EXPERIMENTAL
Ascorbic acid of Analytical Grade (Sarabhai Chemicals, India) was used for the experimental work. Double distilled water cooled to 10-12°C was bubbled with carbon dioxide gas and ascorbic acid was dissolved in it to make a 100 ppm (100 mg/l) stock solution. From this the required concentration of the solution was achieved by dilutions. Fresh solutions of ascorbic acid were prepared just before use. All sprayings were done in the evenings with a plastic hand sprayer. Pre-treatment of the seeds with ascorbic acid was given by moistening the seeds with the solutions (500 ml./1000 gram seeds), for four days as suggested by Chinoy (1967) and sown in the experimental plots on the fifth day. Sowing in all cases was done by hand sowing. Prior to the cultivation of the plants, the soil was thoroughly ploughed, harrowed and mixed with sufficient amount of farm-yard manure. Irrigation was done whenever needed. No synthetic manures were supplied to the plants during the present study. Weeding was done at regular intervals.

_Anethum sowa_ Roxb.:–

Seeds of dark Variyali sowa and pale Variyali sowa were procured from the cultivators at Surat, Gujrat, India. Their authenticity was established with the help of the available literature (Jain, 1975) sowing of the
seeds was done in the month of November. The seeds were divided into four sets of 15 grams each. They were surface sterilized with bromine water for 2 to 3 minutes, washed thoroughly under running tap water and dried. These seeds were given pre-treatment with ascorbic acid solution as mentioned above. Three different concentrations (10, 25 and 50 ppm.) were used for three sets. The fourth set was treated with double distilled water which served as control. After pretreatment, the seeds were directly sown in the plots. A distance of 30 cm. between two rows was maintained. The plant to plant distance was 20 cm.

The seeds were observed for the percentage of germination, general appearance and growth. Growth data such as height of plant and number of branches, were taken in the initial stages.

The plants were sprayed with dithane-M-40, a fungicide as a precautionary measure against possible fungal attack on the plants.

Ascorbic acid solutions in 10, 25 and 50 ppm. concentrations were sprayed on the plants at an interval of every 15 days. The first spray was given to 50 day old plants and the spraying was continued till fruits appeared on the plants.
Plants along with their roots were taken out before they came to flowering. The roots of all the control plants were separated out, and studied for the components in the oil. The herb of control and treated plants in both dark variyali and pale variyali sowa were separately distilled for their volatile oils for qualitative and quantitative studies.

**Distillation of the herb:**

The herb, immediately after collection was cut into small pieces and 100 grams was transferred into a modified Clevenger apparatus. 800 ml. of distilled water was added to it and the oil directly distilled over bunsen burner for 6 hours. The percentage of oil was noted and dried over anhydrous sodium sulphate. These oils were studied for their qualitative characters by Thin Layer Chromatography. The quantitative estimations of the individual components were carried out employing Gas Liquid Chromatographic technique.

**Thin Layer Chromatography:**

0.1 mm. thick plates were prepared by uniformly spreading the slurry of silica gel 'G' (E.merck) and drying in the air for 15 minutes. Afterwards, the plates were activated at 105°C for 45 minutes and stored in a desiccatar. About 20 ul quantities of the oil
were spotted on the plate. Reference standards of Carvone and Dillapiole were also spotted alongwith. Of all the solvent systems tried, benzene gave the better resolution. The developed plates were taken out from the T.L.C. chamber, dried in the air and sprayed with vanillin sulphuric acid reagent (1% w/v vanillin in concentrated sulphuric acid). On heating the plate for ten minutes at 110°C, coloured spots of the various terpene components developed.

