CHAPTER V

Discussion and conclusion

Part I Study on estrogenic activity in plants.

The dose response curves of standard estradiol and estrone revealed that uterotropic activity of estrone in immature female Swiss albino mice is approximately one third to that of estradiol at the same dose while estriol is very much less active in uterotropic activity than the former two. These findings corresponds to the original work of John Evan (19). Estradiol and estrone were simultaneously used as standard in almost every experiment in this study. Standard estrone seemed to be used more frequent in most of the experiment since estrogenic activity of unknowns were rather low in our study.

Estrogenic activity of various plant extracts which mostly comprise of volatile oil of commonly consumed flavoring plants were searched. Those are Ocimum basilicum, Ocimum sanctum, Cymbopogon citratus, Alpinia galanga, Zingiber cassmunar and Piper betel. Alcoholic extracts of Gastrochilus panduratus, Momordica charantia and Vigna sesquipedalis were also included in this study.

Volatile oil of Piper betel seemed to possess estrogenic activity in the first experiment, relative potency when expressed as standard estrone was 0.0257 µg (figure 19 p. 77) in 1.04 mg of oil which is equivalent to 2.4 gm of fresh plants. Thus 100 gm

fresh plant may possess estrogenic activity up to 1.09 µg of standard estrone. In repeated experiment, uterotropic activity expressed as standard estrone was 0.0239 µg in 0.52 mg of extract which is equivalent to 1.2 gm of fresh plant, thus 100 gm of fresh plant possess estrogenic activity equal to 1.99 µg of standard estrone. There was quite difference between results of the two, this may be due to variation in biological responses of different groups of animal itself.

Alcoholic extract of Vigna sesquipedalis showed distinctive uterotropic activity (figure 22 Table 2 p. 80). Relative potency expressed as standard estradiol was 0.0202 µg obtained from 2.15 mg extract which was equivalent to 0.088 gm of fresh plant. Thus 100 gm fresh plant possess estrogenicity equal to 22.95 µg of estradiol. Repeated experiment using standard estrone, relative potency was 0.0335 µg estrone in 0.54 mg of extract which equivalent to 0.022 gm of fresh plant. Thus 100 gm of fresh plant possess estrogenic activity as equal to 152.27 µg. standard estrone

However this plant seem to possess a high degree of estrogenic activity, although no vaginal opening was observed. This result was seemed to confirm the previous finding that majority of plant in Leguminosae are usually found to contain estrogenic activity. This finding may be a warning for the consumer of this plant that ingestion of great quantity may cause pharmacological estrogenic effects since the dose of ethinyl estradiol used for therapeatic purpose in human are ranging between 10–50 µg.

Volatile oil of Cymbopogon citratus did now show uterotropic activity while its alcohol extract showed some evidence of this activity. On the first experiment, low dose of this extract caused uterine enlargement, and relative potency expressed as standard estradiol (E 2) was 0.0259 µg in 3.99 mg of extract which is equivalent to 0.11 gm fresh plant, thus 100 gm of fresh plant possess estrogenicity equal to 23.54 µg of standard estradiol. The higher dose did not show any uterotropic activity and when the experiment was repeated once again using standard estrone, uterine enlargement was not significantly different from that of control in both low and high dose.

Plant estrogens mostly appear in nonsteroidal form of isoflavone type and this substance may be in part contributed to this uterotropic effect. These substances may be transformed into less active metabolite when it was stored for some time. This may be the reason for negative result of the repeated experiment.

Experiment with high dose often showed suppressive effect with great variety of responses of uterine enlargement. This is hard to interprete. However, at high concentration, active substances may not readily be absorbed as well as the dilute one. Other possibility is the substance acting as antagonist to estrogenic activity may be also present in high concentration.

Vagina opening was not observed in those experiments which showed estrogenic activity. However, standard estrogens of low dose did not cause vagina opening. The estrogenic activity of those tested meterials may be too low to cause any effect of this type.

