CHAPTER II

MATERIALS AND METHODS

1. Preparation of Niang extract using ethyl alcohol

Materials

Blender	(General Electric Co.)
Centrifuge	(Hettich Rotamagna Co.)
Flash Evaporator	(Buchi Co.)
Ethyl Alcohol 95%	(The Government Pharmaceutical Organization)
Benzoic Acid	(Carlo-Erba Co.)
Niang bean	(Local market)

Method

The hard outer layer and the thin paper-like inner layer of the testa of Niang beans were first removed exposing 1 pale yellow cotyledons which weighed approximately 5 gm per cotyledon. Each cotyledon was finely chopped and homogenized in a blender for 5 minutes. The homogenate was macerated with 1 ml of 70% ethyl alcohol per 200 mg of the Niang bean for 24 hours at room temperature. The solution was filtered through cotton pad and the filtrate was centrifuged for five minutes at 2000 rpm. The supernatant was separated and concentrated under reduced pressure and preserved with 0.2% Benzoic acid. This portion was alcoholic free, used for feeding experimental animals and for spotting on a chromatogram for the djenkolic acid determination.

2. Separation and determination of djenkolic acid from the Niang bean

Materials and Instruments

Chromatography Tank

Electrical Balance (Mettler Co.)

Paper Whatman No. 1 Specially prepared for Chromatography.

Spectronic 20 (Bausch & Lomb Co.)

Chemicals

Acetone (Carlo-Erba Co.)

Acetic Acid, Glacial (E. Merck Co.)

Ammonia (E. Merck Co.)

Cupric Nitrate (E. Merck Co.)

Djenkolic Acid, Puriss Grade (Fluka Ab, Buchs, Switzerland)

Ethyl Alcohol (BDH Co.)

Methyl Alcohol (E. Merck Co.)

Ninhydrin (E. Merck Co.)

Pyridine (May & Baker Co.)

All chemicals were of analytical grade.

Principle

Djenkolic acid was separated from the Niang bean and quantitatively determined by the chromatographic method using ascending technique.

Method

The substance to be analyzed was applied as a spot

along a base line on a sheet of the Whatman paper No. 1. The spots are dried with a current of air. Space can be saved with a paper sheet by turning it into cylinder, with up to ten samples on one sheet, and standing in a dish containing the developing solvent; and the whole was enclosed in chromatographic tank. When the solvent had run up to sufficient distance, the paper was dried and treated with colour reagents. Position on the chromatogram and colour reactions with reagents were guided to identified.

Chromatographic Procedure

Chromatogram Whatman paper No. 1 was washed with 2 per cent acetic acid for at least 10 hours to get rid of particularly reducing substance. The washed paper was cut into 18 x 10 inches. Samples were usually placed on the paper by a micropipette, or capillary tube. The volume of the standard Djenkolic acid was 10 micro-litres while volumes of Niang extract and urine sample varied from 10-40 micro-litres. The sample spots should be placed at a distance of 2-3 cm apart, so that the chromatogram did not interfere with each other.

The chromatographic tank was made of glass with a round chamber of 9 inches in diameter and 20 inches in depth. The paper was dipped in the developing solvent which was placed in the dish inside the tank. The top edge was ground to contact surface. Air tightness could be completed, by

means of a layer of glycerol along the top edge. The solvent should be seen to rise evenly up. After running the chromatography for 5 hours, the paper was removed from the tank and allowed to dry at room temperature. The chromatogram was developed by dipping technique, dried and then heated at 105°C for 2-3 minutes or left overnight.

Standard solutions. The standard djenkolic acid solutions were prepared by dissolving djenkolic acid powder in the hot water. The standard solutions contain 1, 2, 3, 4, 5, 6, 7 and 8 mg of djenkolic acid in 10 ml.

Solvents. Four volumes of butanol were mixed with 1 volume of glacial acetic acid and 1 volume of water. The mixture was prepared freshly as required. The developing solvent was prepared by mixing 180 volumes of methanol, 4 volumes of ammonia (Sp. gr. 0.880) and 16 volumes of water. The final pH was 8-9. This was let overnight at room temperature before use.

Developing reagent. A few drops of pyridine was added into the 0.2% ninhydrin in acetone immediately before use.

Djenkolic acid gives purple colour with this reagent. The colour may be preserved by dipping the chromatogram in a mixture of 1 ml of saturated aqueous solution of cupric nitrate in 100 ml ethanol and 0.2 ml of 10% nitric acid. By this treatement, the colour changes to a pinkish red, which is stable for many months. The 0.5% aqueous solution of nickel

sulphate may be used instead of copper salt.

