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Effect of *Vitex smillex* on pregnant rats

**Dissertation submitted in partial fulfillment for the
Degree of Bachelor of Science (Health) in Biomedicine**

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2004

CERTIFICATE

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ABSTRACT

The herbal plant *Vitex smillex* was evaluated for its effects on pregnant albino rats. 54 female rats were used for this study. The rats were divided into control group and treatment group. Extract of the plant was prepared with distilled water by using sohlet apparatus in pharmacology laboratories. The plant extract was administered orally by means of a gavage from day 1 to 7 of pregnancy. Out of 12 rats in each group, 6 were laparotomized under ether anaesthesia on day 10 of pregnancy. Both the horns of uterus were observed for number of fetus or resorption sites and allowed to full term pregnancy. Number of fetus was compared with the number of pups delivered. The remaining 6 rats were used for histological study to confirm the activity of the extract. Result of the study showed that aqueous extract of *Vitex smillex* did not show efficacy as an antifertility agent. However, late resorption was observed in rat, which treated with 10mg/kg and 100mg/kg of *Vitex smillex* extract.

INTRODUCTION

From the Dorland's Medical Dictionary term of fertility means 1.The capacity to conceive or induce conception 2.The ratio of the number of births per year to the number of women of child bearing age (Dorland's Medical Dictionary, 2001). The term of antifertility from Dictionary.com Premium means the capable of preventing conception or impregnation; "contraceptive devices and medications". Term contraceptive from the same reference mean capable of preventing conception or impregnation; "contraceptive devices and medications"(Dictionary.com Premium).From the Dorland's dictionary contraception is prevention of conception or impregnancy (Dorland's Medical Dictionary, 2001).

In women, the menstrual cycle can be divided into 3 phases. The phases are follicular phase (days 1-13), ovulation and luteal phase (days 14-28). The initiation of menstrual cycle is regulated by gonadotrophin-releasing hormone secreted by hypothalamus. GnRH is stimulates the production of luteinizing hormone (LH) and follicle –stimulating hormone (FSH). These two hormones function at the ovarian level to stimulate the maturation of ovarian follicle in ovarian level. Estrogen and progesterone act at the level of endometrium to stimulate the growth and differentiation of the endometrium in preparation for embryo implantation. If fertilization fails to occur the corpus luteum spontaneously regress. This followed by reducing the level of estrogen and progesterone resulting in endometrial breakdown and shedding .If pregnancy occurs, estrogens and progesterone are important for maintaining uterine quiescence until term (James H.Liu, 1991).

Modern contraception methods are, intrauterine device (IUD), condom, diaphragm, spermicides and oral contraception. The effect of this contraception method are to prevent pregnancy by various way like, IUD work by causing a change in the lining of the uterus that interferes with the growth of a fertilized egg. The condom used to trapped the sperm and also for diaphragm which sperm is blocked from entering the uterus. Spermicides work by killing the sperms. Oral contraceptives like combination pill contain a fixed ratio of estrogen/progestine (combination BCPs) and progestin pill. Combination BCPs act by suppressing gonadotropin secretion and ovulation. The estrogenic component also act to stabilize the endometrium while the induces decidual change in the endometrial glands which prevents successful embryo implantation. The progestin pill prevents pregnancy by induction of atrophic endometrium and decreased cervical mucus (Asher J.Finkel *et al*, 1982)

Numerous herbs have been used traditionally for contraception. This indigenous method was widely practiced by the rural and urban population but there is very little study to evaluate the contraceptive efficacy of these herbal used. Using the herbal in traditional contraceptive is widely used by the aboriginal community in Malaysia. (Siti Amrah Sulaiman *et al*, 2001) Herbal contraception may never reach the level of contraceptive protection like the modern method, but it offers alternatives for women who have difficulty in reach the modern contraceptive methods.

Herbal contraceptive prevent pregnancy in different ways. Some herbs may affect the ovary, while others act upon the uterus and some plants effect the hormone production. Some other plant block certain hormones, also some herbal work by thickening of the outer covering of the ovum. Some of the herbal plants prevent

implantation or cause resorption or abortion (Sister Zeus, 1998). So these herbal plant are used as emergency contraceptives.

