CHAPTER III

Materials & Methods

Part I Study on estrogenic activity in plants.

Chemicals

1. Ethanol 95% USP from the Government Pharmaceutical organization, Bangkok, Thailand.
2. Ethanol Absolute, Art. 983.
3. Peanut oil BP grade.
4. Standard estradiol, IKAPHARM, RAMAT-GAN, P.O.B. 31, ISRAEL No. 1955.
5. Standard estrone, IKAPHARM, RAMAT-GAN, P.O.B. 31, ISRAEL No. 2020.
6. Anaesthetic ether, May & Baker Ltd. Dagenham, England.

Equipments

1. Set of soxhlet apparatus (figure 9 p.47).
2. Rotary evaporator (Rotavapor-RE), Büchi Laboratory Techniques Limited, Flawil, Switzerland.
3. Set of steam distillation (figure 10 p. 48).
4. Electric blender.
5. Vortex mixer (Vortex Genie^{TM}), Scientific Industries Incorporation, N.Y., U.S.A.
6. Torsion balance, (Roller-Smith precision balances) Federal Pacific Electric Company, N.J., U.S.A.
7. Hot air oven.

8. Two pan balance (Harvard Trip Balance), Ohaus Scale Corporation, N.J., U.S.A.
9. Balance for weighing animals (Autogram® 1000), Ohaus.
10. Auto pipettee.
11. Dissecting instruments i.e. fine & medium scissors and forceps.
12. Insulin syringe.
13. Needle.
14. Phase Contrast microscope (Model BH), Olympus Optical Company Limited, Tokyo, Japan.

Animals

Immature female Swiss Albino mice aged 19-21 days with body weights ranged between 10-13 grams at beginning of experiments.

Sources of animals

Immature female Swiss Albino mice were purchased from the National Laboratory Animal Centre,Thambon Salaya, Nakornpatom, Thailand. They were acclimatized in the animal house of the Department for 1-2 days before use.

Diets

Mouse pellets from Gold Coin, Singapore, distributed by F.E. Zuellig (Bangkok) Ltd., Thailand.

