PART II

EXPERIMENTAL

1. Equipments and Chemicals.

The following equipments were used:

- Extraction apparatus, soxhlet 45/50, Pyrex
- Heating mantle, Electrothermal, London
- pH meter, PHM 61 Laboratory pH meter, Radiometer, Copenhagen
- Vacuum oven, National Appliance Company, U.S.A.
- Rota vapor, Buchi, Switzerland
- Gas chromatograph Perkin-Elmer, model Sigma 3B with flame ionization detector and Perkin-Elmer, model 024, 10 mV recorder ; equipped with stainless steel column (1/16 inch inner diameter (1/8 inch outer diameter) x 12 feet) packed with 3% methyl phenyl polysilicone oil (OV-17) on 100-120 mesh Chromosorb W
- Mill, Arthur H. Thomas Co, Phila, U.S.A.

The experiment was done with analytical reagent grade chemicals and solvents as follows:

- Diethyl ether (May & Baker Ltd. Dagenham, England)
- Chloroform (May & Baker Ltd. Dagenham, England)
- Ammonia (E. merck, Darmstadt, Germany)
- Sulfuric acid (E. merck, Darmstadt, Germany)
- Absolute ethanol (Becthai Bangkok Equipment & Chemical Co. Ltd., Thailand)

- Methyl linoleate standard, for GC (Fluka, Chemische Fabrik, CH-9470 Buchs)

- Methyl phenyl polysilicone oil (OV-17) (Supelco, Inc., Pennsylvania)

- Methyl red (BDH Chemicals Ltd., England)

- Sodium hydroxide (E. merck, Damstadt, Germany)

- Sodium carbonate (Riedel-De Haen, Seelze-Hannover)

- Dibasic potassium phosphate (May & Baker Ltd., Dagenham, England)

- Anhydrous sodium sulphate (May & Baker Ltd., Dagenham, England)

- Atropine standard (Fluka, Chem. Fabrik, CH-9470 Buchs, purum)

- Hyoscine hydrobromide standard (Sigma Chemical Company, St. Louis)

- Urea (Srikrungwatana Co. Ltd., Thailand)

- Triple superphosphate fertilizer (Srikrung-watana Co. Ltd., Thailand)

- Potassium sulphate (Srikrungwatana Co. Ltd., Thailand)

- Chromosorb W 100-120 mesh, acid-washed silanized (dimethyldichlorosilane) (Supelco, Inc., Pennsylvania)

2. Methods.

2.1) Experimental Site.

Seedling preparations and raw material productions had been carried out at the Department of Horticulture, Faculty of Agriculture, Chiangmai University. The plantation was approximately 350 meters above mean sea level. Climatal data on maximum-, minimum- and mean temperature, relative humidity, rainfall and average daily sunshine duration had been collected throughout the experimental period. Data are summarized in Table 5 (p.39).

Soil sampling at the depth to 20 centimeters was carried out before the transplanting for a studying on basic soil properties. Collected soil was analysed at the Department of Soil Science and Conservation. Faculty of Agriculture, Chiangmai University. The results are shown in Table 6 (p.40).

2.2) Growing and Management.

The plants were propagated from seeds obtained from the botanical garden, Faculty of Pharmacy, Chiangmai University. For this study, the seeds were soaking overnight in cold water and sprinkled thinly in the prepared seed-bed (21 th February 1986). Germinating media were sand mixed with coir dust 1:1. The seeds germinated within 8-10 days and ready for transplanting in 50 days

Table 5 Environmental Conditions During the Experimental Period*

Month	Temperature (°C)			Relative humidity, average(%)	Rainfall, average (mm)	Sunshine (hrs)
	Maximum	Minimum	Mean			
February	32.6	15.0	22.5	59.0	0	9.2
March	34.0	15.9	23.7	53.1	0	7.8
April	34.3	21.8	27.1	56.6	0	7.8
May	34.0	23.5	27.0	68.7	82.24	5.7
June	33.1	21.9	27.7	71.7	76.17	4.5
July	32.2	22.4	26.3	75.8	174.65	3.9
August	33.4	22.4	27.1	78.5	189.88	5.1
September	32.2	22.3	26.5	76.0	197.25	5.4

* Data from the Faculty of Agriculture, Chiangmai University, Chiangmai

Table 6 Data on Soil Properties before the Fertilizer Application*

pH	5.97
Organic matter content, percent	1.85
Total Nitrogen content, percent	0.089
Available Potassium, ppm	140.00
Available Phosphorus, ppm	13.00
Available Calcium, ppm	700.00
Available Sulfur, ppm	29.70

* Soil analysis was done at the Department of Soil Science and Conservation, Faculty of Agriculture, Chiangmai University, Chiangmai.

later. The plantation covered an area of 7.9x26.5 square meters. It was divided into 36 small plots of each 3.7 square meters (3.7m long and 1 m wide). Seedling were randomly transplanted to each plot with one spacing of 45x60 centimeters and 14 plants per plot. The planning of experimental plots was completely randomized block design with three blocks (replications) of each 12 plots (treatments). Detail of experimental plots are shown in Figure 4. (p.42).

