Chapter 3

MATERIAL AND METHODS

3.1 Introduction

The term methodology refers to the overall approaches and perspectives to the research process as a whole and is concerned with the following main issues: Why and What collected certain data, Where and How collected it and How analysed it? (Collis and Hussey, 2003). Generally, methodology is a guideline system for solving a problem, with specific components such as phases, tasks, methods, techniques and tools. It can be defined also as “the analysis of the principles of methods, rules, and postulates for regulating a given discipline” and “the organized study of methods that are, can be, or have been applied within a discipline” (Baskerville, 1991).

Thus, methodology is a way to systematically solve the research problems. It may be understood as a science of studying how research is done scientifically. The procedure or course of action is an important phase of the research and design study. In order to achieve the objective of the study, the present research aimed at studying phyto-sociological studies, ethnobotanical studies and chemotypic variation in ‘Oregano’ (Origanum vulgare L.) by applying descriptive and interview survey method. To accomplish this objective, following methods have been followed and a detailed methodology of the study is given in related chapters.

3.2 Phyto-sociological

3.2.1 Study area

A total of thirty three natural sites sustaining Origanum vulgare from Uttarakhand Himalaya were selected. Study area was stratified into three broad climatic zones viz. Lower temperate (LT, 1100 – 1800m asl), Mid temperate (MT, 1801 – 2500m asl) and Upper temperate & sub-alpine (UT/SA, 2501 – 3550m asl) for phyto-sociological and population study. The strata are based on the variation in the geo-morphological features, aspects, altitude and slopes etc. The sampling areas are shown in chapter 1, figure 1.1.
### Climates Zone Altitude Study areas (District) Total sites

<table>
<thead>
<tr>
<th>Climatic Zone</th>
<th>Altitude</th>
<th>Study areas (District)</th>
<th>Total sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower temperate (LT)</td>
<td>1100–1800 m asl</td>
<td>Almora, Bageshwar, champawat, Nainital, Pauri, Pithoragarh, Tehri, Uttarakashi</td>
<td>15</td>
</tr>
<tr>
<td>Mid temperate (MT)</td>
<td>1801–2500 m asl</td>
<td>Almora, Bageshwar, Chamoli, champawat, Nainital, Pithoragarh, Rudraprayag, Uttarakashi</td>
<td>13</td>
</tr>
<tr>
<td>Upper temperate &amp; sub-alpine (UT/SA )</td>
<td>2501–3550 m asl</td>
<td>Chamoli, Pithoragarh, Rudraprayag, Uttarakashi</td>
<td>05</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1100 to 3550m</strong></td>
<td><strong>Districts - 10</strong></td>
<td><strong>33</strong></td>
</tr>
</tbody>
</table>

3.2.2 Population Study

The phyto-sociological analysis of all the selected sites of *Origanum vulgare* species was conducted during rainy season (July-August) when majority of the plants were at the peak of their growth of the year 2009-2010. For this purpose, communities sustaining *Origanum* species were identified and an area of 100 m² was marked. On sampling time, 15 quadrats of 1 m² were laid randomly at each population. All the species present in each quadrat were identified noted and observed. Species were further identified and observed for their presence or absence and individuals of each species were counted. Basal area of each species was determined by clipping ten stems at ground level and diameter of their clipped ends were measured with a screw gauge. In order to determine the distribution pattern and the population status of the species, analytical and synthetic characters were analyzed by the data obtained from this quadrat study.

3.2.3 Quantitative analysis

Data collected after sampling were further used for calculating quantitative characters *viz.*, percentage frequency (%), abundance, density (D, plant m⁻²), total basal cover (TBC, cm²m⁻²) and relative values of per cent frequency, density and dominance were calculated by following the methods of Curtis and McIntosh (1950).
(a) **Density**

Density is an expression of the numerical strength of a species where the total number of individuals of each species in all the quadrats is divided by the total number of quadrats studied.

\[
\text{Density} = \frac{\text{Total number of individuals of a species in all quadrats}}{\text{Total number of quadrats studied}}
\]

(b) **Frequency (%)**

This term refers to the degree of dispersion of individual species in an area and usually expressed in terms of percentage occurrence. It was studied by sampling the study area at several places at random and recorded the name of the species that occurred in each sampling units.

\[
\text{Frequency} (\%) = \frac{\text{Number of quadrats in which the species occurred}}{\text{Total number of quadrats studied}} \times 100
\]

(c) **Abundance**

It is the study of the number of individuals of different species in the community per unit area. By quadrats method, samplings are made at random at several places and the number of individuals of each species was summed up for all the quadrats divided by the total number of quadrats in which the species occurred.

\[
\text{Abundance} = \frac{\text{Total number of individuals of a species in all quadrats}}{\text{Total number of quadrats in which the species occurred}}
\]

(d) **A/F ratio**

Distribution pattern of the species were analyzed on the basis of abundance to frequency, (A/F) ratio calculated according to Curtis and Cottam (1956). Value of A/F <0.025 was categorized regular, between 0.026-0.050 random and >0.050 contiguous type of distribution.

(e) **Importance Value Index**

This index is used to determine the overall importance of each species in the community structure. In calculating this index, the percentage values of the relative frequency, relative density and relative dominance are summed up together and this
value is designated as the Importance Value Index or IVI of the species. To express the dominance and ecological success of any species with a single value Importance Value index (IVI) is calculated according to (Curtis and McIntosh, 1950).

(f) **Relative density**

Relative density is the study of numerical strength of a species in relation to the total number of individuals of all the species and can be calculated as:

\[
\text{Relative density} = \frac{\text{Density of a species}}{\text{Total density of the community}} \times 100
\]

(g) **Relative frequency:**

The degree of dispersion of individual species in an area in relation to the number of all the species occurred.

\[
\text{Relative frequency} = \frac{\text{Percent frequency of species}}{\text{Total percent frequency of the community}} \times 100
\]

(h) **Relative dominance**

Dominance of a species is determined by the value of the basal cover. Relative dominance is the coverage value of a species with respect to the sum of coverage of the rest of the species in the area.

\[
\text{Relative dominance} = \frac{\text{Total basal area of the species}}{\text{Total basal area of all the species}} \times 100
\]

The basal cover of each individual was calculated as, Average basal cover = \( \pi r^2 \) (cm. sq.), where, \( r \) (radius) = Average diameter/2. This average basal cover of each species was multiplied by the respective density to obtain total basal cover (TBC cm\(^2\) m\(^{-2}\)) of each and every species.

\[
\text{TBC} = \text{Mean basal cover} \times \text{density of respective species}
\]

To study the structure of communities sustaining *Origanum* species concentration of dominance (Cd) was computed using Simpson's index (Simpson, 1949), species diversity (H') was determined using Shannon - Wiener information function (Shannon and Wiener, 1963), Species richness was calculated by counting total number of species observed in each habitat (Magurran, 2004) and evenness
Shannon’s diversity index and Simpson’s index of dominance were calculated using important value index (IVI) of species.

(i) **Simpson (1949) index of Dominance (Cd):**

Concentration of dominance is heterogeneity index that measures dominance and weighted towards the abundance of the commonest or abundant species in the sample. Value of Cd varies from 0 to 1.0. A value of 0 indicates the presence of only one species, while 1.0 means that all the species are equally represented.

