CHAPTER-3

MATERIAL AND METHODOLOGY

Scope of Present Study:
The population boom shattered the balance indiscriminately and hunger & disease are the two vital problems threatening the survival of mankind. Again it results in the decline of availability of the per capita cultivated land. In the developing and developed nations, it is most obviously manifested. In India, between 1922 and 1981, these figures have dropped from 1.11 acres to 0.62 acres, suggesting a fall of 44%. Also 40% of medicines now prescribed world over contain chemicals derived from plants. Evidently, we make all our efforts to augment productivity of crops and medicinal plants in respect of both quality and quantity. Therefore, the use of plant growth regulators during commercial production helps to overcome the ever increasing demand of medicinal plants through increased their production.

A perusal of literature reveals that most of the work at the national and international level has been done on the effect of plant growth regulators (PGRs) on the growth aspects along with other physiological and biochemical processes of vegetable crops (fruit vegetables), which have an important role in the human dietary strategy by providing medicines, vitamins and minerals. Plant growth regulators (indoles) may be one of the most important factors to modify the production and biochemical status of the plants. Least work has been carried out regarding the effect of plant growth regulators (indoles) on Kalmegh, a medicinal plant. The present study will add some new aspects on the significance of plant growth regulators (indoles) in cultivation of different types of medicinal crops. This study will add knowledge and information for the scholars, teachers and farmers regarding
application of plant growth regulators (indoles) on medicinal crop Kalmegh; *Andrographis paniculata* (Burm. f.) Wall. ex Nees. It will also be helpful in determining the quality as well as better production of the Kalmegh; *Andrographis paniculata* (Burm. f.) Wall. ex Nees.

**The Study Area:**

The study area is located at Govt PG College, Sector 39, NOIDA, district Gautam Buddha Nagar, Uttar Pradesh, India. The latitude and longitude of NOIDA are 28°35'N and 77°20'E respectively, at an average elevation of 200m above Mean Sea Level and has a flat topography. It is bound on the west and south-west by the river Yamuna, on the north and north-west by Delhi and Ghaziabad and on the north-east, east and south-east by the river Hindon. NOIDA falls under the catchment area of Yamuna and is an old river bed of the same.

**Species Selected:**

In the present study medicinally important plant Kalmegh; *Andrographis paniculata* (Burm. f.) Wall. ex Nees was selected as experimental material. The seeds were procured from F.R.I., Dehradun, and N.B.P.G.R., New Delhi, respectively.

**Morphology:**

*Kalmegh; Andrographis paniculata* (Burm. f.) Wall. ex Nees commonly known as "King of Bitter", and in north-eastern India as *Maha-tita*, is an *Ayurveda* herb. It is known as *Kalmegh* or *Kalamegha*, meaning "dark cloud". It is also known as *Bhui-neem*, meaning "neem of the ground", it has a similar strong bitter taste as that of the large Neem tree (*Azadiracta indica*). In Malaysia, it is known as *Hempedu Bumi*, which literally means 'bile of earth' since it is one of the bitterest plants that are used in traditional medicine. The genus
*Andrographis* consists of 28 species of small annual herb mostly distributed in tropical Asia. Only a few species are medicinal, of which *A. paniculata* is the most popular. It is widely cultivated in southern Asia, Scandinavia, China and some parts of Europe.

*Andrographis paniculata*, is a member of family Acanthaceae. It is an annual herbaceous plant which grows erect to a height of 30–110 cm, stem acutely quadrangular, much branched. Leaves simple, opposite, lanceolate, glabrous, 2–12 cm long, 1–3 cm wide, apex acute, margin entire, slightly undulate, upper leaves often bractiform, petiole short. Leaves have no aroma. Inflorescence patent, terminal and axillary in panicle, 10–30 mm long, bract small, pedicel short. Calyx 5, polysepalous, small, linear. Corolla tube narrow, about 6 mm long; limb longer than the tube, bilabiate, upper lip oblong, white with a yellowish top, lower lip broadly cuneate, 3-lobed, white with violet markings. Stamens 2, inserted in the throat and far exerted; anther basally bearded. Superior ovary, 2-celled, style far exerted. Capsule erect, linear-oblong, 1–2 cm long and 2–5 mm wide, compressed, longitudinally furrowed on broad faces, acute at both ends, thinly glandular-hairy. Capsule contains several very small round seeds, yellowish brown. Flowering time in India is November - December. Propagation is by seeds, cuttings and layering stems. Plant seeds in spring and summer, germination may be in 10-30 days.

In India, it is cultivated as rainy season (Kharif) crop. Kalmegh can be grown on a variety of soil. In the natural habitat, it is found growing in clay to sandy loamy soil rich in organic matter is good for its growth and yield (Farooqui and Sreeramu 2001), Mulikela and Kaliangile, (1995).

