CHAPTER III

MATERIALS AND METHODS

Materials

3.1 Chemicals for Ames test

3.1.1 Sodium nitrite (Sigma Chemical, St Louis, USA)

3.1.2 Ammonium sulfamate ($NH_2SO_3NH_4$) (Sigma Chemical, St Louis, USA)

3.1.3 D- biotin (Sigma Chemical, St Louis, USA)

3.1.4 Sodium dihydrogen phosphate (NaH_2PO_4) (Sigma Chemical, St Louis, USA)

3.1.5 Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) (Merck, Darmstadt, Germany)

3.1.6 Histidine monohydrochloride (Merck, Darmstadt, Germany)

3.1.7 Hydrochloric acid 34% w/v (Merck, Darmstadt, Germany)

3.1.8 Potassium chloride (Merck, Darmstadt, Germany)

3.1.9 Sodium chloride (Merck, Darmstadt, Germany)

3.1.10 Crystal violet (Merck, Darmstadt, Germany)

3.1.11Sodium ammonium hydrogen phosphate tetrahydrate ($NaNH_4HPO_4.4H_2O$) (Fluka, Buchs, Switzerland)

3.1.12 Dipotassium hydrogen phosphate anhydrous (K_2HPO_4) (Fluka, Buchs, Switzerland)

3.1.13 Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) (Fluka, Buchs, Switzerland)

3.1.14 D(+)-glucose anhydrous (Fluka, Buchs, Switzerland)

3.1.15 Aminopyrene (Aldrich, St Louise, USA)

3.1.16 Citric acid (BDH Chemicals, Pool, England)

3.1.17 Ampicillin sodium salt (General Drugs House, Bangkok, Thailand)

3.1.18 Bacto agar (Merck, Darmstadt, Germany)

3.1.19 Oxoid nutrient broth No. 2 (Oxoid, Hants, England)

3.1.20 Acetonitrile (J.T.Baker, Phillipsburg, USA)

3.2 Instruments

3.2.1 Utraviolet Spectrophotometer (PYE Unicam SP 1800)

3.2.2 Filter paper (Whatman No.1)

3.2.3 pH paper (Whatman)

3.2.4 Analytical balance (Oertling model NA 264)

3.2.5 Analytical balance (Sartorius 2200)

3.2.6 Hot air oven (WTB binder No.960462, Germany)

3.2.7 Incubator (WTB binder No.960004, Germany)

3.2.8 Pipette Controller (Brand, Germany)

3.2.9 Transferpipette (Brand Germany , Gilson France, Nichiryo Japan)

3.2.10 Microprocessor pH Tester (Sharp pH, Italy)

3.2.11 Vortex-2 Genie (Scientific Industries, USA)

3.2.12 Pressure steam sterilizers (All American model No.1941, USA)

3.2.13 Colony counter

3.2.14 Rotavapor (Buchi, Switzerland)

3.2.15 Vacuum-system (Buchi B-169, Switzerland)

3.2.16 High vacuum grease (Dow corning corporation, USA)

3.2.17 Shaking water bath (Hotech model 905, Taiwan)

3.2.18 Blender (Imarflex super blender IF-308)

Methods

3.3 Experimental Design

Determination of the mutagenicity modifying activity of herbal drinks on the menstrual regulatory and haematinic traditional preparations were carried out according to the experimental design shown in Figure 1. The concentrated menstrual regulatory and haematinic traditional preparations were interacted with nitrite at pH 3.0-3.5, 37^{O}C for 4 h and determined their mutagenicity through *Salmonella typhimurium* strains TA 98 and TA 100 in the absence of metabolic activation. The mutagenicity of each herbal drink was also assayed without nitrite pre-treatment; it was, then, studied on any modifying effect on the mutagenicity of nitrite treated menstrual regulatory and haematinic traditional preparations.