Before transplanting, the basal fertilizers consist of three doses of Urea (46-0-0) for 25, 50 and 75 kg N/ha and of Phosphorus and Potassium which were added to the soil uniformly as Triple Superphosphate (0-46-0) and Potassium Sulphate (containing 50% K_2O) at 50 kg P_2O_5/ha and 25 kg K_2O/ha respectively. For control no fertilizer was applied to plot number 1,2 and 3. The calculated quantity of each compound equivalent to the required weight of Nitrogen or Phosphorus or Potassium was mixed thoroughly with soil to produce the specific concentration of the elements. The different levels of Nitrogen were applied to soil in a rate of kg/ha were

Plot 1,2,3 : No fertilizer and served as control (F_1)
Plot 4,5,6 : N - P_2O_5 - K_2O 25-50-25 kg/ha (F_2)
Plot 7,8,9 : N - P_2O_5 - K_2O 50-50-25 kg/ha (F_3)
Plot 10,11,12 : N - P_2O_5 - K_2O 75-50-25 kg/ha (F_4)

(Plot numbers are shown in Figure 4, p.42)

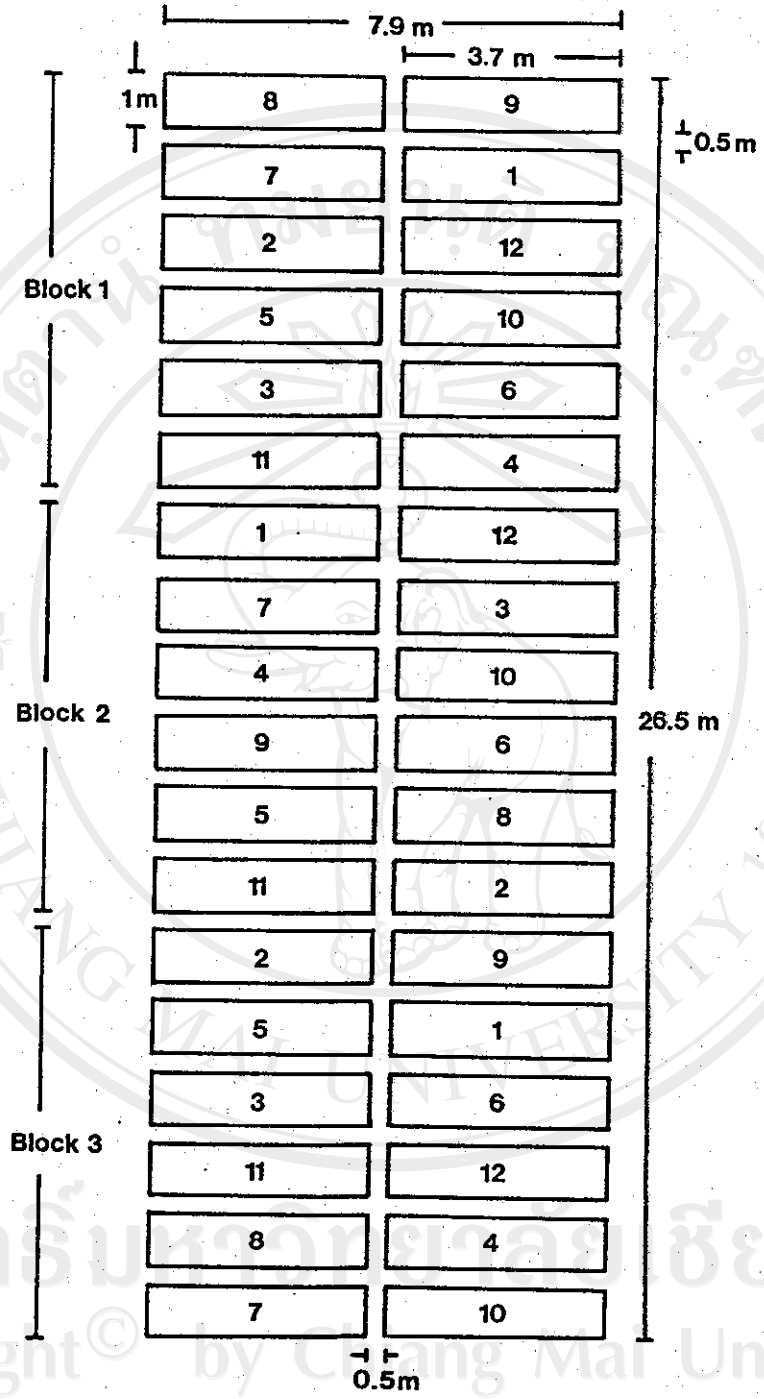

Figure 4 : A Diagram Showing the Planning of Experimental Plots for Datura metel var. fastuosa.

