Chapter II

MATERIALS AND METHODS

Instruments

1. Spectronic 20 (Bausch & Lomb Regulated Model 59)
2. Analytical balance (August-Sauter)
3. Centrifuge (Roto-Uni II)
4. Selectapette (Clay-Adams)
5. Pipettes, 0.1 ml, 1.0 ml, 5.0 ml (Witeg)
6. Micro-beakers, 5 ml (Pyrex)
7. Glass syringes, 25 ml (B-D, Becton, Dickinson Ind. Cirurgicas) with a blunted needle for oral administration of dosed liquid drugs to rabbits.
8. Borosilicate culture tubes, 12 x 125 mm, with teflon-lined screw caps (Pyrex)
9. Burettes, 50 ml (E. Mil)
10. Test tubes, 13 x 100 mm (Kimax)

Chemicals

1. Anhydrous D-glucose (Mallinckrodt Chemical Works)
2. Benzoic acid (Hopkin & Williams)
3. Sodium fluoride (Mallinckrodt Chemical Works)
4. Trichloroacetic acid (Carlo Erba)
5. o-Toluidine (BDH Chemicals)
6. Thiourea (E. Merck AG. Darmstadt)
7. Glacial acetic acid (Carlo Erba)

8. Xylene (Dow Chemicals)

9. Ethanol (BDH Chemicals)

All chemicals were of analytical grade

10. Chlorpropamide USP XVIII (kindly supplied by chas. Pfizer, Inc., New York).

Sources and identification of plant material

The berries of Solanum sanitwongsei Craib. and Solanum trilobatum L. were collected from the waste ground opposite Savangkanivasana, Sumutprakarn and from a private garden at Bangkhungor in Klong Bangkoknoi, respectively.

These berries of S. sanitwongsei and S. trilobatum were identified by Assistant Professor Bumrung Tuntisewie of the Pharmacognosy Department, Faculty of Pharmaceutical Science, Chulalongkorn University.

Experimental subjects

Healthy male and female rabbits, weighing between 1.6 to 3.0 kg, were selected. They were kept individually in clean, well maintained cages, and were well fed with liberal amounts of rabbit food pellets (consisting of corn, soya-bean, mung bean, yeast, peanut and leaves of lead tree) and of water. The rabbits were accustomized in an air-conditioned room for not less than one week before being used in the experiment and were fasted for about 18 hours prior to their use in each experiment. During these hours and

throughout the experimental period only water was allowed to remain in the cage.

Method

1. Method of determination of blood sugar. The blood sugar determinations were carried out using the o-Toluidine Method. (Hyvärinen and Nikkilä, 1962).

1.1 Preparation of anticoagulant. A 2.5% solution of sodium fluoride was prepared using:

Sodium fluoride	2.5	g
Distilled water added to,	100	ml

Into each microbeaker 0.5 ml of sodium fluoride solution was added and the microbeaker was then dried in a hot-air oven at 100°C for 30 minutes.

1.2 Preparation of protein-precipitating reagent. A 3% solution of trichloroacetic acid was prepared using:

Trichloroacetic acid	3	g
Distilled water added to	100	ml

1.3 Preparation of colour reagent. This colour reagent was prepared using:

o-Toluidine	60	ml
Glacial acetic acid	940	ml
Thiourea	1.5	g

The thiourea was dissolved in 940 ml of

glacial acetic acid; and 60 ml of o-toluidine were then added.

o-Toluidine was almost colourless. It was re-distilled at 80°C in vacuum, and the re-distilled o-toluidine was kept in a brown flask in the refrigerator.

The reagent so obtained and used was almost colourless. It was stored in brown container away from direct sunlight and from contact with metal or rubber.

1.4 Preparation of a standard calibration curve (Dubowski, 1962). Standard 25 to 200 mg/100 ml solutions of glucose were prepared, in steps of 25 mg, by diluting a stock solution of glucose (containing 1.0 g of anhydrous D-glucose in 100 ml of 0.2% benzoic acid solution) with appropirate volumes of 0.2% benzoic acid solution.