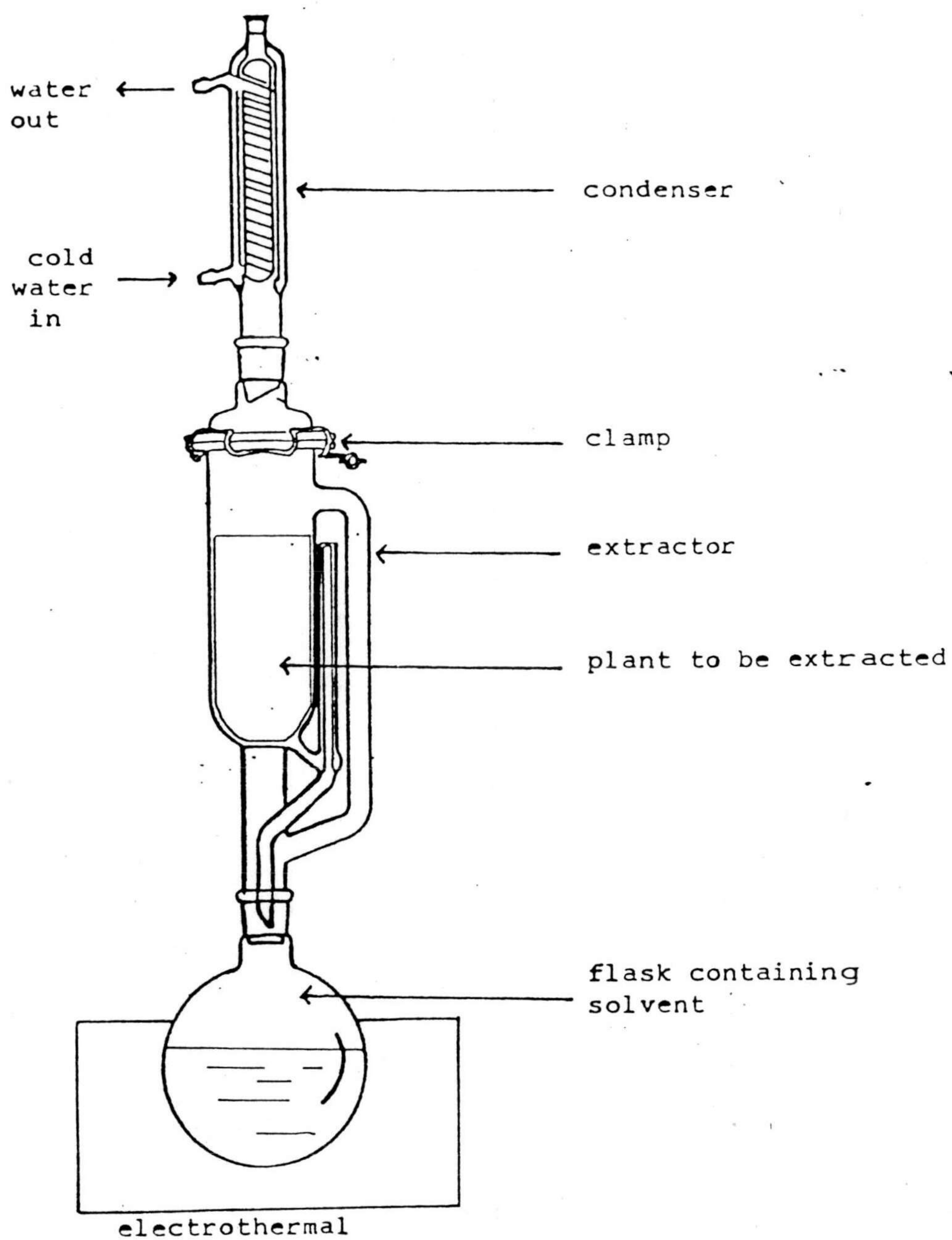

Figure 9 Diagram illustrating Soxhlet apparatus

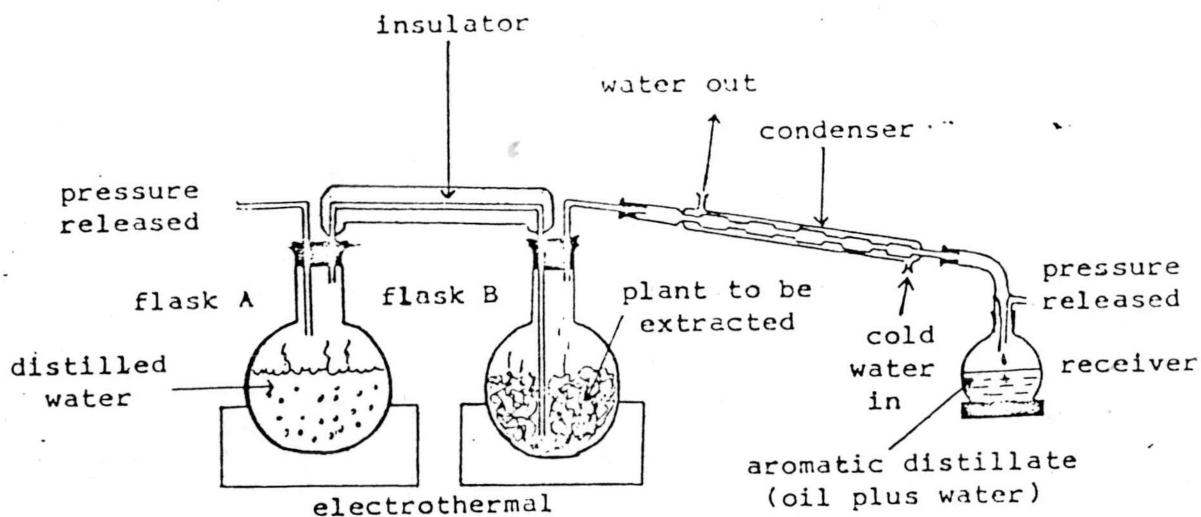

Figure 10 : Diagram showing set of steam distillation. Steam from flask A passed through U-shaped glass tube insulated with cotton wool to flask B. Volatile substances were then extracted & carried by steam passing along the condenser and finally condensed into aromatic distillate (which is composed of aromatic oil & water) and collected in the receiver.

Plant studied

Thai name	English name	Botanical name	Family
1 Kaphrao	Holly basil	Ocimum sanctum	Labiatae
2 Horapha	Sweet basil	Ocimum basilicum	Labiatae
3 Plai	-	Zingiber cassumunar	Zingiberaceae
4 Kha	-	Alpinia galanga	Zingiberaceae
5 Marachin	Bitter gourd	Momordica charantia (large variety)	Curcubitaceae
6 Takai	Lemon grass	Cymbopogon citratus	Graminae
7 Plu	Betel vine	Piper betel	Piperaceae
8 Makrut	Leech lime	Citrus hystrix	Rutaceae
9 Marakenok	Bitter gourd	Momordica charantia (small variety)	Curcubitaceae
10 Tuaphug Yao	Yard long bean	Vigna sesquipedalis	Leguminosae
11 Kachai	-	Gastrochilus panduratus	Zingiberaceae

Sources of plant

All plants were obtained from farms in Nakornpathom and Dhonburi provinces which was sent to Pak Klong Market.

