LITERATURE REVIEW
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2.1 REPRODUCTIVE SYSTEM

Reproduction is the process by which new individuals of a species are produced and the genetic material is passed from generation to generation. This maintains continuation of the species\textsuperscript{13}.

2.1.1. HUMAN BEINGS

The reproductive system has been cloaked in secrecy and cultural inhibitions for centuries. In human beings the process of reproduction is sexual one in which the male and female organs differ anatomically and physiologically\textsuperscript{14}.

The structures of the female reproductive system are the ovaries, fallopian tubes, uterus, and vagina (Fig-1, 2). The ovaries respond directly to stimulating hormones secreted by the anterior pituitary to produce the female sex hormones. Beginning with puberty the females have the ability to reproduce until the menopause\textsuperscript{15}.

The functions of the female reproductive system are\textsuperscript{14}:

- Formation of female gametes, ova
- Reception of male gametes, spermatozoa
- Provision of suitable environments for fertilization of the ovum by spermatozoa and development of the resultant fetus
- Parturition (Child birth)
- Lactation, the production of breast milk, which provides complete nourishment for the baby in its early life
Fig 1: Human Female Reproductive Organs
Fig 2: Human Female Reproductive System
In the male, the urinary and reproductive systems are interrelated. The male reproductive tract consists of testes, which reside in the scrotum; the vas deferens; the prostate gland and the penis (Fig-3). The testes must be external to the body cavity because normal body temperature will damage sperm. Beginning at the puberty, males are capable of reproducing for the rest of their lives. The functions of the male reproductive system are:

- production of male gametes, spermatozoa
- transmission of spermatozoa to the female

Fig 3: Human Male Reproductive System
The male and female sex hormones are necessary for the development and maintenance of secondary sex characteristics and for reproduction\textsuperscript{15}. The reproductive process is highly complex. It begins with the secretion of gonadotrophic hormones from the anterior pituitary gland, which stimulate the development of the sex organs. Follicle-stimulating hormone (FSH) stimulates the growth and the maturation of the graffian follicles in the ovaries and spermatogenesis in the testes. Luteinizing hormone (LH) stimulates the release of an egg by the ovary and the formation of the corpus luteum. In the testes, formation of sperm (spermatogenesis) and the secretion of androgens occur. The male sex hormones are called androgens; the female sex hormones are estrogen and progesterone (Fig-4, 5).

**Hormonal control of the female reproductive system**\textsuperscript{13-16}: The menstrual cycle consists of 3 phases (Fig-6).

1. Menstruation
2. Proliferative/ Follicular phase
3. Secretory phase

The menstrual cycle starts with menstruation which lasts for 3-6 days during which the superficial layer of endometrium is shed. The endometrium regenerates during the follicular phase of the cycle after menstrual flow has stopped.

Hypothalamic gonadotrophin-releasing hormone (GnRH) acts on the anterior pituitary to release the gonadotrophins follicle-stimulating hormone (FSH) and luteinising hormone (LH), which act on the ovary. The gonadotrophins stimulate follicle development. A small group of follicles are developed each containing an
ovum. One follicle develops faster than the others and forms the Graffian follicle and the rest degenerate. The ripening follicle consists of thecal and granulosa cells surrounding a fluid filled centre within which lies an ovum.

Estrogens are produced by the granulosa cells stimulated by FSH, from androgen precursor molecules derived from thecal cells stimulated by LH. Estrogen controls the proliferative phase of the endometrium which occurs from day 5 or 6 until midcycle and has negative feedback effects on the anterior pituitary (Fig-5, 7).

LH stimulates ovulation at midcycle and proliferates the ruptured follicle into corpus luteum which secretes progesterone. Progesterone controls the secretory phase and has negative feedback effects on both hypothalamus and anterior pituitary. The progesterone renders the endometrium suitable for the implantation of a fertilized ovum.

If implantation does not occur, progesterone secretion stops, triggering menstruation (Fig-8). However, if implantation occurs, the corpus luteum continues to secret progesterone and prevents further ovulation by its negative feedback effect. The chorion (an antecedent of the placenta) secretes human chorionic gonadotrophin (hCG), which maintains the lining of the womb during pregnancy. Later in pregnancy, placenta secretes gonadotrophins as well as progesterone and estrogens. Progesterone secreted during pregnancy controls the development of the secretory alveoli in the mammary gland, while estrogen stimulates the lactiferous ducts. After parturition, estrogens, along with prolactin are responsible for stimulating and maintaining lactation.
Fig 4: Hormonal inter-relationships in the control of Male Reproductive System
Fig 5: Hormonal inter-relationships in the control of Female Reproductive System
Fig 6: Hormonal regulation of changes in the ovary and uterus
Fig 7: Changes in conc. of anterior pituitary and ovarian hormones.
Fig 8: Stages of development of ovum and associated hormones.
2.1.2. **ANIMALS**

Reproductive process of all animals requires the coordinated function of several organs & tissues. The process is regulated by the endocrine system through the timely release of hormones to create an environment for initiating and maintaining the reproductive process.

The primary steps in the reproductive process of the female are as follows-

1) Maturation and release of ovum
2) Fertilization of the ovum in the fallopian tubes
3) Transport of the fertilized ovum to the uterus
4) Implantation of the fertilized ovum in the lining of uterus
5) Maintenance of the fetus by the placenta within the uterus
6) Delivery of the litters

A drug that inhibits fertility could interact with one or more of the steps above. Ideally, an antifertility agent should target specifically only at one step of the reproductive process. Rats are commonly used for this purpose, although mice, hamsters, rabbits and monkeys may also be employed.

2.1.2.1. **RAT**

Albino Rat (Rattus norvegicus) is one of the commonest laboratory animals suitable for experimental work because of its small size and greater sensitivity to most drugs. It is also the most standardized of all laboratory animals. It can be bred to obtain pure and uniform strains and is found to be very sturdy to withstand long periods of experimentation under anesthesia.

Body weight of Adult rat is between 150-200 g. Two strains of albino rats are commonly used in experimental pharmacology.
i) Wistar strain: Its body length is longer than tail length. It has wide head. It is not susceptible to infection.

ii) Sprague dawley strain: Its body length is almost equal to tail length. It has long and narrow head. It is susceptible to infection.

A. Anatomy:

The male reproductive system of rat is anatomically and functionally similar to that of human being. However, the female reproductive system consists of ovary, uterine horn and vagina. There are no fallopian tubes in rats (Fig-6-8).

B. Breeding of rats:

Rats should not be mated till they are at least 90-100 days old. Females must be placed in the cage a few days before introducing male. 3 females to 1 male is the general rule. Alternatively one male and one female can be placed together in cage for the duration of their breeding period.

Mating age : 9-10 weeks
Breeding season : all seasons
Estrous cycle : 5 days
Gestation period : 21 days
Litter size : 6-10
Breeding life : 1-1.25 yrs

C. Oestrous cycle in rats:

The cycle makes its appearance at puberty at the age of two to three months, and the whole cycle lasts for about 4-5 days being divided into four stages according to the cell types found in the vaginal smear (Fig-12). These are as follows:
1. **Oestrous**: Its duration is 9-15 hrs. It is characterized by sexual receptivity when the female will allow copulation. During this period there is increased running activity, quivering of the ears, and lordosis in the presence of another rat. The vaginal smear shows 100 per cent cornified epithelial cells.

2. **Met-oestrous**: Its duration is about 20 hrs. It follows oestrous and occurs shortly after ovulation. The vaginal smear is characterized by many leucocytes with a few cornified cells.

3. **Dioestrous**: Its duration is 60-70 hrs. It is the longest of the phases. The vaginal smear consists mainly of leucocytes.

4. **Pro-oestrous**: Its duration is about 12 hrs. It follows dioestrous. It is a preparatory phase preliminary to the next estrous phase. The vaginal smear is characterized by nucleated epithelial cells either singly or in sheets.

**D. Some physiological data on Rat:**

- **Life span**: 2-3 yrs
- **Water consumption**: 35 ml/day
- **Food consumption**: 10-20 g/day
- **Blood volume**: 50-65 ml/kg body wt.
- **Haemoglobin**: 12-17 g%
- **RBC**: 7.2-9.6 million/ cu mm
- **WBC**: 6-12 thousand/ cu mm
Fig 9: Female Rat Anatomy

1. Stomach
2. Kidney
3. Ovary
4. Oviduct
5. Uterus (left horn)
6. Large Intestine
7. Urinary bladder
Fig 10: Reproductive System of Female Rat
Adrenal Gland
Kidney
- Ureter /
Bladder — Seminal Vesicle
Prostate
Gland
Utogenital Prifice
Penis
Vas deferens
Epididymus
Testis
Scrotal Sac

Fig 11: Reproductive System of Male Rat
Fig 12: Oestrous Cycle in Rats.

PE : Proestrous
E : Estrous
ME : Metestrous
DE : Diestrous

Fig 12: Oestrous Cycle in Rats.
2.2 DEMOGRAPHY

It is the scientific study of human population. It focuses its attention on the following three observable human phenomena.

a. Change in population size
b. Composition of population
c. Distribution of population in space

The demographic processes which determine these phenomena are-

a. Fertility
b. Mortality
c. Marriage
d. Migration
e. Social mobility

2.2.1. POPULATION GROWTH

Global population:

The global population in 1830 was 1 billion which was doubled in 1930 with a time gap of 100 years. By 1960, again the world population increased by 1 billion. The time span for increasing total population by 1 billion rapidly became shorter and shorter. The next addition of 1 billion in total population was made within 15 years time. In 1987, the world population crossed the mark of 5 billion and this time the addition of 1 billion was made within 12 years. In 2000, the world population touched the mark of 6 billion and it is estimated that it will touch the level of 7 billion and 8 billion by 2010 AD and 2022 AD respectively.
Indian population at 0.00 hours of 1st March, 2001 stood at 1,027,015,247 comprising 531,277,078 males and 495,738,169 females. Thus India became only the second country in the world after China to cross the one billion mark. India accounts for a mere 2.4% of world surface area of 135.79 million square kms. Yet it supports and sustains whopping 16.7% of the world population.

**Fig 14:** Ten most populous countries

**Fig 13:** Global Population
### Population of selected countries:

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Country</th>
<th>Ref. Date</th>
<th>Total Population in millions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>China</td>
<td>01.02.00</td>
<td>1,277.6</td>
</tr>
<tr>
<td>2.</td>
<td>India</td>
<td>01.03.00</td>
<td>1,027.0</td>
</tr>
<tr>
<td>3.</td>
<td>USA</td>
<td>April, 00</td>
<td>281.4</td>
</tr>
<tr>
<td>4.</td>
<td>Indonesia</td>
<td>01.07.00</td>
<td>212.1</td>
</tr>
<tr>
<td>5.</td>
<td>Brazil</td>
<td>01.07.00</td>
<td>170.1</td>
</tr>
<tr>
<td>6.</td>
<td>Pakistan</td>
<td>01.07.00</td>
<td>156.5</td>
</tr>
<tr>
<td>7.</td>
<td>Russia</td>
<td>01.07.00</td>
<td>146.9</td>
</tr>
<tr>
<td>8.</td>
<td>Bangladesh</td>
<td>01.07.00</td>
<td>129.2</td>
</tr>
<tr>
<td>9.</td>
<td>Japan</td>
<td>01.10.00</td>
<td>126.9</td>
</tr>
<tr>
<td>10.</td>
<td>Nigeria</td>
<td>01.02.00</td>
<td>111.5</td>
</tr>
</tbody>
</table>

It is now estimated that by 2050 India will most likely overtake China to become the most populous country in the earth with 17.2% growth at an annual rate of 1.4%. Chiona registered a much lower annual growth rate of population (1%) during 1990-2000, as compared to India (1.9% during 1991-2000).

**Indian population:**

India's population growth during the 20th century can be chartered and classified into 4 distinct phases as follows:

- **1901-1921**: stagnant population
- **1921-1951**: steady growth
- **1951-1981**: rapid high growth
- **1981-2001**: high growth with definite signs of slowing down
## Population Growth in India:

<table>
<thead>
<tr>
<th>Census Year</th>
<th>Population</th>
<th>Decadal Growth %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1901</td>
<td>238,396,327</td>
<td>-</td>
</tr>
<tr>
<td>1911</td>
<td>252,093,390</td>
<td>5.75</td>
</tr>
<tr>
<td>1921</td>
<td>251,321,213</td>
<td>-0.31</td>
</tr>
<tr>
<td>1931</td>
<td>278,977,238</td>
<td>11.00</td>
</tr>
<tr>
<td>1941</td>
<td>318,660,580</td>
<td>14.22</td>
</tr>
<tr>
<td>1951</td>
<td>361,088,090</td>
<td>13.31</td>
</tr>
<tr>
<td>1961</td>
<td>439,234,771</td>
<td>21.64</td>
</tr>
<tr>
<td>1971</td>
<td>548,159,652</td>
<td>24.80</td>
</tr>
<tr>
<td>1981</td>
<td>686,329,097</td>
<td>24.66</td>
</tr>
<tr>
<td>1991</td>
<td>843,387,888</td>
<td>23.86</td>
</tr>
<tr>
<td>2001</td>
<td>1,027,015,247</td>
<td>21.34</td>
</tr>
</tbody>
</table>

![Population growth in India](image)

**Fig 15:** Population growth in India
Almost half of the country's population lives in 5 states, namely Uttar Pradesh, Maharastra, Bihar, West Bengal and Andhra Pradesh. The population of Orissa was 36,706,920 in 2001 census with 18,612,340 males and 18,094,580 females. It was 3.57% of the total population of India. In Orissa, the decadal growth rate was decreased to 15.94% in 91-01 as compared to 20.06% in 81-91. In Khurdha district, the population was 1,874,405 with decadal growth rate of 24.79% and density of 666 per square km.

**Density of population in India:**

At the beginning of the 20th century, the density of India was as low as 77 and this steadily increased from 1931 to reach 324 in 2001.