The following standard solutions were prepared:

conc. of std. glucose solution (mg %)	ml of 1% stock glucose solution	ml of 0.2% benzoic acid solution
25	2.5	97.5
50	5.0	95.0
75	7.5	92.5
100	10.0	90.0
125	12.5	87.5
150	15.0	85.0
175	17.5	82.5
200	20.0	80.0

Into each individual test tube containing 1.0 ml of trichloroacetic acid solution was added separately 0.1 ml of the different concentrations of the standard solutions of glucose and both solutions in each tube were well mixed. A blank solution tube was prepared containing similar quantities of trichloroacetic acid and of 0.2% benzoic acid solutions. Five-tenths of a milliliter of each of these mixtures was transferred with the aid of a selectapette into another individual test tube, to which were added 4.5 ml of colour reagent from the burette and both solutions in each individual tube were mixed. All of these tubes were simutaneously placed in a boiling water bath for 8 minutes and then cooled. The absorbances (O.D.) of the blue-green coloured solutions containing different concentrations of glucose were determined at 630 nm in a Spectronic 20 (Bausch & Lomb, Regulated Model 59) with corresponding filter against the blank. The spectrophotometric readings were made within 30 minutes after the cooling of the test tubes.

A standard calibration curve of glucose concentrations was obtained by plotting the absorbances against the different concentrations of standard glucose solutions (mg %) (Fig. 3). This standard calibration curve was prepared along with each individual experiment, and was always found to be linear, which confirmed the finding of Hultman (1959) that the calibration curve in his study was found to

