Chapter - 3

MATERIALS AND METHODS

The details of materials and methods used in the present study are given below under the separate headings.

3.1 Materials

The experimental materials used in the present investigations have selected two important medicinal plants, *Chamomile* (*Chamomile recutita*) and Safed musli (*Chlorophytum borivilianum*). The seeds as well as plant materials of these medicinal plants were obtained from the research farm of Bundelkhand University, Jhansi (U.P.). As well for intercropping, Chickpea (*Cicer arietinum*) with *chamomile* and bottle guard, pigeon pea and maize with safed musli were obtained from the farmers of Bundelkhand region.

3.2 Methods-

Experiment (A)

3.2.1. Layout plan for *Chamomile recutita*:

The all experiments were conducted at research farm Bundelkhand University, Jhansi, during rabbit season 2004 in unreplicated design. Intercropping of *Chamomile* with Chickpea (*Cicer arietinum*) has been done in a separate farm from the *Chamomile*
*recutita* itself used as control. Chickpea is also a rabbi crop so that this traditional crop was used for intercropping with *chamomile*.

### 3.2.2. Cultivation of *Chamomile recutita*:

For the cultivation of *Chamomile* the plot size was kept 5 ×10 meter containing two hundred rows with five meter long. Plant to plant distance was maintaining 30 cm and row to row distance was kept 50 cm. All the recommended agronomic and cultural practices were followed to raise a good crop (Figure- 9 a, b).

### 3.2.3. Intercropping (*Chamomile with Chickpea*):

Intercropping of *Chamomile* with chickpea taken for dual benefit, improved production of essential oil of *chamomile* and high yield of chickpea. Chickpea is a legume crop; it helps to fixing atmospheric nitrogen to make good condition of soil. This experiment conducted in 50 meter square plot which contain 200 rows at the distance 50 cm and plant to plant distance 30 cm. At the sowing time 100 rows were sowing in experiment plot in alternative row as Chickpea and *C. recutita*, respectively. The agronomical and cultural practices were flowed as in *chamomile* control. The data were recorded of *Chamomile recutita* in both experiments only but *Cicer aritinum* taken as intercrop for the extra benefit with *chamomile* as symbionts crop (Figure -10 a, b).
3.2.4. Common cultural practices (Chamomile recutita (control) and Intercropping Chamomile with Chick pea (experimental) :

Land Preparation

Chamomile is a shallow-rooted plant; therefore, deep plough was not required. The land was ploughed once followed by four cross-harrowing and leveled properly and flat beds of convenient size were made. At the time of the final harrowing, farmyard manure (FYM or compost) @ 20-25 t/h was applied and mixed well into the soil.

Cultivation:

Propagation

Chamomile was propagated through seeds. One kg of seeds were required to raise seedlings sufficient to plant one hectare of land are required.

Nursery rising

The nursery area was well prepared by repeated ploughing and brought to a fine tilth. Well-rotted FYM was applied to the soil, and nursery-beds of convenient sizes were prepared. As the seeds are small, the seeds were mixed with sand or fine soil in the ratio of 1:4 and sown in the nursery-beds. The nursery should be watered frequently. The seeds germinated in 15-20 days of sowing and the seedlings were ready for transplanting into the main field when they were 4 to 5 weeks old (Figure- 12).
**Transplanting**

In order to obtain the maximum flower and essential oil yield, the crop has to be planted at a spacing of 30 cm x 30 cm and row to row distance 50 cm (Figure- 13).

**Application of Fertilizer**

The crop responds well to the application of fertilizers. Hence, a balanced dose of N, P and K are recommended in order to obtain the maximum yield. Application of recommended for red sandy soils. However, a fertilizer dose of 80 kg N, 40 kg P$_2$O$_5$ and 20 kg K$_2$O/ha was considered optimum for a good crop.

**Irrigation**

When the crops are grown in normal soils, about 3-4 irrigations may be given during the entire crop period.

