal neem oil use before and after coital treatment inhibited conception [45]. Praneem, a refined seed extract of neem, has been shown to induce pregnancy termination in rodents when taken orally; THI cytokines are likely responsible for this effect [46]. It was discovered that NIM-76, a component extracted from neem oil, reduced sperm motility by causing cytosolic LDH to gradually leak out of the

sperm. Banerji et. al. assessed the spermicidal action of 12 sapogenins [66]. Acacic acid from *Acacia Concina* bark, oleanolic acid from *Albizzia procera* root and *angalliogenone* from *Aragallis arvensis* plant were spermicidal at a concentration of 0.004 to 0.008% in structure activity relationship studies with 10 sapogenins; it was demonstrated that spermicidal activity is related to beta- amyrin C-28 carboxylic acid type molecules [42]. In NII, Delhi, Praneem polyherbal cream has been developed as a spermicidal formulation. It contains quinidine hydrochloride and praneem extract from the fruit pericarp of Sapindus species. After intravaginal administration, it has demonstrated great contraceptive efficiency in both rabbits and monkeys [67].

Table 2.1: Spermicidal Plants

Spermicidal Plants			
Sr. No.	**List of Plants**	**Part used**	**Active Principle**
1	*Acacia concina*	Stem bark	Acacic acid [66]
2	*Albizzia procera*	Root Seed	Oleanolic acid Oleanolic acid and Proceric [66]
3	*Anagallis*	Whole	Anagalligenone [66]
4	*Balanites*	Fruit	Diosgenin [66]
5	*Madhuca lantifolia*	Seed	Bassic acid [66]
6	*Madhuca*	Seed	Bassic acid [66]
7	*Mimusops elengi*	Seed	Bassic acid [66]
8	*Mimusops*	Seed	Bassic acid [66]
9	*Mimusops littpratis*	Seed	Bassic acid [66]
10	*Pittosporum dulce*	Seed	Oleanolic acid and Echinocytic acid [66]
11	*Pittosporum nilghirense*	Seed	Pittoside A or B [66]

1. Antispermatogenic Plants:-

From the five plants tested in rodents, dogs at different schedules, eleven plant extracts and 6 compound fractions have been reported to arrest spermatogenesis and also to reduce the weight of accessory organs. None of them had a selective anti-spermatogenic effect. In addition to supporting spermatogenesis, *Allium sativum bulbus* caused hypoglycemia and hypolipidemia [68], Cannabis sativua plant lowered liver glycogen and adrenal ascorbic acid [69], *Hibiscus rosa sinesis* flowers reduced pituitary rats [71, 72], and *Malvaviscus conzattii* flowers stimulated adrenal weight in house rat and gerbil [70].

Table 2.2: Antispermatogenic Plants

Antispermatogenic Plants					
Sr.No.	**List of Plants**	**Part used**	**Species tested**	**Effect on Spermatogenesis (Stage arrested)**	**Effect on accessory sex organ**
1	*Aristolochia indica*	Whole plant	Mouse	Secondary spermatocyte	Reduced [73]
2	*Azardiracht a indica*	Leaf	Mouse	Fall in fertility	Not Reduced [76]
3	*Calatropis procera*	Flower	Gerbil	Primary spermatocyte	Reduced [77]
4	*Carica papaya*	Seed	Rat	40% Fall in fertility	No effect [78]
5	*Embelia ribes*	Berry	Monkey	Adversely affected semen quality and quantity	Testostero ne reduced

					[79]
6	*Hibiscus rosa sinesis*	Flower	Rat	Adversely affected	Reduced [71]
7	*Malvaviscus conzattii*	Flower	Gerbil Rat Mouse	Spermatogonia	Reduced [80]
8	*Ocimum sanctum*	Leaf	Mouse	Spermatid induced sterility	Reduced [72]
9	*Pisum sativum*	Seed	Rat	No effect, Fertility normal	No effect [81]
10	*Vinca rosea*	Leaf	Rat	Spermatogonia	Not Reporter [82]

2. Fertility-inhibiting plants:-

It has been demonstrated that *Embelia ribes* [79] negatively impacts the quantity and quality of semen while also lowering testosterone levels in bonnet monkeys. It has been found that giving mice or rats varying doses of *Azadirachta indica* leaves [76], Carica papaya seeds [78], and paracoumaric acid from the Aristolochia indica plant [73] lowers fertility without affecting spermatogenesis.

PLANTS FOR FEMALE CONTRACEPTION:-

1) ANTI OVULATORY PLANTS:-

Agents disturbing the natural steroid hormone balance by inhibiting the synthesis or the release of gonadotrophins can be widely used as contraceptives of the 24 plants evaluated for inhibition in rabbits [5] or induction of digestrous stage in rats, Albizzia lebbeck seed [83], *Aloe*

barbadensis leaf [84], *Mentha arvensis* leaf [85-87] and *Vitex negundo* plant [83] exhibit 70% more anti-ovulatory activity in rabbits. Leaves of *Butea monosperma* demonstrated anti_ovulatory activity in mice, noted to be significant [88]. *Embelia ribes* berries showed 100% antiovulatory activity in rabbits [89]. The plant of *Solanum khasianum* showed anti-ovulatory activity using alcoholic extract in rabbits. Leaves of *Taxus bacatta* showed anti-ovulatory activity in rabbits when the aqueous extract was screened [90].

Table 2.3: Antiovulatory Plants

Antiovulatory Plants			
Sr. No.	**List of Plants**	**Part used**	**% Activity**
1	*Albizzia lebbeck*	Seed	60 [83]
2	*Annona squamosa*	Plant	40 [83]
3	*Aloe barbadensis*	Leaf	80 [84]
4	*Areca catechu*	Nut	40 [84]
5	*Carica papaya*	Seed	20 [85]
6	*Daucus carota*	Seed	40 [85]
7	*Lepidium sativum*	Plant	40 [83]
8	*Lygodium flexosum*	Plant	20 [91]
9	*Mentha arvensis*	Leaf	60 [85]
10	*Nigelia sativa*	Plant	20 [83]
11	*Polygonum hydro piper*	Root	60 [85]
12	*Randia dymetorum*	Seed	20 [83]
13	*Vitex negundo*	Plant	60 [83]
14	*Butea monosperma*	Leaf	Significant [88]

15	*Embelia ribes*	Berry	Significant [89]
16	*Solanum khasianum*	Plant	Significant [89]
17	*Taxus baccata*	Leaf	Significant [90]

1) Anti-implantation plants:-

Abortifacients are the agents causing expulsion of the fetus from the womb when the fetus is incapable of independent survival. In Indian materia medica, Medicinal plants of India, and Indigenous drugs of India [87], 120 plants from 103 genera and 54 families have been examined for their potential as emmenagogues and abortifacients. Ancient literature mostly mentions plant use for abortion sticks, used locally or as oral decoctions. Abortion sticks are plant twigs wrapped in a piece of rag or cotton wool soaked with the juice of astringent plants like *Abrus precatorius*, *Calotropis procera*, *Calotropis gigantea*, *Semecarpus anacardium*, etc., or irritant twigs of the plant *Plumbago rosea*, Plumbagozylanica, Euphorbiaceous plants, and less frequently of *Nerium odonum* alone or smeared with Femla asafoetida. Oral abortifacients include ergot, quinine, or drastic purgatives like *Aloe barbadensis* and irritant volatile oils from *Juniporus Sabina*, *Mentha pulegium*, *Tenacetum vulgare* etc. These plants are intestinal irritants and produce violet gastro- enteritis leading to uterine contraction and abortion.

23 plants belonging to 23 genera and 21 families have been tested for abortifacient activity in rodents (Table 2.3). All the plants except *Calotropis gigantea* and *Vitex negundo* induced pregnancy in more than 50% animals. The tested plants included 13 mentioned in ancient literature, and of these, *Achyranthus aspera* [75], *Aristolochia indica* [44], Carica papaya [92], and *Daucus carota* [93]. In a preliminary clinical trial, ethanolic extract of *Pucraria tuberosa* has been found to induce abortion in about 50% subjects. [84]

Table 2.3: Abortifacient Plants

Abortifacient Plants			
Sr.No.	**Name of Plants**	**Part used**	**% Activity**
1	*Abroma augusta*	Root	28-90 [75]
2	*Abrus precatorius*	Seed	60 [94]

3	*Achyranthes aspera*	Stem bark	0-100 [95]
4	*Adhatoda vasica*	Plant	60-100 [94]
5	*Azadirachta indica*	Root	65-100 [74]
6	*Carica papaya*	Unripe fruit	100 [92]
7	*Cichorum papaya*	Plant	80 [94]
8	*Daucus carota*	Seed	100 [96]
9	*Embelia ribes*	Seed	67 [94]
10	*Ensete superbum*	Seed	100 [97]
11	*Momordica olifera*	Root	80-90 [99]
12	*Moringa olifera*	Root	60 [94]
13	*Plumbago zylanica*	Plant	88 [5]
14	*Pueraria tuberosa*	Tuber	70 [101]
15	*Sesbania*	Flower	70 [98]
16	*Terminalia arjuna*	Stem bark	60 [94]
17	*Vitex negundo*	Seed	50 [102]
18	*Woodfordia buticosa*	Flower	100 [74]

2.6 Interceptive plants:-

There are many indigenous plants that are reputed to possess interceptive activity when administered orally [90]. Extracts of 113 plants and 48 compound fractions isolated from 26 plants [5, 103, 104, 94, 49] have been tested in rats or mice in a 1-5 or 1-7 days post coital schedule. *Abroma augusta*, *Calotropis gigantea*, *Michaelia champaka*, *Polygonum hydropiper*, and *Plumbago rosea* have been reputedly mentioned in literature as antifertility agents [85-87]. 6T plant species covering 59 genera and 37 families, but not mentioned in ancient literature, have been tested for anti-implantation activity in rodents. The plants showing more than 50% activity, though with equivocal activity reports in some cases, are *Artemisia Scoparia* plant,

Artabotrus orodratissimus leaf, *Cassia fistula* fruit, *Curcuma longa* rhizome, *Embelia ribes* berry, and *Ensete superbum* seed. Coronaridine isolated from *Tabemaemontana heynera*, *Datura lacfone* form *Datura quercifolia* [105], ferujol from *Femla jaeschena* [106], and active fraction from *Lepidium capitatum* [107] and butanol fraction of *Achyranthus aspera* [95] exhibited estrogenic activity, whereas VIDR – 2GD from *Ensete superbum* [97] had weak estrogenic activity but interfered with implantation in mice, Rats, and Guinea pigs. Aristolic acid or its methyl ester extracted from *Aristolochia indica* [74] and oleanolic acid-3-glycoside from *Randia dumetomm* [109] possessed anti-estrogenic activity and were devoid of estrogenicity.

Table 2.4: Interceptive Plants

Interceptive plants			
Sr. No.	**Name of Plants**	**Part used**	**% Activity**
1	*Abroma augusta*	Root	0-100 [110]
2	*Abrus precatorius*	Seed Root	0-100 [114] 75-100 [115]
3	*Acacia nigata*	Bark	20 [74]
4	*Achyranthus aspera*	Seed	0-100 [74]
5	*Acrostichum aureum*	Plant	66.6 [94]
6	*Actiniopteris radiata*	Plant	90 [80]
7	*Adhatoda vasica*	Leaf	50 [94]
8	*Adina cordifolia*	Leaf	100 [116]
9	*Albizzia lebbeck*	Bark	20 [94]
10	*Aloe barbadensis*	Leaf	0-100 [118]
11	*Andropogon monticola*	Root	20 [94]

12	*Annona squamosa*	Seed	16-50 [118]
13	*Anthemis cotula*	Plant	33.3 [94]
14	*Areca catechu*	Nut	0-80 [123]
15	*Aristolochia indica*	Root	100 [73]
16	*Arabotrus odoratissimus*	Leaf	0-67 [75]
17	*Asclepias currassavica*	Plant	40 [84]
18	*Berberis asiatica*	Root	20 [94]
19	*Butea frondosa*	Petals	67 [119]
20	*Butea monosperma*	Seed	0-62 [120]
21	*Syzygium jambos*	Stem bark	Significant [119]

2.7 Future potentials:-

Highly potent synthetic anti-ovulatory agents are under wide clinical usage; hence, further research on ovulation-inhibiting agents may be redundant. Presently, emphasis is on the development of post coital contraceptives. Studies are aimed at identifying plants with anti-implantation activity. The top priority plants for detailed follow-up studies would be those with an inert hormonal profile, such as *Lygodium flexosum* and *Embelia ribes*, followed by those with an anti-estrogenic profile, such as *Randia dumetorum*, or with weak estrogenic and anti-estrogenic property namely *Artabotrys odoratissimus* and *Vitex negundo*. Keeping in view the side effects of using estrogens for contraception, the plants exhibiting estrogenic activity and/or toxicity do not deserve attention for follow-up. Promising plants tested under interceptive plants deserve attention, as enough data on them is not yet available [122].

In order to assess plants for male fertility regulation, coordinated research activities are required. The leading plants appear to be *Carica Papaya* and *Embelia ribes*. Among the

spermicidal plants, *Sapindus mukorossi* saponins are already under clinical evaluation and lead. Follow-up plants include Trigonella foenumgraecum, Madhuca butyracea, and Albizzia lebbeck. The conclusions of Kamboj and Dhawan, Choudhary and Haq, Choudhary et al., Satyavati and Patnaik, and Dhawan were among the published works by Indian researchers that offered useful reference information on the extracts/compounds used, testing schedules, activity profiles, hormonal profiles, and toxicity data of fertility-regulating plants up until 1985 and should be consulted before selecting a plant for additional research [123].

Antiestrogenic activity is usually determined by the ability of a compound to inhibit the increase in uterine weight induced by an estrogen. Antiestrogens may be designated as those compounds that interfere with any of the actions of estrogen. For estrogenic/antiestrogenic activity, uterine weight and vaginal opening are usually taken as endpoints. High doses of estrogen caused premature vaginal opening in immature mice, and cornified epithelial cells prevailed in the vaginal smear [121].

2.8 Unani System of Medicine on Family Planning:-

The Unani system of family planning has prevailed since 4th century A.D. They are broadly classified as:

1. Temporary methods and medicines
2. Permanent methods and medicines

2.8.1 Temporary methods and medicines:-

Hamool is a cloth wetted in medicine used in the vaginal cavity, commonly used in Unani literature, and qatiran is the oil of Sanibar (Pinus roxburgii).

Various temporary methods utilized for contraception are:

a) Hamool of qatiran in the vaginal smear
b) Hamool of rogan-e-balsan
c) Safeda kashgahri (Oxycarbonate of lead)
d) Yellow thin rind of Anar (Punica granatum)
e) Seeds of karamkalla (Brassica oleracia)
f) Hamool of Piper nigrum (kali mirch)

g) Juice of Ocimum sanctum (jungle tusli) (Avicenna)

Himalayan of rock salt (Sendha namak, Euphorbia lathyrus, Salixamophylla gharab before intercourse – Almajury, oil of Sesamum indicum Ceelaqii) is also an effective contraceptive method.

Oil of *Convolvulus Scamonia* (sakmunia) mixed with oil of *Euphorbick lathyrus* and Hamool of *Thalictrum folisum*.

Henna of *Mentha silvesterrs*, *arq-e-gulab* ½ kg orally, and seeds of *Brassica oleracea* can be used for controlling fertility for one year.
Abrus precatorius, Liquidambar orientaliscan check fertility for two years (Antaki). Hamool of *Mentha arvensis* (Pudina), *Querces infectoria* (Mazoo), *Terminalia chebula* black, *Luffa echinata* (bundle), *Brassica oleracea* (karmkalla), *Euphorbia resinifera* (farfuni), *Areca catechu* (supari), *Syzygium aromaticam* (laung)**,** Hamool of convolvulus *scammonia*, *Pinus roxburgii* (sambar), *Ficus religiosa* (dar-e-filfil), *Juniperus communis* (abhal).

Embrocation of dried leaves of *Euphorbia cathyrus* and Bora-e-armania of equal weight is used as a male contraceptive.

Embrocation of the seeds of *Lawsonia inermis* and *Lawsonia alba* mixed with leaves of sudab and fresh water of *Coriandrum sativum*. Vaginal suppositories are made up of *Quercus infectoria* and *Myrthus communis*, both of equal weight, which are mixed with hot water. Medicines for oral use include seeds of *Brassica oleracea*, leaves of *Curcumis melovar utilissimus*, *Ferula narthex*, leaves of *Euphorbia lathyrus*, dried prawn seeds of *Physalis alkekengi*, *Ricinus communis*, *Abrus precatorius*, *Jasminum grandflomm*, *Caparis deciduas*, *Peganum husmala*, *Cumin of Butea monosperma*, Seed of *Tamarix aphylla*. Various pills mentioned in the Unani literature are *Pimpinaca anusum*, *lllicum anisatum*, *Mentha longefolia*, *Apium graviolens*, *Nardo stachys*, Jatamansi, *Cinnamonum zeylanicum*, *Juniperus communis*, and *Saussurea lappa*.

2.8.2 Permanent contraceptive methods:-

Surma, Iron rust, if taken orally, caused permanent contraception. A suppository made up of the seeds of *Moringee oleifera*, powdered and mixed with cow milk, ghee, and honey, caused permanent contraceptive action. Oral preparations include powdered *Cateria lacca*, *Vicia fabu*. Other oral remedies are the oil of seeds of *Indigofera tincteria*, *Curcuma longa*, *Euphorbia norifolia*, powder of *Ficus religosa*, *Embelia ribes*, *Paganum hurmala*, all of equal weight mixed with milk prone to be an effective antifertility agent. *Centratherum anthelmantium*, *Terminalia chebula*, *Zinjjibar zerumble*, *Nigella sativa*, and *Myrica nagi*, all 18 grams powdered and mixed in water, made into a tablet, and one tablet taken daily during menstruation have been proven to be an effective method of permanent contraception as per Unani literature.

2.9 Profile of Plants:-

Herbal remedies, **Unani**, and **Ayurvedic** treatments are used by many alternative medicine therapies because of the remarkable power of herbal therapy. Roughly 25% of all prescription medications come from plants like herbs, trees, or bushes. India is known as the world's medicinal garden due to its wealth of medicinal plants. One of the oldest ancient medicinal plants is *Saraca asoca*. The Sanskrit word asoka, or ashoka, meaning **"without sorrow"** or "without grief." One of India's most revered and mythical trees is the Ashoka. The Ashoka tree, which belongs to the Caesalpinaceae family, is commonly referred to by its Latin binomial name, *Saraca asoca* (Roxb.), De.wild, or *Saraca indica*. This evergreen tree is known as the Asok tree in English. Other names for it include Kankeli (Sanskrit), Ashok (Marathi), Ashoka (Assamese), Ashoka (Bengali), Ashoka (Hindi), Ashoka (Gujrati), Ashokadamara (Kannada), Asokam (Malayalam), Ashok (Punjabi), Ashok (Kashmiri), Ashoka (Oriya), Asogam (Tamil), and Ashokapatta (Telugu). It can be found all over India, but particularly in Bengal, Kerala, the Himalaya, and the entire southern region. Hindu mythology states Gautama Siddhartha (c. 563-483 B.C.) was born under the Ashoka tree. Hindus worship Ashoka on December 27 for Kamadeva, the Hindu God of Love. The current study seeks to explain *Saraca asoca's* pharmacological and therapeutic effects (**Ayurvedic Pharmacopoeia of India, 1986. Vol. I; Part-I:17-18)** [128]**.**

Classification:-

Kingdom: Plantae Division: Magnoliophyta Class : Mgnoliopsida Order : Fabales
Family: Caesalpinaceae Genus: *Saraca*
Species : *Asoca* [128]

Botanic description-

Saraca indica Or Saraca asoca, is a small evergreen tree 7 to 10 cm high. The parpinnate, 15-20 cm long leaves have firmly sub-coriaceous, oblong, 6-12 leaflets. The leaves have a thin lanceolate shape, a cork-like base, and intrapetiolar, fully joined petioles. The bark has a warty surface and is dark brown, gray, or nearly black. The presence of rounded or protruding lenticels causes the stem bark to be rough and uneven. Bark that is traversely ridged, smooth, and occasionally fractured, with round lenticles. A thin, continuous layer of white is visible behind the cork lever, revealing a striated surface due to fracture splinting. The scent of flowers is delightful. Polygamous, apetalous flowers that are yellowish orange before turning crimson, with thin, deciduous bracts, axillary panicles, calyx petaloid, and corymbose positioned horizontally. Four to eight compressed, ellipsoid-oblong seeds are produced. [129 - 131]

Powder characters-

Under a microscope, ashoka bark powder, which has a brown color, contains a lot of fibers, stone cells, sieve tube fragments, parenchyma cells, tracheids, and numerous unknown cells.

Properties and action-

Rasa: Kasaya, tikta, madhura
Guna: Guru **Virya** : Usna **Vipaka** : Katu
Karma : Kaphapittashamaka, varnya, swarya, visa, sothaghna, kusthagahna, pramehaghna, vrsya, krimighna, stambhna, artavjannan, rasayan, sonitsthapna

Biological and pharmacological activity of *Saraca indica:-*

Antimicrobial Activity:-

The antimicrobial potential of Saraca indica (ethanol: water, 1:1) was evaluated using agar plate assays against various bacterial species, including Salmonella typhosa, Bacillus subtilis, Escherichia coli, and Staphylococcus aureus. However, no significant activity was observed against Agrobacterium tumefaciens. In contrast, dried flower buds of Saraca indica exhibited notable antibacterial activity against several pathogenic strains such as Shigella boydii, Salmonella viballerup, E. coli, Shigella flexneri, Vibrio cholerae, and Shigella dysenteriae.

Further investigations on leaf extracts revealed that both aqueous and 95% ethanol extracts demonstrated antibacterial activity against E. coli, while no inhibitory effect was observed against Staphylococcus aureus. Antifungal studies using methanolic extracts of Saraca indica at concentrations ranging from 1000 to 5000 µg/mL showed inhibitory effects against fungal species such as Alternaria cajani, Helminthosporium sp., Bipolaris sp., Curvularia lunata, and Fusarium sp., with significant activity observed particularly against A. cajani, even at lower concentrations.

The antibacterial activity of different bark extracts of Saraca asoca was assessed against a wide range of pathogens, including Salmonella typhi, E. coli, Bacillus cereus, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella aerogenes, Shigella boydii, and Proteus vulgaris. Additionally, enteric pathogens such as Shigella sonnei, E. coli, and Salmonella enteritidis were tested. Among the extracts, all except the aqueous extract exhibited antibacterial activity, with the methanolic extract showing the highest efficacy.

Leaf extracts (methanol and aqueous) of Saraca asoca also demonstrated strong antibacterial activity against Pseudomonas aeruginosa, Bacillus subtilis, and Salmonella typhimurium. Moreover, these extracts exhibited considerable antifungal activity against Alternaria alternata, Colletotrichum gloeosporioides, and Drechslera specifera. The in vitro antibacterial activity of bark extracts was further confirmed using the agar well diffusion method against pathogens

such as Proteus vulgaris, Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus spp., and Klebsiella pneumoniae at a concentration of 4 mg/mL, where both ethanol and aqueous extracts showed broad-spectrum antibacterial effects.

In addition to antimicrobial activity, crude extracts from leaves, flowers, and bark of Saraca asoca were evaluated for larvicidal activity against early fourth-instar larvae of mosquito vectors, including Aedes aegypti, Culex quinquefasciatus, and Anopheles stephensi. At an initial concentration of 1000 ppm and exposure durations of 24–48 hours, significant larval mortality was observed. Notably, after 48 hours, petroleum ether extract of the leaves and chloroform extract of the bark resulted in over 50% mortality in Culex quinquefasciatus larvae. [132].

