Chapter 2

Materials & Methods
MATERIALS

Althea officinalis L. and Calendula officinalis L. were analyzed for their responses to sulphurdioxide exposure.

Althea officinalis L. (Family: Malvaceae)

Vernacular names: Hindi: Gulkairo, Khairo; Sanskrit: Khatmi; English: Marshmallow; German: Eibisch; Spanish: Althea, Malvavisco; Tamil: Simaitutti.

Habitat: The plant, known as khatmi in unani system of medicine, grows in ditches and wet places in the neighbourhood of sea and tidal rivers throughout the Europe with the exception of Scandinavia and North Russia, also in Asia minor, Western Asia (reaching Kashmir) and Algeria (Bentley and Trimen, 1981). In India, it has been successfully introduced into Manali at 2000 m altitude, and is cultivated in the gardens of Kulu valley in Himachal Pradesh. The seeds are sown 15 cm apart in nursery in March, preferably in sandy soils.

Plant description: This herb is 60-180 cm high; stem is thick, elongated, tough, unbranched, cylindrical, and covered with dense velvety stellate hairs; leaves are alternate, 1.5-3 inches long, serrate, covered with hairs; flowers in small axillary clusters of 2-4 or solitary, short-stalked, pale purplish to rose colored, finely veined; stamens numerous with anthers drooping and reniform; fruit brownish-green; seeds kidney-shaped, smooth, embryo curved (Bentley and Trimen, 1981; Weiners, 1990).

Medicinal importance: Marshmallow root is an excellent demulcent and emollient. The decoction is taken internally to relieve irritation and inflammation of the mucous membranes. The crushed leaves and flowers are boiled and applied externally in poultice form as a soothing dressing for scrapes, chafing and other related skin conditions (Weiners, 1990). The flowers, boiled in oil or water with a little honey and alum have proved good as a gargle for sore throats (Caius, 1986).
Decoctions of the plant, especially of the root, is very useful where the natural mucous has been abraded from the coats of intestines, and in painful complaints of the urinary organs, exerting a relaxing effect upon the passage. It is effective in curing bruises, sprains or any aches in the muscles (Caius, 1986). The powdered root boiled in milk is useful in haemorrhage from the urinary organs, and in dysentery. Boiled in wine or milk, it relieves chest diseases, bronchitis, whooping-cough etc. In France, leaves are added to salad for stimulating the kidney’s health (Caius, 1986). The root is used as an emollient in irritability of vagina and rectum. It is also used in making absorbent pills, pastilles, and in ointments and hair-skin protection preparations (Weiners, 1990; Bremness 1988; Graves, 1996).

_Calendula officinalis_ L. (Family: Asteraceae)


_Habitat_: This hardy annual is native to Mediterranean and introduced in India. It is found in Punjab and Sind, extending to Afganistan and westwards to south Europe. In India it is cultivated as an ornamental plant. In some areas it flourishes throughout the year. It likes cool and temperate climate with rich and sandy soil. The seeds are sown during October- November (Caius 1986).

_Plant description_: An aromatic, erect, annual herb, up to 60 cm in height, stem corymbosely branched above; leaves 2.5-7.5 cm, acute, alternate, often hispid on both surfaces, lower leaves spatulate quite entire, upper leaves lanceolate, cordate-amplexicaul, toothed or subentire; heads terminal, heterogamous, rayed, 5 cm diameter, involucral bracts 6 mm, incurved and appressed to the ripe achenes, ligules many, yellow to orange, ray-flowers female, disk-flowers hermaphrodite; anther-bases sagittate, auricles acuminate or tailed; achenes longer than the involucre, all curved boat-shaped dorsally muricate, outer larger ventrally creasted, scarcely beaked (Keville, 1991; Kirtikar and Basu, 1993; Bisset, 1994).
Medicinal importance: *Calendula*, applied locally as a tincture, oil or lotion is considered a 'natural antiseptic' by homeopaths. The crushed petals combined with olive oil form an ointment for external application to cuts, bruises, sores and burns. The infusion is used to soothe watery, irritated eyes and for relief in bronchial complaints. It forms a home remedy in liver disorders and to induce perspiration in fever (Weiners, 1990).

In Europe, the leaves are considered as a resolvent and diaphoretic, the flowers as a stimulant and antispasmodic. Country people employ a decoction of flower in England as a drink in measles, smallpox and jaundice and fresh juice proves a useful remedy against constiveness (Kirtikar and Basu, 1993).

Druggists now make a medicinal tincture, which is advised as a sudorific stimulant in low fevers and to relieve spasm. A saturated tincture of the flowers, when mixed with water, promotes the cure of contusions, wounds and simple sores or ulcers. The plant, specially its flowers, can treat wounds and injuries (Kirtikar and Basu, 1993). The leaves, eaten as salad, are useful in the scrofula of children. The expressed juice given in cases of costiveness proves very efficacious. Snuffed up the nose, it excites sneezing and a discharge of mucous from the head (Caius, 1986).

