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Effects of *Vitex pubescens* on pregnant rats

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

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2004

CERTIFICATE

This is to certify that the dissertation entitled

"Effects of *Vitex pubescens* on pregnant rats "

is the bona fide record of research work done by

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ACKNOWLEDGEMENTS

I gratefully acknowledge with gratitude, the support and guidance given by my Supervisor, Assoc. Prof. Dr. K. Sembulingam and Co-Supervisor Assoc. Prof. Dr. Prema Sembulingam, for their willingness in spending time assisting throughout the research; giving knowledge and advices in handling an appropriate research project. The comments and advices given are most welcome to improve the skills in handling the project and getting a more accurate and precise result.

Not forgotten Assoc. Prof. Dr. Manoharan Madhavan and Deputy Dean, Prof. Syed Mohsin Jamalullail for their full support in completing the research project; by providing photos of the plants and extract drug of the leaves material needed.

My grateful also goes to all the staff of Animal House especially my research Assistant, Mr. Mustakim for his dedicated research assistance, support and commitment given in handling the research project till its completence. I also appreciate all the other staffs for their cooperation in helping out in handling and the data collection of the project.

I'm also indebted my beloved father, mother, brothers and sisters who gave me emotional and financial support in finishing my project.

Lastly I'm grateful to my colleagues, Ms. Nor Hafizah and Ms. Nordalilah who support me throughout the dissertation preparation and completion.

Thank you.

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ABSTRACT

The herbal plant *Vitex pubescens* was evaluated for it 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 soxhlet apparatus in pharmacology laboratory. The plant extract was administered orally by means of a gavage from day 1 to day 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 was observed for number of fetus or resorption site 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 pubescens* 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 pubescens* extract.

INTRODUCTION

As the era of bioinformatics is developing fast in a glance, the number of birth and population is also getting wide. The safety given for lives through bioinformatics, has also caused the development to have a control on the size of population breeding. Thus, bioinformatics is now getting and exploring into resizing number of population by having a control in number of birth given.

For a start, plants were taken as a subject as it is practiced for thousand years of use and well known its magic for contraceptive formulation in preventing birth among our far elderly people (Hiremath SP *et al.*, 1994). Many indigenous plants prepared traditionally have strongly possessed property of preventing conception by preventing implantation or cause resorption or abortion (James H.Li, 1991) when administered orally among rural and urban population. One of the sample is *Striga lutea* plant (Hiremath SP *et al.*, 1990).

In Malaysia, indigenous methods of fertility regulation, regardless its effectiveness in preventing birth, have played an important role in family planning among the society (Siti Amrah Sulaiman *et al.*, 2001). Malay, Chinese and Indian women in rural and even urban areas adhere to indigenous methods though known the presence of modern contraceptives (Siti Amrah Sulaiman *et al.*, 2001). Thus it grabs the interest of researcher to further their collected knowledge in interrogating and investigating the hidden secrets that dragged the massive use of those indigenous plants besides modern contraceptives among the

community. In this study, it was decided to evaluate the effects of *Vitex pubescens* in female albino pregnant rats.

Vitex pubescens is a plant called *halban* and specifically as *Lemuni Hitam* in the local language . It belongs to the family verbenacea. It is well known as a slow acting herb which takes full cycles to work its magic as been noted in thousand years of use. However it is not known whether *Vitex pubescens* is having antifertility effect or help in maintaining pregnancy. Therefore, it was decided to evaluate the effects of aqueous extract of *Vitex pubescens* on pregnant rats.

REVIEW OF LITERATURE

Vitex pubescens is a well known plant as *halban,* or specifically as *Lemuni Hitam* in the local language, belonging to the family of verbenacea. It also known with other common names as *Leban* in Malaya, *Kulim papa* in Sabah, *Teen-nok* in Thailand, *Milia* in India, *Bitum* in New Guinea, *Gupasa* in Indonesia and Chasteberry/Monk's pepper in Mediterranian.

Morphology:

This herbal plant shrubbing with silvery leaves and clusters of purple flowers. It is distributed throughout the Indo-Malayan region including Western Pacific Islands and grow upto 120 feet in height. It varies from small to large tree with boles clear to 50 feet.