<table>
<thead>
<tr>
<th>Census year</th>
<th>Density (per sq.km.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1901</td>
<td>77</td>
</tr>
<tr>
<td>1911</td>
<td>82</td>
</tr>
<tr>
<td>1921</td>
<td>81</td>
</tr>
<tr>
<td>1931</td>
<td>90</td>
</tr>
<tr>
<td>1941</td>
<td>103</td>
</tr>
<tr>
<td>1951</td>
<td>117</td>
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<tr>
<td>1961</td>
<td>142</td>
</tr>
<tr>
<td>1971</td>
<td>177</td>
</tr>
<tr>
<td>1981</td>
<td>216</td>
</tr>
<tr>
<td>1991</td>
<td>267</td>
</tr>
<tr>
<td>2001</td>
<td>324</td>
</tr>
</tbody>
</table>
Among major states, West Bengal (population density 904 in 2001) is the most populated. In Orissa, the population density per sq. km had increased to 236 in 2001 from 205 in 1991.

2.2.2. PROBLEM OF EXPANDING POPULATION

The problem of population expansion in India has led to problems in following areas-

a. **Food production**: The average calorie an Indian gets is only 1400 in place of 2400 despite of bumper crops.

b. **Clothing**: Against the per capita minimum of 25 meters per annum, the supply is only 14 meters. So there is scarcity of clothing.

c. **Employment**: In spite of creation of millions of additional jobs, the number of unemployed has gone up every year. It is over 25 million at present.

d. **Education**: There has been an overall expansion in education. But still millions of children are not attending schools resulting in additional social problems.

e. **Shelter**: With an ever increasing population, shelter and healthy houses are not available to everyone.

f. **Health**: Increase in population has caused an increased pollution of air, water and soil leading to an adverse effect on health.

All the above hazards produced by increasing population have to be tackled effectively. This can be achieved through family planning programmes by controlling fertility.
2.3 FERTILITY

It is the actual bearing of children. If a female is living in married union from menarche to menopause she can bear 14 children or can have 31 abortions or any combination of the two if she is not using any contraceptive anytime in her life.

In India the fertility level is higher as compared to the developed world.

2.3.1. FACTORS REGULATING FERTILITY:

The following are important factors which regulate fertility:

a) age of marriage:

The earlier is the age of marriage, the higher is the level of fertility. Early marriage is a long established custom in India.

b) duration of married life:

Maximum child birth occurs in the first 10-15 years of married life.

c) spacing of children:

Spacing between births reduces fertility rates.

d) Education:

Educated women give birth to less number of children as compared to illiterates. The best example of this inverse relationship is the state of Kerala where there is highest literacy rate & lowest fertility rate.

e) social and cultural factors:

The socio-cultural factors of importance which determine fertility are voice of the females, value of children, customs and beliefs, widow remarriage, desire for a male child, health conditions and housing.
f) **economic status:**
Fertility decreases with an increase in per capita income. Therefore economic development is considered to be the best contraceptive.

g) **religion and caste:**
Muslims show higher fertility rate than Hindus and Christians. Among Hindus, lower caste people have comparatively higher fertility than higher caste people.

h) **Nutrition:**
Well administered societies show lower fertility rate. All the poorly administered communities have higher fertility rates.

i) **family planning:**
It is a way of living and thinking that is adopted voluntarily, upon the basis of knowledge, attributes and responsible decisions by individuals, and couples in order to promote the health and welfare of the family group and thus contributes effectively to the social development of a country.

It is an important factor which can lower fertility. The term ‘Family welfare’ is now used in place of family planning. It signifies the improvement of the quality of health & welfare of the entire family.

2.4. **FERTILITY CONTROL**

Unlike animals, man alone can control the reproduction at will and plan his family. Man's great reproductive potential and the greatly increased survival rate in recent years have posed a major problem of population growth with far reaching consequences. If the world resources are considered, this forecasts a disaster for the human race, unless the fertility is controlled.
Fertility control or birth control is a regimen of one or more actions, devices, or medications followed in order to deliberately prevent or reduce the likelihood of a woman becoming pregnant or giving birth. Methods and intentions typically termed birth control may be considered a pivotal ingredient to family planning. Mechanisms which are intended to reduce the likelihood of the fertilization of an ovum by a sperm may more specifically be referred to as contraception.

Birth control may be accomplished using pharmacological methods such as oral contraceptives, medication-laden implants, and intrauterine devices etc. Nonpharmacological methods include surgical sterilization, mechanical devices, and the rhythm method.

2.4.1. HISTORY

Coitus interruptus (withdrawal of the penis from the vagina prior to ejaculation) probably predates any other form of birth control. Once the relationship between the emission of semen into the vagina and pregnancy was known or suspected, some men began to use this technique.

Arabs were using some intrauterine foreign body (small stones) to prevent conception in saddled camels.

1550 B.C.: In the Ebers Papyrus, the preparation of a tampon treated with a spermicide has been described.

1897 A.D.: Beard had postulated that the corpus luteum serves a necessary function during pregnancy.

1900: The hormonal nature of the ovarian control of the female reproductive system was established by Knauer and Halban.
1903: Fraenkel showed that destruction of the corpora lutea in pregnant rabbits causes abortion.

1909: Intra Uterine Device made of silkworm gut was used.

1923: Allen and Doisy devised a simple bioassay for ovarian extracts based upon the changes produced in the vaginal smear of rats.

1925: Loewe first reported a female sex hormone in the blood of various species.

1926: Loewe & Lange discovered a female sex hormone in the urine of menstruating women. They also observed that the concentration of that hormone varied with the phase of menstrual cycle.

1928: The excretion of large amount of estrogen in the urine during pregnancy was reported by Zondek.

1929: Estrogen was isolated in crystalline form by Butenandt and Doisy.

Corner & Allen established the hormonal function of Corpus luteum.

Gafenburgh, a Gynaecologist used an IUD made of silver

1930s: First physiological method was developed and termed as Rhythm method.

1950s & 60s: Sympto-thermal method was developed.

1955: First clinical demonstration of usefulness of estrogen-progestin combination for oral contraception by Pincus.

1956: The first report on the successful use of orally administered norethynodrel-mestranol combination for inhibition of ovulation.

1959: Ishihama of Japan & Oppenheimer of Israel introduced plastic IUDs.
1962: Jensen and Jacobsen suggested the presence of intracellular receptors for estrogen in the target tissues.

1986: A team of French Physicians reported a new approach to birth control using a drug called RU 486 (mifepristone).

2.4.2. AVAILABLE CONTRACEPTIVE METHODS

Fertility control is very essential for maintaining satisfactory standards of living and for raising the existing standards in developing countries. This can be done by using contraceptive methods. Methods of contraception can be permanent or temporary.\textsuperscript{4,5,13,15-16,23,25}

I. PERMANENT METHODS / STERILIZATION

Surgical sterilization is available in the form of tubal ligation for women and vasectomy for men

a) Male sterilization or vasectomy

b) Female sterilization or tubectomy

Vasectomy: An incision is made in the scrotum, the ducts of Vas deferens are located, each is tied in two places and the portion between the ties is removed. Although sperm production continues in the testis, sperm cannot reach exterior because the ducts are cut. The sperm degenerate and are destroyed by phagocytosis.

Advantages:

a. It is a relatively uncomplicated procedure and is done under local anaesthesia.

b. It has no effect on sexual desire and performance.
Disadvantages:

a. Sometimes an immune response to certain sperm specific proteins appears after vasectomy.

Tubectomy: The uterine tubes are tied, closed and then cut. The ovum is thus prevented from passing to the uterus, and the sperm cannot reach the ovum.

Advantages:

a. Complications are less if done properly.

b. It is a one time method

c. It does not require continued motivation

Laparoscopic sterilization: Another revolutionary technique is laparoscopic sterilization. A small incision is made in the abdominal wall (just 1 cm) and the instrument laparoscope is introduced into it. Uterus is inflated with air so that tubes are visible through the laparoscope. Small clips are applied on both the tubes for occlusion.

Advantages:

a. Short operating time

b. Needs no hospitalization

c. Results only in a small scar

Disadvantages:

a. Requires specially trained surgeon

b. Requires a laparoscope
II. TEMPORARY METHODS

A) Barrier methods: These methods are designed to prevent spermatozoa from gaining access to the uterine cavity and uterine tubes. Various barrier methods are:

i) Physical methods

   Ex: condom
       vaginal pouch/ female condom
       vaginal sponge
       diaphragm
       cervical cap

   These are available for both sexes. The mechanism of action is that they prevent the union of ovum with the sperm mechanically.

   Condom is a male contraceptive made from latex. It is fitted on the erect penis before intercourse. Vaginal pouch or female condom is made of two flexible rings connected by a polyurethane sheath. One ring lies inside the sheath and is inserted to fit over the cervix. The other ring remains outside the vagina and covers the female external genitals. The diaphragm is a rubber dome-shaped structure that fits over the cervix and stops the sperm from passing into the cervix. Variations of Diaphragm are available as cervical caps, vault cap and vimule cap. The cervical cap is thimble-shaped contraceptive device made of latex or plastic that measures about 4 cm (1.5 in.) in diameter. It fits snugly over the cervix of the uterus and is held in position by suction. Vaginal sponges are commonly available in the name of 'Today'. It is a sponge in a chemical
spermicide called nanoxynol-9. It is less effective than diaphragm. It is put into the vagina just before coitus.

Advantages:

a. They are practically free from side effects.
b. They can prevent sexually transmitted diseases like AIDS, Syphilis, gonorrhea etc. and cervical carcinoma.

Disadvantages:

a. Interferes with sex sensation locally
b. Care should be taken for proper or correct use.

ii) Chemical methods

They make use of chemical spermicides. They act as surface active agents. They get attached to the sperm and inhibit oxygen uptake resulting in the death of the sperms.

Ex: nonoxynol-9, octoxynol-9

They are used in the form of foams (foam tablets, foam aerosols), creams, pastes, jellies, suppositories, soluble films and douches. The diaphragm and cervical cap etc are also used with a spermicide.

Disadvantages:

a. High failure rate
b. Requires deep insertion into vagina.
c. Produces burning and irritation

iii) Combined method

Physical or chemical method alone can not be useful. They can be used in combination to reduce the failure rate.
B) Intra Uterine Devices (IUDs)

i) 1st generation: First generation IUDs are those which contain only plastic material
   e.g. lippe's loop

ii) 2nd generation: Second generation IUDs have metallic copper with plastic.
   e.g. copper T, copper 7
   Nova T
   Multi load devices

iii) 3rd generation: Third generation IUDs contain plastic with a hormone.
   e.g. Progestasert

Mechanism:
Earlier it was thought that they prevent the embedding of the fertilized ovum in the uterus but it is now opined that they cause certain cellular and biochemical changes in the uterus which are unfavorable to the gametes. The gametes cannot survive and fertilization is prevented. The metal copper enhances the uterine changes and also affects the motility of the sperms. Hormone releasing devices change the amount and consistency of the cervical mucous which makes the uterine conditions unsuitable for implantation.

Advantages:
   a. Insertion is simple
   b. Once inserted effective for few years
   c. Reversible, safe and effective
d. No systemic side effects

e. Does not require constant motivation or daily administration.

Disadvantages:

a. Less than 5% failure rate

b. Complications like bleeding and pain

C) Oral Hormonal Contraceptives

Hormonal contraceptives are most effective spacing methods of contraception. These hormonal contraceptives can be administered orally or by injection or as implant. But oral pills are more commonly used.

i) Steroidal oral contraceptives

Combined pill

Sequential pill

Progestogen only pill or minipill

Post coital pill

Once a month pill

ii) Nonsteroidal oral contraceptive

Male: gossypol, adzudin

Female: centchroman

iii) Nonsteroidal oral contraceptives with estrogenic activity

Diethyl stilbestrol

Bisphenol A

Genistein
**Combined Pill** contains oestrogen 30-35 µg and progesterone 0.5 to 1mg. The combination is given from the 5th day of menstruation for 21 days.

**Mechanism:**

The combined pill prevents ovulation by interfering the gonadotrophin secretions from the pituitary. The estrogen inhibits follicle stimulating hormone (FSH) release and therefore, follicle development; the progestogen inhibits luteinising hormone (LH) release and, therefore, ovulation, and makes cervical mucus inhospitable for sperm; together they render the endometrium unsuitable for implantation (Fig- 5).

**Advantages:**

a. Easy to use by oral route
b. Almost 100% effectiveness
c. Non contraceptive benefits like protection from breast disorders, ovarian cyst/cancer, iron deficiency anemia and pelvic inflammation.

**Disadvantages:**

a. Many cardiovascular and metabolic complications
b. May lead to carcinogenesis in breast and genitalia
c. Bleeding irregularities
d. Reduce breast milk production
e. Return to fertility may be delayed
f. Drawbacks like weight gain, nausea, mood changes and skin pigmentation.

The combination oral contraceptives occur in monophasic, biphasic, triphasic and estrophasic formulations. In the monophasic regimen, the daily doses of estrogen
and progestin remain constant throughout the menstrual cycle. The biphasic and triphasic combination pills contain various proportions of an estrogen and a progestin. This allows lower doses of both steroids in the early part of the cycle, reducing the total dose per cycle to less than that during conventional oral contraceptive therapy. However, they are more complex to use, offer little advantage over monophasic pills and are more expensive. In the esophagic regimen, the amount of progestin remains constant and the estrogen dose is gradually increased throughout the cycle.

**Sequential pills** consist of only estrogen for 14 to 16 days, then combination for 5 or 6 days and 7 or 8 off days. Sequential pills were removed from the United States market by the FDA because of concerns about their effectiveness relative to other preparations and because of reports of endometrial pathology associated with their use.