Vitex smillex is the scientific name of Tropical rain forest plant from *Verbenaceae* family. It's grown throughout the Indo-Malayan region including Western Pacific islands. This herb locally called halban growth widely in Burma, Thailand, Laos, Vietnam, Cambodia, and Indonesia and Malaysia (world Agrosforestry Centre). Uptill now neither chemical examination no antifertility testing has been carried out on this plant.

However it is not known whether *Vitex smillex* is having antifertility effect or it can help in maintaining pregnancy. Therefore, it was decided to evaluate the effect of aqueous extract of *Vitex smillex* on pregnant rats.

REVIEW OF LITERATURE

Morphology:

Vitex smilax:

Morphology of *Vitex smilax* is quite similar to other *Vitex* SP. This plant can grow up to 4-meter height. It has bigger leaves, which range in opposite direction and compose to seven radiating leaflets. The flowers are purple in colour (Figure 1)

This plant has many common local names. In Malaysia, it is known as 'Leban/ laban /halban' but it is called Kulim Papa in Sabah, in Thailand it is called Teen-nok/ tinnok, in India it is known as Milla, Bitum in New Guinea, Gupasa Indonesia (World Agrosforestry Center).

Figure 1. *Vitex smilax* plant



Classification

Kingdom	<i>Plantae</i> - plants
Subkingdom	<i>Tracheobionta</i> - vascular plants
Superdivision	<i>Spermatophyta</i> - seed plants
Division	<i>Magnoliophyta</i> - Flowering plants
Class	<i>Magnoliopsida</i> - Dicolyledons
Subclass	<i>Asteridae</i>
Order	<i>Lamiales</i>
Family	<i>Verbenaceae</i> - Verbena family
Genus	<i>vitex smilax</i>

(United State Department of Agriculture (USDA))

Components isolated

The component isolated from other *Vitex* species are reported as below:

- Components isolated from *Vitex trifolia* are: 1,8-cineole 23.5%, sabinene 20.8%, α -pinene 14.2%, β -pinene, α -terpinyl acetate 5.7%, β -caryophyllene 5.7%. (Donald J. Brown, 1994), (Tony Burfield, 2001). Two more compound isolated are viteosin –A and vitexicarpin (Alam G *et al*, 2002)
- Component isolated from *Vitex pubescens* are: betulinic acid, oleanolic, acids 4-hydroxybenzoic acid and 5,7,3', 4' – tetrahydroxyflavanone (Farediah Ahmad, *et al*, 2002).
- Component isolated from *Vitex polygama* was rich in flavonoids from fruits and leaves (Goncalves JL *et al*, 2001).
- The β -caryophyllene is the most common sesquiterpene in *Vitex* spp (Tony Burfield, 2001).

But, no component was isolated so far from *Vitex smilax*.

Traditional uses

This plant used traditionally for contraception. Then also as an ornamental plant on the road side. No other uses of *Vitex smilax* had been reported.

Pharmacological action

The pharmacological actions of other species of *Vitex* are reported as below:

- *Vitex polygama* has antiviral properties and was tested against acyclovir –resistance herpes simplex virus type1 (Goncalve *et al*, 2001).
- *Vitex rotundifolia* has analgesic properties where the effect on pressure pain threshold was tested (Okuyama E, 1998).
- *Vitex trifolia* has shown tracheospasmplytic activity (Alam G *et al*, 2002).
- *Vitex pubescens* has anti-inflammatory properties by activity of betulinic acid and oleanolic acids. These properties investigated using the TPS-mouse ear model. (Farediah Ahmad, *et al*, 2002)
- *Vitex agnus cactus* has dopaminergic activities and has been shown in human (Gorkow C, 2002). It also can restore fertility in women (Christies S *et al*, 1997).

Lacuna

Survey of literature reveals that so far no attempt has been made to screen any pharmacological action of the plant *Vitex smilax*.

OBJECTIVE OF STUDY

The objective of the study is to find out whether the aqueous extract of *Vitex smillex* will prevent pregnancy or maintain pregnancy in albino rats.

MATERIAL AND METHODS

Protocol of the experiment was approved by Animal Ethical Committee of the University.

***Vitex smillex* extract**

Water extract of dried ground leaves of the *Vitex smillex* was used in this study. Plant extract was obtained by the following procedure:

The leaves were washed and dried in an oven at 40 - 42°C for a few days until dried completely. By using the blender, leaves were ground into small pieces. Then the ground material extracted used sohlet apparatus based on traditional practices. The extracts were rotavapoured to remove the water. Then the extract was frozen dried at 20°C and reconstituted with distilled water for use.