**EXPERIMENTAL SITE**
The field experiments were conducted at the experimental field of Hamdard University, New Delhi, India.

**Climate and weather conditions**: New Delhi is situated 28.38’ N latitude and 77.11’ E longitude at an altitude of 228 m above the mean sea level. The soil is normally loam and clayey loam with pH around 8-9. It has a semiarid and subtropical climate with extremes of hot weather in summer and cold weather in winter. The maximum rainfall, 80-100 cm, is observed in July and August. Winter showers sometimes are accompanied with high velocity winds and hale storms. The relative humidity increases from 45% in June to 81% in July and August.
Wind velocity is 2 m sec\(^{-1}\). The maximum temperature in summer normally exceeds 45 °C.

**Experimental design and layout of field experiments:** Experiments were laid out in plots (12.5 \times 9 square feet), each having 3 rows of plants, maintaining a row to row distance of 30 cm and a plant to plant distance of 15 cm. Seeds were sown in October when monthly means of the minimum and maximum temperatures lay around 19 °C and 32 °C, while those of relative humidity around 49% and 80%, respectively. Fifteen days old seedlings were transplanted. The plants were then let to grow undisturbed for 35 days and were watered periodically as and when required. The soil at Jamia Hamdard campus is a coarse-textured sandy loam with pH around 8, E.C. 0-198 m mhos cm\(^{-1}\), and the nitrogen and sulphur contents around 10.4 ppm and 2.8 ppm, respectively.

**Fumigation procedure:** The plants were fumigated for half an hour in the morning hours (7:30 to 9:30 am) daily for a week when they were 30 days old. The fumigation chamber used was made of a steel frame with a transparent polythene sheet mounted on it, leaving the bottom side uncovered. Before starting fumigation, base of the frame and ends of the polythene sheet were buried few cm deep in the soil in order to check the possible leakage of SO\(_2\) during fumigation. The chamber, with a capacity of 10.28 m\(^3\), was made air-tight with an inlet at the base and an outlet at the top. To ensure a proper mixing of the gas inside the chamber, a small electrical fan was fixed. The desired concentration of SO\(_2\) gas (1 and 2 ppm) was released from a gas cylinder through a tube and the flow was controlled by a regulator fitted on the cylinder. Sampling for analysis was done 30 days after fumigation (in pre-flowering stage), 60 DAF (in flowering stage) and 120 DAF (in post-flowering stage) in the case of *Althea* and 30 DAF, 60 DAF and 90 DAF in the case of *Calendula*, depending on difference in flowering time (April for *Althea* and March for *Calendula*) of the two species. Thus, the last sampling (in post-flowering stage) was done around the end of May (for *Althea*) and April (for *calendula*) when RH was relatively low (up to a minimum of 23% and 31%,
respectively) and temperature was considerably high (up to a minimum of 38 °C and 34 °C, respectively).

Parameters studied

Data have been collected on the following aspects:

(A) Morphological parameters
1. Number of leaves per plant
2. Single leaf area and total leaf area
3. Dry weight of leaves
4. Shoot length
5. Root length
6. Root length-shoot length ratio
7. Dry weight of shoots
8. Dry weight of roots
9. Root-shoot dry weight ratio
10. Number of flowers per plant
11. Number of fruits per plant
12. Number of seeds per fruit
13. Weight of 100 seeds

(B) Anatomical parameters
1. Length of stomatal aperture
2. Width of stomatal aperture
3. Relative proportions of cortex, vasculature and pith in stems and roots
4. Length of xylem fibres in stem and roots
5. Length of vessel elements in stems and roots
6. Vessel diameter in stems and roots
7. Vessel density in stems and roots
(C) Physiological parameters
1. Length of stomata
2. Width of stomata
3. Stomatal density
4. Stomatal index
5. Epidermal cell density
6. Stomatal conductance
7. Intercellular carbon dioxide concentration
8. Net photosynthetic rate
9. Chlorophyll a & b contents
10. Carotenoid content

(D) Biochemical Parameters
1. Nitrate reductase activity
2. Nitrate content of leaves
3. Total soluble protein
4. Total amino acids
5. Total phenolic contents
6. Proline content
7. Sulphydryl content
8. Lipid peroxidation
9. Ascorbic acid
10. Sulphur content in leaves, stem and root
11. Reducing nitrogen in leaves, stem and root
12. Soluble sugars in stem, leaves, and root

(E) Seed Oil Analysis
METHODS

The various morphological, physiological, anatomical and biochemical parameters were determined by using the following methods:

(A) Morphological parameters:
Five plants at random were sampled 30, 60 and 95 days after treatment at different stages of plant growth. The plants so collected were cut at root-shoot junction. Plant height, root length and shoot length were measured in centimeters. The shoot and root axes were measured from base to apex. Fresh weights (g) of roots, stems and leaves were obtained just after the sampling. For dry mass, the separated plant parts were dried in hot air oven at 65 ± 2 °C for 72 hours and then weighed on a digital balance. The size and area per plant of a leaf and also the total leaf area were measured by a (LI-COR, Lincoln, USA) leaf area meter.