Anticancer Activity:-

The anticancer compound isolated from Saraca asoca flowers demonstrated approximately 50% in vitro cytotoxic activity against Dalton's lymphoma ascites and Sarcoma-180 tumor cell lines at concentrations of 38 µg and 54 µg, respectively. Importantly, the compound did not exhibit cytotoxic effects on normal lymphocytes, while showing selective cytotoxicity toward lymphocytes derived from leukemia patients. [133]

Antimenorrhagic Activity:-

Dried bark from *Saraca indica* has been utilized in India for the treatment of menorrhagia. In India, women suffering from uterine ailments are administered a tonic derived from the desiccated bark and flowers of *Saraca indica*. The stem bark of *Saraca indica* is also utilized to treat any menstrual cycle-related disorders. In Sri Lanka, the bark of *Saraca indica* is traditionally employed in the management of menorrhagia and various menstrual disorders. In India, it is widely utilized as a uterine sedative, and hot aqueous extracts administered to adult females are reported to stimulate uterine activity in a manner comparable to ergot, without inducing tonic contractions. The bark is also utilized in the treatment of menorrhagia, uterine ailments, and as an ingredient in formulations intended for female reproductive health, including contraceptive preparations. In Pakistan, *Saraca indica* bark is traditionally used to

alleviate menorrhagia and uterine discomfort. Additionally, in India, the dried bark is employed as an astringent to control excessive uterine bleeding associated with menorrhagia and is valued for its refrigerant and demulcent properties, as well as for relieving uterine disorders, including common abdominal pain during menstruation. Reports indicate that the aqueous extract of the bark includes active constituents that both stimulate and relax the smooth muscle of guinea pig's ileum. The medication is said to enhance uterine stimulation, resulting in more common and protracted contractions. The crystalline glycoside compound is additionally noted for inducing uterine contractions. [134-139]

Antioxytocic Activity:-

The plant demonstrated oxytocic effects in isolated uterine preparations from both rats and humans. A gravid or estrogen-primed uterus exhibited increased susceptibility to the effects of the alcoholic extract. The oxytocic effect was totally inhibited by pentolinium bitartrate. It has been discovered that seed extract works well against dermatophytic fungi. *S. indica* extracts did not exhibit oxytocic action in rat uterine preparation experiments conducted in vitro. Previous tests on S. indica have shown two negative results and one positive result [136].

Ayurvedic actions:-

Vedana sthapana- Beneficial for treating any painful problem.
Varnya- *Ashoka* helps the body's complexion.
Grahi- *Ashoka helps digestion and absorption.*
Trishanashnam- *Ashoka* alleviates excessive thirst.
Daha shamanam- *Ashoka* alleviates burning sensation.
Krimighna- *Ashoka* kills all pathogenic agents.
Shothajit- *Ashoka* is useful in management of all edematous conditions.
Vish asrajit- *Ashoka is beneficial for all blood diseases and toxicities.*
Apachijit- It is helpful in controlling lymph node inflammation.
Asrigdara nashanam- In the treatment of heavy menstrual bleeding

CHAPTER – 3
AIM & OBJECTIVES

TITLE: Evaluation of Anti fertility Activity of *Saraca Indica* (Ashoka) in Experimental Animals

3: AIM & OBJECTIVES

3.1 Aim:

The primary aim of the present research is to evaluate the anti-implantation, anti-estrogenic, anti-ovulatory, oxytocic and anti-androgenic activities of *Saraca indica* (Ashoka), with special emphasis on ursolic acid as a key triterpenoid constituent associated with its traditional use in gynaecological disorders. The study is intended to determine whether ursolic acid can act as a natural modulator of reproductive physiology in validated *in vitro* and *in viv*o rodent models, thereby generating experimental evidence to support its potential role in fertility regulation.

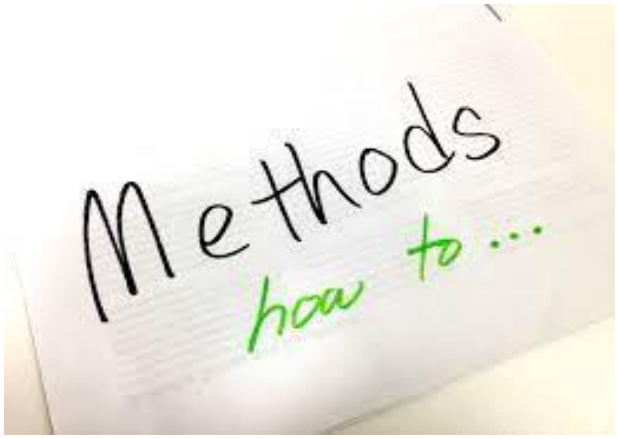
3.2 Objectives:

The study achieved its aim through the following specific objectives:

1. **To conduct a comprehensive literature review** on the ethnomedicinal uses, phytochemistry and reported reproductive or antifertility effects of *Saraca indica* and ursolic acid, in order to identify existing knowledge and gaps that justify the present work**.**
2. **To extract the bark of *Saraca indica* and prepare an ursolic acid enriched fraction, and to identify and quantify ursolic acid in this fraction using chromatographic techniques (TLC and HPLC).**

   This objective includes optimizing extraction conditions, confirming the presence of ursolic acid by TLC through comparison with a standard, and estimating its content in the enriched fraction by HPLC analysis.
3. **To evaluate the *in vitro* oxytocic activity of ursolic acid using** isolated rat uterine preparations, by recording uterine contractions at increasing concentrations of ursolic acid and comparing its contractile response and potency with that of the standard uterotonic agent oxytocin.
4. **To determine the anti implantation potential of ursolic acid in female rats** by employing an established early pregnancy model, quantifying the number of corpora lutea, implantation sites, live fetuses and resorptions, and calculating pre implantation and post implantation losses to assess its impact on implantation success.

5. **To evaluate the anti estrogenic activity of ursolic acid in immature mice** by measuring uterine weight in estrogen challenged animals and performing histopathological examination of uterine tissues, with particular reference to endometrial proliferation, glandular development and stromal changes.
6. **To investigate the anti ovulatory effects of ursolic acid in Wistar female rats using** a cupric acetate based ovulation model, by assessing ovarian organ weights and ovarian histology (follicular maturation, corpus luteum formation and other structural alterations) to determine inhibition or delay of ovulation.
7. **To examine the anti androgenic activity of ursolic acid in male rats by** evaluating reproductive organ weights (testes, and epididymides), sperm parameters (count, motility, viability and morphology) and testicular histology, using cyclophosphamide as a standard reference agent for male reproductive toxicity.
8. **To perform appropriate statistical analysis of all experimental data** (e.g., one way ANOVA followed by suitable post hoc tests) in order to determine the significance of the observed effects and to draw evidence based conclusions regarding the antifertility profile of ursolic acid.



CHAPTER – 4 MATERIALS AND METHODS

TITLE: Evaluation of Anti fertility Activity of *Saraca Indica* (Ashoka) in Experimental Animals

4: MATERIALS AND METHODS

4.1 In Vitro Screening of Ursolic Acid for Oxytocic Activity

4.1.1 Animal Selection and Estrous Cycle Synchronization

Adult female Wistar rats (170-200 g, 2-3 months old) were "housed in a conventional laboratory environment (12:12 h light: dark cycle, 22±2°C, 50 to 60% humidity) with access to a pellet food and water. The estrous cycle, lasting 4-5 days, was observed daily via vaginal smears obtained by gently irrigating the vagina with 0.2mL of normal saline and" inspected under a light microscope at 40× magnification.

Dioestrus: Predominance of leukocytes

Proestrus: Large number of nucleated epithelial cells

Estrus: Cornified epithelial cells (optimal stage for uterus isolation)

Metaestrus: Mixture of keratinized cells, nucleated epithelial cells, and leukocytes

Rats not in estrus were synchronized with stilbestrol (0.1mg/kg, subcutaneously, 24h before experimentation). All animal procedures received approval from the "Institutional Animal Ethics Committee (IAEC Approval No. [ARL/PT/975/2023])".

4.1.2 Materials and Reagents

Animals: Female Wistar rats (170-200 g)

Drugs: Oxytocin (10 µg/mL stock solution), Stilbestrol (0.1 mg/kg), Ursolic acid (purity >98%)

Solutions: De Jalon's solution (NaCl 9.0g, KCl 0.42g, $CaCl_2$ 0.06g, $NaHCO_3$ 0.50g, Glucose 0.05g per liter distilled water)

Vehicle: Dimethyl sulfoxide (DMSO, analytical grade)

Equipment: Student organ bath (20mL capacity), aerator pump, tuberculin syringe (1 mL), Sherrington rotating drum, kymograph, analytical balance, silk threads (4-0)

4.1.3 Preparation of De Jalon's Solution

De Jalon's solution was made daily by dissolving 9.0g NaCl, 0.42g KCl, 0.06g $CaCl_2$, 0.50g $NaHCO_3$, and 0.05g glucose in 1 L of distilled water. To ensure tissue viability, maintaining solution at 32°C with 95% O_2 + 5% CO_2 aeration.

4.1.4 Isolation of Uterine Tissue

- Rats in estrus were euthanized by CO_2 asphyxiation (70% CO_2 in air for 3-5 min).
- The abdominal cavity was accessed through a midline incision, revealing both uterine horns.
- The uterine horns were carefully separated from surrounding adipose and connective tissue.
- Tissues were immediately transferred to a Petri dish containing oxygenated De Jalon's solution at 32°C.
- Each horn was longitudinally sectioned, and 2-3 cm segments were excised, avoiding the cervical and ovarian regions.
- Both ends of the tissue segment were tied with fine silk threads (4-0), leaving a central portion for mounting.

4.1.5 Mounting and Stabilization of Tissue

The uterine segments were placed in 20 mL organ baths of De Jalon's solution at 32°C with continuous aeration. The thread connected the higher end to the frontal writing lever and the lower end to a tissue holder. With a 500 mg weight, resting tension was 500 mg (0.5 g). After 45 minutes of tissue equilibration, spontaneous contractions stabilized.

Tissues showing irregular contractions were discarded.

4.1.6 Preparation of Test Substances

To create a stock solution of ursolic acid, weigh 100mg using an analytical balance and dissolve it in 10 mL of DMSO, resulting in a concentration of 10mg/mL (10,000µg/mL).

Pure ursolic acid (purity 99.2%, Hubei Heilongjiang Biotechnology Co., Ltd,) was used as the test compound. Working doses were formulated through dilution in De Jalon's solution.

Oxytocin standard: Stock solution (10µg/mL) was made in distilled water.

Rationale for DMSO: DMSO was selected as a vehicle due to excellent solubility of ursolic acid, established safety in isolated tissue preparations, and compliance with international guidelines

(OECD, 2008) [111 – 114].

4.1.7 Experimental Protocol

Standardization: Dose-response curve (DRC) for oxytocin was established using 0.1, 0.2, 0.4, 0.8, and 1.6 mL of 10 µg/mL solution (doses: 1, 2, 4, 8, 16 µg).

Test doses: Ursolic acid was tested at 0.3 mL of 10 mg/mL solution (30 µg dose), ensuring responses fell within the linear portion (20-80%) of the oxytocin DRC.

Confirmatory tests: Two additional 30 µg doses were administered after 30-minute washout periods.

Response recording: Contractions were recorded isotonically on Sherrington rotating drum (speed: 2 mm/min) via frontal writing lever (magnification 8×). Response amplitude was measured as height (cm) from baseline.

Data expression: Responses were expressed as a percentage of maximum oxytocin response (7.8 cm = 100%).

4.1.8 Number of Experiments

Each concentration was tested on uterine tissues from 3-5 different rats (n=3-5). Each tissue served as its own control.

4.2. Extraction, fractionation and chromatographic analysis of *Saraca Indica* bark

4.2.1 Extraction procedure

- Approximately 1000 g of air-dried, powdered *Saraca indica* bark was subjected to exhaustive maceration with n-hexane (~1.5 L) at room temperature. The macerated mixture was filtered through Whatman filter paper, and the n-hexane solvent was removed under reduced pressure using vacuum distillation, yielding approximately 1.4 g of crude n-hexane extract.

4.2.2 Fractionation

- The crude n-hexane extract was fractionated by column chromatography over silica gel (60–120 mesh), initially eluted with 100% n-hexane, followed by gradual increases in ethyl

acetate concentration (n-hexane:ethyl acetate 95:5 to 70:30). Fractions were monitored by TLC using anisaldehyde–sulphuric acid spray reagent for visualization. Fractions matching ursolic acid standard (Rf and color) were pooled as the ursolic-acid-rich fraction.

4.2.3 Identification by TLC

- Precoated Merck silica gel 60 F254 TLC plates were used. Standard solution (1 mg/mL ursolic acid in n-hexane) and test solution (1 mg of n-hexane fraction in 1 mL n-hexane) were spotted (5 µL bands). Plates were developed in ethyl acetate:n-hexane (17:3) mobile phase in a saturated chamber, air-dried, sprayed with anisaldehyde–sulphuric acid reagent, and heated at 105°C for 5–10 min. Spots were compared for Rf values and color characteristics.

4.2.4 Quantification by HPLC

- HPLC analysis was performed on an Agilent HPLC system with photodiode array (PDA) detector. Chromatographic separation was achieved on a C18 column (250 × 4.6 mm i.d., 5 µm) using binary gradient elution: Mobile phase A (water + 0.1% trifluoroacetic acid), Mobile phase B (methanol). Gradient program: initial 20% A, programmed to 100% B over 50 min. Flow rate: 0.8 mL/min, column temperature: 27°C, injection volume: 20 µL, total run time: 70 min with 20 min equilibration. Detection wavelength: 278.6 nm. Standard solution (1 mg/mL ursolic acid in n-hexane) and test solution (1 mg/mL fraction in n-hexane) were filtered through 0.45 µm membrane filters. Ursolic acid content was determined by direct peak area comparison with the standard under identical conditions, suitable for identification and semi-quantitative assay in a phytochemical screening context [115].

4.3 Anti-ovulatory Activity in Cupric Acetate-Induced Ovulation Model

4.3.1 Animal Ethics Approval

The Institutional Animal Ethical Committee (IAEC) of Accuprec Research Labs Pvt. Ltd., Ahmedabad, Gujarat, India, approved the study (Protocol No. ARL/PT/691/2023 dated 12-05-2023). All animals had been maintained under standardized laboratory settings (22 ± 3°C, 30-70% relative humidity, 12:12 h light: dark cycle) with unlimited access to a standard pellet meal and

water.

4.3.2 Animal Subjects and Experimental Design

Twenty-four mature female Wistar rats (180-220 g) were isolated individually for 21 days to ensure nulliparity and prevent pregnancy. Following isolation, rats had been randomly allocated to four groups (n=6 per group) as per body weight using stratified randomization to minimize inter-group variation [117]:

- Group 1: Vehicle control (0.5% w/v aqueous CMC, 10mL/kg, p.o.)
- Group 2: Low dose ursolic acid (100mg/kg b.wt./day, p.o.)
- Group 3: High dose ursolic acid (250mg/kg b.wt./day, p.o.)
- Group 4: Recovery high dose ursolic acid (250mg/kg b.wt./day, p.o.) + 21-day recovery period

4.3.3 Dose Selection Rationale

Doses were selected based on safety margins derived from ursolic acid's reported LD_{50} (approximately 1000mg/kg, p.o. in rats). The low dose (100mg/kg = 1/10th LD_{50}) and high dose (250mg/kg = 1/4th LD_{50}) were chosen to evaluate efficacy within therapeutic safety windows while maximizing potential anti-ovulatory effects.

4.3.4 Vehicle Selection Rationale

Aqueous 0.5%w/v carboxymethyl cellulose (CMC) was selected as vehicle due to:

- Formation of homogeneous suspensible formulations
- Successful passage through syringebility testing (19G needle)
- Established safety profile for oral gavage in reproductive toxicity studies
- Minimal impact on gastrointestinal absorption

4.3.5 Dose Formulation Preparation

Dose formulations were prepared fresh daily immediately before administration:

1. Ursolic acid was accurately weighed for each dose group. Pure ursolic acid (purity 99.2%, Hubei Heilongjiang Biotechnology Co., Ltd,) was used as the test compound.
2. Powder was gradually dispersed in 0.5% w/v aqueous CMC using a magnetic stirrer (500 rpm) for 15 minutes to ensure homogeneity.

3. Suspensions were maintained under continuous stirring during dosing to prevent settling.
4. Vehicle control received 0.5% CMC alone.

4.3.6 Dose Administration Protocol

Ursolic acid or vehicle was administered orally via gavage using a 16G curved stainless steel cannula attached to a 1 mL calibrated tuberculin syringe. Dose volume was standardized at 10mL/kg body weight, calculated daily as per individual fasted body weights [116].

Treatment Schedule:

- Days 1-3: Oral administration of vehicle (Group 1), low dose (Group 2), high dose (Groups 3 & 4) once daily at 10:00 AM.
- Day 3 (~30 min post-final dose): Cupric acetate (4mg/kg, i.v.) had been administered to all groups to induce ovulation. Fresh 0.4% w/v cupric acetate solution was prepared in sterile saline.
- Group 4 Recovery: Following treatment, animals were maintained for 21 days without further dosing.

4.3.7 Observations and Monitoring

- Mortality/Morbidity: Monitored twice daily (AM/PM) throughout the investigation.
- Clinical Signs: Recorded daily, including lethargy, piloerection, lacrimation, and behavioral changes.
- Body Weight: Recorded on Days 1, 3, 7, 14, and 21 (Group 4 only).

4.3.8 Necropsy and Tissue Collection

Groups 1-3 (Terminal Necropsy - Day 3):

1. Animals were euthanized by CO_2 asphyxiation (70% CO_2 for 3-5 min) followed by exsanguination via cardiac puncture.
2. Both ovaries were excised, trimmed of extraneous tissue, and weighed immediately (absolute ovary weight).
3. The ovaries were sectioned: one was preserved in 10% neutral buffered formalin for histopathological examination, while the other had been rapidly frozen in liquid nitrogen for prospective biochemical study.

4. Relative ovary weight was assessed as:

   Relative ovary weight=Absolute ovary weight (g)Final body weight" (kg)×1000Relative ovar y weight=Final body weight (kg)Absolute ovary weight (g)×1000

Group 4 (Recovery Necropsy - Day 24):

- Identical procedures performed after a 21-day recovery period.

4.3.9 Histopathological Examination

Ovaries preserved in formalin were subjected to a graded procedure involving alcohols, cleaned with xylene, and subsequently embedded in paraffin. H&E-stained serial sections (5 µm) were examined under a light microscope at 10× & 40× magnification for:

- Follicular development (primordial, primary, secondary, Graafian follicles)
- Corpora lutea count
- Ovulation sites
- Stromal changes and vascularity

4.3.10 Statistical Analysis

Data are expressed as mean ± SD. Statistical comparisons were undertaken employing one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. Considered value of $P<0.05$ statistically significant. Analysis was done utilizing GraphPad Prism version 9.0.

4.4 Anti-Implantation Activity of Ursolic Acid in Female Albino Rats

4.4.1 Animals, Housing, and Ethical Considerations

Healthy adult female *Wistar albino rats* (Rattus norvegicus), weighing 150 to 200g and aged 8 to 10 weeks, along with sexually mature male rats weighing 250 to 300g with confirmed fertility, were obtained from Accuprec Research Labs Pvt. Ltd. All animals underwent a 7-day acclimatization period before research under standardized laboratory conditions: temperature (23±2°C), a 12h light/dark cycle (lights on at 07:00h), relative humidity (50 to 70%), and unrestricted access to standard pelleted rat food and purified water ad libitum. Animals were accommodated in polypropylene cages (30 × 20 × 10 cm) with rice husk bedding, which was replaced biweekly [121].

The Institutional Animal Ethics Committee (IAEC No. [Protocol No. ARL/PT/691/2023 dated 12-

05-2023]) approved all experimental procedures, which followed the CCSEA, Government of India, guidelines. The study followed the 3Rs principle (Replacement, Reduction, Refinement) and used small group sizes (n=6 females/group) depending on power analysis (α=0.05, power=0.80, expected effect size=50% implant reduction).

A total of 32 rats (24 females, 8 males) were used, distributed as detailed in Table 4.1.

Table 4.1: Experimental grouping and treatment schedule for anti-implantation study

Group	Treatment	Dose (mg/kg b.wt.)	Vehicle & Dose Volume	Route	Females (Rat Nos.)	Males (Rat Nos.)
1	Control	-	0.5% CMC, 10mL/kg	p.o.	1–6	31, 32
2	Ursolic acid (Low)	100	0.5% CMC, 10mL/kg	p.o.	7–12	33, 34
3	Ursolic acid (Mid)	125	0.5% CMC, 10mL/kg	p.o.	13–18	35, 36
4	Ursolic acid (High)	250	0.5% CMC, 10mL/kg	p.o.	19–24	37, 38

Ursolic acid was suspended fresh daily in 0.5% w/v carboxymethyl cellulose (CMC, viscosity 400–800 cps, Loba Chemie) aqueous solution. Pure ursolic acid (purity 99.2%, Hubei Heilongjiang Biotechnology Co., Ltd,) was used as the test compound. Dose volume was calculated based on latest body weight and administered orally using a curved stainless-steel gavage needle (16G, 100 mm length) fitted to a 5 mL calibrated syringe between 09:00–10:00 h daily from Day 1 to Day 7 post-coitum.

4.4.2 Vaginal Cytology for Estrous Cycle Monitoring and Pregnancy Confirmation

Daily estrous cycle monitoring: Vaginal smears had been collected daily (08:30–09:30 h) for 10–14 days prior to mating to confirm regular 4–5 day cycles. Rats were gently restrained in ventral recumbency. Using a sterile Pasteur pipette (150 mm × 7 mm, Borosil), ~0.1 mL sterile 0.9% saline (w/v, pH 7.4, autoclaved) was introduced into the vagina, aspirated, and reinstilled 3–5 times to collect exfoliated cells without traumatizing vaginal epithelium (which could cause false-positive leukocytic infiltration).

The collected fluid (~20–30 µL) was immediately transferred to preheated (37°C) glass slides, covered with 22 × 22 mm coverslips (avoiding air bubbles), and examined fresh under a compound microscope (Olympus CX21, 10×/0.25 objective, 45× total magnification) within 2 min. Estrous cycle stages were identified per standard criteria (Hafez; Marston and Chang):

- **Proestrus (~12 h):** Predominantly nucleated epithelial cells (round nuclei, moderate cytoplasm).

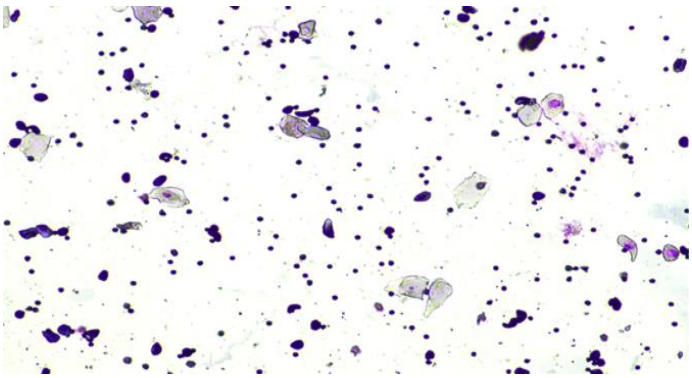


Figure 4.1: Proestrus phase in vaginal smear

- **Estrus (~12 h):** Anucleated cornified squamous cells (>90%, large, flat, eosinophilic).

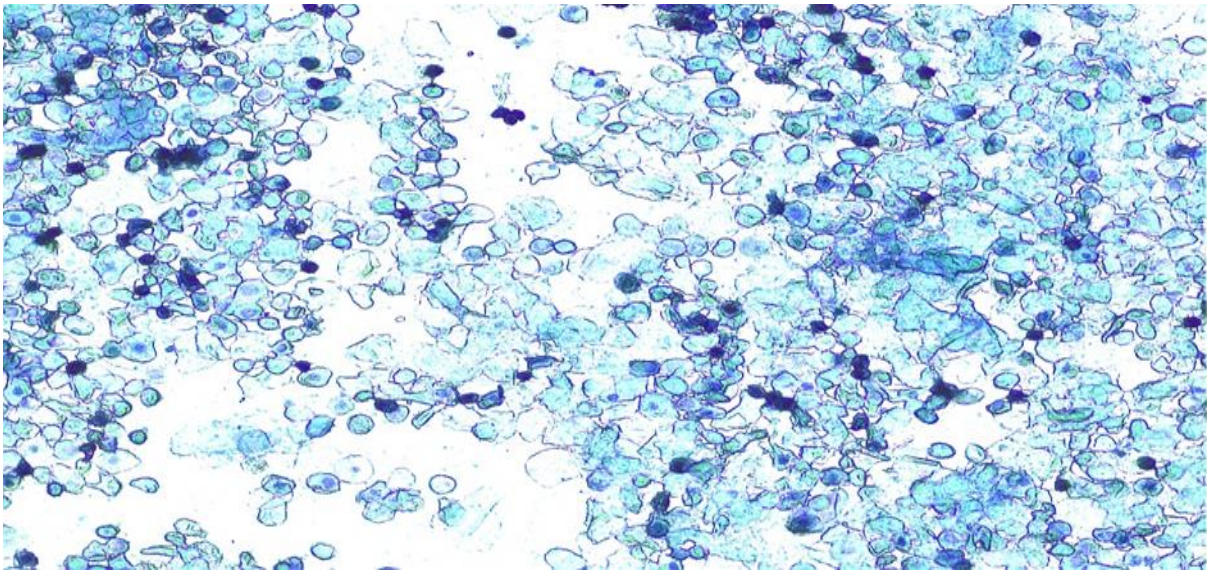


Figure 4.2: Estrus phase in vaginal smear

- **Metestrus (~21 h):** Mixed cornified epithelial cells + leukocytes + few nucleated epithelial cells.
- **Diestrus** (~57 h): Predominantly polymorphonuclear leukocytes.