Two different doses of extract viz. 10mg/kg and 100mg/kg were used for the study. The extract was administered orally by gavages method.

Animals

54 female albino rats of *Sprague dawley* strain were obtained from Animal house, USM Health Campus, Kubang Kerian, Kelantan. All the rats were weighing 180g to 200g. Food and water were available freely for all rats. Vaginal smears were taken from all animals each morning to monitor their estrous cycles. The rats were assigned to experimental groups only after completing a minimum of two consecutive estrous cycles of the same length. Rats with both four-day and five-day estrous cycles were included in the study. On the day of proestrus the female rats were mixed with males. The rats were randomly divided into four group of six each.

Group of animals

1st category - Control groups

Control 1: Normal rats

Control 2: Pregnant rats - Laparotomy

Control 3: Pregnant rats- Sacrifice

2nd Category - Treatment groups

Group 4: Low dose - Laparotomy

Group 5: Low dose - Sacrificed

Group 6: High dose - Laparotomy

Group 7: High dose - Sacrifice

3rd category - Standard drug group

Group 8: Standard drug - Sacrifice

1st category - Control groups

Group 1: Normal rats - Sacrifice

Rats were sacrificed during estrous / metaestrous period. The ovaries and uterus were removed for histological study.

Group 2: Pregnant rats - Laparotomy

Rats were laprotomized under ether anesthesia on 10th day of pregnancy. The numbers of zygots in each horn were noticed. The rats were allowed for full term pregnancy and the number of the pups delivered in 21st day was counted.

Group 3: Pregnant rats - Sacrifice

The rats were sacrificed on 10th day of pregnancy .The number pups in each horns were noticed. The ovaries and uterus were removed for histological study.

2nd Category – Treatment groups

Group 4:Low dose - Laparotomy on 10th day

The pregnant rats were administered with 1mg /kg dose of the extract of *Vitex smillex* orally daily for 7 days from first day of pregnancy. Rats were laparotomized under ether anesthesia on 10th day of pregnancy. The number of zygots in each horn were noticed. The rats were allowed for full term pregnancy and the number of the pups delivered in 21st day was counted.

Group 5:Low dose –sacrifice on 10th day

The rats were sacrificed on 10th day of pregnancy .The number pups in each horns were noticed. The ovaries and uterus are removed for histological study.

Group 6:High dose laparotomy on 10th day.

The pregnant rats were administered with 100mg /kg dose of the extract of *Vitex smillex* orally daily for 7 days from first day of pregnancy. Rats were laprotomized on 10th day. The number of zygots in each horn were noticed. The Rats were allowed for full term pregnancy and the number of the pups delivered on 21st day was counted.

Group 7: High dose sacrificed on 10th day

The rats were sacrificed on 10th day of pregnancy. The number pups in each horn was noticed. The ovaries and uterus were removed for histological study.

3rd category - Standard drug group

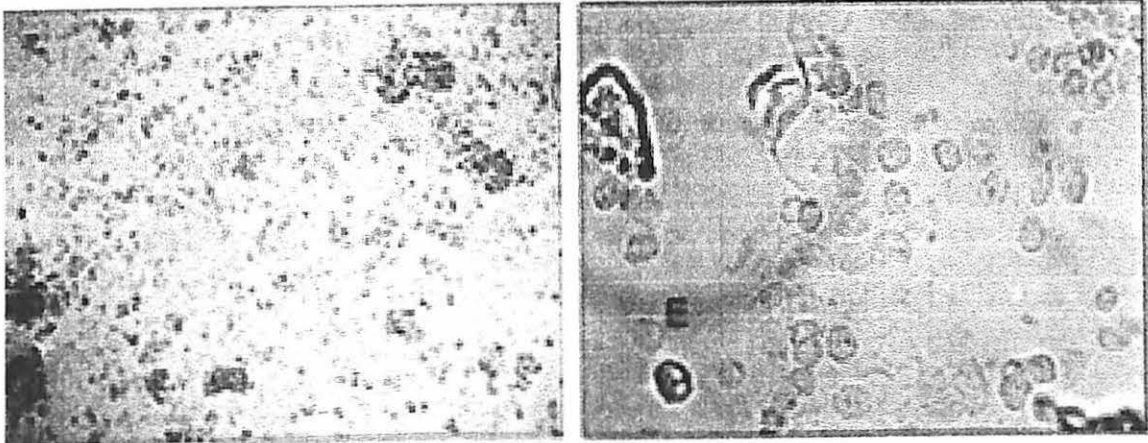
Group 8: Sacrificed on 10th day.