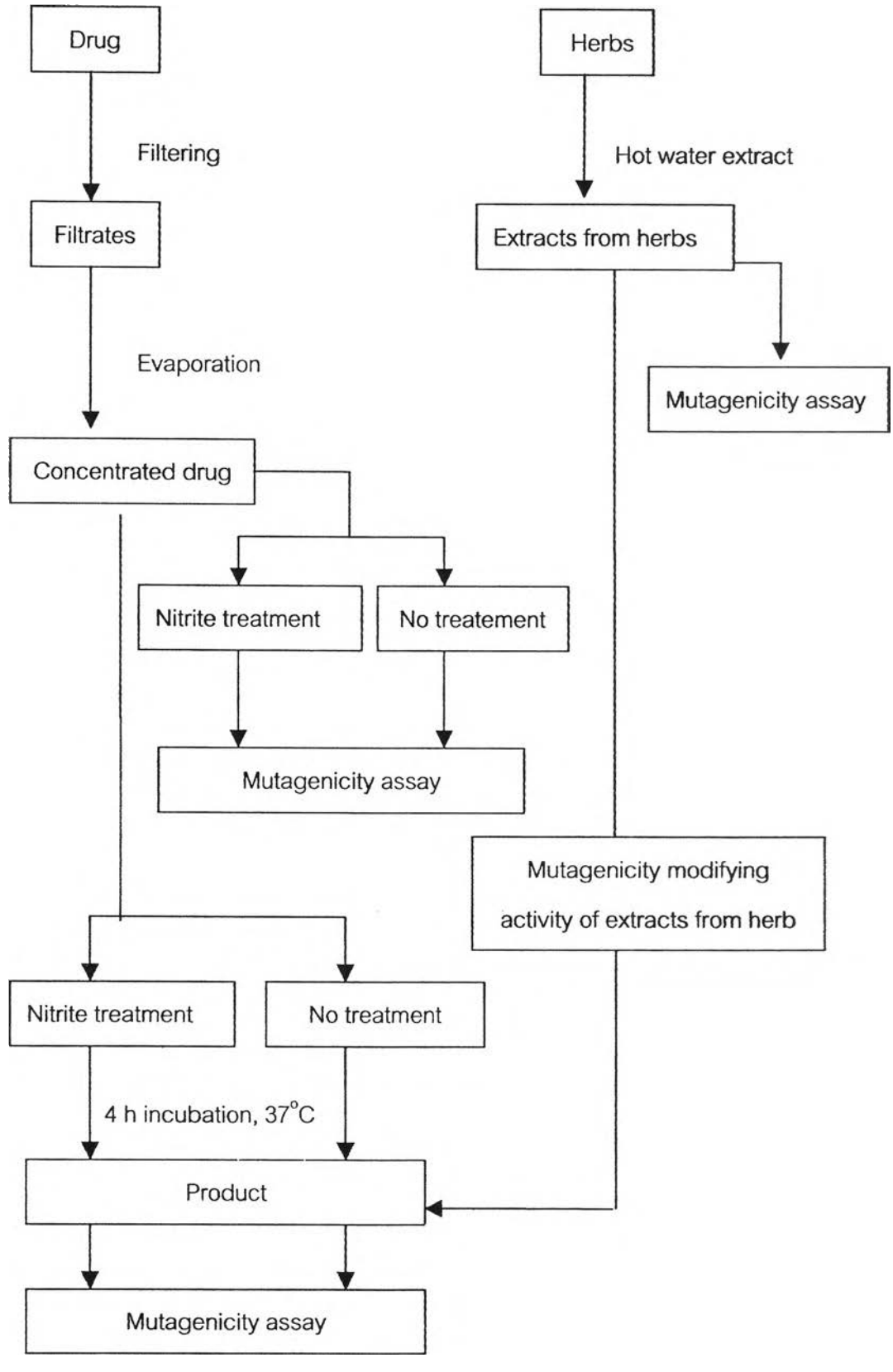

Figure 1. Experimental design of mutagenicity assay

3.4 Ames test

Modified method of Yahagi *et al.* (1975), including pre-incubation of the test sample with *Salmonella typhimurium*, was used throughout this study. Two strains of *Salmonella typhimurium* TA98 and TA100, which required histidine as growth factor, were used. They were provided by Assoc. Prof. Dr. Kaew Kangsadalampai (Institute of Nutrition, Mahidol University). Overnight culture of each strain was prepared in Oxoid nutrient broth No. 2 at 37°C with shaking. Manipulation of the culture was done as suggested by Maron and Ames (1983) (Appendix).