The equation used to calculate Simpson’s index was $Cd = \sum (p_i)^2$

Where, Cd = Simpson index of dominance

$p_i = \text{the proportion of important value of the } i^{th} \text{ species } (p_i = n_i / N, n_i \text{ is the important value index of } i^{th} \text{ species and } N \text{ is the important value index of all the species}).$

(j) **Shannon–Weaver (1963) index of diversity (H’):**

It is measure of information in a group of a species, which have different probabilities of being represented and is based on the assumption that individually sampled from an infinity large population. Information maximum when probabilities (number of individuals) for all the species are equal. The index of diversity was computed by using Shannon–Weaver information index.

The formula for calculating the Shannon diversity index is $H’ = - \sum p_i \ln p_i$

Where, $H’ = \text{Shannon index of diversity}$

$p_i = \text{the proportion of important value of the } i^{th} \text{ species } (p_i = n_i / N, n_i \text{ is the important value index of } i^{th} \text{ species and } N \text{ is the important value index of all the species}).$

(k) **Evenness (E):**

Evenness was calculated as $E = H’/ H_{max}$

Where, $H’ = \text{Shannon index of diversity}$

$H_{max} = \log_2 P_i$
(1) Similarity and dissimilarity indices

Indices of similarity and dissimilarity were calculated by using formulae as Sorensen (1948) as follows:

Index of similarity (S) = \( \frac{2C}{A+B} \)

Where,  
A = Number of species in the community A
B = Number of species in the community B
C = Number of common species in both the communities.

Index of dissimilarity = 1-S

3.2.4 Nativity and Endemism

The nativity of the species has been identified for their origin or reported first recording from the Himalayan region (Anonymous, 1883-1970; Samant and Dhar, 1997; Samant et al., 1998b) whereas the species with wide distribution or left over species was considered as non-natives. The species with their restricted distribution to the Himalayan Region have been considered as endemic (Dhar and Samant, 1993; Samant, 1999; Samant and Dhar, 1997).

3.2.5 Threat Assessment

Threat categories of the species was identified based on six attributes like habitat specificity, population size, distribution range, use values, extraction, nativity and endemism of the taxon (Samant et al., 1996b; Samant, 1998a, Ved et al., 2003 and Samant et al., 2007). The Conservation Priority Index (CPI) is the cumulative value of all the attributes presented in Table-1. Based on the CPI values, each accession of three climatic zones was categorised under different threat categories. The conservation attributes used were divided into three grades/scores: highest (10 marks); followed by 06 marks and the lowest score of 02 marks. Species with scoring ≥ 60% were identified as critically endangered; 56–60% as endangered; 51–55 as vulnerable; 46–50 as near threatened; and 46 as of least concern. During the study, only healthy or vigour plants were consider and taken further for population assessment. Furthermore, status was assigned separately for each natural site of different climatic zones as well as for entire Indian Himalayan Region.
Table 3.1: Threat assessment Attributes used for Conservation Priority Index (CPI)

<table>
<thead>
<tr>
<th>Distribution Range (m)</th>
<th>Habitat</th>
<th>Population (Ind./Location)</th>
<th>Use Pattern</th>
<th>Extraction</th>
<th>Native and Endemic</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 500</td>
<td>1</td>
<td>≤ 50 Ind./2 locations</td>
<td>≥ 4</td>
<td>Commercial</td>
<td>Native and Endemic</td>
<td>10</td>
</tr>
<tr>
<td>500-1000</td>
<td>2-3</td>
<td>50-250 Ind./3-5 locations</td>
<td>3-4</td>
<td>Self use</td>
<td>Native/Endemic</td>
<td>6</td>
</tr>
<tr>
<td>≥ 1000</td>
<td>≥ 3</td>
<td>≥ 250 Ind./≥ 5 locations</td>
<td>≤ 3</td>
<td>No extraction</td>
<td>Non Native</td>
<td>2</td>
</tr>
</tbody>
</table>

3.3 Taxonomical study (Morphological and Anatomical variation)

For the taxonomic study following heads have been taken:

3.3.1 Exploration and bulk collection of plant material

Intensive survey in Uttarakhand hills identified the locations/areas, where Oregano is found in natural habitats. On the basis of the preliminary study in ten districts of Uttarakhand were identified. Genuine plant materials (*Origanum vulgare* L.) were randomly collected from different 33 natural habitats before flowering and flowering period. Properly identified voucher herbarium specimens along with voucher drug samples were deposited in the NBPG, (ICAR), Regional Station, Bhowali and NBRI, (CSIR), Lucknow, UP, for future reference. The passport data of collected accessions have been generated and documented. The live plants were established at in Field Gene Bank, NBPG, (ICAR), Regional Station, Bhowali for assigning authentication identity Indigenous Collection (IC) number NBPG, (ICAR), Pusa campus, New Delhi.

3.3.2 Processing of plant material for study

The plant materials were properly dried in shade or at 40° C and powdered for *In-vitro* studies. The fresh materials were preserved in FAA (Formalin-acetic acid-alcohol) for microscopic studies.

3.3.3 Taxonomic Study

To study the variations among the populations of Oregano, taxonomic studies were carried out. Morphological observations include morphological attributes in
Field Gene Bank. Besides, soil features of all the populations were also observed to identify better edaphic requirements during domestication.

### 3.3.4 Morphological Variations

Selected collections of ‘Oregano’ grown and maintained in Field Gene Bank, NBPG, (ICAR), Regional Station, Bhowali. Morphological variations among the plants of different populations/ accessions were studied during flowering season. For this purpose, 10 mature individuals were randomly selected in each populations/ accessions and observed for parameters viz., plant growth habit, plant height, lamina length and lamina width, number of flowers/ whorl, fresh herbage yield per plant, dry herbage yield per plant, surface characters, colour etc. These parameters were recorded according to NBPG (ICAR), Descriptor (Singh et al., 2003).

#### 3.3.4.1 Phenotypic Traits

For detail phenotypic observations, seeds and live plants or germplasm of Oregano were randomly collected from mature plants and newly sprouted plant from different 33 natural habitats of ten districts of Uttarakhand and planted in Field Gene Bank, NBPG, (ICAR), Regional Station, Bhowali. This Field Gene Bank served as an experimental site for preliminary characterization, evaluation, multiplication, domestication along with various agricultural and horticultural crops and to develop agro-techniques for temperate MAPs. For the experiment, plots of 3m² were prepared by levelling and digging. Eight seedlings of each population/accessions in triplicates were transplanted in Random Block Design (RBD) without any treatments (no manure, fertilizer inputs). Special care was taken viz., plant growth habit, plant height, lamina length and lamina width, number of flowers/ whorl, fresh herbage yield per plant, dry herbage yield per plant, surface characters, colour etc. have been recorded (according to NBPGR Descriptor). Based on best performances for survival, plant growth and economic yield, an elite population was identified for the purpose of domestication, multiplication of germplasm and ultimately to wide cultivation.

### 3.3.5 Histological and micro-anatomical feature

Histological and micro-anatomical studies of the collected accessions of Oregano will be helpful for proper identification of elite germplasm to the industries for maintaining batch to batch uniformity of the raw materials.
3.3.5.1 Quantitative estimation

Quantitative assessment of leaves and stem of *Origanum vulgare* were estimated as per Khandelwal (2005), Kokate (1997), Ahmad *et al.* (1984) and Ayurvedic Pharmacopoeia of India (2001).