The pregnant rats were administered with 7 µg /kg dose of the stilbestrol daily for 7 days from first day of pregnancy by intramuscular injection (IM). The rats were sacrificed on 10th day of pregnancy. The number pups in each horn was noticed. The ovaries and uterus were removed for histological study.

Study of vaginal smears of female rats for estrous cycle

Vaginal smear was observed for each rat in the morning between 8:00 and 10:00 a.m. Samples for making a vaginal smear were collected by inserting a cotton tipped swab moistened with normal saline into the vaginal cavity of a rat but not deeply. The swab was applied gently against the vaginal wall and rolled slightly before withdrawing. The moist swab was then rolled onto a clean glass microscope slide. Unstained material was observed under a light microscope with 10 and 40 x objective lenses (Marcondes, 2002). The figure 2 - 5 shows the microphotographs of different phase of estrus cycle with 10 and 40x objective lenses (Marcondes, 2002).

Figure 2: Proestrus phase

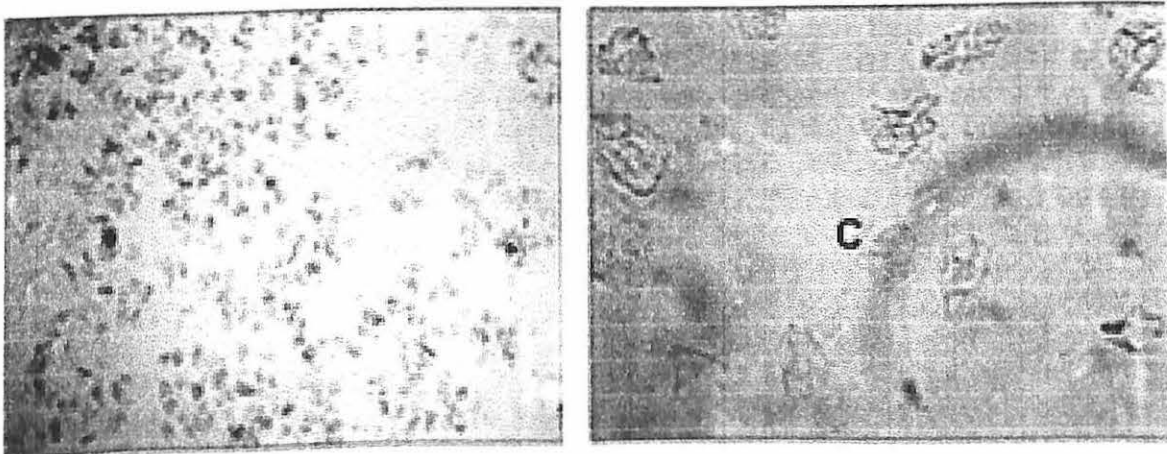


2a

2b

Figure 2a (10x) and 2b(40x)- Proestrus smear consists of a predominance of nucleated epithelial cells