(B) Anatomical parameters:
The collected samples were fixed in FAA (formaldehyde : acetic acid : alcohol : : 5 : 5 : 90) for a week, and then transferred for preservation either to an alcoglycerol mixture (1:1 mixture of ethyl alcohol and glycerol) or to 70% ethyl alcohol only, depending on the hardness of the material.

Maceration of vascular tissues: To study the cell size variation in vascular tissues, small tangential slices of roots and stems were macerated in hot nitric acid (Ghouse and Yunus, 1972). The macerated wood elements, i.e., fibres and vessel elements, were measured by a compound microscope and the data obtained were put to statistical analysis.

Microtomy and microscopy: The fixed materials (roots and stems), washed with distilled water, were sectioned in transverse plane at a thickness of 10 μm on a Reicherts Sliding wood Microtome. The sections from the root and the third internode of the stem, were dehydrated in ethanol series, stained with safranin or Bismarck brown and mounted in Canada balsam. Microphotography was done on Venox-S microscope (Olympus, Tokyo, Japan).
(C) Physiological parameters:

Epidermal peels: The epidermal peels were obtained by the method of Ghouse and Yunus (1972) using hot nitric acid. The peels, processed in the customary ethanol series for dehydration and stained with safranin or Bismarck brown, were mounted in Canada balsam. Stomata and trichomes were measured with an ocular scale in a light microscope. The stomatal index (S.I.) was calculated using the following formula of Salisbury (1927).

\[
S.I. = \frac{S}{S + E} \times 100
\]

wherein, \( S \) indicates number of stomata and \( E \) indicates number of epidermal cells in a microscopic field of a compound microscope.

SEM study: For electron microscopy, the leaf samples of 1 cm\(^2\) size from the normal and treated plants were fixed in 2.5% glutaraldehyde. This is normally supplied as a 25% or 50% solution. To prepare a 500 ml of 2.5% glutaraldehyde solution, 50 ml of 25% glutaraldehyde was made up to 500 ml with 200 ml of DDW and 250 ml of 0.2 M phosphate buffer (7.2 pH). The fixed plant samples were kept at 4 °C overnight. The samples were transferred into phosphate buffer (pH 7.2) and then washed thoroughly with buffer. The samples were trimmed to bring them to proper orientation. Critical points for the material were CO\(_2\) - 31.5 °C at 1100 p.s.i. and Freon 13 - 28.8 °C at 560 p.s.i. (pound per square inch).

Metal film coating of SEM samples was carried out by the Blazeres SCD 020 sputter device under vacuum in an inert atmosphere using Argon gas, coating of uniform thickness was obtained by evaporating the metal (target) on to the specimen kept at the specified distance (cathode specimen distance). Gold was used as a target; coating of about 35 nm (350 Å) thickness was obtained under the following conditions:
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Current : 21.5 mA
Pressure : 0.05 m bar
Gas (atmosphere) : Argon
Distance : 30 nm
Time : 1 minute

The samples were observed under a Leo 435 VP Scanning Electron Microscope. Pictures obtained on 35 mm fast films (125, 200, or 400 ASA) were developed in D-76 fine grain developer, fixed in Agfa 301 fixer, before washing and drying.

During the field study of normal and treated plants, parameters like stomatal conductance, intercellular carbondioxide concentration and net photosynthetic rate were measured by clamping the given leaf in the leaf chamber (6000-13, quarter litre) of portable LI-6200 Photosynthesis System (LI-COR, Inc. Lincoln, USA) that measured the transient exchange rate of water vapour and carbondioxide in a closed system. The equipment was calibrated before the experiment, using the calibrated standard CO\textsubscript{2} supplied with the system. All measurements were taken between 9-11 O’clock in the morning from ten leaves of each plant and five plants from each plot.

Estimation of chlorophyll and carotenoid: The chlorophyll and carotenoid contents of fresh leaves were estimated by the method of Hiscox and Israelstam (1979), using dimethyl sulphoxide (DMSO). Vials containing 100 mg of finely chopped fresh leaves in 7 ml DMSO were covered with black paper and kept in oven at 65 °C for an hour. The reaction mixture was transferred to a graduated tube and the final volume was made up to 10 ml by adding DMSO. This chlorophyll extract (3 ml) was taken in cuvette and absorbance was read at 480, 510, 645 and 663 nm on DU-64 Spectrophotometer (Beckman).