**Progestogen Only Pill (POP)** is also called micro or minipill and contains small amount of norethisterone or levonorgestrel. It is taken daily throughout the month. POP is useful in selected women to whom oestrogen is contraindicated. These pills are having high failure rate and limited use.

**Mechanism:**

Progestin-only contraceptives prevent ovulation in 70% to 80% of cycles largely by slowing the frequency of the GnRH pulse generator and blunting the LH surge. The micropills also change the quality of cervical mucus and inhibit the motility of fallopian tubes thereby preventing the union of sperm and ovum.
Post-coital pill is recommended within 48 hours of an unprotected intercourse. It contains either a high dose of estrogen or double the dose of standard combined pills.

Once-a-month pill contains a long acting estrogen in combination with a short acting progestogen. It is not popular since it has a high pregnancy rate and bleeding is irregular.

Gossypol: It is a polyphenolic compound obtained from the cotton plant. Given orally in male rats, gossypol acetic acid causes dose dependent damage to germinal cells of testes. Long term treatment damages spermatogonia leading to sterility.

Clinical studies carried out in China indicate that Gossypol is an effective male antifertility agent. It takes 2-3 months to achieve the desired therapeutic effect. However, it is not widely used since it is highly toxic and produces permanent azoospermia.

Centchroman: It is a selective estrogen receptor modulator (SERM). It causes ovulation to occur asynchronously with the formation of the uterine lining, preventing implantation of a zygote. It has been widely available as a birth control method in India since early 90s, marketed under the trade names of Centron and Saheli. It has been developed by the Central Drug Research Institute (CDRI), Lucknow. It is not legally available anywhere outside of India.

The drug appears to be well tolerated. The main side effect is prolongation of the menstrual cycle. It may cause enlargement of ovaries. It should be avoided in polycystic ovarian disease, renal and hepatic disorders, tuberculosis and lactating mothers. Its long term toxicity needs evaluation.
**Diethyl stilbestrol**: One of the first nonsteroidal estrogens to be synthesized was diethyl stilbestrol or DES. DES is as potent as estradiol in most assays. DES no longer has widespread use, but historically is important, because its introduction as a cheap, plentiful, orally active estrogen at a time when the natural products were scarce was a milestone in the development of effective endocrine therapy.

**D) Depot formulations (slow release):**

They include

i) **Injectables**

   e.g. depot medroxyprogesterone acetate (DMPA),

   norethisterone enanthate (NET-EN)

They are progestogens and require administration once every 3 months. The active compound is slowly released which inhibit ovulation, thickens cervical mucus and produce a thin atrophic endometrium.

ii) **Subdermal implants**

   e.g. norplant containing levonorgestrel

These capsules are implanted below the skin of forearm or upperarm. They provide effective contraception for 5 years. The contraceptive effect is reversible on removal of the capsules. However it may lead to irregularities of menstrual bleeding.

iii) **Vaginal rings containing levonorgestrel**

It is solely absorbed through the vaginal mucosa. It bypasses the digestive system and liver. So an effective concentration is available even with a lower dose.
E) Post conceptional methods / Termination of pregnancy

i) Menstrual regulation
It is a relatively simple method of birth control. It consists of aspiration of uterine contents within 1 to 2 weeks of a missed period but before tests can confirm pregnancy. Usually no aftercare is required.

ii) Menstrual induction
In this method prostaglandin F₂ is administered into the uterus. Uterine contractions start after a few minutes and uterus is spontaneously evacuated. Later bleeding starts and continues for 7 to 8 days.

iii) Abortion
Termination of pregnancy before the fetus becomes viable (upto 28 weeks) is called abortion. The use of abortion is a controversial issue, subject to ethical issue.

 Abortions can be done with surgical methods, usually suction-aspiration abortion (in the first trimester) or dilation and evacuation (in the second trimester).

F) Behavioural Methods

i) Abstinence/ Celibacy
It is refraining from sexual intercourse. It is an impractical method since it is very difficult to suppress a natural desire.

ii) Avoiding vaginal intercourse
The risk of pregnancy from non-vaginal sex is low, such as outercourse (sex without penetration), anal sex or oral sex. With this method, great discipline is required from both partners to prevent the progression into intercourse. This
method may be considered unreliable, due to the level of discipline required while in a passionate state.

iii) Coitus interruptus (withdrawal)
It means withdrawal of the penis before semen is ejaculated into the vagina. This method is not much effective due to its high failure rate.

G) Physiological methods / Natural family planning methods

i) Rhythm method (safe period)/ Statistical method/
   Standard Days method
This method is based on the principle that ovulation occurs 12 to 16 days before the menstruation. Roughly, 7 days before and 7 days after the menstrual bleeding are considered safe for coitus. This method has a high failure rate.

ii) Sympto-thermal method/ Fertility awareness method
It includes monitoring of basal body temperature and cervical mucus. The cervical mucus changes in quantity and consistency at the time of ovulation and the basal body temperature also rises by 0.5 °C. This knowledge can be applied in identifying the ovulation and to avoid coitus accordingly.

iii) Breast feeding / Lactation
Breast feeding can prevent pregnancy for 6 months in 50% females. Hence it is not reliable.

Of the contraceptive methods available, oral contraceptives have the largest spectrum of adverse effects- from nausea to menstrual abnormalities to rare thromboembolic disorders. The lowest mortality rate is seen with barrier methods, but oral contraceptives are relatively safe in non-smoking women with normal cardiovascular function.
2.4.3. GENERAL MECHANISM OF ACTION

Fertility can be controlled by using drugs which can act by the following mechanisms:

- Inhibiting ovulation
- Modifying the cervical mucus
- Interfering with the implantation
- Slowing down the rate of egg transport
- Preventing the ovum maturation and sperm capacitation
- By immunological methods
- Inhibiting spermatogenesis in males

The drugs which are currently in use act mainly by the first three mechanisms. However, the mechanism of combined pills may be summarized as follows:

- Estrogen inhibits secretion of FSH via negative feedback on the anterior pituitary and thus suppresses development of the ovarian follicle.
- Progestogen inhibits secretion of LH and thus prevents ovulation; it also makes the cervical mucus less suitable for the passage of sperm.
- Estrogen and progestogen act in concert to alter the endometrium in such a way as to discourage implantation.
- The combination synergistically decreases plasma gonadotrophin levels and suppresses ovulation more consistently than either alone.
- They may interfere with the coordinated contractions of cervix, uterus and fallopian tubes, which facilitate fertilization and implantation.
2.4.4. SHORTCOMINGS IN CURRENT DRUGS

The oral contraceptives are associated with following shortcomings which need to be checked.\textsuperscript{15-16,23-25}

- The estrogen component of oral contraceptives have been associated with-
  - Venous and arterial thromboembolism causing myocardial infarctions and strokes
  - Hypertension which increases with prolonged use and increasing age
  - Endometrial cancer

- Oral contraceptives containing progestins can elevate blood sugar levels

- Oral contraceptives may also cause gall bladder disease, acne, hirsutism, and hair loss.

- Oral contraceptives are contraindicated in women who have had disorders that are known to cause disease processes such as emboli and coronary diseases.

- Women with diabetes, those who are heavy smokers, and those with risk factors for cardiovascular disease are discouraged from using oral contraceptives.

- Oral contraceptives may reduce breast milk production and may produce bleeding irregularities.

- Estrogen and progestin may cause a sunburn-like reaction on exposure to sunlight or ultraviolet light.
Estrogens can cause genital abnormalities in male fetus and vaginal cancer in the female fetus, if used during pregnancy. Diethylstilbestrol and related compounds are contraindicated during pregnancy.

### 2.4.5. IDEAL ANTIFERTILITY DRUG

An ideal antifertility drug should possess following characteristics:

- It should be orally active.
- It should show reversible antifertility effect. Return to fertility should be there just after withdrawal of oral administration.
- It should show 100% efficacy. In other words, it should have 0% failure rate.
- It should possess non contraceptive benefits like protection from breast disorders, ovarian cyst/cancer, iron deficiency anemia and pelvic inflammation etc.
- It should be free from associated complications. It should have minimum adverse effect.
- It should not require daily administration. The frequency of administration should be as low as possible.
- It should be used in all human being whether suffering from any disease or not.
- It should not have any effect on milk production.
- It should not affect the menstrual cycle.
- It should have no effect on sexual desire and performance.
- It should have no effect on the fetus, if used by chance during pregnancy.
1. **Contraceptive patches:** Contraceptive patches for both men and women are expected to be approved by the FDA in the near future. Nesterone is the active drug in a patch being tested for use by women. It is thought that women will be more likely to follow the weekly administration of medication than to remember to take a pill daily.

2. **New implants:** Researchers are also pursuing new implants with fewer rods than Norplant. The long term (5 years) implantable contraceptive levonorgestrel (Norplant 2) is under development.

3. **Vaccines:** Vaccines for both the sexes are being searched.

   **Birth control vaccine:** Human chorionic gonadotrophin (hCG) has been used in preparing a vaccine for females. Antibodies develop within 4-6 weeks and reach to the peak after 5 months of administration and decline after 6 to 11 months. There are many uncertainties about such vaccine.

   The **male vaccine** has been shown to be 99% effective in suppressing sperm production. The vaccine requires weekly injections of testosterone at present, but scientists are looking at implants or longer acting injections for this use.

4. **Experimental contraceptives for males**

   Research is being done into a variety of substances that have potential as male oral contraceptives or implants or injections that may be used as male hormonal contraceptives.

   **RISUG (Reversible Inhibition of Sperm under Guidance),** is an injection into the vas deferens that coats the walls of the vas with a spermicidal
substance. This method can be reversed by washing out the vas deferens with a second injection. Vaso-occlusive contraception would be analogous to intrauterine contraception in women.

Heat-based contraception involves heating the testicles to a high temperature for a short period of time.

2.4.7. HERBAL OPTION TO CONTROL FERTILITY

There are a large number of indigenous plants, used as oral contraceptives especially by tribal people. Such plants are even recommended in folk medicines & ayurvedic medicines from very ancient times. Already several scientific papers have been published related to fertility control. Some of these are-8,27,34