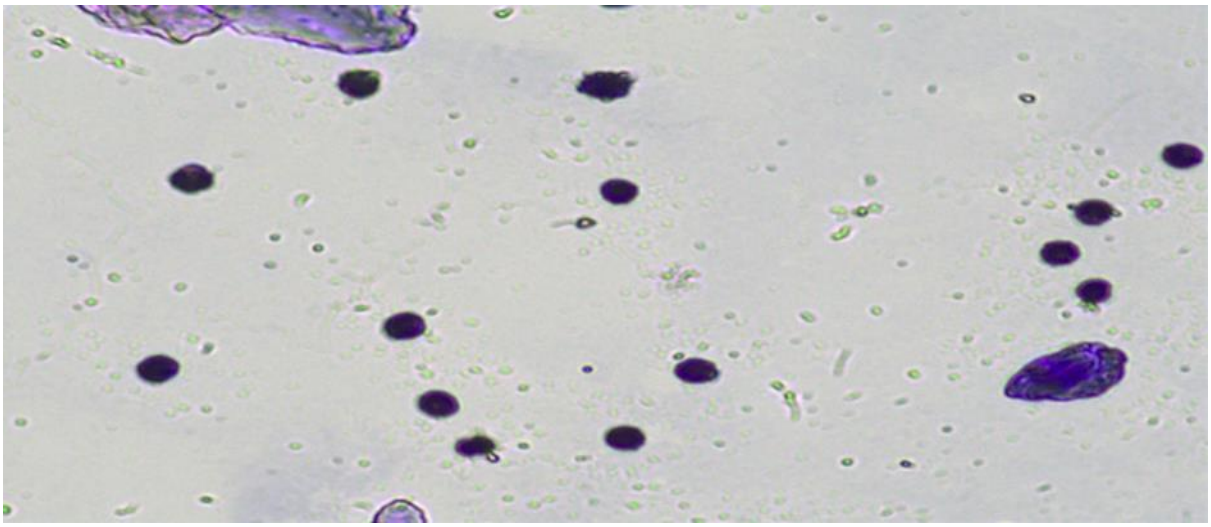


Figure 4.3: Metestrus and Diestrus phase in vaginal smear

Permanent smear preparation: Select smears were air-dried (5 min), fixed in absolute methanol (AR grade, 5 s immersion), air-dried (10 min), and examined without coverslip under phase contrast (40×). Slides were stored at room temperature for records.

Mating confirmation: Only females in verified proestrus/estrus showing regular cyclicity were paired with males (3:1 ratio) from 17:00 h to 08:30 h (over night). Vaginal smears collected in the early morning (08:30 h) were analyzed for spermatozoa, characterized by specific motility and morphology under 100× oil immersion, marking that day as Day 1 of pregnancy. Positive females had been segregated and assigned to groups utilizing a random number table.

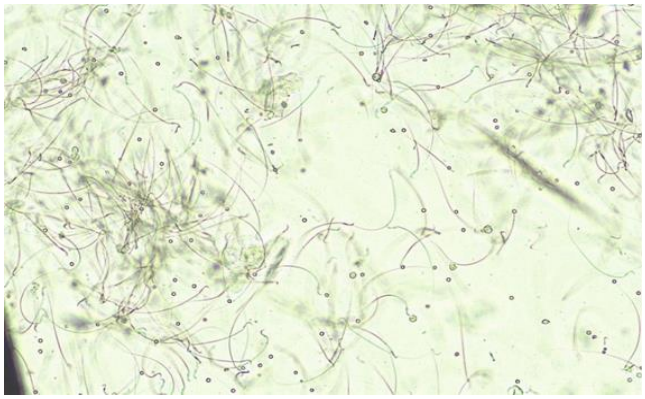


Figure 4.4: Sperm in vaginal smear

4.4.3 Mid-Pregnancy Laparotomy (Day 10 Post-Coitum)

On Day 10 (±2 h), rats were subjected to overnight fasting and thereafter anesthetized intraperitoneally with ketamine hydrochloride (80mg/kg, Neon Laboratories) and xylazine (10mg/kg, Indian Immunologicals; total volume of 0.5 mL/kg). Anesthesia depth was confirmed by pedal reflex absence and stable respiration (60–80 breaths/min).

Surgical preparation: Ventral abdominal hair was clipped (2 × 4 cm midline area) using sterile curved scissors. Skin was sequentially cleaned with 70% ethanol → povidone-iodine → 70% ethanol. Rats were positioned supine on a heated pad (37°C) under aseptic conditions.

Laparotomy procedure:

1. A 2–2.5 cm midline incision was made through skin and linea alba using sterile scalpel (#11 blade).
2. Peritoneum was incised, and intestinal coils gently displaced laterally with moist sterile gauze (0.9% saline soaked).
3. Bilateral uterine horns were exteriorized onto saline-moistened gauze. Each horn was examined under incident light for:

- Implantation sites: Discrete nodular swellings (1–2 mm diameter) producing "beaded" appearance.
- Resorption sites: Hemorrhagic/degenerating implants (pale, irregular).

4. Ovaries were exteriorized; corpora lutea counted as distinct yellow corpora hemorrhagica (×2 magnification loupe).
5. Data recorded per animal/horn/ovary in proforma.

Closure: Organs returned to peritoneal cavity, dusted with neomycin sulphate powder (100 mg, Albert David). Muscular layer closed with continuous 3-0 absorbable catgut (Ethicon, Pain 2-0, 3.5 metric, NW-4241, 75 cm length). Skin closed with 4-0 silk (non-absorbable, interrupted sutures). Wound dressed with povidone-iodine ointment. Rats recovered in individual cages on heating pads (37°C) with ad libitum access to liquid diet (Ensure, Abbott) until voluntary feeding resumed (Agrawal and Aravind, 1994).

Post-operative care: Animals monitored q2h × 6h for pain (tail pinch response), infection, or dehiscence. Buprenorphine (0.05mg/kg s.c.) provided if distress observed. Surviving rats observed

until parturition.

4.4.4 Parturition Monitoring and Litter Evaluation

Rats were allowed to deliver normally (gestation: 21–23 days). Cages checked twice daily (08:00, 20:00 h) from Day 20. Live litters (pupil-present, pink, moving) counted on postpartum Day 1 (08:00 h). Dead pups, cannibalized litters, or dystocia noted. Pups were weighed collectively (±0.1 g) and sexed by anogenital distance.

4.4.5 Calculation of Anti-Implantation Parameters

Anti-implantation efficacy calculated per established formulae (Shafiq et al., 2003; Agrawal and Kachroo, 2009):

1. Pre-implantation loss = No. of corpora lutea (Day 10) – No. of implants (Day 10)
2. Post-implantation loss = No. of implants (Day 10) – No. of live litters delivered
3. % Pre-implantation loss = [(No. of corpora lutea – No. of implants) / No. of corpora lutea]×100
4. % Post-implantation loss = [(No. of implants – No. of litters) / No. of implants]×100
5. Implantation efficiency = (No. of implants / No. of corpora lutea)×100
6. Fertility index=(No. of litters delivered / No. of pregnant rats)×100

SEM represents mean±standard error of mean. One-way ANOVA with Dunnett's multiple comparison test (GraphPad Prism v9.0). Significant differences were assessed at $p<0.05$ in comparison to control group.

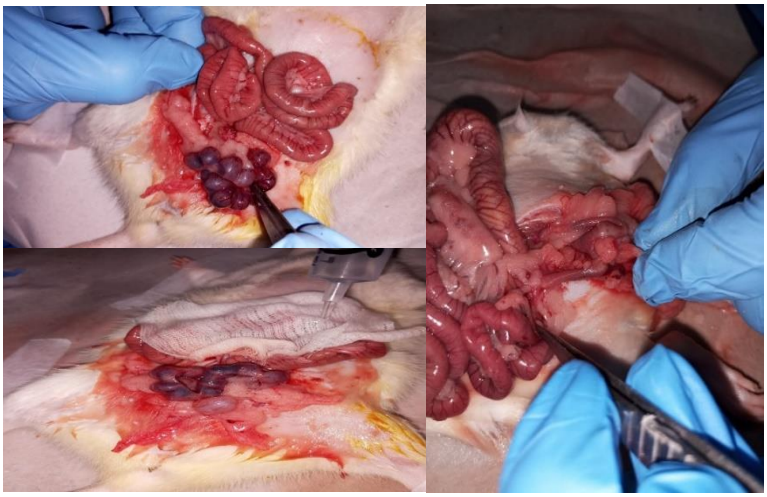


Figure 4.5: Images of Laparotomy

4.5 Anti-Estrogenic Activity of Ursolic Acid in Immature Female Mice

4.5.1 Animals, Housing, and Ethical Approval

Immature female Swiss albino mice (Mus musculus, 18–22 g body weight, 21–23 days old) were procured from Accuprec Research Labs Pvt. Ltd. Animals were exposed to controlled laboratory conditions for seven days, including a temperature (23 ± 2°C), relative humidity (50 to 70%), a 12-hour light/dark cycle (lights on at 07:00), and unlimited access to pelleted mouse chow and purified water. Mice had been kept in 29 × 22 × 14 cm polypropylene cages (5-6 per cage) with rice husk bedding, replaced biweekly.

All methods received approval from the Institutional Animal Ethics Committee (IAEC No. [Protocol No. ARL/PT/691/2023 dated 12-05-2023]) in accordance with CCSEA rules, Government of India. The study complied with the 3Rs principles, utilizing a minimal group size (n=6/group) determined by power analysis (α=0.05, power=0.80, anticipated effect size=40% reduction in uterine weight). Table 4.2 shows that 30 immature female mice were randomly assigned to 5 groups (n=6).

Table 4.2: Experimental grouping for anti-estrogenic activity study

Group	Treatment	Dose	Route & Schedule	No. of Mice
1	Control	Vehicle (0.5% CMC)	10 mL/kg, p.o., Day 1–5	6
2	Standard (Estrogen)	0.05 µg/animal	s.c., Day 1	6
3	Ursolic acid (Low dose)	200mg/kg b.wt.	10 mL/kg, p.o., Day 1–5	6
4	Ursolic acid (High dose)	500mg/kg b.wt.	10 mL/kg, p.o., Day 1–5	6
5	Estrogen + Ursolic acid	0.05 µg (s.c., Day 1) + 200 mg/kg (p.o.)	s.c. + p.o., Day 1–5	6

Dose selection rationale: Ursolic acid doses were calculated based on reported oral LD_{50} of 2000 mg/kg b.wt. in Swiss albino mice. Low dose (200mg/kg=1/10th LD_{50}) and high dose (500mg/kg=1/4th LD_{50}) were selected to evaluate dose-dependent anti-estrogenic effects within a safe therapeutic range.

Test agents:

- Pure ursolic acid (purity 99.2%, Hubei Heilongjiang Biotechnology Co., Ltd,) was used as the test compound suspended fresh daily in 0.5% w/v carboxymethyl cellulose (CMC, Loba Chemie).
- 17β-Estradiol (0.05 µg/animal in 0.1 mL sesame oil) as positive control.
- Oral doses administered (10 mL/kg) using 20G curved gavage needles (100 mm length) between 09:00–10:00 h. Subcutaneous injections given in dorsal neck region.

4.5.2 Treatment Protocol and Body Weight Monitoring

Treatment commenced on Day 1 (age 28–30 days). Animals were weighed daily (animal weighing balance) prior to dosing to calculate individual dose volumes and monitor growth. Body weight gain had been computed as:

% Body weight gain=[(Final body weight – Initial body weight) / Initial body weight] × 100

4.5.3 Uterine Weight Assessment

On Day 6 (24 h post-final treatment), mice were humanely sacrificed by CO_2 asphyxiation followed by exsanguination. Both uterine horns were promptly removed, excised of adherent adipose tissue and mesenteries, wiped on filter paper, and weighed utilizing an analytical scale [117].

Uterine weight parameters:

1. Absolute uterine weight (mg)
2. Relative uterine weight = [Uterine weight (mg) / Final body weight (g)] × 100
3. Uterine weight index = [Uterine weight (mg) / 100 g body weight]

Uteri from Groups 2 and 5 had been preserved in 10% neutral buffered formalin for histopathology.

4.5.4 Serum Estrogen Quantification

Trunk blood (~0.5–0.8 mL) was collected post-sacrifice in EDTA-coated tubes, centrifuged (3000 × g, 4°C, 10min), and serum was kept at -80°C. Serum 17β-estradiol levels were evaluated using ELISA kits (Mouse Estrogen ELISA Kit, [Fine Test, Code: EM 1501, Batch Number: FN241009 A) per manufacturer protocol.

Assay specifications:

- Range: 12.5 to 800pg/mL
- Sensitivity: 4.7pg/mL
- Precision: Intra-assay CV <8%, Inter-assay CV <10%
- Sample volume: 50 µL serum (diluted 1:5)
- Wavelength: 450 nm

Concentrations calculated from the standard curve using four-parameter logistic regression (GraphPad Prism). All samples were assayed in duplicate.

The anti-estrogenic effects of ursolic acid were assessed by quantifying estrogen levels in the serum of mice with the help of a Fine Test ELISA kit. For this assay, the concentrated wash buffer supplied with the kit was first diluted with distilled water based on the manufacturer's instructions. A series of standard estrogen solutions was then prepared using the dilution buffer at successive dilutions of 1/2, 1/4, 1/8, 1/16, 1/32, and 1/64 to generate the calibration curve. One vial containing only dilution buffer was kept as the blank control to correct for background absorbance.

Biotin-labelled detection antibody working solution was prepared freshly before use to ensure optimal binding to the estrogen present in the serum samples. In addition, the horseradish peroxidase (HRP)–streptavidin conjugate (SABC) working solution was prepared, which binds to the biotinylated antibodies and enables colorimetric detection. The schematic representation of the assay procedure delineates the following steps: following the incubation of samples and standards on the coated plate, detection antibody and SABC are added in succession, subsequently introducing the TMB substrate, which generates a blue color that transitions to yellow upon adding stop solution, and is determined at 450nm to quantify estrogen levels.

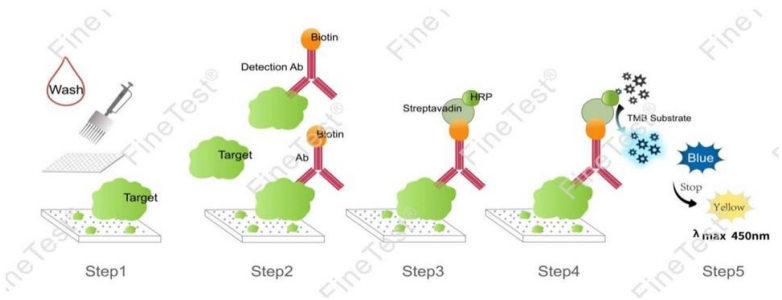


Figure 4.6: FineTest ELISA Kit

4.5.5 Uterine Histopathology

Formalin-fixed uteri (Groups 2, 4, 5) were processed for paraffin embedding:

1. Dehydration through graded ethanol (50–100%, 1 h each)
2. Clearing in xylene (2 changes, 45 min each)
3. Infiltration with molten paraffin wax (58–60°C, 2 h)
4. Embedding in rectangular blocks (Leica EG1150H)

Sectioning and staining:

- Cut 4-5µm sections using Leica RM2245, rotary microtome.
- Mounted on poly-L-lysine coated slides
- Stained with Hematoxylin & Eosin (H&E): Harris hematoxylin (5 minutes), acid alcohol differentiation (2 seconds), 1% eosin Y (3 minutes)
- Dehydration, clearing, DPX mounting

Slides examined under light microscope (Olympus BX51, 10×, 40× objectives). Quantitative morphometry performed using ImageJ software (v1.53):

- Endometrial thickness (µm, 5 random measurements/horn)
- Luminal epithelial height (µm)
- Stromal edema score (0–3 scale)
- Glandular development score (0–4 scale)

Qualitative parameters: Epithelial stratification, stromal cellularity, vascular congestion, leukocytic infiltration.

4.5.6 Statistical Analysis

Data are obtainable as mean ± SEM. Normality was evaluated employing Shapiro–Wilk test. For normally distributed data, one-way ANOVA was performed, followed by Dunnett's post hoc test for comparisons with the control group or Tukey's test for multiple group comparisons. Estrogen + ursolic acid group compared with estrogen control using unpaired t-test. Differences measured significant at $p<0.05$ (GraphPad Prism v9.0). Uterine weight reduction ≥30% vs. estrogen control considered biologically significant anti-estrogenic activity.

Expected anti-estrogenic endpoints:

- Reduced uterine wet weight
- Lower serum estrogen levels
- Attenuated estrogen-induced histological changes (endometrial proliferation, stromal edema)

4.6 Screening of Ursolic Acid as an Anti-Androgenic Agent

- The current investigation attempts to screen the potential anti-androgenic activity of ursolic acid in male Wistar rats using cyclophosphamide as a standard reference compound for induction of testicular and reproductive toxicity. Ursolic acid (UA), a pentacyclic triterpenoid, was selected as the test item because earlier rodent studies have indicated endocrine-modulatory, anti-androgenic, anti-inflammatory, and antifertility properties, suggesting that UA may interfere with androgen-dependent reproductive functions. Cyclophosphamide monohydrate (CP) of analytical grade was obtained from a certified manufacturer (HiMedia Laboratories Pvt. Ltd., India); the accompanying certificate of analysis described it as a white crystalline powder having a melting range (47–53°C) and an assay purity of approximately 97.59% by HPLC, confirming its suitability as a positive control toxicant. The batch used in this work had defined quality-control release and expiry dates, ensuring that the compound was used within its validated stability period.
- Twenty-four healthy adult male Wistar albino rats were used as experimental subjects in order to provide sufficient statistical power while respecting ethical constraints on animal use. Animals were kept in clean polypropylene cages under controlled conditions comprising a 12:12hour light–dark cycle, maintaining ambient temperature (22 ± 2°C), and relative humidity of 55 ± 5%, which are considered optimal for maintaining normal physiological and

reproductive status in this strain. Standard pelleted rodent feed and potable water were supplied ad libitum throughout the research to avoid confounding nutritional effects. All rats were acclimatized to the animal facility for a minimum of five days prior to dosing to minimize stress-related variability. All experimental procedures were performed in compliance with the guidelines prescribed by the Committee for Control and Supervision of Experiments on Animals (CCSEA), Government of India. The study protocol was reviewed and approved by the Institutional Animal Ethics Committee of Accuprec Research Labs Pvt. Ltd., Ahmedabad, Gujarat (Protocol No. ARL/PT/691/2023; dated 12-05-2023).

- Dose levels for ursolic acid were selected on the basis of previously published reports demonstrating significant endocrine and reproductive effects of UA in male rodents without overt systemic toxicity at comparable doses. Pure ursolic acid (purity 99.2%, Hubei Heilongjiang Biotechnology Co., Ltd,) was used as the test compound. Two dose levels were employed: a low dose of 100mg/kg body weight and a high dose of 250mg/kg body weight, allowing assessment of dose-dependent anti-androgenic responses. Cyclophosphamide was administered at 30mg/kg body weight via the intraperitoneal route on days 17–21, a regimen widely recognized to induce oxidative stress, germ-cell depletion, and testicular damage, thereby providing a robust positive control model for male reproductive toxicity against which the effects of UA could be compared.

  Because ursolic acid is lipophilic and exhibits poor solubility in water, it was formulated using 0.5% carboxymethyl cellulose (CMC) in distilled water as a suspending vehicle. At this concentration, CMC is pharmacologically inert and commonly used in preclinical toxicology studies, providing adequate viscosity to maintain a homogeneous suspension of poorly soluble compounds during oral dosing without influencing reproductive endpoints. This choice of vehicle ensured accurate and reproducible administration of UA while minimizing potential interference with the evaluated anti-androgenic parameters.

  Dose formulations were prepared each day freshly for all treatment groups for preserving chemical stability and biological activity of ursolic acid. For each dose level, the required quantity of UA was accurately weighed and gradually dispersed into a measured volume of 0.5% CMC under continuous stirring with a magnetic stirrer until a uniform suspension was

obtained; the suspension was kept under gentle stirring during dosing to prevent sedimentation. The administration volume for all oral treatments was standardized at 10 mL/kg body weight, resulting in final UA doses of 100mg/kg (low dose) & 250mg/kg (high dose), whereas cyclophosphamide was set as per manufacturer's recommendations and administered intraperitoneally at 30mg/kg body weight in a volume (5mL/kg).

Randomly, animals were allocated into four experimental groups (n=6 per group) to reduce selection bias and to distribute baseline characteristics evenly across groups. Group 1 (Vehicle control) received only 0.5% CMC orally at 10mL/kg body weight once daily for 21days and served as normal reference group. Group 2 (Positive control) received cyclophosphamide 30mg/kg body weight intraperitoneally from day 17 to day 21 in addition to the oral vehicle, thereby modeling drug-induced testicular and reproductive toxicity. Group 3 (UA low dose) was treated with ursolic acid 100mg/kg body weight orally once daily for 21 consecutive days, while Group 4 (UA high dose) received ursolic acid 250mg/kg body weight orally for the same duration; these groups were used to evaluate the anti-androgenic potential of UA at two dose levels, with or without comparison to CP-induced changes. All oral administrations were performed using a calibrated oral gavage fitted with a smooth stainless-steel feeding needle to ensure accurate dosing and minimize trauma.

Table 4.3 : Experimental design and treatment protocol for vehicle, cyclophosphamide, and ursolic acid–treated groups

Group	Treatment	Dose	Dose volume & route	Animal numbers
1	Vehicle control	–	10mL/kg body weight, P.O.	1–6
2	Cyclophosphamide (Archana M. Navale et al., 2023)	30mg/kg body weight (standard)	5mL/kg body weight, I.P.	7–12

Group	Treatment	Dose	Dose volume & route	Animal numbers
3	Ursolic acid	Low dose, 100mg/kg body weight	10mL/kg body weight, P.O.	13–18
4	Ursolic acid	High dose, 250mg/kg body weight	10mL/kg body weight, P.O.	19–24

During the 21-day treatment period, animals were monitored daily for clinical symptoms, behavioral changes, and evidence of morbidity or mortality, yielding qualitative data on the systemic acceptance of UA and CP. Each animal's weight was recorded on day 1 before treatment as well as at 3-day intervals using a calibrated electronic balance, creating a longitudinal profile of weight changes. These data were subsequently used to calculate organ-to-body-weight ratios and to interpret treatment-related effects on reproductive organ weights.

On day 22, 24hrs after last treatment, all animals were kindly terminated using carbon dioxide asphyxiation and exsanguination through major blood vessels. The scrotal region was opened immediately, and both testes, along with the cauda epididymides, were carefully dissected free of surrounding tissues, blotted to remove surface blood, and weighed individually on a precision balance to obtain absolute organ weights. Relative testicular weights (mg testis per 100g body weight) were then evaluated for each rat, enabling quantitative comparison of treatment effects across groups. The harvested testes and epididymides were further processed for assessment of spermatogenic indices, motility, sperm count, viability, and morphology, as well as for histopathological examination of testicular architecture, providing comprehensive endpoints for evaluating the screening of UA as an anti-androgenic agent in this experimental model.

