CHAPTER 3

MATERIALS AND METHODS

3.1 Collection of *Artemisia annua* Seeds.

CIMAP, Lucknow, has developed a new variety of *A. annua* ‘Jeevanraksha’ containing high levels of artemisinin (Kumar et al., 1999; Tondon et al., 2003). Two gram seeds of *Artemisia annua* ‘Jeevanraksha’ were procured from CIMAP for the process development under the present research work under Material Transfer Agreement.

3.2 Surface Sterilization and Germination of Seeds:

Seeds of *A. annua* ‘Jeevanraksha’ are very small in size (average size ~1.0mm). It is very difficult to handle these seeds and therefore, conventional methods of surface sterilization were not suitable. The desired number of seeds were immobilized/captured between two sterile Nitro-Cellulose Membranes (pore size 0.2 μm, diameter 45mm) using regular syringe assembly. The assembly was then flushed with three rounds of sterile distilled water (10 ml for each wash). It was followed by a wash of 0.1% Mercuric Chloride (HgCl₂) solution. This solution was allowed to remain in the syringe assembly for 10 min. It was then flushed with six to seven washes of sterile distilled water (10 ml for each wash). The assembly was disassembled and the membranes along with seeds were blotted on sterile filter paper. The pair of membrane was carefully opened so as to expose the seeds. They were then carefully inverted on the solid growth medium (Gamborg’s B₅ + 0.25% Phytagel) without any growth hormones in tissue culture jars.
This complete process was carried out on clean bench (Kirloskar Dyne). These vessels were further incubated at 15°C in dark condition till the germination of seeds. Seeds were germinated on Gamborg’s B5 medium without any plant hormone and > 95% germination was observed after 5-6 days of incubation at 18°C (Gamborg et al., 1968).

Individual seedlings of *A. annua* were then transferred to tubes containing B5 medium and 0.25% phytagel, a solidifying agent and incubated at 15°C, 13 hours light and 11 hours dark period. Temperature was gradually increased up 25°C within 2 weeks. Plants were grown well under these conditions. At the same time to find out the best medium for the germination of seeds of *Artemisia*, another two media i.e. Murashige Skoog medium (MS) and half strength MS were tested for germination percentage and time required for germination were noted as per Table 1 and Figure 1 (given in results and discussion).

### 3.3 Maintenance of Shoot Culture

During the experiments aseptic conditions were maintained by using UV Laminar Air flow cabinet. The working surface of laminar was cleaned with 70% ethanol and it was irradiated with UV for 20 min. After the formation of seedling the leaves, shoot tips/axillary buds were used as explants for the development of shoot culture. The MS media, half Strength MS and B5 medium were used with different combination of hormones for the optimization of suitable media for the shooting. The combination were

1. MS medium with BA-0.5mg/L and NAA-0.05 mg/L,
2. MS medium with BA-0.1mg/L and IAA- 1mg/L,
3. MS with BA-1mg/L and Kin-10mg/L,
4. Half Strength MS with BA-0.5mg/L and NAA-0.05 mg/L,
5. Gamborg’s B5 with BA-0.5mg/L and NAA-0.05
mg/L. Axillary buds obtained from shoot culture were used as explants (inoculum) for multiplication of shoot on optimized MS medium. Shoots were well developed along with initiation of callus. This callus was transferred on MS medium with same hormonal combination and concentration.

