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Kalmegh (*Andrographis paniculata* Nees) was selected as a medicinal plant for study on heavy metal impact. The source of heavy metal was copper, cobalt, cadmium and mercury in the form of their chloride salt i.e. CuCl₂, CoCl₂, CdCl₂ and HgCl₂. Chart 1 represents the experimental design for the heavy metal impact assessment study on growth and metabolism of medicinal plant (*Andrographis paniculata* Nees). The following experiments were carried out.

**EXPERIMENT I: IMPACT OF HEAVY METAL ON GROWTH AND METABOLISM OF SEEDLINGS OF MEDICINAL PLANT-KALMEGH (Petriplate Experiment)**

The seeds of Kalmegh (*Andrographis paniculata* Nees) were germinated in sterilized petriplates lined with (Whatmann) filter paper. The media for germination were:

- DW (control)
- 10ppm CuCl₂ / CoCl₂ / CdCl₂ / HgCl₂
- 50ppm CuCl₂ / CoCl₂ / CdCl₂ / HgCl₂
- 100ppm CuCl₂ / CoCl₂ / CdCl₂ / HgCl₂
- 150ppm CuCl₂ / CoCl₂ / CdCl₂ / HgCl₂
- 200ppm CuCl₂ / CoCl₂ / CdCl₂ / HgCl₂

The experiment was conducted at 28±2°C under laboratory conditions up to 216 hours. The impact of heavy metal on Kalmegh seedlings were studied as follows:

**(A) Study on seedling growth:**

10 seedlings from each treatment i.e. DW, 10, 50, 100, 150 and 200 ppm CuCl₂, CoCl₂, CdCl₂ and HgCl₂ were analysed for growth. The elongation of root and shoot was measured and mean was expressed as cm/seedling. The
HEAVY METAL IMPACT ASSESSMENT STUDY ON GROWTH AND METABOLISM OF MEDICINAL PLANT

KALMEGH (Andrographis paniculata Nees)

IMPACTS

Vegetative Growth
- Seedling growth
  - Root length
  - Shoot length
  - Fresh weight
  - Dry weight
- Plant growth
  - Root length
  - Stem height
  - Leaf number
  - Fresh weight
  - Root
  - Stem
  - Leaf
  - Whole Plant
- Dry weight
  - Root
  - Stem
  - Leaf
  - Whole plant

Reproductive Growth
- Inflorescence branch number
- Flower bud number
- Flower number
- Fruit number
- Fresh weight
- Inflorescence
- Flower bud
- Flower
- Fruit
- Dry weight
- Inflorescence
- Flower bud
- Flower
- Fruit

Photosynthetic Pigments
- Chlorophyll 'a'
- Chlorophyll 'b'
- Total Chlorophyll
- Carotenoids

Metabolism
- Carbohydrate metabolism
  - Invertase activity
  - Nonreducing Sugar content
  - Reducing Sugar content
- Protein metabolism
  - Protease activity
  - Protein content
  - Total Amino acid content
  - Proline content
- Antioxidant enzymes and IAA oxidase activity
  - Polyphenol oxidase activity
  - Peroxidase activity
  - IAA oxidase activity
- Phenolic substances
  - Phenol content
- Andrographolide
  - Above ground parts

Heavy Metal Uptake and Soil Analysis
- Heavy metal Uptake
  - Above ground parts
- Soil analysis
  - EC
  - pH
  - Carbonate
  - Bicarbonate
  - Ca-hardness
  - Mg-hardness
  - Chloride
  - Potassium
  - Phosphorus
  - Organic carbon
Fresh weight of 10 seedlings in two lots was noted and transferred to the oven at 80° C for dry weight determination. Fresh weight and dry weight were expressed as mg/seedling.

Heavy metal effects were evaluated with the help of RRG, Percent Phytotoxicity and Tolerance Index Value. Following formulas were used:

◆ **Relative Root Growth (RRG)**

Relative Root Growth was calculated according to the formula given by Ouzounidou *et al.* (1992). The formula is given below:

\[
RRG = \frac{\text{Mean length of longest root in toxic solution}}{\text{Mean length of longest root of control}} \times 100
\]

◆ **Percent Phytotoxicity**

Percent Phytotoxicity was calculated following bioassay technique suggested by Chou and Muller (1972).