Minimal agar plate, containing 30 ml of minimal glucose agar medium consisting of 1.5% Bacto-Difco agar and 2% glucose in Vogel-Boner medium E and top agar, containing 0.6% agar and 0.5% NaCl, were prepared as described by Maron and Ames (1983) (Appendix). Ten ml of a sterile solution of 0.5 mM L-histidine/biotin was added to each 100 ml of the top agar and mixed thoroughly by swirling before use (Appendix).

3.5 Preparation of Menstrual Regulatory and Haematinic Traditional Preparations

Two drugs were selected on the basis that they were shown nearly the same mutagenicity after being treated with nitrite (แก้ว กังสดาลอำไพ และวรรณี โรจณโพธิ์, 2531) but their compositions were different. Drug number 1 was composed of *Piper nigrum* Linn. (พริกไทย), *Piper longum* Linn. (ดีปลี), *Myristica fragrans* Houtt. (ดอกจันทน์, ลูกจันทน์), *Plumbago* sp. (เจตมูลเพลิง), *Cinnamomum* sp.(อบเชย) while drug number 2 was composed of *Angelica sinensis* Diels (โกฐเชียง), *Ligusticum chuanxiong* Hort. (โกฐ

หัวบัว), *Cinnamomum* sp. (อบเชย), *Salvia miltiorrhiza* Bunge (ตันเซียม), *Astragalus membranaceus* (Fisch.) Bunge (อิ๋งชิ). Amounts of main ingredients of each drug are shown in Table1. Each drug was filtered through Whatman filter paper No.1 to remove insoluble material. Then, the filtrate was evaporated to about 10% of its original volume under reduced pressure at 50°C and each concentrated drug was autoclaved at 121°C for 15 min before use.

Table 1. The composition of the menstrual regulatory and haematinic traditional preparations in this study

Botanical name	Thai name	g. in 1000 ml
Drug 1		
Piper nigrum Linn.	พริกไทย	30
Piper longum Linn.	ดีปลี	30
Myristica fragrans Houtt.	ดอกจันทน์, ลูกจันทน์	30
Plumbago sp.	เจตมูลเพลิง	30
Cinnamomum sp.	อบเชย	30
Drug 2		
Angelica sinensis Diels	โกฐเชียง	26.67
Ligusticum chuanxiong Hort.	โกฐหัวบัว	30
Cinnamomum sp.	อบเชย	16.67
Salvia miltiorrhiza Bunge	ตันเซียม	16.67
Astragalus membranaceus (Fisch.) Bunge	อิ๋งชิ	20

3.6 Preparation of Herbal Drinks

Herbs (*Hibiscus sabdariffa* Linn. (กระเจี๊ยบ), *Chrysanthemum morifolium* Hemsl. (เก๊กฮวย), *Carthamus tinctorius* Linn. (คำฝอย), *Morus alba* Linn. (ใบหม่อน), *Aegle*

marmelos (Linn.) Corr. (มะตูม), *Centella asiatica* (Linn.) Urban (บัวบก)) were purchased from a local market in Bangkok. Each sample was washed with tap water and dried in a hot air oven at 50°C. Dried herb was minced and boiled in distilled water (1:10 W/V) for 20 min. The aqueous solution was filtered through a cotton mesh and the filtrate was filtered through filter paper No.1 to remove insoluble material. The filtrate was evaporated to about 10% of its original volume under reduced pressure at 50°C and then the concentrate was autoclaved at 121°C for 15 min before use.

3.7 Mutagenicity of Concentrate from Menstrual Regulatory and Haematinic Traditional Preparations

The concentrate from each sample in various amounts (50,100 and 200 µl) was introduced into the tube containing appropriate volume of distilled water to obtain the final volume of 200 µl, dilute HCl containing sufficient acid to acidify the reaction mixture to pH 3.0-3.5 was added until the pH of mixture was stable. The distilled water was added to obtain the final volume of 1000 µl. Then, the tube with stopper was shaken at 37°C for 4 h and stopped by placing the tube in an ice bath for 1 min. Distilled water was added to the reaction mixture to obtain the final volume of 1250 µl., and the reaction tube was immersed in an ice bath for another 10 min.

Direct mutagenicity of the reaction mixture was assayed according to the preincubation (at 37°C for 20 min) method of Yahagi *et al.* (1975), using *Salmonella typhimurium* in absence of S-9 mix.

