MATERIALS AND METHODS
MATERIALS AND METHODS

Isabgol (Plantago ovata Forsk) var Guj 2, Garden Cress (Lepidium sativum L) and Kalmegh (Andrographis paniculata Nees) were selected for the study. The source of fluoride was sodium fluoride (NaF). Chart – 1 represents the experimental design for the study on the effects of fluoride on growth, development and metabolism of medicinal plants. The following experiments were carried out.

EXPERIMENT - 1: STUDY ON EFFECTS OF NaF ON GROWTH AND METABOLISM IN SEEDLINGS OF MEDICINAL PLANTS

Isabgol experiment

The seeds of Isabgol (Plantago ovata Forsk) var Guj 2 were germinated in sterilized petri plates lined with sterilized filter paper. The media used were DW (control), NaF (200 ppm), NaF (400 ppm), NaF (600 ppm), NaF (800 ppm) and NaF (1000 ppm). Experiment was conducted at 28 ± 2°C under laboratory conditions up to 120h. The effects of fluoride on Isabgol seedlings were studied as follows:

(A) Study on growth

20 seedlings from each treatment i.e. DW, 200, 400, 600, 800 and 1000 ppm NaF were analysed for growth. The elongation of the root and shoot was measured and mean was expressed as cm/seedling. The fresh weight of 20 seedlings in two lots each containing 10 seedlings were noted and transferred to an oven at 80°C for dry weight determination. Fresh weight and dry weight were expressed as mg/seedling.

Fluoride effects were evaluated with the help of Relative Root Growth (RRG). RRG was calculated according to the following formula given by Ouzounidou et al. (1992).
### EXPERIMENTAL DESIGN FOR A STUDY ON EFFECTS OF FLUORIDE ON GROWTH, DEVELOPMENT AND METABOLISM OF MEDICINAL PLANTS

**FLUORIDE APPLICATION**

- **SEED**
  - (Lab Experiments)
  - (Media)

- **ROOT**
  - (Pot Experiments)
  - (Soil Application)

- **LEAF**
  - (Plot Experiments)
  - (Foliar Application)

**MEDICINAL PLANTS**
- (Isabgol, Garden Cress, Kalmegh)

**EFFECTS**

#### VEGETATIVE GROWTH
- Seedling Growth
  - Root Length
  - Shoot Length
  - Fresh Weight
  - Dry Weight
- Plant Growth
  - Root Length
  - Shoot Height
  - Leaf Length
  - Leaf Number
  - Root Fresh Weight
  - Stem Fresh Weight
  - Leaf Fresh Weight
  - Whole Plant Fresh Weight
  - Root Dry Weight
  - Stem Dry Weight
  - Leaf Dry Weight
  - Whole Plant Dry Weight

#### REPRODUCTIVE GROWTH
- Spike
- Inflorescence
- Fruit Number
- Spike Length
- Stock Length
- Spike
- Inflorescence
- Fruit Fresh Weight
- Stock Fresh Weight
- Spike
- Inflorescence
- Fruit Dry Weight
- Stock Dry Weight

#### PHOTO-SYNTHETIC PIGMENTS
- Chlorophyll 'a'
- Chlorophyll 'b'
- Total Chlorophyll
- Carotenoids

#### METABOLISM
- Carbohydrate Metabolism
  - α amylase
  - β amylase
  - Invertase
- Nonreducing sugar
- Reducing sugar
- Protein Metabolism
  - Protease
  - Protein
  - Total aminoacid
- Proline
- Oxidizing Enzymes and Phenol Content
  - Peroxidase
  - IAA oxidase
  - Polyphenol Oxidase
  - Phenol
  - Mucilage
  - Andrographolide

#### FLUORIDE UPTAKE & SOIL ANALYSIS
- Fluoride Uptake
  - Seeds
  - Vegetative Parts
  - Soil Analysis
  - EC
  - pH
- Carbonate
- Bicarbonate
- Ca hardness
- Mg hardness
- Sodium
- Chloride
- Fluoride
- Potassium
- Phosphorus
- Organic Carbon
Percent phytotoxicity was calculated by the following bioassay technique as suggested by Chou and Muller (1972).

\[
\text{Percent Phytotoxicity} = \frac{\text{Radicle length of control} - \text{Radicle length of test}}{\text{Radicle length of control}} \times 100
\]

(B) Study on metabolism

The control and treated seedlings were analysed for following metabolism.

- **Carbohydrate metabolism**
  1. α-Amylase and β-Amylase
  2. Invertase
  3. Reducing and Nonreducing sugar

- **Protein metabolism**
  4. Protease
  5. Protein
  6. Proline
  7. Total Amino acid

- **Oxidizing enzymes and Phenolic compound**
  8. Peroxidase
  9. IAA oxidase
  10. Polyphenol oxidase
  11. Total phenol

For enzymic activities, seedlings were crushed in ice-cold DW and centrifuged at 2000 rpm. The supernatant was the source of enzyme. The enzymic activities were expressed on the basis of protein. The following is the method for enzyme protein determination.
Enzyme protein

Enzyme protein was estimated by the method of Lowry et al (1951). To 1 ml aliquot, 4 ml 12.5% sodium carbonate and 1 ml 0.1% copper sulphate were added, incubated for 30 minutes and 0.5 ml folin-phenol reagent was added. After incubating for 10 mins, the optical density (OD) was recorded at 660 nm on Systronics 106 spectrophotometer. The following regression formula was prepared using the known concentration of casein.

\[ X = 236.60Y - 35.22 \]

Where \( Y = \) Optical Density

Enzyme protein was calculated and expressed as \( \mu g/mg \) fr. wt.

The metabolites were calculated on a dry weight basis.