Assessment of epididymal sperm characteristics

Total sperm count

A small amount of the right cauda epididymis was carefully removed and minced in 5 mL of 0.9% normal saline at room temperature to release spermatozoa, producing solution A, a homogeneous solution. The tissue fragments were gently incubated in this medium for approximately 2–5 minutes with occasional agitation to allow complete dispersion of sperm. A 100 µL aliquot of Suspension A was subsequently extracted and diluted with 1.9 mL of normal saline, resulting in a 20-fold dilution appropriate for counting using a hemocytometer. The diluted sample was then loaded into a clean Neubauer hemocytometer, and sperm cells were counted under a 10× objective lens in the four standard white-blood-cell (WBC) counting squares.

The concentration of spermatozoa in the original suspension was expressed as million sperm per milliliter using the following formula:

Sperm count (million/mL)=N×20×1000×dilution factor0.4Sperm count (million/mL)=0.4N×20×1000×dilution factor

where NN represents the total number of sperm counted in four WBC squares, 20 is the hemocytometer depth correction factor, 1000 converts from cubic millimeters to milliliters, and 0.4 mm33 is the total counted chamber volume. The resulting value provided the epididymal sperm concentration for each animal, which was used to compare the effects of the different treatments on spermatogenesis.

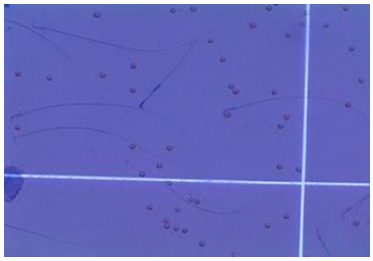


Figure 4.7: Total Sperm Count

Sperm viability

The vitality of sperm was assessed using trypan blue dye exclusion. A 10 µL Suspension A aliquot was mixed with 10 µL normal saline and 10 µL 0.4% trypan blue solution, then gently homogenized. A small volume of the stained suspension was mounted onto a clean glass slide, overlaid with a coverslip, and subsequently examined using a light microscope at 40×

magnification. Each sample contained at least 200 spermatozoa. Cells that rejected the dye and stayed uncolored were viable, whereas those that absorbed trypan blue and became pink to red were non-viable. The percentage viability has been computed according to the formula:

Viability (%)=Number of viable spermTotal sperm counted×100Viability (%)=Total sperm countedNumber of viable sperm×100

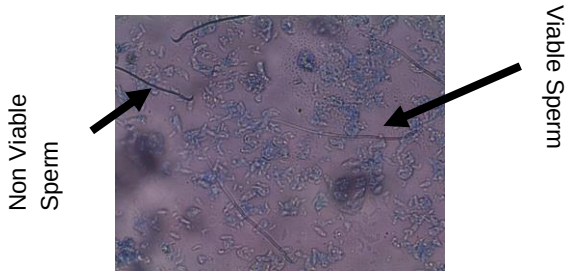


Figure 4.8: Sperm Viability

Sperm motility

Sperm motility was assessed using an aliquot from Suspension A without staining. A 10µL drop of the well-mixed suspension was loaded into a hemocytometer chamber, ensuring even distribution over the grid. The preparation was then examined immediately under 40× magnification to avoid artifacts arising from drying or temperature changes. Motile and non-motile spermatozoa were counted in four WBC squares, and at least several hundred cells were evaluated per animal. The percentage motility was determined using the following expression:

Motility (%)=Number of motile spermTotal sperm counted×100Motility (%)=Total sperm counte dNumber of motile sperm×100

This parameter reflected the functional competence of epididymal sperm and the potential influence of the test treatments on flagellar activity and energy metabolism.

Sperm morphology

For morphological assessment, 100 µL of Suspension A was transferred into a microcentrifuge tube containing 1 mL of 10% neutral buffered formalin to fix the spermatozoa and preserve cellular details. Subsequently, two to three drops of 1% eosin dye were introduced, and the mixture was incubated at ambient temperature for 30 to 45 minutes to facilitate optimal staining of sperm structures. Following incubation, several drops of the stained suspension were applied to clean

glass slides, distributed to create thin smears, air-dried, and subsequently viewed under a light microscope at high magnification. At least 100 spermatozoa per animal were evaluated systematically for structural abnormalities, including head defects (e.g., headless forms, amorphous heads) and tail anomalies (e.g., tailless forms, bent or coiled tails).

The proportion of abnormal sperm was determined by employing the formula: Abnormal sperm (%)=Number of morphologically abnormal spermTotal sperm counted×100Abn ormal sperm (%)=Total sperm countedNumber of morphologically abnormal sperm×100

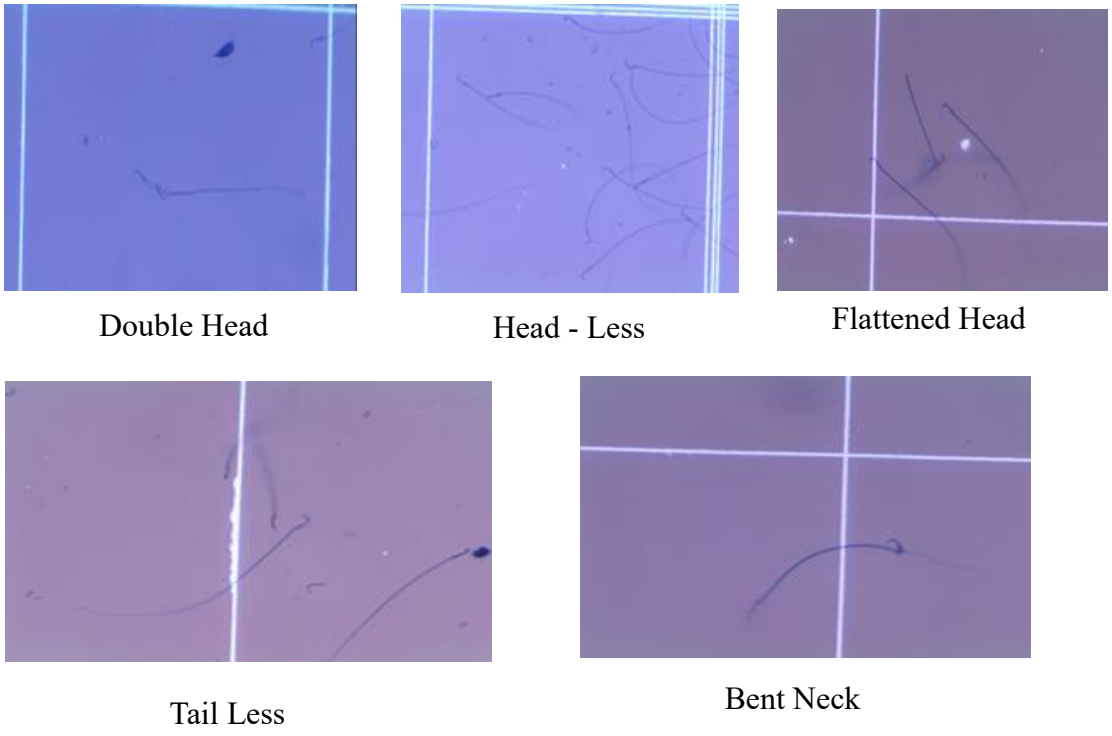


Figure 4.9: Sperm Morphology

Statistical analysis

All quantitative data generated from sperm count, viability, motility, morphology, and related reproductive parameters were first summarized as mean ± standard deviation for each experimental group. One-way ANOVA was employed to assess the statistical significance of differences between the vehicle control group, the ursolic acid-treated groups, and the cyclophosphamide-positive control group. Tukey's post hoc multiple comparison test was used to find group pairs that differed while adjusting for type I error after the ANOVA showed significant

variation. P<0.05 indicates a statistically significant difference, with less than a 5% possibility of the observed changes occurring by chance.

4.7 Histological studies:-

Histopathology involves autopsy or biopsy tissue preparation for microscope diagnosis. The uterus and ovaries were preserved in 10% Neutral buffer fromaline (NBF). The following sections were cut and processed:

❖ **Preparation of slides:-**

The tissue can be observed after suitable preparation. It is kept in a preservative named a preparation of tissues **Fixative,** to prevent its decay and autolysis. Then it is cut into single-cell sections and stained to identify tissue constituents.

a) Fixation:-

Fixation preserves and solidifies tissue. Fixation begins with rapid tissue death and ends with tissue hardening. To preserve tissue component arrangement in vivo, tissue must be submerged in fixative immediately after extraction. The hardening process must be rigorous enough to protect tissue components and architecture against further treatments. The tissue is sliced thinly to ensure that the fixing fluid can permeate it within a relatively brief period, and submerged in a minimum of 20 times its volume of fixative. 10% formalin is the most commonly utilized fixative due to its compatibility with the majority of strains. Following fixation, the tissue is rinsed in running water for a duration of 3 to 24 hours prior to dehydration, cleaning, and embedding.

10% formalin (buffered) solution:

37 - 40% Formaldehyde - 100 cc Distilled water - 900cc Sodium phosphate monobasic - 4g Sodium phosphate dibasic - 6.5 g

b) Processing of tissues – Dehydration, Clearing and Embedding:-

Embedding with paraffin is executed most quickly and yields optimal results when thin pieces of soft tissues are required. Paraffin is water-insoluble. The tissue is required to undergo dehydration followed by clearing employing paraffin-compatible solution.

❖ **The schedule for paraffin processing is as follows:**

1)	Alcohol 80%	- 1 to 2 hours
2)	Alcohol 95% (2 changes each)	- 1 to 2 hours
3)	Alcohol absolute	- 1 to 2 hours each
4)	Xylene-2 changes	- 1 to 2 hours each
5)	Melted paraffin	- 1 to 2 hours each

The treated tissues were then embedded in paraffin within shallow pans exhibiting slightly sloping edges, measuring 1×2 inches and ¾ inch in depth. A Bunsen burner steadily heated the pan with filtered, melted paraffin. A string tag with the animal group and number was attached to each tissue specimen in the pan. Paraffin cooled and solidified in these unallocated pans. Paraffin solidified and retreated from pan edges. Then the pile was lifted and cut into suitable blocks.

c) Preparing and Cutting sections:-

The outcome generated by histological procedures is greatly impacted by the knife employed for sectioning. The knife must be exceedingly sharp and devoid of nicks. An optimal edge for a microtome knife can be characterized as a flat surface inclined at around 14˚. The paraffin blocks were positioned correctly in the microtome and aligned with the blade. A Petri dish placed adjacent to the microtome contained a cotton swab moistened with tap water along with an ice cube. Following initial rough trimming, the damp cotton was applied to the paraffin block to facilitate smooth sectioning. The block was then cooled using an ice cube, after which sections of 5 micron thickness were cut. After gathering in a water bath, a fine-tipped camel hair brush gently unfurled the segment ribbons.

d) Attaching sections to slide:-

The animal's identification number was written on the glass slides that were to be used for mounting tissue sections after they had been covered with albumin.

The floating part was carefully collected, placed centrally on the slide, and allowed to air-dry before staining procedure.

e) Staining:-

The sections were stained using hematoxyline – eosin stain.

Solutions used:

Harri's alum hematoxylin

Hematoxylin crystals - 5 g

Alcohol absolute - 50 g

Ammonium or potassium alum - 100 g Distilled water

Mercuric oxide (red) - 25 g

Stock 1% alcoholic eosin solutions:-

Eosin Y_1 water - 1 g

soluble - 20 cc

Distilled water

Dissolved and add alcohol 95% - 80 cc

The schedule followed for staining was:

Xylene – 3 changes - 30 min each

Xylene + Alcohol - 30min

Alcohol 50% - 30min

Alcohol 70% - 30min

Alcohol absolute - 30min

Distilled water - 20min

Hematoxylin - 10min

Running tap water -1 hour

Acid alcohol - 3-10 quick dips

Alcohol 50% - 10 min

Alcohol 95% - 10 min

Eosin - 45 – 60 secs

Alcohol absolute (2 changes) - 5min each

Xylene + Alcohol - 5min

Xylene (3 changes) - 10min each.

The stained sections were finally mounted in D.P.X. mountant.

CHAPTER – 5
RESULTS

TITLE: Evaluation of Anti fertility Activity of *Saraca Indica* (Ashoka) in Experimental Animals

5. RESULTS

5.1 *Invitro* Screening of Ursolic Acid as an oxytocic activity

The oxytocic activity of ursolic acid was evaluated using the isolated rat uterine preparation method and compared with the standard drug oxytocin. The dose–response curve (DRC) for oxytocin was first established to serve as a reference for determining relative uterine contractile responses.

The tissue's sensitivity and the bioassay's dependability were confirmed by a graduated increase in the amplitude of uterine contractions caused by incremental dosages of oxytocin (1–16 μg/mL). The maximal contraction height (7.8 cm) was obtained with 1.6 mL of oxytocin solution (16 μg), which was considered 100% response. Lower doses showed progressively smaller contractions corresponding to the concentration–response relationship (Table 1).

When the test compound, ursolic acid (10 mg/mL in DMSO), was administered at a dose of 3 mL (equivalent to 30 mg), it produced a mean contraction height of 0.5–0.6 cm. This response corresponded to approximately 6.4–7.7% of the standard oxytocin-induced maximal contraction, indicating that ursolic acid exhibits measurable but comparatively weak oxytocic activity under in vitro conditions. The confirmatory trials of the same dose yielded consistent results, demonstrating reproducibility.

These findings suggest that ursolic acid possesses partial agonistic activity on uterine smooth muscle, capable of eliciting contraction but markedly less potent than oxytocin. The activity may be attributed to its triterpenoid structure, which could interact with uterine receptors mediating contraction.

Table 5.1: In vitro dose–response evaluation of oxytocin and ursolic acid

Sr. No.	Drug Concentration	Dose (mL)	Dose (µg)	Response in	
				Height (cm)	%
1	Oxytocin (10µg/mL)	0.1	1	1.5	19.23
2		0.2	2	2.4	30.76
3		0.4	4	4.0	51.28
4		0.8	8	6.2	79.48
5		1.6	16	7.8	100
6	Ursolic Acid (Test) (10 mg/mL)	3	30 mg	0.5	6.41
7	Ursolic Acid (Confirmatory) (10 mg/mL)	3	30 mg	0.6	7.69
8	Ursolic Acid (Confirmatory) (10 mg/mL)	3	30 mg	0.5	6.41

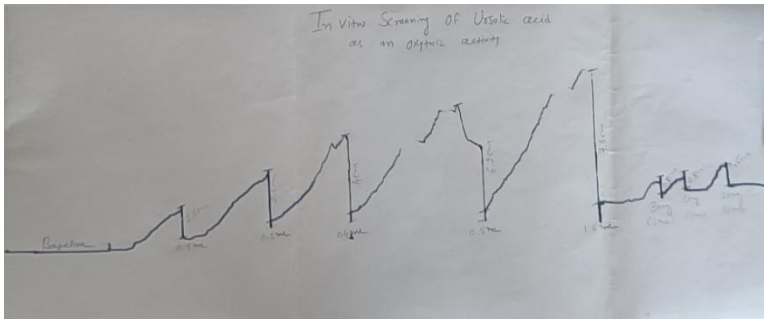


Figure 5.1: Dose Resposne Curve of Ursolic Acid

5.2 Results of phytochemical analysis of *Saraca Indica* bark

5.2.1 Extraction yield

n-Hexane exhaustive maceration of 1000 g air-dried *Saraca indica* bark powder yielded 1.4 g crude extract (0.40% w/w) after vacuum evaporation, representing the non-polar triterpenoid-enriched fraction.

5.2.2 Identification by TLC

TLC analysis of the n-hexane fraction (1 mg/mL) showed a prominent spot at Rf value identical to ursolic acid working standard (1 mg/mL) when developed in ethyl acetate:n-hexane (17:3) and visualized with anisaldehyde–sulphuric acid reagent (Figure 5.2). Both spots exhibited characteristic purple color upon heating, confirming the identity of ursolic acid in the Saraca indica bark fraction.

5.2.3 Quantification by HPLC

HPLC analysis confirmed a major peak in the n-hexane fraction at the retention time of standard ursolic acid under the specified gradient conditions. From 1000 g of air-dried *Saraca indica* bark powder processed, n-hexane exhaustive maceration yielded 1.4 g of crude extract, corresponding to an extraction yield of 0.40% w/w relative to the dried bark. TLC analysis showed a prominent spot in the fraction at Rf value identical to ursolic acid working standard when developed in ethyl acetate:n-hexane (17:3) and visualized with anisaldehyde–sulphuric acid reagent (Figure 5.2). The ursolic acid content was determined as 90% w/w of the enriched fraction by peak area comparison. For the purposes of this PhD phytochemical screening study, direct standard comparison provided sufficient method suitability for ursolic acid identification and semi-quantitative assay, consistent with established practices for natural product analysis where full pharmacopoeial validation is not required.

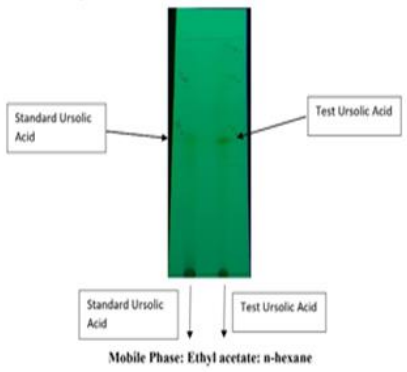


Figure 5.2: Indetification of Ursolic Acid

(Note: TLC identification of ursolic acid in Saraca indica bark n-hexane fraction (Lane 1: ursolic acid standard; Lane 2: test fraction; ethyl acetate:n-hexane 17:3; anisaldehyde–sulphuric acid reagent)

5.3 Screening of Usrsolic Acid as an Anti-Ovulatory Activity

General Observations

No treatment group reported any death or morbidity during the trial period. All animals remained clinically normal, showing no abnormal behaviors or external signs of toxicity. The daily clinical observations did not reveal any significant adverse changes across treated or control groups.

Body Weight and Organ Weight Analysis

Comparative analysis of mean body mass and ovarian weights demonstrated that treatment with ursolic acid at the higher dose (250 mg/kg body weight/day) produced a statistically significant reduction ($P < 0.05$) in overall body weight as well as in both the absolute and relative weights of the ovaries, in comparison with the vehicle-treated control group. In contrast, animals receiving the low dose (100 mg/kg b.wt./day) did not exhibit any significant changes in these parameters. Similarly, animals in the recovery high-dose group, after a 21-day post-treatment recovery period, showed weight values comparable to controls, indicating reversibility of effects associated with the high-dose treatment.

Gross Pathology

None of the groups had any evident pathological abnormalities at necropsy. The ovarian morphology across groups appeared intact, although a decrease in the number and size of mature

follicles was observable in the high-dose group compared to the vehicle control animals. Recovery group ovaries appeared largely comparable to controls.

Histopathological Findings

Microscopic examination of ovarian tissues confirmed the presence of normal ovarian structures in the control animals, including the surface epithelium, cortical and medullary stromal regions, and follicles in various developmental stages (primordial, primary, secondary, tertiary, Graafian, and atretic). Prominent corpora lutea and corpora albicantia were evident, along with well-developed Graafian follicles and luteinized granulosa and theca cells (Fig. 5.3: G1 Vehicle Control).

In the low-dose group (G2), the ovarian sections predominantly displayed primary follicles with fewer secondary or mature follicles, indicating a mild inhibitory effect of ursolic acid on folliculardevelopment (Fig. 5.4: G2 Low Dose).

In the high-dose group (G3), numerous early-stage follicles were visible, while mature Graafian follicles and ruptured corpora lutea were notably reduced compared to controls (Fig. 5.5: G3 High Dose). These findings suggest a dose-dependent suppression of follicular maturation and ovulatory activity. In the recovery high-dose group (G4), ovarian sections demonstrated the reappearance of developing follicles, indicating partial restoration of normal histoarchitecture and folliculogenesis following the 21-day recovery period (Fig. 5.6: G4 Recovery High Dose).

Table 5.2: Mortality and Morbidity Record

Group	Treatment	Animal No.	Observation
G1	Vehicle Control	1	No Mortality and Morbidity observed during the treatment period
		2	
		3	
		4	
		5	
		6	
G2	Low Dose	7	No Mortality and Morbidity observed during the treatment
		8	
		9	

		10	period
		11	
		12	
G3	High Dose	13	No Mortality and Morbidity observed during the treatment period
		14	
		15	
		16	
		17	
		18	
G4	Recovery High Dose	19	No Mortality and Morbidity observed during the treatment period
		20	
		21	
		22	
		23	
		24	

Table 5.3: Clinical Sign Record

Group	Treatment	Animal No.	Observation
G1	Vehicle Control	1	No abnormal clinical sign observed during the treatment period
		2	
		3	
		4	
		5	
		6	

G2	Low Dose	7	No abnormal clinical sign was observed during the treatment period
		8	
		9	
		10	
		11	
		12	
G3	High Dose	13	No abnormal clinical sign was observed during the treatment period
		14	
		15	
		16	
		17	
		18	
G4	Recovery High Dose	19	No abnormal clinical sign was found during the treatment period
		20	
		21	
		22	
		23	
		24	

Table 5.4: Individual Body Weight Record

Group	Treatment	Animal No.	Prior treatment Body weight (g)	Terminal treatment Body weight (g)
G1	Vehicle Control	1	228.40	232.54
		2	240.64	243.87
		3	240.10	245.41
		4	238.60	241.98
		5	242.65	246.78
		6	246.16	250.14

G2	Low Dose	7	230.61	234.15
		8	238.91	240.17
		9	237.92	241.74
		10	243.14	248.19
		11	244.66	246.72
		12	245.81	248.65
G3	High Dose	13	233.60	226.17
		14	230.49	226.47
		15	238.27	237.46
		16	240.61	232.67
		17	245.67	240.22
		18	240.64	233.87
G4	Recovery High Dose	19	230.01	244.47
		20	241.53	250.98
		21	238.24	246.78
		22	237.94	251.32
		23	238.91	254.14
		24	239.37	256.56
G1	Vehicle Control	Mean	239.43	243.45
		SD	6.00	6.02
		N	6	6
G2	Low Dose	Mean	240.18	243.27
		SD	5.64	5.66
		N	6	6
G3	High Dose	Mean	238.21	232.81 *
		SD	5.44	5.69
		N	6	6

G4	Recovery High Dose	Mean	237.67	250.71
		SD	3.96	4.49
		N	6	6

Note: Values are mean ± SD (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test. *P < 0.05 compared to G1 (vehicle control)

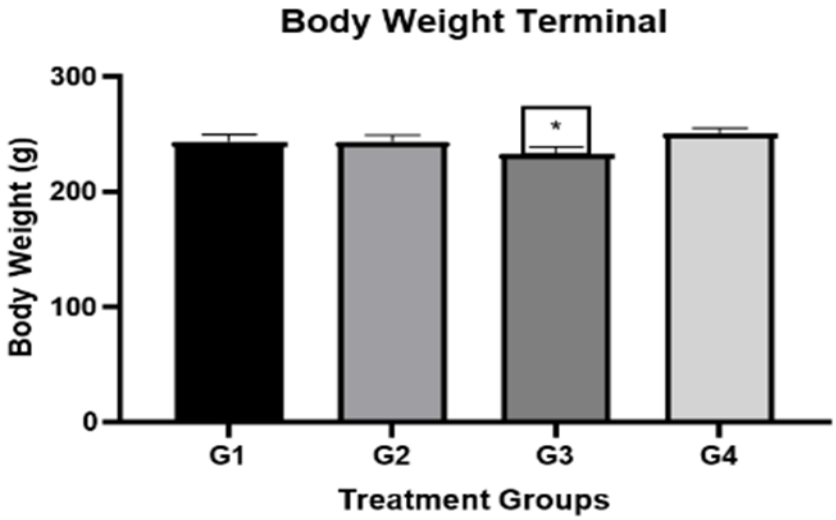


Figure 5.3 – Body weight of all four groups from anti-ovulatory study. Values are mean ± SD (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test. *P < 0.05 compared to G1 (vehicle control).