Gas Liquid Chromatography :-

Toshniwal Gas Chromatographic instrument fitted with a flame ionization detector and two meter long copper column of polyethylene glycol (carbowax) 20 M (5%) with 0.25 inch diameter was employed for analysis of the volatile oils. The conditions maintained for the analysis were as follows:-

Column: PEG 20 M (5%) on chromosorb W

<table>
<thead>
<tr>
<th>Column temperature</th>
<th>110°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injector temperature</td>
<td>250°C</td>
</tr>
<tr>
<td>Detector temperature</td>
<td>230°C</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Nitrogen</td>
</tr>
<tr>
<td>Gas flow rate</td>
<td>80 cc. per minute</td>
</tr>
<tr>
<td>Attenuation</td>
<td>16</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>$10^{-9}$</td>
</tr>
<tr>
<td>Chart speed</td>
<td>1 cm. per minute</td>
</tr>
</tbody>
</table>
The oils were diluted in hexane and 2 microlitre was injected with care into the injector port and the peaks were recorded by the X-Y recorder.

When the plants started flowering, the young buds, flowers young fruits and matured fruits were studied by T.L.C. They were crushed in n-hexane and small quantities spotted on the silica gel plate and the plates developed as described earlier.

Plants bearing young and mature green fruits were collected and the young, fully mature but still green fruits and herbs were distilled for oils. Growth parameters such as number of compound umbels, umbellules, number of flowers/fruits per umbellule, length of the primary and the secondary rays were noted at this stage. These studies were undertaken only in control and treated plants of the dark variyali sowa.

The yield of oils from the young and mature green fruits was noted and T.L.C. and G.L.C. studies were carried out as mentioned before.

Finally when the fruits on the plants turned brown and the plants were drying which occurred in the month of April, the whole crop was harvested from the control and treated plots, of both pale and dark
variety sowa. Growth parameters and yield data were compiled. The fruits were separated by thrashing and were cleared of the broken pieces of primary and secondary rays. The dried herb was cut into small pieces and distilled for its volatile oil. 100 grams of the seeds were crushed to a coarse powder and immediately distilled in a Clevenger's apparatus. The oils were dried over anhydrous sodium sulphate. The specific gravity of the oils of dark and pale variety fruits was determined at 25°C. Refractive index of the oils was determined at 25°C using Abbe Refractometer (Model: WK 53). Thin layer chromatographic and Gas Liquid Chromatographic studies were undertaken for the above distilled oils to study their chemical constituents.

The percent composition of the individual components in the oils was calculated from the G.L.C. peaks using the peak areas. Peak areas were calculated as peak height multiplied by peak width at half the height of the peak. The peaks on the GLC chromatograms were identified by comparison of their retention times with those of authentic samples.

As good resolution of the dillapiole could not be obtained by GLC, the percentage of dillapiole was determined by spectroscopic method as described by Chauhan (1974).
As dillapiole exhibits maximum light absorbance in the ultraviolet range, a standard curve of the reference solution of pure dillapiole in methanol (Analar, BDH) was prepared using Bockman DU spectrophotometer and taking the absorbance at 288 nm.

The dark and pale Varioyali sowa oils were appropriately diluted in methanol and their absorbance readings were taken and dillapiole contents calculated using the standard curve.

*Trigonella foenum-graecum* Linn.:

Seeds of *Trigonella foenum-graecum* Linn. were procured from the local source and identified and their diosgenin content was determined according to the acid hydrolysis method.

**Estimation of diosgenin content in the seeds:**

5 grams of the seeds were taken and hydrolysed in 2 N H\textsubscript{2}SO\textsubscript{4} at 15 lb. pressure for one hour. The hydrolysate was washed first with distilled water followed by another wash with 10% sodium carbonate solution to remove the acid. This was further washed thoroughly with distilled water till the sodium carbonate was completely eliminated. The material was dried in oven at 55°C overnight, powdered and extracted thrice with chloroform (3 x 30 ml) for two hours each time. The
chloroform extracts combined, evaporated and the concentrate taken in a volumetric flask and volume made with chloroform up to the mark. Silica gel 'G' plates activated for 45 minutes at 105°C were used for TLC. Measured amount of the fenugreek seed extract was applied as a spot one centimeter above the base line of the plate. 99% pure diosgenin in chloroform solution was also spotted along with the test sample. The plates were developed in n-hexane:ethyl acetate (4:1) solvent system. After the solvent had travelled to 3/4 the height of the plate, it was removed from the chamber, solvent evaporated at room temperature and sprayed with vanillin phosphoric acid reagent (1% vanillin in 50% phosphoric acid). When the plates were heated for ten minutes at 100°C, diosgenin initially appeared as yellow to yellow orange spot which on further heating or by keeping overnight at room temperature turned to brownish violet. Of all the solvent systems tried, n-hexane:ethyl acetate (4:1) as suggested by Blunden et al. (1967) gave the best resolution. Hence this system was used throughout our studies. The $R_f$ value of the diosgenin coincided with the authentic sample and it was found to be 0.295. In another experiment diosgenin spots obtained from the test sample were scraped out from the T.L.C. plate before spraying with the spray reagent and eluted with chloroform.
This eluate when analysed by T.L.C. using different solvent systems gave a single spot thereby confirming the homogeneity of the sample.