- Carbohydrate Metabolism
  1. \( \alpha \) Amylase and \( \beta \) Amylase activities

These activities were determined by estimating total amylase activity and \( \alpha \) amylase activity. Total amylase activity was determined using the method of Paleg et al (1962). 1.0 ml enzyme aliquot was mixed with 1.0 ml citrate buffer \((pH 5)\) and 1.0 ml of 1% starch solution and incubated for 30 minutes at room temperature. The blank had 1.0 ml of DW instead of the enzyme extract. The enzyme activity was determined using the method of Paleg et al (1962) and it was as follows:

- 1.0 ml enzyme aliquot was mixed with 1.0 ml citrate buffer \((pH 5)\) and 1.0 ml of 1% starch solution and incubated for 30 minutes at room temperature. The blank had 1.0 ml of DW instead of the enzyme extract. The enzyme activity was stopped by adding 0.5 ml of cold iodine solution \((2.5 \text{ g of iodine and } 25 \text{ g of potassium iodide in } 1 \text{ L of } 0.05 \text{ N HCl})\). The final volume was made up to 20 ml and optical density \((OD)\) was read at 600 nm. The difference in OD between the blank and reaction i.e. starch hydrolyzed was calculated using the regression formula

\[ X = 331.49Y - 2.34 \]

Where \( Y = \) Optical Density

The amylase activity was expressed as \( \mu g \) starch hydrolyzed/h/\( \mu g \) protein

\( \alpha \) Amylase activity was determined by killing the \( \beta \) amylase. For this, the enzyme aliquot was heated at \( 70^0 \text{ C} \) for 20 min., then it was used for determination of \( \alpha \) amylase. The same procedure was used for the determination and calculation of enzyme activity. The value of \( \beta \) amylase activity was calculated by subtracting the value of \( \alpha \) amylase activity from value of total amylase activity.
2. **Invertase Activity**

Invertase activity was determined by the method of Hatch and Glasziou (1963). To 1 ml enzyme aliquot, 1 ml acetate buffer (pH 4.8) and 1 ml 0.25% sucrose (250 mg sucrose in 100 ml acetate buffer (pH 4.8) was added. The blank had 1 ml acetate buffer instead of 0.25% sucrose. This was incubated for one hour at room temperature. Enzyme activity was stopped by adding 2 ml 5% perchloric acid and made up to 10 ml. 1 ml of this mixture was taken to develop colour by the Nelson-Somogyi's method (Wharton and McCarty, 1972 as in case of estimation of sugar) OD was read at 540 nm on a Systronics 106 spectrophotometer. The invertase activity was calculated using the following regression equation prepared from glucose standard curve.

\[ X = 426.67Y - 15.25 \]

Where \( Y = \) Optical Density

The activity was expressed as \( \mu g \) glucose liberated/h/\( \mu g \) protein.

3. **Nonreducing and reducing sugar content**

Weighed oven-dried plant material was boiled in 80% ethanol for 4 to 5 minutes, homogenized with sand and centrifuged. The residue was again extracted with 5 ml 80% ethanol. After centrifugation, the ethanol was extracted and residual sugar was dissolved in a fixed volume (20 ml) of DW. From 20 ml of the extract, 10 ml each was taken for reducing sugar and total sugar. To the extract of total sugar, 3 ml 1N HCl (8.75 ml conc. HCl + 91.2 ml DW) was added and kept in a boiling water bath for 20 mins to hydrolyse nonreducing sugar. It was cooled and neutralized by adding 3 ml 1N NaOH. 1 ml of 25% lead acetate and 1ml of 25% sodium carbonate were added in both the sets and the volume was made up to 20 ml. After filtering, 1 ml of the aliquot was taken for sugar estimation by the method of Nelson-Somogyi (Wharton and McCarty, 1972). To 1 ml of the above aliquot was added 1 ml Nelson-Somogyi reagent (Nelson reagent A: 12.5 g sodium carbonate, 12.5 g sodium potassium tartarate, 10 g sodium bicarbonate and 100 g sodium sulphate were dissolved and volume made up to500 ml with DW. The Nelson reagent B: 15 g copper sulphate in 100 ml DW and 2 drops of conc. \( \text{H}_2\text{SO}_4 \)). Nelson reagent was prepared by mixing 50 parts of A with 2 parts of B. The tubes were capped with glass marbles and heated in a water bath at 100° C for 20 min, then cooled rapidly. 1 ml arsenomolybdate reagent (25 g ammonium
molybdate dissolved in 400 ml DW. To this was added 21 ml conc. H₂SO₄, 3 g sodium arsenate in 25 ml DW and volume was made to 500 ml) was added and shaken thoroughly for 5 minutes to dissolve the red ppt. This was made up to a final volume of 25 ml, the OD was read at 540 nm on a Systronics 106 spectrophotometer. By referring to the regression equation of glucose, the amount of reducing sugar and total sugar were calculated and expressed as mg/g dry weight. Nonreducing sugar was calculated by subtracting the value of reducing sugar from total sugar.

\[ X = 426.67Y - 15.25 \]

Where \( Y \) = Optical Density

- **Protein Metabolism**

4. Protease Activity

The method of Premgupta and Mukherjee (1982) was followed for the protease assay. 100 mg of fresh plant material was homogenized using sodium phosphate buffer (pH 6.0) and centrifuged at 2000 rpm for 15 min. The supernatant was collected and the volume made up to 10 ml. To 1 ml of aliquot were added 0.25 ml 0.1 % casein and 1 ml sodium acetate buffer (pH 7) and incubated at 30° C for 30 mins. The enzyme activity was then stopped by adding 2 ml of 15% trichloroacetic acid. The tubes were incubated for 20 min. at room temperature and then centrifuged. The supernatant was used to determine the amount of tyrosine liberate. To 1 ml of reaction aliquot, 1 ml DW, 3 ml of 0.5N NaOH, 0.7 ml folin phenol reagent were added and allowed to stand for 20 min. to develop the blue colour. The OD was read at 660 nm against a blank. The following regression equation was prepared using tyrosine as the standard.

\[ X = 232.14Y - 0.29 \]

Where \( Y \) = Optical Density

Protease activity was calculated and expressed as \( \mu g \) tyrosine liberated/30 min/\( \mu g \) proteins.

