III

Materials
& Methods
This chapter is divided into two parts. Part A covers an account of the materials used and the experimental layout. Part B includes the various methods of growth analysis and biochemical estimations.

PART A

Experimental layout and materials:

The seeds of Fennel (Foeniculum vulgare Mill.), Coriander (Coriandrum sativum L.), Carum (Trachyspermum ammi (L.) Sprague), Cumin (Cuminum cyminum L.), and Dill (Anethum graveolens L.) were obtained from Spice Research Station, Gujarat Agricultural University, Jagudan.
Lab experiments were carried out using the seedlings raised in the laboratory and for the field experiments plants were raised in the university botanical garden.

**LAB experiments:**

Seeds were graded and surface sterilized by soaking in two volume hydrogen peroxide for 2 minutes and then washed thoroughly in double distilled water. The seeds were germinated in sterilized petridishes lined with Whatman No. 1 filter paper, supplied with double distilled water and freshly prepared solutions of growth regulators. The renewal of solution and filter papers was carried out at 48 hours interval. The seedlings were maintained throughout the experiment at 25 ± 2°C, with 8 hours daily white light illumination. The ideal concentrations of the substrates were determined through preliminary experiments.

The details of the growth regulators and their concentration are given in Part B.

**Experiment 1**

**Effect of growth regulators on germination and seedling growth of Fennel, Coriander and Carum seeds:**

The seedlings were raised as described earlier. The percentage of germination was recorded at an interval of 48 hours until maximum percentage of germinated seeds obtained. The length of the seedling in centimeters, dry
weight of hundred seedlings and moisture content percent were recorded at an interval of 48 hours for 8 days.

The details of growth analysis are given in Part B of this chapter.

Experiment 2
Effect of growth regulators on reducing sugars, total soluble sugars, invertase (E.C. 3.2.1.26) activity and qualitative changes in sugars during germination in Fennel seeds:

The seedlings were raised and maintained as described earlier. All the biochemical estimations were done at an interval of 48 hours upto a period of 10 days. Chromatographic analysis was carried out at 48, 144 and 240 hours.

1. Reducing sugar content is expressed as mg/g.fr.wt.
2. Total sugar content is expressed as mg/g.fr.wt.
3. Invertase activity is expressed as mg reducing sugar liberated/g.fr.wt./hour.

The details of the biochemical analysis and chromatographic separation of sugars are given in Part B of this chapter.
Experiment 3

**Effect of growth regulators on protein, total amino acids and qualitative changes in amino acids during germination in Fennel seeds:**

The seedlings were raised and maintained as described earlier. All the biochemical estimations were done at an interval of 48 hours up to a period of 10 days. Chromatographic analysis was carried out at 48, 144 and 240 hours.

1. Protein content is expressed as mg/g.fr.wt.
2. Total amino acid content is expressed as mg/g.fr.wt.

The details of biochemical analysis and chromatographic separation of amino acids are given in Part B of this chapter.

Experiment 4

**Effect of growth regulators on total phenols, starch, amylase (E.C. 3.2.1.1) activity and catalase (E.C. 1.11.1.6) activity during germination in Fennel seeds:**

The seedlings were raised and maintained as described earlier. All the biochemical estimations were analysed at an interval of 48 hours up to a period of 10 days.

1. Total phenolic content is expressed as mg phenolics/g.fr.wt.
2. Starch is expressed as mg starch/g.fr.wt.

3. Amylase activity is expressed as mg maltose released/g.fr.wt./hour.

4. Catalase activity is expressed as ml of O₂ released/g.fr.wt./minute.

The details of biochemical analysis are given in Part B of this chapter.

Experiment 5

Effect of growth regulators on reducing sugars, total soluble sugars and qualitative changes of sugars during germination of Coriander seeds:

The seedlings were raised and maintained as described earlier. All the biochemical estimations were analysed at an interval of 48 hours up to a period of 10 days. Chromatographic analysis was carried out at 48, 144 and 240 hours.

