The present investigation will be focused on “Osmoprotectant mediated salinity stress tolerance in the Himalayan medicinal plants: *Silybum marianum* L. for its morphological, physiological and biochemical consequences”. A brief account of the materials used and methodologies adopted are discussed in this chapter.

### 3.1 MATERIALS

The seeds of *S. marianum* will be obtained from Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan (H.P). These will be grown in petriplates to check the vigor and germination. The morphological characters of the seedlings will be recorded at different intervals.

### 3.2 STUDY SITE

The Shoolini University which is falls in the mid hill zone of Himachal Pradesh and extend from 76°3.3'E longitude and 31°0.15' N latitude, at an average elevation of 1290 m above sea level. The climate of the region is humid and the location is transitional between sub–tropical to sub temperate in the mid hill of Shivalik ranges of the Himalayas. Temperature ranges from 22° C to 37.8° C. The annual rainfall ranges between 850-1300 mm of which 75% is received during mid-June to mid-September.

### 3.3 METHODS

#### 3.3.1 Seed germination and seedling growth assay

Seeds of *S. marianum* will be selected for uniformity (on the basis of color and size). Before germination, damaged and insect infected seeds will be discarded and the empty ones will be eliminated using floating method in distilled water. Surface sterilization of seeds of *S. marianum* will be done with 0.1% HgCl₂ for 2-5 minutes, after which the seeds will be rinsed three times with distilled water. The surface sterilized seeds will be soaked in distilled water (control), 25mM, 50mM, 100mM, 150mM, and 200mM, NaCl for 24 hours. Thereafter, the seeds were transferred to petriplates lined with three layers of filter paper moistened by distilled water (control) and different concentration of NaCl. The seeds will be allowed to germinate in an incubator at 18 ± 2°C under continuous illumination provided by fluorescent white light. Emergence of 2-5
mm radical will be taken as seed germination (ISTA, 1966). The seed germination will be recorded at periodic intervals for 24 days. After 24 days of germination, seedlings growth will be measured in terms of root length, shoot length, plant height, dry weight and seedling fresh weight. The shoot and root lengths will be measured with scale while seedlings weight will be measured by weighing balance.

### 3.3.2 Hydroponic culture

Prior to determining the responses of *Silybum marianum* L. to salt stress the seeds selected for uniformity will be surface sterilized and soaked in distilled water for 24 hour. Thereafter, they will transfer to petriplates lined with three layers of filter paper moistened with distilled water. The petriplates will be placed at 18 ± 2°C for 15 days in an incubator for germination under continuous illumination. The 15 days old seedling will be shifted to hydroponic culture containing Hoagland nutrient solution. The composition of nutrient medium will be: KNO$_3$-101g/l, Ca(NO$_3$)$_2$.H$_2$O-236.1g/l, KH$_2$PO$_4$-136.1g/l, MgSO$_4$.7H$_2$O-246.5g/l, Trace elements (H$_3$BO$_3$-2.8g/l, Mncl$_2$.4H$_2$O-1.8g/l, ZnSO$_4$.7H$_2$O-0.2g/l, CuSO$_4$.5H$_2$O-0.1g/l, NaMoO$_4$.0.025g/l, FeEDTA:EDTA.2Na-10.4g/l, FeSO$_4$-7.8g/l, KOH-56.1g/l) Plastic pots of 300 ml capacity will be used for plant culture. Three plants per pot will be grown by holding the plants in holes into the thermocol lids with the help of cotton wool. The nutrient medium will be appropriately replaced by fresh one at regular intervals during the growth of the plants. The plastic pots along with plants will be shifted to the BOD incubator at 18 ±2°C for further growth. After 15 days of shifting to BOD the plants will be treated with 25mM, 50mM, 100mM, 150mM, and 200mM, NaCl through appropriate addition to the nutrient medium and different concentration of sorbitol, mannitol and trehalose 50ppm, and100ppm each. After 15 days of treatment, various physiological and biochemical analysis will be done.

### 3.4 MORPHOLOGICAL ANALYSIS

In order to determine the status of growth after 24 days of applying salt stress the following growth parameters were recorded in untreated (control), 25mM, 50mM, 100mM, 150mM, and 200mM, NaCl.

- Plant height
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- Shoot length
- Root length
- Fresh weight
- Dry weight

3.5 BIOMASS AND PRODUCTIVITY

Fresh and dry weight of leaves was recorded. Shoot, root length and plant height is measured to determine the effect of Salt stress at 24 days after applying treatment.

3.6 PHYSIOLOGICAL ASPECTS

3.6.1 Relative Water Content (RWC)

The fresh weight of top leaves from each treatment was recorded. The leaves were then immersed in distilled water in beakers and left for 24 h. Thereafter, fully turgid leaves were weighed again. The leaves were dried in oven for 72 h at 70 °C, until constant weight of leaves was obtained. Relative water content (RWC) of leaves was calculated according to (Wheatherley, 1950).