<table>
<thead>
<tr>
<th>SI No</th>
<th>Name of the plant &amp; Family</th>
<th>Parts used</th>
<th>Pharmacological activity</th>
<th>Chemical constituents</th>
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<tbody>
<tr>
<td>1</td>
<td>Abelmoschus manihot Malvaceae</td>
<td>Whole plant</td>
<td>Anti-implantation activity</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Abrus precatorius Linn Papilionaceae</td>
<td>seeds</td>
<td>Antifertility activity in mice &amp; rats Oxytocic activity in vitro in guinea pigs</td>
<td>Abrine Abralin Hypaphorine Choline trigonelline Precatorine Carbohydrate Aminoacid</td>
</tr>
<tr>
<td></td>
<td>Plant Name</td>
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<td>Part</td>
<td>Activity</td>
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<tr>
<td>3</td>
<td><em>Abutilom persicum</em></td>
<td>Malvaceae</td>
<td>Aerial parts</td>
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</tr>
<tr>
<td>4</td>
<td><em>Acacia farnesiana</em></td>
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<tr>
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<td><em>Achyranthes aspera</em></td>
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<tr>
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<td></td>
<td></td>
<td>stem, bark</td>
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<tr>
<td>6</td>
<td><em>Actiniopteris radiate</em></td>
<td>Actinopteridaceae</td>
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<td><em>Adhatoda vasica</em> Nees</td>
<td>Acanthaceae</td>
<td>Whole plant</td>
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<tr>
<td>7</td>
<td><em>Achyranthes aspera</em></td>
<td>Amaranthaceae</td>
<td>Leaves</td>
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<td>Uterotonic activity in human myometrium strip</td>
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<td>Activity</td>
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<td>8</td>
<td>Aeschynomene indica Linn</td>
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<td>Spermicidal activity in human and rat semen</td>
<td>Vicenin-2, Reynoutrin, Rutin, Myricitrin, Robinin, Saponin</td>
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<tr>
<td></td>
<td>Papilionaceae</td>
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<td>9</td>
<td>Albizia lebbeck Linn</td>
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<td>Saponin, Lebbekanin-A,B,C,D, Echinocystic acid, β-sitosterol</td>
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<tr>
<td></td>
<td>Mimosaceae</td>
<td>Roots</td>
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<tr>
<td></td>
<td></td>
<td>Pods</td>
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<td>10</td>
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<td>Bulb</td>
<td>Ecbolic effect in mice and rats</td>
<td>Cycloallin, Propyl-sulphonic acid</td>
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<td></td>
<td>Liliaceae</td>
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<td>11</td>
<td>Allium sativum Linn</td>
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<td>Ecbolic effect in mice and rats</td>
<td>Scordinin A1, Saponin, Allicin, Allinase</td>
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<td>Liliaceae</td>
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<td>12</td>
<td>Aloe vera Linn</td>
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<td>Estrogenic activity in rats</td>
<td>Aloe-emodin, Isobarbaloin, Emodin</td>
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<tr>
<td></td>
<td>Liliaceae</td>
<td>Roots</td>
<td>Antiimplantation and antiovulatiry activity in rats and rabbits</td>
<td>Aloe-emodin, barbaloin</td>
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<tr>
<td>No.</td>
<td>Species</td>
<td>Part</td>
<td>Activity/Substance</td>
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<td>Aloe indica Royle</td>
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<td>Aloin, Isobarbaloin, barbalin, Aloe-emodin, β-barbaloin</td>
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<td>Alstonia scholaris</td>
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<tr>
<td>15</td>
<td>Anagallis arvensis Linn</td>
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<td>Uterine stimulant activity on isolated uterus of guinea pig</td>
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<td>Primulaceae</td>
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<td>Saponin, Cucurbitacin-B,D,E,I,L &amp;R, Arvenin-I,II,III &amp;IV</td>
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<td>Ananas comosus Linn Bromeliaceae</td>
<td>Unripe fruits, leaves, rhizomes</td>
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<td>Ergosterol peroxide, β-sitosterol, campesterol, campestanol, stigmastanol</td>
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<td>Anisomeles malabarica R.Br Lamiaceae</td>
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<td>Ovatodiolide, Anisomelolide, Diterpenes</td>
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<td>Anona squamosa</td>
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<td>Ardisia nerifolia Wall</td>
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<td>Areca catechu Linn</td>
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<td>Arecaein, Arecoline, Guvacoline, Guvacine, Saponins, Avenacines- A,B</td>
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<td>Aristolochia indica Linn</td>
<td>roots</td>
<td>Abortification, estrogenic and antiimplantation activity in mice</td>
<td>Aristolochine, Ishwarene, Ishwarone, Ishwarol, Isoaristolochic acid</td>
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<td>Artabotrys odorantissimus</td>
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<td>Azadirachta indica A. Juss</td>
<td>Barks leaves</td>
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<td>Nimbin, Nimbidin, Nimbosterol</td>
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<td>24</td>
<td>Bambusa burmanica</td>
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<td>Bombax malabaricum DC</td>
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<td>Gum, Arabinose, Galactose, Rhamnose</td>
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<td>26</td>
<td>Bridelia retusa Spreng</td>
<td>Bark</td>
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<td>Tannin</td>
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<td>Butea monosperma Kuntze</td>
<td>Leaves, flowers</td>
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<td>Fixed oil, Resin, Glycosides</td>
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<tr>
<td></td>
<td></td>
<td>seeds</td>
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<td>29</td>
<td>Caesalpinia bonducella</td>
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<td>Caesalpin</td>
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<td>(Flem)</td>
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<td>Antiestrogenic activity in mice and rabbits</td>
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<tr>
<td>30</td>
<td>Calamintha umbrosa</td>
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<td>(Fisch)</td>
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<td>Activity</td>
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<td>31</td>
<td><em>Calendula officinalis</em></td>
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<td>ß-carotene, lycopene, violaxathin, rubixanthin, hentriacontane, phytosterols</td>
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<td>32</td>
<td><em>Caltha palustris</em></td>
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<td>Saponin, Heueborin, Veratrin</td>
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<td><em>Canscora decussata</em></td>
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<td>Sitosterol, Stigmasterol, campesterol</td>
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<td><em>Capsella bursa-pastoris</em></td>
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<td>Impedes ovulation in mice</td>
<td>Bursin, Fatty oil, Flavonoid</td>
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<td><em>Carica papaya</em></td>
<td>Unripe fruit and seeds</td>
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<td>Papain, Phytofulene, 2-carotene, carbohydrate</td>
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<td>36</td>
<td><em>Cardiospermum Halicacabum</em></td>
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<td>Saponins, Glycosides, Flavonoids, Steroids</td>
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<td>Activity</td>
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<td>37</td>
<td>Cedrus deodara G.Don</td>
<td>stem</td>
<td>Antifertility activity in female rats</td>
<td>Flavonoids Oleoresin essential oil</td>
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<td>Celsianol Celsiosides A,B,C</td>
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<td>Centratherum anthelminticum Kuntze</td>
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<td>Fixed oil Stigmastadienol Stigmasterol Vernodalol</td>
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<td>41</td>
<td>Cicer arietinum Linn Papilionaceae</td>
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<td>Estrogenic activity in rats. Ecbolic property in mice and rats</td>
<td>Isoliquiritinigenin Daidzein Pratensein</td>
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<td>42</td>
<td>Cichorium intybus Linn Asteraceae</td>
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<td>Ammirin, Oxypeucedanin</td>
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<td>Apiaceae</td>
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<td><em>Piper aurantiacum</em> Wall.ex.hook.f</td>
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<td><em>Piper betle</em> Linn</td>
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<td><em>Pisum sativum</em> Linn</td>
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<td>Ecbolic property in mice and rats. Anti-implantation activity in rats.</td>
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<td><em>Pittosporum nilghirense</em> W&amp; A</td>
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<td>118</td>
<td><em>Pluchea lanceolata</em></td>
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<td>Plumbago indica Linn</td>
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<td>Plumbagin, 3-chloro Plumbagin</td>
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<td>fruits</td>
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<td>Uterine stimulant activity on isolated guineapig uterus</td>
<td>ß-sitosterol fulvoplumierin, plumericin</td>
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<td>Tannin, Essential oil, anthraquinones</td>
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<td>Psoralone, Isopsoralone, Monoterpene, Isoflavone</td>
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<td>Part</td>
<td>Activity</td>
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<td><em>Punica granatum</em>  Linn Punicaceae</td>
<td>Fruit skin</td>
<td>Antifertility activity in female rats and guineapigs</td>
<td>Iso-pelletierine Sitosterol Ursolic acid Punicalagin Punicalin</td>
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<td><em>Randia dumetorum</em> Poir Rubiaceae</td>
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<td>Sesbania sesban L. Marr</td>
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<td>antifertility activity in female mice &amp; rats abortifacient effect in mice</td>
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<td>143</td>
<td>Solanum xanthocarpum Schard &amp; Wendl</td>
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<td>144</td>
<td>Solidago virgaurea Linn</td>
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<td>No.</td>
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<td>Part</td>
<td>Activity/Effect</td>
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<td><em>Syzygium jambos</em> Myrtaceae</td>
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<td><em>Taxus baccata</em> Linn</td>
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<td>Anti-implantation activity in rats. Antiovulatory activity in rabbits</td>
<td>Flavonoid</td>
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<td><em>Terminalia bellerica</em> Roxb</td>
<td>fruits</td>
<td>Spermicidal activity in human &amp; rat semen</td>
<td>Cardiac glycoside: bellericanin</td>
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<td>Estrogenic activity in rats</td>
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<td>Triqonella foenum-graecum</td>
<td>seeds</td>
<td>Spermicidal activity in human, bovine &amp; rat semen. Ecbolic effect in mice and rats</td>
<td>Steroidal Saponin, Flavonoid-glycosides</td>
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<td>Uraria lagopodioides</td>
<td>Whole plant</td>
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<td>Verbera bonariensis</td>
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<td>155</td>
<td>Vitex negundo</td>
<td>Roots</td>
<td>Antiovulatory activity in rats</td>
<td>n-Trihexacosanol, n-hentriacontane, n-nonacosane, β-sitosterol</td>
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<td>156</td>
<td>Withania somnifera</td>
<td>Roots, Tuber</td>
<td>Delays the estrous or mating, Caused a decrease in litter size</td>
<td>Phytosterol, Steroid lactone, Nonsteroid lactone</td>
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<td>Woodfordia fruticosa</td>
<td>flowers</td>
<td>Abortifacient effect in mice</td>
<td>Diglucoside, Octacosanol, β-sitosterol</td>
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<td>158</td>
<td>Zingiber roseum</td>
<td>Whole plant</td>
<td>Abortifacient effect</td>
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</table>
2.5. PLANT PROFILE

2.5.1. ZIZYPHUS JUJUBA

It is a small deciduous tree with dense spreading crown, commonly 0.6m girth and 6m high. It is usually armed with sharp straight or hooked thorns, which are transformed stipules; thorns solitary or in pairs, usually one straight, the other curved.

SYSTEMATIC POSITION:

Division: Spermatophyta
Sub-division: Angiospermae
Class: Dicotyledonae
Subclass: Polypetalae
Series: Disciflorae
Order: Celastrales
Family: Rhamnaceae

DESCRIPTION:

Leaves: alternate, subdistichous, 3-5 ribbed.

Flowers: small greenish and yellowish, in axillary fascicles or in sessile or peduncled cymes. Calyx with broadly obconic tube and 5 triangular acute lobes keeled within, lobes valvate. Petals 5 or rarely 0, cucullate, deflexed. Stamens 5, opposite to and enclosed in the petals and usually longer than them. Disk 5-10 lobed, flat or pitted, the margin free. Ovary sunk in or adnate at the base to the disk, 2-4 celled; styles 2-3, rarely 4, free or connate; stigmas small papillose.
Fruit: a globose or oblong drupe, with a woody or bony 1-4 celled and seeded store.

Bark: blackish to grey or brown, rough, regularly and deeply furrowed.

SYNONYM:

Sanskrit: badari
English: jujuba fruit
Hindi: ber
Bengali: kul
Telegu: regu
Tamil: ilandai
Oriya: barakoli

DISTRIBUTION:

This plant is found wild and cultivated in many parts of India and Burma.

LOCAL USES:

Fruit: Stomachic
Mild laxative
Expectorant
Improve digestion (appetiser)
Purifies blood

Leaves: Anti diarrheal
Antipyretic
Anti obesity
Anthelmintic
Heal wounds and syphilitic ulcers

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Bark: Astringent
Antidirrheoeal
A domestic dressing to old wound and ulcers

Root: In fever and delirium
In gout and rheumatism
Purgative

Twigs: As an application to boils and abscesses.

Seed: Aphrodisiac
Cure eye diseases
Cough, asthma
In leucorrhoea

**PHARMACOLOGICAL ACTIVITY:**

**Anti-steroidalgenic activity:**

Gupta et al\(^7\) have evaluated anti-steroidalgenic activity of ethyl acetate extract of *Zizyphus jujuba* bark in adult female mouse. It reduced the wet weight of ovaries significantly and arrested the normal estrous cycle of adult female mouse at diestrous stage at a dose of 120 & 240 mg/kg body wt. It also inhibited the activity of both ovarian steroidealgenic enzymes i.e. glucose-6-phosphate dehydrogenase (G-6-PDH) and \(\Delta^5\)-32-hydroxysteroid dehydrogenase (\(\Delta^5\)-32-HSD) along with the elevation of cholesterol & ascorbic acid content in ovaries after 18 days of treatment. Normal estrous cycle & ovarian steroidogenesis were restored after withdrawal of treatment with this extract on average 32 days.
Miscellaneous actions:

1. Immuno-modulatory effects:
   a. *Zizyphus jujuba* is one of the components of CKBM, a Chinese herbal formulation. CKBM is capable of triggering the release of IL-6, TNF-alpha from human peripheral blood mononuclear cells. CKBM may exhibit its immuno-modulatory effects by regulating intracellular signaling as well as cytokine production in different lymphocytic cell types. 39
   b. Protojujuboside A and Jujuboside A, B & C were found to show potent immunological adjuvant activity. 40
   c. 3-O-cis-p-coumaroylmaslinic acid, 3-O-trans-p-coumaroylmaslinic acid, and Oleanolic acid exhibited significant anti-complement activity against the classical pathway of the complement system with IC$_{50}$ values of 101.4, 143.9 and 163.4 µM. This suggests that the oleanane-structure plays an important role in inhibiting the hemolytic activity of human serum against erythrocytes. 41

2. Inhibit the histamine release: Jujuboside A, C and acetyl jujuboside B were found to inhibit the histamine release from rat peritoneal exudate cells induced by antigen-antibody reaction. 42

TOXICITY STUDY:

1. The LD$_{50}$ of ethyl acetate extract of barks of *Zizyphus jujuba* determined by Litchfield & Wilcoxon was found to be 2.5 g/ kg body wt. in mice by i.p. administration7.

2. Lee et al have studied the cytotoxic effect of triterpenoic acids obtained from fruits of *Zizyphus jujuba* Mill against K562, B16(F-10), SK-MEL-2, PC-3, LOX-IMVI and A549 tumor cell lines by the sulforhodamin B (SRB) method. They
found that the coumaroyl moiety at the C-3 position of the lupine type triterpene may play an important role in enhancing cytotoxic activity. 43

CHEMICAL CONSTITUENTS:

Seeds of *Zizyphus jujuba*:

1. The seed of *Ziziphus jujuba* Mill. var spinosa (Bunge) contains 2 saponins and 9 fatty acids.44

The *saponins* are-

- Jujuboside A
- Jujuboside B

The *fatty acids* are-

- Lauric acid
- Palmitoleic acid
- Linoleic acid
- Myristic acid
- Stearic acid
- Arachidic acid
- Palmitic acid
- Oleic acid
- Docosanoic acid

2. Guil-Guerrero et al have found fatty acids and *carotenes* in some ber (Zizyphus jujuba Mill) varieties.45

3. Yoshikawa et al have isolated 3 new dammarane-type triterpene oligoglycosides together with 3 known saponins from the seeds of *Zizyphus jujuba* Mill var spinosa.42 The 3 new *dammarane-type triterpene oligoglycosides* are-

- Jujuboside A₁
- Jujuboside C
- Acetyl jujuboside B₁

4. Liu et al have isolated 5 compounds from the seeds of *Zizyphus jujuba* Mill var spinosa.46 They are-
a. Jujuboside D

b. Jujuboside A

c. 5,7,4'-trihydroxy flavonol-3-O-beta-D-rhamno-pyranosyl-(1-6)-Beta-D-glucopyranoside

d. 6''''-coumaroylspinosin

e. Phenylalanine

Compound (a) was a new compound, (d) was reported as rotamer for the first time and (c) and (e) were isolated for the first time from the plant.

5. Bai et al have isolated 7 compounds from the seeds of *Zizyphus jujuba* Mill var spinosa. 47

   a. Jujuboside E
   b. Jujuboside B
   c. Jujuboside A
   d. Betulic acid
   e. Stearic acid
   f. Sucrose
   g. inosine

Compound (a) was a new compound, and (e), (f) and (g) were isolated for the first time from the plant.

6. Matsuda et al have isolated novel protojujubogenin type triterpene bisdesmosides from the seeds of *Zizyphus jujuba* Mill var spinosa. 40 They are-

   Protojujuboside A
   Protojujuboside B
   Protojujuboside B₁
Barks of *Zizyphus jujuba*:

1. The dried bark powder of *Zizyphus jujuba* was extracted with 80% methanol in a soxhlet extractor. This solution was filtered & dried under vacuum. The solid mass obtained was partitioned with water and successively extracted with chloroform, ethyl acetate & methanol. The solvent was completely removed under reduced pressure and a semisolid mass was obtained. The yields of chloroform, ethylacetate and methanol extract of *Zizyphus jujuba* were 6.9, 5.9 & 6.7% respectively.  