3.4 Establishment and Maintenance of Root Organ Culture

Root organ system is used for the production of many plant based secondary metabolites. In our study to establish root organ culture following procedure was carried out. For the initiation of roots, axillary buds/leaves were used as explants. More than one month old seedling was chosen for collecting the leaves. As they were cultivated in aseptic condition, surface sterilization of leaves was not done. To optimize the media and best hormonal combinations, we have tested two different media, half strength MS and B₅ with hormone combination IAA and IBA, NAA and KIN. Firstly, leaves and axillary buds were scratched at several location with the help of sterile scalpel and inoculated on half strength solid MS medium containing hormones IBA-0.2 mg/L and IAA-0.5 mg/L and NAA-1mg/L and KIN-0.1mg/L. It was incubated at 25°C in dark and observed for developments of roots. At the same time another set was maintained by using B₅ media and same hormonal combination and concentrations. Fully developed roots from leaf piece were obtained after incubation of 3 weeks at 25°C, 13hrs light and was taken to develop root organ system. The roots were separated aseptically from explants and nutrient medium sticking on the roots was removed by washing with sterile distilled water. Thereafter 1-2 cm long pieces of roots (200 mg fresh weight) were inoculated in 30 ml aliquots of half strength MS medium supplemented with IBA-0.2 mg/L and IAA-
0.5 mg/L in 150 ml Erlenmayer flask and were kept on gyratory shaker (Orbitek) for continuous agitation at 90 rpm and at 25°C in dark. Regular subculturing was performed on interval of 3 week for getting bulk mass.

3.5 Growth Kinetics of Roots in Shake Flask

Growth kinetics experiment was designed and carried out to find the doubling time of the roots as well as the exact period for next sub-culturing. To study growth kinetics, MS ½ medium with hormones in optimized concentration without phytagel was sterilized and 50ml was carefully added into pre-sterilized Erlenmayer flasks (250 ml) in aseptic condition. Root tips of 1-2 cm long (10 mg fresh weight) were inoculated and flasks were transferred to orbital shaker at 100 rpm and at 27°C in dark. The weight of the flask was measured before and after the inoculation, that could be used for calculation of amount of inoculums initially added. The weight of every flask was taken at the time of harvesting of biomass. The control flasks without inoculums were maintained to determine the weight loss due to evaporation of liquid media.

The specific growth rate of the roots in flask was calculated by using following mathematical model.

\[
\mu = \frac{ln X_t - ln X_0}{t_1 - t_0}
\]

Where,

- \(ln X_t\) --- Biomass at Time \(t_1\) (g)
- \(ln X_0\) --- Biomass at Time \(t_0\) (g)
- \(\mu\) --- Specific growth rate (day⁻¹)
The yield constant $Y_x/s$ was calculated by using the biomass produced

$$Y_x/s = \text{Biomass produced (g)} / \text{Sucrose utilized (g)}$$

The doubling time was calculated by formula,

$$\ln 2$$

$$td = \frac{\ln 2}{\mu}$$

Where,

$td$ -- Doubling time (day)

Flasks (3 No.) were removed every week and roots were harvested. After harvesting, roots were washed 3-4 times with sterile distilled water and then blotted dry on filter paper and weighed and fresh weight was recorded. They were then oven dried at 60-70°C till the weight became constant. The dried powders of shoot, root and callus were kept in airtight vial for further analysis. Wet weight and dry weight as well as concentration of glucose, sucrose and phosphorus was noted.

3.5.1 Estimation of Glucose

The concentration of residual substrate (glucose) in the media was measured by DNSA method (Miller, 1959), as follows:

1) 0.2 ml of liquid media was diluted in 9.8 ml of sterile distilled water.

2) In 1 ml of above mixture 0.5 ml of distilled water and 1.5 ml of DNSA solution was added. That mixture is then heated at 90°C for 15 min followed by addition of 0.5 ml of sodium potassium tartarate and cooling.
3) Optical density was measured at 575 nm.

### 3.5.2 Estimation of Sucrose

Sucrose concentration from media flask was measured by DNSA method.

1) 1ml of liquid media was diluted in 1.4 ml of sterile distilled water and 20 μl of concentrated HCl were added.

2) The mixture was heated at 90°C for 5 min to allow hydrolysis and 1.5 ml of DNSA solution was added in it. This mixture was then heated at 90°C for 15 min followed by addition of 0.5 ml of Sodium Potassium Tartarate and cooled immediately. Optical density was measured at 575 nm.