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

◆ **Tolerance Index Value (TIV)**:

Tolerance Index Value (TIV), was calculated using the following formula:

\[
\text{TIV} = \frac{\text{Value of particular parameter of heavy metal treated plant}}{\text{Value of same parameter of control plant}} \times 100
\]

**(B) STUDY ON METABOLISM:**

The control and treated seedlings of 120, 144, 168, 192 and 216 h old were analysed for carbohydrate metabolism, protein metabolism, antioxidative enzymes and IAA oxidase activity as well as total phenol. The biochemical parameters estimated were as follows:

(i) **Carbohydrate Metabolism**

1. Invertase Activity
2. Reducing sugar and Nonreducing sugar content.
(ii) Protein Metabolism

3. Protease Activity
4. Protein Content
5. Total Amino Acid Content
6. Proline Content

(iii) Antioxidative enzymes and IAA oxidase activity

7. Polyphenol Oxidase Activity
8. Peroxidase Activity
9. IAA Oxidase Activity

(iv) Phenolic Substances

10. Total Phenol Content

For enzymic activities, seedlings were crushed in ice-cold DW and centrifuged at 200 rpm. The supernatant was the source of enzyme. The enzymic activities were expressed on the basis of protein. Following is the method for enzyme protein determination.

Enzyme protein:

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

\[ X = 236.60Y - 35.22 \]

Where, \( Y \) = optical density (OD).

The protein content was expressed as \( \mu g/mg \) fresh weight.

(i) Carbohydrate Metabolism

1. Invertase Activity:

Invertase activity was determined by the method of Hatch and Glasziou (1963). To 1ml enzyme aliquot, 1ml acetate buffer (pH 4.8) and 1ml 0.25%
sucrose (250mg sucrose in 100ml acetate buffer pH 4.8) was added. Blank had 1ml acetate buffer instead of 0.25% sucrose. This was incubated for one hour at room temperature. Enzyme activity was stopped by adding 2ml 5% perchloric acid and volume was made upto 10ml. 1ml of this mixture was taken to develop colour by Nelson-Somogyi method (Wharton and McCarty 1972). Method is mentioned under the method for reducing and nonreducing sugars. OD was taken at 540nm on Systronics 106 spectrophotometer, by using the regression equation prepared from known concentration of glucose,

\[ X = 426.67 Y - 15.25 \]

Where, \( Y \) = optical density (OD).

Amount of reducing sugar released by invertase activity was calculated and expressed as \( \mu g \) glucose liberated/h/\( \mu g \) protein.

2. Reducing sugar and Nonreducing sugar content

Weighed plant material (oven dried) was boiled in 80% ethanol for 4 to 5 minutes, homogenized with sand and centrifuged. Residue was again extracted with 5ml ethanol. After centrifugation, the ethanol was evaporated and the residual sugars were dissolved in a fixed volume (20ml) of DW. From the 20ml of the extract, 10ml each was taken for reducing sugar and total sugars. To the extract of total sugars, 3ml 1N HC1 (8.75ml of conc. HC1 + 91.25ml DW) was added and kept in boiling water bath for 20 minutes to hydrolyse non-reducing sugar. It was cooled and neutralized by adding 3ml 1N NaOH. 1ml of 25% lead acetate and 1ml of 25% sodium carbonate were added in both the sets and volume was made upto 20ml, then it was filtered, 1ml of the aliquot was taken for sugar estimation by the method of Nelson-Somogyi (Wharton and McCarty 1972). To 1ml of the above aliquot was added 1ml of Nelson-Somogyi reagent (Nelson reagent A : 12.5g sodium carbonate, 12.5g sodium potassium tartarate, 10g sodium bicarbonate and 100g sodium sulphate were dissolved and volume was made upto 500ml
with DW and Nelson reagent B: 15g copper sulphate in 100ml DW and 2 drops of conc. H₂SO₄). Nelson reagent was prepared by mixing 50 parts of A with 2 parts of B. Tubes were capped with glass marbles and heated in a water-bath at 100°C for 20 minutes then cooled rapidly. 1ml arsenomolybdate reagent (25g ammonium molybdate dissolved in 400ml DW. To this was added 21ml conc. H₂SO₄ + 3g sodium arsenate 25ml DW and volume was made to 500ml) was added and shaken thoroughly for 5 minutes to dissolve the red precipitates. Final volume was made to 25ml with DW. OD was taken at 540nm on Systronics 106 spectrophotometer. By referring to the regression equation of glucose