5. Protein Content

Protein was estimated by the method of Lowry et al (1951). Weighed amount of oven dried plant material was ground in 80% ethanol and extracted twice. The residue was first washed with cold 5% perchloric acid (to remove sugars and soluble protein fractions) and centrifuged. The residue was washed again with a
mixture of ethanol: ether: chloroform in the ratio of 2: 1: 1 (to remove acid soluble fractions and lipids) and centrifuged. The protein was dissolved in 1 N sodium hydroxide and kept for one hour before centrifuging. The supernatant was made 5 ml with NaOH and used as an aliquot. To 2 ml of the above aliquot was added 5 ml of Lowry reagent C (prepared by mixing 50 ml reagent A which is 2% sodium carbonate in 0.1 N NaOH and 1 ml reagent B which is 0.5% copper sulphate in 1% sodium potassium tartarate) and incubated at room temperature for 30 mins. The colour was developed by adding 0.5 ml folin phenol reagent. After 10 mins, the OD was read at 660 nm on a Systronics 106 spectrophotometer. By using casein following regression was prepared as standard. The protein content was calculated and expressed as mg/g dry weight.

\[ X = 236.6Y - 35.22 \quad \text{Where } Y = \text{Optical Density} \]

6. Proline content

Proline was determined by the method of Bates et al (1973). Weighed oven dried plant material was extracted in 3% sulphosalicylic acid. After centrifugation, 2 ml of the aliquot was added to 2 ml of acid ninhydrin reagent (1.25 g of ninhydrin dissolved in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid) and 2 ml of glacial acetic acid. The tubes were stoppered and the reaction mixture was allowed to boil in water bath at 100°C for 1h. The reaction was terminated using an ice-bath. Then the reaction mixture was extracted with 6 ml toluene with a separating funnel. The absorbance of the toluene extract was read on a Systronics 106 spectrophotometer at 520 nm against toluene blank. The following regression equation was prepared using proline as standard

\[ X = 49.776Y - 0.286 \quad \text{Where } Y = \text{Optical Density} \]

The proline content was calculated and expressed as mg/g dry weight.

7. Total Amino acid Content

The content of total amino acids was determined following the method of Harding and Maclean (1916). The reaction system contained 1 ml ethanol extract of the plant material, 1 ml 10% pyridine and 1 ml 2% ninhydrin reagent. The tubes were stoppered, heated in a water bath at 100°C for 30 mins, the violet blue colour was developed, later it was cooled and diluted with DW to a final volume of 10 ml. OD
was read at 570 nm on Systronics 106 spectrophotometer. The following regression was prepared using isoleucine as standard.

\[ X = 413.42 \, Y + 19.23 \]

Where \( Y = \) Optical Density

The total amino acid content was calculated and expressed as mg/g dry weight.

- **Oxidizing Enzymes and Phenolic compounds**

8. Peroxidase Activity

The method of George (1953) and Maehly (1954) was employed to assay peroxidase activity. The reaction mixture with 2 ml enzyme aliquot, 2 ml 0.2 M phosphate buffer (pH 7.0) and 2 ml 20 mM guaicol reagent (0.22 ml guaicol in 100 ml DW, prepared 24 hours before carrying out the estimation) was taken in cuvette. The cuvette was placed in the Systronics 106 spectrophotometer. The OD was read at 540 nm. 2 drops of 10 \( \mu \)M hydrogen peroxide (0.4 ml H\(_2\)O\(_2\) in 9.6 ml DW) was added to the reaction mixture in the cuvette and again the OD was noted after 30 seconds. The difference in OD before and after adding H\(_2\)O\(_2\) to the reaction mixture was used to calculate peroxidase activity. The activity was expressed in terms of OD developed/min/mg protein.

9. IAA Oxidase Activity

IAA oxidase activity was assayed according to the method of Hare (1964). To 1 ml of enzyme aliquot, 1 ml IAA solution (200 ppm), 1 ml 0.15 M phosphate buffer and \( 10^{-3} \) M 2, 4 dichlorophenol and 1 ml \( 10^{-3} \) M MnCl\(_2\) were added and incubated at room temperature for 1h in the dark. To 2 ml of the above reaction mixture, 4 ml Salkowski reagent (1 ml of 0.5 M FeCl\(_2\) and 50 ml 35% perchloric acid) was added and incubated at 30 mins in the dark, the pink colour was developed. The OD was read at 530 nm on a Systronics 106 spectrophotometer. The following formula was prepared using IAA as standard.

\[ X = 101.98 \, Y + 7.79 \]

Where \( Y = \) Optical Density

The activity was calculated and expressed as \( \mu \)g IAA oxidized/h/\( \mu \)g protein.

10. Polyphenol Oxidase Activity

The activity was estimated according to the method of Kar and Mishra (1976). To 1 ml of enzyme aliquot, 1 ml DW, 2 ml 0.15 M phosphate buffer (pH 6.3) and 2 ml
pyrogallol (50 μM = 12.6 mg in 100ml DW) were added and incubated at room temperature for 5 mins. After incubation 0.5 ml 5% H₂SO₄ was added. The OD was read at 420 nm and expressed as OD developed/5 min/μg protein.

11. Total Phenol Content
Phenol was determined using the method of Farks and Kiraly (1962). The reaction mixture containing 0.5 ml of the ethanol extract, 1 ml of 20% sodium carbonate and 0.5 ml folin phenol reagent was heated in a water bath for 10 mins, cooled and diluted to a fixed volume of 5 ml with DW. It was filtered using Whatman filter paper No. 1 to remove the precipitates. The OD of the blue colour filtrate was read at 660 nm on a Systronics 106 spectrophotometer. The following regression formula was prepared using gallic acid as the standard.

\[ X = 96.05 \times Y + 10.03 \]

Where \( Y \) = Optical Density

The phenol content was calculated and expressed as mg/g dry weight.