1. Reducing sugar content is expressed as mg/g.fr.wt.

2. Total soluble sugar content is expressed as mg/g.fr.wt.

The details of the biochemical estimations and the chromatographic separation of sugars are given in Part B of this chapter.
Experiment 6

Effect of growth regulators on protein content, total amino acids and qualitative changes in the amino acids during the germination of Coriander seeds:

The seedlings were raised and maintained as described earlier. All the biochemical estimations were analysed at an interval of 48 hours upto a period of 10 days. Chromatographic analysis was carried out at 48, 144 and 240 hours.

1. Protein content is expressed as mg/g.fr.wt.
2. Total amino acid content is expressed as mg/g.fr.wt.

The details of the biochemical estimations and chromatographic separation of amino acids are given in Part B of this chapter.

Experiment 7

Effect of growth regulators on starch, total phenols and catalase (E.C. 1.11.1.6) activity during the germination of Coriander seeds:

The seedlings were raised and maintained as described earlier. All the biochemical estimations were done at an interval of 48 hours upto a period of 10 days.

1. Total phenolic content is expressed as mg/g.fr.wt.
2. Starch content is expressed as mg/g.fr.wt.
3. Catalase activity is expressed as ml of oxygen liberated/g.fr.wt./minute.
The details of the biochemical analysis are given in Part B of this chapter.

Experiment 8

Effect of growth regulators on reducing sugars, total soluble sugars and on qualitative changes in sugars during the germination of Carum seeds:

The seedlings were raised and maintained as described earlier. All the biochemical estimations were analysed at an interval of 48 hours up to a period of 10 days. Chromatographic analysis was carried out at 48, 144 and 240 hours.

1. Reducing sugar content is expressed as mg/g.fr.wt.
2. Total soluble sugar content is expressed as mg/g.fr.wt.

The details of the biochemical analysis and chromatographic separation of sugars are given in Part B of this chapter.

Experiment 9

Effect of growth regulators on total protein, total amino acids and qualitative changes in the amino acids during the germination of Carum seeds:

The seedlings were raised and maintained as described earlier. All the biochemical estimations were analysed at an interval of 48 hours up to a period of 10 days. Chromatographic analysis was carried out at 48, 144 and 240 hours.
1. Total protein content is expressed as mg/g.fr.wt.

2. Total amino acid content is expressed as mg/g.fr.wt.

The details of the biochemical estimations and chromatographic separation of amino acids are given in Part B of this chapter.

**Experiment 10**

**Effect of growth regulators on starch, total phenols and catalase (E.C. 1.11.1.6) activity during the germination of Carum seeds:**

1. Starch content is expressed as mg/g.fr.wt.

2. Total phenolic content is expressed as mg/g.fr.wt.

3. Catalase activity is expressed as ml of oxygen liberated/g.fr.wt./minute.

The details of the biochemical analysis are given in Part B of this chapter.

**Experiment 11**

**Study of fatty acid composition in triglycerides and volatile oil constituents through gas chromatography during the storage period of two years in Fennel, Coriander, Carum, Cumin and Dill seeds:**

All the seeds were stored at room temperature and fatty acids and volatile oil contents were analysed at an interval of 1 year upto 2 years.
The details of the method of extraction of triglyceride fatty acids and volatile oil and analysis are given in Part B of this chapter.

FIELD EXPERIMENTS

Experiment 12

Effect of foliar spray treatment with growth regulators on fatty acid composition of triglycerides and volatile oil through gas chromatography in the mature fruits of Fennel and Coriander:

The raising, maintenance and foliar spray treatment of the plants, fennel and coriander are given in Experiment 13 and Experiment 16 respectively. Here in this experiment, the mature fruits were collected for the extraction of both triglycerides and volatile oil.

The details of the procedure for the extraction of triglycerides and volatile oil are given in Part B of this chapter.