The trunk is straight a cylindrical, often fluted and irregular trunk with diameters upto 6 to 7 feet over. And it has a large buttresses. The gravity varying with species 0.58 to 0.72 (overdry weight/green volume) and air dry density 45 to 55 pcf.

Figure 1: *Vitex pubescens*

Classification:

Kingdom - Plantae (plants)

Subkingdom - Tracheobionta (vascular plants)

Superdivision - Spermatophyta (seed plants)

Division - Magnoliophyta (flowering plants class) / Magnoliopsida (dicotyledons)

Subclass - Asteridae

Order - Lamiales

Family - Verbenaceae (verbena family)

Genus - *Vitex L.*(chastetree)

Spesies - *Vitex pubescens*

Components Isolated

Phytochemical studies of the petroleum ether extract of barks of *Vitex pubescens* have afforded betulnic acid and oleanolic acids. Isolation and purification of the ethyl acetate extract gave 4-hydroxybenzoic acid and 5,7,3',4'-tetrahydroxyflavanone (Farediah A *et al.*, 2002).

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

- V*itex trifolia*

  1,8-cineole 23.5%, sabinene 20.8%, α-pinene 14.2%, ß-pinene, α-terpinyl acetate 5.7% and ß-caryophyllene 5.7% (Chandramu C, 2003.

  Two more compounds isolated are viteosin –A and vitexicarpin (Alam G *et al.*, 2002).

- *Vitex polygama*

  Rich in flavonoids from fruits and leaves (Goncalves JL *et al.*, 2001).

- *Vitex* species.

  The most common sesquiterpene found is ß-caryophyllene (Chandramu C, 2003).

Traditional Uses

Vitex pubescens is used traditionally as herbal plants for contraception (Wochenschr, 2002).. It is also used as an ornamental plant on the road side (Wochenschr, 2002). No other uses of *Vitex pubescens* had been reported.

Pharmacological Action

Vitex pubescens compounds show an anti-inflammatory activity, using the TPS-mouse ear model. The most active compound was betulinic acid which inhibited edema by 101% followed by oleanolic acid (65%), 5,7,3',4'-tetrahydroxyflavanone (21%) and 4-hydroxybenzoic acid (19%) at a dose of 0.5mg/ear (Farediah A *et al.*, 2002)

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

- ***Tracheospasmplytic activity***

  Vitex trifolia has shown tracheospasmplytic activity (Alam G *et al.*, 2002).

- ***Dopaminergic activities***

  Vitex agnus cactus has dopaminergic activities and has been shown in human (Gorkow C *et al.*, 2002).

- ***Antiviral properties***

  Vitex polygama has antiviral properties and was tested against acyclovir –resistance herpes simplex virus type 1 (Goncalves JL *et al.*, 2001).

- ***Analgesic properties***

  Vitex rotundifolia has analgesic properties where the effect on pressure pain threshold was tested (Okuyama E, 1998).

Lacuna found in Literature

Review of literature reveals that so far no attempt was made to evaluate the antifertility effects of *Vitex pubescens* in experimental animals.

OBJECTIVES

The objectives of this research project study are:-

1. To evaluate whether this aqueous extract of *Vitex pubescens* plant prevent pregnancy or maintain pregnancy in female albino pregnant rats.

MATERIALS AND METHODS

The protocol of the experiment was approved by the Animal Ethical Committee of the University Science Malaysia.

Collection of plant materials

Dried leaves of the plant grown in flourishing soil of the Taman Herba of University Science Malaysia, were collected during the hot season in December. The leaves obtained were dried and stored.

Figure 2: Leaves material from *Vitex pubescens* plant.

Preparation of *Vitex pubescens* extract for preleminary animal study.

1. Leaves material from *Vitex pubescens* plant was washed throughly and carefully.
2. It was dried in an oven at 40-42°C for few days until complete dry.
3. The dried leaves were then grind into small pieces using a blender.
4. 24g of the grounded leaves was weighed into a boiling flash (96g).
5. 750ml of distilled water was added into the flash (3000ml).
6. The mixture was then boiled at 100°C for 4 hours until the volume reduced to 300-350ml. The aqueous was the allowed for cooling at room temperature (1000ml).
7. The decoction was filtered using muslin cloth.
8. The mixture was centrifuged at 1000G for 5 minutes. The supernatant is then transferred into a clean container (70°C, 5 min, 2800rpm).
9. The extract was rotavaporised until the volume is reduced to about 30ml and allowed to cool.
10. The extract was kept frozen in multiple small clean container (-20°C) and freeze-dried.
11. The dried extract was weighed and kept at 4-6°C (in freezer) until the use for the experiment.