Garden Cress experiment

A similar experiment was done with Garden Cress (Lepidium sativum L). The seeds were germinated in sterilized petriplates having sterilized filter paper under laboratory conditions. The media were DW and graded concentrations i.e. 200, 400, 600, 800 and 1000 ppm of NaF, experiment was continued up to 96h. The 24h, 48h, 72h and 96h old seedlings were analyzed for growth and metabolism. The parameters of growth and metabolism were same as mentioned in EXPT. 1

Kalmegh experiment

The seeds of Kalmegh (Andrographis paniculata Nees) were also germinated in sterilized petriplates lined with sterilized filter paper. The media for germination were DW (control), NaF (100 ppm), NaF (200 ppm), NaF (400 ppm) and NaF (600 ppm). Seeds did not germinate in 800 ppm. The experiment was continued for eight days. Seedling growth was recorded on completion of 5th, 6th, 7th and 8th day. The parameters of growth were same as mentioned in EXPT. 1
EXPERIMENT - 2: STUDY ON EFFECTS OF SOIL APPLICATION OF NaF ON GROWTH, PHOTOSYNTHETIC PIGMENTS AND METABOLISM IN MEDICINAL PLANTS

Isabgol experiment

Seeds of Isabgol (Plantago ovata Forsk, var Guj 2) were sown in earthen pots containing 0 NaF (control), 200 mg NaF/kg soil, 400 mg NaF/kg soil, 600 mg NaF/kg soil, 800 mg NaF/kg soil and 1000 mg NaF/kg soil. Seeds did not germinated in the pots having 800 mg NaF/kg soil and 1000 mg NaF/kg soil, so the experiment focused on the responses up to 500 mg NaF/kg soil. Plants were cultivated using normal practice, and the effects of soil application of NaF on Isabgol plants were studied as follows:

(A) Study on growth

The method of growth analysis used by Gregory (1921, 1926) and Hunt (1978) was used for the growth study. 10 plants at random from each treatment i.e. control, 200, 400 and 600 NaF were selected for the growth study. The measurements were carried out at fortnightly intervals and the study period was 105 days. Plants were carefully uprooted, brought to the laboratory and washed under slow running tap water. The root length and shoot height of each plant were measured and expressed as cm/plant. The length of the longest leaf was also measured and expressed as cm/plant. The leaf number was noted and expressed as no/plant. The fresh weight of roots, stems and leaves were recorded and these were transferred to the oven for dry weight determinations. All were expressed as g/plant. Reproductive growth was studied in terms of spike number (number/plant), spike length (cm/plant), stock length (cm/plant), fresh weight and dry weight of spike and stock (g/plant). Yield was recorded in terms of seed weight, vegetative parts weight and expressed as g/plant.

Harvest Index was calculated using following formula

\[
\text{Harvest Index} = \frac{\text{Dry weight of economic yield i.e. seed}}{\text{Dry weight of biological yield i.e. whole plant}} \times 100
\]
The fluoride effect was evaluated on the basis of relative root growth (RRG), percent phytotoxicity and root shoot ratio. RRG and percent phytotoxicity were calculated as described earlier. R/S ratio was calculated on elongation base. Percent allocation of dry matter in roots, stems and leaves were calculated using the following formula.

\[
\text{Percent allocation} = \frac{\text{Dry matter of particular organ}}{\text{Dry matter of whole plant}} \times 100
\]

Relative Growth Rate (RGR) was another parameter used to evaluate the effects of fluoride on plant growth. RGR was determined as the difference between Naperian logarithms of dry weight of successive days sample, (RGR was calculated on dry weight basis). The formula for RGR (Blackman, 1919) is as follows:

\[
\text{RGR} = \log_e W_1 - \log_e W_0
\]

Where, \( W_0 \) and \( W_1 \) are successive dry weight

Leaf Weight Ratio (LWR) and Net Assimilation Rate (NAR) were calculated by Gregory’s method (1926) as below:

\[
\text{LWR} = \frac{L_1 - L_0}{\log_e L_1 - \log_e L_0}
\]

\[
\text{NAR} = \frac{W_1 - W_0}{L_1 - L_0 / \log_e L_1 - \log_e L_0}
\]

Where \( L_0 \) and \( L_1 \) were successive dry weight of leaves of the plant and \( W_0 \) and \( W_1 \) were the successive dry weights of whole plant.
Fluoride was considered as a stress for the plants and fluoride toxicity on Isabgol growth was evaluated with the help of a stress index. The following formula was used for calculating the fluoride stress index.

\[
\text{Fluoride Value of particular parameter of fluoride treated plant} \\
\text{Stress Index =} \frac{\text{Value of same parameter of control plant}}{\text{X100}} \\
\text{Stress index values were calculated from yield data.}
\]

(B) Study on Photosynthetic Pigments

The photosynthetic pigments were determined using the method of Arnon (1949). The fourth leaf of control and treated plants (in replicate) were analyzed for photosynthetic pigments viz. chlorophyll ‘a’, chlorophyll ‘b’, total chlorophyll and carotenoids. The photosynthetic pigments were estimated from 30, 45, 60, 75 and 90 days old plants. Weighed fresh leaf material was crushed in 80% acetone. The homogenate was filtered using Whatman filter paper No. 1 and the filtrate was made up to 25 ml with 80% acetone. The absorbance of pigment suspension was read on a spectrophotometer (Systronics 106) at 480, 510, 645 and 663 nm.