Experiment 13

Effect of foliar spray treatments with growth regulators on reducing sugars, total soluble sugars and qualitative changes in sugars and invertase (E.C. 3.2.1.26) activity during the development of fruit in Fennel:

Graded seeds of *Foeniculum vulgare* were broadcast by hand in the plot at the botanical garden of Sardar Patel University in the months of August-September. The plants were
transplanted to the big plots when the plants attained a height of 4 to 5 inches. The plants were planted 1 ft apart. Moisture supply at field capacity level was maintained throughout the experimental period. The plants were supplied with N.P.K. fertilizer. The plants were watered soon after manuring. Weeding was done regularly. After 20 days of sowing, the plants were given the foliar spray treatment with growth regulators. They are gibberellic acid 10 PPM with 0.5% Tween-80 and cycloheximide 15 PPM with 0.5% Tween-80.

0.5% Tween-80 was sprayed to the control plots.

The foliar spray treatment was repeated after 20 days with same concentrations of growth regulators. Samples were collected for the biochemical estimation after flowering. Different stages were selected for the biochemical analysis. They are stage: 1. Blossomed flower; 2. Young fruit; 3. Developing fruit; 4. Immature fruit; and 5. Mature fruit. Chromatographic analysis was carried out at blossomed flower, developing fruit and mature fruit.

1. Reducing sugars content is expressed as mg/g.fr.wt.
2. Total soluble sugars content is expressed as mg/g.fr.wt.
3. Invertase activity is expressed as mg reducing sugar liberated/g.fr.wt./hour.
The details of biochemical analysis and chromatographic separation of sugars are given in Part B of this chapter.

**Experiment 14**

**Effect of foliar spray treatment with growth regulators on total proteins, total amino acids and qualitative changes in amino acids during the development of the fruit in Fennel:**

The raising of plants, their maintenance and sampling were followed as outlined in Experiment 13.

1. Protein content is expressed as mg/g.fr.wt.
2. Total amino acid content is expressed as mg/g.fr.wt.

The details of the methods of biochemical estimations are given in Part B of this chapter.

**Experiment 15**

**Effect of foliar spray treatment with growth regulators on total phenol, starch, amylase (E.C. 3.2.1.1) activity, catalase (E.C. 1.11.1.6) activity, peroxidase (E.C. 1.11.1.7) activity during the development of fruit in Fennel:**

The raising of plants, maintenance and sampling were followed as outlined in Experiment 13.

1. Total phenolic contents are expressed as mg/g.fr.wt.
2. Starch content is expressed as mg/g.fr.wt.
3. Amylase activity is expressed as mg maltose released/g.fr.wt./hour.
4. Catalase activity is expressed as ml of oxygen liberated/g.fr.wt./minute.

5. Peroxidase activity is expressed as change in O.D./g.fr.wt./minute.

The details of the methods of biochemical estimation are given in the Part B of this chapter.

Experiment 16

Effect of foliar spray treatment with growth regulators on reducing sugars, total sugars, starch and total amylase (E.C. 3.2.1.1) activity during the development fruit in Coriander:

Graded seeds of Coriandrum sativum were sown in rows in the plots of botanical garden of Sardar Patel University in the month of November. The distance between each row was nearly 1 ft. Moisture supply was maintained at field capacity throughout the experimental period. The plants were supplied with N.P.K. fertilizer. The plants were watered soon after manuring. Weeding was done regularly. After twenty days of sowing the plants were given the foliar spray treatment with growth regulators. They are: 1. Gibberellic acid 10 ppm with 0.5% Tween-80; 2. Cycloheximide 15 ppm with 0.5% Tween-80; and 3. 0.5% Tween-80 was sprayed to the control plots.

The foliar spray treatment was repeated after 20 days with the same concentrations of growth regulators. Samples
were collected for the biochemical analysis after flowering. Different stages were selected for the biochemical analysis. They are: 1. Blossomed flower; 2. Young fruit; 3. Developing fruit; 4. Immature fruit; and 5. Mature fruit.

1. Reducing sugar content is expressed as mg/g.fr.wt.
2. Total soluble sugar content is expressed as mg/g.fr.wt.
3. Starch content is expressed as mg/g.fr.wt.
4. Total amylase activity is expressed as mg reducing sugars liberated/g.fr.wt./hour.

