The soil was thoroughly ploughed, harrowed and mixed with farm-yard manure. The plots were properly irrigated before sowing the seeds of *D. innoxia* and planting the stem cuttings of *A. vasica*. No artificial fertilizers were used during the present experiments. Weeding and irrigation were done whenever required. A distance of one foot was kept between two plants in a row and between two rows of plants.

Chloramphenicol was obtained from Cadila Pharmaceutical Company Pvt. Ltd., Ahmedabad. MNNG was obtained from Fluka AG, Chem. Fabrik, CH-9470, Buchs - supplied by Glaxo Laboratories Pvt. Ltd., Bombay. EMS was obtained from Koch-light laboratories Ltd., England. L-Phenylalanine-$^{14}$C(U) was obtained from Bhabha Atomic Research Centre, Bombay, India. Universal liquid scintillation cocktail for aqueous and non-aqueous samples - Insta-Gel, Packard, was used for the liquid scintillation counting.

**Datura innoxia Mill.**

The crop of *Datura innoxia* was grown in two seasons - one during monsoon and the second in summer. The monsoon crop was raised in July. The seeds were collected from the plants growing in the Pharmacognosy garden, L. M. College of
Pharmacy, Ahmedabad. For each crop four sets of seeds, 15 gms each, were soaked in 100 ml distilled water for 24 hours. The water was then removed, the seeds were washed thoroughly and sown in different plots.

After the plants started growing well, first foliar application of chloramphenicol was given in the month of August for monsoon crop. 200 mg of chloramphenicol was dissolved in a minimum quantity of alcohol and then it was made upto 1000 ml using glass distilled water, to prepare 200 ppm solution. From this stock solution 50 ppm and 100 ppm concentrations were made by proper dilution using distilled water. The treatment with 50, 100 and 200 ppm was given as a foliar spray with a plastic hand sprayer in the evening hours. The plants were collected at four stages of growth - young herb, mature herb, flowering and fruiting stages. The collections were made in the morning hours. Roots, stems and leaves were separated immediately after collection and dried at 60°C and powdered to 40 mesh. Seeds collected from the control group of plants were used to raise summer crop.

Summer crop was raised in the month of March. Foliar spray with 50, 100 and 200 ppm chloramphenicol was started in the month of April. A fourth group of plants was given
2% urea treatment as foliar spray and a fifth group of plants were given 2% urea treatment as foliar spray followed by 200 ppm chloramphenicol treatment after one week. A sixth group of plants served as control. The treatments were repeated at intervals of fifteen days, till the fruiting stage was reached. The plants were collected at four stages of growth — young herb, mature herb, flowering and fruiting stages. The morphological parameters, namely, plant height, root length, number of branches per plant, length of leaf, breadth of leaf, number of flowers per plant, number of fruits per plant, and weight of 100 seeds were also recorded in this summer crop. Immediately after each collection root, stems, leaves, pericarp and seeds were separated, dried at 60°C and powdered to 40 mesh. The samples were stored in cool dry place.

**Extraction of alkaloids**

One gram of the powdered drug was moistened with 10% ammonia solution and then dried at room temperature. This air dried powder was then transferred to a reflux flask and 40 ml of chloroform was added. The flask with its contents was weighed, covered and macerated overnight. After maceration it was extracted under reflux for four hours.
in a water bath. The flask was cooled to the room temperature and its weight was adjusted by adding chloroform. The extract was filtered and the filtrate was measured. The filtrate was taken into a separator and extracted first with 5N sulphuric acid and alcohol (3:1) mixture and then twice with 1N sulphuric acid and alcohol (3:1) mixture (2 x 15ml).

The acid extracts were pooled and washed with 10 ml chloroform to remove colouring matter. This chloroform was rinsed with a small amount of 1N sulphuric acid. This acid extract was combined with the original acid extract and basified first with sodium carbonate and then with ammonia to bring its pH to 9-10. The free alkaloids from this aqueous extract were extracted with chloroform (3 x 15ml). The combined chloroform extract was concentrated, dried over anhydrous sodium sulphate, transferred to a 25ml volumetric flask and the volume was adjusted with chloroform. This chloroform extract was used for the estimation of total alkaloids and for thin layer chromatography.