The following formula was used to calculate the quantity of photosynthetic pigments

\[
\begin{align*}
\text{Chlorophyll 'a'} & = 12.7 \times (D663) - 2.6g \times (D645) \\
\text{Chlorophyll 'b'} & = 22.9 \times (D645) - 4.68 \times (D663) \\
\text{Total Chlorophyll} & = 20.2 \times (D645) + 8.02 \times (D663) \\
\text{Carotenoids} & = 7.6 \times (D480) - 1.49 \times (D510)
\end{align*}
\]

Where D = Optical Density

The photosynthetic pigments were expressed on the basis of mg/g fresh weight.

For evaluating the fluoride stress effect, the ratio of Chlorophyll ‘a’: Chlorophyll ‘b’ and Total chlorophyll : Carotenoids were also calculated.
(C) Study on metabolism
The fourth leaf of 30, 45, 60, 75, 90 and 105 days old control and treated plants (in 2 replicate) were analyzed for following metabolism. The methods were the same as mentioned in EXPT.1 (B)

- **Carbohydrate metabolism**
  1. $\alpha$-Amylase and $\beta$-Amylase
  2. Invertase
  3. Reducing and Nonreducing sugar

- **Protein metabolism**
  4. Protease
  5. Protein
  6. Proline
  7. Total Amino acid

- **Oxidizing enzymes and Phenolic compound**
  8. Peroxidase
  9. IAA oxidase
  10. Polyphenol oxidase
  11. Total phenol

**Mucilage content**
The seeds of control and treated plants (120 DAS) were analyzed for mucilage content by the method mentioned in Indian Herbal Pharmacopoeia (1998). 1 g of Isabgol seeds was introduced into 25 ml glass stoppered measuring cylinder and then 20 ml DW was added. The mixture was shaken thoroughly at intervals of every 10 min. for 1 hr. Then it was kept for three hours at room temperature. The volume in ml occupied by the plant material including any sticky mucilage was measured. Mean values of the individual determination were calculated in relation to 1 g of the seeds, which indicates the 'Swelling Index'.

(D) Study on fluoride uptake
Seeds and vegetative parts (120 DAS) were analyzed for fluoride uptake using the Ion selective electrode method (AOAC, 1998). The methodology is described below:
For fluoride analysis of plant material, samples were oven-dried in paper sacks at 80°C for 48 hours, finally ground and used for further analytical procedure.

A potentiometric method was followed in preparing the sample for fluoride determination and in making fluoride standard curves. 0.25 g of previously-ground sample was placed in an acid-cleaned plastic beaker to which was added 1 ml of analytical grade acetone for wetting the dry material. Most of the acetone was allowed to evaporate from the sample. Next, 20 ml of 0.05 N nitric acid solution was added. The mixture was stirred for at least 30 min with a magnetic stirrer, following which 20 ml of 0.1 N potassium hydroxide solution was added and stirred for an additional 30 min. Finally, 5 ml of 0.2 N nitric acid solution was added along with 5 ml of 0.4 M sodium citrate solution (pH 5.5) containing 1 ppm fluoride. Samples from at least two different digests were analyzed in duplicate using two or more different fluoride electrodes. The amount of fluoride in each sample was calculated as ppm dry weight of plant material from the equation:

\[
\text{ppm F (µg/g)} = \frac{(C - 0.1)50}{W}
\]

Where: \( C \) = ppm F from standard curve, \( W \) = gram of sample used
\( 0.1 \) = ppm of F present in the sodium citrate solution
\( 50 \) = total ml of solution

**Preparation method for fluoride standard curve**

25.0 ml of 0.1 ppm fluoride working standard solutions were placed in plastic containers containing stirring bar. Electrodes (aprox. 12 mm) were inserted into the solution and stirred magnetically. The mV readings were recorded at 1 min intervals until change of < 0.2 mV/min. Electrodes were removed, blotted lightly with absorbent paper, and the procedure was repeated with 0.2, 0.5, 2.0 and 10.0 ppm standard solution. Electrodes were placed in 0.2 ppm standard solution until samples were analyzed. Potential (mV) on vertical arithmetic axis and fluoride concentration (µg/ml; ppm) on horizontal (logarithmic) axis was plotted on of 2-cycle semi log graph paper.

- Nitric acid: 10 N: Add 63 ml HNO₃ to DW, cool and dilute to 100 ml
- Nitric acid: 0.2 N: Dilute 5.0 ml 10 N to 250 ml.
- Nitric acid: 0.05 N: Dilute 5.0 ml 10 N to 1 L
Potassium nitrate solution: 0.4 M: Dissolve 4.0 g KNO₃ in DW and diluted to 100 ml.

Sodium citrate solution: 0.8 M: Dissolve 58.8 g sodium citrate·2H₂O in 200 ml DW, adjusted to pH 5.5 by drop wise addition of 10 N HNO₃, using pH meter, and diluted to 250 ml with H₂O.

0.4 M sodium citrate containing 1 ppm fluoride: Dilute 125 ml 0.8 M sodium citrate solution and 25.0 ml 10 ppm fluoride standard solution to 250 ml with DW.

Fluoride standard solutions

Fluoride stock solution: 100 ppm F⁻: 0.221 g NaF dissolve in DW and dilute to 1 L.

Intermediate solution: 10 ppm: Dilute 10.0 ml stock solution to 100 ml with DW.

Working solution: prepare as follows in 100 ml volumetric flask:

<table>
<thead>
<tr>
<th>ppm</th>
<th>Cons.</th>
<th>0.4 M KNO₃</th>
<th>0.8 M Sodium citrate</th>
<th>100 ppm Fluoride solution</th>
<th>10 ppm Fluoride solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>10.0</td>
<td>5.0</td>
<td>10.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>10.0</td>
<td>5.0</td>
<td>2.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>10.0</td>
<td>5.0</td>
<td>0.0</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>10.0</td>
<td>5.0</td>
<td>0.0</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>10.0</td>
<td>10.0</td>
<td>0.0</td>
<td>0.0+10.0 ml 0.4 M Sodium citrate solution containing 1 ppm fluoride</td>
<td></td>
</tr>
</tbody>
</table>

(E) Study on soil analysis

At the end of the experimental period control and fluoride contaminated soils were analyzed for EC, pH, Chloride, Carbonate, Bicarbonate, Calcium hardness, Magnesium hardness, Potassium, Phosphorous, fluoride and Organic Carbon. EC, pH, chloride, carbonate, bicarbonate, calcium, magnesium and sodium were estimated using the method of Saxena (1987). 10 g of air dried soil was taken and 100 ml DW added to it. The suspension was prepared and it was filtered through a filter paper (Whatman No.44). The filtrate was used for following determinations.