\[ X = 426.67 Y - 15.25 \]

Where, \( Y \) = optical density (OD).

Amount of reducing sugar and total sugars were calculated, and expressed as mg/g dry weight. Nonreducing sugar was calculated from the data of total and reducing sugars.

(ii) Protein Metabolism

3. Protease Activity:

The method of Penner and Ashton (1967) was followed for protease activity. The reaction mixture containing 2ml enzyme extract, 1ml of 0.5% casein solution (pH 7.0) and 3ml of 0.1M phosphate buffer (pH 7.0) was incubated for one hour at room temperature. Then 2ml of the reaction mixture was mixed with 2ml of 15% TCA. After 20 minutes, the precipitated protein was discarded by centrifugation. To 1ml of enzyme aliquot, 1ml of DW, 4ml of 0.5N NaOH and 1ml of folin-phenol reagent were added. After 30 minutes, the OD was recorded at 660nm on spectrophotometer. The following regression formula was made by reacting known amounts of tyrosine with folin-phenol reagent and activity was expressed in terms of \( \mu g \) tyrosine liberated/h/\( \mu g \) protein.
X = 232.14 Y - 0.71
Where, Y = optical density (OD).

4. Protein Content:
Protein was determined using 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), centrifuged and secondly, it was washed with mixture of ethanol: ether : chloroform in the ratio of 2:1:1 (to remove acid soluble fractions and lipids) and centrifuged. The protein fraction was dissolved in 1N sodium hydroxide and kept for one hour and centrifuged. The supernatant was made up to 5ml with sodium hydroxide and used as an aliquot. To 2ml of the above aliquot was added 5ml of Lowry reagent C (prepared by mixing 50ml reagent A which is 2% sodium carbonate in 0.1N NaOH and 1ml of reagent B which is 0.5% copper sulphate in 1% sodium potassium tartarate) and incubated at room temperature for 30 minutes. The colour was developed by adding 0.5ml folin phenol reagent. After 10 minutes, OD was read at 600nm on Systronics 106 spectrophotometer. The following regression equation was prepared by using known concentrations of casein.
X = 236.6 Y - 35.22
Where, Y = optical density (OD).
The protein content was expressed as mg/g dry weight.

5. Total Amino Acid Content:
The content of total amino acid was determined following the method of Harding and McClean (1916). The reaction system containing 0.5ml of ethanol extract from the dried material, 1ml 10% pyridine and 1 ml of 2% ninhydrin reagent was stoppered and heated in water-bath at 100°C for 30 minutes. Violet blue colour was developed. Later it was cooled and diluted
with DW to a final volume of 10ml. OD of the violet blue colour was read at 570nm on spectrophotometer. The following regression formula was prepared using isoleucine as standard.

\[ X = 413.42Y - 19.23 \]

Where, \( Y \) = optical density (OD).

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

6. Proline Content:
The method described by Bates et al (1973) was followed to estimate free proline. Weighed oven dried plant material was extracted in 3% sulphosalicylic acid. After centrifugation, 2ml of the aliquot was added to 2ml of acid ninhydrin reagent (1.25g of ninhydrin dissolved in 30ml of glacial acetic acid and 20ml of 6M phosphoric acid) and 2ml of glacial acetic acid. The tubes were stoppered and the reaction mixture was allowed to boil in water-bath at 100°C for 1 hour and the reaction was terminated in ice-bath. Then the reaction mixture was extracted against 6ml toluene with a separating funnel. The absorbance of the toluene extract was read at 520nm against toluene blank. The proline content was calculated using the regression equation prepared from known concentrations of proline.

\[ X = 49.776Y - 0.286 \]

Where, \( Y \) = optical density (OD).