Table 5.5: Individual Absolute and Relative Ovary weight

Group	**Treatment**	**Animal No.**	**Absolute Ovaries weight (g)**	**Terminal treatment Body weight (g)**	**Relative Ovaries weight (%)**
G1	Vehicle Control	1	0.9737	232.54	0.4187
		2	0.9826	243.87	0.4029
		3	1.0892	245.41	0.4438
		4	0.9912	241.98	0.4096
		5	1.0986	246.78	0.4452
		6	1.0756	250.14	0.4300

G2	Low Dose	7	0.8272	234.15	0.3533
		8	0.8845	240.17	0.3683
		9	0.9426	241.74	0.3899
		10	0.9776	248.19	0.3939
		11	1.0621	246.72	0.4305
		12	0.9945	248.65	0.4000
G3	High Dose	13	0.6625	226.17	0.2929
		14	0.5746	226.47	0.2537
		15	0.7810	237.46	0.3289
		16	0.4654	232.67	0.2000
		17	0.8842	240.22	0.3681
		18	0.8801	233.87	0.3763
G4	Recovery High Dose	19	1.0612	244.47	0.4341
		20	0.9781	250.98	0.3897
		21	1.0889	246.78	0.4412
		22	1.0757	251.32	0.4280
		23	1.0627	254.14	0.4182
		24	0.9845	256.56	0.3837
G1	Vehicle Control	Mean	1.04	243.45	0.43
		SD	0.06	6.02	0.02
		N	6	6	6
G2	Low Dose	Mean	0.95	243.27	0.39
		SD	0.08	5.66	0.03
		N	6	6	6
G3	High Dose	Mean	0.71***	232.81	0.30 ***
		SD	0.17	5.69	0.07
		N	6	6	6
G4	Recovery High Dose	Mean	1.04	250.71	0.42
		SD	0.05	4.49	0.02
		N	6	6	6

Note: Values are mean ± SD (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test.

***P < 0.001 compared to G1 (vehicle control).

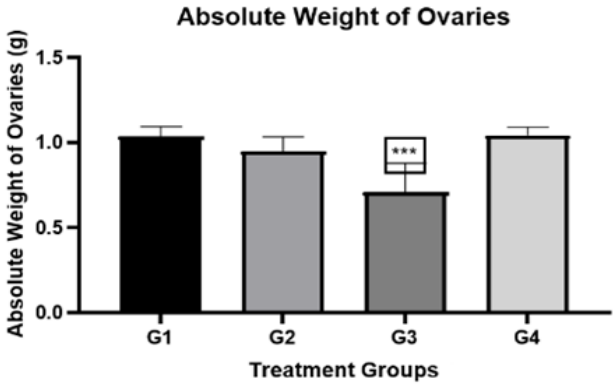


Figure 5.4 – Absolute ovaries weight of all four groups from anti-ovulatory study. Values are mean ± SD (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test. ***P < 0.001 compared to G1 (vehicle control).

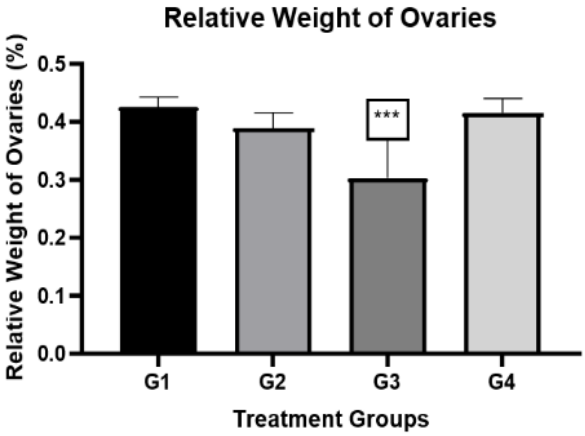


Figure 5.5 – Relative ovaries weight of all four groups from anti-ovulatory study. Values are mean ± SD (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test. ***P < 0.001 compared to G1 (vehicle control)

Histopathology

Figures 5.6-5.9: Representative photomicrographs of ovarian histology from female Wistar rats in the anti-ovulatory activity study (cupric acetate model). All images prepared with Haematoxylin & Eosin (H&E) stain at 10× magnification.

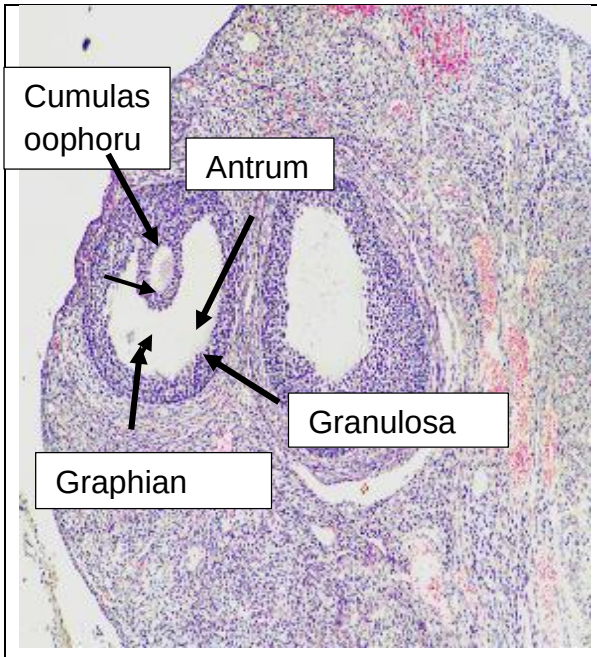


Figure 5.6 - G1 (Vehicle Control)
(H&E) stain at 10× magnification

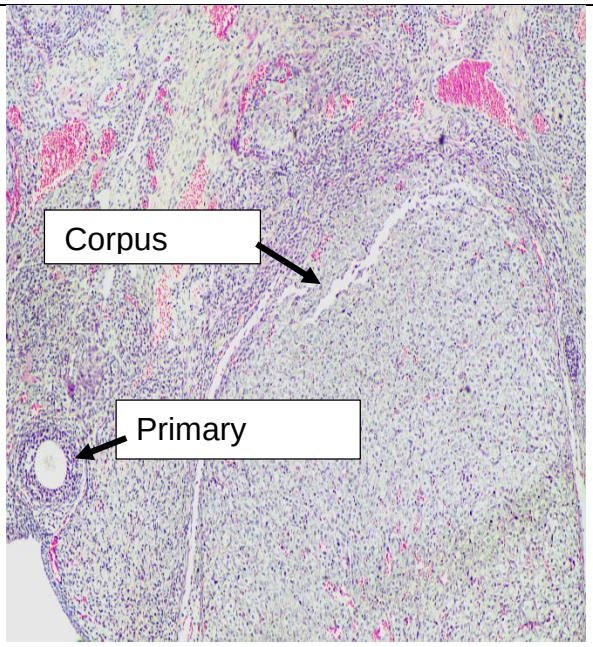


Figure 5.7- G2 (Low Dose)
(H&E) stain at 10× magnification

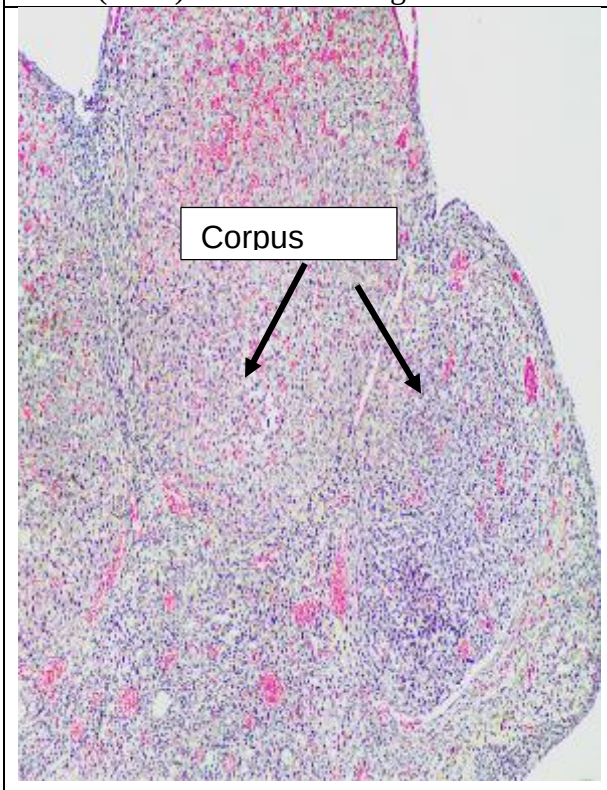


Figure 5.8 - G3 (High Dose)
(H&E) stain at 10× magnification

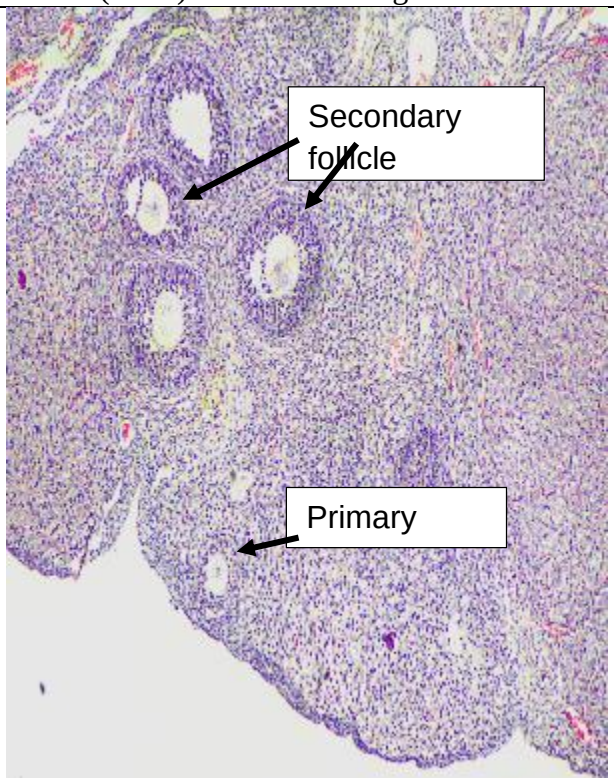


Figure 5.9 - G4 (Recovery High Dose)
(H&E) stain at 10× magnification

5.4 Screening of Usrsolic Acid as an Anti Implantation Activity

Results

The four treatment groups of 24 female albino rats had no mortality or morbidity during the 21-23-day experiment. Detailed twice-daily clinical observations, including general behavior, activity levels, and external signs of toxicity, revealed no abnormalities across the vehicle control, low dose (100 mg/kg b.wt.), mid dose (125 mg/kg b.wt.), or high dose (250 mg/kg b.wt.) groups, confirming the overall tolerability of ursolic acid administration.

Body Weight Changes

Body weights were monitored regularly and remained stable across all groups, exhibiting no statistically significant differences. Mean body weights at the time of laparotomy (day 10 post-coital) were 189.27 ± 7.10 g for the vehicle control (G1), 189.82 ± 5.13 g for the low dose (G2), 186.27 ± 6.05 g for the mid dose (G3), and 189.20 ± 3.90 g for the high dose (G4). These values indicate that ursolic acid, even at the highest dose of 250 mg/kg b.wt. administered orally from days 1-7 post-coital, did not induce systemic toxicity or weight loss attributable to treatment.

Ovarian and Uterine Reproductive Parameters

Laparotomy on day 10 of pregnancy allowed direct visualization and quantification of corpora lutea (CL), implantation sites, and subsequent litter counts after allowing gestation to complete. The vehicle control group exhibited normal reproductive performance, with 13.17 ± 2.64 CL per animal, translating to 11.17 ± 2.23 implantation sites and 9.67 ± 1.75 live litters delivered, reflecting typical fertility rates.

The low dose group (100 mg/kg) showed no disruption in reproductive endpoints, recording 15.17 ± 2.48 CL, 12.50 ± 1.52 implants, and 10.17 ± 0.75 litters—values statistically comparable to controls. In contrast, the mid dose (125 mg/kg) demonstrated clear anti-implantation effects, with 14.00 ± 2.28 CL but markedly reduced implants (8.33 ± 1.86) and litters (5.67 ± 1.51). The high dose (250 mg/kg) produced the most pronounced inhibition, yielding 15.17 ± 2.64 CL alongside drastically fewer implants (5.50 ± 1.38) and litters (2.33 ± 1.03), indicating substantial interference with early pregnancy maintenance.

Implantation Loss Quantification

Pre-implantation loss, calculated as [(Number of CL - Number of Implants) / Number of CL] × 100,

remained low and non-significant in the low dose group (16.81 ± 7.55%) compared to controls (15.17 ± 3.82%). However, mid and high doses induced dose-dependent elevations, reaching 38.20 ± 19.57% (mid) and 63.47 ± 8.30% (high; both $P < 0.05$), suggesting interference with ovum transport, fertilization, or blastocyst development prior to implantation.

Post-implantation loss, computed as [(Number of Implants - Number of Litters) / Number of Implants] × 100, followed a similar pattern: 18.21 ± 4.83% (low, non-significant), 32.49 ± 9.54% (mid, $P < 0.05$), and 57.66 ± 15.24% (high, $P < 0.05$). These marked increases at higher doses point to enhanced embryonic resorption or developmental arrest after implantation, collectively demonstrating robust anti-implantation and anti-fertility activity of ursolic acid.

Statistical graphs confirmed these trends, with one-way ANOVA after that Tukey's test highlighting significant elevations in both loss types for G3 and G4 relative to G1, while G2 remained indistinguishable from controls.

Table 5.6: Individual Animal Data of Anti-Implantation Activity

Group	Treatment	Animal No.	Body Weight (g)	No. of Corpora Lutea	No.of Implants	No. of Litters	% Pre Implantation Loss	% Post Implantation Loss
G1	Vehicle Control	1	182.45	14	12	10	14.29	16.67
		2	190.41	15	13	11	13.33	15.38
		3	188.74	11	10	9	9.09	10.00
		4	195.36	17	14	12	17.65	14.29
		5	180.21	10	8	7	20.00	12.50
		6	198.47	12	10	9	16.67	10.00
G2	Low Dose (100 mg/Kg. b.wt.)	7	194.45	11	10	9	9.09	10.00
		8	184.22	18	14	11	22.22	21.43
		9	191.54	17	12	10	29.41	16.67
		10	184.14	14	12	10	14.29	16.67
		11	196.24	16	14	11	12.50	21.43
		12	188.33	15	13	10	13.33	23.08

G3	Mid Dose (125 mg/Kg. b.wt.)	13	179.14	14	8	6	42.86	25.00
		14	180.02	12	10	7	16.67	30.00
		15	194.11	17	6	3	64.71	50.00
		16	189.41	14	11	7	21.43	36.36
		17	190.52	11	8	6	27.27	25.00
		18	184.41	16	7	5	56.25	28.57
G4	High Dose (250 mg/Kg. b.wt.)	19	186.54	17	6	2	64.71	66.67
		20	190.34	14	7	4	50.00	42.86
		21	187.14	18	5	3	72.22	40.00
		22	184.24	17	7	2	58.82	71.43
		23	192.32	11	4	2	63.64	50.00
		24	194.59	14	4	1	71.43	75.00

Table 5.7: Effect of ursolic acid on implantation parameters in female rats

Group		Body Weight (g)	No. of Corpora Lutea	No.of Implants	No. of Litters	% Pre Implantation Loss	% Post Implantation Loss
G1 Vehicle Control	Mean	189.27	13.17	11.17	9.67	15.17	13.14
	SD	7.10	2.64	2.23	1.75	3.82	2.79
	N	6	6	6	6	6	6
G2 Low Dose (100 mg/Kg. b.wt.)	Mean	189.82	15.17	12.50	10.17	16.81	18.21
	SD	5.13	2.48	1.52	0.75	7.55	4.83
	N	6	6	6	6	6	6
G3 Mid Dose (125 mg/Kg. b.wt.)	Mean	186.27	14.00	8.33	5.67	38.20 *	32.49 **
	SD	6.05	2.28	1.86	1.51	19.57	9.54
	N	6	6	6	6	6	6
G4 High Dose (250 mg/Kg. b.wt.)	Mean	189.20	15.17	5.50	2.33	63.47 ***	57.66 ***
	SD	3.90	2.64	1.38	1.03	8.30	15.24
	N	6	6	6	6	6	6

Note: Values are mean ± SD (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test. * P < 0.05 compared to G1 (vehicle control); ***P < 0.001 compared to G1 (vehicle control).

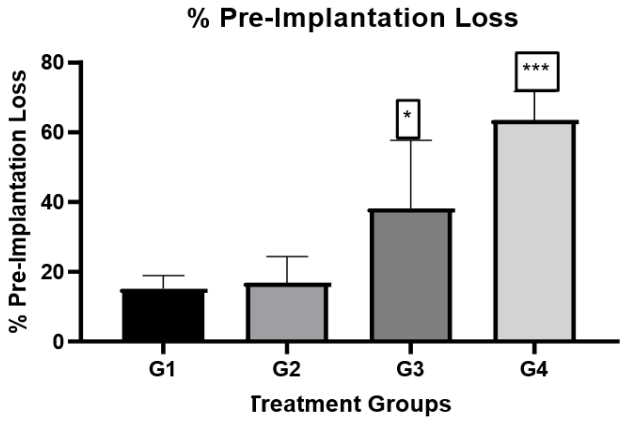


Figure 5.10 – Comparison of % Pre-Implantation Loss of all four groups from Anti-Implntation Activity; Values are mean ± SD (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test. * P < 0.05 compared to G1 (vehicle control); ***P < 0.001 compared to G1 (vehicle control).

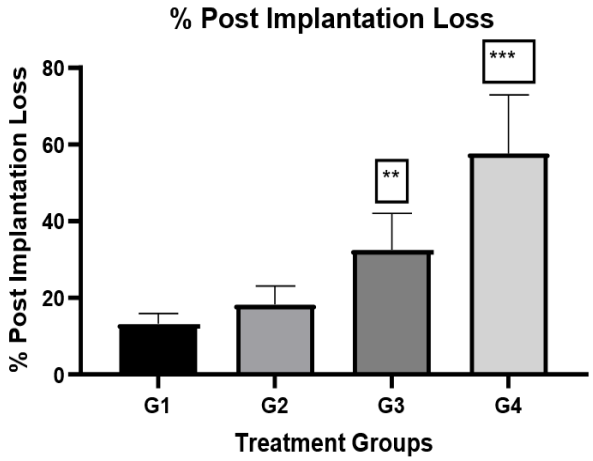


Figure 5.11 – Comparison of % Post-Implantation Loss of all four groups from Anti-Implntation Activity Values are mean ± SD (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test. ** P < 0.01 compared to G1 (vehicle control); ***P < 0.001 compared to G1 (vehicle control).

5.5 Screening of Usrsolic Acid as an Anti Estrogenic Activity

Results

Over the course of the 5-day treatment period, none of the six experimental groups, which included 36 immature Swiss albino female mice, had any morbidity, mortality, or abnormal clinical indications. Animals in the vehicle control (G1: 0.5% CMC, p.o.), estrogen standard (G2: 0.05 g/animal, s.c.), ursolic acid low dose (G3: 200 mg/kg b.wt., p.o.), high dose (G4: 500 mg/kg b.wt., p.o.), estrogen + low dose (G5), and estrogen + high dose (G6) groups exhibited normal grooming, locomotion, food intake, and external appearance, confirming the tolerability of all treatments up to the highest dose tested.

Body Weight Progression

Body weights were noted on Day 1 (pre-treatment) and Day 5 (terminal sacrifice) to assess systemic growth effects. Initial weights showed no intergroup differences, ranging from 10.08 ± 0.87 g (G1) to 10.55 ± 0.92 g (G6). During the treatment period, vehicle controls gained weight normally (13.09 ± 1.06 g on Day 5), reflecting physiological growth in immature mice.

Estrogen administration (G2) induced pronounced weight gain to 15.79 ± 1.03 g ($P < 0.001$ vs. G1), attributable to estrogen-mediated fluid retention and uterine/anogenital hypertrophy. Ursolic acid monotherapy prevented this hyper-response: G3 animals reached 11.24 ± 0.66 g, and G4 reached 10.59 ± 0.80 g, both comparable to controls and significantly lower than G2 ($P < 0.001$). In combination regimens, ursolic acid dose-dependently attenuated estrogen effects: G5 (11.31 ± 0.64 g) and G6 (11.02 ± 0.68 g), both markedly reduced versus G2 ($P < 0.001$), indicating effective counteraction of estrogen-driven somatic changes.

Table 5.8: Effect of estrogen and ursolic acid treatment on body weight changes in experimental groups

Group	Treatment	Day 1 Body Weight (g) Mean ± SD	Day 5 Body Weight (g) Mean ± SD	% Change from Day 1
G1	Vehicle Control (0.5% CMC, p.o.)	10.08 ± 0.87	13.09 ± 1.06	+29.9%

G2	Estrogen Standard (0.05 µg/animal, s.c.)	10.50 ± 0.78	15.79 ± 1.03*	+50.4%
G3	Ursolic Acid Low (200 mg/kg, p.o.)	10.12 ± 0.55	11.24 ± 0.66	+11.1%
G4	Ursolic Acid High (500mg/kg, p.o.)	10.27 ± 0.70	10.59 ± 0.80	+3.0%
G5	Estrogen + Ursolic Acid Low	10.41 ± 0.62	11.31 ± 0.64†	+8.7%
G6	Estrogen + Ursolic Acid High	10.55 ± 0.92	11.02 ± 0.68†	+4.4%

Note: Values are mean ± SD (n = 6 per group)*Significant increase (P < 0.001) vs. G1; †Significant decrease (P<0.05) v/s G2 by one-way ANOVA followed by Tukey's post-hoc test.

Absolute Uterine Weight: Primary Endpoint

Uterine weight at necropsy provided the gold-standard measure of estrogenic/anti-estrogenic activity in this immature rodent model. Vehicle controls displayed baseline uterine weights of 15.13 ± 0.55 mg. Estrogen challenge elevated this ~9-fold to 136.32 ± 1.70 mg (P < 0.001 vs. G1), confirming robust estrogenic stimulation of endometrial proliferation, myometrial hypertrophy, and vascularization.

Ursolic acid alone exerted mild inhibitory effects on baseline uterine growth: G3 (12.63 ± 0.64 mg) and G4 (7.30 ± 0.45 mg), suggesting intrinsic anti-proliferative activity on uterine tissue. Most strikingly, concurrent ursolic acid administration antagonized estrogen potently and dose-dependently: G5 reduced estrogen effects to 57.85 ± 5.28 mg (~58% inhibition relative to G2; P < 0.001), while G6 achieved near-complete blockade at 23.67 ± 3.74 mg (~83% inhibition; P < 0.001 vs. G2), approaching vehicle control levels. These data unequivocally demonstrate ursolic acid's anti-estrogenic properties.

Table 5.9: Effect of estrogen and ursolic acid treatment on body weight changes in experimental groups

Group	Treatment	Absolute Uterine Weight (mg) Mean ± SD	% of Estrogen Control (G2)
G1	Vehicle Control	15.13 ± 0.55	11.1%
G2	Estrogen Standard	136.32 ± 1.70***	100% ***
G3	Ursolic Acid Low Dose	12.63 ± 0.64	9.3%
G4	Ursolic Acid High Dose	7.30 ± 0.45	5.4%
G5	Estrogen + Low Dose	57.85 ± 5.28###	42.4% ###
G6	Estrogen + High Dose	23.67 ± 3.74###	17.4% ###

Note: Values are mean ± SD (n = 6 per group) ***Significant increase (P<0.001) v/s G1; ### Significant decrease (P<0.001) v/s G2 one-way ANOVA followed by Tukey's post-hoc test.

Serum Estrogen Concentrations

Quantified estrogen levels (pg/mL) corroborated uterine findings. Basal levels in G1 averaged 27.64 ± 5.75 pg/mL. Exogenous estrogen spiked G2 to 548.85 ± 4.69 pg/mL (P < 0.001). Ursolic acid monotherapy maintained low circulating estrogen: G3 (35.21 ± 30.10 pg/mL) and G4 (15.51 ± 22.02 pg/mL). Combinations yielded intermediate antagonism: G5 (298.85 ± 33.73 pg/mL, ~46% reduction vs. G2) and G6 (151.88 ± 76.42 pg/mL, ~72% reduction; both P < 0.001 vs. G2).

Statistical analyses (one-way ANOVA with Tukey's post-hoc) across body weight, uterine weight, and estrogen endpoints confirmed highly significant (P < 0.001) estrogen-induced changes and dose-dependent ursolic acid blockade, establishing its efficacy as an anti-estrogenic agent in this validated model.