The chlorophyll concentration in mg/g of fresh sample was calculated, using the following formulae given by MacLachlan and Zalik (1963), and Duxbury and Yentsch (1956).

\[
\text{Chlorophyll } a \ (\text{mg } g^{-1}\text{ f.w.}) = \frac{12.3_{663} - 0.86_{545}}{D \times 1000 \times W} \times V
\]
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#### Chlorophyll $b$ (mg g$^{-1}$ f.w.)

\[
\text{Chlorophyll } b \ (\text{mg g}^{-1} \text{ f.w.}) = \frac{19.3_{d545} - 3.60_{d63} \times V}{D \times 1000 \times W}
\]

#### Carotenoids (mg g$^{-1}$ f.w.)

\[
\text{Carotenoids} \ (\text{mg g}^{-1} \text{ f.w.}) = \frac{7.6_{d480} - 1.49_{d510} \times V}{D \times 1000 \times W}
\]

#### Total chlorophyll (mg g$^{-1}$ f.w.)

\[
\text{Total chlorophyll} \ (\text{mg g}^{-1} \text{ f.w.}) = \frac{20.2_{d465} - 8.02_{d663} \times V}{D \times 1000 \times W}
\]

wherein, $D$ = distance travelled by the light path; $W$ = weight of the material taken; and $V$ = volume of the extraction medium.

(D) **Biochemical parameters:**

**Estimation of total soluble protein:** Following the method of Bradford (1976), half a g fresh leaves were ground in mortar and pestle with 1.5 ml phosphate buffer (pH 7.0) and then centrifuged at 5000 rpm for 10 minutes at 4 °C. Half ml supernatant added with a 0.5 ml TCA was centrifuged at 3300 rpm for 30 minutes. The supernatant was discarded and the left pellet was washed twice with DDW and dissolved in 1 ml 0.1 N NaOH. Then 0.1 ml aliquot was added with 5 ml Bradford reagent and optical density was measured at 595 nm. The protein concentrations were determined by using bovine serum albumin as the standard.

**Preparation of reagents:**

(a) Bradford reagent: 100 mg coomassic brilliant blue G250 (sigma) was dissolved in 50 ml of 90% ethanol. To this solution, 100 ml of 85% phosphoric acid was added and stirred well. This was diluted to a final volume of 1 litre by adding DDW.

(b) Phosphate buffer: 3.56 g of 0.2M Na$_2$HPO$_4$ was dissolved in 100 ml DDW to make solution A, In another flask, 3.12 g of 0.2M NaH$_2$PO$_4$ was dissolved in
100 ml DDW to make solution B. The solution B was added drop by drop in solution A so as to get pH 7.0 for the buffer.

(c) 10% trichloro acetic acid (TCA) was prepared by dissolving 10 g of TCA in 100 ml DDW.
(d) 1N sodium hydroxide was prepared by dissolving 0.4 g NaOH in 100 ml DDW.

Estimation of nitrate: Nitrate extraction was done by the method of Grover et al. (1978). Half a gram fresh leaves taken in a conical flask were added with 50 mg charcoal and 10 ml distilled water and boiled for 10 minutes. After cooling and filtration the volume of the filtrate was made up to 50 ml by adding distilled water. One ml of this aliquot was mixed with 0.5 ml CuSO₄-ZnSO₄ solution, 0.25 ml hydrazine sulphate, 0.25 ml 0.1N NaOH and 1.5 ml distilled water. The vials were kept in water bath incubator for 10 minutes at 33°C and then transferred to ice. Chilled acetone (0.5 ml), was added to the aliquot to stop reaction and remove extra hydrazine sulphate. In 2.0 ml of this mixture, 1.0 ml of each of sulphanilamide and [NEDD N(1-naphthyl)ethylene diamine dihydrochloride] were added and the vials were kept for 30 min. for colour development. The volume was made up to 6.0 ml with water and absorbance was read at 540 nm on Beckman, DU 640 B Spectrophotometer (Evans and Nason, 1953).

Preparation of reagents:
(a) 0.1 N NaOH was prepared by dissolving 0.4 g NaOH in 100 ml of distilled water.
(b) Hydrazine sulphate was prepared by dissolving 152 mg hydrazine sulphate in 100 ml of DDW.
(c) 10% acetone was prepared by dissolving 10 ml acetone in 100 ml of DDW.
(d) 1% sulphanilamide was prepared by dissolving 1 g sulphanilamide in 100 ml of 1N HCl.
(e) 0.02% NEDD was prepared by dissolving 0.02 g NEDD in 100 ml of DDW.
(f) Catalyst solution prepared by dissolving 31.36 mg CuSO$_4$ in 100 ml of distilled water to get solution A, of which 10 ml was taken and added with 10 mg of ZnSO$_4$. The final volume was made up to 100 ml.