Proline was expressed as mg/g dry weight.

(iii) Antioxidative enzymes and IAA oxidase activity:

7. Polyphenol Oxidase Activity:
Polyphenol oxidase activity was determined by the method of Kar and Mishra (1976). For this, 0.5ml enzyme extract was mixed with 2ml phosphate buffer (pH 7.0) 2ml of pyrogallol (50\( \mu \)m = 12.6mg in 100ml DW) and incubated at room temperature for 5 minutes. Similarly blank had 0.5ml enzyme extract and 4ml phosphate buffer, OD was taken at 420nm.
Polyphenol oxidase activity was calculated and expressed as OD/10 min/μg enzyme protein.

8. Peroxidase Activity:
George (1953) and Maehly (1954) method was employed to assay peroxidase activity. The reaction mixture having 2ml enzyme aliquot, 2ml 0.2M phosphate buffer (pH 7.0) and 2ml 20mM guaicol reagent (0.22ml guaicol in 100ml DW, was prepared 24 hours before carrying out the estimation) was taken in a cuvette. The cuvette was placed in the spectrophotometer. The wavelength was adjusted to 470nm and OD was noted. 2 drops of 10μM hydrogen peroxide (0.4ml H2O2 of 20 volumes in 9.6ml 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 H2O2 to the reaction mixture was used to calculate peroxidase activity. Peroxidase activity was expressed as difference in OD/30sec/μg protein.

9. IAA Oxidase Activity:
IAA oxidase activity was assayed according to the method of Hare (1964). To 1ml of enzyme aliquot, 1ml IAA solution (200μg/ml.), 1ml 0.15M phosphate buffer (pH 6.3) and 1ml, 1mM 2,4-dichlorophenol and 1ml 1mM MnCl2 were added and incubated at room temperature for 1 hour in dark. To 2ml of above reaction mixture, 4ml Salkowski reagent (1ml of 0.5M FeCl3 and 50ml 35% HClO4) was added and incubated for 30 minutes in dark. Pink colour was developed. OD was read at 530nm on Systronics 106 spectrophotometer. The following regression formula was prepared using IAA as standard.

\[ X = 101.98Y + 7.79 \]

Where, \( Y \) = optical density (OD).

The activity was calculated and expressed as μg IAA oxidized/h/μg protein.
(iv) Phenolic Substances

10. Total phenol content:
It was determined using the method of Farks and Kiraly (1962). The reaction mixture containing 0.5ml of the ethanol extract, 1ml of 20% sodium carbonate and 0.5ml folin phenol reagent was heated in a water-bath for 10 minutes, cooled and diluted to a fixed volume of 5ml with DW. It was filtered using Whatmann filter paper No. 1 to remove the precipitates. The OD of the blue coloured filtrate was read at 660nm. Phenol content was expressed as mg/g dry weight. The following regression formula was prepared using gallic acid as standard.

\[ X = 96.05 Y + 10.03 \]

Where, \( Y \) = optical density (OD).

EXPERIMENT II: IMPACT OF HEAVY METAL AS A SOIL POLLUTANT ON GROWTH AND METABOLISM OF MEDICINAL PLANT - KALMEGH

The earthen pots were filled with garden soil (5kg in each pot). The graded concentrations i.e. 50, 100, 150, 200 mg kg\(^{-1}\) of each heavy metal salt i.e. CuCl\(_2\), CoCl\(_2\), CdCl\(_2\) and HgCl\(_2\) were added separately before sowing. 10 pots were kept for each treatment. Following were the treatments:

* # Control
* # 50ppm CuCl\(_2\) / CoCl\(_2\) / CdCl\(_2\) / HgCl\(_2\)
* # 100ppm CuCl\(_2\) / CoCl\(_2\) / CdCl\(_2\) / HgCl\(_2\)
* # 150ppm CuCl\(_2\) / CoCl\(_2\) / CdCl\(_2\) / HgCl\(_2\)
* # 200ppm CuCl\(_2\) / CoCl\(_2\) / CdCl\(_2\) / HgCl\(_2\)

The pots without any heavy metal were considered as control. 10 pots were kept for each treatment. Kalmegh saples having 4-5 leaves were collected from Anand Agriculture University Anand and were transplanted in the pots (10 saples/pot). Plants were grown with normal practice. Heavy metal
induced impacts on growth, photosynthetic pigments, and metabolism were studied from 15, 30, 45, 60, 75, 90 and 105 days old plants. Impact of heavy metal as a soil pollutant on Kalmegh were studied as follows:

(A) STUDY ON GROWTH:
Following parameters were studied:

(1) Vegetative growth:
Root length – cm/plant
Stem height – cm/plant
Leaf number – no/plant
Fresh weight of root, stem, leaf and whole plant – g/plant
Dry weight of root, stem, leaf and whole plant – g/plant

(2) Reproductive growth:
Inflorescence branch number – no/plant
Flower bud number – no/plant
Flower number – no/plant
Fruit number – no/plant
Fresh weight of inflorescence, flower bud, flower and fruit – g/plant
Dry weight of inflorescence, flower bud, flower, and fruit – g/plant

The method of growth analysis used by (Gregory 1921, 1926) and Hunt (1978) were used for the study. Ten plants at random from each treatment were selected for growth study. These plants were carefully uprooted (minimizing the damage to the root), brought to the laboratory, washed thoroughly under slow running tap water and gently pressed against blotting sheets to remove moisture from the surface. The root length and stem height of each plant was measured and expressed as cm/plant. The leaf number was noted and expressed as no/plant. Parts viz root, stem and leaf were separated and their fresh weight was recorded as g/plant. They were then packed in the paper bags and transferred to the oven at 80°C for a period of one week for
complete drying. Dry weight of each part was determined and expressed as g/plant. Reproductive growth was studied in terms of inflorescence branch number, flower bud number, flower number and fruit number (no/plant). Their fresh and dry weight was determined and noted as g/plant.

Impact of heavy metal as a soil pollutant on growth was evaluated with the help of Relative Root Growth (RRG) and percent phytotoxicity. The formulas are mentioned in EXPERIMENT I.

* Percent Allocation:

Heavy metal as a pollutant also affects the distribution of dry matter in various parts of plants. Thus percent allocation was also considered as an important aspect for studying the responses of Kalmegh to copper, cobalt, cadmium and mercury.

Percent Allocation was calculated as follows:

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

* Growth indices viz. Relative Growth Rate (RGR), Leaf Weight Ratio (LWR) and Net Assimilation Rate (NAR) are important parameters for the growth study in plants (Blackman 1919, Gregory 1921, Nilsen and Orcutt 1996). RGR, LWR and NAR were calculated as follows from the data of dry weight.

RGR : It was calculated as the difference between Naperian logarithms of dry weight of successive samples. The formula for RGR is as follows :

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

Where, \( W_0 \) = initial dry weight of the plant

\( W_1 \) = dry weight of the plant on succeeding sampling date
LWR: The following formula was used for determining the Leaf Weight Ratio (LWR):

\[ \frac{L_1 - L_0}{\log_e L_1 - \log_e L_0} \] \[ \frac{W_1 - W_0}{\log_e W_1 - \log_e W_0} \]

Where \( L_0 \) and \( L_1 \) represent the successive dry weights of leaves. \( W_0 \) and \( W_1 \) represent the successive dry weight of the whole plants.

NAR: From the data of dry matter production of whole plant and leaf, Net Assimilation Rate (NAR) was calculated as follows:

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

Where, \( W_0 \) and \( W_1 \) represent the successive dry weights of the whole plant, \( L_0 \) and \( L_1 \) represent dry weights of the leaves of corresponding samples.