Preparative Thin Layer Chromatography:-

Thin layer chromatographic glass plates (20 x 20 cm.) were washed thoroughly with a detergent and then with water to make it grease free and finally rinsed with distilled water. The plates were dried and coated with silica gel 'G' slurry (5 grams in 15 ml. distilled water) to give a thickness of about 0.25 mm. Silica gel 'G' of B.D.H. Laboratory reagent grade containing 13% calcium sulphate was used in the experiments. The prepared chromatoplates were left on a leveled surface for about 15 minutes or until the surface of the layer becomes dull and then activated by heating at 105°C for 15 minutes. Measured amounts of chloroform extracts containing diosgenin were applied in the form of a thin band using a micropipette of 0.1 ml. separately on 20 plates. A chloroform solution of standard diosgenin was also applied as small spot on each plate along with the seed extract. The plates were developed by giving a single run with n-hexane:ethyl acetate (4:1) in a glass chamber already saturated with the same solvent system. The plates were removed when solvent front reached to about 3/4 the height of the plates. Visuali-
zation of bands was achieved by exposure to iodine vapours after allowing the solvents to evaporate. Sample diosgenin band was marked and scrapped out. The scrapped out diosgenin bands from all the plates were pooled together, iodine completely evaporated at room temperature and the silica gel was eluted with chloroform for about 8 hours, centrifuged and the supernatant filtered through Whatman No. 42 filter paper. Evaporation of the chloroform filterate left white powder of diosgenin which on recrystallization in acetone yielded chromatographic pure crystals of diosgenin (m.p. 195°).

Quantitative estimation of diosgenin:-

The percentage of diosgenin in the fenugreek seeds was determined by using the procedure of Akira-Akahori et al. (1966).

Preparation of Standard curve for Diosgenin:--

100 mg. of recrystallised diosgenin was dissolved in chloroform in 100 ml. volumetric flask and the volume was made up to the mark with chloroform. Out of this, 10 ml. was further diluted to 100 ml. to give a concentration 100 ug/ml. From this, 0.1 ml., 0.2 ml., 0.3 ml., 0.4 ml., 0.5 ml., 0.6 ml., 0.7 ml., and 0.8 ml. were spotted as a narrow band on Thin Layer Chromatographic plates. The plates were developed, diosgenin
bands scraped out and eluted as mentioned earlier. The colour was developed using Akira-Akahori (1966) method. The absorbance was measured at 485 nm. A standard curve was obtained by plotting absorbance against concentration of diosgenin.

**Akira-Akahori method:**

1 ml. of ferric chloride solution (800 mg. of ferric chloride in 10 ml. water) was mixed with ortho-phosphoric acid to make up totally 50 ml. volume.

10 ml. of this reagent was transferred into the beakers containing diosgenin to be estimated. The solution mixture was cooled for 10 minutes in an ice water bath. 1 ml. of concentrated sulphuric acid was added to the contents of the beakers and again cooled for another 10 minutes. The mixtures of the above solutions were mixed thoroughly and heated at 70°C in an oven for 15-20 minutes. During heating, a pinkish violet colour has developed. The beakers were taken out and kept for one hour. Absorbance was measured at 485 nm. on a colorimeter.

For determining the percentage of diosgenin in the seed extract measured amount of the extract was spotted on the T.L.C. plates and proceeded as described above. The per cent diosgenin was calculated from the standard
curve of pure diosgenin.