Figure 3: Estrous phase

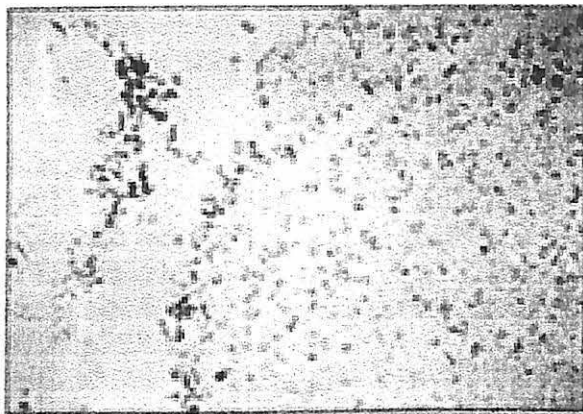


3a

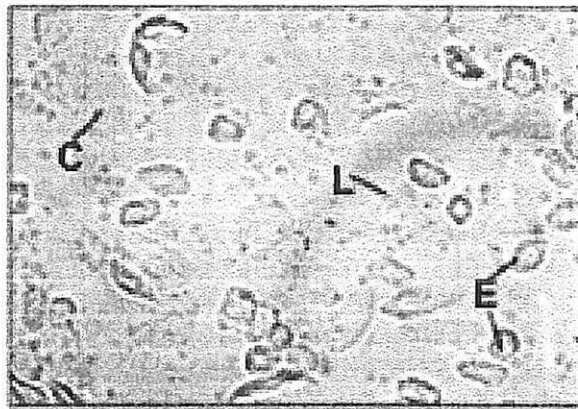
3b

Figure 2a (10x) and 2b (40x)-Estrous smear primarily consists of nucleated cornified cells

Figure 4: Metestrus phase



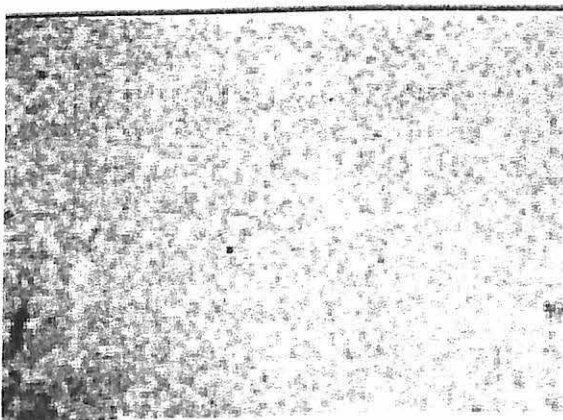
4a



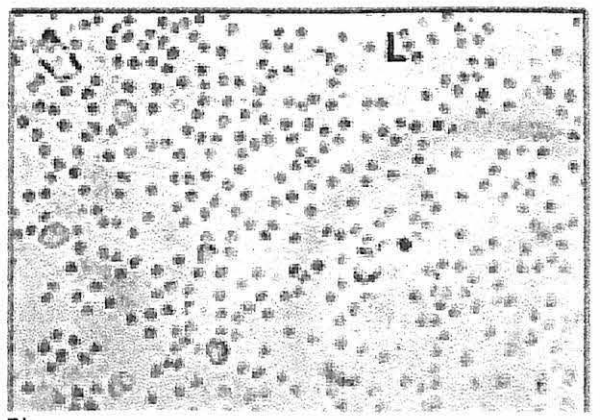
4b

Figure 4a (10x) and 4b(40x)-Metestrus smear consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells.

Figure 5: Diestrus phase



5a



5b

Figure 5a (10x) and 5b(40x)- Diestrus smear primarily consists of a predominance of leukocytes.

Vaginal smears of female rats were studied microscopically for determining estrous cycle. Estrous cycle of the rats can be divided into four stage diestrus, proestrus, estrus and metestrus. The characterization of each phase is based on the proportion among three types of cells observed in the vaginal smear: epithelial cells, cornified cells and leukocytes (Marcondes, 2002). Using the 10x objective lens, it was easier to analyze the proportion among the three cellular types, which are present in the vaginal smear. Using the 40x objective lens, it is easier to recognize each one of these cellular types (Marcondes, 2002). Diestrus has abundant neutrophils and a few non-cornified epithelial cells, proestrus has abundant nucleated non-cornifid epithelial cells, estrus begins with about 75% nucleated and 25% cornified cells, with cornified cells predominating as estrus continues. Metestrus has large numbers of neutrophils and scattered squamous epithelial in the vaginal Smear. (Dennis F Kohn, 1984)

Each stage has different duration where for diestrus lasts up to 57 hours, last 3-12 hours for proestrus, around 12 hours for estrus and 21 hours for metestrus. The rats found in proestrus phase will be caged with the males. (Dennis F Kohn, 1984)

Caging with males during proestrus phase

The reason for mating at proestrus stage was the female rat accepts the male for mating only at the end of the 12-hour preliminary period of proestrus and during the 12 hours of estrus. Ovulation occurs about 10 hours after the onset of estrus (Dennis F.Kohn, 1984). The best times for mating are at evening in order to prevent any disturbance to the rat .The ratio for male to a female rat was 1:2 or 1:1. Males used for mating were experienced males tested for fertility.