*Andrographis paniculata* contains active principle andrographolide shown in Figure (i). It is a diterpenelactone with a very bitter taste and colourless crystalline in appearance. Other
active components include 14-deoxy-1, 12 didehydroandrographolide (andrographolide-D), Homoandrographolide, andrographan, andrographon, andrographosterin and stigma sterol. Besides these compounds, the chemicals isolated from leaves are diterpenoids viz-deoxyandrographolide, D-glucoside and neo-andrographolide (Wiming and Xiaotion, 1982). The leaves contain the highest amount of andrographolide (2.39%) while the seeds contain the lowest (Sharma et al., 1992). The roots contain Apigenin-7, 4’-di-O-methyl ether, andrographolide and a natural flavone, 5-hydroxy 7, 8, 2’, 3’-tetramethoxyflavone. It also contains a monohydroxy trimethyl flavone, andrographin and a dihydroxy-di-methoxyflavone, panicolin.

![Molecular structure of Andrographolide](image)

**Fig. (i) - Molecular structure of Andrographolide**

**Medicinal Uses:**

*Andrographis paniculata* extract is traditionally used as a medicine to treat different diseases in India, China and Southeast Asia including Malaysia. The leaves and roots have been traditionally used over the centuries in Asia and Europe as a folklore medicine for a wide variety of ailments or as herbal supplements for health. In traditional Chinese medicine, it is widely used to get rid of body heat, extract form for diseases of the throat, as in fevers and to dispel toxins from the body. *Cold infusion of the drug is mentioned in ‘Sushruta Samhita’*
for fever and liver disorders and recommended in ‘Charaka Samhita’ for treatment of Jaundice. It is also used in the treatment of Hepatitis-‘B’. The leaves contain the maximum active principle content while in the stem it is in lesser amount. According to ‘Ayurveda’ the plant is very useful in treatment of many type of diseases.

**Documental properties of Andrographis paniculata** -

Adaptogen (helps to normalise a physical function, depending on what the individual needs, e.g. it will lower high blood pressure, but raise low blood pressure), Antibacterial, Antibiotic, Analgesic (pain reliever), Anti-inflammatory (Shen *et al.*, 2002), Antioxidant, Anti-diabetic, Anti-acne, Anti-carcinogenic (activity against different types of cancer) (Zhou *et al.*, 2006) and leukemia, Anti-thrombotic (blood clot preventative), Anti-viral (Wiart *et al.*, 2005), Anti-microbial, Antiperiodic (counteracts periodic/intermittent diseases such as malaria) (Rahman *et al.*, 1999), Antipyretic (reduces fever, usually caused by multiple infections or toxins), Bitter tonic, Blood purifier, Cardio-protective (protects heart muscles), Choleretic (alters the properties and flow of bile), Digestive, Depurative (acts to clean and purify the body, particularly the blood), Expectorant (promotes mucus discharge from respiratory system), Hepato-protective (Trivedi and Rawal, 2001), Hypoglycemic (blood sugar reducer), Immuno-stimulant, Laxative, Prophylactic (helps prevent disease), Sedative, Thrombolytic (blood clot buster), Vermicidal (used to kill intestinal worms and helps support the intestines), antidiarrhoeal (Gupta *et al.*, 1990), antihuman immunodeficiency virus (HIV) (Calabrese *et al.*, 2000), immune stimulatory (Iruretagoyena *et al.*, 2005) and anti-snakebite activity. Diterpenoids and flavonoids are the main chemical constituents of *A. paniculata* which are believed to be responsible for the most biological activities of this plant.
Other reported therapeutic uses includes coughs, headaches, edema, pain conditions, muscular pain, arthritis, rheumatism, fibro myalgia, multiple sclerosis, depression, dysentery, cholera, candida, lupus, piles, fatigue, herpes, leprosy, loss of appetite, swollen lymph nodes and other lymphatic conditions, dyspepsia, dermatitis, eczema, burns, pneumonia, bronchitis, tuberculosis, chicken pox, mumps; sluggish liver, spleen, kidneys and adrenal glands; sleeplessness, vaginitis, and constipation. It has had use as a replacement for quinine in treatment of malaria (still a prevalent disease in many tropical and subtropical countries).

**Climate:**

The climatic conditions of NOIDA are average. The general annual rainfall is 732 mm with minimum and maximum temperature varying between 3°C and 45°C. The soil is sand and clay mixed (Khadar) and is suitable for growing seasonal vegetables, grains like wheat and cash crops like sugarcane.