**Weeding and Intercultural**

About 3-4 weeding and hoeing were required for raising a good crop. Only one thorough weeding and hoeing one month after planting, was sufficient. The application of herbicides, 2, 4-D Sodium salt @ 1 to 1.5 kg/ha, 4 weeks after transplanting or Oxyflouren @ 0.6 kg/ha reported to control weeds.
**Pests and Diseases**

No serious pests or diseases were observed on this crop. However, black bean aphids (*Aphis febae*); and an insect, *Nysius* minor, which attacks flowers and cause their shedding, and a defoliating insect, *Antographis chryson*, were noticed on this crop. The black bean aphid was controlled by spraying Phosdrin at 0.1 % and Brevinyl at 0.5% concentration. *Nysius* minor was controlled by spraying the crop with Endosulphon (0.2%).

**Harvesting and Yield**

Flowering starts from the second fortnight of February and it continues till the middle of April. Fully-opened flowers were picked immediately, as delay in harvesting might be result in the shedding of seeds. The picking of individual flowers was done by hand. About 4-5 harvests were taken at 10-15 days' intervals. The harvests of flowers were maximum in the 3rd or 4th flush of flowering. (Figure- 14 a, b).

**Drying and Storage**

The freshly harvested flowers have a moisture content of 60 to 85%. Hence, their drying was required. For drying, the harvested flowers were spread in thin layers in the shade. The flowers are dried at a temperature ranging between 22°C to 24°C. While drying, the flowers were turned once or twice. Temperature-controlled dryers were used for drying the flowers in European countries (Figure -15 a and b).
The dried flowers were packed in moisture-proof containers and stored under moisture-free conditions, so as to avoid spoilage by insects and fungi.

3.2.5. Observations were recorded on following characters:

The ten randomly selected competitive plants in each plot were utilized for recording on the following yield contributing characters.

(i) **Plant height (cm).** The plant was measured at the time of maturity February to April in five years (2005, 2006, 2007, 2008 and 2009) from the surface of the soil to the tip of the main capitula.

(ii) **Branches of plants.** Total numbers of the branches of a plant were count at the time of maturity.

(iii) **Spread area (Sq cm).** Area covered by a plant at the time of maturity.

(iv) **Area of capitula.** Area calculated at the time of maturity.

(v) **Fresh flower weight capitula g/plant.** Weight of fresh flower capitula in grams at the time of maturity.

(vi) **Dry weight of capitula g/plant.** Weight of dry flower capitula in grams at the time of maturity.

(vii) **Oil content (%)**. Percentage of oil present in plant at the time of maturity.

(viii) **Oil yield g/plant.** Oil obtained in grams from a plant at the time of maturity.
3.3 Isolation of Chemical Constituents:

3.3.1. Isolation of essential oil from the capitula of *Chamomile recutita*:

*Chamomile recutita* flowers (capitula) and normal rich and their essential oil was extracted by hydro distillation technique using mini-Clevenger apparatus (Clevenger 1928). The essential oil was also extracted from developing *Chamomile* flower at regular 10 days interval. The volume of oil was measured and collected in small –Stoppard tubes containing anhydrous sodium sulphate to remove the trace of moisture. The oil density was determined to convert the volume yield in to their respective weight.

3.3.2 Gas Chromatography (GC) Analysis of Essential Oil:

3.3.2.1. GC Analysis of Major Constituents:

The essential oil samples were analyzed for the major constituents using gas liquid chromatography (GLC). The GLC analysis was performed using a perking Elmer (model 3920 B) apparatus equipped with FID (Flame Ionization detector) stainless steal column (2mx 3mm I. d.) packed with 10% FFAP (Free fatty acid phase) on chromosorb WAW (80-100mesh) . The operating condition was as follows: temperature isothermal at 165°C, injector and detector temperature 60°C and 220°C, respectively. Nitrogen was used as a carrier gas and its flow rate adjusted to 30 ml/min. The major constituents were identified by co-injecting authentic standards and quantified using a Varian integrator (model 4400). The quantitative value of the various oil constituents was compared from the relative percentage data.
3.3.2. GC Analysis of Minor Constituents:

GC analysis of *C. recutita* for minor constituents were carried out a Perkin Elmer 8500 gas chromatography equipped with FID (Flame Ionization detectors) Using BP-1 (Diethyl polysiloxane) capillary column (30m X 0.32 mm i.d. and 0.25 micron film thickness) and nitrogen was used as carrier gas at 7 psi inlet pressure. Temperature programming was performed from 60°C to 220°C at 5°C/min and split ratio was 1:80.