Two different doses of the extract viz. 10 mg and 100 mg were screened by adding 10 ml of distilled water with 100 mg of the dried extract for Low dose extract and 1000 mg of dried extract for High dose extract for antifertility effects. The extract was administered orally by gavage method.

Animals.

Cyclic female Albino rats of Spraque Dawley strain were choosen, as it is the preferred strain rats used for the antifertility effects study. A total of 54 female rats of same age (nine weeks) and weighing 180-200g were selected. As for the mating purpose, nine fertile male rats of same age (nine weeks) and weighing about 200g with proven fertility were also selected. Food and water were available freely for all the experimental rats.

Vaginal smears were taken in each female rat every 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 days and five days of estrous cycles were included in the study. On the day of proestrous, the female rats were mixed with males. The rats were randomly divided into nine groups, six in each group.

Group 1: Control (Normal rats) group- Sacrifice.

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

Group 2: Control (Pregnant rats)- Sacrifice.

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

Group 3: Control (Pregnant rats)- Laparotomy.

The pregnant rats were laparotomized under ether anesthesia on the tenth day of pregnancy. The number of live fetuses in both the horns of the rats uterus were recorded. The rats were allowed for full term pregnancy and the number of the pups delivered was counted and recorded.

Group 4: Low dose 10mg/kg group- Sacrifice.

The pregnant rats were administered with 10mg/kg dose of the extract of *Vitex pubescens* orally daily for seven days from the first day of pregnancy. The pregnant rats were sacrificed on 10th day of pregnancy. The number of pups in each horns was noticed. The ovaries and uterus were removed for histological study.

Group 5: Low dose 10mg/kg group- Laparotomy.

The pregnant rats were administered with 10mg/kg dose of the extract of *Vitex pubescens* orally daily for seven days from the first day of pregnancy. Rats were laparotomized on the tenth day. The number of live fetuses and resorption sites in each horn was noticed. The rats were allowed for full term pregnancy and the number of the pups delivered were counted and recorded.

Group 6: High dose 100mg/kg group- Sacrifice.

The pregnant rats were administered with 100mg/kg dose of the extract of *Vitex pubescens* orally daily for seven days from the first day of pregnancy. The pregnant rats

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

Group 7: High dose 100mg/kg group- Laparotomy.

The pregnant rats were administered with 100mg/kg dose of the extract of *Vitex pubescens* orally daily for seven days from the first day of pregnancy. Rats were laparotomized on the tenth day. The number of live fetuses and resorption sites in each horn was counted. The rats were allowed for full term pregnancy and the numbers of the pups delivered were counted ands recorded.

Group 8: Standard group (Stilbesterol drug)- Sacrifice.

The pregnant rats were administered with Stilbesterol drug by intramuscular injection, daily for seven days from the first day of pregnancy. The pregnant rats were sacrificed on 10th day of pregnancy. The number of pups in each horns was noticed. The ovaries and uterus were removed for histological study.

Group 9: Standard group (Stilbesterol drug)- Laparotomy.

The pregnant rats were administered with Stilbesterol drug by intramuscular injection, daily for seven days from the first day of pregnancy. Rats were laparotomized on the tenth day. The number of live fetuses and resorption sites in each horn was counted. The rats were allowed for full term pregnancy and the numbers of live pups and the number of late resorption sites were counted ands recorded.

Housing of experimental animals.

This Spraque Dawley rats were quarantined and isolated according to vendor subpopulations that have a similar microbial flora. This is one of the sophistication taken from various levels to provide barriers to the spread of infections in rat colony, which can trigger to unaccurate results. Since many rat pathogens are spread by aerosol, ventilation control is taken by housing the rats in a nonrecirculating room air, with room temperature of 22°C and evenly distributed light source for 12 hours per day (David L and Hoffman, 2003).