1. Electrical Conductivity (EC) of Soil

Electrical Conductivity was determined from soil solution using a Systronics digital conductivity meter 303 and expressed as mMho.

2. pH of Soil

The pH of soil solution was determined using a Systronics digital pH meter.
3. Chloride (Cl⁻) Determination
10 ml soil solution was taken in a flask and 5 to 6 drops of 0.5% K₂CrO₄ was added. The color of the sample became yellow, it was titrated against 0.01 N silver nitrate solution until a persistent brick red color appeared. Chloride content as ppm was calculated by using the following formula:

\[
\text{Chloride (ppm)} = \frac{V \times N \times 35.457}{S} \times 1000
\]

Where, 
- \( V \) = Volume of titrant (ml)
- \( N \) = Normality of titrant (0.01N)
- \( S \) = Volume of sample (ml)

4. Carbonate and Bicarbonate Determination
10 ml of the sample was taken in a flask and 2-3 drops of phenolphthalein indicator was added. The appearance of pink color indicates the presence of carbonate. The sample was titrated against 0.01N HCl until the pink color disappeared. Carbonate content (ppm) was calculated by using the following formula:

\[
\text{Carbonate (ppm)} = \frac{2V \times N \times 30 \times 1000}{S}
\]

Where, 
- \( V \) = Volume of titrant (ml)
- \( N \) = Normality of titrant (0.01N)
- \( S \) = Volume of sample (ml)

Then 2-3 drops of methyl orange indicator was added to the same flask and titrated against 0.01N HCl continued until the yellow color of the solution turned orange (end point). Bicarbonate content (ppm) was calculated using the following formula:

\[
\text{Bicarbonate (ppm)} = \frac{V \times N \times 61 \times 1000}{S}
\]

Where, 
- \( V \) = Volume of titrant [Volume of methyl orange end point--2xVolume of phenolphthalein end point (ml)]
- \( N \) = Normality of titrant (0.01N)
- \( S \) = Volume of sample (ml)
5. Calcium Determination

10 ml of the sample was taken in a flask and 1 ml of 4N sodium hydroxide solution and 1 grinded calcium hardness tablet was added and then titrated against 0.02N EDTA solution until the pink color turned purple. Calcium content (ppm) was calculated by using the following formula:

\[
\text{Calcium (ppm)} = \frac{V \times N \times 20 \times 1000}{S}
\]

Where, \( V \) = Volume of titrant (ml)
\( N \) = Normality of titrant (0.02N)
\( S \) = Volume of sample (ml)

6. Magnesium hardness

10 ml of the sample was taken in a flask and 1 ml of Ammonia buffer pH 10 solution was added. To it one grinded total hardness tablet was added. Sample was titrated against EDTA solution (0.02 N) until the wine red color of the solution turned blue (end point). Total hardness in terms of Calcium carbonate (CaCO₃) was determined using the following formula:

\[
\text{CaCO₃ (ppm)} = \frac{V \times N \times 50 \times 1000}{S}
\]

Where, \( V \) = Volume of titrant (ml)
\( N \) = Normality of titrant (0.02N)
\( S \) = Volume of sample (ml)

Magnesium hardness was determined by subtracting the value of calcium hardness from the value of total hardness and presented as ppm.

7. Sodium Determination

Sodium (ppm) was determined using a Systronics 125 digital flame photometer. Sodium chloride was used for the preparation of a standard sodium curve.

Phosphorus, potassium and organic carbon were determined using the methods described by Tandon (1993).
8. Phosphorus Determination

2.5 gm air dried soil was placed in a 150 ml Erlenmeyer flask. A small amount of phosphorus free activated charcoal was added and then 50 ml of Olsen reagent (84 g NaHCO₃ was dissolved in water and made up to 2 liter, pH 8.5 was adjusted with 1 M NaOH it was stored in glass bottle) was added and shaken on a reciprocating shaker for 30 min. Blanks were run without soil. The soil suspension was filtered through Whatman No. 42 filter paper into a clean dry flask and it was shaken immediately. 5 ml aliquot of this extract was placed in a 25 ml volumetric flask and acidified with 2.5 M H₂SO₄, adjusted to pH 5. DW was added and the volume was made up to 20 ml then 4 ml of following reagent was added. (1.056 g ascorbic acid was dissolved in 200 ml reagent A, for reagent A, 12 g ammonium molybdate was dissolved in 250 ml DW, 0.2908 g antimony potassium tartrate was dissolved in 100 ml water. Both the solutions were added to 1000 ml of 2.5 M H₂SO₄. The solutions were thoroughly mixed and made up to 2000 ml and stored in Pyrex bottles in a dark and cool place. After waiting for 10 min, the intensity of blue color was measured in a spectrophotometer. The standard solution was prepared by dissolving 0.439g potassium dihydrogen ortho phosphate AR Grade (dried in oven at 60°C for 1h and cooled in desiccator) was dissolved in 500 CC of DW. 25 ml of 7 N H₂SO₄ was added. Finally 1 L was made up with distilled water. These give 100 ppm P standard stock solution. From this, a 2 ppm solution was made by diluting it 50 times. 1, 2, 3, 4, 5 and 10 ml of 2 ppm phosphorous solution were placed in a 25 ml volumetric flask. 5 ml of Olsen’s reagent was added. Each 5 ml aliquot was acidified with 2.5 M H₂SO₄ adjust to pH 5, and DW added to make up the volume to 20 ml. 4 ml reagent B was added. After 10 min the OD was recorded at 730 nm on spectrophotometer and standard curve was prepared.