The corresponding concentration of nitrate was prepared by using sodium nitrate (NaNO$_3$) solution.

**Estimation of nitrate reductase activity:** It was done by the method of Klepper et al. (1971). Vials containing 0.3 g fresh leaves with 3 ml phosphate buffer (pH 7.2) and 3 ml of KNO$_3$ (0.4 M) each were kept in vacuum desiccator and vacuum infiltration was done for 30 sec. The vials were kept in water bath incubator for 1 hour at 33 °C under dark conditions and then in hot water for 5 minutes to stop the reaction. One ml of sulphanilamide (1% in 1N-HCl) and 1 ml of NEDD (0.02%) were added into a 0.2 ml aliquot. The vials were kept in dark for 30 minutes for colour development and the final volume was then made up to 6 ml with water. Absorbance was read at 540 nm on DU B640 Spectrophotometer. The corresponding concentration of nitrite was determined against the standard curve prepared using sodium nitrite (NaNO$_2$) solution.

**Preparation of reagents:**

(a) 0.4 M KNO$_3$ was prepared by dissolving 4.04 g KNO$_3$ in 100 ml DDW.
(b) 1% sulphanilamide was prepared by dissolving 1 gm sulphanilamide in 100 ml 1N HCl.
(c) 1 N hydrogen chloride was prepared by dissolving 8.4 ml HCl in 91.6 ml DDW.
(d) 0.02% NEDD was prepared by dissolving 0.2 g NEDD in 100 ml DDW.
(e) Phosphate buffer: Solution A and B were prepared and mixed as described earlier.

**Estimation of soluble sugar:** The soluble sugar was estimated by the method of Dey (1990). 0.1 g material of each of the fresh leaf, stem and root was kept separately in 10 ml alcohol for one hour at 60 °C in incubator. The extract was then decanted into a 25 ml volumetric flask and the residue re-extracted. Final volume
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was made up to 25 ml by adding alcohol. One ml of the aliquot (containing 20-100 μg sugar) was transferred to a thick-walled test tube and 1.0 ml of 5% phenol was added to it and mixed thoroughly. Five ml of analytical grade sulphuric acid was then added to it and mixed thoroughly by vertical agitation with a glass rod. For exothermic reaction the test tubes were cooled in the air. Optical density was measured at 485 nm on Beckman, DU 640 Spectrophotometer.

The corresponding concentration was determined against a standard curve prepared by using a glucose solution.

Estimation of amino acids: Amino acids were estimated by the method of Lee and Takahashi (1966). Half a gram fresh leaves were ground in a mortar and pestle with 5 ml absolute alcohol and centrifuged at 5500 rpm for 1 minutes at 4 °C. The alcohol was evaporated by boiling in a hot water bath. The pellet was dissolved in 10 ml of 0.5 M citrate buffer (pH 5.6). Half ml aliquot from this solution, added with 1.2 ml of 55% glycerol and 0.5 ml of ninhydrin, was boiled for 20 minutes. A purple blue colour appeared. A cool and final volume was made up to 6 ml by adding the citrate buffer. Optical density was measured at 570 nm. The corresponding concentration of amino acids was determined against the standard curve prepared using different concentration of glycine.

Preparation of reagents:

(a) 55% glycerol was prepared by mixing 55 ml glycerol in 100 ml DDW.
(b) 1% ninhydrin solution was prepared by dissolving 1 g ninhydrin in 100 ml 0.5 M citrate buffer (pH 5.6).
(c) Citrate buffer was prepared by dissolving 21.02 g of citric acid monohydrate in 100 ml DDW to make solution A. In another flask, 29.41 g of trisodium was dissolved in 100 ml DDW to make solution B. The solution A is added to solution B drop by drop to get pH 5.6 for the buffer.

Estimation of ascorbic acid: It was carried out by the method of Keller and Schwager (1977). Half a gram of fresh leaf was homogenized in mortar and pestle
with 20 ml extracting solution and centrifuged for 15 minutes at 6000 x g. One ml supernatant from this solution was added with 5 ml of dichlorophenol indophenol (DCPIP). Absorbance of this pink solution was read at 520 nm. It was taken as Es. Then one drop of 1% ascorbic acid was added to it to bleach the pink colour and optical density was measured again at the same wavelength. This was considered as Et. Optical density of DCPIP solution was also measured at 520 nm. This was considered as Eo. The concentration of ascorbic acid (mg g⁻¹ f w.) was calculated using the formula:

\[
\frac{[E_o - (E_s - E_t)] \times V}{W \times V_1 \times 1000}
\]

Wherein, \(E_o\) = Optical density of DCPIP solution; \(E_s\) = Optical density of pink solution; \(E_t\) = Optical density of solution after bleaching the pink colour; \(V_1\) = Volume of the supernatant; \(V\) = Volume of the aliquot and \(W\) = Weight of fresh leaves taken.