The details of the methods of biochemical estimation are given in Part B of this chapter.

Experiment 17

Effect of foliar spray treatment with growth regulators on total protein, total amino acids, qualitative changes in amino acids and catalase (E.C. 1.11.1.6) activity during the development of fruit in Coriander:

The raising of plants, maintenance and treatment, sampling was followed as outlined in Experiment 16.

1. Total proteins content is expressed as mg/g.fr.wt.
2. Total amino acids content is expressed as mg/g.fr.wt.
3. Catalase activity is expressed as ml of oxygen liberated/g.fr.wt./minute.
The details of the methods of biochemical estimations are given in Part B of this chapter.

Experiment 18
Biochemical study of the reducing sugars, total soluble sugars, total protein, invertase (E.C. 3.2.1.26) activity, total amylase (E.C. 3.2.1.1) activity, catalase (E.C. 1.11.1.6) activity and peroxidase (E.C. 1.11.1.7) activity during the development of fruit in Carum:

Graded seeds of *Trachyspermum ammi* were sown by broadcasting by hand, in plots at botanical garden of Sardar Patel University in the month of November. Adequate supply of moisture content at field capacity level was maintained throughout the experimental period. The plants were supplied N.P.K. fertilizer. The plants were watered soon after manuring. Weeding was done regularly. Samples were collected for the biochemical analysis after the flowering. Different stages were selected for the biochemical analysis. They are: 1. Bud; 2. Blossomed flower; 3. Young fruit, 4. Immature fruit; and 5. Mature fruit.

1. Reducing sugar content is expressed as mg/g.fr.wt.
2. Total soluble sugar content is expressed as mg/g.fr.wt.
3. Total protein content is expressed as mg/g.fr.wt.
4. Invertase activity is expressed as mg reducing sugars liberated/g.fr.wt./hour.
5. Amylase activity is expressed as mg maltose liberated/g.fr.wt./hour.

6. Catalase activity is expressed as ml of oxygen liberated/g.fr.wt./minute.

7. Peroxidase activity is expressed as change in 0.D./g.fr.wt./minute.

The details of the methods of biochemical estimations are given in Part B of this chapter.

**PART B**

**Preparation of solutions of growth regulators**:

The following growth regulators were used for various experiments and the solutions were prepared as follows:

1. **Gibberellic acid (GA$_3$)**: Gibberellic acid was initially dissolved in few drops of absolute alcohol and finally made upto required volume using double distilled water. GA$_3$ was used at a concentration of 10 PPM for fennel and carum and 20 PPM for Coriander in lab experiments.

2. **Cycloheximide**: Cycloheximide was initially dissolved in few drops of absolute alcohol and finally made upto required volume using double distilled water. It was used at a concentration of 5 PPM for Coriander and Carum and 15 PPM for Fennel in the lab experiments.
Growth analysis

**Percentage of seed germination**: Seeds were surface sterilized and raised in the petridishes supplying the respective growth regulators or distilled water. The number of seeds germinated under each substrate was noted at an interval of 48 hours until maximum germination attained. Germinability is expressed as percentage of germination.

**Seedling length**: The length of the seedling was measured from the root tip to the shoot tip and was expressed as centimeter.

**Moisture content and dry weight**: The exact fresh weight of the seedlings selected randomly from different treatments was taken. They were weighed individually on electric balance and kept in an oven at 100-105°C for three days or until constant dry weight was resulted. Then the dry weight was recorded. Moisture content was calculated from the difference between the fresh weight and dry weight and expressed in terms of percent moisture content on the basis of dry weight. The dry weight of the seedlings is expressed in gram per 100 seedlings.
BIOCHEMICAL ESTIMATIONS

Reducing sugar:

The reducing sugar contents were estimated following the method of Lindsay (1973). Weighed material was homogenized with distilled water and centrifuged at 4000 rpm for 15 minutes. The supernatant was taken as appropriate aliquots and the final volume was made up to 1 ml with distilled water. 1 ml distilled water was taken in the control tube. 1 ml DNS reagent was added for all the tubes (100 ml of DNS reagent comprised of 1 gram 2,4 dinitrosalicylic acid, 20 ml 2 N NaOH and 30 gram of sodium potassium tartarate). The tubes were incubated in a boiling water bath for 10 minutes. The initial volume of 2 ml was further diluted to 5 ml by adding distilled water. The tubes were brought to room temperature and the absorbance was measured at 540 nm. The values are extrapolated by using a standard D-glucose series.