The chemical constituents of ethylacetate extract of *Zizyphus jujuba* were identified by qualitative analysis & confirmed by Thin Layer Chromatography. This revealed presence of tannins, flavonoids, and phenolic compounds.

Fruits of *Zizyphus jujuba*:

1. Lee et al have isolated 11 triterpenoids\(^4,43\) from the fruits of *Zizyphus jujuba* Mill:

   1. Ceanothane type triterpenes:
      a. Colubrinic acid
      b. Zizyberenalic acid

   2. Lupane type triterpenes:
      a. Alphitolic acid
      b. 3-O-cis-p-coumaroylalphitolic acid
      c. 3-O-trans-p-coumaroylalphitolic acid
      d. Betunic acid
      e. Betu'onic acid
3. Oleanane type triterpenes:
   a  3-O-cis-p-coumaroylmaslinic acid
   b  3-O-trans-p-coumaroylmaslinic acid
   c  Oleanolic acid
   d  Oleanonic acid

2.5.2. **STEPHANIA HERNANDIFOLIA**

It is a slender climber, 2-10 m long.

**SYSTEMATIC POSITION:**

Division: Spermatophyta

Sub-division: Angiospermae

Class: Dicotyledonae

Subclass: Polypetalae

Series: Thalmiflorae

Order: Ranales

Family: Menispermaceae

**DESCRIPTION:**

Leaves: triangular-ovate or ovate, peltate. Petioles 3-6.5 cm long

Flowers: both male and female flowers green, white or yellow, in axillary, compound, umbelliform cymes.

Fruit: red, obovate to suborbicular

Roots: tuberous.
SYNONYM:
Sanskrit: ambastha, patha, vanatikita
English: tape-vine
Bengali: akanadi
Oriya: musakani, ckanobhindi

DISTRIBUTION:
It is found in some parts of the north, east and south India.

LOCAL USES:
Root: In fever and stomachache
In urinary diseases
In diarrhea
As astringent

Leaves: leaf paste is applied to infections of the breast,
Boils and septic inflammations
As depilatory

The roots are used as a substitute for the roots of Cissampelos pareira in many ayurvedic drugs.

PHARMACOLOGICAL ACTIVITY:

a. Spermicidal activity:

Paul et al. have determined the contraceptive spermicidal activity of a composite extract of Acharyranthes aspera and Stephania hernandifolia on human semen. They have taken 50% ethanolic extract of the leaf of Stephnia
hernandifolia & the root of Achyranthes aspera in a ratio of 1:3 by weight at different concentrations & studied the sperm motility and function.

Conc. of 0.8 g/ml of the extract affected the motility and at a conc. of 0.16 g/ml the sperm motility was reduced to 20% immediately (within 20 sec). At a conc. of 0.32g/ml, this composite extract showed the most promising results by complete sperm immobilization within 2min after the application of the extract. The effects were spermicidal but not sperm static as sperm immobilization effect was found to be irreversible. Sperm viability was decreased significantly and was found to be nonviable after 30min. When treated with the composite extract at a conc. of 0.32 g/ml. The hypo-osmotic swelling of these sperm was reduced significantly at this highest conc. indicating that the crude extract may probably cause injury to the sperm plasma membrane. A low conc. of 0.04 g/ml is ineffective.

The findings indicated that this composite plant extract possesses potential contraceptive spermicidal activity in vitro.

b. Effect on testicular gametogenesis:

1. The testicular inhibitory effect of the aqueous fraction of methanolic extract of Stephania hernandifolia leaf was studied in male wistar rats by Jana et al.12

The supernatant and precipitate part of of aqueous fractions of the methanol extract of the leaf was gavaged separately to rats at a similar dose of 200 mg/ml per 100g body wt per day for 28 days.

In both treated groups, there was significant decrease in the relative weights of the sex organs, the testicular key androgenic enzyme
activities, the plasma level of testosterone, the no. of different germ cells at stage VII of seminiferous epithelial cell cycle and the seminiferous tubular diameter in comparision to the controls. Neither of the parts had somatic, renal and hepatic toxicity.

The aqueous fraction of methanolic extract of *Stephania hernandifolia* leaf possesses certain testis-inhibitory substances, which may be steroid like agents.

2. The dose dependent effects of aqueous extract of *Stephania hernandifolia* leaf on testicular activities in albino rats was studied by Ghosh et al.

Adult male wistar rats, maintained under standard laboratory conditions, were forcefully administered with the aqueous extract of these leaves at the dose of 2g or 4g of leaves/ ml distilled water/ 100g body weight per day for 28 days. All the animals alongwith vehicle treated controls were killed on the day 29 of the experiment. Treatment with this leaf extract at both doses resulted in significant reduction in relative weight in the testis, the seminal vesicles, the prostate, the epididymis without any significant change in the liver and kidney weight in comparison to control.

Activities of testicular steroidogenic key enzymes and plasma testosterone level were decreased significantly alongwith a significant reduction in the no. of germ cells at the stage VII of the spermatogenic cycle and in the seminiferous tubular diameter in both treated groups in comparision to control. Activities of glutamate oxaloacetate transaminase, glutamate pyruvate
transaminase, acid phosphatase, alkaline phosphatase were not altered significantly in the liver and kidney in both treated groups compared with control.

So they concluded that treatment with an aqueous extract of leaves resulted in diminution in the activities of testicular androgenic key enzymes and plasma level of testosterone along with inhibition of spermatogenesis without any induction of hepatic & renal toxicity.

c. Post-coital interceptive activity:

Mukherjee et al have evaluated post-coital pregnancy interceptive activity of aqueous extract of leaves of *Stephania hernandifolia* and an ethnomedicinal formulation of tape-vine leaves (EF) in female wistar rats. The aqueous extract at these dose levels have not interfered in pregnancy but significant anti-implantation property was observed at 1g/kg & 2g/kg.

EF at 500 & 250mg/kg doses induced 66.7% and 33.3% post coital pregnancy interception respectively and the higher dose exhibited significant reduction in number of litters born & also anti-implantation property.

EF probably acts by restriction of implantation by alteration of gonadal hormone levels (significant increase in serum estradiol, LH & FSH but decrease in progesterone level) & decline in blood glucose levels that possibly disrupts oxidative energy metabolism in Uterus during implantation. This preparation also seems to be free from cardiovascular risk factors.

d. Effect on ovarian gametogenesis:

Maiti et al have reported that the leaf extract of *S. hernandifolia* inhibited ovarian gametogenesis.
e. **fertility promoting activity:**

The petroleum ether extract of the rhizome is reported to exhibit fertility promoting activity, whereas its alcoholic extract showed antifertility activity.\textsuperscript{49}

f. **Miscellaneous activity\textsuperscript{49}**:

- **Antispasmodic activity:** The alkaloid aknadine is reported to possess significant antispasmodic activity on uterine spasms brought about by the posterior pituitary lobe extract.

- **Cytotoxic activity:** d- and dl-tetrandrine, fangchinoline and d-isochondrodendrine are reported to exhibit significant cytotoxicity against human carcinoma of the nasopharynx carried in tissue culture, and d- and dl-tetrandrine against Walker-256 intramuscular carcinoma in rats.

- d-tetrandrine has a marked irritating action on the mucous membrane of the rabbit, and it acts slightly as an antipyretic. It causes vomiting in pigeons, and depresses cardiac activity and lowers blood-pressure in frogs and cats. It depresses the movements of the smooth muscles of the intestines and uterus of guineapigs and rabbits.

**TOXICITY STUDY:**

1. Both supernatant and precipitate part of the aqueous fraction of methanol extract of the leaves of *Stephania hernandifolia* does not show somatic, renal and hepatic impairment after administration for 28 days at a dose of 200 mg/ kg body wt. per day.\textsuperscript{12}

2. The aqueous extract of the leaves at the dose of 2g/4g of leaves/ kg body wt. for 28 days does not show any hepatic and renal toxicity.\textsuperscript{52}
CHEMICAL CONSTITUENTS:

Whole plant:

*Stephania hernandifolia* contains alkaloids. In addition, steroids and fats are also reported to be present in the plant.

Roots and rhizomes:

The alkaloids are concentrated mostly in the rhizomes and roots.

- Stepholidine
- Sinoacutine
- Isocorydine
- L-tetrahydropalmatine
- Crebanine
- Fanchinoline
- Tetrandrine
- Alkadinine
- Epistephanine
- 4-demethylhasbuanonine
- Hypoepistephanine, C₃₆H₃₆N₂O₆ (m.p. 257°C)

Ray et al had isolated the alkaloid epistephanine. Kupchan et al had isolated and structurally elucidated a new alkaloid 4-demethylhasbuanonine from this plant.

Leaf:

In an investigation, the leaves are reported to contain the alkaloids.
The active molecules present in the aqueous fraction of methanol extract of *Stephania hemandifolia* leaves might be steroids as indicated by thin layer chromatography using specific staining substance for steroid molecules.\textsuperscript{12}

\textbf{2.5.3. CISSAMPELOS PAREIRA}\textsuperscript{50,57-58}

It is a climbing shrub.

**SYSTEMATIC POSITION:**

Division: Spermatophyta  
Sub-division: Angiospermae  
Class: Dicotyledonae  
Subclass: Polypetalae  
Series: Thalmiflorae  
Order: Ranales  
Family: Menispermaceae

**DESCRIPTION:**

Leaves: peltate. 3.8 to 10 cm in diameter, orbicular or reniform, truncate at the base, margins ciliate, petiole 3.8 to 10 cm long
Flowers: minute, yellowish
male flowers cymose. Sepals 4, petals 4, connate into a sub entire short cup. Anthers 2-4, sessile, connate, encircling the top of the peltate staminal column, bursting transversely.

Female flowers racemose, crowded in the axils of roundish, leafy bracts. Sepals and petals 1 of each, 2-nerved, adnate to the bracts. Staminoids 0. ovary 1; style short, 3-fid or 3-toothed.

Drupe: sublobose, endocarp compressed, hairy, red, dorsally tubercled, the sides excavated.
Seed horseshoe-shaped; embryo linear; cotyledons appressed.

Bark: blackish to grey or brown, rough, regularly and deeply furrowed.

SYNONYM:

Sanskrit: venivel; laghu patha
English: velvet leaf
Hindi: akanadi; harjori
Bengali: akanadi
Telugu: pata
Tamil: appatta
Oriya: patha, okanobindhi

DISTRIBUTION:

It is found in tropical and subtropical India from Sind and the Punjab to South India and Ceylon.
LOCAL USES:

Whole plant: astringent

Antidiarrhoeal

In indigestion, dyspepsia

In nephritis

Root: as a Diuretic in acute and chronic cystitis

Antiseptic of the urinary bladder

In later stages of bowel complaints

In unhealthy sores, sinuses and itches for its cooling quality

Externally in snake-bites and scorpion sting

A valuable stomachic

Leaves: In unhealthy sores, sinuses and itches

CHEMICAL CONSTITUENTS:

*Cissampelos pareira* contains following alkaloids.

- Cissampeline or pelosine
- Seperine
- Bebeerines
Fig. 16:  *Zizyphus jujuba* Lamk.  

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Fig. 17: *Stephania hernandifolia* Walp.⁶⁰
Fig. 18: *Cissampelos pareira* Linn.\textsuperscript{61}
2.6. HERBAL DRUG EVALUATION

Evaluation of a drug means confirmation of its identity and determination of its quality and purity and detection of nature of adulteration. The evaluation of a crude drug is necessary because of 3 main reasons.

- Biochemical variation in the drug
- Deterioration due to treatment and storage
- Substitution and adulteration, as a result of carelessness, ignorance or fraud

Over the years the nature and degree of evaluation of crude drugs have undergone a systematic change. Initially, the crude drugs were identified by comparison only with the standard description available. Due to advancement in the chemical knowledge of crude drugs, at present, evaluation also includes method of estimating active constituents present in the crude drug, in addition to its morphological and microscopic analysis. With the advent of separation techniques and instrumental analysis, it is possible to perform physical evaluation of crude drug, which could be both qualitative and quantitative in nature. The biologic behavior of crude drug extracts constitutes pharmacologic evaluation.

The crude drugs can be identified on the basis of their morphological, histological, chemical, physical and biological studies.

2.6.1. MORPHOLOGICAL EVALUATION:

Morphological or organoleptic evaluation refers to evaluation of drugs by color, odour, taste, size, shape and special features, like touch, texture etc. It is a
technique of qualitative evaluation based on the study of morphological and sensory profiles of whole drugs.

2.6.2. MICROSCOPIC EVALUATION:

This method allows more detailed examination of a drug and it can be used to identify the organized drugs by their known histological characteristics. It is mostly used for qualitative evaluation of organized crude drugs in entire and powdered form.

Other important histological aspect is the quantitative microscopy and linear measurements. The various parameters studied here are stomatal number and index, palisade ratio, veir-islet number, size of starch grains, length of fibres, etc.

2.6.3. CHEMICAL EVALUATION:

It comprises of different chemical tests and chemical assays. The isolation, purification and identification of active constituents are chemical methods of evaluation. Quantitative chemical tests such as acid value, saponification value, etc., are also covered under this technique. Some of these test are useful in evaluation of resins (sulphated ash, acid value); balsams (acid, saponification and ester values); volatile oils (acetyl and ester values); and gums (methoxy determination and volatile acidity).

The purity of crude drugs is ascertained by quantitative estimation of active chemical constituents present in them. The method may be useful in determining single active constituent or the group of related constituents present in the same drug.
The chemical evaluation also covers phytochemical screening carried out for establishing chemical profile of a crude drug.

**PHYTOCHEMICAL INVESTIGATIONS:**

The systematic investigations of plant material for its phytochemical behaviour involve 4 different stages.