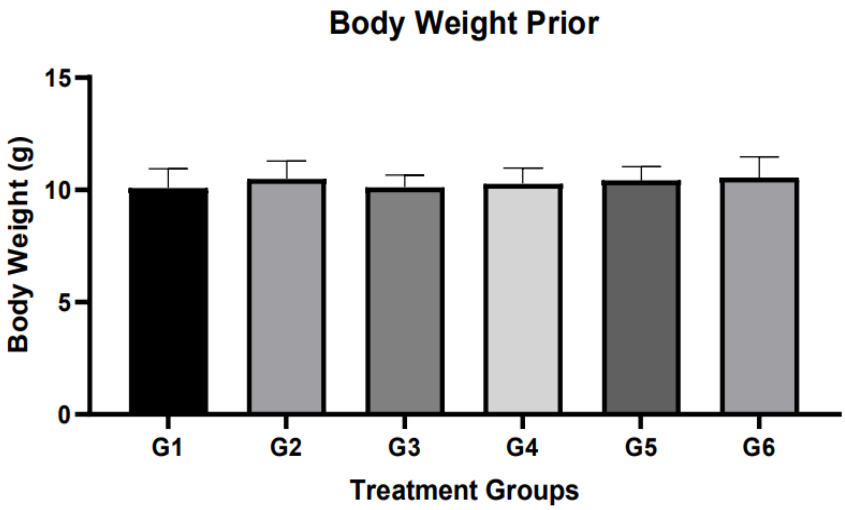


Figure 5.12 – Body weight prior to treatment of all six groups of animals from Anti Estrogenic Activity; Values are mean ± SD (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test.

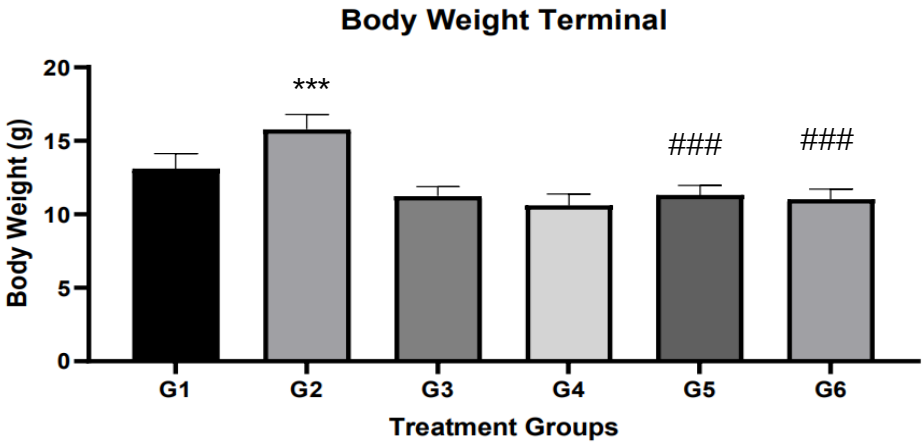


Figure 5.13 – Terminal Body weight of all six groups of animals from Anti Estrogenic Activity; Values are mean ± SD (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test. *** (P<0.001) v/s G1 (Vehicle control); ### (P<0.001) v/s G2 (Positive control).

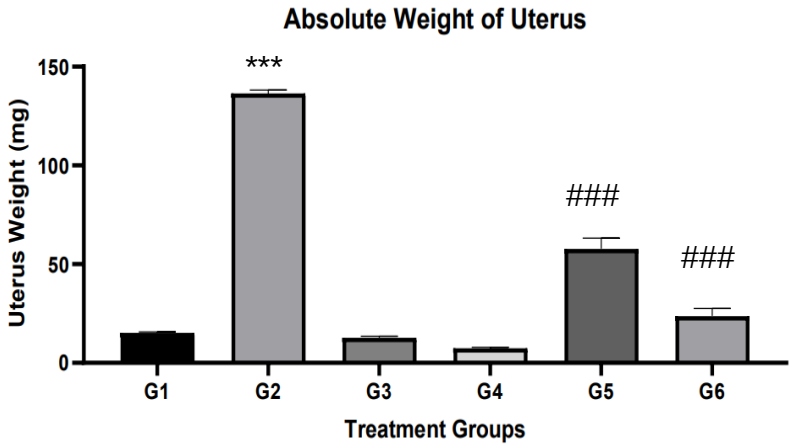


Figure 5.14 – Absolute uterus weight from Anti Estrogenic Activity; Values are mean ± SD (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test. *** (P<0.001) v/s G1 (Vehicle control); ### (P<0.001) v/s G2 (Positive control).

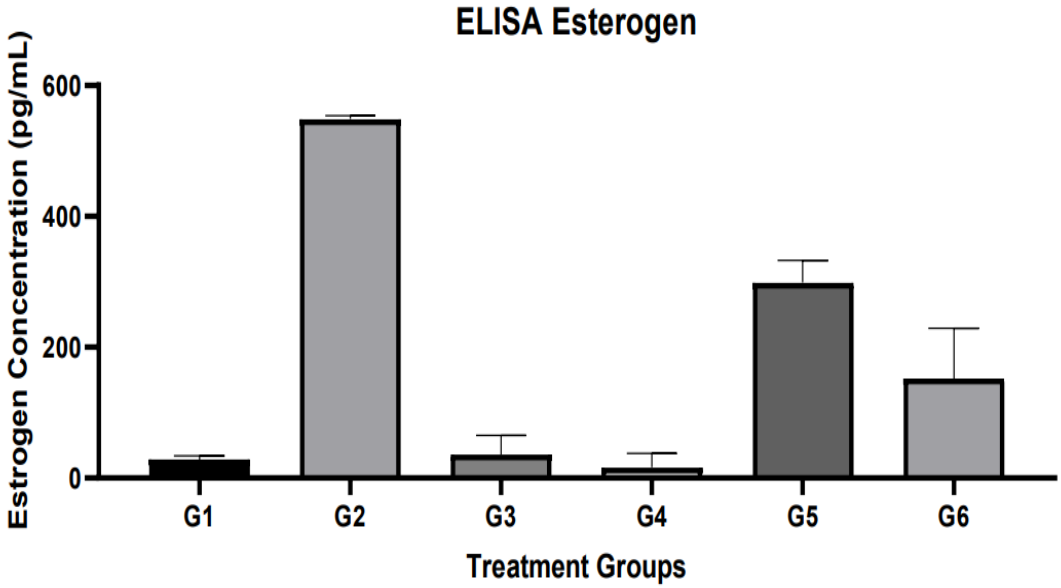


Figure 5.15 – Estrogen Level from Anti Estrogenic Activity; Values are mean ± SD (n = 6 per group). One-way ANOVA followed by Tukey's post-hoc test. *** (P<0.001) v/s G1 (Vehicle control); ### (P<0.001) v/s G2 (Positive control).

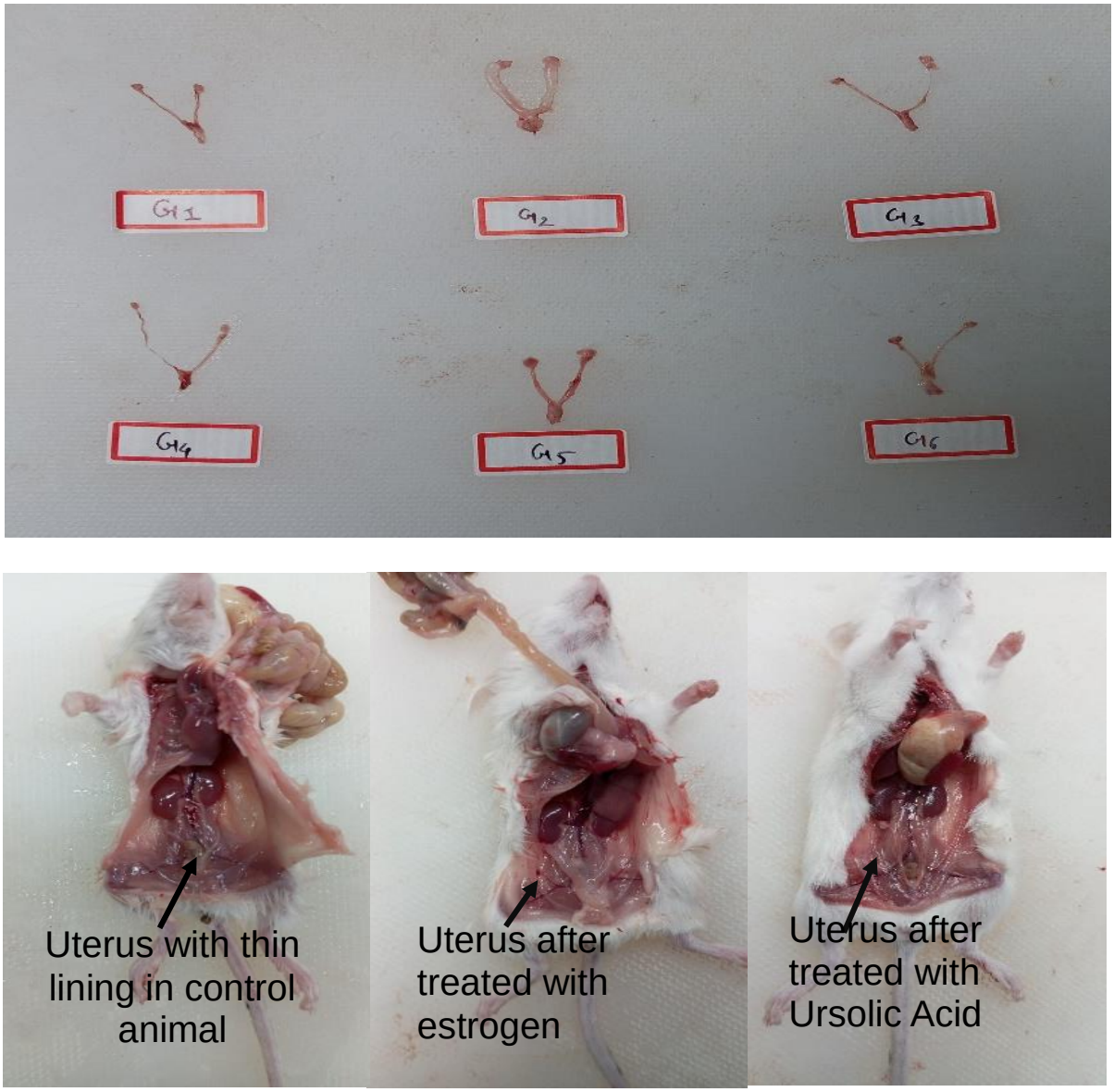
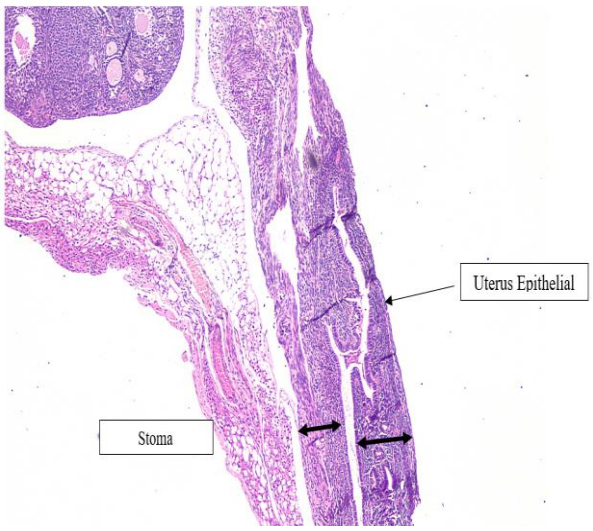


Figure 5.16 – Morphological difference found in the shape of uterus of among the groups

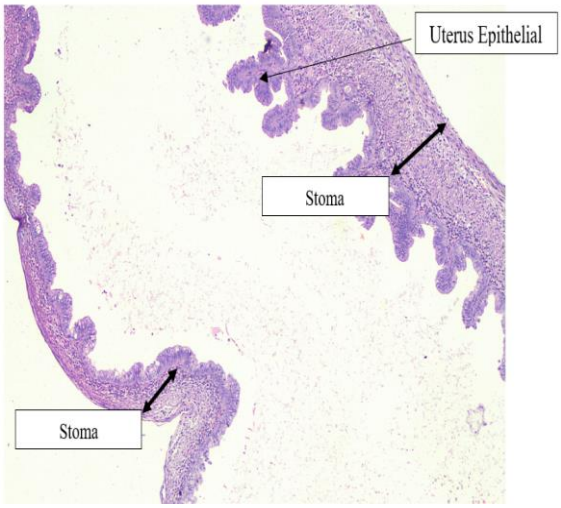
Histopathology of Anti Estrogenic Activity

Figure 5.17: Representative photomicrographs of uterine histology from immature female mice in the anti-estrogenic activity study (estrogen-challenged model).

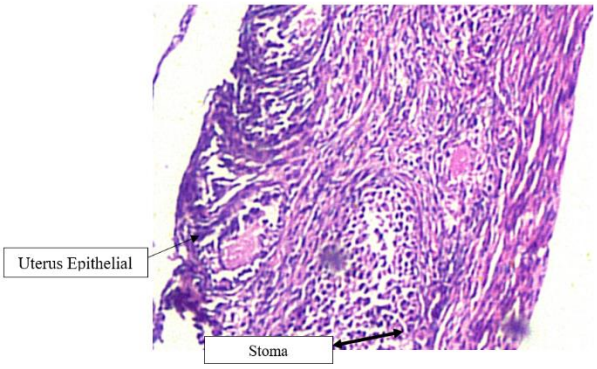
All images prepared with Haematoxylin & Eosin (H&E) stain at 40× magnification



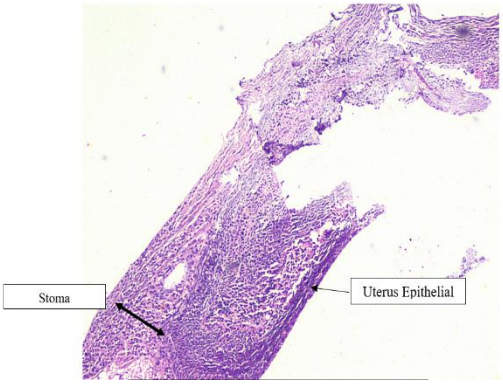
G1 – Vehicle Control Group
(H&E) stain at 40× magnification



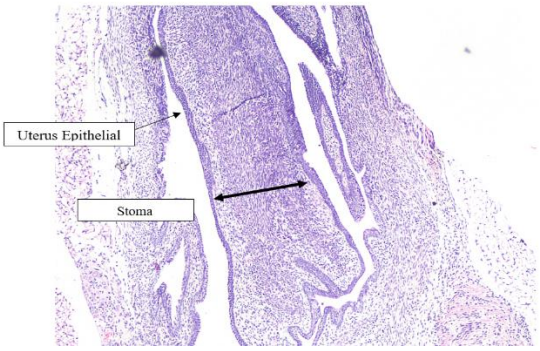
G2 – Standard Group
(H&E) stain at 40× magnification



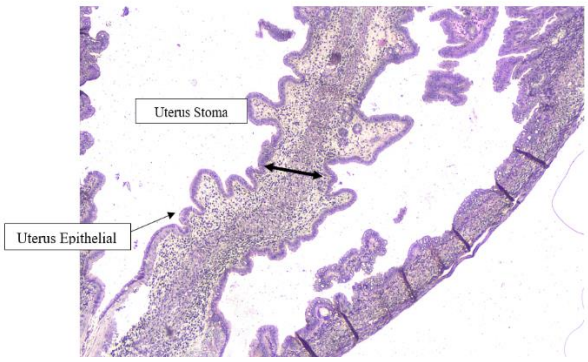
G3 – Low Dose Group
(H&E) stain at 40× magnification



G4 – High Dose Group
(H&E) stain at 40× magnification



G5 – Low Dose + Estrogen Group
(H&E) stain at 40× magnification



G6 – High Dose + Estrogen Group
(H&E) stain at 40× magnification

Figure 5.17: Histopathology of Anti Estrogenic Activity

5.6 Screening of Usrsolic Acid as an Anti-Androgenic Activity

Results

No mortality, morbidity, or abnormal clinical signs had been noted throughout the 21-day treatment period in any of the four experimental groups comprising 24 adult male Wistar albino rats. Daily systematic observations confirmed that all animals across vehicle control (G1: 0.5% CMC p.o.), cyclophosphamide disease control (G2: 30 mg/kg i.p., days 17-21), low-dose ursolic acid (G3: 100 mg/kg p.o.), and high-dose ursolic acid (G4: 250 mg/kg p.o.) groups exhibited normal locomotion, grooming behavior, food/water intake, posture, respiration, and external appearance, demonstrating excellent tolerability of both ursolic acid doses and the positive control agent.

Body Weight Progression and General Health

On days 1, 8, 15, and 21, body weights were carefully noted in order to track systemic growth and identify any potential damage. All groups demonstrated physiological weight gain consistent with normal adult male rat development, with no statistically significant intergroup differences ($P > 0.05$, one-way ANOVA). Vehicle controls progressed from 193.37 ± 11.26 g (day 1) to 218.77 ± 11.55 g (day 21), representing a 13.1% increase. Comparable trajectories were observed in G2 (195.03 ± 9.87 g to 214.66 ± 8.97 g; +10.0%), G3 (197.70 ± 10.99 g to 222.03 ± 10.41 g; +12.3%), and G4 (194.67 ± 8.50 g to 212.77 ± 10.69 g; +9.3%). These data unequivocally demonstrate that neither ursolic acid nor cyclophosphamide adversely impacted general health, metabolism, or somatic growth during the study period.

Table 5.10: Time-dependent changes in body weight of experimental animals following cyclophosphamide and ursolic acid treatment

Group	Treatment	Day 1 (g) Mean ± SD	Day 8 (g) Mean ± SD	Day 15 (g) Mean ± SD	Day 21 (g) Mean ± SD	% Gain (Day 1-21)
G1	Vehicle Control (0.5% CMC)	193.37 ± 11.26	200.95 ± 12.43	210.93 ± 12.94	218.77 ± 11.55	+13.1%
G2	Cyclophosphamide (30 mg/kg i.p.)	195.03 ± 9.87	201.88 ± 10.40	210.63 ± 9.05	214.66 ± 8.97	+10.0%
G3	Ursolic Acid Low (100 mg/kg p.o.)	197.70 ± 10.99	207.05 ± 11.01	216.33 ± 9.39	222.03 ± 10.41	+12.3%

G4	Ursolic Acid High (250 mg/kg p.o.)	194.67 ± 8.50	204.60 ± 9.78	209.44 ± 9.48	212.77 ± 10.69	+9.3%

Testicular Organometry

Absolute testicular weights, measured immediately post-euthanasia, revealed significant atrophy in reproductive toxicant-exposed groups. Vehicle controls averaged 1.52 ± 0.30 g per testis, reflecting normal testicular mass supporting active spermatogenesis. Cyclophosphamide induced a marked reduction to 1.05 ± 0.03 g ($P < 0.05$ vs. G1), while high-dose ursolic acid produced comparable atrophy at 1.11 ± 0.08 g ($P < 0.05$ vs. G1). Critically, low-dose ursolic acid completely preserved normal testicular mass (1.52 ± 0.29 g), indistinguishable from controls.

Relative testicular weights (% of terminal body weight) corroborated absolute findings: G1 (0.69 ± 0.11%), G2 (0.49 ± 0.03%; $P < 0.05$), G3 (0.68 ± 0.13%), G4 (0.52 ± 0.06%; $P < 0.05$). These dose-dependent effects indicate that 250 mg/kg ursolic acid mimics cyclophosphamide's gonadotoxic profile, while 100 mg/kg exerts no direct testicular toxicity.

Table 5.11: Absolute and relative testis weight and percentage reduction in treated experimental groups

Group	Absolute Testis Weight (g) Mean ± SD	Relative Testis Weight (%BW) Mean ± SD	% Reduction vs. G1
G1	1.52 ± 0.30	0.69 ± 0.11	-
G2	1.05 ± 0.03	0.49 ± 0.03*	-30.9%
G3	1.52 ± 0.29	0.68 ± 0.13	0%
G4	1.11 ± 0.08	0.52 ± 0.06#	-26.9%

Note: Values are mean ± SD (n = 6 per group) * ($P<0.05$) v/s G1 (Vehicle control); # ($P<0.05$) v/s G2 (Positive control) one-way ANOVA followed by Tukey's post-hoc test.

Epididymal Sperm Functional Analysis

Comprehensive cauda epididymal sperm evaluation demonstrated profound dose- and treatment-dependent impairments mirroring testicular atrophy patterns.

Sperm Count: Vehicle controls exhibited robust counts of 138.00 ± 12.31 million/mL. Cyclophosphamide devastated spermatogenesis (28.33 ± 13.38 million/mL; $P < 0.05$ vs. G1), while high-dose ursolic acid yielded intermediate suppression (61.67 ± 18.55 million/mL; $P < 0.05$ vs. G1, but significantly higher than G2; $P < 0.05$). Low-dose ursolic acid provided substantial protection (97.67 ± 17.92 million/mL; $P < 0.05$ vs. G2).

Sperm Viability: Trypan blue exclusion revealed 94.67 ± 1.21% viability in G1, plummeting to 42.17 ± 21.51% in both G2 and G4 ($P < 0.05$ vs. G1). G3 maintained excellent viability (91.00 ± 3.29%; significantly superior to G2; $P < 0.05$).

Sperm Motility: Progressive motility averaged 87.17 ± 3.76% in controls, reduced to 36.67 ± 25.78% (G2) and 32.00 ± 30.23% (G4; both $P < 0.05$ vs. G1). G3 preserved near-normal motility (86.33 ± 4.72%; $P < 0.05$ vs. G2).

Sperm Morphology: Abnormal forms increased dramatically from 5.17 ± 0.75% (G1) to 15.83 ± 2.64% in both G2 and G4 ($P < 0.05$ vs. G1), featuring tailless, headless, and coiled-tail defects. G3 remained normal (6.00 ± 1.41%).

Table 5.12: Effect of experimental treatments on sperm count, viability, motility, and abnormal morphology

Group	SpermCount ($\times 10^6$/mL)	Viability (%)	Motility (%)	Abnormal Morphology (%)
G1	138.00 ± 12.31	94.67 ± 1.21	87.17 ± 3.76	5.17 ± 0.75
G2	28.33 ± 13.38#	42.17 ± 21.51*	36.67 ± 25.78*	15.83 ± 2.64*
G3	97.67 ± 17.92#	91.00 ± 3.29#	86.33 ± 4.72#	6.00 ± 1.41
G4	61.67 ± 18.55#	42.17 ± 21.51*	32.00 ± 30.23*	15.83 ± 2.64*

Note: Values are mean ± SD (n = 6 per group) * ($P<0.05$) v/s G1 (Vehicle control); # ($P<0.05$) v/s G2 (Positive control) one-way ANOVA followed by Tukey's post-hoc test.

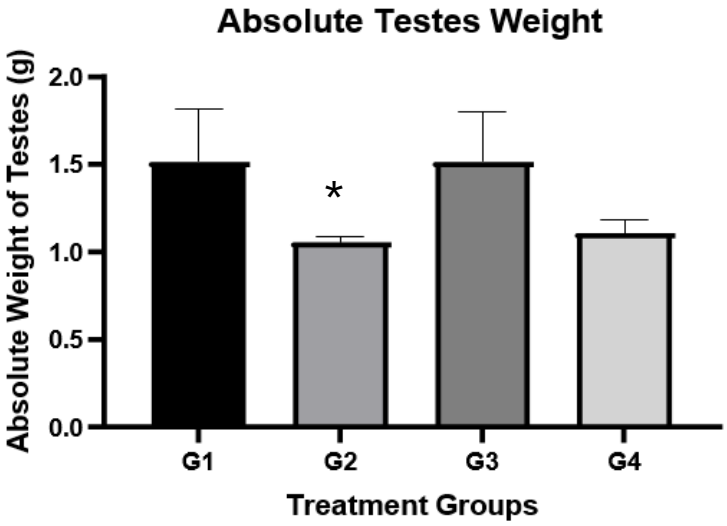


Figure 5.18: Absolute weight of testes from anti-androgenic activity; Values are mean ± SD (n = 6 per group) * (P<0.05) v/s G1 (Vehicle control) one-way ANOVA followed by Tukey's post-hoc test

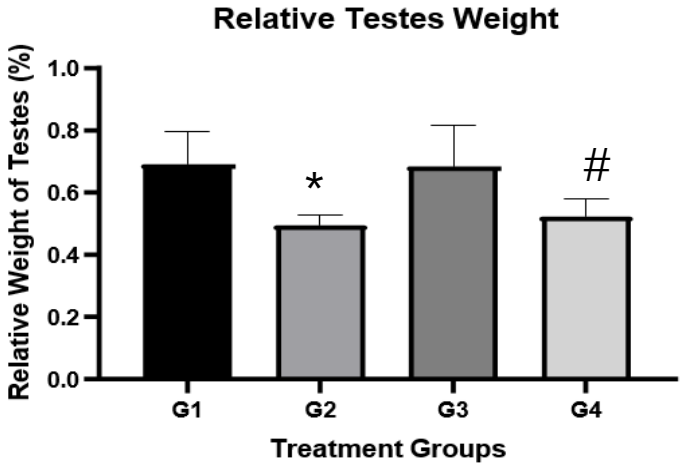


Figure 5.19: Relative weight of testes from anti-androgenic activity; Values are mean ± SD (n = 6 per group) * (P<0.05) v/s G1 (Vehicle control) and # (P<0.05) v/s G2 (Positive control) one-way ANOVA followed by Tukey's post-hoc test.