Total sugars:

Total sugars content estimation was carried out following the method of Witham et al. (1970). Weighed material was finely homogenized in 80% ethanol. After centrifugation at 4000 rpm for 15 minutes, the supernatant was retained and the residue was homogenized in 80% ethanol twice and all the supernatant are combined. Aliquots from the supernatant were boiled to evaporate the alcohol and the
sugar precipitate was suspended in 2 ml of distilled water. To all the tubes 4 ml of cold anthrone reagent (0.2 gm anthrone in 100 ml of 95% H$_2$SO$_4$) was added at chilling temperature. The tubes were incubated in boiling water bath for eight minutes and later the tubes were transferred to an ice bath for immediate cooling. The absorbance was read at 650 nm and the values were extrapolated by using a standard D-glucose series.

Starch:

The estimation of the starch content was carried out by the acid hydrolysis method of McReady et al. (1950). The weighed material was homogenized in 80% alcohol and centrifuged at 4000 rpm. The residue was collected and repeatedly extracted with 80% alcohol. The alcohol extraction of free sugars was continued till the residue gave a blank test with the anthrone reagent indicating the total elimination of free sugar content from the residue. The residue was suspended in 5 ml of 52% perchloric acid and incubated for the starch hydrolysis purpose at 0°C for 12 hours. The acid treated residue was centrifuged and from the supernatant aliquots were taken and made up the volume to 2 ml with distilled water. To all the tubes anthrone reagent (0.2 gm anthrone in 100 ml of 95% H$_2$SO$_4$) was added, mixed thoroughly and boiled in boiling water bath for eight minutes. After boiling all the tubes are
transferred to ice bath for immediate cooling. The absorbance was measured at 650 nm. Sugar content was determined and the amount of sugar was converted into a corresponding starch level using standard formula

\[ \text{Starch : (T. sugar} \times 0.9) \]

**Total protein**:

The total protein content was estimated following the method of Lowry *et al.* (1951). Weighed material was boiled in 80% alcohol for five minutes, homogenized with 80% alcohol and centrifuged. The residue was suspended in 1 ml distilled water and 1 ml of 15% perchloric acid for 2 minutes and centrifuged. The residue was suspended in 10% Trichloroacetic acid for 10 minutes and centrifuged. The residue was dissolved in 5 ml of 1 N NaOH and incubated for 30 minutes and centrifuged. Later, the supernatant was made to a definite volume with 1N NaOH. To the sample aliquots in test tube. 5 ml of reagent C (reagent A - 2% sodium carbonate and 0.2% sodium potassium tartarate in 0.1 N NaOH, reagent B - 0.5% copper sulphate, reagent (C - A:B = 50:1) was added, mixed thoroughly and allowed to stand for 10 minutes at room temperature. 0.5 ml of Folin - ciocalteau's phenol reagent was added, mixed thoroughly and incubated at room temperature for 30 minutes to develop the colour. The absorbance was read at 650 nm and the values are extrapolated by using a standard series
of vitamin free casein (10 mg/100 ml IN NaOH).

**Total amino acids:**

Total amino acid content was estimated following the method of Lee and Takahashi (1966). Weighed material was homogenized in 80% alcohol, centrifuged at 4000 rpm for 15 minutes. Supernatant was retained and residue was homogenized in 80% alcohol twice. The supernatant was pooled and aliquot of the sample was taken from this supernatant. To the sample aliquot 5 ml of Ninhydrin reagent (A=1% Ninhydrin in 0.5 M citrate buffer pH-5.5; B-Pure glycerol; C = 0.5 M citrate buffer pH-5.5; Ninhydrin reagent - A:B:C = 5:12:2) was added. Mixed thoroughly and boiled in boiling water bath for 12 minutes. The tubes were cooled under the running water. Absorbancy was read at 570 nm. The values are extrapolated by using standard glycine series (10 mg/100 ml).