1. The procurement of raw material and quality control.
2. Extraction, purification and characterisation of the constituents of pharmaceutical interest and in-process quality control
3. Investigations of biosynthetic pathways to particular compounds and
4. Quantitative evaluation

The commonly employed technique for separation of active substance from crude drug is called 'Extraction' which involves the use of different solvents. The plant material for extraction depends on its nature and the components required to be isolated. The dried powdered plant material is commonly used for extraction. The fresh plant parts when used are homogenized or macerated with a solvent such as alcohol which is a general solvent for many potential constituents and as such may give problem in subsequent elimination of pigments, resins, etc. Water immiscible solvent, such as light petroleum ether is used for the extraction of fixed and essential oils, steroids and aglycones. Chloroform and ether are used for separation of alkaloids and quinines. The extraction of organic bases like alkaloids usually necessitates basification of plant material if a water immiscible solvent is to be used, whereas for aromatic acids and phenols, acidification may be required. The glycosides are soluble in water and alcohol, but are insoluble in non-polar solvents. Tannins are phenolic matter.
soluble in water, alcohol and ethyl acetate. Extraction itself may be performed by repeated maceration with agitation, percolation or by continuous extraction using Soxhlet extractor.

**Preliminary Phytochemical Screening:**

The plant is a biosynthetic laboratory, not only for chemical compounds such as carbohydrates, proteins and lipids that are utilized as food by man, but also for a multitude of compounds like glycosides, alkaloids, volatile oils, tannins, etc., that exert a physiological and therapeutic effect. The compounds that are responsible for medicinal property of the drug are usually secondary metabolites. A systematic study of a crude drug embraces, through consideration of primary and secondary metabolites derived as a result of plant metabolism. The plant material is subjected to preliminary phytochemical screening for the detection of various plant constituents on following lines.

**Successive solvent extraction:** The air-dried plant material is extracted in Soxhlet assembly successively with petroleum ether, benzene, solvent ether, chloroform, acetone, ethanol and methanol. Finally, the drug is macerated with chloroform water. Each time before extracting with the next solvent, the powdered material is dried in hot-air oven at a temperature below 50°C. Each extract is concentrated by distilling off the solvent and then evaporating to dryness on water bath. The extract obtained with each solvent is weighed. Its percentage is calculated in terms of air-dried weight of plant material. The color and consistency of the extract are noted.

The extracts with different solvents can also be prepared by successively macerating (cold extraction) the powdered drug in order of increasing polarity.
It is quite obvious that the extract of phytoconstituents prepared by maceration or percolation method must be as pure as possible and unless it is reasonably so, the test reaction may no: be accurate. Therefore, some purification procedures are usually adopted prior to characterization of individual components. The extract may contain, along with actually desired compound, some other substances such as chlorophyll or other kinds of pigments, inorganic and organic acids, resins, fatty substances etc. Depending upon the type of impurities present the method of purification varies, but, by and large, separation of constituents by partitioning between two immiscible solvents in which the compound dissolves preferentially or precipitation of either the desired medicinal product or impurity by a certain reagent, are quite widely used. Such partially purified extract may still contain very closely related constituents in traces. This necessitates further purification of extract, which is done by various means such as sublimation, distillation, fractional crystallization, fractional liberation etc. However, the most convenient modern technique for purification is chromatography.

Sublimation is possible for purification of materials present in a crude extract. Fractional distillation has been traditionally used for separation of components of volatile oils. Steam distillation is used for extraction of volatile oils and hydrocyanic acid from plant material. Some types of compounds lend themselves to fractional liberation from a mixture of constituents. For example, a mixture of alkaloidal salts in aqueous solution, when treated with aliquots of alkali, gives first the weakest base in the free state followed by base liberation in ascending order of basicity. If the mixture is shaken with an organic solvent after addition of aliquot of alkali, a fractionated series of bases shall be obtained. The technique
of fractional crystallization exploits the differential solubility of components of a mixture in a particular solvent.

**Qualitative chemical examination:**

The extracts obtained as above are then subjected to qualitative tests for the identification of various plant constituents. In addition, 50 g of air-dried or fresh plant material is also subjected to hydrodistillation to detect the presence of volatile oil.

**Chemical constituents**

<table>
<thead>
<tr>
<th>Organic</th>
<th>Inorganic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>calcium</td>
</tr>
<tr>
<td>Proteins, aminoacids</td>
<td>magnesium</td>
</tr>
<tr>
<td>Fats and oils</td>
<td>sodium</td>
</tr>
<tr>
<td>Steroids</td>
<td>potassium</td>
</tr>
<tr>
<td>Volatile oils</td>
<td>iron</td>
</tr>
<tr>
<td>Glycosides</td>
<td>sulphate</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>phosphate</td>
</tr>
<tr>
<td>Tannins</td>
<td>chloride</td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td></td>
</tr>
<tr>
<td>Saponins</td>
<td></td>
</tr>
<tr>
<td>Oxygenic acids</td>
<td></td>
</tr>
<tr>
<td>Enzymes</td>
<td></td>
</tr>
</tbody>
</table>
Chemical tests for organic compounds are performed on the extracts obtained using nonpolar and polar solvents like petroleum ether, ether, benzene, chloroform, acetone, alcohol, water etc.

Chemical tests for inorganic compounds are performed on the ash.

Test for carbohydrates:

a. Molisch's test:

Extracts required: aqueous, alcoholic

Small quantities of extract is dissolved in 4 ml of distilled water and filtered. Then the filtrate is treated with alpha-naphthol and conc. sulphuric acid.

With a soluble carbohydrate this appears as a ring if sulphuric acid is gently poured to form a layer below the aqueous solution. With insoluble carbohydrate (cellulose) the color will not appear until the acid layer is shaken to bring it in contact with the material.

<table>
<thead>
<tr>
<th>Sugar</th>
</tr>
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<tbody>
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</table>

Reducing sugar  |  non reducing sugar |
-----------------|---------------------|
All monosaccharides | some disaccharides |
Many disaccharides  | sucrose |
Lactose            | trehalose |
Maltose            | polysaccharides |
Cellobiose         |           |
Gentiobiose        |           |
Tests for reducing sugars:

a. Fehling's test:
1ml Fehling's A & 1ml Fehling's B solutions are mixed for 1 minute. Equal volume of test solution is added and heated in boiling water bath for 5-10 minutes. First a yellow, then a brick red precipitate is observed for a reducing sugar.

b. Benedict's test:
Equal volume of Benedict's reagent and test solution are added in a test tube and heated in boiling water bath for 5 minutes.
Solution appears green, yellow or red depending on amount of reducing sugar present in test solution.

Test for monosaccharides:

a. Barfoed's test:
Equal volume of Barfoed's reagent and test solution are mixed and heated for 1-2 minutes in boiling water bath and cooled
A red precipitate is observed for monosaccharides.

Tests for nonreducing sugars:
Test solutions do not give response to Fehling's and Benedict's tests.

Test for gums:
Extracts required: aqueous
The test solution is hydrolysed using dilute HCl. Then Fehling's and Benedict's test are performed.
Red colour is developed for gums

Test for mucilage:
Extracts required: aqueous
About 10 ml of aqueous extract is added slowly to 25 ml of absolute alcohol with
constant stirring. The precipitate is filtered and dried in air. The precipitate is
examined for its swelling property and for the presence of carbohydrate.
Powdered drug material swells in water or aqueous KOH.
Powdered drug material also shows red color with ruthenium red.

**Test for glycospdes:**

The free sugar content of the extract is determined. Then the extract is hydrolyzed
with mineral acid (dil. HCl/ dil H₂SO₄). Again the total sugar content of the
hydrolyzed extract is determined.

Increase in sugar content indicates presence of glycoside in the extract.

**Test for cardiac glycospdes:**

a. **Bäljet's test:**

To a thick section of the leaf, sodium picrate solution is added.

It shows yellow to orange color for cardiac glycosides.

b. **Legal test:**

Extracts required: aqueous, alcoholic

To the extract, 1ml pyridine and 1ml sodium nitroprusside is added.

Pink to red color appears for cardiac glycosides.

c. **Keller-Kiliani test for deoxy sugars:**

To 2ml extract, glacial acetic acid, one drop of 5% FeCl₃ and conc. H₂SO₄ are
added.

At the junction of the liquids a reddish-brown color is produced which generally
becomes blue for cardiac glycosides.
Test for anthraquinone glycosides:

a. Borntrager's test:
To 3 ml extract, dil H₂SO₄ is added, boiled and filtered. To the cold filtrate, equal volume of benzene or chloroform is added and shaken. The organic solvent is separated and ammonia is added. Ammoniacal layer turns pink or red.

Test for saponin glycosides:
Extracts required: aqueous, alcoholic

a. Foam test:
1 ml of the extract is diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes.
A 1 cm layer of foam indicates the presence of saponins.

b. Hemolytic test:
The drug extract or dry powder is added to 1 drop of blood placed on glass slide. Hemolytic zone appears.

Test for flavonoids:

a. Shinoda test:
To dry powder or extract, 5 ml 95% ethanol, few drops of conc. HCl and 0.5 g magnesium turnings are added. Pink color is observed for presence of flavonoids.
To small quantity of residue, lead acetate solution is added. Yellow color precipitate is formed for presence of flavonoids. Addition of increasing amount of NaOH to the residue shows yellow coloration, which decolorizes after addition of acid.
Test for Phenolic compounds and tannins:

Extracts required: aqueous, alcoholic

Small quantity of the extract is taken in water and

a. 5% FeCl₃ solution is added.
   Deep blue-black color appears for phenolic compounds and tannins.

b. 1% gelatin solution containing 10% NaCl is added.
   White precipitate occurs for phenolic compounds and tannins.

c. 10% lead acetate solution is added
   White precipitate occurs for phenolic compounds and tannins.

d. Aqueous bromine solution is added
   Decoloration of bromine water occurs for phenolic compounds and tannins.

e. Acetic acid solution is added
   Red color solution occurs for phenolic compounds and tannins.

f. Dil. Potassium permanganate solution is added
   Decoloration occurs for phenolic compounds and tannins.

Test for Proteins:

Extracts required: aqueous, alcoholic

Small quantities of the extract is dissolved in water and subjected to following tests.

a. Biuret test:
   4% NaOH and few drops of 1% CuSO₄ solution is added to the test solution.
   Violet or pink color appears for presence of proteins.

b. Millon's test:
   3 ml test solution is mixed with 5 ml Millon's reagent.
White precipitate appears. Warm precipitate turns brick red or the precipitate dissolves giving red color solution.

Test for amino acids:

a. Ninhydrin test:

3 ml test solution and 3 drops of 5% ninhydrin solution are mixed and heated in boiling water bath for 10 minutes.

Purple or bluish color appears for presence of amino acids.

Test for alkaloids:

Extracts required: aqueous, alcoholic, chloroform

The extract is evaporated and to the residue dil. HCl is added, shaken well and filtered. The filtrate is tested carefully with various alkaloidal reagents-

a. Mayer's reagent: cream precipitate
b. Dragendorff's reagent: orange brown precipitate
c. Hager's reagent: yellow precipitate
d. Wagner's reagent: reddish brown precipitate

Test for steroids/ phytosterols:

Extracts required: petroleum ether, acetone, alcoholic

The extract is refluxed with solution of alcoholic KOH till complete saponification takes place. The saponification mixture is diluted with distilled water and extracted with ether. The ethereal extract is evaporated and the unsaponifiable residue is subjected to following tests-

a. Libermann's test:

3 ml extract is mixed with 3 ml acetic anhydride, heated and cooled. Few drops of conc. H₂SO₄ are added.
Blue color appears for the presence of steroids.

b. **Libbermann-Burchard reaction:**

2 ml extract is mixed with chloroform. 1-2 ml of acetic anhydride and 2 drops of conc. $\text{H}_2\text{SO}_4$ are added from the side of the test tube.

First red, then blue and finally green color appears for the presence of steroids.

c. **Salkowski reaction**

To 2 ml of the extract, 2 ml of chloroform and 2 ml of conc. $\text{H}_2\text{SO}_4$ are added and shaken well.

Chloroform layer appears red and acid layer shows greenish yellow fluorescence.

**Test for fixed oils and fats:**

Extracts required: petroleum ether, benzene

a. A small quantity of extract is pressed between two filter papers.

Oil stains on the paper indicate the presence of fixed oil.

b. A few drops of 0.5 N alcoholic KOH is added to a small quantity of the extract along with a drop of phenolphthalein. The mixture is heated on a water bath for 1-2 hrs.

Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

**Test for volatile oil:**

About 50 g of powdered material is taken in a Clavenger's apparatus and subjected to hydrodistillation. The volatile oil is detected by following tests-

a. volatile oils have characteristic odour

b. filter paper is not permanently stained with volatile oil

c. volatile oils are soluble in 90% alcohol.
Chromatography:

Among the various methods of separating plant constituents, the chromatographic procedure originated by Tswett is one of the most commonly used techniques of general application.

Chromatography represents a group of methods for separating molecular mixtures that depend on the differential affinities of the solute between two immiscible phases. One of the phases is a fixed bed of large surface area which is called a stationary phase, while the other is a fluid or gas which moves through or over the surface, which is called the mobile phase. The stationary phase may be a porous or finely divided solid or liquid that has been coated as thin layer on an inert support material. The mobile phase may be a pure liquid or a mixture of liquids or it may be a gas or mixture of gases.

The different chromatographic methods used are:

1. Thin Layer Chromatography
2. High Performance Thin Layer Chromatography
3. Gas Liquid Chromatography
4. High Performance Liquid Chromatography
5. Column Chromatography
6. Gel Permeation Chromatography
7. Affinity Chromatography
8. Paper Chromatography

For the separation of some bio-constituents, it is necessary to use a two dimensional chromatography using two different solvent systems. The resolved components of original mixture can be separately eluted from chromatogram by
treating the cut-out spots with a suitable solvent and then determined quantitatively by some suitable instrumental method of analysis.