Identification Djenkolic acid was identified by chromatography along with standard samples. The presence of djenkolic acid in the sample was observed by visual comparison of the ninhydrin colour of the spots on paper with standard djenkolic acid which was run on the same chromatogram (Fig. 4).

Quantitative estimation of djenkolic acid Ninhydrin colour spots on the paper were cut out and eluted from paper with 5 ml distilled water at room temperature for half an hour. The colour was determined by Spectronic 20 at wave length of 570 mμ . This wave length gives an optimally high ratio of absorbance. The standard curve was obtained by plotting the optical densities against the concentrations of the standard djenkolic acid solutions which were run in every determination. The concentration of djenkolic acid in each sample was estimated from this standard curve.

3. Analysis of the chemical state of djenkolic acid in the Niang bean

Materials	Niang bean	(Local market)
Instrument	Blender	(G.E. Co.)
Chemicals		
	Trichloracetic acid	(Carlo-Erba Co.)
	Hydrochloric acid	(May & Baker Co.)
	Sodium bicarbonate	(May & Baker Co.)

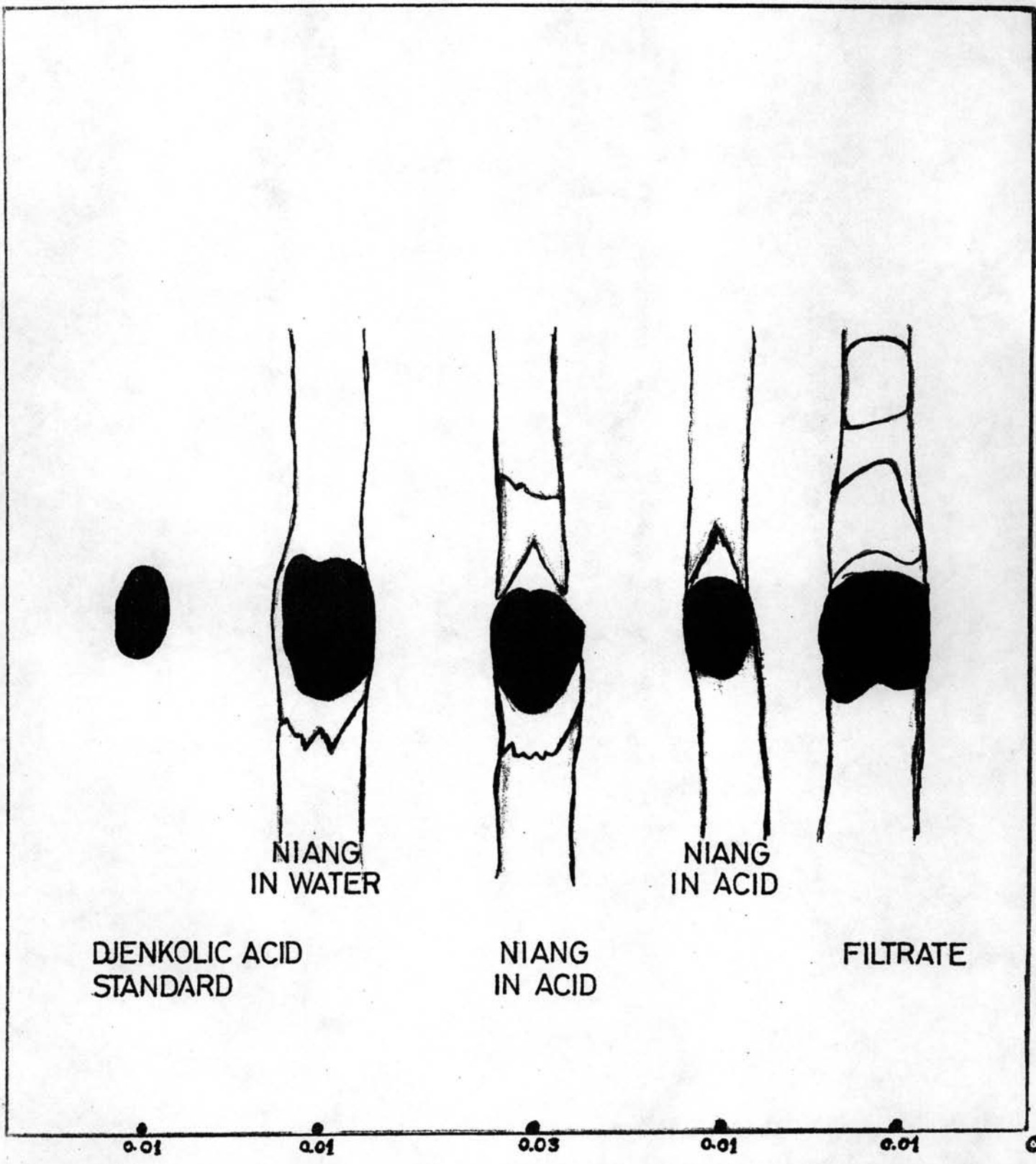

Fig. 4 Paper chromatogram of standard and samples after spraying with 0.2% ninhydrin reagent.