**Estimation of total alkaloids**

One ml of the chloroform extract was pipetted out into
a clean dry 100 ml beaker and heated in an oven at 100°C for one hour. When it was cooled to room temperature, the residue was dissolved in 1 ml methanol. To it 5 ml of acetate buffer (pH 4.6) and 3 ml of tropeolin '00' solution were added. This mixture was transferred to a separator and the colour complex formed was extracted thrice with chloroform (3 x 10 ml). The combined chloroform extract was dried over sodium sulphate and taken into a 50 ml volumetric flask containing 3 ml of acid reagent. A deep violet colour was developed in the flask. The volume was made up to 50 ml with chloroform. The absorbance of the extract was measured at 530nm. The total alkaloid content was calculated as hyoscyamine from the standard curve.

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 and dry 100 ml beakers and chloroform was evaporated. Colour was developed using tropeolin '00', described as above. A graph was plotted with absorbance against concentration of atropine, which gave a linear curve.
Preparation of the reagents

1. **Acetate buffer (pH 4.6):**

   Acetate buffer was prepared by taking 5.4 gms of sodium acetate and 2.4 grams of glacial acetic acid and making the volume to 100 ml with distilled water. The pH was then adjusted to exactly 4.6 with the help of pH meter using standard buffer of pH 4.0.

2. **Tropeolin '00':**

   A saturated solution of tropeolin '00' in double distilled water was prepared, filtered and stored in an amber coloured bottle.

3. **Acid reagent:**

   One ml concentrated sulphuric acid was taken in 100 ml volumetric flask and 99 ml of methanol was added to it to make up the volume to 100 ml.

**Thin Layer Chromatography:**

The glass plates (20 x 20 cm) that were to be used for thin layer chromatography were washed free of any grease with detergent, rinsed with water and then with distilled water.
and dried. These clean plates were coated with silica gel 'G' (BDH Laboratory Reagent grade, 13% calcium sulphate) slurry (5 gms in 15 ml distilled water) to give a uniform thickness of about 0.25 mm. The prepared plates were kept on a levelled surface for drying. When the plates were dried, they were activated by heating at 105°C for 45 minutes in an oven.

The chloroform extract containing total alkaloids was applied on TLC plate along with standard hyoscyamine and scopolamine, one inch from the base line of the plate. The plate was then developed in chloroform : methanol : ammonia (80:20:1) solvent system in a glass chamber already saturated with the same system. The plate was removed from the chamber when the solvent front reached to about three fourths the height of the plate and the solvents from the plate were allowed to evaporate. The spots developed on the plate were visualized by spraying with modified Dragendorff's reagent.

Estimation of hyoscyamine and scopolamine

One ml of the chloroform extract containing total alkaloids was applied as a band on the activated TLC plate and developed in chloroform:methanol:ammonia (80:20:1)
system. Along with the sample, reference standards of hyoscyamine and scopolamine were also applied on the plate. The bands developed on the plate were visualized by exposing the plate to iodine vapours. The bands of hyoscyamine and scopolamine were marked and the iodine was allowed to evaporate completely. The bands of hyoscyamine and scopolamine were scrapped and extracted with chloroform for four hours under reflux in a water bath. The chloroform extract was filtered using Whatman No. 42 filter paper. The chloroform was evaporated off and colour was developed using tropeolin '00'. The amount of hyoscyamine and scopolamine were calculated from the standard curves prepared by the same method for both the compounds.