Preparation of the standard Estrogens

Stock standard estradiol and estrone solution contained 10 ug each per 1 ml. in absolute ethanol were kept in the refrigerator at 4°C before use. Different concentrations of standard estrogens were prepared. Absolute ethanol was evaporated rapidly and carefully in a boiling water bath. Complete dryness is essential and achieved by evaporating under the nitrogen jet and then they were dissolved in peanut oil and mixed well by vortex mixer.

Preparation of various plant extracts for testing the estrogenic activity

After cleansing, the selected parts, the leaves, roots or fruits of the plants were sliced and dried in drying oven at 50°C. The dried slices were then pulverized in an electric blender. Each prepared plant was successively soxhletted with 95% ethanol until the overflowing solvent in the extractor become colorless. This process took about 3 days. The extracts were then evaporated in a rotary evaporator until no solvent remained in the constitution of the extract, then it was stored in the refrigerator 4°C.

Volatile oils of various plants were obtained by a steam distillation process. Selected part of fresh plant was put in a steam distillation apparatus (fig.10p.48). The extracting process continue for 6 hours. Anhydrous sodium sulfate was then added to the volatile oil to absorb residue of water which may be contaminated during extraction procedure.

Before using, the extracts were dissolved in peanut oil. Volatile oils are completely dissolved in peanut oil but alcoholic extracts are incompletely dissolved. The maximum dissolution concentration of alcoholic extract would be assigned as the highest dose level of the experiment.

Experimental procedures

Mouse uterine weight method as described by John evans (19) was used. Immature female swiss albino mice, 19–21 days old and 10–13 grams in weight, were subcutaneously injected daily with 0.05 cc. peanut oil solution containing tested materials for 3 days successively. Control animals were injected in the same manner with peanut oil only. Five to eight animals were used at each dose level. 18 hours after the last injection the animals were sacrificed under light ether anaesthesia. The uteri were separated from the vagina by cutting through the cervix, the surrounding tissue was stripped off and the uterotubal junction severed. The uteri were weighed immediately after the intra-uterine fluid had been pressed out on moistened blotting paper. Weights were read to tenths of a mg. on a Roller-Smith torsion balance. Estrogenic effects were confirmed by presence of vagina opening and vagina cytology.

Long-range stand curves of estradiol and estrone were performed and two doses of each standard were selected for using in all experiments. Results of two standard doses and two unknown doses were compared and check for parallelism when there was increase in uterine weight. The relative potency was then calculated in term of μg of standard either estradiol and estrone. The results are shown as mean ± standard deviation.

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

Chemicals for semen analysis

1. Reagents for preparing Hanks' balanced salt solution (80).

   a. NaCl, analytical grade, BDH Chemical Limited, Poole, England.

   b. $NaHCO_3$, analytical grade. BDH, England.

   c. KCl, analytical grade, E. Merck, Darmstadt, Germany.

   d. $Na_2HPO_4 \cdot 2H_2O$, analytical grade, Merck, Germany.

   e. KH_2PO_4, analytical grade, Merck, Germany.

   f. $MgSO_4 \cdot 7H_2O$, analytical grade, Merck, Germany.

   g. $MgCl_2 \cdot 6H_2O$, analytical grade, Merck, Germany.

   h. $CaCl_2$ (anhydrous), analytical grade, J.T. Baker Chemical Company, Phillipsburg, N.J., U.S.A.

   i. D (+)-glucose, laboratory grade, May & Baker Limited, Dagenham, England.

   j. Albumin-bovine, fraction V (95-99% albumin), Sigma Chemical Company, St. Louis, M.O., U.S.A.

Reagents in item (a) to (h) were prepared as stock solution in concentrations shown in p. 59. Glucose and BSA were added into stock solution shortly before use.

2. Reagents for sperm diluting agent.

   a. $NaHCO_3$, analytical grade, BDH. England.

   b. Formalin (Formaldehydlosung 35 Gew %) analytical grade, E. Merck, Darmstadt, Germany.