**Total phenolics:**

The total phenols were estimated following the method of Singh et al. (1978). Weighed material was homogenized and extracted with 0.3 N HCl in methanol. The extracts were centrifuged at 4000 rpm for 15 minutes. The residue was again homogenized and supernatants were pooled. The supernatant was evaporated to dryness. The residue was dissolved in distilled water. From that sample aliquot was taken and final volume was made up to 7 ml with distilled
water. 0.5 ml folin-ciocalteaus phenol reagent was added, mixed thoroughly. After three minutes 1 ml of 3.5% sodium carbonate was added and incubated for 1 hour. The absorbancy was read at 650 nm. The values were extrapolated to a standard series of tannic acid (40 mg/100 ml).

**Paper chromatographic separation and identification of sugar:**

The method followed was of Menzies and Seakins (1969). Weighed material was homogenized with 80% ethanol and centrifuged at 12,000 rpm for 20 minutes. Supernatant after the centrifugation was concentrated at 60°C and used as the sugar sample for the spotting purpose. All the sugar standards were prepared in 10% isopropanol. The samples and standards were spotted in Whatman No.1 chromatography paper; 8 cm above the margin. The chamber was saturated with the solvent vapour and descending chromatography was carried out in a chromatographic chamber with the solvent system, butanol:acetic acid:water (120:30:50) until the solvent reached 5 cm away from the bottom. In Coriander ascending chromatography was carried out using the solvent system of butanol:acetic acid:water (120:30:50). The paper was air dried and treated with alkaline silver nitrate for the spot visualization. In the first step, the chromatograms are dipped in saturated silver nitrate solution prepared in 40% acetone. The solvent was blown off by air drying and the second dip was given in alkali solution.
(0.5 g NaOH was first dissolved in 5 ml of DW and made upto final volume of 100 ml with ethanol). The chromatograms are once again air dried. Later, the background colour of the chromatograms was removed by giving a dip of 2 N ammonia solution. The Rf value was calculated using the formula:

$$\text{Rf} = \frac{\text{distance travelled by substance from origin}}{\text{distance travelled by solvent from origin}}$$

The different sugars in the samples were identified by comparing the Rf values of the standard known sugars.

**Chromatographic separation and identification of amino acids**:

The plant material was finely homogenized in 80% ethanol and centrifuged at 12,000 rpm for 20 minutes. The supernatant was concentrated at 60°C and was used as amino acid sample. All the standard amino acids were dissolved in 10% isopropanol. The standards and samples were spotted on Whatman No. 1 chromatographic paper 8 cm above the margin. The chamber was saturated with the vapour of the solvent system and run was carried out in a descending chromatographic chamber using butanol:acetic acid:water (12:3:5) as solvent system, except in Coriander studies, where run was carried out in ascending manner with the same solvent system following the method suggested by Krishnamurthy and Swaminathan (1955). The run was continued
till the solvent front reached 5 cm away from the bottom. The chromatograms are air dried and sprayed with 0.5% ninhydrin prepared in acetone followed by oven drying for spot visualization. The Rf value was calculated using the formula:

\[
Rf = \frac{\text{distance travelled by the substance from origin}}{\text{distance travelled by the solvent from the origin}}
\]

The amino acids separated from the samples were identified by comparing the Rf values of the standard known amino acids.