**Seed sowing:**

Seeds were selected uniformly and surface sterilized with 0.1% Hgcl₂ for one minute, thoroughly rinsed with distilled water. The seeds were pre-soaked for 24 hour in various concentrations (25, 50 and 100 ppm) of IAA, IBA and IAA+IBA with a control set (seed pre-soaked in distilled water). After thorough washing with distilled water, seeds were placed in Petri dishes, each lined with moistened filter paper at room temperature for seed germination and seedling stage study. For further growth pattern study, seed sowing was carried out in the month of July (Kharif season) at Govt. P.G. College, Sector 39, Noida (U. P.). Seeds were sown in 1x1 meter plots in rows. Plant to plant distance was 10 cm and row to row distance
was 15 cm. Nearly 50 seeds were sown in each plot of 1x1 m$^2$. There were ten such type of plots designed and the plots were marked as T$_1$ (Control), T$_2$ (IAA 25 ppm), T$_3$ (IAA 50 ppm), T$_4$ (IAA 100 ppm), T$_5$ (IBA 25 ppm), T$_6$ (IBA 50 ppm), T$_7$ (IBA 100 ppm), T$_8$ (IAA+IBA 25 ppm), T$_9$ (IAA+IBA 50 ppm) and T$_{10}$ (IAA+IBA 100 ppm).

**Plant Growth Regulators (Indoles) Treatment:**

Plant growth regulators (IAA and IBA) spray solution of different concentration (25, 50 and 100 ppm) was prepared. IAA and IBA in powder/crystal form was dissolved in 2-5 drops of ethyl alcohol (C$_2$H$_5$OH) by light heating, then make up final volume of 1000 ml with distilled water. For IAA 25 ppm solution preparation, 25mg IAA was dissolved with some drops of ethyl alcohol by light heating and make up final volume of 1000 ml with distilled water. Various other concentrations of growth regulators (IAA and IBA), alone and in combinations were also prepared by the same method. Plant growth regulators treatment was given at a regular interval of crop growth. First time spray treatment was given after one month of seed sowing. The following spray treatment of plant growth regulators in various concentrations (25, 50 and 100 ppm) of IAA, IBA and IAA+IBA were given for T$_1$ (Control = sprayed with distilled water), T$_2$ (IAA 25 ppm), T$_3$ (IAA 50 ppm) and T$_4$ (IAA 100 ppm), T$_5$ (IBA 25 ppm), T$_6$ (IBA 50 ppm), T$_7$ (IBA 100 ppm), T$_8$ (IAA+IBA 25 ppm), T$_9$ (IAA+IBA 50 ppm) and T$_{10}$ (IAA+IBA 100 ppm) up to maturity of crop.

**Seed Germination and Seedling Growth:**

For the seed germination and seedling growth studies, seeds were selected uniformly and surface sterilized with 0.1% Hgcl$_2$ for one minute, thoroughly rinsed with distilled water. The
seeds were pre-soaked for 24 hour in various concentrations (25, 50 and 100 ppm) of IAA, IBA and IAA+IBA with a control set (seed pre-soaked in distilled water). After thorough washing with distilled water, seeds were placed in Petri dishes, each lined with moisted filter paper at room temperature (25°C±2°C). Germination percentage was calculated on the basis of radicle emergence at 2 mm in length and considered as germinated. After seed germination, eight-day’s old seedlings were dissected into epicotyl and radicle for growth measurement. Different growth parameters viz- length, fresh weight and dry weight were measured and compared with control. The averages of 5 seedlings were calculated with standard deviation (S.D.) and represented in the results. Germination percentage was calculated with the following formula.

\[
\text{Germination Percentage} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds plotted}} \times 100
\]

**Growth Pattern:**

After seedling emergence, plants of plots T₁ (distilled water sprayed), T₂, T₃, T₄, T₅, T₆, T₇, T₈, T₉ and T₁₀ were sprayed with various concentrations (25 ppm, 50 ppm and 100 ppm) of IAA, IBA and IAA+IBA at a regular interval of 30 days till maturity of crop. The samples for growth analysis were taken regularly at 30 days intervals after the seedling stage till maturity. For each study, 5 plants from each plot randomly were taken carefully without damaging the root system. The plants were transported to laboratory where they were washed in running water to remove the soil particles. The growth measurements were taken on parts per plant basis for the leaf, stem, root, flower and pod etc. on monthly basis (Kumar, 1981).
The mean values of 5 plants from each plot were calculated, represented the results with standard deviation and test of significance at 5% level with the help of SPSS 15.0 software.

**Biomass:**

**Dry Matter:**

For the estimation of biomass and productivity “short term harvested method” (Odum, 1960) has been applied in the present study. The samples were collected on the basis of morphological similarities and standing crop was estimated on per plant basis. 5 plants were selected and harvested from each plots at regular interval of 30 days. The different plant parts viz. root, stem, leaf, flower and fruits were separated at different stages of growth. The samples were dried at 80°C for 48 hours after fresh weight measurement and then weighed for dry matter content.