Immature female mice used in this assay procedure did not differ in body weight greater than 3 mg and in ages which lies between 21-22 days. At the end of experiment the animals will reach the age of 25-26 days old and still be free from endogeneous hormone production especially estrogens. Despite this strict control, large variation in uterotropic responses to estrogens was often observed. Since animals were purchased for use, errorneous effects in animal age may be possible. The best alternative way is to raise animals in one's own animal house for use in the assay but this was not practical since it involves agreat deal of work, manpower, space and budget.

Since estrogens are often more conviniently administered in oil solution, uses of vegatable oil such as sesame oil and peanut oil are widely practised. Plant extracts studied for estrogenic activity were also dissolved in peanut oil. Volatile oil of plant were always completely dissolved in peanut oil, thus any required doses could be obtained. However, a problem of incomplete dissolvig in peanut oil of alcoholic extracts of plant studied existed due to limited dissolution capacity. The extracts often appeared as

sticky and syrupy mass eventhough after dissolution in peanut oil. Therefore, difficulties in injection to mice i.e. obstruction of needle etc. occurred. Primary purification process may be needed for better results in future experiment.

Estrogenic activity found in some of those plant extracts was generally low except those of Vigna sesquipedalis. This low incidence of estrogenicity may be partly derived from using crude extract for study. Other substances in the crude extract components may interfere with the uterotropic responses.

This uterine weight method for assay of estrogen is rather easy and practical since it took only four days. The technique to perform uterine incision is simple but this require a uniformity in weight and age of animal and a lot of animals must be used for each assay. The method itself is suffered from criticism of being not highly specific but this problem could be overcome by estimating other indicators of specific estrogenic activity i.e. the opening of vagina and change of vaginal epithelium in conjunction with the mouse uterine assay.

Part II Study on antispermatogenic effect of various plant extracts in rats and mice.

95% alcoholic extract of both large and small variety of M. charantia fruit did not show distinctive antispermatogenic effect. However, extract of large variety at dose level of 400 mg/kg/day significantly reduced weight of seminal vesicle & prostate in both groups of mice treated for 15 and 60 days. According to the work of Dixit V.P. et al (74) in which alcoholic extract of M. charantia fruit was orally given to dogs at this dose level, a significant reduction in testicular weight was observed within 40-60 days. This discrepancy may be due to species difference in response to testing material. The climatoric condition for cultivating plant may be also different and this probably affect chemical constituent of plant. M. charantia, small variety at dose level of 400 mg/kg/day given for continuous 60 days showed significant reduction in total sperm count but at a higher dose it did not affect any parameters measured. Animals may resist to the suppressive effect of the high dose. For this instance, there might be an increase also in some antagonistic substances of the active ingredient. Anyhow, M. charantia fruit seemed to possess some degree of antispermatogenic effect and further study should be carried on. The appropriate dose level should be selected according to LD_{50} dose. Generally animals could tolerate to a dose level of one fifth or to one forth

of LD_{50} dose and this dose is assigned as the highest dose.

Nearly all studies on reproduction and contraceptive must be conducted using animal models before human trials can begin. The number and type of animal studies may vary, depending upon potential risks and benifits to the recipient human clinical trial. Mice&rats are usually employed at first, rabbit and dog are followed and finally animal species that are closely related to human such as monkey are used.

Quite a large quantity of plant extract is required for antifertility testing in the male since experimental period usually prolongs up to 60 days. Therefore experiment with large animals such as rat and rabbit requires a larger amount of extract and the extracting process is also rather laborious procedure.

Mice are selected as animal model for antifertility testing of M. charantia fruit extract because of their low body weight; thus less amount of plant extract was required. However, form experience gained in handling with mice, mating has some problems, vagina smear of female might cause pain and trauma to animals especially when it was repeatedly performed and this may cause abortion. Difficulties in oral administration of plant extract to mice was also encountered.

Oil of O. basilicum demonstrated a marked antifertility effect in male rats, summarized data were shown in table 18 p. 113. Originally, doses level of 145.6, 291.3 and 582.6 mg/kg/day for

continuous 15 and 60 days were used in this study and it was found that a 582.6 mg/kg/day dose showed toxic effect (table 16) in reduction of body weight gain. So, we tried to perform the LD_{50} and about one fifth of LD_{50} dose was selected for further study. This was a dose level of 320 mg/kg/day for 60 days. The 15 days experiment was not done since mating effect of the group having same dose level was still highly positive (table17 p. 112).