**Thin-layer Chromatography**

Thin-layer Chromatography is a method of flat bed chromatography which involves the passage of a solvent (mobile phase) across a uniform layer of finely divided insoluble adsorbent (stationary phase). When different substances are applied to the adsorbent layer, they migrate across it at different rates with the passage of the solvent and are separated. As an analytical technique it was developed by Stahl in 1958, and it is now recognized as a rapid and sensitive system for the identification of compounds and for the separation and detection of trace amount of impurities.

**Stationary Phase:**

The most common adsorbents used are silica gel, kieselguhr, alumina, cellulose and cellulose derivatives, ion-exchange resins and polyamides. A thin layer of the appropriate adsorbent is supported on a rigid base of glass, plastics or metal foil and adherence to the base may be ensured by the inclusion of a binding agent such as calcium sulphate. As an aid to visualization of the separated compounds, a fluorescence indicator may be mixed with the adsorbent.

The activity of a chromatplate is influenced by the amount of moisture present, and the activity may be increased, if necessary, by heating the spread plate in an oven usually at 110 °C for about 1 hr. During the heating process the plate should be placed in a vertical position.
Mobile Phase:
The choice of mobile phase, which may be a single solvent or a mixture of solvents, will depend on the polarity of the compounds to be separated and on the particular stationary phase which is being used.

Method:
A development chamber or tank is prepared by placing sufficient of the mobile phase in it to produce a depth of 5 to 10 mm after which the chamber is sealed to establish vapour saturation (equilibration). In order to facilitate this process, the tank is usually lined with filter paper which dips into the mobile phase. Equilibration is usually established in 1 hr.

A volume of a solution, normally containing up to 10 \( \mu \text{g} \) of the substance under investigation in 1 to 20 \( \mu \text{l} \) of a suitable solvent, is applied to the stationary phase as a closely confined spot, preferably less than 5 mm in diameter and about 20 mm from the bottom edge of the chromatoplate. The solutions are applied with a micro-pipette or a melting point capillary tube and are allowed to dry, either naturally or in a current of warm air from a blower.

A line is scored across the chromatoplate, or a suitable mark is made, at an appropriate distance (usually 150 mm) from and parallel to the line of application, to indicate the extent to which the mobile phase should run. The chromatoplate is then placed vertically in the tank, ensuring that the line of application is above the solvent level. The tank is then sealed and allowed to stand, preferably in a constant temperature atmosphere, until the solvent has traveled up the plate to the predetermined level. The chromatoplate is then removed from the tank and
the solvent is allowed to evaporate, either in the air or under prescribed conditions of warming.

After drying, the separated components are visualized on the chromatoplate by the use of a suitable spray reagent. If the coating of the plate contains a fluorescence indicator, screened ultraviolet radiation (254 or 366 nm) may be used in addition to spraying for the detection of compounds; the former wavelength is used for compounds which quench the fluorescence of the indicator, and the latter wavelength for those compounds which fluoresce or can be induced to fluoresce.

**Evaluation:**

The movement of a compound across the chromatoplate can be expressed as an $R_f$ (relative to front) value

Where, $R_f = \frac{\text{distance moved by compound}}{\text{distance moved by solvent front}}$

This value is less than 1, and hence the value of $100 \times R_f$ is often used in order to preclude the use of decimal fractions.

When two compounds are run on the same chromatoplate, each will have its own $R_f$ value, and if one of them (compound 1) is regarded as a standard or marker, the other (compound 2) can be assigned a relative $R_f$ value ($R_{Rf}$).

Where, $R_{Rf} = \frac{R_f \text{ value of compound } 2}{R_f \text{ value of compound } 1}$

The relative $R_f$ value is independent of the distance moved by the solvent front.

**Factors which affect the $R_f$ value:**

The following are the most important factors which affect $R_f$ values and which should be carefully controlled in order to achieve reproducibility.
stationary phase
i. the quality of the adsorbent
ii. the presence or absence of a binder
iii. the nature of the chromatoplate
iv. the degree of activation
v. thickness and uniformity of adsorbent layer

mobile phase
i. the composition and nature of the mobile phase
ii. the degree of vapour saturation of the tank

temperature
tank dimensions
development distance
weight loaded on to the chromatoplate

Types of Thin-layer Chromatography:

- Adsorption Thin-layer Chromatography
- Partition or Reverse Phase Thin-layer Chromatography
- Ion exchange Thin-layer Chromatography
- Gel Thin-layer Chromatography

Thin-layer Chromatographic Systems:

A number of thin-layer chromatographic systems suitable for use in the identification of specified drug substances are described in the British Pharmaceutical Codex.

A general system (system 1) is also described which can be used for all the substances.
**System 1: General System**

Stationary Phase: silica gel 60F254, 0.25 mm.

Mobile Phase: methyl alcohol, 100 volumes + strong ammonia solution, 1.5 volumes.

Sample Solution & Visualisation: for any particular group of substances, the sample solution and the visualization reagents are the same as are stated for the alternative systems.

**System 10: Steroids**

Systems 10a and 10b:

Stationary Phase: Kieselguhr F254 impregnated with a mixture containing acetone, 9 volumes + formamide, 1 volume.

Mobile Phase:

System 10a: chloroform

System 10b: toluene, 3 volumes + chloroform, 1 volume.

Sample Solution: 0.1 % in methyl alcohol: 1 and 10 μl applied to the chromatoplate.

Visualization:

1. examination under ultraviolet radiation, 254 nm
2. Spraying with a 10% solution of sulphuric acid in alcohol (95%) and heating the chromatoplate for 10 minutes at 105 °C.
3. examination under ultraviolet radiation, 366 nm

Systems 10c, 10d and 10e:

Stationary Phase: Kieselguhr F254 impregnated with a mixture containing acetone, 9 volumes + propylene glycol, 1 volume.
Mobile Phase:

System 10c: toluene

System 10d: cyclohexane, 1 volume + toluene, 1 volume.

System 10e: cyclohexane, 1 volume + petroleum ether (40-60 °C), 1 volume.

Sample Solution & Visualisation: As for Systems 10a and 10b

Systems 10f and 10g:

Stationary Phase: Silica Gel 60F254

Mobile Phase:

System 10f: dichloromethane, 77 volumes + ether, 15 volumes + methyl alcohol, 8 volumes + water, 1.2 volumes

System 10g: ethylene chloride, 95 volumes + methyl alcohol, 5 volumes + water 0.2 volume.

Sample Solution & Visualisation: As for Systems 10a and 10b

**Spectrophotometry:**

The ways in which the measurements of radiation frequency (emitted or absorbed) are made experimentally and the energy levels deduced from these comprise the practice of spectroscopy. Spectroscopy is one of the most powerful tools available for the study of atomic and molecular structure and is used in the analysis of a wide range of samples.

**Ultra-violet and Visible Spectrophotometry:**

This technique encompasses analytical methods based upon measurement of light absorption by substances in the wavelength region from 190-900 nm. The region from 190 to 380 nm is known as the UV region and from 380 to 900 nm,
the visible region of the spectrum. Absorption in the UV-Visible region arises from electronic transitions within the molecule.

A variety of instruments are available for measuring light absorption in the UV-Visible region of the spectrum. In a single beam instrument, light passes through a monochromator and then through the sample and into the detector. A dual beam instrument measures ratio of the intensity of beam coming through sample cell to a second beam which does not pass through the sample. A rapid scanning spectrophotometer employs multichannel detectors. Differential spectroscopy is a method of compensating for the presence of extraneous materials in a sample, which would otherwise interfere with the spectrum of the drug being determined. In dual-wavelength spectrophotometry, two monochromatic beams at different wavelengths are passed through the same sample. One wavelength is generally characteristic of the drug, while the other is carefully selected so that the absorbance is equivalent to the level of absorptive interference anticipated at the analytical wavelength. Thus, the second radiation beam is analogous to the reference cell employed in conventional difference spectrophotometry. In derivative spectroscopy, the absorbance of a sample is differentiated with respect to wavelength to generate the first, second or higher order derivatives. This method has found significant application in forensic and bio-medical analysis.

The use of a separation step followed by measurement of absorption in UV-visible region is the most widely used analytical procedure in pharmaceutical analysis today. Often, the UV spectrum serves as a confirmatory evidence of identity in support of other analytical data and is also used as detector in HPLC.
**Infra red Spectroscopy (IR):**

Infra red (IR) Spectroscopy is the study of the reflected, absorbed or transmitted radiant energy in the range of electromagnetic spectrum ranging from wavelength, 0.8 to 500 nm. A more commonly used measurement is the frequency and is expressed in wave number. The IR spectrum is usually divided into 3 regions namely- near IR (12500 to 4000 cm\(^{-1}\)), mid IR (4000 to 400 cm\(^{-1}\)), far IR (400 to 20 cm\(^{-1}\)). Only the mid IR region is usually referred to simply as infra red and is widely used in the analysis of drugs and pharmaceuticals.

IR spectrophotometers can be single or double beam instruments. Fourier transform spectrophotometer is the recent advancement in the field of IR spectroscopy, which has a number of advantages over dispersive instruments. They can scan quickly and therefore be used along with gas or liquid chromatography for recording spectra, as compounds are eluted.

Identical IR spectra of two samples (super-imposable spectra) are very good evidence that the two samples have the same chemical structure. IR spectrometric analysis is a very useful tool in the detection of functional groups of bio-molecules, thus aiding in their structural elucidation. The quantitative analysis of antibiotics, alkaloids, quinine and strychnine, steroidal sapogenins etc is also possible.

**Nuclear Magnetic Resonance Spectroscopy (NMR):**

NMR is the branch of spectroscopy dealing with the absorption of radio-frequency radiation by substances held in a magnetic field. Absorption results from interaction of radiation with magnetic moment of nuclei in the sample and it occurs at different frequencies for nuclei with chemically different environments.
within a molecule. NMR spectra are normally prepared using minimal resonance signals. Many solvents are available like water, chloroform, acetone etc as their deuterated analogues.

NMR has been an extremely important tool for elucidation of molecular structure, especially the stereochemistry and configuration. The technique reveals position of protons in a complex molecule. It is an excellent choice for identification test in pharmaceutical analysis. In quantitative analysis, it is an absolute technique, in the sense, that a reference standard of the substance being analyzed is not required.

NMR has found many applications in the determination of impurities and minor components in mixtures because of ease, speed and specificity of the analysis.

**Mass Spectrometry (MS):**

Mass spectroscopy is concerned with the electron ionization, subsequent fragmentation of molecules, determination of the mass to charge ratio (m/e) and relative abundancies of ions which are produced. Knowing the fragmentation pattern, a possible structure of the original molecule can be suggested.

The most important application of mass spectrometry is in determination of molecular weight of compounds. Mass spectrometry, either alone or in combination with a chromatographic separation, is probably the most effective method for the identification of drug constituents. The complete mass spectra for about 100 drugs, metabolites and other compounds of pharmaceutical interest are reproduced in *Pharmaceutical Mass spectra*, London, published by Pharmaceutical Press London, 1985. Mass spectrometer has been successfully coupled to both gas chromatograph and HPLC.
2.6.4. PHYSICAL EVALUATION:

Physical standards are to be determined for drugs, wherever possible. These are rarely constant for crude drugs, but may help in evaluation, specifically with reference to moisture content, specific gravity, density, optical rotation, refractive index, melting point, viscosity, and solubility in different solvents.

2.6.5. BIOLOGICAL EVALUATION:

In evaluation of herbal drugs, assessment of biological efficacy is found to be most assuming method. In biological evaluation, requirements are a suitable animal model for testing the drug and control, methodology for experiment and assessment of results.

2.6.5.1. SCREENING OF ANTIFERTILITY AGENTS

A screening programme should clearly demonstrate the potentiality of a compound to produce temporary and fully reversible sterility in laboratory animals. The initial routine fertility tests may be carried out on both sexes followed by special tests to determine the mechanism of action. Rats are commonly used though mice, rabbits and monkeys are also employed for the purpose.

Tests in the female rat:

Antifertility action in the female may result from the following:

I. Inhibition of ovulation
II. Prevention of fertilization
III. Interference with the transport and/or implantation of the fertilized ovum
IV. Destruction and resorption of the early implanted embryos
The following tests are usually carried out for screening of antifertility drugs.

1. **Cohabitation test:**

Female rats of established fertility (having produced two consecutive litters) are examined on **seven** consecutive days by vaginal smear for the presence of normal estrous cycle. After administration of the test compound or the solvent, the females are paired with males of proven fertility. The females are examined for the following:

- **Absence of mating** may indicate possibly the lack of oestrogen and/or progesterone.
- Sign of mating as indicated by the presence of sperm in vagina.

**Prolongation of the average time for pairing to the first insemination** is indicative of antifertility effect.

- Oestrous cycle changes by vaginal smear examinations
- Inspection of uterus for number of implants. Number of litters at birth.

**Resorption of fetuses** revealed by the difference between number of implants and number of litters.

- Bleeding or the loss of gained weight may be due to **abortifacient** effect.

2. **Ovarian weight in unilateral overiectomized immature female rat**

The immature female rats weigh less than 60 g. The ovarian weight increases in control animal 7-14 days after removal of other ovary. A decrease in the ovarian weight in the treated animal compared to the control will indicate an **inhibition of ovulation** through suppression of follicular stimulating hormone. Ovarian histology may show corpus luteum, etc.
3. Changes in the uterine weight:

Any change in uterine weight will indicate antioestrogenic action.

**Anti-oestrogenic activity:** Immature female Sprague-Dawley rats weighing about 55 g are ovariectomized. Groups of 5-10 animals are injected daily with estradiol 0.03 to 0.06 µg per animal s.c or several doses of the test compound or estradiol alone for 7 days. The test compound is administered orally or subcutaneously in 0.5% solution of carboxymethylcellulose or in cotton seed oil either orally or subcutaneously. Controls receive the vehicle only. On the 8th day, the animals are sacrificed and uterine weights determined. Mean values of each group are calculated. The anti-oestrogenic effect is expressed as percent reduction of estrogen-stimulated uterine weight by test compounds compared to rats treated with estradiol alone.