For impact assessment study of heavy metals as a soil pollutant on growth of Kalmegh, Tolerance index as per formula given in EXPT I was calculated from the growth parameters of 15 and 105 days old plants.

**B) STUDY ON PHOTOSYNTHETIC PIGMENTS:**

The photosynthetic pigments were determined using the method of Arnon (1949). The third leaf in replicate of control and treated plants was analysed for photosynthetic pigments viz chlorophyll 'a', chlorophyll 'b', total chlorophyll and carotenoids. The photosynthetic pigments were estimated from 15, 30, 45, 60, 75, 90 and 105 days old plants. Weighed fresh leaf material was crushed in 80% acetone (80ml acetone + 20ml DW) with a pinch of sand. The homogenate was filtered using Whatmann filter paper No. 1 and the filtrate was made up to a specific volume. The absorbance of the chlorophyll suspension was read on Systronic 106 spectrophotometer at 480, 510, 645 and 663nm wavelength. The following formula were used to calculate the quantity of photosynthetic pigments:

\[
\text{Chlorophyll 'a' (mg / g fr wt)} = 12.7 \times (D663) - 2.69 \times (D645)
\]
Chlorophyll ‘b’ (mg / g fr wt) = 22.9 (D645) - 4.68 (D663)
Total Chlorophyll (mg / g fr wt) = 20.2 (D645) + 8.02 (D663)
Carotenoids (mg / g fr wt) = 7.6 (D480) - 1.49 (D510)

Where,  \( D = \text{Optical Density.} \)

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

For evaluating the heavy metal stress effect, the ratios of chlorophyll ‘a’ to chlorophyll ‘b’ and total chlorophyll to carotenoids were also calculated.

(C) STUDY ON METABOLISM:
The third leaf (in replicate) of 15, 30, 45, 60, 75, 90 and 105 days old control and treated plants were analysed for carbohydrate metabolism, protein metabolism, antioxidative enzymes and IAA oxidase activity, total phenol. The methods for metabolism were the same as mentioned in EXPERIMENT 1 B. Andrographolide content (from the above ground parts of the plant) was estimated using the method of Gaind *et al.*, (1963). The details are as follows:

(11) Andrographolide content:
One g of the finely powered drug of above ground parts of 105 days old plant, accurately weighed was refluxed with 50 ml of benzene on a water bath for 1 hour. It was kept for 3-4 hours and then filtered under suction. The residue was washed with cold benzene two to three times till no more of the green colouring matter was extracted. The residue was again treated with hot benzene and filtered to ensure complete removal of the chlorophyll. It was dried to remove the traces of benzene and then mixed thoroughly with 10 g if Kieselghur. The mixed powder was then extracted in a soxhlet apparatus with pure chloroform for 4-5 hours. The chloroform was completely distilled off and the residue dissolved in 50ml of methyl alchohol. Further dilution was made so that the optical density of the resulting solution falls with in the range of the standard curve. The tubes were subjected to read at
226 nm against methyl alcohol as blank. The percentage andrographolide was calculated using standard graph factor prepared by dissolving pure compounds (Gaind et al., 1963).

(D) STUDY ON HEAVY METAL DETERMINATION:
The heavy metal was determined from leaves using the method of Trivedi et al., (1987). One g dried plant material was taken into a 100ml Kjeldahl flask. 1ml of 60% HClO₄, 5ml HNO₃ and 0.5ml H₂SO₄ were added and heated at low temperature. Heat was increased and material was digested for 10-15 minutes. After appearance of white fumes, it was cooled and final volume 100ml was made up, with several washings of filter paper by small portions of water. The filtrate is usually colourless or occasionally pink. This solution was used for determination of Cu, Co, Cd and Hg in plants using inductively coupled plasma Atomic Emission Spectrometer.