**Feeding experiment with L-phenylalanine - \(^{14}\text{C}(U)\)**

*D. innoxia* seeds were soaked in distilled water for 24 hours and sown in two pots in the month of November. When the seeds germinated, two plants were kept in each pot and rest of the plants were removed. In mid-January, when the plants were mature and bearing flower buds, the plants of one pot were given a foliar spray with 200 ppm chloramphenicol. The two plants in the second pot served as control group. After three days after chloramphenicol
treatment, cotton wicks were inserted in the stems near ground level in all the four plants and L-phenylalanine-\(^1^{14}\)C(U) was fed to the plants. One ml of L-Phenylalanine-\(^1^{14}\)C(U) of high specific activity (387 mci/m mole), solution in 0.01 N hydrochloric acid was obtained from BARC, Bombay. This solution (0.1mCi) was diluted ten times by adding 9ml of glass distilled water. From this, one ml was taken in a small glass vial and the loose end of the cotton wick from the plant was dipped in the amino acid in the vial. The other three plants were also given L-phenylalanine-\(^1^{14}\)C(U) in the same way. By the next day (in 24 hrs) the amino acid in the vial was absorbed by the plant through the cotton wick. Foliar spray with 200 ppm chloramphenicol was repeated once more. One ml of glass distilled water was added in the vials for two days to ensure that all the amino acid added was taken up by the plants. The plants were allowed to metabolize phenylalanine for ten days. Then the plants were collected along with the roots in the morning hours. All parts of the two plants of each group were dried at 60°C and powdered to 40 mesh.

2.5 grams of the drug was extracted for total alkaloids using the method described earlier. The chloroform extract
containing total alkaloids was used - (1) to estimate hyoscyamine and scopolamine, (2) to isolate hyoscyamine and scopolamine by preparative thin layer chromatography (3) for autoradiography and (4) for measuring the radioactivity.

One ml of the chloroform extract was applied on thin layer chromatographic plate and the amount of hyoscyamine and scopolamine were estimated as described earlier, using tropeolin '00'. Thin layer chromatographic plates were prepared with silica gel 'G' (E.Merk) and activated as described earlier. Measured amount of the chloroform extract containing total alkaloids was applied in the form of a thin band. Reference standards of hyoscyamine and scopolamine were also applied as small spots on each plate along with the sample. The plates were developed by giving a single run with chloroform:methanol:ammonia (80:20:1) solvent system. The bands were visualized by exposing the TLC plates to iodine vapour. The bands of hyoscyamine and scopolamine were marked and after the iodine was completely evaporated at room temperature they were scrapped out. The scrapped out bands of hyoscyamine and scopolamine from
all the plates were pooled and extracted with chloroform, under reflux, for four hours. All the four extracts were filtered through scinter glass funnels. The chloroform from the filtrate was evaporated. Both the alkaloids from the two groups were diluted ten times by adding additional non-radioactive hyoscyamine and scopolamine to each of the alkaloid and purified as picrates by adding picric acid and recrystallizing from methanol.

Radio-active assay of hyoscyamine and scopolamine

5 mg of hyoscyamine picrate was dissolved in 10 ml of 10% sodium carbonate solution. It was transferred to a separator and the free alkaloid was extracted with chloroform (3 x 10ml). The chloroform extract was concentrated, dried over sodium sulphate and transferred to a small aluminium disc. The chloroform was evaporated completely at room temperature. In the same way the other three samples were also made. The thin film of hyoscyamine and scopolamine formed in the aluminium discs were used to measure on Geiger counting system for their radioactivity. Another set of samples were made in same method and the free alkaloids were transferred into counting vials and universal liquid scintillation cocktail for aqueous and non-aqueous
samples (Insta-Gel, PACKARD) was added to each of the vials. The samples were counted with a liquid scintillation counter (Beckman LS 7000 Microprocessor controlled) for 10 minutes. Background count rates were subtracted and the specific radio activity and total activity in the alkaloids were calculated taking into consideration the efficiency of counting of $^{14}\text{C}$ (which was around 70%).

**Autoradiography**

Measured amount of the chloroform extract containing total alkaloids was applied as a thin band on TLC plate and developed in chloroform:methanol:ammonia (80:20:1) system. The developed chromatogram was exposed to an X-ray plate in dark for one month. Bands of hyoscyamine and scopolamine were observed on the X-ray plate after development.