Watering was made once a day in the morning. The mixture of fertilizers were used only at the beginning in the preparation of the plots. No chemical insecticide was employed. Hand weeding was carried out fortnightly.

Within two months, the plant grew to 50-60 centimeters tall and the first collection of leaves was made at the beginning of flowering stage (S_1). The S_2-plants started to flower at 3 1/2 4 months after transplanting. The start-blooming flowers were gradually collected and dried in 45° hot air until the required raw materials for extraction were achieved. Leaves of the flowering plants (S_2-plants) were then harvested. In 120-150 days after transplanting the fruits of S_s-plants began to ripen and dehisce. Plants were harvested at this stage, only seeds from dehisced fruits and leaves were dried for active substances content analysis. Planting and harvesting date are shown in Table 7 (p.44).

2.3) Evaluation of the Products

2.3.1) Preparation of the Samples for Analysis.

Each plant part collected was dried in an oven at a constant temperature of 45° for at least 48 hours until it was thoroughly dried. It was then pulverized by a milling machine to coarse powder. Each sample was kept in the brown glass bottle, tightly stoppered.

Table 7 Planting and Harvesting Date of *D. metel* var. *fastuosa*.

Development	Date
Seed sowing	February 21, 1986
Transplanting of the seedling	April 11, 1986
Harvesting of S_1-stage (preflowering)	June 8, 1986
Harvesting of S_2-stage (flowering)	August 7, 1986
Harvesting of S_3-stage (fruit-ripening)	September 9, 1986

For the seed samples, the fixed oil was extracted with petroleum ether and the marc was dried at 40° before analysis was performed.

2.3.2) Quantitative Determination of Total Alkaloids Using Titrimetric Method.

The total alkaloids were extracted from the sample by the process described in the United States Pharmacopoeia XX (1979) and the alkaloid extracted were then determined by residual titration, described in the United States Pharmacopoeia XVIII (1970).

Procedure : A portion of 12 g. of the coarsely powdered sample was accurately weighed and moistened with a mixture of 8 ml of ammonium hydroxide, 10 ml of alcohol and 20 ml of ether. The moistened drug was placed in a continuous-extraction thimble and stood overnight, then extracted with ether until it was exhausted. The ether extract was evaporated to 50 ml under reduced pressure and transferred quantitatively to a separater with the aid of ether. The solution was exhaustively extracted with successive portions of dilute sulfuric acid (1 in 70) and filtered into a 100-ml volumetric flask. The filter was washed with dilute sulfuric acid (1 in 70) and collected the washings in the flask. The dilute sulfuric acid (1 in 70) was added to volume and mixed. Fifty ml of this solution was transfered to a separator and chloroform was added, shaked vigorously, allowed the layers to separate and discarded the

chloroform layer. Another portion of chloroform was added and repeated the process in the same manner. Twenty ml of pH 9.5 phosphate buffer and sufficient 1N Sodium hydroxide was added to yield a final pH between 9.0 and 9.5. Then, the alkaloids were completely extracted with chloroform. The combined extracts were filtered through anhydrous sodium sulfate, previously washed with chloroform. The final combined extracts were evaporated to dryness under reduced pressure, at a temperature below 45', then 12 ml of chloroform was added and mixed to dissolve the alkaloids ("solution X").

Five ml of "solution X" was transfered to erlenmeyer flask and 10ml of 0.02 N sulfuric acid was added. The chloroform was removed by evaporation and the excess acid was determined by titration with 0.02 N sodium hydroxide using methyl red T.S. as indicator. The content of total alkaloids was calculated in term of alkaloid hyoscyamine (or atropine). Each ml of 0.02 N sulfuric acid is equivalent to 5.788 mg of hyoscyamine.

Then for another portion of 5 ml of "solution X" was set aside for the determination of individual alkaloid; atropine (hyoscyamine) and hyoscine by gas liquid chromatographic method. Each experiment was performed in duplicate.

2.3.3) Quantitative Determination of Atropine and Hyoscing Using GLC

In GLC separation, the optical isomers of atropine are not separated from each others so the combined atropine-hyoscyamine peak is expressed solely as atropine herein for convenience.

Operating Condition : The column* was equilibrated at 240° for 1 hour before injecting the sample. Then the column temperature was programmed from 220° to 227° at the rate of 1°/min. The injection port and flame-detector block temperature were 260° and 265° respectively. Nitrogen gas was used as carrier gas at a flow rate 25 ml/min. Air and hydrogen were maintained at 45 and 35 psig. inlet pressure, respectively.