Experiments with the germination of seeds of *Trigonella foenum-graecum* :-

Two grams of accurately weighed fenugreek seeds were taken in four sets and were germinated on moistened filter papers in petri plates in double distilled water and 10, 25 and 50 ppm. solutions of ascorbic acid. The solutions in the petri dishes were changed every 24 hours. The germinating seeds were taken out at 24, 48 and 72 hours of germination and analysed for their diosgenin contents.

Pretreatment of the seeds :-

Four sets of seeds (15 grams each) were taken in petri dishes and given pretreatment with distilled water (control) and 10, 25 and 50 ppm. solutions of ascorbic acid for four days. The method involves wetting the seeds with measured amount of the ascorbic acid solutions followed by drying as suggested by Chinoy (1967). Out of each set, two grams of seeds were taken, dried to their original weight and analysed for their diosgenin content according to the procedure described earlier. The remaining seeds were sown in four separate plots (15' x 15') in the month of October.
The seeds were sown in rows 30 cm. apart. A distance of 15 cm. was maintained between the plants. After germination of the seeds, 8 days old seedlings with only cotyledonary leaves were taken out along with the root. The growth data of the seedlings was recorded. Their cotyledonary leaves and the hypocotyl parts were separated out and dried. The diosgenin content of these two parts was determined separately.

The remaining plants in the plots were sprayed with 10, 25 and 50 ppm. solutions of ascorbic acid, the fourth unsprayed set of plants served as control. First spraying was done one month after sowing of the seeds. Further foliar spray treatments were given at a regular interval of 15 days till the plants started fruiting. Leaves from the plants were collected at monthly intervals, dried, powdered and analysed for diosgenin. The final crop was collected in the month of March when the fruits were fully mature. Yield data was taken and the seeds were separated by thrashing the plants and winnowing. The diosgenin content in these seeds was determined.

*Adhatoda vasica* Nees. :-

Two different varieties of *Adhatoda vasica* plants were found to be growing in the Pharmacognosy garden of L.M. College of Pharmacy, Ahmedabad, India. They have
distinct morphological traits and show a wide variation especially in the leaf size. One of them has usual leaves and designated as the small leaf variety. The other variety possesses abnormally large leaves as compared to the usual plants. Trial studies showed high alkaloidal concentrations in the plants with big leaves.

Both the plants were selected to study the seasonal variation of total alkaloids during the first year in plants raised from stem cuttings. Ascorbic acid solution of 10 ppm. concentration was given to the plants and its effects on the total alkaloids of the roots and leaves of the two varieties of plants was also studied.

The leaves of both big and small leaf varieties were taken for the measurement of length and breadth, number of veins and for the study of the surface preparations. The upper and lower epidermal peels of both the leaves were separated by heating the pieces of leaves at the mid rib region in potassium chlorate and concentrated nitric acid mixture. The peelings were then washed in distilled water, mounted in glycerine and observed under the microscope for the distribution of glandular and covering trichomes, length of the trichomes, number of cells, stomatal index etc.
Plants were raised from stem cutting in the second week of July, in plots measuring 12' x 8'. Both big and small leaf v8saka plants were raised through stem cuttings only. Pieces of v8saka stem of about 20 cm. long and 0.6 to 1.0 cm. in diameter containing 3-4 nodes, were cut just a little below one of the nodes and transplanted at a distance of 1.5 feet keeping the nodal region in the ground. All stem cuttings of each variety were taken from a single mother plant to avoid possible plant to plant variation. All stem cuttings started rooting within 15 days of transplantation. The young primary roots were taken for their histochemical and microscopic studies. The roots, especially the tips, of both big and small varieties were taken. The roots tips were treated with colchicine (0.2%) for three hours and fixed in acetic acid: alcohol (1:3). These tips were used for determination of chromosome number and DNA content in the actively growing undifferentiated root tip cells. The chromosomes were prepared using acetocaramine reagent.