4. Deciduomata:

The development of deciduoma in the endometrium will indicate progestational action.

5. Oestrogenicity:

Immature female rats are given daily doses of herbal extract. Increase in weight of uterus, cornification of vagina etc shows the estrogenicity of herbal extract. Increase in the glycogen contents of reproductive organs especially uterus of cyclic as well as ovariectomized rats shows the estrogenicity of herbal extract.

**Vaginal cornification:** Immature female Sprague-Dawley rats weighing about 55 g are ovariectomized. They are kept for about one week on standard laboratory diet and water ad libitum. The test compounds are administered orally or subcutaneously in 0.5% solution of carboxymethyl-cellulose or in cotton seed
oil injected at several doses to groups of 10-20 rats. Doses of 0.02, 0.1, and 0.5 
µg estradiol per animal are used as standard. The compounds are dosed e.g. 
twice daily on two following day and at 10:00 a.m. and 5:00 p.m. At 5:00 p.m. of 
the third day and at 10:00 a.m. of the fourth day vaginal smears are taken using 
cotton swabs moistened with saline. The smears are transferred to a glass slide 
and stained for 10 min with 5% aqueous methylene blue solution. They are 
evaluated microscopically according to the following scores:

0 diestrus smear, mainly leukocytes, few epithelial cells
1 mixture of leukocytes and epithelial cells
2 proestrus smear, nucleated or nucleated plus cornified cells
3 estrus smear, cornified cells only.

Only animals showing score 2 or 3 are considered to be positive.

**Uterus weight:** Immature female Sprague-Dawley rats weighing about 55 g 
are ovariectomized. Groups of 5-10 animals are injected daily with several doses 
of the test compound or the standard (estradiol 0.03 to 0.06 µg per animal s.c.) 
for 7 days. The test compound is administered orally or subcutaneously in 0.5% 
solution of carboxymethylcellulose or in cotton seed oil. Controls receive the 
vehicle only. On the 8th day, the animals are sacrificed and uterine weights 
determined.

**Increase in glycogen content:** The increased estrogenic level promotes the 
glycogen vis-à-vis glucose synthesis in the reproductive organs especially the 
uterus. Glycogen is mainly located in the circular muscles in the rats and its 
physiological function may be involved in the rhythmic uterine contractions. This 
is further supported by the fact that many of the potent estrogenic compounds
increased the uterine contractility and propel the blastocysts from the pregnant uterus and thus provoke their antiimplantation action. Therefore, the increased uterine glycogen level may be involved in providing the readymade energy for the uterine contractions and thus helps in the expulsions of fertilized eggs from the uterus. This explanation is further strengthened by the fact that the administration of exogenous glycogen in the uterus of pregnant rats terminates the pregnancy. Investigators are in progress to confirm these metabolic events and also the changes in the pattern of glycogen mobilization in different reproductive organs of rats during various phases of implantation.

6. Anti-implantation studies:

The female rats of established fertility in proestrous phase of the estrous cycle are left overnight with known fertile males in a ratio of 1:2. The vaginal smears of those rats are examined on the following morning to determine the presence of sperm. The day on which sperms appeared in vaginal smear is labeled as day 1 of gestation and the female is considered mated. The drug is given for 7 days to the mated animals and the animals are laparatomised under mild ether anesthesia on 10th day. The two horns of the uterus are examined for the number of implantations. The number of corpora lutea on each ovary is counted. The abdominal wound is sutured in layers and the animals are allowed to go to full term. After delivery the number of litters born is noted.

Calculation of antifertility activity:

Pre-implantation loss = no. of corpora lutea on day 10 - no. of implantations on day 10

Post-implantation loss = no. of implantations on day 10 - no. of litters delivered
% Pre-implantation loss = Pre-implantation loss X 100 / no. of corpora lutea on
day 10

% antifertility activity = Post-implantation loss X 100 / no. of implants.

**Tests in the male rat:**

In the *male*, there are two possible mechanisms by which an antifertility agent
may act:

- **Suppression of spermatogenesis** at any of the stages resulting in
  sterility associated with oligospermia or aspermia.

- A qualitative change in spermatozoa rendering them nonfunctional.

The following tests are usually carried out for screening of male antifertility drugs.

1. **Routine testing** of male fertility is most conveniently carried out in rats,
since tests extending over 12 weeks after treatment cover possible effects on
any stage of spermatogenesis normally of 9 weeks duration.

2. **Cohabitation test:**

Treated males are mated with two females of known fertility and examined for the
following:

- sign of mating as indicated by the presence of sperm in vagina of females;
calculation of the date of insemination from the date of birth (gestation
period approximately 21 days) permits an estimate of the duration of
sterility; pseudopregnancy (presence of leucocytes in vaginal smear for
10-14 days) is indicative of aspermic copulation

- normal oestrous cycle is an indication of failure to mate due to an effect on
  libido

- Motility test of sperm from base of epididymis in males.
3. **Antispermatogenic activity in male rats:**

After acclimatisation, adult male rats are given a daily dose of herbal drug extract for 60 days. Between days 12-15, each male rat is mated with a female rat undergoing proestrous or estrous cycle. Subsequent mating is arranged between days 56-60. After this, testis of each male rat is morphologically studied and weight is noted. Sperms are examined under microscope. Besides, histopathological studies of testes are also carried out. If there is no fertilization due to mating between days 12-15, it is considered as **functional sterility**; while that between days 56-60 will be due to **antispermatogenic activity** of herbal drug extract.

4. **Spermicidal activity:**

It is a simple method and can be carried out in vitro directly on **human semen**. The fresh sample of human semen taken on slide is added with two drops of herbal drug extract in Sorensen's phosphate buffer, mixed well and observed under microscope for **motility** of sperms. Immobility of sperm occurs, in case of spermicidal herbal drug extract. Besides motility, other parameters such as sperm **viability**, **acrosome status and function**, **hypo-osmotic swelling** and **nuclear chromatin decondensation** are now being increasingly assessed to predict a successful outcome with different techniques followed in assisted reproductive technology to challenge infertility.

An **occlusion in vas deferens in male** can occur due to granuloma type tissue formation, leading to male sterility. It can be assessed by carrying in vitro testing in male rats.
The histological study involves the following steps:

1. **Tissue preparation:**

   It is the initial step in histology studies. After receipt of the specimens they should be properly labeled and numbered.

2. **Fixation:**

   To preserve the cell's physical and chemical characteristics in a near life-like state, certain chemical substances are added; these are called fixatives.

   Amount of fixative to be added is 15 – 20 times the bulk of the specimen.

   Reagents used as fixatives can be classified as:

   a. **simple fixatives**

      i. aldehydes: formaldehyde, glutaraldehyde
      
      ii. oxidizing agents: potassium permanganate, potassium dichromate
      
      iii. protein denaturing agent: ethyl alcohol, methyl alcohol, acetic acid
      
      iv. unknown mechanism: mercuric chloride, picric acid

   **Formaldehyde:**

   Formaldehyde, a gas when dissolved in water to 40% by weight is called FORMALIN. Formalin is a polymerized form of formaldehyde unsuitable for fixation. On dilution to 10% formalin, depolymerisation occurs and it becomes suitable for fixation.

   Mode of action: It forms cross links between amino acids of proteins, thereby making them insoluble. It fixes 4mm thick tissue in 8 hours.
Advantages:

- Rapid penetration
- Easy availability and cheap
- Does not overharden the tissues. Fixes lipids for frozen sections
- Ideal for mailing

Disadvantages:

- Irritation of nose.
- Forms white precipitate of paraformaldehyde.

b. **Compound fixatives:** This is a combination of simple fixatives.

i. Microanatomical fixatives: These are used to preserve the anatomy of the tissue. Fixatives for routine use are from this group. They never harden the tissue.

ii. Cytological fixatives: These are used when intracellular structures are to be preserved.

iii. Histochemical fixatives: These are used to demonstrate chemical constituent of the cell e.g. enzymes.

3. **Dehydration:**

Paraffin is not miscible with water. Hence, removal of water from the tissue sections is essential before paraffin embedding. This must be carried out by the use of a reagent which mixes with water thus penetrating easily between the tissue cells. Total dehydration time depends on the volume and type of the tissue and on dehydrating agent. The amount of the agent used in each stage should not be less than 10 times the volume of the tissue to be dehydrated.
Example: Ethyl alcohol, Acetone, Isopropyl alcohol, Dioxane

**Ethyl Alcohol:** It is the best dehydrating agent.

**Advantages:**
- Nontoxic
- Graded alcohols starting from 70%, if used, avoid shrinkage of tissues
- Ideal for delicate tissues

**Disadvantages:**
- Hardening of tissue if left for long period, requiring 3-4 changes

**Dehydrating time for alcohol:**
1. 70% alcohol: 1 hour
2. 95% alcohol: 1 hour
3. Absolute alcohol: 1 hour

4. **Clearing (Dealcoholisation):**

This is a procedure wherein, the alcohol in the tissue is replaced by a fluid which will dissolve the wax used for impregnating the tissues.

The substances used also have the ability of making the tissue transparent or clear, because of approximately similar refractive indices.

**Properties of a good clearing agent:**
1) It should remove alcohol quickly
2) It should clear quickly without hardening
3) It should not dissolve aniline dyes
4) It should not evaporate too quickly on the wax baths

**Example:** Clearing agents in use are:

Cedar wood oil, benzene, xylene, chloroform, toluene, clove oil, phenol etc
5. **Embedding media:**

Impregnation with embedding media takes place in an oven heated to 54 - 60°C, the temperature depending on the melting point of the wax used. The ovens may be electrically heated or glass heated, with or without water jacket. They should have a temperature range of 50 - 60°C. The volume of the impregnating medium should be at least 25 times the volume of the tissue.

Example: The commonly used embedding media are-
Paraffin wax, paraplast and paraplast plus, celloidin, carbowax etc

**Paraffin wax:**

It is cheap and is solid at room temperature with a melting point of 45-60 °C. In tropical countries, wax with a melting point of 58-60 °C is used. All waxes should be filtered routinely before use, by keeping it in an enamel jug in an embedding oven with a funnel of ordinary filter paper.

Paraffin wax should be free of dust, dirt, water. It is melted and filtered before use and kept in the embedding oven at a temperature maintained 2°C above the melting point of the wax. Dense tissues are impregnated under negative atmospheric pressure by vacuum embedding. Processing of the tissues can be done manually or by automatic tissue processor.

In the manual method tissue is placed in 3 baths after clearing; first 2 baths for an hour each and overnight in the third bath. Processing in the automatic processor depends on the time set. Ideally the tissue is placed in first 2 baths for a minimum period of half an hour and the last bath for at least 2 hours.
6. **Blocking:**

The method of blocking involves transfer of tissue from the final wax to a mould filled with melted wax, inverting it so that the surface to be cut is free from air bubbles. The surface of the tissue should be oriented to rest on the base of the mould. The block is then cooled to solidify.

7. **Decalcifying agents:**

The presence of calcium salts in the tissues hardens them, preventing preparation of good sections by routine methods. Incomplete removal of these salts results in torn and ragged sections and in damage to the cutting edge of the knife. This is prevented by decalcification.

The criteria of a good decalcifying agent are-

1) Complete removal of calcium
2) Absence of damage to tissues
3) Non impairment of subsequent staining techniques
4) Reasonable speed of decalcification

Decalcifying agents are of 2 types:

**a) Acids**

i. **Strong:** nitric acid, hydrochloric acid

ii. **Weak:** formic acid, picric acid, acetic acid, citric acid.

Most frequently used decalcifying agents are nitric acid and formic acid.

**b) Chelating agents:** These are organic compounds, which have the power of binding certain elements. For example, disodium salts of EDTA binds to calcium.
8. Section cutting:

The machines or instruments designed for the accurate cutting of thin slices (sections) of tissues are called Microtomes. Section cutting is also known as microtomy.

There are 5 classes of Microtomes.

1) Slicing
2) Rotary
3) Rocker
4) Freezing
5) Ultra thin section microtomes for electron microscopy work.

Basic principles of a microtone:

A panel is brought into contact with a ratchet wheel, which is connected to micrometer screw. This action turns the wheel and rotates the screw. By this means, the block is moved towards the knife at a predetermined thickness.

Microtome knife is a very important tool for getting good sections. They are made of high carbon tool steel. The hardness of the cutting edge is 400-900 on Vickers hardness scale. Glass knives and diamond knives are available for electron microscopy. The other instruments required for microtomy are block holders, water bath with 43-47 °C water, slide warmer, forceps, and albuminized slides.

9. Mounting of sections:

Adhesives are used to fix the sections on the slides so that sections are not washed off during subsequent staining. Various adhesives used are-
10. **Staining:**

Staining of tissue sections enables to study the characteristics of the tissue and their constituent cells. This is true because different components of the cells have varying affinity for most of stains or dyes. The purpose of tissue staining is to facilitate the pathologist in identifying the disease process, if any, in the given tissue sample. Two contrasting dyes/stains are used for this purpose in routine practice: Haematoxylin, which stains the nuclear detail and eosin which stains the cytoplasm and therefore the tissue structure. Haematoxylin and eosin are therefore the most commonly used routine stains in most laboratories worldwide.

11. **Mounting of stained sections:**

Stained elements of the tissue sections will be visible only if the sections are impregnated by a transparent medium with a refractive index close to that of glass. These media are known as mounting media. Media used for most routine techniques are natural resins like Canada balsam, synthetic resins like DPX, glycerin jelly, fructose syrup etc.

The function of mounting media is to protect the section from physical injury or deterioration of stain due to oxidation. It also avoids distortion of tissue and loss of staining over long periods.