Conformation of copulation by vaginal smear

The females were examined in the following morning for presence of spermatozoa in the vaginal smear. The day when spermatozoa were detected in the smear was considered as day one of pregnancy (SP Hiremath, Sembulingam K *et al*, 1990).

Administration of plant extract orally for 7 days

Plant extract was administered orally by means of a gavage from day one to day seven of pregnancy.

Laparotomy on 10th day

The animals were laparotomized under ether anaesthesia on day 10 of pregnancy. Ether was used to anaesthetize the rats to laparotomy using the technique of Feldmen and conforti (1980) to avoid stress (Feldman, 1980). Both horns of the uterus were observed for number of live fetuses and resorption site. Then the rats were allowed to full term.

On delivery day

On the day of delivery, the number of live pups delivered was noted.

Sacrificed on 10th day for histological studies

The antifertility effect of the extract was confirmed by histological studies of the ovary and uterus. The rats were sacrificed. Ovary and uterus were followed for tissue preparation and the samples were cut into thin sections of 6µm after embedding in paraffin. Tissues were stained with suitable stains.

Histological studies

In order to confirm effect of *Vitex smillex* extract on antifertility. Histological study was doing on ovary and uterus of normal pregnant rats and Pregnant rats that treatment with a different dose of extract. In this study the specimens were process by the following procedures:

There are four steps in tissue preparation:

1. Fixation stabilizes and preserves the tissue.
2. Embedding converts the tissue into a solid form, which can be sliced ("sectioned").
3. Sectioning (slicing) provides the very thin specimens needed for microscopy.
4. Staining provides visual contrast and may help identify specific tissue components.

(Stanley S. Raphel, 1983)

10% formalin was used as preservatives. Tissues was embedded in paraffin. In sectioning process tissue were cut into 6µm. Tissues were stained with Haematoxiline and Eosin (H&E) stains.

After all procedure applied to the specimen, it was observed under electron microscope. Histological study of ovary was to observe the corpus lutea and atretic follicles. Corpus luteum in the ovaries was associated with the number of the fetus in uteruses. Regression of a corpus luteum in ovaries was shown reabsorption of implantation of a fetus. Histological studies of the uterus are to observe the tropoblastic cells. Tropoblastic cells were remained from the placenta after abortions occur. (Hiremath SP *et al*, 1990)

Statistical analysis

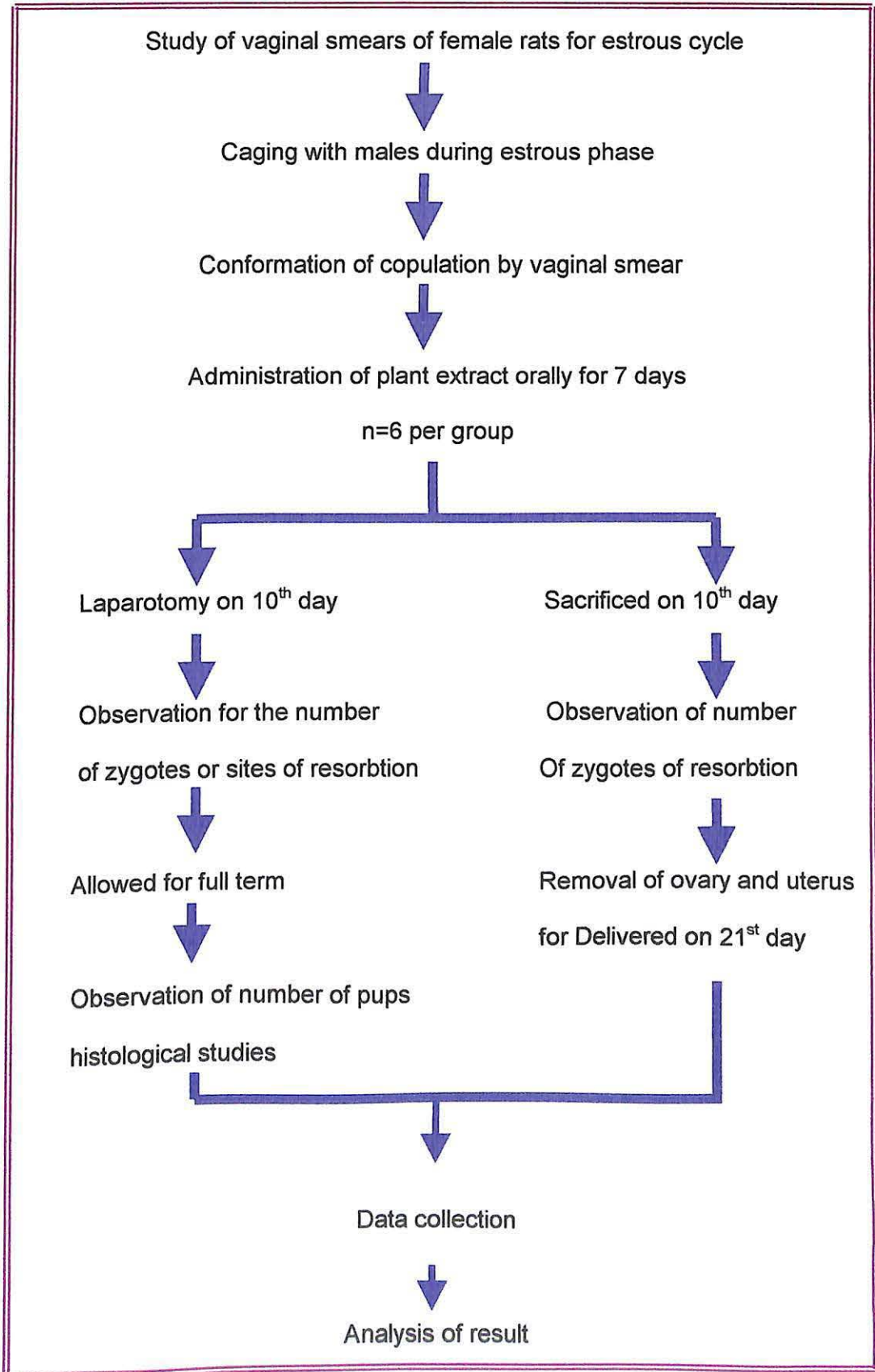
Statistic study was done for efficacy of the plant extract in preventing pregnancy .By observing number of pups in control animals and in treated groups.