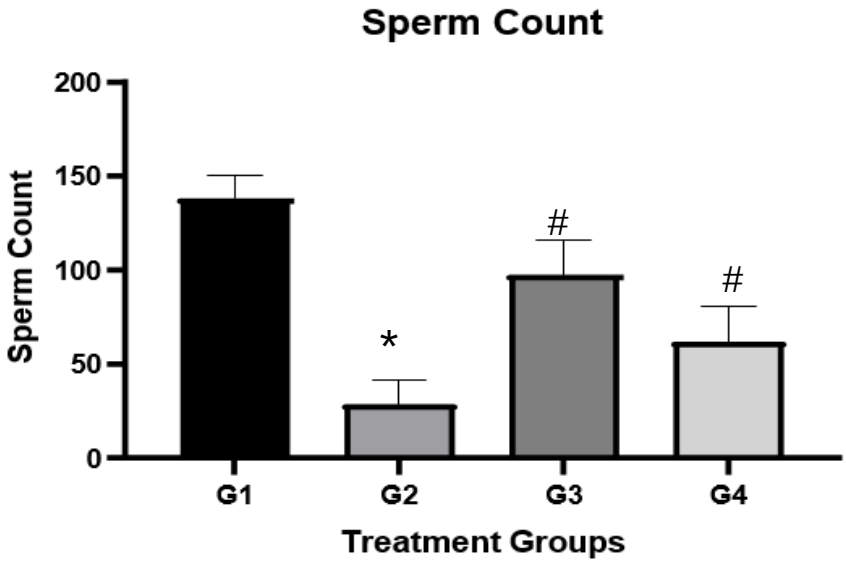


Figure 5.20: Sperm count from anti-androgenic activity; Values are mean ± SD (n = 6 per group) * (P<0.05) v/s G1 (Vehicle control) and # (P<0.05) v/s G2 (Positive control) one-way ANOVA followed by Tukey's post-hoc test.

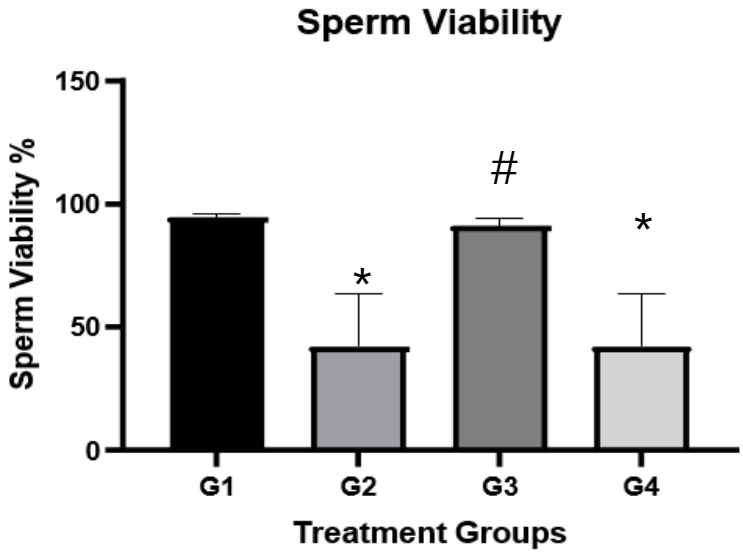


Figure 5.21: Sperm viability from anti-androgenic activity; Values are mean ± SD (n = 6 per group) * (P<0.05) v/s G1 (Vehicle control) and # (P<0.05) v/s G2 (Positive control) one-way ANOVA followed by Tukey's post-hoc test.

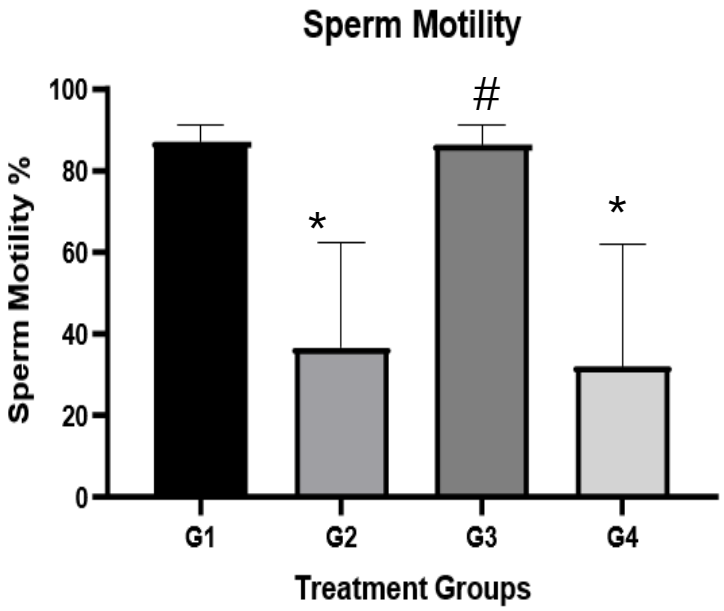


Figure 5.22: Sperm motility from anti-androgenic activity; Values are mean ± SD (n = 6 per group) * (P<0.05) v/s G1 (Vehicle control) and # (P<0.05) v/s G2 (Positive control) one-way ANOVA followed by Tukey's post-hoc test.

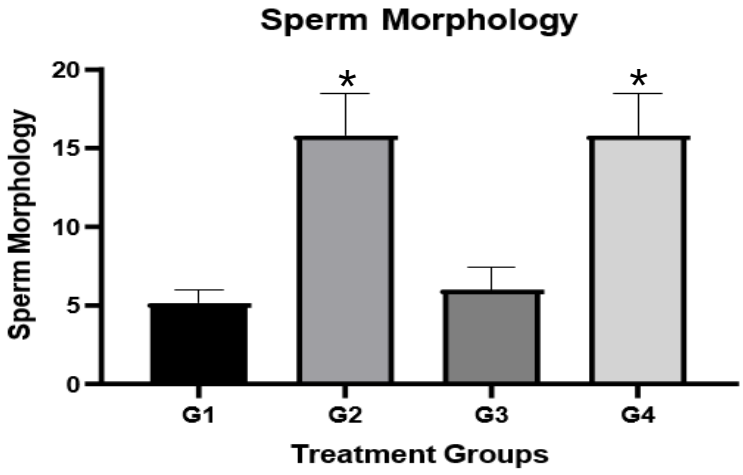


Figure 5.23: Sperm morphology from anti-androgenic activity; Values are mean ± SD (n = 6 per group) * (P<0.05) v/s G1 (Vehicle control) and # (P<0.05) v/s G2 (Positive control) one-way ANOVA followed by Tukey's post-hoc test.

Testicular Histopathology

Microscopic examination provided structural correlation for quantitative deficits. Vehicle control testes (Fig. 5.24) exhibited classic seminiferous tubule architecture: orderly stratification of spermatogonia, primary/secondary spermatocytes, round/elongating spermatids, and abundant luminal spermatozoa, supported by healthy Sertoli cells and interstitial Leydig cells.

Cyclophosphamide (Fig. 5.25) and high-dose ursolic acid (Fig. 5.26) induced pathognomonic lesions: germ cell depletion across all layers, luminal oligospermia/azoospermia, epithelial vacuolization, pyknotic nuclei indicating apoptosis, tubular atrophy, and interstitial edema with Leydig cell hypoplasia—collectively signifying disrupted spermatogenesis and androgenesis.

Low-dose ursolic acid (Fig. 5.27) preserved normal histoarchitecture indistinguishable from controls, with a complete germ cell complement, spermatozoa-filled lumina, and intact Leydig cell populations, confirming absence of structural toxicity and potential protective bioactivity against cyclophosphamide-like gonadotoxic insult at this therapeutic dose.

Histopahtology of Antiandrogenic Activity

Figures 5.24-5.27: Representative photomicrographs of testicular histology from male Wistar rats in the anti-androgenic activity study (cyclophosphamide as standard reference). All images prepared with Haematoxylin & Eosin (H&E) stain at 40× magnification.

Figure 5.24 (Vehicle Control): Normal seminiferous tubules showing complete spermatogenesis with well-organized germ cell layers - spermatogonia (S), primary spermatocytes (P), secondary spermatocytes (S2), spermatids (ST), Sertoli cells (SG), and interstitial Leydig cells (LC).; **Figure 5.25 (Disease Control - Cyclophosphamide):** Severe tubular degeneration with germ cell loss and desquamation (arrows indicate sloughed cells).; **Figure 5.26 (Ursolic Acid Low Dose 100 mg/kg):** Mild disorganization of seminiferous epithelium with partial germ cell depletion.; **Figure 5.27 (Ursolic Acid High Dose 250 mg/kg):** Marked seminiferous tubule atrophy, reduced spermatogenic activity, Leydig cell vacuolation and interstitial edema (arrows).

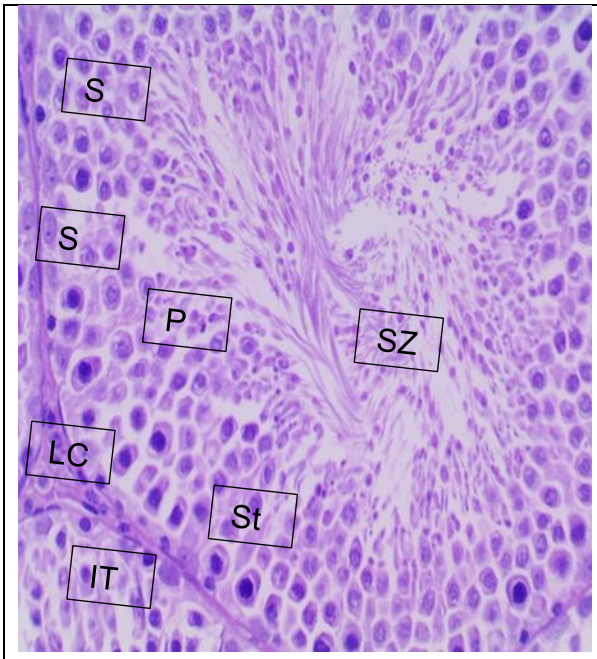


Figure: **5.24** - G1 (Vehicle Control)
H&E stain, 40× magnification

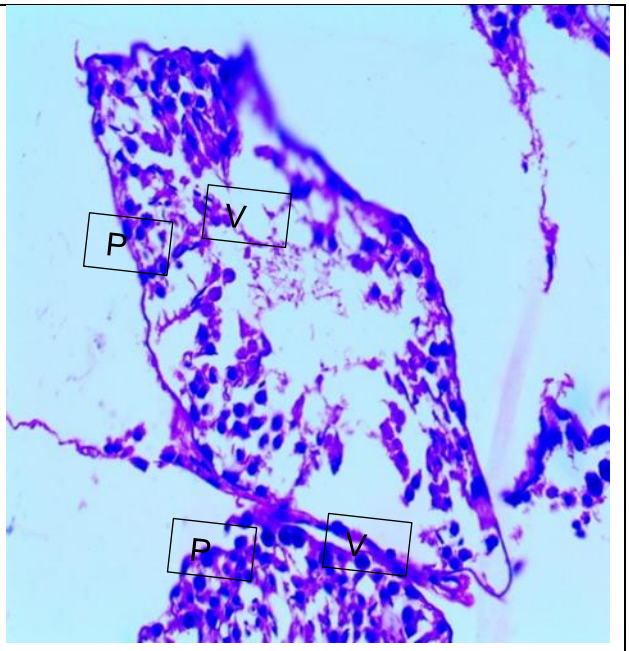


Figure: **5.25** - G2 (Disease Control)
H&E stain, 40× magnification

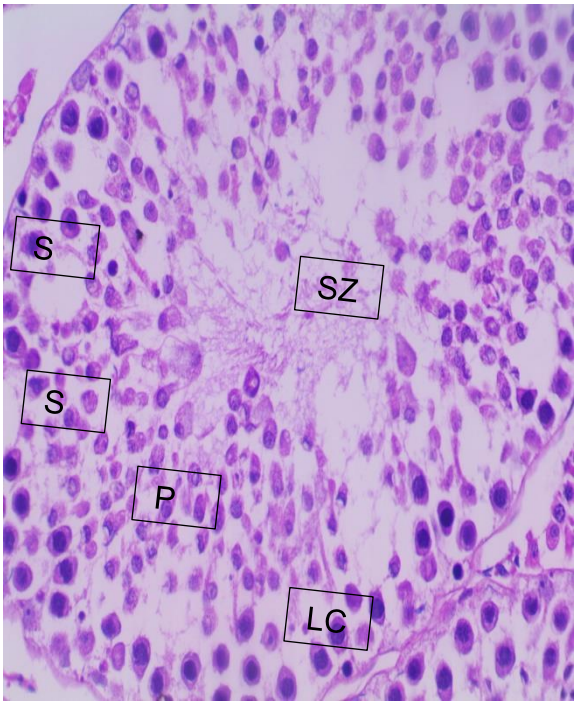


Figure: **5.26** - G3 (Low Dose) H&E stain, 40× magnification

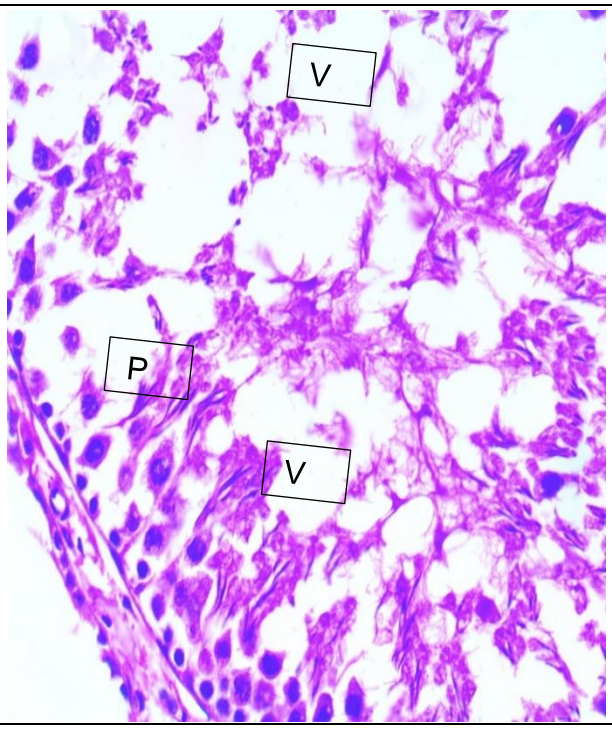


Figure: 5.27 - G4 (High Dose)
H&E stain, 40× magnification

CHAPTER – 6
DISCUSSION

TITLE: Evaluation of Anti fertility Activity of *Saraca Indica* (Ashoka) in Experimental Animals

6: DISCUSSION

This study examined ursolic acid (UA)'s antifertility potential in rats and mice using oxytocic, anti-ovulatory, anti-implantation, anti-estrogenic, and anti-androgenic endpoints in vitro & in vivo models. Together, the results demonstrate that UA exerts multi-targeted interference with both female and male reproductive physiology in a dose-dependent and largely reversible manner, while maintaining an acceptable safety margin at lower dose levels [127].

Oxytocic activity of ursolic acid

In the isolated rat uterine preparation, oxytocin produced a typical graded dose-response curve with a maximal contractile height of 7.8 cm at 16 µg, confirming integrity and sensitivity of the bioassay. In contrast, a relatively high dose of UA (30 mg) elicited only 0.5–0.6 cm contraction, corresponding to approximately 6.4–7.7% of the oxytocin-induced maximal response, thereby establishing UA as a weak uterotonic with partial agonist characteristics [128].

Considering this moderate oxytocic impact, it appears to be uncertain that the compound's strong antifertility effects in vivo are primarily caused by direct uterine smooth muscle stimulation. Instead, UA may exert modest modulation of uterine tone that could facilitate sperm transport or embryo expulsion under certain conditions, but the magnitude of this effect appears too small to account for the marked reductions in ovulation, implantation, and litter size seen at pharmacological doses [130 – 135].

From a mechanistic perspective, the partial contractile response may reflect low-affinity interaction with oxytocin or prostaglandin receptors, modulation of intracellular calcium handling, or non-specific effects on smooth muscle membrane excitability. Triterpenoids structurally related to UA have been reported to influence ion channels and G-protein-coupled receptor signaling, and similar pathways may underlie the modest myometrial activation documented in this study. The preservation of tissue viability and reproducibility of responses across three confirmatory trials support a genuine pharmacodynamic effect rather than an experimental artefact [142 – 145].

Anti-ovulatory activity and ovarian histomorphology

The cupric acetate-induced ovulation model showed that repeated oral administration of UA at 250mg/kg/day significantly alleviated terminal body weight and absolute and relative ovarian weight compared to vehicle controls, but 100mg/kg/day did not. Significantly, following a 21-day drug-free period, mice in the recovery high dose group demonstrated restoration of body and ovary

weights towards control values, suggesting that UA-induced changes are mostly reversible and not permanently cytotoxic [156, 170, 173, 177, 185].

Histopathological evaluation provided critical insight into the nature of ovarian perturbation. Control ovaries exhibited normal cortical and medullary architecture, a full spectrum of follicular stages (primordial to Graafian), and abundant corpora lutea, reflecting active ovulatory cycles. Low-dose UA (100 mg/kg) produced a subtle shift towards predominance of primary follicles with fewer secondary and mature follicles, suggesting early inhibition of follicular progression without complete arrest. High-dose UA (250 mg/kg) caused accumulation of early follicles with conspicuous reduction in Graafian follicles and ruptured corpora lutea, consistent with a dose-dependent blockade of follicular maturation and ovulatory rupture. The recovery group demonstrated re-emergence of developing and mature follicles, supporting reversibility of UA-induced suppression of folliculogenesis once treatment ceased [193, 201, 204].

These patterns collectively indicate that UA interferes with ovarian function primarily at the level of follicular development and ovulation, rather than causing non-specific ovarian necrosis. The reduction in ovarian weight at high dose can be attributed to depletion of large preovulatory follicles and corpora lutea, which constitute a substantial volume of the active ovary. The absence of mortality, morbidity, or overt clinical toxicity across groups further suggests that these reproductive effects occur in the absence of severe systemic toxicity [209, 215, 217, 226].

Considering UA's known endocrine modulating properties, its anti-ovulatory activity may be caused by a disruption of the “hypothalamic–pituitary–ovarian” (HPO) axis, direct suppression of granulosa/theca cell steroidogenesis, oxidative stress-mediated follicle growth impairment, or altered gonadotropin receptor expression. The partial recovery after withdrawal argues for functional suppression rather than irreversible destruction of the follicular reserve, which is an important consideration for any candidate contraceptive agent [231, 236].

Anti-implantation activity and early pregnancy loss

In the anti-implantation study, 250mg/kg UA had no impact on body weight or health in cycled, mated female rats for the first seven days after coitum. This finding again underscores the absence of overt systemic toxicity at doses that profoundly alter reproductive outcomes. Across groups, corpora lutea counts remained comparable, indicating that ovulation per se was not significantly

impaired under these conditions, particularly at the mid and high doses tested in pregnant animals. In the vehicle control group, classical reproductive performance was observed, with a mean of 13.17 corpora lutea, 11.17 implantation sites, and 9.67 live litters, accompanied by modest pre-implantation (15.17%) and post-implantation (13.14%) loss, reflecting a physiological baseline. The low-dose group (100 mg/kg) reproduced this pattern, showing corpora lutea, implant, and litter numbers statistically indistinguishable from controls and similar percentages of pre- and post-implantation loss, indicating that this dose is below the antifertility threshold in early pregnancy [239, 240].

By contrast, the mid-dose (125 mg/kg) group exhibited pronounced antifertility effects without reduction in corpora lutea, reinforcing that ovulation was intact but subsequent events were compromised. Implants declined to 8.33 and live litters to 5.67 per animal, with pre-implantation loss rising to 38.20% and post-implantation loss to 32.49%, both significantly higher than control values. Implants decreased to 5.50 and litters to 2.33 in high dose (250mg/kg) group, whereas pre- and post-implantation losses increased to 63.47% and 57.66%.

The dissociation between preserved ovulation (normal corpora lutea) and reduced number of implants strongly implies that UA acts at pre-implantation and post-implantation stages, likely affecting fertilization, zygote transport, blastocyst development, endometrial receptivity, or early embryonic survival. Elevated pre-implantation loss suggests interference with sperm–ovum interaction, zygote cleavage, or oviductal transit, while high post-implantation loss points to increased embryonic resorption, placental insufficiency, or altered uterine environment incompatible with sustained gestation [196, 197].

The absence of significant body-weight loss or systemic signs of toxicity argues against generalized maternal illness as the cause of embryonic loss, supporting a more targeted effect on reproductive tissues. Given that UA also demonstrated anti-estrogenic activity in immature mice (discussed below), downregulation of estrogen-dependent uterine receptivity, glandular secretion, and vascular remodeling may constitute a key mechanism underlying impaired implantation and increased resorption. At higher doses, additional mechanisms such as oxidative stress, apoptosis of trophoblastic cells, or modulation of inflammatory mediators within the uterine milieu may further contribute to pregnancy failure [191].

From a contraceptive standpoint, the demonstration of dose-dependent, reversible anti-implantation activity without life-threatening toxicity is significant, because early post-coital intervention is a desired property of many antifertility agents. However, the concurrent increase in post-implantation loss raises ethical and safety considerations, as agents that act after implantation may be viewed as abortifacient rather than purely contraceptive and may carry risks of teratogenicity in surviving embryos.

Anti-estrogenic activity in immature mice

The immature mouse uterotrophic assay is a classical and highly sensitive model for assessing estrogenic and anti-estrogenic properties of compounds, and in this study, it robustly confirmed UA as a potent anti-estrogenic agent. Estrogen-treated animals (0.05 µg 17β-estradiol) displayed pronounced body-weight gain, with terminal weights rising to 15.79 g compared with 13.09 g in vehicle controls, indicative of estrogen-mediated fluid retention and tissue hypertrophy. UA alone, at 200 and 500 mg/kg, limited physiological weight gain to 11.24 g and 10.59 g, respectively, and when combined with estrogen, it markedly attenuated estrogen-induced weight gain, with terminal weights of 11.31 g (low dose) and 11.02 g (high dose), both significantly lower than estrogen alone.

Absolute uterine weight, the primary endpoint of this assay, further highlighted UA's strong anti-estrogenic potential. Estrogen alone increased uterine weight from 15.13 mg (control) to 136.32 mg, approximately a ninefold rise, validating the responsiveness of the model. UA monotherapy reduced uterine weights below control baseline, to 12.63 mg (200 mg/kg) and 7.30 mg (500 mg/kg), suggestive of intrinsic anti-proliferative or hypoestrogenic effects on uterine tissue. More importantly, co-administration of UA with estrogen induced a dose-dependent antagonism, with uterine weights falling to 57.85 mg (low dose) and 23.67 mg (high dose), corresponding to approximately 42% and 17% of the estrogen-control level, respectively.