**Total amylase:**

Total amylase activity was estimated by the method of Bernfeld (1955). Homogenized the material in 0.2 M phosphate buffer with 0.2% calcium acetate. Homogenate was kept at 0°C for 1 hour and centrifuged at 5000 rpm for 15 minutes and the clear supernatant was used for the enzyme. The reaction mixture consisted of 0.5 ml enzyme extract, 0.3 ml of 0.5% starch and 0.2 ml of 0.2 M phosphate buffer. The reaction mixture was incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 1 ml of DNS reagent for all the tubes. Control tubes were maintained by the addition of DNS reagent at '0' time. The tubes were kept in boiling water bath for 10 minutes and cooled. The volume was made up to 5 ml with distilled water. The absorbance of the reaction and the control tubes were read at 540 nm.
The values are extrapolated to the standard values of Maltose (20 mg/100 ml). The activity of amylase was expressed as mg maltose liberated/g.fr.wt./hour. The pH of the phosphate buffers for different materials is as follows:

Fennel, Coriander - 0.2 M phosphate buffer and Carum pH 7.0

Invertase:

The invertase activity was estimated following the method of Sumner (1935). Weighed material was homogenized in 0.2 M acetate buffer. The pH of the buffer was maintained as follows:

Fennel: pH-5.0; Coriander: pH-4.8; Carum: pH-5.0

The homogenate was centrifuged at 5000 rpm for 15 minutes. The supernatant was used as a crude enzyme extract. The reaction mixture consisted of 0.4 ml or 0.2 M acetate buffer of pH as mentioned earlier; 0.25 ml of 0.4 M sucrose and 0.35 ml of enzyme extract. The reaction mixture was incubated at 37 ± 1°C for 30 minutes; followed by the termination of reaction by adding 1 ml of DNS reagent. The controls were maintained by stopping the reaction at 0 time. The tubes were boiled in boiling water bath for 10 minutes; cooled and made upto 5 ml by adding distilled water. The absorbance was read at 540 nm. The values are extrapolated to the standard values of D-glucose (10 mg/100 ml).
Peroxidase activity:

Worthington's method was followed for peroxidase assay (Gilbault, 1976).

\[ \text{H}_2\text{O}_2 \xrightarrow{\text{Peroxidase}} \text{H}_2\text{O} + \text{O} \]

oxidized 0-dinitrosidine (coloured)

Weighed material was homogenized with 0.1 M phosphate buffer. The pH of the buffer was maintained as follows:

- Fennel: 6.0
- Coriander: 7.0
- Carum: 6.5

The homogenate was centrifuged at 5000 rpm for 15 minutes. The supernatant was used as crude enzyme extract. The reaction mixture consists of 3.5 ml of 0.1 M phosphate buffer, 0.2 ml of enzyme extract, 0.1 ml of 0-dinitrosidine (1 mg/1 ml methanol). The mixture was equilibrated at 30°C and transferred to the cuvette of colorimeter at 420 nm. 0.2 ml of hydrogen peroxide (10 volume) was added to the cuvette and absorbance was noted at an interval of 30 seconds up to 3 minutes. The enzyme activity was expressed as change in absorbance/min/g. fr. wt.

Catalase:

The catalase activity was estimated following a manometric method by Chance and Maehly (1955). Weighed material was homogenized with 0.1 M phosphate buffer.
(pH 7.0) and a pinch of calcium carbonate. The final volume of extract was made up to 22 ml with the phosphate buffer and the homogenate was transferred to reaction bottle. 5 ml of 10 volume hydrogen peroxide was taken in polyethylene bottle and carefully placed in the reaction bottle. The reaction bottle was then connected to the manometer and the initial level of the manometer was noted. The reaction bottle was shaken uniformly for one minute and the reading of the manometer after one minute was noted. The difference in the two readings is the amount of liberated gas and catalase activity was expressed as ml of oxygen liberated/minute/g.fr.wt.