(E) STUDY ON SOIL:
At the end of the experiment control and heavy metal contaminated soils were analysed for EC, pH, Chloride, Carbonate, Bicarbonate, Calcium hardness, Magnesium hardness, Phosphorus, Potassium and Organic carbon. The methods of Saxena (1987) were used for estimating these parameters. 10g of air-dried soil was taken and 100ml DW was added to it. Suspension was prepared and it was filtered through the filter paper (Whatmann No. 44). The filtrate was used for following determination. The following parameters were studied.

1) Electrical Conductivity (EC)
2) pH
3) Chloride
4) Carbonate (CO₃) and Bicarbonate (HCO₃)
5) Calcium (Ca⁺⁺)
6) Magnesium (Mg⁺⁺)
(7) Sodium and Potassium.
The details are as follows:

(1) Electrical Conductivity (EC) of Soil:
A soil suspension (1:10 w/v) was used for EC determination. The EC was recorded with the help of Digital Electrical Conductivity meter-303 and expressed as μmho.

(2) pH:
The pH of this soil suspension was determined using the Digital pH meter.

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

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

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

(4) Carbonate (CO₃) and Bicarbonate (HCO₃):
10ml of soil solution was taken in flask and 2-3 drops of phenolphthalein indicator was added. The appearance of pink colour indicates presence of carbonate. The sample was titrated against 0.01N HCl until pink colour disappeared. Carbonate content (ppm) was calculated by using the following formula:

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

Where,
\[ V = \text{Volume of titrant (ml)} \]
\[ N = \text{Normality of titrant (0.01 N)} \]
\[ S = \text{Volume of soil solution (ml)} \]

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

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

Where,

\[ V = \text{Volume of titrant (volume of methyl orange end point - 2 X volume of phenolphthelin end point) (ml)} \]
\[ N = \text{Normality of titrant (0.01N)} \]
\[ S = \text{Volume of soil solution (ml)} \]

(5) Calcium (Ca\(^{++}\)):

10ml of soil solution was taken in a flask and 1ml of 4N sodium hydroxide solution and one grinded calcium hardness tablet was added. Then titrated against 0.02N EDTA (Ethylene Diamine Tetra Acetic acid) solution until the pink colour turns purple. Calcium content (ppm) was determined by using the following formula:

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

Where,

\[ V = \text{Volume of titrant (ml)} \]
\[ N = \text{Normality of titrant (0.02 N)} \]
\[ S = \text{Volume of soil solution (ml)} \]

(6) Magnesium (Mg\(^{++}\)):

10ml of soil solution was taken in a flask and 1ml of Ammonia buffer pH 10 solution was added. To it one total hardness tablet was added. Sample was titrated against 0.02N EDTA (Ethylene Diamine Tetra Acetic acid) solution
until the wine red colour of solution turns blue (end point). Total hardness in terms of Calcium carbonate (CaCO₃) was determined by using the following formula:

\[
\text{CaCO}_3 \text{ (ppm)} = \frac{V \times N \times 50 \times 1000}{S}
\]

Where,
- \(V\) = volume of titrant (ml)
- \(N\) = Normality of titrant (0.02N)
- \(S\) = Volume of soil solution (ml)

Magnesium content was determined by subtracting the value of calcium hardness from value of total hardness \([\text{CaCO}_3 \text{ (ppm)}]\), and it was represented as ppm.

(7) Phosphorus determination

2.5 g 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 liters, 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 Whatmann No. 2 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 acids was dissolved in 200 ml 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 solution 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 colour was measured in a spectrophotometer. The standard solution was prepared
by dissolving 0.439 g 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 gives 100ppm P standard solution. From this, a 2ppm solution was made by diluting it 50 times. 1, 2,3,4,5 and 10 ml of 2ppm phosphorus 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 make up he 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.

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

Where R= μg P in the aliquot (obtained from standard curve)

**(8) Potassium determination:**

Potassium (ppm) was also determined using Systronics digital flame photometer 125. Potassium chloride was used for the preparation of standard potassium curve, this was used for calculation of potassium.