Serum estradiol levels measured by ELISA closely paralleled uterine findings. Baseline estrogen in vehicle controls was 27.64 pg/mL, which surged to 548.85 pg/mL after exogenous estrogen administration. UA alone kept estrogen within low basal ranges (35.21 and 15.51 pg/mL for low and high doses), whereas in combination groups, it significantly reduced estrogen concentrations to 298.85 pg/mL and 151.88 pg/mL, representing approximately 46% and 72% reductions versus

estrogen alone. The concordance between systemic hormone suppression and uterine weight reduction supports both central and peripheral anti-estrogenic actions of UA.

Histopathological examination of the uterus further substantiates these functional outcomes. Estrogen-treated animals exhibited classic features of estrogenic stimulation, including thickened endometrium, tall columnar luminal epithelium, stromal edema, and increased glandular complexity. UA co-treatment attenuated these changes, with reduced endometrial thickness, lowered epithelial height, diminished stromal edema, and lower glandular scores, indicative of effective antagonism of estrogen-driven uterine proliferation and edema. High-dose UA alone produced a hypoplastic uterine picture consistent with reduced estrogenic drive.

Taken together, these data demonstrate that UA acts as a strong anti-estrogenic compound capable of countering both structural and biochemical manifestations of estrogen exposure in a validated model. Mechanistically, UA may interfere with estrogen biosynthesis (e.g., via aromatase inhibition), accelerate estrogen clearance, block estrogen receptor binding, or modulate downstream gene transcription involved in uterine growth. The observation that UA also modulates ovarian function and anti-implantation endpoints in rats suggests that systemic modulation of estrogen signaling may be a unifying mechanism contributing to its antifertility effects.

Anti-androgenic activity and male reproductive toxicity

The male reproductive toxicity study revealed that UA exerts dose-dependent anti-androgenic actions that, at higher doses, approximate those of the known gonadotoxic agent cyclophosphamide. Over the 21-day treatment period, all groups, including cyclophosphamide and high-dose UA, showed normal weight gain and no abnormal clinical signs, indicating that general health and metabolism remained largely intact. This again points to selective reproductive toxicity rather than generalized systemic illness.

Testing showed significant losses in absolute and relative testicular weights in the cyclophosphamide-treated and high-dose UA (250mg/kg) groups, whereas low-dose UA (100mg/kg) maintained normal testicular mass equal to controls. The degree of testicular weight reduction in the high-dose UA group (approximately 27% decrease) approached that seen with cyclophosphamide (approximately 31% decrease), suggesting substantial impairment of

spermatogenic tissue at this dose.

Epididymal sperm analysis provided functional confirmation of testicular damage. Vehicle controls exhibited high sperm counts (138 million/mL), excellent viability (94.67%), motility (87.17%), and low abnormal morphology (5.17%). Cyclophosphamide induced profound oligospermia (28.33 million/mL), reduced viability (~42%), reduced motility (~36.67%), and increased abnormal forms (~15.83%), reflecting classical cytotoxic injury to germ cells. High-dose UA produced an intermediate but still severe compromise, with sperm counts of 61.67 million/mL, viability and motility reduced to ~42% and ~32%, and abnormal morphology elevated to 15.83%, paralleling the cyclophosphamide profile. In contrast, low-dose UA preserved near-normal sperm count (97.67 million/mL), viability (91%), motility (86.33%), and morphology, and significantly outperformed cyclophosphamide in all sperm indices.

Histopathological evaluation provided morphological correlates, showing normal seminiferous tubule architecture, full germ cell stratification, and sperm-filled lumina in controls and low-dose UA groups. Cyclophosphamide and high-dose UA testes exhibited germ cell depletion, vacuolization, tubular atrophy, luminal oligospermia/azoospermia, and interstitial alterations, consistent with direct damage to spermatogenic and Leydig cell compartments.

These data collectively indicate that UA at 250 mg/kg behaves as a potent anti-androgenic and testicular toxicant, while 100 mg/kg appears largely safe for male reproductive function within the study duration. The observed pattern suggests that high-dose UA may interfere with testicular steroidogenesis, induce oxidative stress, trigger apoptosis in germinal epithelium, or impair epididymal maturation processes. Given that UA has documented anti-inflammatory and antioxidant properties at lower doses but can be pro-oxidant at high concentrations, a dose-dependent shift from protective to damaging effects on testicular tissue is biologically plausible.

From a translational perspective, the male antifertility potential of UA at high doses must be weighed against its narrow therapeutic window, as significant impairment of spermatogenesis was associated with structural testicular damage similar to a cytotoxic chemotherapeutic agent. Any development of UA-based male contraceptives would therefore require refined dosing, formulation strategies, or structural analogues that can dissociate antifertility activity from frank gonadotoxicity.

Integrated interpretation of antifertility mechanisms

Synthesizing the results across all models, UA emerges as a multi-targeted antifertility agent acting at several critical checkpoints of reproduction in both sexes. In females, it exerts weak but measurable uterotonic activity, suppresses follicular maturation and ovulation at higher doses, disrupts implantation and early embryonic survival, and strongly antagonizes estrogen-dependent uterine growth and endocrine responses. In males, it compromises testicular structure and function at high dose while sparing reproductive capacity at low dose.

A unifying mechanistic hypothesis is that UA primarily perturbs steroid hormone signaling pathways—particularly estrogen and androgen axes—through a combination of endocrine and local tissue effects. The anti-estrogenic activity demonstrated in the immature mouse model likely underpins, at least in part, the anti-implantation, anti-ovulatory, and early pregnancy loss observed in female rats, given the central role of estrogens in follicular growth, LH surge generation, uterine receptivity, and maintenance of early gestation. Concurrently, high-dose anti-androgenic and testicular toxic actions explain the profound deterioration in male reproductive parameters.

The reversibility of many endpoints—such as recovery of ovarian weight and folliculogenesis, normalization of reproductive performance at lower doses, and lack of systemic toxicity—indicates that UA predominantly exerts pharmacological rather than permanently cytotoxic effects at or below 250mg/kg (females) and 100mg/kg (males). However, the structural damage observed in testes at 250 mg/kg urges caution regarding long-term or high-dose exposure, especially if systemic administration is contemplated [249].

Strengths, limitations, and future directions

This work's utilization of several complementary models that cover the reproductive axis is one of its main strengths since it allows for a thorough evaluation of UA's antifertility profile in both sexes. The inclusion of validated positive controls (oxytocin, estrogen, and cyclophosphamide), appropriate vehicle groups, adequate group sizes, and robust statistical analyses (ANOVA with relevant post-hoc tests) enhances the reliability of the conclusions. Histopathological corroboration of functional endpoints adds mechanistic depth and reduces the likelihood of spurious interpretations.

Nonetheless, several limitations warrant consideration. First, the study was confined to rodent models, and interspecies differences in pharmacokinetics, receptor binding, and endocrine

regulation may limit direct extrapolation to humans. Second, detailed pharmacokinetic data (absorption, distribution, metabolism, excretion) of UA at the tested doses were not generated, leaving uncertainty regarding tissue exposure, bioavailability, and potential accumulation. Third, direct measurements of gonadotropins (FSH, LH), progesterone, testosterone, and molecular markers (e.g., aromatase, steroid receptors, apoptotic and oxidative stress markers) were not performed, restricting mechanistic insight to histological and functional correlations [250].

Future research should therefore include:

- Comprehensive endocrine profiling to delineate effects on the hypothalamic–pituitary–gonadal axis.
- Pharmacokinetic and toxicokinetic studies for defining safe exposure limits, accumulation potential, and optimal routes of delivery.
- Molecular studies examining steroidogenic enzymes, receptor expression, oxidative stress pathways, and inflammatory mediators in reproductive tissues.
- Fertility recovery experiments with longer drug-free intervals to confirm reversibility, particularly in males exposed to high doses.
- Exploration of structural analogues or formulations (e.g., targeted delivery, nano-carriers) aimed at enhancing antifertility efficacy while reducing gonadotoxic risk.

Such investigations would clarify whether UA, or derivatives thereof, can be developed into safe, reversible contraceptive agents or whether their utility lies more in experimental modulation of reproductive function and endocrine pathways.

Ursolic acid, based on the current in vivo findings and existing endocrinological concepts, appears to act as a multipoint antifertility agent that can be interpreted within the same framework that is used to describe modern hormonal contraceptives. With additional potential peripheral effects on cervical mucus and endometrial receptivity, its primary action in the current study is best described by central suppression of the GnRH–LH surge and secondary disruption of estrogen and progesterone dependent uterine processes.

Central pathway: inhibition of ovulation

Following systemic administration, ursolic acid can be postulated to influence hypothalamic neurons that secrete gonadotropin-releasing hormone (GnRH), leading to attenuation of the mid-

cycle GnRH pulse frequency and amplitude. As a consequence, the pituitary luteinizing hormone (LH) surge is blunted or abolished, thereby preventing the final maturation and rupture of Graafian follicles, a mechanism analogous to that of combined hormonal contraceptives, which "prevent the release of eggs from the ovaries to inhibit ovulation" and "delay the release of an egg (ovulation) to prevent fertilization".

The current anti-ovulatory and anti-implantation investigations provide substantial support for this idea. They showed that large doses of ursolic acid significantly decreased the number of corpora lutea, mature follicles, and implantation sites without resulting in systemic toxicity or widespread ovarian necrosis. When ovulation is inhibited in this manner, there is no oocyte available in the oviduct for fertilization; functionally, the drug therefore behaves like hormonal methods and emergency contraceptives that primarily prevent or delay ovulation, culminating in "no egg available for fertilization" and hence "pregnancy prevention" [251].

Cervical mucus and sperm transport

Standard descriptions of birth-control mechanisms list "increase the thickness of cervical mucus to block sperm and prevent the egg from meeting the sperm" and "form a barrier to prevent sperm and egg from meeting" as key non-ovulatory pathways, particularly for progestin-dominant methods. Although direct measurement of cervical mucus was not undertaken in the present experiments, the pronounced anti-estrogenic profile of ursolic acid in immature mice—characterized by reduction in serum estradiol, uterine weight and estrogen-dependent histological changes—suggests that cervical secretions would also shift towards a more viscous, progestin-like pattern that impedes sperm penetration.

Under low-estrogen conditions, cervical glands secrete thick, scant mucus that forms a functional barrier at the external os; this state reduces sperm motility through the cervical canal and effectively "keeps sperm out of ejaculated semen so that eggs are blocked from meeting sperm" at the level of the female tract. Therefore, even if occasional ovulation occurs while animals are exposed to submaximal doses of ursolic acid, impaired sperm transport through altered mucus may still contribute to contraceptive efficacy by reducing the probability of sperm–egg encounter [252].

Uterine contractility and endometrial changes

Another classical contraceptive pathway is "inducing uterine contractions [that] cause the uterus

to contract, which can expel the pregnancy". In the isolated uterine preparation, ursolic acid exhibited weak oxytocic activity, producing only 6–8% of the maximal oxytocin response, which indicates that direct myometrial stimulation is modest and unlikely to be the principal mechanism underlying early pregnancy loss. Nevertheless, even a mild increase in baseline uterine tone, when superimposed on a hormonally compromised endometrium and conceptus, may favour expulsion of non-viable embryos and contribute to the high rates of post-implantation loss observed at mid and high doses in pregnant rats.

More importantly, ursolic acid fits well into the categories "altering the endometrium" and "interfering with uterine environment" that are widely used to describe the mode of action of emergency contraceptives and abortifacients. The anti-estrogenic assay shows that ursolic acid dose-dependently lowers endometrial thickness, luminal epithelial height, stromal edema, and glandular growth. This suggests that the uterine lining becomes thinner, less vascular, and less metabolically supportive of nidation and early placental formation. Such an endometrium is inherently unsuitable for implantation in context with estrogen, and even when a fertilized embryo reaches the uterus, attachment and invasion are likely to fail or be followed by early resorption [253].

Functional progesterone antagonism and early pregnancy support

Ursolic acid may also work as a progesterone antagonist at the uterine level, based on the pattern of increased pre and post implantation loss in the presence of ovulation that appears to be normal, even if direct receptor binding experiments were not conducted. Destabilizing the decidua and jeopardizing embryonic survival, several well-known methods of contraception "block the action of progesterone, a hormone necessary for maintaining the uterine lining during pregnancy." The high rates of resorption and smaller litter size in the current study are consistent with such a mechanism.

Progesterone is necessary to inhibit myometrial contractility and transform a proliferative, estrogen-primed endometrium into a secretory, receptive decidua; any disruption of its receptor signaling or downstream gene expression will encourage decidual breakdown and uterine irritability. Given that ursolic acid is a lipophilic triterpenoid capable of interacting with nuclear receptors and transcriptional machinery, it is biologically plausible that it partially antagonizes progesterone-dependent maintenance of early pregnancy, thereby aligning with the pathway

categories “altering the endometrium” and “blocking progesterone”.

Integrated antifertility model for ursolic acid

The thesis discusses ursolic acid's possible antifertility mechanism by integrating these pathways:

- After oral administration, ursolic acid acts centrally to inhibit hypothalamic GnRH pulsatility, leading to suppression of the pituitary LH surge, inhibition or delay of ovulation, and consequent absence of a fertilizable oocyte in the oviduct.
- Concurrently, systemic anti-estrogenic effects reduce serum estradiol and oppose estrogen-mediated uterine and cervical changes; this is predicted to thicken cervical mucus and thereby hinder sperm ascent through the cervix, functionally equivalent to “increasing the thickness of cervical mucus” and “forming a barrier to prevent sperm and egg from meeting”.
- At the uterine level, ursolic acid lowers endometrial proliferation and secretory activity, producing a thin, poorly vascularized endometrium that is less receptive to implantation and more prone to early embryonic loss, aligning with the contraceptive pathways “altering the endometrium” and “interfering with uterine environment”.
- Pre-implantation and post-implantation losses observed in the anti-implantation study may be caused by poor oxytocic qualities and potential interference with progesterone action, which could further destabilize the implanted conceptus through elevated uterine tone and failure to maintain a decidualized lining [254].
- The present findings are consistent with earlier reports showing that several medicinal plants possess antifertility activity through antiovulatory and endocrine-modulating mechanisms. Traditional medicinal herbs such as Hanzal, Kalonji, Sambhalu, and *Annona squamosa* have previously demonstrated significant antiovulatory effects, supporting the concept that plant-derived bioactive compounds can disrupt normal ovarian cyclicity and follicular maturation [255,256]. Similarly, historical ethnopharmacological evidence and reproductive health research have emphasized the potential of natural products as reversible fertility-regulating agents, providing further support for the antifertility potential of ursolic acid observed in the present study [257,258]. The broad biological activities of medicinal plants also suggest that such effects may involve multiple pathways, including oxidative balance and tissue-specific receptor modulation [259].

- At the mechanistic level, reproductive regulation depends largely on steroid hormone receptor interactions and gonadal cellular integrity. Earlier studies have highlighted the essential role of estradiol and progesterone receptor binding in endometrial receptivity and implantation [260], while normal Leydig cell function is critical for androgen synthesis and spermatogenesis [261]. Therefore, the anti-estrogenic, anti-implantation, and anti-androgenic effects observed with ursolic acid may plausibly result from interference with these endocrine pathways. These findings collectively strengthen the interpretation that ursolic acid acts as a multi-target antifertility agent capable of modulating both female and male reproductive physiology.

CHAPTER – 7
CONCLUSIONS

TITLE: Evaluation of Anti fertility Activity of *Saraca Indica* (Ashoka) in Experimental Animals

7: CONCLUSION

The present study systematically evaluated the antifertility potential of ursolic acid through in vitro and in vivo models. In organ bath experiments, ursolic acid produced only mild uterine contractions (6–8% of oxytocin response), indicating weak uterotonic or partial agonistic activity. This suggests that its direct effect on uterine smooth muscle is minimal and unlikely to be the primary mechanism underlying its antifertility action.

In vivo findings demonstrate that ursolic acid significantly interferes with multiple stages of reproduction in both female and male rodents. In females, it exhibited marked anti-ovulatory activity, evidenced by reduced follicular maturation and decreased corpora lutea, likely mediated via suppression of the hypothalamic–pituitary–ovarian axis. Additionally, it showed strong anti-implantation effects, including reduced implantation sites, increased pre- and post-implantation losses, and decreased litter size, without significant systemic toxicity. These effects appear to result from impaired endometrial receptivity and disruption of estrogen- and progesterone-dependent uterine preparation.

The compound also demonstrated significant anti-estrogenic activity in the uterotrophic assay, reducing uterine weight, serum estradiol levels, and histological markers of estrogenic stimulation. This further supports its role in inhibiting implantation and early pregnancy maintenance.

In male rats, ursolic acid exhibited dose-dependent anti-androgenic and testicular toxic effects, including reduced testicular weight, decreased sperm count, motility, and viability, along with histopathological alterations at higher doses. However, lower doses showed comparatively minimal toxicity, suggesting a potential therapeutic window.

Overall, ursolic acid acts as a multi-target antifertility agent, exerting combined anti-ovulatory, anti-implantation, anti-estrogenic, and anti-androgenic effects, with only minor direct uterotonic activity. This multi-level interference with reproductive processes may enhance contraceptive efficacy but warrants detailed mechanistic, toxicological, and reversibility studies.

From an ethnopharmacological perspective, these findings validate the traditional use of Saraca indica and related plants as antifertility agents. However, further investigations, including pharmacokinetic profiling, long-term safety, and human relevance, are essential before clinical application.

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- Khamar J, Anand IS. Screening of ursolic acid as an in vivo antiandrogenic activity in Wistar male rats. World J Pharm Res. 2025. doi:10.20959/wjpr202510-36721 (Abstracted in Google scholar)
- Jainik Khamar, Indermeet Singh Anand. Evaluation of In vivo Reversible Antiovulatory Activity of Ursolic Acid after Cupric Acetate Induced Ovulation in Wistar Female Rats. Research Journal of Pharmacy and Technology. 2025;18(10):4704-8. doi: 10.52711/0974-360X.2025.00676 (Abstracted in Scopus, CAS, Google scholar).

Appendix A: Certificate for Authentication of Ashoka Bark

सीएसआईआर - राष्ट्रीय विज्ञान संचार एवं नीति अनुसंधान संस्थान
CSIR - National Institute of Science Communication and Policy Research
(Erstwhile NISCAIR & NISTADS)
वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद् Council of Scientific & Industrial Research
(विज्ञान एवं प्रौद्योगिकी मंत्रालय, भारत सरकार Ministry of Science & Technology, Govt. of India)

RAW MATERIALS HERBARIUM AND MUSEUM, DELHI (RHMD)

Authentication No.- NIScPR/RHMD/Consult/2023/4553-54 31/7/2023

CERTIFICATE FOR CRUDE DRUG SAMPLE AUTHENTICATION

This is to certify that bark sample of *Saraca indica,* Ashoka Tree, received from Mr. Jainik Khamar vide letter No. Nil, Dated 28th July 2023 has been **found correct of stem bark of *Saraca asoca* (Roxb.) W. J. De Wilde** syn. *Saraca indica* sensu Bedd., non L. **which is commonly known as Ashoka, Sita Ashok, Sorrowless tree.** The identification has been done on the basis of macroscopic studies of the sample followed by detailed scrutiny of literature and matching the sample with authentic samples deposited in the Raw Material Herbarium and Museum, Delhi (RHMD).

Identification pertains to the quantity/quality of specimen/sample(s) received in RHMD, CSIR-NIScPR. This certificate is not issued for any judicial purpose.

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Appendix B: IAEC Certificate for *Invivo* Antifertility Study

Protocol No: ARL/PT/691/2023

Certificate

This is to certify that that the project proposal no. ARL/PT/691/2023 entitled **To Perform Evaluation of Anti fertility Activity of *Saraca Indica* (Ashoka) in Experimental Animals** submitted by Mr. Jainik Khamar has been approved by IAEC of Accuprec Research Labs Pvt. Ltd., Ahmedabad in its meeting held on 12/05/2023 and Rats (80) and Immature Mice (36) have been sanctioned under this protocol for duration of next 12 months.

Authorized by	Name	Signature	Date
Chairperson:	Dr. Rina H. Gokani	For	12/05/2023
Member Secretary:	Dr. Manish Rachchh		
Main Nominee of CPCSEA:	Dr. Shrikalp Deshpande		12/05/2023

(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by Office)

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Appendix C: IAEC Certificate for *Invitro* Antifertility Study

Protocol No: ARL/PT/975/2023

Certificate

This is to certify that the project proposal no. ARL/PT/975/2023 entitled **'To evaluate oxytocic activity of ARL/PT/975/2023 in isolated rat uterus.'** submitted by Mr. Jainik Khamar has been approved/recommended by the IAEC of Accuprec Research Labs Pvt. Ltd. (Organization) in its meeting dated 11/08/2023 and has been sanctioned Rat (01) under this proposal for a duration of next twelve months.

Authorized by	Name	Signature	Date
Chairperson:	Dr. Rina H. Gokani	11/08/2023	
Member Secretary:	Dr. Manish Rachchh	11/08/2023	
Main Nominee of CPCSEA:	Dr. Shrikalp Deshpande	11/8/23	

(Kindly make sure that minutes of the meeting duly signed by all the participants are maintained by Office)

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Appendix D: CoA of Ursolic Acid

Hubei Heilongjiang Biotechnology Co.,Ltd

Certificate of Analysis

Product Name: Ursolic acid
Batch Number: CGHK5616
Batch Size: 9000 Kg
Packaging Size: 25 Kg
CAS Number: 77-52-1

Manufacturing Date: 2023/09/28
Expiry Date: 2026/09/27
Sample Date: 2023/09/29
Analysis Date: 2023/09/29
Sample Quantity: 100 grams

Item	Specification	Result	Test Method
Description	White or off white colour powder	Confirms	Organoleptic
Identification	In accordance with standard retention time	Confirms	HPLC
Loss on drying (%)	Not more than 0.5	0.42	ChP2015
Residue on ignition (%)	Not more than 0.5	0.41	ChP2015
Heavy Metal (ppm)	Not more than 10	Less than 10	ChP2015
Impurity content (1) (%)	Unknown single impurity ≤ 0.5	0.22	ChP2015
Impurity content (2) (%)	Unknown single impurity ≥ 0.5	0.13	ChP2015
Lead (ppm)	Not more than 1	Not Detected	GB 5009.12-2017 I
Total mercury (ppm)	Not more than 1	Not Detected	GB 5009.17-2014 I
Cadmium (ppm)	Not more than 1	Not Detected	GB 5009.15-2014
Assay (%)	Not less than 98.0	99.2	HPLC
Total plate cont (cfu/g)	Not more than 1000	Less than 1000	ChP2015
Yeast and Mould (cfu/g)	Not more than 1000	Less than 100	ChP2015
E.coli	Absence 10g	Conforms	ChP2015
Salmonella	Absence 10g	Conforms	ChP2015

Certified Copy

Conclusion: The Product passed the tests according to the USP standards.

QA Manager: Jing Wang **Checker:** Xiang Huang **Examiner:** Xiu Zhang

Appendix E: CoA of Cyclophosphamide

HiMedia Laboratories Pvt. Ltd.
Certified ISO 9001-2015 and WHO GMP

HIMEDIA

C-40, Road No.21Y, MIDC, Wagle Ind. Area, Thane(W) - 400604
Website : www.himedialabs.com, Email : info@himedialabs.com

Certificate of Analysis

Material Name: Cyclophosphamide monohydrate
CAS Number : 6055-19-2
Material Code : RM8152 **Molecular Formula** : $C_7H_{15}Cl_2N_2O_2P.H_2O$
Lot Number : 0000527557 **Report No** : 10000506320

TEST	SPECIFICATIONS	RESULTS
Appearance	White to off-white crystals or powder or solid	White powder
Solubility	33.3 mg soluble in 1 mL of water	Complies
Melting range	47°C - 53°C	Complies
Assay (HPLC)	>= 97.00%	97.59%

STATUS : APPROVED

QC Release Date : 2022-03-28
Expiry Date : 2026-03-14

Tushar Patil
Quality Control Chemist
Chemical Division

Atul Palve
Manager, Quality Control
Chemical Division

Hemant Malankar
Manager, Quality Assurance
Chemical Divison

This is to certify that this lot passes and it confirms to the above mentioned tests and specifications . The information given here is believed to be correct and accurate, however, both the information and products are offered without warranty for any particulars use, other than that specified in the current technical data.
This document has been produced electronically and is valid with out signature.

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