3.3.5.2 Anatomical feature

The fresh or preserved (in FAA) materials were taken for microscopic studies. Section taken by moving the blade back and forth and placed in watch glass containing water. Thin section were selected and placed in Chloral hydrates and cleared by boiling. Cleaned sections were stained by using Phloro-glucinol and concentrated hydrochloric acid. One thin section was taken out and mounted on a clean glass slide. A drop of glycerine was added and covered with the cover slip. This slide was observed under microscope and photomicrographs of with different magnifications were taken with Olympus CX 31 Lica ATC 2000 (digit 3) microscopic unit.

3.3.5.3 Determination of Stomatal number

Stomatal number is the average number of stomata per sq. mm of the epidermis of the leaf. Piece of *Origanum vulgare* leaf (middle part) was cleaned by boiling with Chloral hydrate solution. The upper and lower epidermis was peeled separately. The peeled epidermis was placed on slide and mounted with glycerine water. Camera Lucida and drawing board was arranged for the drawing with the help of stage micrometer 1 sq. mm was drawn. The prepared slide was placed on the stage, epidermal cell and stomata are traced. The number of stomata lying in the area of 1 sq. mm are counted including the cell if at least half of its area lying with in the square. Average number of stomata per sq. mm is calculated by tracing ten different fields.

3.3.5.4 Determination of Stomatal Index (SI)

Stomatal number is affected by various factors like age of the plant, size of the leaf, environmental conditions etc., stomatal index is not much affected by these factors. It is relatively constant. Hence it is more significant in the evaluation of a leaf drug. Stomatal index is the percentage which the numbers of stomata form to the total
number of epidermis cells, each stomata being counted as one cell. Stomata index can be calculated by using following equation.

\[
\text{Stomatal Index} = \frac{S}{E + S} \times 100
\]

Where \( S \) = the number of stomata in a given area of leaf.

\( E \) = the number of epidermal cells in the same area of leaf.

The number of stomata and the number of epidermal cells in each field were counted. Stomatal index was calculated using the above formula. Values for upper and lower surface (epidermis) were determined separately.

### 3.3.5.5 Determination of Palisade ratio

Palisade ratio is the average number of palisade cells beneath one epidermal cell of a leaf. It is determined by counting the palisade cells beneath four continuous epidermal cells. \textit{Camera lucida} and drawing board was arranged for the drawing. A piece of leaf was cleaned by boiling in chloral hydrate solution. The four cell of epidermis are traced off. By focusing down to palisade layer, sufficient cells are traced off to cover the epidermal cells. The number of palisade cells under the four epidermal cells is counted (including the palisade cells in the count when more than half is covered in the area of epidermal cells). Average number of cells beneath a single epidermal cell are calculated which gives the palisade ratio. By focusing different part of the leaf, the same of traced and the average was calculated to get the palisade ratio of the leaf.

### 3.3.5.6 Determination of vein-islet number

A vein-islet is the small area of green tissue surrounded by the vein-lets. The vein-islet number is the average number of vein-islets per sq. mm of a leaf surface. It is determined by counting the number of vein-islets in an area of sq. mm of the central part of the leaf between the midrib and margin. A piece of leaf is cleaned by boiling in chloral hydrate solution. Camera Lucida and drawing board was arranged for the drawings with the help of stage micrometer 1 sq. mm were drawn. The cleaned leaf was mounted on the slide and a drop of glycerine water was added then covered with cover slip. The above prepared slide was placed on the stage of the microscope. Veins
are traced which are included with in the square. The outlines of those islets which overlap two adjacent sides of the square are also traced. The number of vein-islet in the sq. mm is counted. The islets which are intersected by the sides of square are included on two adjacent sides and excluded on other two sides. The average number of vein islet is calculated.

3.3.5.7 Determination of vein-let termination number

It is defined as the number of the vein let termination per sq. mm of the leaf surface, midway between midrib of the leaf and its margin. A vein termination is the ultimate free termination of vein let. Same as, for the determination of vein-let number. The average number of vein terminations present within the square was counted from ten different squares to get the value of one sq. mm.

3.4 Ethno-botanical and Socio-economic study

Ten districts with 30 villages of Uttarakhand were identified for collection of plant material and study of relevant traditional knowledge. A semi-structured interview schedule/questionnaire were developed. Data were collected from the primary sources by interviewing and recording of statements of farmers and locals during September, 2008 to October, 2011. Sample households were randomly selected from the villages, for collection of information. The Participatory Rural Appraisal (PRA) and observation methods were also deployed for collection of data. In each village 5-10% households were interviewed. After careful recording of the information and traditional knowledge, the findings were shared with the local healers and medicine men for validation of information.

The present study was brought to record several uses of ethno-medicinal properties among inhabitants of Kumaon and Garhwal region of Uttarakhand Himalayas to highlight ethno-botanical notes and economy of this taxon.

3.5 Biochemical and Phytochemical analysis

3.5.1 Biochemical estimations

To study the biochemical attributes of 33 accessions of *O. vulgare*, the medicinally important part of the plant (leaves/aerial parts), were used. Carbohydrates
(total soluble sugars and starch), total soluble protein and total free amino acid estimations were carried out by using fresh leaves which were collected from Field Gene Bank of NBPR, Regional Station, Bhowali, Niglat, District- Nainital, during pre-flowering period (May). Freshly harvested leaves/ aerial parts were brought to laboratory in ice and stored in refrigerator for various estimations. For phytochemical analysis crud drug (aerial part/leaves powder) were obtained from natural conditions (NT).

3.5.1.1 Carbohydrates (soluble sugars and starch)

For the extraction of sugars, the homogenates were prepared by grinding 100 mg of fresh leaves in 80% warm ethanol with the help of mortar and pestle. The extract was centrifuged at 3000 rpm for 10 minutes and the supernatant was taken for the estimation of soluble sugars while the pellet was kept for starch estimation.

The Anthrone method described by Mc Gready et al. (1950) was followed for the determination of both soluble sugars and starch.

2.5.1.1a Soluble sugars

The anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 ml of 95% sulphuric acid, which was then kept in refrigerator and the cold reagent was used for the colour development. To the known value of supernatant, the 4 ml anthrone reagent was gently passed through the walls of the test tube containing the sugar solution, mixed thoroughly and heated in boiling water bath for 7-8 minutes.

After allowing the solution to cool down to room temperature, the absorbance of the colour was measured against a suitable blank (1 ml 80% ethanol + 4 ml anthrone) at 620 nm using spectrophotometer (perkin-elmer UV double beam). Standard curve was prepared with the help of dextrose.

2.5.1.1b Starch

The pellet left behind was suspended in 52% perchloric acid, centrifuged at 3000 rpm for 10 minutes and the supernatant was taken for the estimation of Starch content in the supernatant was estimated by following the above mentioned Anthrone method.
3.5.1.2 Total free amino acids

Total free amino acid contents in sample were determined by the method of Moore and Stein (1954). 100 mg plant material was homogenized in 5 ml of 80% cold ethanol in a mortar with the help of a pestle. After centrifugation at 3000 rpm for 10 minutes, the supernatant obtained was used for amino acid estimation.