A dose of 145.6 mg/kg/day, no antispermatogenic effect was observed either in 15 or 60 days experiments. Mating effect in these early experiments was not successful i.e. a high percentage of positive in control animal was not obtained. However, in latter experiments, much more experience was gained and nearly 100 % mating positive in control group were always obtained. The anti-spermatogenic effect occured from a dose level of 291.3 mg/kg/day of both 15 and 60 days of treatment. The 60 days treatment of this dose, sperm count was not changed but percentage of sperm motility was reduced and mating effect was also reduced to 50%. Body weight was not decreased at this dose. The higher dose i.e. 582.6 mg/kg/day seemed to possess a marked antispermatogenic effect in long period of treatment. Body weight of treated animal decreased 5% while in control group it increased up to 11%. Both grade and percentage of sperm motility were decreased. It seemed that effect on sperm motility required a long duration as well as high dose of treatment.

Unfortunately, experiment at dose level of 582.6 mg/kg/day scheduled for 60 days study was not finished (table16 p.110). The amimals had to be sacrified at day 40th instead of 60th and mating was not performed due to toxic effect of the extract, if it had been done it would show a reduction in number of mating positive since sperm motility both in percentage and grade of forward movement significantly changed. The sperm count was strikingly lowered down to 31% of the control.

In group recieving 320 mg/kg/day sperm count was significantly decreased and only slightly reduction in percentage of sperm motility was observed but mating positive was reduced only 14.3%.

The water retention effect was inconsistently observed in some experiment groups. Only rats given 582.6 mg/kg/day for continuous 15 days (table 15 p. 107) and 320 mg/kg/day for continuous 60 dyas (table 17 p.111) demonstrated a significant increase in testicular weight. The reason for this is difficult to interprete. Reduction in body weight of treated animals probaby due to toxic effect of plant extract since it was consistent and correlate with the total dose given and the degree of antispermatogenic effect. The active principle of the extract and the toxic fraction are the same or different moieties need further study.

Histological examination of testis which showed a significant reduction in number of spermatids and spermatozoa in seminiferous tubules of treated animals may be due to direct suppression of spermatogenic cells since the number and character of Leydig's cells seemed to be normal. It is likely that testing plant extract

inhibit a differentiation process from spermatogonia to spermatid and to spermatozoa. Sertoli cell may also be somewhat affected since a small reduction in cell numbers seemed apparent.

Testosterone production by Leydig cells and libido may not be interferred since Leydig cells seemed normal and were in active form. Further studies should be performed in detail including biological examination and toxicity. Further purification of the crude fraction may help to separate the toxic fraction from the antispermatogenic fraction.

Though histological examination of group having / 320 mg/kg/day for 60 days treatment showed marked devoid of spermatozoa in about 90% of lumen of seminiferous tubule of treated animals. However, high percentage of mating positive was still occured. It seemed that a reduction to azoospomia may be needed to cause complete infertility.

Volatile oil of O. sanctum did not show any antispermatogenic effect. Though a dose level calculated in term of gram of fresh plant/kg/day of O. sanctum studied was the same as O. basilicum. Body weight gain during experimental period was not changed from control group. The dose level studied may be too low to show any effect. However, this fraction of O. sanctum may itself not possess antifertility effect. The previous work on O. sanctum by Seth, S.D. et al (70), nevertheless, revealed a marked antispermatogenic effect i.e. reduction in sperm count, sperm motility and in weight of genital sex organ of benzene extract of dried leaves at dose level of 100-150 mg/kg/day for 15 days. Eventhough, the dose was equal to the dose used in this study but different extracts were applied.

In conclusion, oil of O.basilicum possess a marked antifertility effect in male rats but with some toxic effect as shown by lossing of body weight gain. Great quantity of extract could easily be produced for study since O.basilicum is widely and easily grown in Thailand and a technique of preparation of volatile oil is simple. Further detail study of this plant should be carried out in the hope that new contraceptive drug for human male may be developed in the future.