The following formula was used for calculating available phosphorus.

\[
P (\text{kg/hectare}) = \frac{R \times \text{Volume of extract}}{\text{Volume of aliquot}} \times \frac{2.24 \times 10^6}{\text{Wt. (g) of soil} \times 10^6}
\]

Where \( R = \mu g \) P in the aliquot (obtained from standard curve)
9. Potassium Determination

5 g soil was placed in a Erlenmeyer flask and 25 ml of neutral ammonium acetate pH 7 (57 ml 99.5% glacial acetic acid was added to 700 ml of DW and then 69 ml of concentrated ammonium hydroxide was added to it. The volume was diluted to 900 ml and the pH was adjust to 7.0 by the addition of 3N ammonium hydroxide or 3N acetic acid and made up to 1 L) was poured into the flask. The flask was shaken on a reciprocating shaker (180 + oscillations/min) for 5 min and then filtered immediately through Whatman No. 1 filter paper. The first few ml of the filtrate was rejected. The filtrate was analyzed using flam photometer. A KCl standard (1000 ppm stock solution of KCl was made by dissolving 1.908 g of AR grade potassium chloride in DW and diluted up to 1 liter. Graded range of various standard was prepared by diluting stock solution with DW) was used for preparing the standard curve.

The following formula was used to calculate Potassium (kg/hector)

\[
K \text{ (kg/ hectare)} = \frac{R \times \text{Volume of extract} \times 2.24 \times 10^6}{\text{Wt. (g) of soil} \times 10^6}
\]

Where \( R = \) ppm of K in the extract (obtained from standard curve)

10. Fluoride determination

The Ion selective electrode method (Expt-1D) used to estimate the fluoride concentration from control and treated soil and expressed as mg/g.

11. Organic Carbon by Wet Digestion

Organic matter (OM) in the soil was oxidized with a mixture of potassium dichromate (K\(_2\)Cr\(_2\)O\(_7\)) and concentrated H\(_2\)SO\(_4\) utilizing the heat of dilution of H\(_2\)SO\(_4\). Unused K\(_2\)Cr\(_2\)O\(_7\) was back titrated with ferrous sulphate (FeSO\(_4\). 7H\(_2\)O) or ferrous ammonium sulphate (FeSO\(_4\). (NH\(_4\))\(_2\)SO\(_4\).6H\(_2\)O).

Reagent Needed

1. Standard potassium dichromate solution (0.1667 M = 1N): Dissolve exactly 49.04 g reagent grade K\(_2\)Cr\(_2\)O\(_7\) (dried at 105°C for 2h) in DW and diluted to 1 litre in a volumetric flask
2. Ferrous sulphate or Ferrous ammonium sulphate solution (0.5M = 0.5N): Dissolve 140 g of FeSO₄. 7H₂O or 196.1 g reagent grade FeSO₄. (NH₄)₂SO₄.6H₂O in about 800 ml DW, add 20 ml conc. H₂SO₄, cool and diluted to 1 litre in a volumetric flask.

3. Diphenylamine indicator: Dissolve 0.5 g diphenylamine in a mixture of 20 ml water and 100 ml of conc. H₂SO₄.

4. Sulphuric acid: concentration not less than 96% (Sp.Gr.1.84). If a high amount of Chloride (Cl⁻) was present in the samples, Ag₂SO₄ was added at the rate of 15 g/litre to the acid.

5. Orthophosphoric acid (85%) and/or sodium fluoride chemically pure/pure grade.

Procedure:

1 g of 0.2 mm (dia.) soil was accurately weighed, and kept in a dry 500 ml Erlenmeyer flask. 10 ml of dichromatic solution was added and the flask was swirled gently then rapidly 20 ml of concentrated H₂SO₄ was added into the suspension and swirled the flask 2 to 3 times and allowed the flask to stand for 30 minutes followed by addition of 200 ml distilled water. Then 10 ml of phosphoric acid or 0.5 g sodium fluoride and 1 ml of diphenylamine indicator were added and titrated with ferrous sulphate solution till the color changed from blue violet to green. Procedure was repeated without soil for blank. The organic carbon was determined using the following formula

\[
\text{Organic Carbon (\%) = } \frac{10 (B-T)}{B} \times \frac{0.003 \times 100}{\text{Wt. of soil (g)}}
\]

Where B = Volume (ml) of ferrous sulphate solution required for blank titration.

T = volume of ferrous sulphate solution needed for titration of soil sample.

**Garden Cress experiment**

The effects of soil applications of NaF were also studied on Garden Cress (Lepidium sativum L). Seeds of Garden Cress were sown in earthen pots filled with garden soil. Before sowing 0.0 NaF (control), 200 mg NaF/kg soil, 400 mg
NaF/kg soil, 600 mg NaF/kg soil, 800 mg NaF/kg soil and 1000 mg NaF/kg soil were added separately to the pots. Plants were cultivated using normal practices. The effects of soil application of NaF on growth, photosynthetic pigments and metabolism were studied from 15, 30, 45, 60, 75, 90 and 105 days old control and treated plants. The parameters were the same as mentioned for Isabgol EXPT. 2. Chemical analysis of soil and fluoride uptake by vegetative parts and seeds were also carried out as in EXPT. 2.

**Kalmegh Experiment**

The effects of NaF on Kalmegh (*Andrographis paniculata* Nees) was also studied. Kalmegh saples having 3 to 4 leaves were transplanted in the pots having 0.0 (control), 100 mg NaF/kg soil, 200 mg NaF/kg soil, 400 mg NaF/kg soil and 600 mg NaF/kg soil. Plants were grown with normal practice. NaF induced effects on growth and photosynthetic pigments were studied from 15, 30, 45, 60, 75, 90 and 105 days old plants. The parameters were the same as mentioned for EXPT.2.