For colour development 0.1 ml supernatant was made 1 ml by adding 0.9 ml of 80% ethanol and finally 2 ml ninhydrin reagent (prepared by dissolving 0.750 g ninhydrin in 26.25 ml ethylene glycol (ethanediol) with 1.25 ml 0.2 M acetate buffer of pH 5.0) was added. The contents were mixed well and the test tubes containing the solution were capped with aluminum foil and kept for 20 minutes in boiling water bath. After cooling the contents to room temperature, absorbance was measured at 570 nm against a suitable blank (1 ml 80% ethanol + 2 ml ninhydrin). Standard curve was prepared by glycine.

3.5.1.3 Total soluble proteins

For protein estimation the plant material was homogenized in 1:5 ratio, ice chilled 0.1 M tris HCl extraction buffer (pH 7.5). The homogenates were centrifuged at 10000 rpm for 20 minutes at 4°C. Soluble protein was estimated by the supernatant obtained, following the Lowry method (Lowry et al., 1951). The Lowry method is sensitive to low concentrations of protein. Dunn (1992) suggests concentrations ranging from 0.10- 2 mg of protein per µl while Price (1996) suggests concentrations of 0.005 - 0.10 mg of protein per µl.

Principle:

The blue colour developed by the reduction of the Phosphomolybdic-Phosphotungastic components in the Folin-ciocalteau reagent by the amino acids Tyrosine and Tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry’s method.

Preparation of Reagents:

Regents ‘A’: 2% sodium carbonate (Na₂CO₃) in 0.1N NaOH.
Regents ‘B’: 0.5% copper sulphate (CuSO₄) solution in 1% Sodium potassium tartarate solution (to be prepared fresh). Or 0.5% CuSO₄·5H₂O in H₂O + 1% Sodium potassium tartarate in H₂O.

Regents ‘C’ (Lowry Reagent): Mix 50 ml of Regents ‘A’ with 1 ml of Regents ‘B’ (just prior to use).

Regents ‘D’ (Folin-Ciocalteau reagent): This is commercially available and has to be diluted with equal volume of water just before use.

Standard protein solution: Dissolve 10 mg of BSA (Bovine Serum Albumin) in 100 ml of distilled water in a volumetric flask. (Concentration-0.1mg/ml).

Procedure:

- Set up eleven sets of three (16 x 150) mm test tubes in rack.
- Add BSA [0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µl] to these tubes.
- Add 5 mL of Regents ‘C’ to each test tube.
- Incubate for 10 minutes at room temperature.
- Add 0.5 mL of Regents ‘D’ to each tube.
- Vortex each tube immediately.
- Incubate at room temperature for 30 minutes and the blue colour formed.
- Determine absorbance of each sample at 660 nm.
- Set up triplicate assays for all "unknowns".
- Prepare a blank with 1ml of distilled water, instead of protein solution and with 1ml of unknown solution and proceeds as per standards. Prepare a calibration curve with mg of protein on X-axis and O.D. on Y-axis and determine the amount of protein present in a given unknown sample.

3.5.1.4. Photosynthetic pigments

For chlorophyll estimation, 100 mg or 0.1gm of fresh plant material/tissue, taken from the fourth and fifth leaf from aerial parts of plants, and homogenized in 5 or 10 ml 80% acetone, using mortar and pestle. The homogenate was centrifuged at 5000 rpm for 5 min and the supernatant is collected in a volumetric flask. The residue
was re-extracted using the same method, until it became devoid of colour (Lichtenthaler and Wellburn, 1983). Then, the optical density (O.D.) or absorbance of the supernatant was measured at wave lengths 663, 645, and 440.5 nm (Smith and Benitez, 1955) to estimate chlorophyll ‘a’ and ‘b’, and carotenes respectively, using a Spectrophotometer. Three replicates were used for each treatment, and amount of chlorophyll a, b, total chlorophyll and total carotenoids in each sample was calculated according to the following equations:

- Chlorophyll a mg/g FWB:
  \[12.7 \times A(663\text{nm}) - 2.69 \times A(645\text{nm}) \times \frac{V}{W \times 1000}\]

- Chlorophyll b mg/g FWB:
  \[22.9 \times A(645\text{nm}) - 4.68 \times A(663\text{nm}) \times \frac{V}{W \times 1000}\]

- Total Chlorophyll (a + b) mg/g FWB:
  \[20.2 \times A(645\text{nm}) + 8.02 \times A(663\text{nm}) \times \frac{V}{W \times 1000}\]

- Total carotenoid mg/g FWB:
  \[46.95 \times A(440.5\text{nm}) - 0.268 \times \text{Chlorophyll (a + b)}\]

Where \(A\) = absorbance; \(V\) = Final volume; \(W\) = Fresh weight (g)

3.5.2 Phytochemical Estimation

3.5.2.1 Determination of antioxidant activity with 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method

The antioxidant activity of the oregano extract was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH (Brand-Williams et al., 1995). DPPH is a free radical which when dissolved in methanol has a blue-violet colour. The loss of colour indicates radical scavenging activity and the objective is to inhibit the loss of colour with oregano extracts. The effect of extract on DPPH radical was estimated using the method of Liyana-Pathirana et al. (2005).
Chemicals requirement:

- 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Ascorbic acid, Rutin, methanol
- Standard DPPH solution: 0.135 mM solution
- Sample stock solution: 0.1 mg/ml solution for all sample methanolic extract (1 mg/10 ml methanol)

Method:

A methanolic stock solution (Conc. 0.1 mg/ml) of each accessions (plant extracts) at different concentrations was mixed in a test tube with a mixture of 2.0 ml of methanol and 2 ml of 0.135 mM methanolic solution of DPPH (2,2-diphenyl-1-picrylhydrazyl), (stable free radical). Methanol was used to zero the spectrophotometer. The absorbance of the DPPH radical without antioxidant, i.e. the control, was measured daily. Special care was taken to minimize the loss of free radical activity of the DPPH radical stock solution (Blois, 1958). The reaction mixture was set-aside in the dark at room temperature for 30 min and absorbance was recorded at 517 nm by UV-1 Double beam spectrophotometer. The experiment was done in triplicate. Ascorbic acid and rutin were used as references. IC50 value is the concentration of sample, required to scavenge 50% of DPPH free radicals. IC50 values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm. The scavenging of DPPH was calculated according to the following equation (Yen and Duh 1994).

\[
\text{% scavenging} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}}\right) \times 100
\]

Where, \(\text{Abs}_{\text{control}}\) = absorbance of DPPH radical + methanol;
\(\text{Abs}_{\text{sample}}\) = absorbance of DPPH radical + wheat extract/standard.

3.5.2.2 Determination of total phenolic contents

A total phenolic compound in the methanolic extract of leaves of *Origanum vulgare* was determined by Folin–Ciocalteu’s calorimetric method (Singleton *et al.*, 1999).

Extract preparation: 1 gram air dried powdered drug percolated with pure methanol, three time filter the extract and lyophilized to dry and was weighted.
Regents used:

A. 20% Sodium carbonate saturated solution: It was prepared by adding 20g anhydrous sodium carbonate dissolved in 100 ml distilled water, and get cool.

B. Gallic acid standard solution: (1mg/ml) dissolve 10 mg gallic acid in 10 ml of deionized water.

C. Folin & Ciocalteu’s phenol reagent

Standards:

Stock Solution: Weight 1 mg of Gallic acid and mix in 10 ml of Methanol to get the final concentration of 0.1 mg/ml.