The corresponding concentration of ascorbic acid was determined against the standard curve prepared by pure ascorbic acid.

**Preparation of reagents:**

(a) Extraction solution was prepared by dissolving 5 g oxalic acid and 0.75 g EDTA in 1000 ml of distilled water.

(b) DCPIP (2,6-dichlorophenol indophenol) was prepared by dissolving 100 mg of DCPIP in 500 ml DDW which was heated for perfect dissolution. The final volume of the solution was made up to 1000 ml by adding DDW. Twenty ml of this solution was made into a final volume of 100 ml by adding DDW.

(c) 1% ascorbic acid was prepared by dissolving 1 g ascorbic acid in 100 ml DDW.
Estimation of total phenolics: The phenolic content was estimated by the method of Bray and Thorpe (1954). Fresh leaves (0.1 g) were ground in a mortar and pestle with 10 ml of 70% acetone and centrifuged at 6000 x g for 10 minutes. One ml aliquot was then mixed with 1 ml of 1 N Folin ciocalteu reagent. To this was added 2 ml of sodium carbonate (2% wt/vol) and the final volume was made up to 10 ml with DDW. The mixture was boiled for 1 minute in a boiling water bath and then cooled. Optical density was measured at 650 nm. Concentration of phenol (mg g\(^{-1}\) f. w.) was calculated using the following formula.

\[
\text{Phenol content (mg g}^{\text{f. w.}}\) = \frac{\mu g \text{ phenol x } V}{W \times V_1 \times 1000}
\]

wherein,

- \(\mu g\) = Phenol obtained from standard curve.
- \(V\) = Total volume of mixture
- \(W\) = Weight of leaf sample in gram
- \(V_1\) = Volume of extract taken.

Estimation of proline: It was estimated by the method of Bates et al. (1973). Fresh leaves (0.3 g) were ground in 10 ml of 3% sulphosalicylic acid and the solution was filtered. To a 2 ml filtered supernatant 2 ml acetic acid and 2 ml acid ninhydrin were added and the mixture was kept in a boiling water bath for one hour. The test tubes were then kept in ice for terminating the reaction. To this was added 6 ml of toluene and the mixture was vortexed vigorously. After settlement, the suspension was divided in two layers. The upper layer was taken out in another test tube and its optical density was measured at 520 nm.

Preparation of reagents:
(a) Acid ninhydrin was prepared by dissolving 1.25 g of ninhydrin in a mixture of 30 ml of glacial acetic acid and 20 ml of 6N orthophosphoric acid. The final volume was made up to 100 ml by adding DDW.
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(b) 6 N orthophosphoric acid was prepared by adding 38.1 ml of orthophosphoric acid in 61.9 ml of DDW.

The corresponding concentration of proline was determined against the standard curve prepared by using Proline (sigma).

Estimation of sulphydryl group: Following the method of (Sedlak and Lindsay, 1968). Two gram fresh leaves were homogenized with 20 ml of distilled water containing 0.15% ascorbic acid. The homogenate was centrifuged at 20,000 \( \times \) g for 10 minutes. Two ml of DTNB reagent was added to 2 ml of clear supernatant. Optical density was measured at 412 nm against a blank consisting of 2 ml phosphate buffer, giving the absorbance \( E_1 \). In a second experiment, the absorbance of 2 ml \( H_2O \) + 2 ml DTNB reagent was measured against 2 ml \( H_2O \) + 2 ml phosphate buffer, giving the absorbance \( E_2 \). The \(-\)SH content of the supernatant was then calculated by the following formula:

\[
\text{Mole SH/litre extract} = (E_1 - E_2) \times \frac{2}{13,100}
\]

Wherein, 13,100 = Molar extinction co-efficient

Preparation of reagents:

(a) DTNB (55'-dithiobis-nitrobenzoic acid) was prepared by dissolving 3.96 mg of DTNB in 10 ml of 0.2 M phosphate buffer with pH 7.0.

(b) Phosphate buffer (pH 7.0) was prepared as described earlier.

Estimation of lipid peroxidation: Following the method of Heath and Packer (1968), fresh leaves (0.3 mg) were homogenized in 3 ml of 0.1% TCA solution. The homogenate was centrifuged at 1500 g for 10 minutes and 0.5 ml of the supernatant obtained was added with 1.5 ml of 0.5% TBA in 20% TCA. The mixture was incubated at 90 °C in a shaking water bath for 20 minutes and then reaction was stopped by placing the tubes in an ice water bath. The samples were then centrifuged at 10,000 g for 5 minutes. Absorbance of the supernatant was read
at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm.