Mutagenic assay

To a tube containing 0.1 ml of the reaction mixture was added 0.5 ml of Na_3PO_4-KCl buffer (pH 7.4), and then 0.1 ml of fresh overnight nutrient broth culture of the tester strain (1-2x10^8 cells). After incubation, 2 ml of molten top agar (45°C) containing 0.5 mM L-histidine / biotin was added. It was mixed well and poured onto a minimal glucose plate. The plate was rotated to achieve uniform colony distribution and incubated at 37°C in the dark for 2 days. The procedure, step by step, of the test in shown in Figure 2.

Induced his^+ revertant colonies were counted and compared to the established spontaneous mutation. Duplicate plates were carried out for each sample and all experiments were performed at least twice.

For each experiment, positive control and negative control plates were included in the assay. Negative control plate containing the bacteria and distilled water was used to establish the spontaneous mutation of each tester strain.

Aminopyrene treated with nitrite in acid solution was used as a positive control. Briefly explain, appropriate volume (20 µl for testing on *Salmonella typhimurium* TA98 and 40 µl for TA100) of aminopyrene (0.075 mg/ml) in a tube fitted with a plastic stopper was mixed with 0.2 N hydrochoric acid (sufficient to acidify the reaction mixture to pH 3.0-3.5) and 0.25 ml of 2 M sodium nitrite. The volume was adjusted to be 1,000 µl giving the final concentration of 500 mM. nitrite.