**Measurement of total protein content of the plants that received L-phenylalanine $^{14}\text{C}$(U)**

2.5 grams of the powdered sample was extracted with 80% ethanol (2 x 25ml). Ethanol extract was filtered off and the residue was washed first with cold 5% HClO$_4$ solution (20ml) and then with 20ml of a mixture of ethanol:ether:chloroform (2:1:1) to remove acid soluble fractions and lipids.
The residue was then washed with cold 1M trichloro acetic acid (25 ml). The protein fraction present along with the residue was suspended in 30ml of 1N sodium hydroxide for one hour and centrifuged. The pellet was given two more washings (2 x 15 ml). The supernatant, containing dissolved total proteins, was taken in a clean 100ml volumetric flask and the volume was made upto 100ml with 1N NaOH. Using this aliquot, colour was developed following the method of Lowry and co-workers (1951).

One ml of the above aliquot was taken in a clean test tube and to it 1ml of distilled water and 5 ml of reagent C were added and incubated for 10 minutes at room temperature. To this 0.5ml of 1N Folin-Ciocalteu reagent was added and incubated for 30 minutes at room temperature. Blue colour developed was measured at 660 nm. Total protein content was calculated from standard curve prepared using casein.

10 mg of casein was dissolved in 10ml of glass distilled water. One ml of this solution was diluted to 10ml with distilled water. From it 0.1ml, 0.25ml, 0.5ml, 0.75ml, 1.0ml, 1.25ml, 1.5ml were pipetted out into clean test
tubes and distilled water was added to each of these test tubes to make the total volume in each test tube to 2ml. One test tube containing 2ml of distilled water served as blank. Colour was developed using Folin-Ciocalteu reagent as described above. A graph was plotted with absorbance against concentration of casein, which gave a linear curve.

Radio active assay of protein

One ml of total protein extract of each sample were taken and mixed with scintillation fluid and the radio activity was measured in Liquid scintillation counter. The total activity in the total proteins extracted was calculated.

Preparation of reagents

Reagent A : 2% sodium carbonate in 0.1N sodium hydroxide.

Reagent B : 0.5% copper sulphate. 5H2O in 1% sodium or potassium tartrate.

Reagent C : 50ml of reagent A + 1ml of reagent B.

Folin-Ciocalteu's Reagent:

Commercially available reagent diluted to make 1N solution (obtained from Glaxo Laboratories (India) Pvt. Ltd.).
Adhatoda vasica Nees.

Two varieties of *Adhatoda vasica* were found to be growing in the Pharmacognosy garden, L.M. College of Pharmacy, Ahmedabad, small leaf variety and big leaf variety (Pundarikakshudu, 1985). We selected the big leaf variety for our experiments.

The crop of *A. vasica* was raised from stem cuttings. Stem cuttings of about 20-25 cm in length, with 3-4 nodes were collected from a single mother plant to avoid any plant to plant variation. The stem cuttings were planted in the last week of July in four plots with a distance of about 1.5 feet between two plants in a row and between two rows. Within fifteen days the stem cuttings started rooting. One and half months later, in the first week of September, when the plants were growing well, treatment with chloramphenicol was started. Treatment with 50, 100 and 200 ppm chloramphenicol solutions was given as a foliar spray in the evening hours. The plants of the fourth plot served as control. The spray was repeated ten times at intervals of fifteen days. Fifteen days after each spray, leaf samples were collected from all the plants of each treatment. The leaves were collected from all over the
plant except the youngest pair of leaves. The leaves were dried at 60°C, powdered to 40 mesh and stored in glass containers in a cool dry place. Two year old plants were given 200 ppm chloramphenicol treatment from the third week of October to first week of February to see its effect on older plants. Another two year old plants served as control. Leaf samples were collected from these plants also at an interval of fifteen days and foliar spray was repeated every fifteen days.