Dilutions: From stock make at least five different dilutions-

0.2, 0.4, 0.6, 0.8 and 1.0 ml of stock and diluted as below procedure.

Procedure:

1. Take the stock concentration in the 25 ml of volumetric flask.
2. Add 10 ml of distilled Water and 1.5 ml of Folin Ciocalteu reagent.
3. Keep it for 5 mins.
4. Add 4 ml of 20% Na$_2$CO$_3$ solution.
5. Make up the volume with dist. Water up to 25 ml.
6. Keep it for 30 mins then take the absorbance at 765 nm at UV spectrophotometer.

Final conc. of std. in 25 ml of Vol. flasks used for the absorbance-

1. 0.2 ml in 25 ml = 0.2 x 0.1 mg/25 ml = 0.0008 mg/ml
2. 0.4 ml in 25 ml = 0.4 x 0.1 mg/25 ml = 0.0016 mg/ml
3. 0.6 ml in 25 ml = 0.6 x 0.1 mg/25 ml = 0.0024 mg/ml
4. 0.8 ml in 25 ml = 0.8 x 0.1 mg/25 ml = 0.0032 mg/ml
5. 1.0 ml in 25 ml = 1.0 x 0.1 mg/25 ml = 0.0040 mg/ml

Plot a Std. curve of Abs./conc. in UV spectrophotometer, note the correlation coefficient and then measure the sample content by using that standard curve.
**Procedure for Sample:**

1. Make the extract of the crude drug that may be alcoholic, hydro-alcoholic or methanolic and then dry it properly to get residue.

2. Take the dried extract of 1mg/ml concentration in the 25 ml of volumetric flask.

3. Add 10 ml of dist. Water and 1.5 ml of Folin- Ciocaltue reagent.

4. Keep it for 5 minutes.

5. Add 4 ml of 20% Na$_2$CO$_3$ solution.

6. Make up the volume with dist. Water up to 25 ml.

7. Keep it for 30 minutes then take the absorbance at 765 nm at UV-1 Double beam spectrophotometer.

**Calculation:** % of Total Phenolics or Gallic acid equivalents in sample crude drug

\[
\text{Calculation} = \frac{\text{Conc. of sample by obtained by UV mg x Extract obtained in mg x 25ml} \times 100}{\text{Wt. of extract taken mg x Wt. of crude drug taken for extraction mg}}
\]

The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of gallic acid and the calibration line was construed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in extracts or crude drug was expressed in terms of gallic acid equivalent (mg of GA/g of extract or crude drug) per gram of a sample on dry weight basis.

### 3.5.2.3 Determination of flavonoid concentrations:

The content of flavonoids in the examined plant extracts was determined us in spectrophotometric method (Quettier *et al.*, 2000).

**Extract preparation:** 5 gram of dried powdered leaf, cold percolated with known volume of methanol.

**Reagent used:** 2% Aluminum chloride, Methanol, Rutin

**Standards:**

Stock Solution: Weight 1 mg of Rutin and mix in 10 ml of Methanol to get the final concentration of 0.1 mg/ml.
Dilutions: From stock make at least five different dilutions-
0.2, 0.4, 0.6, 0.8 and 1 ml of stock and diluted as below procedure.

Procedure:
1. Take stock concentration in 10 ml test tube.
2. Add 0.5 ml of 2% AlCl₃ in Methanol.
3. Make up the volume with Methanol up to 5 ml.
4. Keep it for 5 mins then take the absorbance at 420 nm at UV spectrophotometer.

Final conc. of std. in 5 ml of methanol used for the absorbance-
1. 0.2 ml in 5 ml = 0.2 x 0.1 mg/5 ml = 0.004 mg/ml
2. 0.4 ml in 5 ml = 0.4 x 0.1 mg/5 ml = 0.008 mg/ml
3. 0.6 ml in 5 ml = 0.6 x 0.1 mg/5 ml = 0.012 mg/ml
4. 0.8 ml in 5 ml = 0.8 x 0.1 mg/5 ml = 0.016 mg/ml
5. 1.0 ml in 5 ml = 1.0 x 0.1 mg/5 ml = 0.020 mg/ml

Plot a Std. curve of Abs./conc. in UV spectrophotometer, note the correlation coefficient and then measure the sample content by using that standard curve.

Procedure for Sample:
6. Make the extract of the crude drug that may be alcoholic, hydro-alcoholic or methanolic and then dry it properly to get residue.
7. Dried extract of 1mg/ml concentration in methanol.
8. Take 0.5 ml of above.
9. Add 0.5 ml of 2% AlCl₃ in Methanol.
10. Make up the volume with methanol up to 5 ml.
11. Keep it for 5 mins then take absorbance at 420 nm at UV-1 Double beam spectrophotometer.

Calculation: % of Total Flavonoids or Rutin equivalents in sample crude drug

\[
\% = \frac{\text{Conc. of sample by obtained by UV mg} \times \text{Extract obtained in mg} \times \frac{25\text{ml}}{\text{Wt. of extract taken mg} \times \text{Wt. of crude drug taken for extraction mg}}}{} \times 100
\]
The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure was repeated for the standard solution of rutin and the calibration line was construed. Based on the measured absorbance, the concentration of flavonoids was read (mg/ml) on the calibration line; then, the content of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract or crude drug) per gram of a sample on dry weight basis.

3.5.2.4 Physiochemical Analysis (According to Ayurvedic Pharmacopoeia of India)

3.5.3.1. Determination of Moisture Content (Loss on Drying)

Procedure: For determining the amount of volatile matter (i.e., water drying off from the plant). For substances appearing to contain water as the only volatile constituent, the procedure given below, was appropriately used. About 10g of aerial parts/leaves powder (without preliminary drying) after accurately weighing (accurately weighed to within 0.01 g) it in a tared evaporating dishes. Avoid the use of high speed mills in preparing the samples, and exercise care that no appreciable amount of moisture is lost during preparation and that the portion taken is representative of the official sample. After placing the above said amount of the aerial parts/leaves in the tared evaporating dish, dry at 105°C for 5 hours, and weigh. Continue the drying and weighing at one hour interval until difference between two successive weighing corresponds to not more than 0.25 per cent. Constant weight is reached when two successive weighing after drying for 30 minutes and cooling for 30 minutes in a desiccator, show not more than 0.01 g difference.

3.5.3.2 Determination of Total Ash values

After incineration of crude drug, remaining residue is the ash content of the drug, which simply contains mostly inorganic salts, naturally occurring in drug or adhering to it or deliberately added to it as a form of adulteration. Ash content is used to determine quality and purity of a crude drug. Many a time, the crude drugs are admixed with various mineral substances like sand, soil, calcium oxalate, chalk powder or other drugs with different inorganic contents. For determination of total ash, the powdered drug is incinerated so as to burn out all organic matter. Ash value is a criterion to judge the identity or purity of crude drugs. Total ash usually consists of carbonates phosphates, silicates and silica.
**Procedure:** 2 gram of air dried powdered drug was accurately weighted and put in pre-weighted completely dried silica crucible and place in the muffle furnace at 550 °C 24 hours. After complete incinerations it was kept in desiccators. The weight of ash with Silica crucible was noted. Then the total ash was calculated in terms of percentage.