For the determination of DNA per cell, the young roots were fixed in Carnoys' fluid (ethanol:acetic acid, 3:1, v/v) for one hour, washed in 3 changes of 70% ethanol and then brought to distilled water. They were placed in 5 N HCl at room temperature for 20 minutes,
rinsed twice with distilled water and placed in freshly prepared Schiff's reagent for 30 minutes in dark (Feulgen and Rossenbeck, 1924). The stained root tips were then washed in freshly prepared sulphur dioxide water, to remove any unspecific staining. They were later placed on albuminised slides and squashed under a cover slip, until the slide appeared transparent. Later the cover slip was removed with the help of a blade and the slide was allowed to air dry for over night. The localization of DNA was done by observing the slides under microscope. The site of DNA appeared to be stained of magenta colour. The colour intensities of the DNA per whole nucleus in both big leaf and small leaf varieties were measured on a cytophotometer (Chinoy et al. 1976). The extinction values of DNA per nucleus in the roots of both varieties were represented in arbitrary units.

After the plants were well established, a 10 ppm. ascorbic acid solution was sprayed on the leaves. First spray was given in mid August. Further sprays were given at every monthly intervals for one year. In both big and small vasaka, plants that did not receive any spraying treatment served as controls. Whole plants along with their roots of control and treated sets in both varieties were collected at monthly intervals, in the first week of every month starting with September.
The leaves and roots of the control and treated plants were separated and oven dried at 60°C. They were reduced to a fine powder in a Remi Mixer and the powder passed through a 40 mesh sieve. Care was taken not to exclude any hard parts of the leaves or the roots.

**Extraction of the alkaloids:**

One gram of the accurately weighed drug was moistened with 10% ammonia solution and the powder dried till free from moisture. It is then transferred to a 100 ml. volumetric flask. 40 ml. of chloroform:isopropanol (3:1) mixture was added to the above powder, the flasks along with the contents weighed, slightly warmed on a water bath and kept for maceration overnight (Groger and Johna, 1965b). The weight was adjusted with the same solvent mixture after maceration and the extract filtered. The volume of the filtrate was noted. This filtrate was taken into a separator and extracted first with 5 N sulphuric acid (15 ml.) followed by two successive extractions with 1 N sulphuric acid (15 ml. each time). The combined acid extractions were rinsed with chloroform to remove colouring matter. This chloroform extract in turn was extracted with a few ml. of 1 N sulphuric acid. This acid extract was combined with the original acid extract. The acid extract was made alkaline first with sodium carbonate and
then with a few ml. of ammonia to bring the pH to 9.5 to 10.0. Later it was extracted thrice with chloroform using (3 x 15 ml.). The chloroform extracts were combined, dried over anhydrous sodium sulphate, and taken into a volumetric flask of definite volume. The volume was made up with chloroform. This represents the total alkaloid content of the drug.

**Thin Layer Chromatography:**

Silica gel 'G' plates were prepared and activated as described earlier. The above chloroform extract containing the total alkaloids was spotted on the plates one centimeter above the base line of the plate. Reference standards of vasicine and vasicinone were also spotted along with the test sample. The plates were developed by a single run in dichloromethane:methanol (3:1) solvent system in a glass chamber saturated with the same solvent system. After the solvent front has travelled nearly 3/4th the height of the plate, the plate was removed from the chamber, dried at the room temperature and sprayed with modified Dragendorff's reagent. The coloured spots of the alkaloids were marked and their Rf values measured.

**Colorimetric determination of total alkaloids of Adhatoda vasica:**

The method followed was that described by Groger and
Johne (1965). 50 mg. of pure vasicine was accurately weighed and dissolved in chloroform and the volume made to 100 ml. with chloroform. From this 0.1 ml., 0.2 ml., 0.3 ml., 0.4 ml., 0.5 ml., 0.6 ml., 0.7 ml. and 0.8 ml. were pipetted out into clean 100 ml. beakers. The chloroform was evaporated and one ml. of methanol was added to the residue. To this 5 ml. of acetate buffer (pH 4.6) and 3 ml. of tropeolene '00' were added. The solvent mixture was transferred into a separating funnel and the alkaloid tropeolene colour complex was extracted thrice with dichloromethane using 10 ml. each time. The combined dichloromethane extracts were dried with anhydrous sodium sulphate and transferred into a 50 ml. volumetric flask containing 3 ml. of acid reagent. A sharp violet developed with acid reagent. The volume was made up to 50 ml. with dichloromethane. Blank was simultaneously prepared by omitting the vasicine solution and following the same steps. The absorbance of the colour was measured at 530 nm. on a colorimeter. The absorbance observed was plotted against concentration to obtain a linear curve. This served as the standard curve.