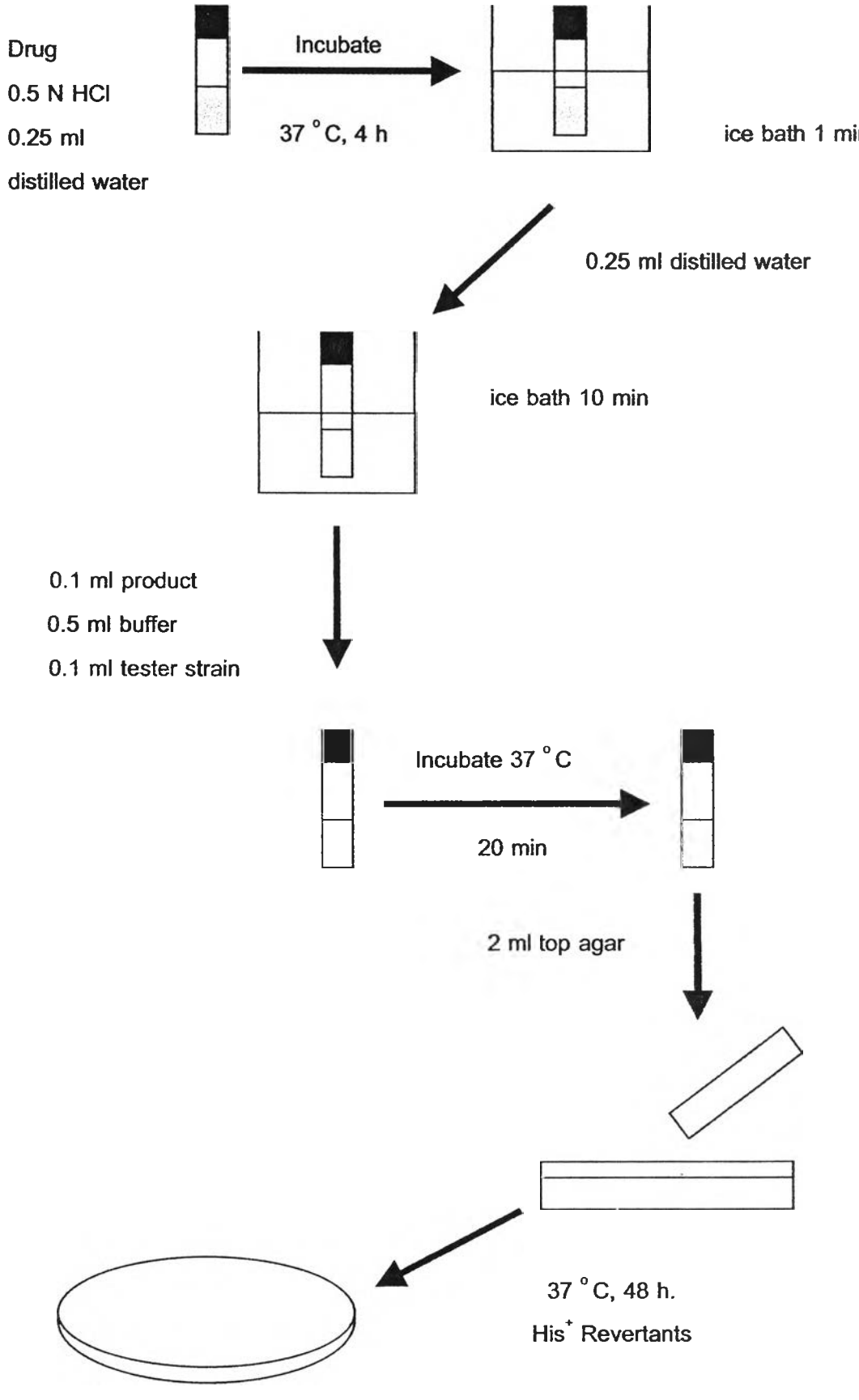

Figure 2. Direct mutagenicity evaluation of menstrual regulatory and haematinic traditional preparations using the Ames test (pre-incubation modification)

3.8 Mutagenicity of Nitrite Treated Concentrates from Menstrual Regulatory and Haematinic Traditional Preparations

To determine the mutagenicity of concentrates from menstrual regulatory and haematinic traditional preparations treated with nitrite, an aliquot of each concentrate 50,100 and 200 µl was introduced into the tube containing 150, 100 and 0 µl of distilled water respectively. Dilute HCl, which contain sufficient acid to acidify the reaction mixture to pH 3.0-3.5, was added. Adding 0.25 ml of 2 M sodium nitrite started the reaction. The final volume was 1,000 µl. Then the tube was incubated at 37°C with shaking for 4 h. The reaction was stopped by allowing the mixture to stand for 1 min in an ice bath. Ammonium sulfamate (2M, 0.25 ml) was added to the reaction mixture to decompose the residue nitrite and the reaction tube was immersed in an ice bath for another 10 min. Finally, the mixture was determined for its mutagenicity by Ames test as described in Figure 3.

3.9 Mutagenicity of Extracts from some Herbs

An aliquot of 50, 100 and 200 µl of each extract from herbs was adjusted to 200 µl by distilled water and the mutagenicity of the extracts without nitrite treatment was conducted follow the procedure shown in Figure 2.