% Ash value calculated by the formula:

\[ \frac{2 \text{ gram powdered drug contain}}{100\text{ gram powdered drug contain}} = \frac{X}{2} = 50X \]

Where \( X \) = difference in the weight

### 3.5.3.3 Determination of total acid insoluble ash value

Ash contains inorganic radicals like phosphates, carbonates and silicates of sodium, potassium, magnesium, calcium oxalate, silica carbonate content of the crude drug which affects ‘total ash’. Such variable were then removed by treating with acid and then acid insoluble ash value was determined. Acid-insoluble ash which is a part total ash insoluble in diluted hydrochloric acid is also recommended for natural drugs. Adhering dirt and sand may be determined by acid-insoluble ash contain.

**Procedure:** Ash obtain after incineration was dissolved in the 10% 25 ml of the hydrochloric acid, heat for 5 minutes than solution was filtered with the ash less filter paper, after filtration filter paper made to neutralized by washing with the hot water. Took pre-weight of crucible and placed filter paper in the crucible incinerate at 550 °C till white ash obtain.

**Formula used for calculation:**

\[ \frac{2 \text{ gram powdered drug contain}}{100\text{ gram powdered drug contain}} = \frac{X}{2} = 50X \]

\( X \) = difference in the weight

### 3.5.3.4 Determination of extractive values

Extractive value is a measure of the content of the drug extracted by solvents. It is useful for the evaluation of crude drug as it gives idea about the nature of the chemical constituents present in a crude drug and is also useful for the estimation of chemical constituents, soluble in that particular solvent used for extraction. Extractive value can be water soluble, alcohol soluble and hexane soluble.
a) Water extractive:

**Procedure:** Air dried 2 gram accurately weighted powdered drug placed in 250 ml of conical flask, macerated 24 hr, with 100 ml of water as a solvent in duplicate with proper marking and kept aside for 24 hours with shaking frequently. After extraction process filters out and precociously for solvent loss and arranges 2-2 petri plate for each conical flask with proper coding. These plates were pre-weighted previously, pipette out 10 ml extract for each petri-plate from the respective conical flask. Evaporated to dryness on water bath and dried completely in an oven at 90°C. Kept in desiccators to cool, then percentage w/w of extractive with reference to air dried drug was calculated.

**Formula used for calculation:**

\[
\begin{align*}
10 \text{ ml of extract solution contain} &= X \text{ gram extract} \\
100 \text{ ml of extract solution contain} &= X \times 100/10 = 10X \text{ gram extract} \\
2 \text{ gram powdered drug contain} &= 10X \text{ gram extract} \\
100 \text{ gram powdered drug contain} &= 10X \times 100/2 \text{ gram extract} = 500X \%
\end{align*}
\]

Where, \( X \) = difference in pre weight and final weigh

b) Alcohol extractive:

**Procedure:** Air dried 2 gram accurately weighted powdered drug placed in 250 ml of conical flask, macerated 24 hr, with 100 ml of alcohol as a solvent in duplicate with proper marking and put on shaker for 6 hr for extraction allow to stand for 18 hr. After extraction processes filter out and precociously for solvent loss and arrange 2-2 petri plate for each conical flask with proper coding. These plates were pre-weighted previously, pipette out 10 ml extract for each petri plate from the respective conical flask and dried till the solvent loss. Steps of calculating the percentage w/w of extractive was followed similar to those mentioned in the previous procedure.

c) Hexane extractive:

**Procedure:** Air dried 2 gram accurately weighted powdered drug placed in 250 ml of conical flask, macerated 24 hr, with 100 ml of hexane as a solvent in duplicate with proper marking and put on shaker for 6 hr for extraction allow to stand for 18 hr. After extraction processes filter out and precociously for solvent loss and arrange 2-2 petri plate for each conical flask with proper coding. These plates were pre-weighted
previously, pipette out 10 ml extract for each petri plate from the respective conical flask and dried till the solvent loss. Steps of calculating the percentage w/w of extractive was followed similar to those mentioned in the previous procedure.

3.6 Antimicrobial assay

3.6.1 Extraction of essential oil

Aerial plant part materials (stems, leaves and flowers) were collected from Oregano sustaining natural habitat and dried in shade. The air dried plant samples were subjected to Clevenger Hydro-distillation for 4 hours, 1:10 with water to obtain oregano essential oil and to evaluate for their bioactive potential.

3.6.2 Growth & Maintenance of Test Micro-organism for Anti-microbial Studies

The in vitro anti-bacterial activities of the essential oils were evaluated against a total of six bacteria including four gram negative bacteria viz., Pseudomonas aeruginosa (MTCC-424), Aeromonas hydrophila ssp. hydrophila (MTCC-646), Klebsiella pneumonia (MTCC 3384), Escherichia coli (MTCC-443) and two gram positive, Bacillus subtilis (MTCC-441) and Streptomyces candidus subspp. Azaticus or Kitasatospora azatica (MTCC-703). The antifungal activity of the oil was performed against Candida albicans (MTCC-227). The test strains were purchased from the Institute of Microbial Technology (IMTECH), Chandigarh, India. MTCC (Microbial Technology Culture Collection) numbers represents the standard strain numbers assigned to these microorganisms. The bacteria were maintained on nutrient broth (NB) at 37°C and fungus was maintained on Malt yeast broth at 28°C.

Table I: Strains used for anti-bacterial and anti-fungal activity

<table>
<thead>
<tr>
<th>Strains</th>
<th>MTCC No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram Positive Bacterial Strain</td>
<td></td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>441</td>
</tr>
<tr>
<td>Streptomyces candidus subspp. Azaticus or Kitasatospora azatica</td>
<td>703</td>
</tr>
<tr>
<td>Gram Negative Bacterial Strain</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>424</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>443</td>
</tr>
<tr>
<td>Aeromonas hydrophila ssp. hydrophila</td>
<td>646</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>3384</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
</tr>
<tr>
<td>Candida albicans</td>
<td>227</td>
</tr>
</tbody>
</table>
3.6.3.1 Determination of zone of inhibition

The antimicrobial activity of the essential oils was investigated by the agar well diffusion method (Perez et al., 1990) using 24–48 h grown strains reseeded on nutrient broth (bacterial strains) and Malt yeast agar (fungal strains). The cultures were adjusted to $1 \times 10^6$ CFU/mL with sterile water. 100 µL of the suspensions were spread over nutrient agar and malt yeast plates to obtain uniform microbial growth. A well was prepared in the plates with the help of a cork-borer (0.85cm). 20 µl of the test compound was introduced into the well. The plates were incubated at 37 (24 h) and at 30 °C (48 h) for bacterial and fungal strains, respectively. Microbial growth was determined by measuring the diameter of zone of inhibition (ZOI). For each bacterial strain controls were maintained where pure solvents were used instead of test compound. The result was obtained by measuring the zone diameter. The experiment was done three times and the mean values with standard deviation are presented.