The identification of several oil constituents was done by comparison of the Kovats retention indices on BP -1 column (relative to C8 –C23 alkenes) with literature values, and peak enrichment on co-injection with authentic samples wherever possible. The peak area percentages were calculated on BP -1 column without use of correction factors.

3.4. Method Experiment (B):

3.4.1. Layout plan *Chlorophytum borivilianum*:

Another experiment was conducted at research farm Bundelkhand University Jhansi during kharif 2005-2009 on the same plot which is empty by the harvesting of experiment (A).

3.4.2. Cultivation of Safed musli (*C. borivilianum*) as control and also Maize, Bottle guard and Pigeon pea as intercrop:

For the cultivation of Safed musli and its intercrops the plot was taken in which size was kept 5×10 meter containing 200 rows with 5 meter long. Sowing plan of control
and intercrop are maintain 50 rows for control and other 50 rows for each inter crop with Safed musli i.e. maize, bottle guard and pigeon pea, respectively.

### 3.4.2.1. Cultivation of Safed musli (*C. borivilianum*)

At the time of sowing Safed musli plot contains 50 rows with 5 meter long and 50 cm distance between rows and also plant to plant distance maintain 30 cm (Figure- 16 a, b).

### 3.4.2.2. Intercropping of Safed musli (*C. borivilianum*) with Maize, Bottle guard and Pigeon pea:

The next 50 rows of plot use for the sowing of inter crop of maize as row to row distance 50 cm and plant to plant distance kept 30 cm. The again 50 rows sowed of bottle guard crop taken as intercrop with Safed musli containing 5 seeds of bottle guard sowed in 5 meter leaf length in each line. In the last 50 rows of experiment showed pigeon pea in the form of same manner as maize. All recommended agronomic and cultural practices followed of each crop and in each plot separately (Figure- 19, 20 and 21 a, b).
3.4.2.3. Common cultural practices Safed musli (control) and Intercropping Safed musli + Maize, Bottle guard and Pigeon pea (Experimental):

Land preparation

Land selected for Safed musli (C. borivilianum) cultivation was thoroughly prepared by several deep ploughing, disking and harrowing. Thorough land preparations are essential on accounts of two, firstly, to eradicate perennial weeds and secondary, to minimize seasonal weed population that make soil loose to facilitate root development. Depending upon the slope, soil texture and amount of rainfall, suitable sized beds were prepared. In high rainfall areas, planting on raised beds (10-15 cm high) was suggested for a quick drain of excess water. In seedbeds, provision of both irrigation and drainage channels was made. In sloppy lands, planting was generally done in flat beds (Figure - 17).

Propagation

Chlorophytum borivilianum was propagated by vegetative means; roots contained some portion of disc where buds were located. In vitro, micro propagation using stem disc was done successful. Micropropagation also helps in ensuring the production of uniform plants, thereby restricting the variation in commercial population and quality of roots, which, at present, are a major problem encountered with seed and root propagated materials (Figure-18).

Vegetative means

Vegetative propagation was done through fleshy roots which were produced and stored from previous year's crop. Depending upon the planting material requirement, whole or a
portion of bunches of root, after digging in March-April, were stored. If the requirement of planting material is high, the root bunches were stored at a cool and dry place where day temperature remain in the range of 25-30°C. These root bunches were stored till they start sprouting in the month of May- June. Sometimes sprouting starts as early as end of April, if the humidity in the store increases beyond 60-65%. Splitting of root bunches was also be done before spore setting of bud on crown. When the planting material was required in limited quantity, lengthy and thick roots (60-70% of the total weight of roots bunch) were removed from the disc with the help of sharp knife, and small fingers were left to remain with disc. Before planting, bunches were taken out from store, kept for few days at room temperature to allow the buds on the disc to sprout. Fingers containing some portion of disc were separated with the help of sharp blade/knife. Splitting of disc was done in such a way that each disc or sprout contains 1-3 fleshy roots and weighs about 3.5- 5 gram.