**Measurement of Water Content (%):**

Moisture percentage on dry weight basis of each plant component was calculated using fresh and dry weight as follows.

\[
\text{Water Content (\% on D.W. Basis)} = \frac{\text{Fresh Weight} - \text{Dry Weight}}{\text{Dry Weight}} \times 100
\]

**Net Productivity:**

Net productivity was calculated by subtracting the values of biomass from that of subsequent stage (Singh et al., 1975). Summation of underground and aboveground values represents net
productivity of each component. Total sum of values of each part gives the values for total net productivity for a growth season.

**Yield Attributing Parameter:**

Harvesting Index and Shelling Percentage were calculated as described by Francis *et al.*, (1978). Harvesting index shows the contribution of economically useful part over the total production.

1. **Harvest Index**

Harvest Index shows the contribution of economically useful part over total production.

\[
\text{HI} = \frac{\text{Economically important part (leaves, gm/plant)}}{\text{Total dry matter production (gm/plant)}} \times 100
\]

2. **Shelling Percentage (%) Seed**

It represents % seed production over fruit weight.

\[
\text{Shelling Percentage} = \frac{\text{Seed Dry Weight}}{\text{Total Fruit Dry Weight}} \times 100
\]

**Chemical Analysis:**

(a) **Chlorophylls:**

200 mg fresh, fully mature and expanded leaves were homogenized with 80 % acetone. The homogenate was centrifuged at 4000 rpm for 5 minutes and final volume of supernatant was made to 10 ml with 80 % acetone. The optical density of the extract was taken at 626, 645
and 663 nm with the help of spectrophotometer and the amount of chlorophyll a, chlorophyll b and protochlorophyll was calculated (Koski and Smith, 1948).

\[
\text{Chl-a (mg/l)} = 12.67 \times A_{663} - 2.65 \times A_{645} - 0.29 \times A_{626}
\]

\[
\text{Chl-b (mg/l)} = 23.60 \times A_{645} - 4.23 \times A_{663} - 0.33 \times A_{626}
\]

\[
\text{Protochlorophyl (mg/l)} = 29.60 \times A_{626} - 2.99 \times A_{663} - 6.75 \times A_{645}
\]

(b) **Nitrogen Estimation (Micro-Kjeldahl Method)**

Nitrogen has been estimated by Micro kjeldhal method proposed by (Piper, 1942). .250 mg oven dried powder of plant sample was digested with 5 ml of H₂SO₄ and 0.10 gm of catalyst mixture (CuSO₄, K₂SO₄ and Selenium di-oxide in ratio of 1: 8: 1). First heat the mixture gently, after some time raised the temperature till digests and became light green. After clearing the digest, mixture was transferred to a 100 ml volumetric flask and the final volume was made upto 100 ml. 10 ml of aliquot was mixed with 10 ml of 40% NaOH. Transfer this mixture into distillation unit. Ammonia was evolved and trapped in a 100 ml sterilized conical flask containing 5 ml of 2% boric acid and mix indicator. Mix indicator contains 6 ml methyl red solution (0.16% in 95% ethyl alcohol), 12 ml Bromocresol green (0.04% in distilled water) and 6 ml 95% ethyl alcohol. Distillation was done till 30 ml of distillate collected. From the collected distillate, titration process starts, till the end point (pink colour). The same process was followed for the blank which was prepared by 0.25 gm sucrose. Nitrogen percentage was calculated as follows:

\[
N \, (\%) = \frac{(T-B) \times 10 \times N \times 1.4}{S}
\]
Where:

T – Sample titration, ml standard acid consumed
B – Blank titration, ml standard acid consumed
N – Normality of hydrochloric acid, and
S – Weight of the plant material taken for digestion (g)

(c) Estimation of total crude protein content:

Total protein contents were estimated by using the method of Misra, (1968).

\[ \text{Protein} = \text{Nitrogen value} \times 6.25 \text{ mg/gm DW.} \]

Data Collection and Analysis:

Germination percentage was calculated on the basis of radicle emergence at 2 mm in length and considered as germinated. After seed germination, eight- day’s old seedlings were taken out from all the plots for the seedling stage studies. While for the later growth pattern studies, five plants were randomly selected from each plot at an interval of 30 days. The last sample was taken when the fruits were mature at an age of 150 days. Plants were carefully uprooted and washed thoroughly to make free from adhered soil in running tap water. These were kept in polythene bags to prevent evaporation of moisture and brought to laboratory for various studies. Data obtained for various aspects have been analyzed statistically.
Plate 1. The Study Area
The study area at Govt. P. G. College Noida Sec-39, U.P.