3. Anesthetic ether B.P. May & Baker Limited, Dangenham, England.

4. Bouin solution.

Equipments for semen analysis

1. Glasswares for general use including medicine droppers which was standardized to the size of about 2 mm. in diameter at their tip, test-tube, watchglass etc.
2. Pipetman Gilson France.
3. Hemocytometer (Improved Neubauer Chamber, 0.1 mm. deep) American Optical Corporation, N.Y. U.S.A.
4. Plastic moist chamber.
5. Dissecting instruments i.e. fine & medium scissors and forceps.
6. Vortex mixer (Vortex-Genie^{TM}), Scientific Industries Incorporation. N.Y., U.S.A.
7. Phase contrast microscope (Model BH), Olympus Optical Company Limited, Tokyo, Japan.
8. Laboratory counter, Clay Adams, N.J., U.S.A.
9. Torsion balance, (Roller-Smith procision balances), Federal Pacific Electric Company, N.J., U.S.A.

Equipments and chemicals for preparing various plant extracts

1. 95% ethanol U.S.P.
2. Acacia powder B.P.
3. Set of soxhlet apparatus Pyrex® (figure 9 p.47).
4. Set of steam distillation (figure 10 p.48).
5. Rotary evaporator (Rotavapor-RE), Buchi Laboratory Techniques Limited, Fawil, Switzerland.
6. Hot air oven.
7. Electric blender.
8. Syringe with feeding metalic needle.
9. Balance for weighing animals (Autogram® 1000), Ohaus.
10. Two pan balance (Harvard Trip Balance), Ohaus Scale Corporation, N.J., U.S.A.

Animals

1. Rats.

1.1 Sexually mature male Wistar strain rats aged 32 months old with the body weight ranged between 250-350 grams at the beginning of experiments.

1.2 Sexually mature female Wistar strain rats aged 2-3 months old with the body weights ranged between 170-250 grams.

2. Mice.

2.1 Sexually mature male Swiss Albino mice aged 3½ months old with the body weights ranged between 25-30 grams at the beginning of experiments.

2.2 Sexually mature female swiss Albino mice aged 2-4 months old with the body weights ranged between 25-40 grams.

Sources of animals

Rat

Wistar strain rats were purchased from National Laboratory Animal Centre. Thambon Salaya, Nakornpatom, Thailand. They were acclimatized in our laboratory for 1 month before they entered the experiments.

Mice

Swiss albino mice raised in our laboratory were used.

Diets

Mouse pellets from Gold Coin, Singapore, distributed by F.E. Zuellig (Bangkok) Ltd., Thailand.

Plants studies.

Momordica charantia fruit both large and small varieties (fig.11p.57) Ocimum basilicum (fig.12p.58) and Ocimum sanctum (fig.13p.58).

Sources of Plants.

M. charantia fruit of both varieties and O. sanctum were purchased from the market (Pak Khlong Market). Essential oil of O. basilicum was kindly received from Thailand Institute of Scientific and Technological Research (TISTR) Bangkren, Bangkok Thailand.

(1)

(2)

Figure 11 The photograph of <u>Momordica charantia</u>

(1) small variety (2) large variety

Figure 12

The photograph of

Ocimum basilicum

(Horapha)

Figure 13

The photograph of

Ocimum sanctum

(Kaphrao)

Preparation of Hanks' balanced salt solution. (80)

Various chemicals was prepared in various concentration as follows :-

1.	NaCl	8.0	grams
2.	KCl	0.4	"
3.	$MgSO_4.7H_2O$	0.2	"
4.	$MgCl_2$	0.1	"
5.	$CaCL_2$	0.14	"
6.	$Na_2HPO_4.2H_2O$	0.06	"
7.	KH_2PO_4	0.06	"
8.	$NaHCO_3$	0.35	"
9.	Glucose	1.0	"
10.	BSA	4.0	"

Reagents in the list no 1-8 were all dissolved in deionized water to make up a final volume of 1 litre and kept as stock solution in the refrigerator ($4^{o}C$) for months. Glucose and BSA were added just before use.

Preparation of the plant extracts.

1. Preparation of Momordica charantia fruit extract.

1.1 The large size variety.

The plants were sliced and then dried in $50^{o}C$ hot air oven. They were powderized by electric blender. The powderized material of plant was extracted in a soxhlet apparatus using 95% ethanol as vehicle until the overflowing ethanol in the

extractor became colorless. The extract was then concentrated under vacuum reduced pressure by Büchi rotatory evaporator to give a thick syrupy mass.

1.2 The small size variety.

The fresh fruit excluding seed was homogenized by blender and macerated using 95% ethanol as menstrum. The extract obtained was concentrated under vacuum reduced pressure by Büchi rotatory evaporator.