The data of all the result obtained in the study were analysis by computerized statistical software SPSS 11.0.Mean and standard errors of the mean was calculated for all the parameters of different group.

Result of each parameter in different group was analysis by the application of one-way analysis of variance –ANOVA.

The multiple comparisons to elicit the significant differences between various groups were performed by means of Tukey's test. Values of $p < 0.05$ was considered statistically significant.

EXPERIMENTAL PROTOCOL



RESULTS

Table 1: Effect of *Vitex smillex* extract in female rats

Group	Laparotomy		Delivery	
	Total live fetus	Total Resorbtion site	Total live pups	Total late resorbtion
Group 2 (control)	50	0(0%)	50(100%)	0(0%)
Group 5 (Low Dose - 10mg/kg)	58	0(0%)	47(81.03%)	11(18.97%)
Group 7 (High Dose - 100mg/kg)	50	9(18.00%)	43(86.00%)	7(11.86%)

Table 2:Total number of live fetus and resorption site in sacrificed rats

Group	Total live fetus	Total resorption site
Group 3 (Control)	56	0
Group 4 (Low Dose - 10mg/kg)	49	0
Group 6 (High Dose - 100mg/kg)	46	0
Group 8 (Standard Drug - stilbestrol) 7µg/kg	0	0

The results of number of live fetus and number of resorption site during laparotomy on 10th day, number of live pups and number of late resorption after delivered are given in separate tables and figures. The three tables are used to describe each parameter. The first table shows Mean \pm Standard error of mean (SEM), the F-test ratio and its significance. The second table shows the results of Tukey's Multiple Comparison Test and for the third table shows the summary of the result. The data of result for each parameter are presented in a bar diagram. The bar diagram are used to differentiate result of each group for each parameter.

Confirmation of the result was done by histological study and was shown by figure 10 – 19.

Number of live fetus on 10th day during laparotomy

The result of ANOVA revealed that treatment with *Vitex smillex* extract did not decrease the number of live fetus significantly. The Tukey's multiple comparison tests showed that there also no significant between control group, low dose group and High dose group. (Table 3,4,5; Figure 6)

Number of resorption sites during laparotomy

The result of ANOVA showed that there is no significant difference in number of resorption site cause by *Vitex smillex*. According to the Tukey's multiple comparison test, there also no significant between different groups.

(Table 6,7,8 ; Figure 7)