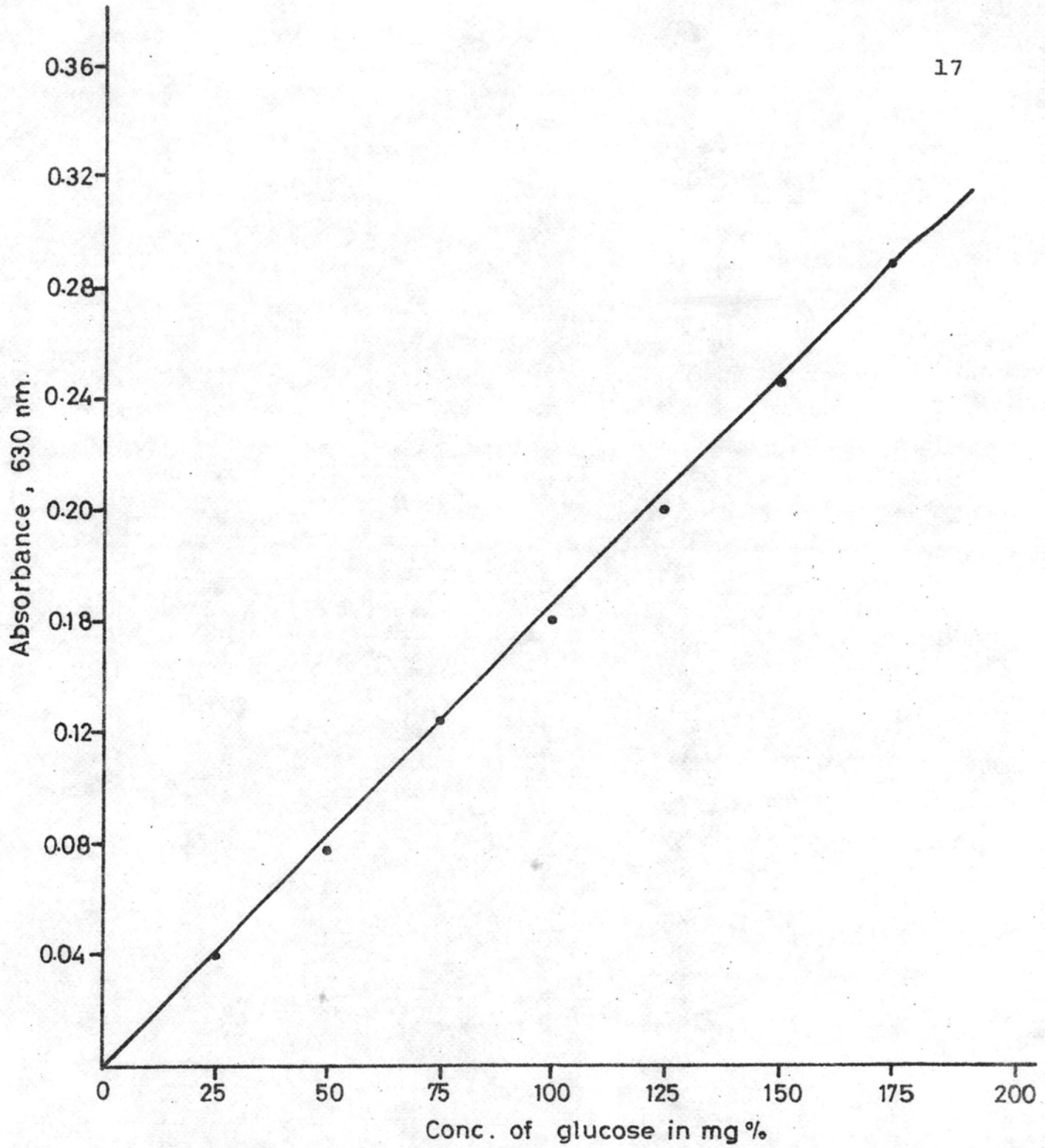

Fig.3 Calibration curve for o-toluidine blood glucose determination.

be linear over a wide range of glucose concentrations (from 0 to 800 mg/100 ml).

1.5 Determination of the glucose content in blood sample. About 0.5 ml of blood was drawn from the rabbit's marginal ear vein which was dilated with the externally applied xylene and this blood was collected in a microbeaker which had a thin film of dried sodium fluoride.

One-tenth of a milliliter of the blood was pipetted into a test tube containing 1.0 ml of trichloroacetic acid solution and mixed; and after a few minutes of standing the mixture was centrifuged. Only 0.5 ml of the clear supernatant liquid was measured out and put into another test tube, and 4.5 ml of colour reagent were added and mixed. This tube was then immersed in a boiling water bath for 8 minutes and then cooled. The absorbance at 630 nm of the resulted blue-green solution was then determined against a blank using Spectronic 20 with corresponding filter.

The glucose content of the blood was obtained from the calibration curve and it was expressed as mg of glucose per 100 ml of blood.

2. Preparation of aqueous extract of S. Sanitwongsei berries. Fresh young and ripe berries of S. sanitwongsei, after removal of pedicels, were washed and dried in open air. One hundred grammes of the dried berries were blended with 80 ml of distilled water in the blender at a

medium speed for 5 minutes. The contents in the blender were filtered and washed through muslin cloth, and the filtrate was made up to 100 ml with distilled water. One ml of the filtrate so obtained represented 1 g of the berries.

3. Preparation of alcoholic extract of S. sanitwongsei berries. Fresh green and red berries of S. sanitwongsei, after removal of pedicels, were washed and dried in open air. Three hundred grammes of the dried berries were blended with 900 ml of 60% ethanol in the blender at a medium speed for 5 minutes. The contents in the blender were transferred to a closed container and allowed to stand at room temperature for 4 hours. During the maceration period, the contents were shaken from time to time. The extract was filtered under reduced pressure and washed with fresh alcoholic solution. The filtrate was evaporated at 65°C in a rotary vacuum evaporator to dryness. The residue was redissolved in distilled water and the extract so obtained was made up to 200 ml, 1 ml of which represented 1.5 g of the fresh berries.

4. Preparation of aqueous extract of S. trilobatum berries. This aqueous extract of S. trilobatum was prepared in the same manner and had the same concentration as the aqueous extract of S. sanitwongsei.