2. Preparation of volatile oil of O. basilicum and O. sanctum.

Volatile oil of both plants were prepared by a steam distillation procers as described in p.48 (figure 10).

Administration of the extracts.

All the extracts were prepared in the form of emulsion or suspension with the aid of gum acacia as suspending agent and were orally fed to experimental animals using metalic needle modified in size and shape in order to suit rats and mice respectively (figure 14 p.61).

The ratio of plant extract : water : gum acacia for each plant extroct is as follow:

O. basilicum = 2 : 15 : 1

O. sanctum = 4 : 8 : 1

M. charantia extract was suspended in 2% gum acacia solution.

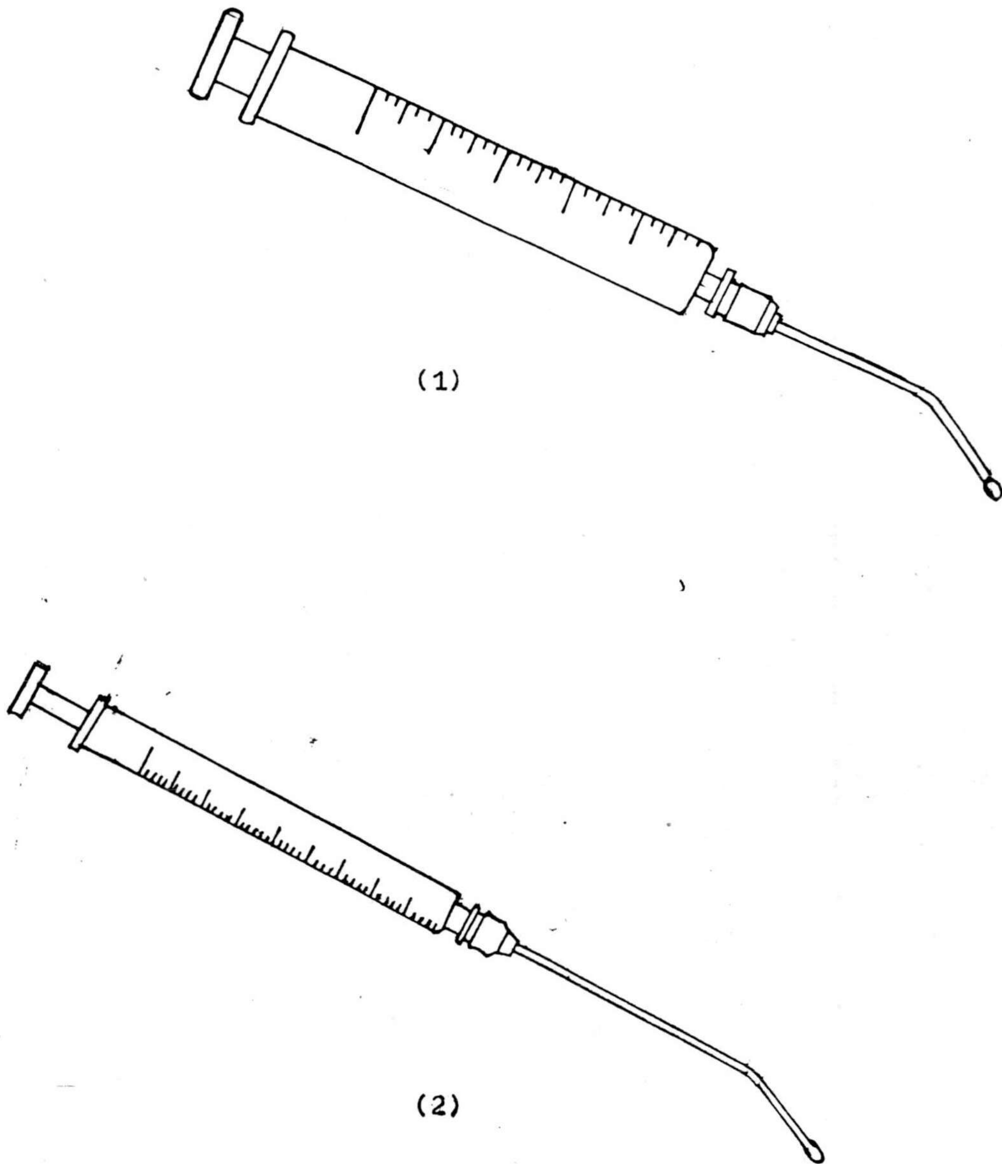

Figure 14 Demonstrate metalic needle and syringe for oral administration of plant extract

1) for rat

2) for mice

<u>Experimental procedures</u>.