The amount of nmoles MDA-TBA complex formed per hour was calculated by using the following formula:

\[
\frac{O.D \times V \times 10^9}{1.56 \times 10^5 \times 10^3 \times W}
\]

wherein, \( V \) = Total volume of the mixture; \( W \) = Weight (g) of fresh leaves; \( 1.56 \times 10^5 \) = Molar extinction co-efficient, and \( 10^3 \) = conversion factor (molar to mole)

**Estimation of sulphur:** Following the method of Patterson (1958), oven dried samples of leaves were ground separately and passed through 72 mm mesh screen. The screened powder (0.3 g) of each sample was digested using 10 ml of HNO\(_3\) and 1 ml of HCl in digestion tubes. The digested material was then filtered in 100 ml volumetric flask. The volume of digested solution was made up to 100 ml with DDW. Ten ml of 3% glycerol was added with 5 ml of 2% BaCl\(_2\) in the above solution just before measuring optical density at 420 nm.

The actual sulphur concentration was determined with the help of a standard curve of the potassium sulphate solution.

**Estimation of nitrogen content:** It was estimated by the method of Linder (1944). The oven-dried samples of leaf, stem and root were ground and passed through 72 mm mesh screen. The screened powder (0.1 g) of each sample was digested with 4 ml of a mixture of concentrated sulphuric acid and perchloric acid (2:1). The sample was heated till it turned brown. The suspension was cooled. Then 0.5 ml of 30% H\(_2\)O\(_2\) was added to it dropwise. It was further heated till the sample turned light yellow. Then the material was cooled and 0.5 ml of H\(_2\)O\(_2\) (30%) was added. The sample was again heated for 15 minutes and this rendered the solution colourless. The digested material was transferred to 100 ml volumetric flask and the final volume was made 100 ml with DDW. Ten ml of it was added with 2 ml of
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NaOH (2.5N) and 1 ml of sodium silicate (10%). The final volume was made 10 ml with DDW. To 5 ml of it was added with 0.5 ml of Nessler’s reagent and the final volume was made up to 10 ml using DDW. The solution was kept for 5 min for colour development. Optical density was measured at 440 nm using a Beckman DU 640B Spectrophotometer.

Preparation of reagents:
(a) 2.5N NaOH was prepared by dissolving 10 g sodium hydroxide in 100 ml of distilled water.
(b) 10% sodium silicate was prepared by dissolving 10 g of sodium silicate in 100 ml of distilled water.
(c) Nessler’s Reagent: 100 g NaOH was dissolved in 400 ml of DDW to make solution A; in another flask 100 g HgI₂ and 70 g KI were dissolved in 400 ml of DDW to make solution B. Solution A was then mixed with a cool solution B with a continuous stirring. The final volume was made 1000 ml with DDW and mixture was kept at room temperature for 24 hours. The solution was then filtered and used.

The corresponding nitrogen concentration was determined against a standard curve prepared by using ammonium carbonate.

(E) Fatty acid composition of oil:

Extraction of oil: The oil was extracted using Soxhlet apparatus (A.O.A.C; 1965). The solvent used was hexane (b.p. 65-70 °C) for *Althea* oil and petroleum ether (b.p. 60- 80 °C) for *Calendula* oil. Two g seeds were weighed and crushed in a mortar and pestle with anhydrous sodium sulphate. Hundred ml n-hexane was filled in the round bottom flask. The crushed seeds, along with sodium sulphate, were put in the Soxhlet. The whole set was placed on a mantle at 80 °C for 3 hours. The mixture of n-hexane and oil was collected in the round bottom flask. The oil was separated from n-hexane using a rota-vapour and collected in vials.
Preparation of methyl esters of fatty acids: 0.1 ml of the oil was taken in air-tight, capped glass tubes and added with 5 ml of 0.5 N sodium methoxide prepared in methanol. The tubes were capped and checked for being air-tight. The contents of the tubes were mixed thoroughly and heated in boiling water bath for 10 minutes. The tubes were removed and cooled in ice cold water. Two drops of boron trifluoride were added to the tubes. The caps of the tubes were tightened and 5 ml of hexane was added to each of the tubes. The caps were again tightened firmly and the contents mixed thoroughly. After 10 minutes, the content of the tubes got separated in two layers. The upper layer which contained methyl esters of fatty acids was separated and concentrated.

Preparation of reagent:
(a) Sodium methoxide (0.5 N) was prepared by dissolving 2.7 g of sodium methoxide in 100 ml of methanol.

Estimation of fatty acid methyl esters (FAME): The methylated fatty acids were quantified by gas chromatography (Perkin Elmer 8700, U.S.A.), equipped with a flame ionization detector (FID). A stainless steel column (2 m long, 3.12 mm width and 2.2 mm thickness) containing 3% OV-17 chromosorb (WHP-100-120 mesh) was used. The following experimental conditions were maintained for estimating the fatty acids of both the oils.