**Weeds control**

Generally two weeding of the crop were required to free it from the weeds, the first within 20-25 days of sowing and the other 10-25 days later. The fields must be kept free from weeds to obtain the proper yield for the crop.

**Fertilizers and pesticides required for the crop**

Apart from the farmyard manure (FYM) used during the preparation of fields, and apply NPK (50:40:40 kg. per acre) in the field after the first rain fall. The crops must be free from most of the pests and diseases. Sometimes water logging causes damage to tuber roots which can be cured by stopping further irrigation. For the control of illi and
other pests, one has to spray thrums 5 ml liter every month. This could be applied as a precautionary measure also. The fungus that attacks Safed musli “Fusarium” *Trichoderma viridi* was used to destroy the attack of this fungus.

**Harvesting of the crop**

After 3-3.5 months of sowing (in the month of Oct/Nov the leaves of safed musli started yellowing), subsequently they become dry and fall off and get detached from the tuber/disc. These were the maturity stage of the crop. When the crop attains this stage then it means the crop cycle are complete, hence the tubers were dug out (Figure- 22 a, b, c, d). During this time the moisture leveling the soil was also maintained. By Jan-Feb the skin of tubers matured and it turned to dark brown. These were the right time to dig out all tubers thus on attaining this stage; the tubers dug out.

After digging out the tubers they were properly washed. Of these tubers, the longer and healthy fingers were detached from the tubers, whereas, the smaller ones were kept to be used as planting material for the next season. The longer and healthy fingers already detached from the tubers were then taken for processing. During processing the skin of the fingers were peeled out with a stainless steel knife and then they were kept in the sun for drying. Within 3 days the fingers dry up. These were then packed in the polythene bags (Figure- 23).
3.4.2.4. Observations were recorded on following characters

The ten randomly selected competitive plants were utilized for recording of the data on the following yield contributing characters.

(i) **Number of leaves /plant:** The total number of leaves per plant was counted after inflorescence emergence five years (2005, 2006, 2007, 2008 and 2009).

(ii) **Leaf length (cm):** Five leaves were selected randomly on each selected plant. Their length was measured in centimeter.

(iii) **Leaf width (cm):** Maximum leaf width of the same leaves selected for leaf length was measured in centimeter.

(iv) **Number of well developed roots/plant:** Number of fully developed fleshy roots was counted.

(v) **Number under developed roots /plant:** The plants also had some exceptionally small fleshy roots which had less than one centimeter length. These roots were counted as under developed root were counted in each of the selected plant.

(vi) **Well length of well developed roots:** Length of all the well developed fleshy roots of each selected plant was recorded in centimeter and the average was worked out.

(vii) **Length of main roots:** Length of main fleshy root was measured in centimeter in each selected plant.

(viii) **Fresh roots weight /plant (g):** Fleshy roots obtained from each selected plant were weighed in grams.
(ix) **Dry roots weight/plant:** Fleshy roots of each selected plant were oven dried at 60°C till the weight become constant. This weight was recorded in grams.

(x) **Carbohydrate content (mg/100g):** Carbohydrate content was estimated by Anthrone method of Lindner (1944).

(xi) **Protein content (mg/100g):** Protein content was estimated by Nessler’s reagent method of Lindner (1944).

(xii) **Saponin content (mg/100g):** Estimation of saponin content was done by the method of Brik et al. (1975).

### 3.5. Estimation total water soluble carbohydrates by Anthrone method:

**Material:** 1- Standard glucose solution (10 mg per 100 ml of distilled water).
2-Safed musli powder 200 mg per 10 ml distilled water mixed a centrifuged.

**Reagents:** Anthrone-0.2% Solution in concentrated H<sub>2</sub>SO<sub>4</sub>.

**Procedure.** Prepare standard glucose solution at different concentrations in different test tubes. To this 4 ml of Anthrone regent was added. The test tubes (become hot) were placed in ice water. Now optical Density (O.D.) was recorded at 620 nm. At the same 1.0 ml of test solution (Safed musli) was also taken which gave green color.