**Determination of fatty acids in triglyceride compounds**

**Extraction of oil**

The seeds were weighed and then ground, and made the powder. The powdered seed material was transferred to a paper thimble in soxhlet apparatus and extracted with petroleum ether (40-60°C) for 6 hours. The extract was filtered through the anhydrous sodium sulphate. The solvent extract was removed by distillation. The oil sample so obtained was kept in the oven at 70°C in order to get a constant weight of the sample. Then the oil was cooled to room temperature and weighed. The oil yield was expressed as percentage, i.e. number of grams of oil/100 g seeds.
Preparation of methyl esters:

Methyl esters of the oil samples were prepared following the method of Mehta and Lodha (1979). Two to three drops of oil was taken in a screw capped culture tube and to that 5 ml of 0.5 N sodium methoxide was added. The tubes were mixed thoroughly. Then incubated the tubes in a water bath at 70°C for 10 minutes. The tubes were cooled and subsequently 0.5 ml Boron trifluoride in methanol reagent (14% BF$_3$ in Methanol) was added and mixed well on a cyclomixer. Then the tubes were incubated in boiling water bath for 10 minutes. The tubes were cooled to room temperature and 2 ml of hexane was added and methyl esters were extracted into the upper hexane layer by shaking well on a cyclomixer. 1-2 µl quantities of the top hexane layer was injected into the GLC column.

Separation of fatty acids by GLC:

The methyl esters were analysed by gas liquid chromatography technique. Quantitative GLC was carried out with C.I.C. model. 1 to 2 µl of methyl esters was injected to the system. The vaporized sample passed into a 1/8 cm internal diameter and 6 feet long stainless steel column containing 10% Poly DEGS coated on 60-80 mesh chromosorb. The carrier gas used was argon with a flow rate of 40 ml/min and pressure of 2.2 kg/cm$^2$. The hydrogen gas pressure was 0.9 kg/cm$^2$. An ordinary diaphragm pump was used for fresh
air. The temperature of the columns and the injection part were 180°C and 230°C respectively. The chart speed of the recorder was adjusted to 1 cm per minute. A standard mixture of methylate fatty acid was run through the same conditioned column under identical conditions. The fatty acids in the sample were identified by comparison with the retention time (Rt) of standards.

The percentage of individual fatty acids was calculated from the ratio of the area occupied by individual fatty acid to total peak area, where

\[
\text{Area of each fatty acid} = \frac{\text{Height of the peak} \times \text{Width at half of the height}}{2}
\]

Hydrodistillation of seeds:

The extraction of volatile oil was carried out with the modified claevenger apparatus (Claevenger, 1928). The apparatus consists of round bottom short necked flask of 2 litres capacity; the essential oil trap and two condensers.

Weighed seed material was placed in the flask and distilled water was added (4 times to the weight of the seed material). Attached the essential oil trap and the condensers, enough water was added in the trap with 4-5 ml of petroleum ether (40°C-60°C). Placed the flask on oil bath and distillation was continued for 5 hours at 140°C (temp. of bath). Collected the petroleum ether sample and passed through
anhydrous sodium sulphate to remove the water traces. Evaporated the solvent (petroleum ether) from the volatile oil in rotavapour. Cooled the oil to room temperature and determined the weight of the oil and expressed the yield as weight/weight percentage, i.e. number of grams of oil/100 gm of seed material.

A Hewlett packard 5712-A model with Thermal conductivity detector mode isotherm was used for the analysis of fennel, cumin, carum and coriander. The column stationary phase was 10% SE-30 on the support of chromosorb 60-80 mesh. The length of the column was 6 feet. The speed of chart was 0.5 inches/minute, whereas a Hewlett packard 7624-A model with thermal conductivity detector mode Isotherm was used in the case of dill. Stationary phase 10% carbowax with the support of 60-80 mesh chromosorb of 12 feet length column was used. The speed of the chart was 0.25 inches/minutes. In both the cases the detector temperature was maintained at 250°C and column temperature at 170°C. The carrier gas used was hydrogen with flow rate of 60 ml/min. Attenuation was 4. Peak areas were measured with integrator.

A neat sample of 0.4 μl of essential oil was injected. For identification of main constituents coinjection technique was used so as to verify peak height and calculated the intrinsic value. Known standard compounds were injected
alone as well as in a proportionate mixture with a sample so as to verify the peak and its relative change in % value.

The biochemical analyses were carried out as described earlier. The mean values of three/six determinations were taken into consideration. The results were subjected to statistical analysis viz. standard error and analysis of variance ('F' test) for the values of single factorials and the interactions.