<table>
<thead>
<tr>
<th>Material &amp; Method</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oven temperature</td>
<td>215 °C</td>
</tr>
<tr>
<td>Injector temperature</td>
<td>240 °C</td>
</tr>
<tr>
<td>Detector temperature</td>
<td>240 °C</td>
</tr>
<tr>
<td>Isothermal time</td>
<td>35 minutes</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>N₂</td>
</tr>
<tr>
<td>Carrier gas flow</td>
<td>30 ml/min</td>
</tr>
<tr>
<td>Ramp rate 1</td>
<td>0.0</td>
</tr>
<tr>
<td>FID 2 sens</td>
<td>high</td>
</tr>
<tr>
<td>FID 2 zero</td>
<td>on</td>
</tr>
<tr>
<td>Gas mixture for flame ignition</td>
<td>hydrogen + zero air</td>
</tr>
</tbody>
</table>
The sample (0.5 μl) was injected and the retention time and % area were noted.

Calculation of fatty acid content in oil: The fatty acid content was calculated as follows:
Peak area (%) of total fatty acid = 100 - Peak area (%) of the solvent
Quantity of individual fatty acid (%) = \( \frac{\text{Peak area}(\%) \text{ of individual fatty acid} \times 100}{\text{Peak area}(\%) \text{ of total fatty acids}} \)

Statistical analysis:
The data collected on different parameters were analyzed statistically to determine the degree of authenticity of the results.

Mean (\( \bar{X} \)): The arithmetic mean or the so called average value is counted by taking the sum of a number of values \( (x_1 + x_2 + x_3 + \ldots + x_n) \) and dividing it by the number of values \( n \) involved. Thus;
\[
\bar{X} = \frac{(x_1 + x_2 + x_3 + \ldots + x_n)}{n}
\]
OR
\[
\bar{X} = \frac{\sum X_n}{n}
\]
Where,
\( x_1 + x_2 + x_3 + \ldots + x_n \) = individual observations, and
\( n \) = total number of observations involved.

Standard deviation (S.D.): Standard deviation or standard range of observation was utilized in determining the statistical significance of the values obtained. It was calculated by the following formula:

SD for large samples (\( \sigma n \)):
Materials & Methods

SD for large samples (σn)-

\[
SD = \pm \sqrt{\frac{(X - x_1)^2 + (X - x_2)^2 + (X - x_3)^2 + \ldots + (X - x_n)^2}{n}}
\]

SD for small samples (σn-1)-

\[
SD = \pm \sqrt{\frac{(X - x_1)^2 + (X - x_2)^2 + (X - x_3)^2 + \ldots + (X - x_n)^2}{n-1}}
\]

Wherein,

\[X = \text{mean of the observations involved}
\]

\[x_1, x_2, x_3, \ldots, x_n = \text{individual observations}
\]

\[n = \text{number of observations involved}
\]

Per cent variation (P.V.) : To show and compare the relative variability of two or more sets of measurements, per cent variation was calculated. This is a unitless number and measures the magnitude of variation per cent between the mean of two plots relative to the average of the plots selected as control for comparison.

\[
P.V. = \frac{X_c - X_t}{X_c} \times 100
\]

where,

\[X_c = \text{arithmetic mean of values of control plants}
\]

\[X_t = \text{arithmetic mean of values of treated plants}
\]

Test of significance: This is a device to find out whether two observed means of values differ significantly from each other, or whether this difference is merely a result of chance influence. A test of significance confirms the validity of a result.
This was applied in the present study to test significance of the difference between two sample means.

The following formula was used to compute t-values, which were compared with the table values of ‘t’ at their particular degree of freedom. If the calculated t-value, exceeded the table ‘t’ value, difference between the two samples was taken to be significant, otherwise the differences was attributed to a chance factor.

\[ t = \frac{\text{Difference of two sample means}}{\text{Standard error of the difference}} \]

OR

\[ t = \frac{\bar{X}_c - \bar{X}_t}{\sqrt{\frac{(SD_c)^2}{n_1} + \frac{(SD_t)^2}{n_2}}} \]

where,

- \( \bar{X}_c \) = Arithmetic mean of control sample
- \( \bar{X}_t \) = Arithmetic mean of treated sample
- \( SD_c \) = Standard deviation of control sample.
- \( SD_t \) = Standard deviation of treated sample.
- \( n_1 \) = Number of observations of control sample
- \( n_2 \) = Number of observations of treated sample.

**Degree of freedom (D.F.):** The degree of freedom, to be applied to number of data, in t-test was calculated.

\[ D.F = n_1 + n_2 - 2 \]

Where,

- \( n_1 \) = number of observations of control plants
- \( n_2 \) = number of observations of treated plants
If the difference between any two sample means exceeds the Student's t-test values obtained at 5% or 1% level, the difference between the two means is said to be significant at the 5% or 1% level, respectively.