Treatment with MNNG and EMS

294 mg of MNNG was dissolved in 1000ml of phosphate buffer (pH 6.2) to give 2mM solution. From this stock solution 0.5mM and 1.0mM dilutions were prepared in phosphate buffer (pH 6.2). 0.5ml and 1.0ml EMS solution were taken into 1000ml volumetric flasks and the volume was made up to the mark by adding glass distilled water to give 0.05% and 0.1% concentrations respectively. 200ml of each solution was taken in 250ml beakers (5 x 200ml). In each beaker five freshly collected stem cuttings were kept dipping in the solution. A fifth set of stem cuttings kept in distilled water served as control. The treatment was given for 12 hours, at the end of which the stem
cuttings were washed with water and planted in individual plots. Within fifteen days the stem cuttings started rooting. When the plants were well established, in the last week of October, collection of the leaf samples was started. Leaves were collected from all the plants of each plot and from each plant leaves were taken from all over the plant, except the youngest pair of leaves. The samples were dried at 60°C, powdered to 40 mesh and stored in glass bottles in a cool dry place. The samples were collected once in a month for two years at monthly intervals. When the mutagen-treated plants were one year old a second crop was raised from these plants by transplanting the stem cuttings taken from the individual mutagen treated plants. From this second generation crop also leaf samples were collected at monthly intervals for one year at an interval of one month.

Extraction of total alkaloids of Adhatoda vasica

One gram of leaf powder was moistened with 10% ammonia solution and dried at room temperature. This air-dried drug was then transferred to a 100ml volumetric flask, to it 40ml of chloroform and isopropanol (3:1) mixture was added and the flask was weighed along with its contents.
The contents were warmed slightly on water bath and kept overnight for maceration (Groger and Johne, 1965). After maceration, the weight was adjusted with the same mixture of solvents. The extract was then filtered and the volume of the filtrate was measured.

The filtrate was taken into a separator and extracted first with 5N sulphuric acid (15ml) followed by two successive extractions with 1N sulphuric acid (2 x 15ml). The combined acid extracts were washed with 10ml of chloroform to remove colouring matter. This chloroform extract in turn was rinsed with 10ml of 1N sulphuric acid. The combined acid extract was basified first with sodium carbonate and then with ammonia to bring the pH to 9-10. This aqueous extract was taken into a separator and extracted with chloroform (3 x 15ml). The chloroform extract was pooled, concentrated, dried over anhydrous sodium sulphate and transferred to a 25ml volumetric flask. The volume was made up the mark with chloroform. This extract was used for the estimation of total alkaloids and thin layer chromatography.

**Estimation of total alkaloids of A. vasica**

The tropeolin '00' method that was adopted for estimation of total alkaloids was described by Gröger and Johne (1965).
One ml of the chloroform extract containing total alkaloids was pipetted out into a clean dry 100ml beaker and from it the chloroform was evaporated off. The alkaloid residue was dissolved in 1ml of methanol. To this 5ml of acetate buffer (pH 4.6) and 3ml of tropeolin '00' solution were added. The mixture was transferred to a separator and extracted with dichloromethane (3 x 10ml). The combined dichloromethane extract was dried over anhydrous sodium sulphate and taken into a 50ml volumetric flask containing 3ml of acid reagent. The volume was made up with additional dichloromethane. The violet colour developed was measured at 530nm. The total alkaloid content was calculated as vasicine from standard curve. To prepare the standard curve 50mg of pure vasicine was dissolved in chloroform and made upto 100ml with chloroform. From this 0.1ml to 0.8ml were pipetted out into clean 100ml beakers. From this chloroform was evaporated off and colour was developed using tropeolin '00' as described above. The absorbance readings were plotted against concentration to obtain a linear curve from which total alkaloid content of the samples was calculated.

Preparation of the reagents is same as was given for D.innoxia.
Thin layer chromatography

The chloroform extract containing total alkaloids was applied on the activated plates of silica gel 'G'. Reference standards of vasicine and vasicinone were applied along with the sample. The plate was developed in chloroform:methanol: ethylacetate (8:2:1). The spots developed were visualized by spraying with modified Dragendorff's reagent and the Rf values were compared.