3.6.3.2 Determination of the minimum inhibitory concentration (MIC)

The performed agar dilution susceptibility test was based on modified methods of NCCLS (2000) and CLSI. To determine the minimum inhibitory concentration (MIC) of the potent oils a series of dilutions of each potent oil, ranging from 10–50 µl ml$^{-1}$ were prepared. In the agar-well diffusion technique, serial dilutions of the essential oils were prepared by diluting oil with DMSO to achieve a decreasing concentration range from 50 to 10 µL/mL using 100 µl of a suspension containing $1 \times 10^6$ CFU/ml of bacteria spread on nutrient agar plates, whereas the fungal strains were reseeded on Potato dextrose agar (PDA) plates. The wells were filled with 20 µl of essential oil solutions in the inoculated nutrient/malt yeast extract agar plates. The bacterial plates were incubated at 37±2 °C for 24–72 h., while fungal cultures were incubated at 30±2°C for 48 h. The MIC was defined as the lowest concentration of the oil inhibiting the visible growth of each bacterium on the agar plate so the least concentration of each essential oil showing a clear zone of inhibition was taken as the MIC. DMSO was used as the negative control. Ciprofloxacin (Cf), Nalidixic (Na) and Amikacin (Ak) were used as positive controls for bacteria and fungi, respectively. Each test was replicated three times.
3.7 Chemotaxonomic study (Identification of the elite and distinct chemotypes)

3.7.1 Extraction of Essential Oil

Aerial parts of all accessions of *Origanum vulgare* were harvested and collected from natural habitat and Field Gene Bank during the flowering stage. After collection all accessions are dried in shade (room temperature), subjected to Clevenger hydro-distillation up to 04 hours for extraction of essential oil (Clevenger, 1928). The essential oil were collected in borosil glass vials, added anhydrous Sodium sulphate (Na$_2$SO$_4$) and kept in refrigerator at 4° C up to chemical analysis.

3.7.2 Gas chromatography (GC)

The chemotypic study (quantitative analysis of chemical compositions) of the essential oil was carried out at NBPGR (ICAR), New Delhi and NBRI (CSIR), Lucknow, U.P. on a Perkin Elmer (clarus 500 model) gas chromatograph apparatus equipped with a flame ionization detector (FID) and factor four capillary column VF-5 (60 m x 0.32 mm I.D., film thickness 0.25 µm). The GC oven temperature was set at 60°C for 3 min and then programmed to rise from 60°C to 280°C at a rate of 3°C /min, and held isothermally for 3 min at 280°C. Nitrogen was used as carrier gas at a flow rate of 1 ml/min a split ratio of 1:40, an injection size of 0.05 µL, heat and the injector and detector temperatures were maintained at 290°C and 300°C respectively. Component identifications were made by comparison of their retention indices with those of authentic compounds or standards (MP Biomedical Ltd. India) used. The relative amounts of individual components were calculated based on GC peak areas without applying any correction factors.

3.8 Growth pattern, Agro-techniques, Conservation and Domestication strategies of elite and distinct Chemotypes

3.8.1 Growth Analysis

Growth is an irreversible increase in size, mass or number. For growth analysis, seeds of *Origanum vulgare* accessions were obtained from the Medium Term Storage (MTS), NBPGR, Regional Station, Niglat, Bhowali, Nainital and sown in the month of March in the Glass House of Regional Station, Niglat, Bhowali. Thirty days after the seedlings were transplanted in Random Block Design (RBD)
experimental field without any treatments (no manure, fertilizer inputs). Data on the leaf area and dry weight were recorded at 20 days intervals during crop growth.

The method of growth analysis involved the calculation of various mean rate changes in plant dry weights ($W_2$ and $W_1$) and leaf areas ($LA_2$ and $LA_1$) observed at two sampling periods ($T_2$ and $T_1$). The following growth parameters (Radford, 1967) were calculated as follows:

3.8.1.1 Absolute growth rate (AGR)

Absolute growth rate (AGR) is the total gain in height or weight by a plant within a specific time interval. It is generally expressed as cm/day in case of plant height and g/day in case of dry matter accumulation per plant (Wareing and Philips, 1981). It was computed by the following formula:

$$AGR = \frac{W_2 - W_1}{T_2 - T_1}$$

Where, $W_1$ & $W_2$ are total dry weight of plant at time $T_1$ & $T_2$ respectively.

3.8.1.2 Relative Growth Rate (RGR)

The relative growth rate indicates the dry weight increase in plant matter over a time interval in relation to the initial weight. It is a commonly used parameter to measure crop plant growth over time. It is generally expressed as g g⁻¹ day⁻¹.

$$RGR = \frac{\text{Log}_e W_2 - \text{Log}_e W_1}{T_2 - T_1}$$

Where, $W_2$ and $W_1$: Dry weight of plant (g) at time $T_2 – T_1$, respectively

$\text{Log}_e$: Logarithm to the base ‘e’

3.8.1.3 The Net Assimilation rate (NAR)

The net assimilation rate is the increase of total dry weight per unit time per unit leaf area. It defines the efficiency of biomass production specifically in terms of the amount of photosynthetic tissue (Gregory, 1926). It is expressed as mg cm⁻² d⁻¹. It is calculated using the following formula.

$$NAR = \frac{W_2 - W_1}{T_2 - T_1} \times \frac{\text{Log}_e LA_2 - \text{Log}_e LA_1}{LA_2 - LA_1}$$

Where, $LA_1$ & $LA_2$ represent the leaf area at $T_2 – T_1$ respectively.
3.8.1.4 Leaf Area Growth Rate (LRGR)

Leaf Area Growth Rate is the dry-matter accumulation per unit area per unit time in plants. Units are \( \text{cm}^2 \text{ cm}^{-2} \text{ day}^{-1} \). LRGR implies that on increasing proportion of the products of photosynthesis as time proceeds. It is calculated by following formula:

\[
LRGR = \frac{\log_e LA_2 - \log_e LA_1}{T_2 - T_1}
\]

3.8.1.5 Leaf area duration (LAD)

Leaf area duration is a measurement that expresses the magnitude and persistence of leaf area during the period of crop growth. It is expressed as \( \text{cm}^2 \text{ day}^{-1} \text{ plant}^{-1} \). It is calculated by following formula:

\[
LAD = \frac{(LA_2 + LA_1) \times (T_2 - T_1)}{2}
\]

3.8.2 Conservation, Domestication and Agro-technique strategies

For the conservation, domestication and agro-technique of the taxon following steps were taken:

3.8.2.1 Seed Biology

Seeds of Oregano were collected in small gunny bags or cotton bags in the month of September-October from all accessions or populations. Immediately after collection, seeds were dried at room temperature and kept in the Medium Term Storage (MTS) as a working collection.

3.8.2.1.1 Seed moisture content

Oven dry method i.e. 103°C for 17 hrs given by International Seed Testing Association (ISTA, 1999) was used to determine the percent moisture content of seeds and Evans (1972) was followed for the calculation.

\[
\text{Moisture Content (\%) = } \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100
\]

3.8.2.1.2 Viability test

The viability of seeds was calculated by following Moore (1962). In this method 25 seeds of all the accessions or populations were taken in triplicates and
soaked in 0.1% solution of TTC (2, 3, 5 triphenyl tetrazolium chloride) with pH 6.0 and incubated at 37°C for 24 hrs. Colour change was observed, red colour of embryo and cotyledons indicate viability while non-viable seeds do not attain any colour.