**(9) Organic Carbon by Wet Digestion:**

Organic matter (%) in the soil was oxidized with a mixture of potassium dichromate (K₂Cr₂O₇) and concentrated H₂SO₄ utilizing the heat of dilution of H₂SO₄. Unused K₂Cr₂O₇ was back titrated with ferrous sulphate (FeSO₄.7H₂O) or ferrous ammonium sulphate (FeSO₄. (NH₄)2SO₄.6H₂O).

**Reagents Needed**

1) Standard potassium dichromate solution (0.1167 M = 1N). Dissolve exactly 49.04g reagent grade K₂Cr₂O₇ (dried at 105°C for 2h) in DW and dilute it to 1 litre in a volumetric flask.
2) Ferrous sulphate or Ferrous ammonium sulphate solution (0.5 M = 0.5 N). Dissolve 140g of FeSO₄·7H₂O or 196.1g reagent grade FeSO₄·(NH₄)₂SO₄·6H₂O in about 800ml DW, and 20ml conc H₂SO₄, cool and dilute it to 1 litre in a volumetric flask.

3) Diphenylamine indicator: Dissolve 0.5g diphenylamine in a mixture of 20ml water and 100ml 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%) / sodium fluoride chemically pure / pure grade.

Procedure:

1g of 0.2mm(dia) soil was accurately weighed and kept in a dry 500ml Erlenmeyer flask. 10ml of dichromatic solution was added and the flask was swirled gently then rapidly 20ml 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 DW. Then 10ml of phosphoric acid or 0.5g sodium fluoride and 1ml of diphenylamine indicator were added and titrated with ferrous sulphate solution till the colour changed from blue violet to green. Procedure was repeated without soil for blank. The organic carbon was determined using the following formula.

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

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

\(T\) = Volume of ferrous sulphate solution needed for titration of soil sample.
EXPERIMENT III: IMPACT OF FOLIAR APPLICATION OF HEAVY METAL ON GROWTH AND METABOLISM OF MEDICINAL PLANT - KALMEGH

The earthen pots were filled with garden soil (5kg in each pot). Kalmegh saples having 4-5 leaves were collected from Anand Agriculture University Anand and were transplanted in the pots (10 saples/pot). Plants were grown with normal practice, 10 pots were kept for each treatment. After 7 days of transplantation, plants were sprayed with following heavy metals separately.

- Control
- DW
- 50 ppm CuCl₂ / CoCl₂ / CdCl₂ / HgCl₂
- 100 ppm CuCl₂ / CoCl₂ / CdCl₂ / HgCl₂
- 150 ppm CuCl₂ / CoCl₂ / CdCl₂ / HgCl₂
- 200 ppm CuCl₂ / CoCl₂ / CdCl₂ / HgCl₂

The pots without any spray were considered as control. DW was sprayed as a control measure of spray treatment. The hand sprayer was used for spray. The foliar spray was given at regular interval of 7 days from 15 till 105 days after transplantation. Impact of foliar application of heavy metal on Kalmegh were studied as follows

(A) STUDY ON GROWTH:
Control, DW and heavy metal sprayed plants of Kalmegh were regularly studied for growth. The growth parameters were the same as mentioned in EXPT II [A]. Percent allocation of dry matter, growth indices and tolerance index values were also calculated.

(B) STUDY ON PHOTOSYNTHETIC PIGMENTS:
The effects of foliar application of heavy metal on photosynthetic pigments were studied from third leaf (in replicate) of Kalmegh. Method was same as described in EXPT II [B].
(C) STUDY ON METABOLISM:

Third leaf in replicate from 15, 30, 45, 60, 75, 90 and 105 days old plants of control and sprayed was analyzed for various enzymes and metabolites. The parameters and methods were the same as mentioned in EXPT II [C]. Andrographolide was estimated from the above ground parts of control and sprayed plants. Method was the same as described in EXPT II [C].

(D) STUDY ON HEAVY METAL DETERMINATION:

Heavy metal contents from above ground parts of control and sprayed plants were estimated as per EXPT II [D].

Statistical analysis

The growth data, data on photosynthetic pigments, enzymes and metabolites (EXPT- I, II, III) were analyzed statistically using ANOVA (Fisher, 1954).