The whole plant (120 days) from control and fluoride treatments were estimated for andrographolide using the HPTLC technique (Indian Herbal Pharmacopoeia, 1998).

**Andrographolide Determination**

The control and NaF (100, 200, 400 and 600 mg NaF/kg soil) treated dried whole plants (105 DAS) of Kalmegh were used for estimation of andrographolide - a diterpene lectone. Andrographolide was estimated using the HPTLC (High Performance Thin Layer Chromatography) technique (Indian Herbal Pharmacopoeia, 1998) with some modifications. The details are as follows:

**Preparation of plant sample**

For quantitative estimation of andrographolide, the plants were dried in an oven at 40-45°C for 48h. For each sample, the dried material of the whole plant was powdered finely and passed through 40 mesh sieve. The following extraction procedure was carried out for quantifying andrographolide in each sample.
Pre weighed sample + 50 ml methanol

\[ \text{Reflux for 15 minutes at } 60^\circ \text{C and then filter through vacuum. Collect the filtrate} \]

\[ \text{Evaporate i.e. concentrate up to 10 ml} \]

Make up volume up to 25 ml with Methanol

Clear solutions from the above 25 ml was used for spotting the HPTLC plates[spot volume ranging from 2 – 30 μl] Thin layer chromatography (TLC) plates of precoated Silica Gel 60 F\(_{254}\) Art 5543 DC Alufoline Kieselgel F\(_{254}\), E.Merck, Darmstadt, Germany were used for analysis.

Standard preparation

Andrographolide procured from Sigma was used as the standard. A stock solution of andrographolide was prepared in methanol (a concentration of 1 mg/ml). Stock solution farther diluted to 100 μg/ml with methanol. The range of spot volume was from 2 to 8 μl. Plate width was 8 mm and spacing between two spot was 8 mm.

Procedure

Aliquots of the sample from the 25 ml clear solution were spotted on TLC plates with the help of Camag Linomat IV Auto sampler. Andrographolide reference standards, ranging from 200 – 600 ng, were spotted on TLC plates and developed in a glass chamber using a solvent system with benzene : acetone : formic acid in the ratio of 60 : 40 : 0.4 up to 10 cm, where the spot were clearly separated as concentrated zones. The chamber was saturated with the solvent system before development and the TLC plates were dried completely using hot air after development. The spots of andrographolide were visualised under UV \( \lambda_{\text{max}} \) 233 nm at Rf value 0.37 + 0.05. Determination of andrographolide was done by scanning the quenching of fluorescence of TLC plates with the help of Camag TLC Scanner 3 using the ratio of the peak area of unknown to that of a standard curve of andrographolide was obtained by plotting the area under the peak of andrographolide against the concentration over a range of 200 – 600 ng. The lowest detection limit of andrographolide was found to be 100 ng.
Chemical analysis of soil and fluoride uptake from whole plant was also studied as in the method given in EXPT. 2.

**EXPERIMENT - 3: STUDY ON EFFECTS OF FOLIAR APPLICATION OF NaF ON GROWTH, PHOTOSYNTHETIC PIGMENTS AND METABOLISM IN MEDICINAL PLANTS**

**Isabgol experiment**

The plants of Isabgol var Guj 2 were grown in plots (1m X 1m), after 30 days plants were sprayed with NaF. The concentrations of NaF were 200, 400, 600, 800 and 1000 ppm. The foliar spray was given every third day from 30 - 90 days after sowing. A hand sprayer was used for exogenous application and spraying was done until the leaf surface became wet on both the sides. The group of plants which did not receive any spray was considered as a control. DW was also sprayed as control of spray treatment. The effects of NaF were studied as follows.
(A) Study on growth
The control and treated plants (45, 60, 75, 90 and 105 days) were examined at regular intervals for various growth parameters as mentioned in EXPT. 2(A).

(B) Study on photosynthetic pigments
The fourth leaf of plants after 45, 60, 75 and 90 days (in 2 replicate) were analyzed for photosynthetic pigments as described in EXPT. 2(B).

(C) Study on metabolism
The fourth leaf of plants (in 2 replicate) after 45, 60, 75, and 90 days was analyzed for the following:

- Carbohydrate metabolism
  1. α-Amylase and β-Amylase
  2. Invertase
  3. Reducing and Nonreducing sugar

- Protein metabolism
  4. Protease
  5. Protein
  6. Proline
  7. Total Amino acid

- Oxidizing enzymes and Phenolic compound
  8. Peroxidase
  9. IAA oxidase
  10. Polyphenol oxidase
  11. Total phenol
The methods were described in EXPT.2 (C)

Mucilage content
The mucilage was estimated from seeds of control and treated plants. Method was same as mentioned in EXPT.1 (B).
Study on fluoride uptake

Seeds and whole plants (120 days) were analyzed for fluoride uptake using the Ion selective electrode method as per EXPT.2 (D)

Garden Cress experiment

The plants of Garden Cress var local were raised in plots, and after 30 days were sprayed with NaF. The concentrations of NaF were 200, 400, 600, 800 and 1000 ppm. The foliar spray was given every third day from 30 - 90 days after sowing. The group of plants which did not receive any spray was considered as control. DW was also sprayed as a control of spray treatment. The effects of NaF on growth, photosynthetic pigments and metabolism were studied on 45, 60, 75, 90 and 105 day old plants. The parameters were the same as these mentioned in EXPT. 2.

Seeds and whole plants were analyzed for fluoride using an Ion selective electrode method as th described in EXPT. 2 (D)

Statistical Analysis

The data on growth and biochemical parameters were subjected to Analysis of Variance using Fisher's method (1954).