**Precaution:** Unknown solution should be free from protection.

### 3.6 Estimation of total protein by Nessler’s reagent:

**Principle:** The conc. H<sub>2</sub>SO<sub>4</sub> in the presence of catalyst H<sub>2</sub>O<sub>2</sub>, break up the protein polypeptide chain into the form of NH<sub>3</sub> and CO<sub>2</sub>. The amounts of NH<sub>3</sub> were then
estimated by adding Nesller’s reagent and measuring the yellow color at 620 nm. The nitrogen then multiplied by value 6.25 to get crude protein.

**Reagent:** Conc. H$_2$SO$_4$, 3% H$_2$O$_2$, 10% sodium silicate, 10% NaOH and Nesller’s reagent.

**Procedure:** (a) Taken 100 mg dry sample in kjeldal flask and added 2.0 ml of conc. H$_2$SO$_4$ and digest for half an hour and cool. (b) Added 0.5 ml (5 drops) of 30 percent H$_2$O$_2$ and digest again till color disappears, if still color added 5 more drops and heated. (c) Made up 100 ml by adding distilled water. (d) Taken 5 ml aliquot in 50 ml volumetric flask. Added to this 2 ml 10% NaOH and 1 ml 10% sodium silicate and made up volume to 50 ml with distilled water. (e) To this (50 ml), added 1.6 ml of Nesller’s reagent color develops, read readings at 620 nm. For blank, taken conc. H$_2$SO$_4$.

3.7 Isolation of Saponin. (Estimation of saponin content by the method of Brik et al., 1975):

**Material:** Safed musli, (*Chlorophytum borivilianum*) powder, aqueous ethanol, ethyl acetate, chloroform, acetone etc.

**Procedure:** Taken 100g of dry powder of Safed musli (*Chlorophytum borivilianum*) and suspended in 500 ml 85% aqueous ethanol and kept for over night. Residue was reek traced with 85% ethanol and refluxed for half an hour. This procedure was repeated three times. All the extracts were combined and ethanol was removed by distillation. The soft extract remained was extracted 500 ml (60-80$^\circ$C) petroleum ether and refluxed for 30 minute, cooled, poured of the solvents. Remaining soft extract refluxed with 500 ml of
ethyl acetate followed by 500 ml of chloroform for 30 min successively. The organic solvents were removed from the separating funnel. The soft extracts were dissolved in 500 ml methanol, filtered and concentrate to 100 ml. The hot extract was added drop by drop to the 500 ml of acetone. A white colored precipitated was formed, filtered, dried and weighted till it gave constant weight. On drying it was collected as light brown powder. The nature of the saponin and its hydrolytic products are being studied for their identification.

3.8. Statistical Analysis:

The following statistical analysis was carried out as follows:

3.8.1 Mean:

The average value of each character was calculated by following formula

\[
\text{Mean} = \frac{\sum X}{N}
\]

Where \( \sum X \) = Summation of variables

\( N \) = Number of observations
Figure-12. Nursery of Chamomile recutita

Figure-13. Transplanting of Chamomile with chickpea (Intercropping)
Figure 14a, b. Plucking of Capitula of *Chamomile recutita*
Figure-15a, b. Shade dried of Capitula flower (*C. recutita*)
Figure-11a, b. Intercropping *Chamomile* with Chickpea
(a)

Figure-9a, b. Cultivation of *C. recutita* (control)

Figure-17. Bed preparation for Safed musli
Figure-18. Cutting of Tubers of Safed musli
Figure-16 a, b. Cultivation of Safed musli (control)
Figure 19a, b. Intercropping of Safed musli with maize
Figure-20a, b. Intercropping of Safed musli with bottle guard
Figure-2 a, b. Intercropping of Safed musli with pigeon pea
Figure-23. Dry Tubers of Safed musli
Figure-22 a, b, c, d. Different structures of Tubers of Safed Musli