2.3.3.1) Calibration Graphs of Atropine and Hyoscine

The following solutions were prepared :

Atropine Standard Stock Solution : Atropine (previously dried over silica gel for 8 hours) 125 mg accurately weighed, was dissolved in chloroform and made up to volume in a 25 ml volumetric flask. Freshly prepared.

Hyoscine Standard Stock Solution : Hyoscine hydrobromide (previously dried at 105° for 3 hrs), 125 mg accurately weighed,

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* Column preparation shown in Appendix (p.112)

was dissolved in dilute sulfuric acid (1 in 350) and made up to volume in a 25 ml volumetric flask. Twenty ml of this solution was transferred into separator and proceeded as directed under "Procedure" (in 2.3.2, page 45), beginning with ".. and chloroform was added, shaked vigorously, allowed the layers to separate and discarded the chloroform layer.." until "...the final combined extracts were evaporated to dryness under reduced pressure, at a temperature below 45°". Then 20 ml of chloroform was added and mixed thoroughly. Freshly prepared.

Internal Standard Solution : Methyl linoleate, 20 mg accurately weighed, was dissolved in chloroform and made up to volume in a 10 ml volumetric flask.

Procedure : Two series of five atropine and five hyoscine standard solutions were taken from their stock solutions ; containing 5.0-25.0 mg of atropine and 2.5-7.5 mg of hyoscine respectively. The solutions were evaporated to dryness, and 1 ml of internal standard solution (2 mg/ml), was added to each, mixed thoroughly. Using the previous described GLC conditions, each soluion was injected (2.5 µl) in duplicated, on the column.

Blank Ten ml of 0.1 N sulfuric acid was transferred to a separator and proceeded as described under "Procedure" (in 2.3.2, p.45) beginning with "... and chloroform was added, shaked vigorously, allowed the layers to separate and discareded the chloroform layer." The blank chromatogram should cantain no significant interferences at the peaks of atropine, hyoscine and methyl linoleate (internal standard).

A linear relationship was obtained by plotting the mean peak height ratio (H) of the determined substance to internal standard versus the mass ratio (M) of that substance to internal standard. The equation of the straight line (H = a+bM, where a is the intercept and b is the slope) was calculated by using the least-squares method. The mass ratios of atropine to methyl linoleate were calculated by the formula $0.4 \times W_a \times V_a / W_m \times V_m$ and the mass ratios of hyoscine to methyl linoleate by the formula $0.5 \times W_h \times V_h \times 0.6921 / W_m \times V_m$ where W_a, W_h and W_m are the weight, in milligrams, of atropine, hyoscine and methyl linoleate standard, respectively ; V_a, V_h and V_m are the volume of atropine, hyoscine and methyl linoleate standard solutions, respectively and 0.6921 is the ratio of the molecular weight of hyoscine to hyoscine hydrobromide.

2.3.3.2) Determination of Samples

Internal Standard Solution : Methyl linoleate, 30 mg accurately weighed, was dissolved in chloroform and made up to volume in a 10 ml volumetric flask.

Procedure : A 5 ml aliquot of each "Solution X" (from 2.3.2) was evaporated to dryness. The residue was dissolved in suitable volume of chloroform in order to obtain the appropriated peak height in the chromatogram. Then 0.1 ml of this solution was transferred into a small screw-capped glass bottle and 0.05 ml of internal standard solution (3 mg/ml)was added and mixed thoroughly. After mixing, a 5 µl of the assay preparation was injected, in duplicate, on to GLC column. The blank was done in the same manner as in the preparation of calibration graphs.

The yield of the determined substance was obtained by using the formula:

$$\text{precent W/W} = [(H-a)/b] \times (m_1/m_2) \times (v_1/v_2) \times 0.3$$

where H is the mean of peak height ratio of substance to internal standard.

a is intercept of calibration graph.

b is slope from calibration graph.

m_1 is the weight, in microgram, of internal standard.

m_2 is the weight, in gram, of dried sample.

v_1 is the suitable adjusted volume, in millitre, of chloroform added to sample residue before injection.

v_2 is the volume, in microlitre, of sample solution injected on to GLC column.

2.3.3.3) Estimation of Atropine and Hyoscine Recovery from the Method Used.

Atropine and hyoscine hydrobromide standard solutions were prepared in chloroform (40mg/10 ml) and in 1 in 150 dilute sulfuric acid (28.8980 mg/10 ml) respectively. One ml of standard atropine solution plus 1 ml of hyoscine hydrobromide standard solution were used to treat with the accurately weighed powdered materials (12 g. from 3 out of 4 samples) and the same procedure (in 2.3.2, p.45) was adopted to these two groups of samples to obtain the crude alkaloid-containing extracts. After extraction, the four assays were completed in the usual way using

gas chromatograph, performed in duplicate in the manner described above and the mean of both was taken for the interpretation of the results. The difference between the two groups of samples was calculated as the recovery of the added substances.