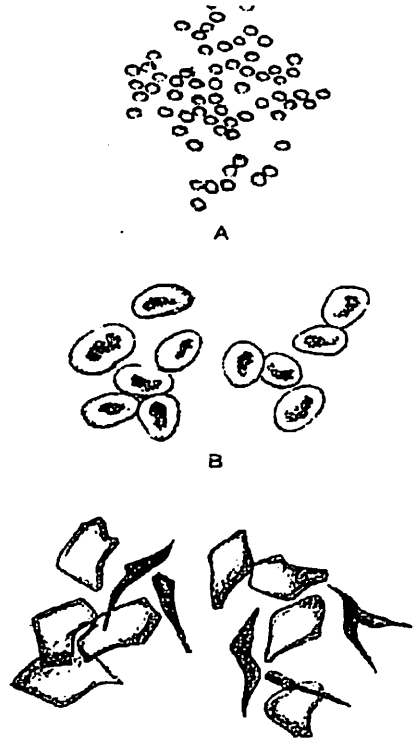
The rats were caged in solid bottom cages with wood shavings as bedding materials. The sanitation and litter pan changing were done two to three times per week (Choudhary DN *et al.,* 1991). To avoid distraction between sexes and stress, the female rats cages are placed at 90° to the male rats cage.

Study of vaginal smears of female rats for estrous cycle.

Vaginal smear was observed for each rat in the morning between 8:00a.m. to 10:00 a.m. The same time is set daily to obtain an estrous cycle with regular stages (William DG *et al.*, 1982).

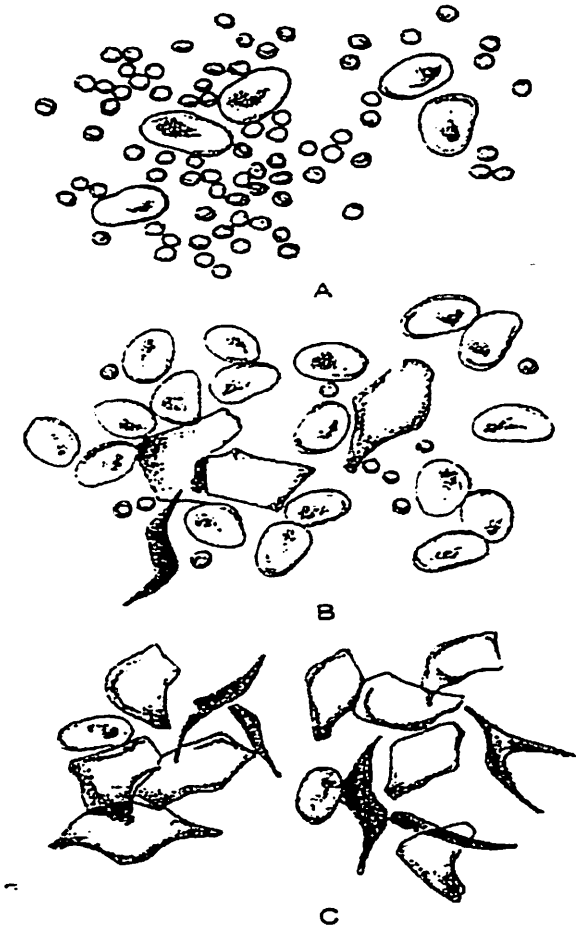
Samples for making a vaginal smear were collected by inserting a cotton tipped swab moistened with normal saline into the vaginal cavity of the rat. The swab was applied gently against the vaginal wall and rolled lightly before withdrawing. The moist swab is then rolled onto a clean glass microscope slide. Unstained material was observed under a light microscope with 10x and 40x objective lenses (Marcondes *et al.*, 2002).

Figure 3: Cell types seen in the vaginal smears of Control rats.



- Presence or absence of cell types and relative portion of each cell type were used to determine the stage of the estrous cycle of each rat. The realistic proportions of epithelial cells (A), cornified cells (B), and leukocytic cells (C) for both 4- and 5-day estrous cyclic rats are given in Figure 1 (Annamarie M *et al.*, 1984).

Figure 4: Appearance of typical smears from stages of rat estrous cycle (Annamarie M *et al.*, 1984).