3.8.2.1.3 Seed germination

To examine the seed germinability in laboratory, seeds were sown in glass petri plates, lined with Whatman No. 1 filter paper and moistened with distilled water. In all the germination treatments, 3 replicates with 50 seeds in each replica were used. For germination, seeds were kept in a germinator (company name) and distilled water was used for moistened the seeds whenever necessary. Prior to the initiation of experiment, all the seeds were surface sterilized by 0.1% aqueous solution of HgCl₂ for two minutes followed by washing with distilled water 2-3 times. The similar process was repeated with ethanol before sowing to avoid microbial contamination. For all the treatments, the seeds germination percentage (SGP) was calculated by following International Seed Testing Association (ISTA, 1999) and other germination associate parameters were calculated by using following formulas:

a. Seeds germination percentage (SGP)

\[
\text{Germination \% (SGP) = } \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \times 100
\]

b. Mean germination Time (MGT)

Mean germination time was calculated by the formula given by (Ellis and Roberts, 1981).

\[
MGT = \frac{\text{n1 x d1 + n2 x d2 + n3 x d3 + - - - - - - - -}}{\text{Total number of days}}
\]

Where, \( n = \) number of germinated seed

\( d = \) number of days

c. Mean daily germination (MDG)

Mean daily germination can be calculated by the following formula given by (Czabator, 1962).

\[
\text{MDG} = \frac{\text{Total number of germinated seeds}}{\text{Total number of days}}
\]
3.8.3 Effect of chemical compounds on seed germination

The effect of Plant growth regulators and nitrogenous compounds on seed germination was determined and following compounds were used. For this purpose, 50 seeds were sown at 25±2°C fixed temperature with alternating photoperiod of 16/8 hrs. The seeds without any treatment served as control.

i. **Auxins:** To determine the effect of auxins on seed germination, the seeds were dipped in different concentrations (500 ppm, 250 ppm, 100 ppm, 50 ppm and 25 ppm) of auxins *viz.*., IAA, IBA and NAA for 24 hrs, 12 hrs, 06 hrs, 03 hrs and 01 hrs, followed by washing with distilled water 2-3 times.

ii. **Gibberellins:** To determine the effect of gibberellins on seed germination, the seeds were dipped in different concentrations (500 ppm, 250 ppm, 100 ppm, 50 ppm and 25 ppm) of GA$_3$ for 24 hrs, 12 hrs, 06 hrs, 03 hrs and 01 hrs, followed by washing with distilled water 2-3 times.

iii. **Nitrogenous compounds:** To determine the effect of nitrogenous compounds on seed germination, the seeds were dipped in (as per above mentioned concentration) Potassium nitrate (KNO$_3$) for 24 hrs, 12 hrs, 06 hrs, 03 hrs and 01 hrs, followed by washing with distilled water 2-3 times.

3.8.4 Agronomic Practices

Germplasm of *Origanum vulgare* collected from wild/natural habitat and planted at Field Gene Bank, NBPGRT, Regional Station, Bhowali, Nainital. The Field Gene Bank is situated at an elevation of 1450 m asl and surrounded by *Quercus, Pinus, Rhododendron, Myrica*, and many shrubs like *Berberis, Rubus* sp. etc. mixed forest. The land was deep dug up or ploughed twice or thrice until a fine tilth was obtained. After removing gravels and weeds, plain the field after than raised beds were prepared for nursery establishment. Experimental beds of 2 x 1.5 m area were prepared for each accessions of Oregano. Before plantation, these nursery beds were supplemented with organic fertilizers like Farm Yard Manure (FYM) and forest litter for seeing their effects on the survival, growth and yield. In order to study for agrotechniques and other related parameters the experiment was designed in Random Block Design (RBD).
3.8.5 Soil Profile of the Experimental Site

Before starting of the agronomic practices, examination of the soil profile of the experimental field site. Analysis of the soil profile (physical and chemical) by following standard procedure of Jackson (1958).

3.8.5.1 Soil Sampling and Analysis

Composite soil samples (250 g) were collected from all the sites from 0-10, 10-20 and 20-30 cm depths using 50 x 50 x 30cm monolith by following the method of Jackson (1967). The soil samples kept in air-tight polythene bags and brought to the laboratory for physical and chemical analysis. Soil pH, total organic carbon, total nitrogen, available phosphorus and potassium were determined by using standard procedure as outlined in Jackson (1958 and 1973) using the facilities available at the Soil Testing Laboratory, Uttarakhand Tea Board, Bhowali, Nainital.

3.8.5.2 Climate of the Experimental Site

Climatic condition of the experiment was observed by Computer Controlled Automatic Weather Station (Max-100m) at NBPGR, Regional Station, Bhowali, Nainital.

3.8.5.3 Selection of elite chemotypes for propagation

Germplasm of *Origanum vulgare* collected from natural habitat were analyzed by preliminary characterization, evaluation and chemo-taxonomic programme at field condition and *in-vitro* condition and selection of elite chemotypes for further conservation, domestication and agro-technique study.

3.8.5.4 Sowing time and methods of Sowing

The germplasm (seeds) of the Oregano were sown in the month of October – November in the low altitude and during in high altitude region inside poly house or green house. 50 to 100 no. of seeds or 20 to 30 newly sprouted soft or semi- wood (5-8 cm long aerial parts) cuttings or 5 to 10 no. of perennial sprouted roots cut (3 to 5 cm small pieces) were directly sown in poly bags (12 x 09 inch and 150 gauge). In the low and high altitude, development of nursery through stems cutting and sprouted root divisions in the month of March and April.

3.8.5.5 Best method and time of raising the plants

After the development of nursery in poly house or green house the seedlings of Oregano planted in raised beds of nursery in the month of March and April.
3.8.5.6 Optimum spacing for plantation

Row to row: 40, 50 and 60cm; Plant to plant: 20, 30 and 40cm.

3.8.5.7 Nutrient Application

Two types of organic fertilizers viz. Farm Yard Manure (FYM) and forest litter along with a control set (beds without fertilizers) were used to check their effect on the survival, growth and yield. These fertilizers were added in the beds @ 100 qt/ha at the time of bed preparation.

3.8.5.8 Water and Weed Management

At the time of nursery establishment the field was well irrigated to keep moist. During propagation through stem or root division cuttings, watering every 24 to 48 hours. At the time of transplanting in the field, 2 to 3 times irrigation required in a week. In summer 3 to 4 times per month and winter season 2-3 times per month. The beds were kept free from weeds manually. The green weed is used as green compost or litter in the cultivated field.

3.8.5.9 Harvesting and Post- harvest Management

To observe economic yield of the plants, aerial parts were harvested 2-3 times annually. Collected aerial parts in different harvesting periods, dried in shade and mild warm air which maintained its natural colour and fragrance. Complete dry leaves and flowering spike stored kept in a gunny bags and air tight bags in the form of whole leaves and powder form.

3.8.6 Data recording

To see the growth performance, survival rate, yield and other parameters were frequently recorded according to NBPGR (ICAR), Descriptor and Descriptor state. Observations were recorded in 10 days interval and pooled over two years.

3.8.7 Statistical Analysis

ANOVA and correlation between different phytosoiglcal study, morphological traits, bio-chemical and phyto-chemical variations and growth analysis of Oregano was calculated by using data analysis tool of Window 2007, STPR software (developed by Panttnagar University, Uttarakhand) and cluster analysis was done by using PAST software version 2.1 Ward methods.
HABITAT ASSESSMENT AND PHYTO-SOCIOTOLOGY