Body weights of animals (mice or rats) of both control and experiment groups were taken at the start and at the end of each experiment. The animals were fed with the extracts through 15 and or 60 days according to WHO protocol MB 50 (81).

Mating was performed with one or two female animals (mice or rats) according to MB 50 of WHO at the end of experiment. Three days after mating, the male animals (mice or rats) were sacrificed with excess ether. The left cauda epididymis was weighed by torsion balance and then transfered into a watch glass containing 2 ml. of Hanks' solution & then chopped into small pieces to release all the spermatozoa into the solution, this suspension is ready for sperm motility assessment. The sperm suspension was mixed using vortex mixer at the highest speed (speed 10) and a drop was transfered onto a glass slide for sperm motility examination under phase contrast microscope using X400 magnification. The motile and nonmotile spermatozoa were counted up to total of at least 100 spermatozoa by random scanning around the central area of the slide. Grade of motile sperms was estimated to be good, fair and poor represented by grade 3, 2 or 1 respectively. In mice the whole epididymis was prepared in 0.5 ml. of Hanks' solution. The weight of left testis and of seminal vesicles and prostate were also recorded. In experiment which gave a positive result, the testis was fixed in Bouin' solution for further histological examination.

Sperm count determination.

Sperm concentration was determined by hemocytometer. A volume of 10/μl of well mixed sperm suspension in Hanks' solution was pipetted with automatic pipette into sperm diluting agent. Two dilution were selected for each specimen, either 1 : 25 & 1 : 50 & 1 : 200, depending on the estimated concentration. The dilute specimens were thoroughly mixed by the vortex mixer at the highest speed (speed 10). A drop of each diluted specimen was immediately transfered to hemocytometer. The hemocytometer was placed in a moist chamber for at least 15 minutes to allow all cells to sediment and was then counted under the light microscope at X 200 magnification. Only morphologically mature spermatozoa with tail intact were counted from the 1st, 2nd, 3rd and 4th square (fig. 15p.64) which held the total volume of 4 X 0.1 mm^3 of diluted semen. The difference between the total counts of spermatozoa in the two dilutions should not exceed 10% at low sperm density ($<$60 mill/ml.) or 20% at high density ($>$60 mill/ml.) (82). Sperm concentration was expressed in term of number of spermatozoa per millilitre of diluted solution. Total sperm count was equal to sperm concentration (mill/ml.) multiplied by total volume (ml.) of diluting fluid.

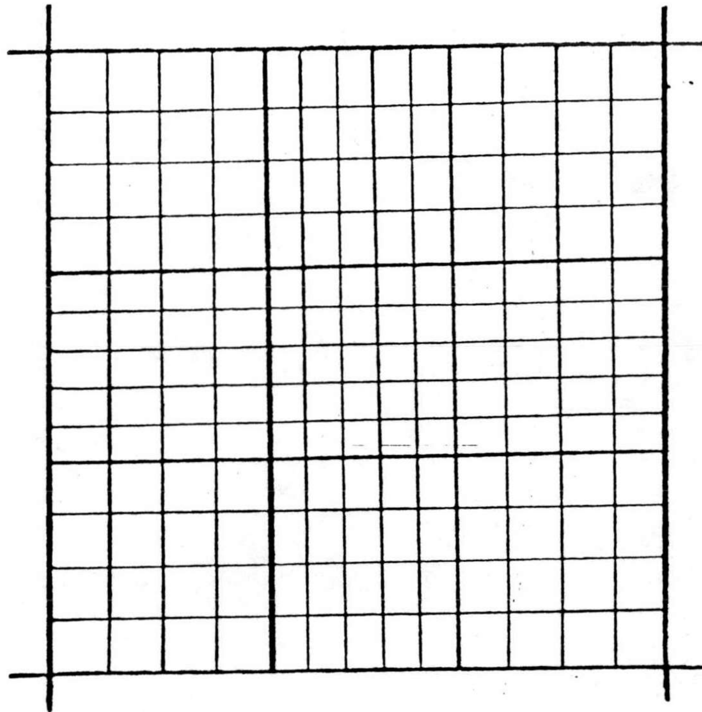

Figure 15 Grid markings on a standard Neubauer hemocytometer. Only morphologically mature spermatozoa with tail were counted from the square 1, 2, 3 and 4 which held the total volume of 4 x 0.1 mm^3 of diluted semen.