A : Metestrous and Diestrous

B : Proestrous

C : Estrous

- The type of phase was indicated by observing the relative amounts of cell types observed in the smear.

Vaginal smears of female rats were studied microscopically for determining estrous cycle. Estrous cycle of the rats can be divided into four stage diestrous, proestrous , estrous and metestrous . 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 *et al.*, 2002; Annamarie M *et al.,* 1984). 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 of these cellular types (Marcondes *et al.*, 2002). Diestrous stage has abundant neutrophils and a few non-cornified epithelial cells, proestrous has abundant nucleated non-cornified epithelial cells, estrous begins with about 75% nucleated and 25% cornified cells, with cornified cells predominating as estrous continues. Metestrous has large numbers of neutrophils and scattered squamous ephithelial in the vaginal smear (Dennis FK, 1984)

Each stage has different duration. For diestrous stage, it lasts up to 57 hours, proestrous lasts 3-12 hours, around 12 hours for estrous and 21 hours for metestrous. The rats found in proestrous phase was caged with the males (Dennis FK, 1984).

Caging with males during Proestrous phase

Female rats found in proestrous stage were mixed into a male rat's cage in ratio of 1:2 or 1:1 in the evening at five in the evening. This is due to Estrous or "heat" period of sexual receptivity of the female, which occurs spontaneously at night after day 3 of the cycle. Proestrous stage which lasts for 12 to 14 hours and as ovulation occurs spontaneously within 8 to 12 hours after the onset of estrous (Dennis FK, 1984), helps in copulation and obtaining a pregnant rat. Evening is also appears to be the best time for mating as it prevents any disturbance for the rats as well.

Male rats with tested Fertility

A colony of male rats with proven fertility were obtained by selecting experienced male rats from Breeding stock of the Animal House, Health campus of University Science Malaysia.

Conformation of copulation by vaginal smear

The female rats were examined for evidence of copulation in the following morning by studying the vaginal smears. The day when spermatozoa are detected in the smear were considered as day 1 of pregnancy (Hiremath SP *et al.*, 1990).

Administration of plant extract orally for 7 days

Plant extracts were administered orally by means of a gavage from day one to day seven of pregnancy according to the group at eight to ten in the morning.

Laparotomy on 10^{th} day

The female pregnant rats were laparotomized under ether anesthesia on tenth day of pregnancy. During laparotomy, observations were done on both horns of the uterus for number and size of implantation, as well as for other signs of abnormal changes. The rats are then allowed for full term pregnancy.

Sacrifice on 10^{th} day

The female pregnant rats were sacrificed on 10th day of pregnancy. The number of pups in each horns was noticed.. The ovaries and uterus are removed and fixed in 10% formalin for histological studies.

Standard procedures were followed for tissue preparation and the samples were cut into thin sections of 6 micrometer after embedding in paraffin. The tissues were stained using H and E staining. Ovaries were observed for the presence of corpus lutea and atretic follice. The uterus were observed for the presence of trophoblastic cells which indicates partial resorption of implants, and endometrial decidualization.

On delivery day

The number of pups delivered on the 21^{st} day was noticed and recorded.

The efficacy of *Vitex pubescens* extracts in preventing pregnancy or maintaining pregnancy were determined by:-

1. Observing number of pups delivered by control rats and the rats treated with plant extracts.
2. Observing the number of resorption sites during laparotomy.
3. Ratio between number of fetuses observed during laparotomy and number of pups delivered in treated rats.

The efficacy of *Vitex pubescens* extracts in preventing pregnancy or maintaing pregnancy were supported by:-

1. Histological studies of ovary- observation of corpus lutea and atretic follicles.
2. Histological studies of uterus- observation of trophoblastic cells.

Statistical Analysis

The data of all the results obtained in the study were analyzed by using computerised statistical software SPSS 11.0. Mean and Standard errors of the mean were calculated for all the parameters of different group:-

1. Number of live fetus.
2. Number of resorption sites.
3. Number of live pups.
4. Number of late resorption.

Results of each parameter in different groups were analyzed by the application of One way-Analysis of Variance (ANOVA).

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

Figure 5: Experimental Protocol.