One ml. of the chloroform extract containing the total alkaloids of vasaka was pipetted out into a beaker and colour was developed proceeding in the same way as described for standard curve. The absorbance was converted
to vasicine using the standard curve. Percentage of total alkaloids was calculated as vasicine.

Preparation of Reagents :-

1. Troeolene '00' :-
   A saturated solution of tropoelene '00' in double distilled water was prepared, filtered and stored in an amber coloured bottle.

2. Acid Reagent :-
   One ml. concentrated sulphuric acid and 99 ml. methanol were mixed in a 100 ml. volumetric flask.

3. Acetate Buffer (pH 4.6) :-
   Acetate buffer was prepared by dissolving 5.4 grams of sodium acetate in 2.4 grams of glacial acetic acid and making the volume to 100 ml. with distilled water. The pH was adjusted to exactly 4.6 with the help of a pH meter using standard buffer of pH 4.0.

Datura innoxia Mill. :-

The crop was cultivated in summer season and in monsoon. Seeds of Datura innoxia were collected from well identified plants grown in the Pharmacognosy garden, L.M. College of Pharmacy, Ahmedabad, India. Summer crop was raised in February. Two sets of 15 grams of the seeds were taken and soaked in 100 ml. distilled water for 24 hours. The water was decanted, seeds were thoroughly
washed and pressed between blotting papers. They were
dried at room temperature. Of the two, one set was pre-
treated with a 10 ppm. (10 mg./1) solution of ascorbic acid
and the other set was given distilled water which served
as control. After pretreatment for four days, the seeds
were sown in experimental plots on the fifth day. The pre-
paration of the plots and cultivation practices were same
as described earlier for other plants.

After the plants grew well, first foliar application
of 10 ppm. ascorbic acid was given in the first week of
April. Further applications of the ascorbic acid solution
were done at an interval of every 15 days. The other set
of plants raised from seeds pretreated with distilled
water were not given any spray and they served as control
plants. Plants were collected along with roots at four
stages before flowering, plants bearing flower buds,
plants in full bloom with very young fruits and plants
with fully mature fruits. The roots, stem (below 5 mm.
diameter), leaves, young and mature fruits were immediately
separated from the plants, dried at 50°C and reduced to a
fine 40 mesh powder. The powdered samples were stored in
cold dry conditions. Final harvest of the plants i.e.
plants bearing mature dry fruits, was obtained in the first
week of August. The seeds from these plants were used for
raising next crop.
For the monsoon crop, seeds from the previous crop were pretreated with ascorbic acid and were sown in the experimental plots in the last week of August. Foliar spray of ascorbic acid (10 ppm.) was started in the third week of September followed by a spray at every 15 days interval. Plants were collected at 5 stages. Two collections were made before the flowering of the plants, one at young stage of herb and the other at full grown herb stage. Third collection was done when most of the plants were showing flower buds. Fourth collection was divided into plants in full bloom without any fruits and plants bearing young fruits. The final collection was made in the third week of December when the fruits were fully mature and started dehiscing. Growth data was also taken in the monsoon crop. The roots, leaves, stem (below 5 mm. diameter) pericarp and seeds are separated immediately after collection of the plants, dried and powdered. The powders were extracted for alkaloids and analysed for total alkaloid content, percent of hyoscyamine and hyoscine.