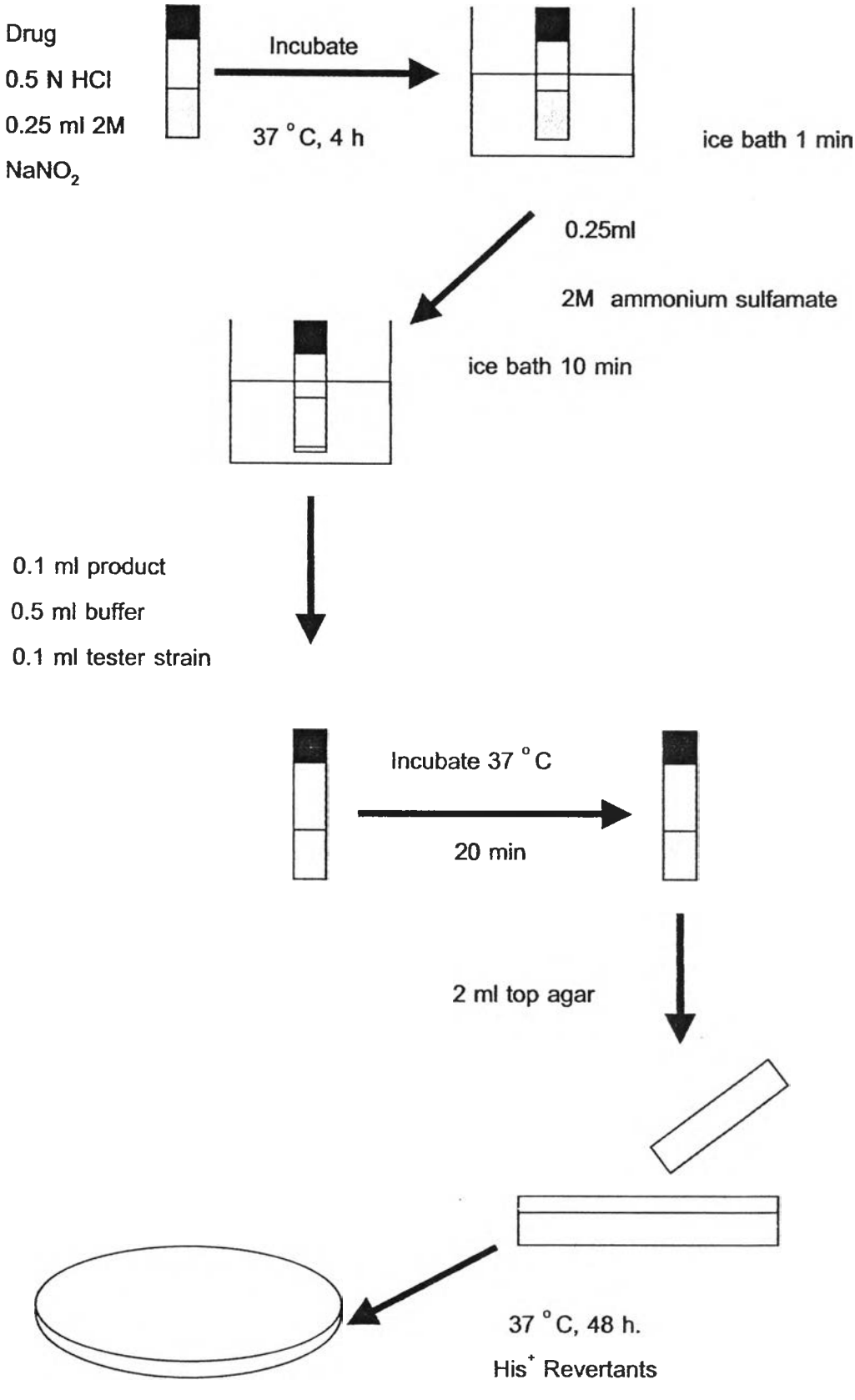

Figure 3. Steps to determine the mutagenicity of nitrite treated menstrual regulatory and haematinic traditional preparations using the Ames mutagenicity test (pre-incubation modification) in the absence of metabolic activation.

3.10. Mutagenicity modification of extract from some herbs on mutagenic product of nitrite treated concentrates from menstrual regulatory and haematinic traditional preparations.

3.10.1 Incubation without nitrite

An aliquot of 100 µl of concentrate of medicinal sample was used in this study and the experiment was performed following the steps in Figure 2. After 4 h of incubation period, aliquot (50, 100 and 200 µl) of each extract from herbs were added (duration of interaction = 0 h). Then each sample- herb was determined for its mutagenicity by Ames test as described previously (experiment 3.7).

3.10.2 Incubation with nitrite

The reaction between an aliquot of 100µl of concentrate of medicinal sample and 0.25 ml of 2 M sodium nitrite was conducted in this study following the step in Figure 3. Before the reaction mixture was subjected to the mutagenicity test, it was added with aliquot (50, 100 and 200 µl) of each extract from herbs.

3.11 Data Manipulation

The mutagenicity of each sample was presented as number of histidine revertants per plate. The data were reported as means with standard deviation of four plates from two different experiments. And for easier to compare the degree of mutagenicity, the results are also expressed as mutagenicity index (MI) which was calculated from the average value of number of histidine revertants per plate of sample

divided by average value of spontaneous revertants. Sample expressed its mutagenicity higher than 2 times of spontaneous revertants with a dose-response relationship was evaluated mutagenic. Percentage of modification (either increase or decrease on mutagenicity of sample) was calculated as following:

Percentage of modification = (A – B) / (A - C) x 100

A= Number of histidine revertants induced by nitrite treated samples

B= Number of histidine revertants induced by mutagen in the present of herbs extracts

C= Number of histidine revertants induced in the presence of water alone (negative control).

The inhibition (or enhancement) of mutagenicity may be divided into four classes (Calomme *et al.*, 1996)

±0-20% inhibition (or enhancement) : no effect

±20-40% inhibition (or enhancement) : weak activity

±40-60% inhibition (or enhancement) : effective or moderate activity

more than 60% inhibition (or enhancement) : strong or potent activity

+ or – indicates that the extract decreased or increased the mutagenicity of the model.