Method

Since there was the possibility that djenkolic acid existed in the bean as a moiety of a larger molecule, the beans were analysed. The hard husk-like outer and the thin paper like inner layer of the testa of the bean were first removed exposing two flattened cotyledons. The orange-yellow epidermis of the cotyledon was then removed exposing the pale yellow contents which weighed approximately 5 gm per cotyledon. Three such cotyledons from different beans were then analysed. Each cotyledon was finely chopped using a hand held blade and then macerated with five volumes of water in a homogeniser. The homogenate was filtered and the filtrate was examined by chromatography as described above.

Large molecular weight material in the filtrate was precipitated by adding equal volume of trichloracetic acid 10% w/v. The precipitate obtained was washed on the filter with trichloracetic acid 5% (w/v) then suspended in 6 M hydrochloric acid and heated in a boling water bath for 1 hour, this bean material was also estimated for the amounts of djenkolic acid by chromatography.

4. Effects of boiling Niang beans with different solvents

It has been suggested that djenkolic acid occurs as the free acid in the Niang bean. It seems therefore possible to remove the acid content of the bean by boiling in the solution.

In the present experiment, the Niang bean was boiled in the following solvents:

a. 5% Sodium bicarbonate

b. 5% Hydrochloric acid

c. Distilled water

The procedure in detail was as follow

1. Boiled 10 gm of chopped Niang beans without testa with 100 ml of 5% Sodium bicarbonate in a 250 ml beaker for ten minutes.

2. Filtered and measured the volume of the filtrate.

3. Added 100 ml of distilled water to boiled Niang beans and blended for five minutes, filtered with a filter paper.

4. Collected filtrate.

5. Boiled the blended bean with alkali solution from step 2 for ten minutes.

6. Again filtered and measured the volume of the alkali solution.

7. Blended the unfiltrate beans with 100 ml distilled water for five minutes.

8. Filtered and collected filtrate.

By means of the chromatographic method, djenkolic acid in the solutions from step 2, 4, 6 and 8 was determined.

The same procedure was carried out for hydrochloric acid and distilled water.

5. Experiment in animals

Materials

Urine collector	
pH meter	(Beckman Co.)
Electrical balance	(Mettler Co.)
Microscope	(Leitz Co.)
Urine specific gravity	(Atago Opticalworks Co. Ltd.)
Centrifuge	(International Co.)

Chemicals

Benzidine	(E. Merck Co.)
Nitric acid	(BDH Co.)
Magnesium sulphate	(BDH Co.)
Glacial acetic acid	(E. Merck Co.)
Ether	(BDH Co.)
Hydrogen peroxide	(E. Merck Co.)

Animals

The Niang bean extract was fed to mice, rhesus monkeys and albino rats.

Methods

1. Experiment in mice Albino mice of 20-25 gm weight were used in this experiment. They were fed with 2 ml

of Niang extract containing about 5.0 mg of djenkolic acid for 2 weeks. Urine was collected for 24 hours and examined for:

1.1 Volume, odor, colour, turbidity, pH, specific gravity and albumin.

1.2 Microscopic examination for red blood cells, white blood cells, epithelial cells, cast, amorphous, mucus, bacteria and crystal.

1.3 Chromatography method for djenkolic acid.

2. Experiment in monkeys Rhesus monkeys (Macaca mulatta) were fed with 15 ml of Niang extract containing 38-65 mg of djenkolic acid for 4 weeks; 24-hour urine was collected and examined as described above.

3. Experiment in rats Albino rats weighing 150-200 gm were fed with 6 ml of Niang extract containing 14-29 mg of djenkolic acid for 6 weeks. Their urines were collected for 24 hrs and examined as described above.

Some animals died during the experiment and their kidneys and livers were examined histologically.

6. Determination of nutritive values and amino acids contents in Niang beans.

Nutritive values of Niang beans were analysed by the Department of Medical Sciences, Ministry of Public Health.

Amino acids were determined by an Amino Acid Analyzer (Hitachi, KLA-38) by Suanpan, S. of the Nutrition Division, Ministry of Public Health.