Extraction of the alkaloids :-

One of the powdered material was taken and moistened with a 10% solution of ammonia. The ammonia was completely evaporated and the powders dried till free from any moisture. The powder transferred into a refluxing flask and 40 ml. of chloroform was added to it. The flask along with the
contents was weighed, fitted to the reflux condenser and kept for maceration overnight. Next day, the drug was extracted by refluxing for four hours in a water bath. After refluxing, the flask was adjusted to its original weight using chloroform. The contents of the flask were filtered through a filter paper and volume of the filtrate was noted.

The filtrate was transferred into a separator, and shaken first with 5 N sulphuric acid containing alcohol (3:1 ratio) and then twice with 1 N sulphuric acid containing alcohol in the same ratio. The acid extracts were combined and colouring material removed by shaking with a few ml. of chloroform. This chloroform was again rinsed with a few ml. of the acid, the acid mixed with the other acid extract and chloroform discarded. The acid extract was basified first with sodium carbonate and then with concentrated ammonia till then pH of the solution reached 9-10. The liberated alkaloids were taken into chloroform by thoroughly shaking the aqueous extract with chloroform (3 x 10 ml.). The chloroform extracts were combined, concentrated and dried with anhydrous sodium sulphate. This chloroform containing the total alkaloids was concentrated and was taken in a 25 ml. volumetric flask, where the volume of the extract was adjusted to the mark with chloroform. This extract was later used for the estimation of the total alkaloids and for thin layer chromatography.
Thin Layer Chromatography :-

Silica gel 'G' was used to prepare the thin layer chromatographic plates and activated for 45 minutes at 105°C as mentioned earlier. Of all the solvent systems tried, the one containing chloroform:methanol:ammonia (80:20:1) suggested by Bhavsar (1971) was found to give the best resolution of the spots. The chloroform extract containing the total alkaloids and reference standards of hyoscyamine and hyoscine were spotted one cm. above the base line of the plate and developed in the chloroform: methanol:ammonia (80:20:1) solvent system. Revelation of the various spots was achieved by spraying the plates with modified Dragendorff's reagent. The various spots were marked and their Rf values measured.

Estimation of total alkaloids by using tropeolin 'OO' :-

1 ml. of the total alkaloid extract was taken into a clean and dry 100 ml. beaker. The extract was evaporated and heated for one hour at 100°C. To this 1 ml. of methanol was added. After the residue was completely dissolved in methanol, 5 ml. of acetate buffer (pH exactly 4.6) and 3 ml. of tropeolin 'OO' were added. The contents of the beaker were well mixed and transferred to a separating funnel and extracted with chloroform (3 x 10 ml.). The combined chloroform extracts were dried with anhydrous sodium sulphate and taken into a 50 ml. volumetric flask
containing 3 ml. of acid reagent.

A deep violet colour was developed which was measured at 530 nm, after making the volume to 50 ml. with chloroform. The total percentage of the alkaloids was calculated as hyoscyamine from the standard curve prepared with authentic sample of pure atropine.

100 mg. of accurately weighed atropine was dissolved in 100 ml. of chloroform. Out of this 0.1 ml., 0.2 ml., 0.3 ml., 0.4 ml., 0.5 ml., 0.6 ml., and 0.7 ml. were taken into clean dry 100 ml. beakers and the colour developed as previously described. A graph was plotted with absorbance against the concentration of atropine which gave a linear curve.

The preparation of the various reagents was the same as detailed for Adhatoda vasica.

Estimation of Hyoscyamine and Hyoscine :-

Measured amount of the chloroform extract containing the total alkaloids was spotted as a band on the silica gel '5' chromatographic plates and the plates were developed in the chloroform:methanol:ammonia (80:20:1) solvent system. Reference standards of atropine and scopolamine were also spotted beside the test sample. The visualization of the various alkaloids was obtained by exposing
the plates to iodine vapours. The bands of atropine and squirolamine were marked. The iodine was completely evaporated, bands scrapped, treated with alcoholic barium hydroxide and eluted with chloroform by refluxing for four hours. The chloroform extract from the silica gel was filtered through a Whatman No. 42 filter paper and the filtrate was taken for development of colour using tropeolin '00'. Amount of atropine and squirolamine were calculated from the standard curve of the respective substances prepared in the same way.