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RWC = \frac{\text{fresh mass} - \text{dry mass}}{\text{saturated mass} - \text{dry mass}} \times 100
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3.7 NUTRIENT ANALYSIS

Potassium and sodium in the acid-digest of plant sample (leaf and root) was determined using Flame photometer. Weighed 500 mg dried plant sample in 100 ml conical flask. Added 10 ml of conc. HNO₃ placed funnel on the flask and kept for about 6-8 hrs or overnight at a covered place for pre-digestion. After pre-digestion when the solid sample was no more visible, added 10 ml of conc. HNO₃ and 2-3 ml HClO₄. Kept on a hot plate in acid proof chamber having fume exhaust system and heated at about 100°C for first 1 hr and then raised the temperature to 200°C. Continued digestion until the contents became colorless and only white dense fumes appeared. Reduced the acid contents to about 2-3 ml by continuing heating at the same temperature. Filtered through Whatman No. 42 filter paper into a 100 ml volumetric flask. Gave 3-4 washings of 10-15 ml portions of distilled water and made the volume to 100 ml. Measured Na⁺ and K⁺.
concentrations in the filtrate using Flame photometer. Recorded the flame photometer readings for each of the working standards of Na and K after adjusting blank to zero. Drawn a standard curve by plotting the readings against Na and K readings.

3.8 PLANT ANTIOXIDANT SYSTEM

3.8.1 Enzymatic Antioxidants

The enzymatic antioxidant analyzed in the leaves of *Silybum marianum* L. were CAT, POD,

3.8.1.1 Catalase

Catalase (EC 1.11.1.6) activity was assayed following the method of Luck (1974). Homogenized plant tissue (leaf and root) in a blender with (0.067 M, pH 7.0) phosphate buffer (assay buffer diluted 10 times) at 1-4°C and centrifuged. Stirred the sediment with cold phosphate buffer, allowed standing in the cold with occasional shaking and then repeating the extraction once or twice. The final volume for the assay mixture was approximately 3 ml, 240 nm wavelength read against a control cuvette containing enzyme solution as in the experimental cuvette, but containing H$_2$O$_2$-free PO$_4$ buffer. Pipette into the experimental cuvette 3ml H$_2$O$_2$-PO$_4$ buffer. Mixed in 0.01-0.04 ml sample with a glass or plastic rod flattened at one end. Noted the time required for a decrease in absorbance from 0.45 to 0.4. This value is used for calculations. One g tissue is homogenized in a total volume of 20 ml, diluted 1 to 10 volumes with water and taken 0.01ml for assay. Calculated the concentration of H$_2$O$_2$ using the extinction coefficient 0.036/m mole/ml.

3.8.1.2 Peroxidase

The method proposed by (Reddy *et al.* 1995) was adopted for assaying the activity of peroxidase. A 20% homogenate was prepared in 0.1 M phosphate buffer (pH 6.5) from the plant sample (leaf and root), centrifuged and the supernatant was used for the assay. To 3.0 ml of pyrogallol solution, 0.1 ml of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 430 nm. To the test cuvette, 0.5 ml of H$_2$O$_2$ was added and mixed. The change in absorbance was recorded every 30 seconds up
to 3 minutes in a spectrophotometer. One unit of peroxidase is defined as the change in absorbance/minute at 430 nm.

3.8.2 Non – Enzymatic Antioxidant

The non-enzymatic antioxidants analyzed were total phenols and lipid peroxidation.

3.8.2.1 Phenols

Phenol content was estimated by (Malick and Singh 1980). Weighed 0.5 g of the sample (leaf and root) and grind it with a pestle and mortar in 10-time volume of 80% ethanol. Centrifuged the homogenate at 10,000 rpm for 20 min saved the supernatant. Re-extracted the residue with five times the volume of 80% ethanol, centrifuged and pooled the supernatants. Evaporated the supernatant to dryness. Dissolved the residue in a known volume of distilled water (5 ml). Pipette out different aliquots (0.2 to 2 ml) into test tubes. Made up the volume in each tube to 3 ml with water. Added 0.5 ml of Folin-Ciocalteau reagent. After 3 min, added 2 ml of 20% Na₂CO₃ solution to each tube. Mixed thoroughly. Placed the tubes in a boiling water bath for one minute, cooled and measured the absorbance at 650 nm against a reagent blank. Prepared a standard curve using different concentrations of gallic acid. From the standard curve the concentration of phenols in the test sample was determined and expressed as mg phenols/100g material.

3.9 BIOCHEMICAL ANALYSIS

3.9.1 Lipid Peroxidation

The extent of lipid peroxidation was estimated according to the method of (Dhindsa et al., 1981). Lipid peroxidation was estimated from the accumulated malondialdehyde (MDA) following the method given by (Dhindsa et al., 1981). In brief, the plant tissue (approx. 200 mg) (leaf and root) was homogenized with 0.1% trichloroacetic acid (TCA) (2 ml). The homogenate was centrifuged at 10,000 rpm for 10 min. And supernatant collected. The supernatant (2 ml) was reacted with 4 ml of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was then heated at 95°C for 45 min. And rapidly cooled in an ice bath for 5 min. Absorbance was read at 532 nm. Measurements were corrected for unspecific turbidity by subtracting the absorbance at
600 nm. MDA contents were determined using the extinction coefficient of 155 mM$^{-1}$cm$^{-1}$ (Heath and Packer, 1968).

3.10 STATISTICAL ANALYSIS

The data was analyzed statistically using Graph Pad Prism® 5.2. Mean values were calculated from measurements of six replicates and the standard error of means were determined. One-way and two-way analysis of variance (ANOVA) was applied to determine the significance of results between different treatments and Tukey’s multiple comparison test and Bonferroni’s post tests were performed at the significance level of P<0.05. In order to verify statistically the significance level in different treatments, null hypothesis (H0) was postulated that there is no significant difference due to the treatments and assumed that there is no significant difference in parameter studied (e.g., germination %, stem length, etc.) between treatments.