3) The calibration curve was generated to correlate the absorption with sucrose concentration at 540 nm by using Spectrophotometer (Shimandzu, Mumbai)

### 3.5.3 Estimation of Phosphorus

Phosphorus concentration from media was measured by using Fiske-Subbarao method published in the year 1929.

1) 0.2 ml of liquid media was diluted in 9.8ml of sterile distilled water.

2) 1 ml of above mixture was taken and 0.4 ml of 10 % Trichloro acetic acid, 0.2 ml of molybdate solution and 0.2 ml of Amino Naptho Sulfuric Acid (ANSA) and 4 ml distilled water was added.

3) It was kept for 5 min at room temperature and optical density was measured at 640 nm.
3.6 Optimization of efficient extraction procedure for Artemisinin - Solid phase extraction

3.6.1 Solid phase extraction of crude artemisinin

Solid phase extraction (SPE) technique is intended for quick, discriminatory sample preparation and purification prior to chromatographic analysis. SPE uses principles of liquid chromatography to control selectivity, provides the sample clean-up, recovery, and concentration necessary for accurate quantitative analysis. The extraction of artemisinin is carried out by using Kumar method (2004) (U.S. Patent for artemisinin isolation). In vitro generated Artemisia plant parts i.e. Shoot, Roots and Callus were firstly dried in oven at 60°C and fine powder was made by grinding with the help of mortar and pestle. Dry powder of root, shoot and callus sample were extracted in methanol as follows.

Different samples (500 mg of each root, shoot and callus) were added in 10 ml of methanol and mixture is sonicated for 30 min at pulse of 40 sec. Then the mixture was heated at 60°C for 1 hour. The methanol extract was concentrated and filtered through 0.2 μm syringe filter and filtrate allowed to evaporate still white needle shaped crystals were obtained. Sesquiterpenes were extracted from root organ culture in methanol and fractionated using LiChosep C18 (Reverse phase column) using solvent system Methanol: Water (75:25) and fractions were monitored at 280 nm. A fraction corresponding to artemisinin was separated in faction 1. The same procedure was carried out for shoot and callus culture.
3.6.2 Qualitative Detection of Artemisinin by using TLC

The qualitative analysis of artemisinin was done by using Kumar’s method (2004). Silica gel plate G60 (ready to use silica coated aluminum plates Himedia, Mumbai) was used and 20 µl of each sample was loaded. TLC was carried out by using ethylacetate: methanol: water (100:13.5:10) solvent system. Plate was air dried and observed under UV light.

3.6.3 Qualitative Detection of Artemisinin by using HPTLC

3.6.3.1 HPTLC Analysis

HPTLC analysis was carried out for detection of artemisinin from crude extract of root, shoot, and callus of A. annua. The protocol developed by CAMAG (Widmer et al., 2007) for HPTLC analysis of artemisinin was used. CAMAG (Muttenz, Switzerland) Laboratory was brought in to develop a reliable method of determining the content of artemisinin while the plant is growing and throughout the processing phase.

3.6.3.2 Preparation of Samples

HPTLC was carried out for eight different samples of in vitro cultivated A. annua viz. shoot extract, callus extract and roots extract. The HPTLC analysis is also carried out to find out the concentration of artemisinin root samples which were harvested at every week in growth kinetics experiment i.e. 0th day root sample (R1), 7th day root sample (R2), 14th day root sample (R3), 21th day root sample (R4), 28th days root sample (R5).
Studies on Production of Antimalarial Compound Artemisinin Through Root Organ Culture of Artemisia annua.

Powder of shoot, root and callus samples (500 mg of each) were mixed with 20 ml of methanol and extracted by sonication by using pulse of 40 for 30 min at room temperature and heating at 60°C for 1 hrs. Supernatant was filtered through 0.2 μ PTFE filters (Axiva) and was evaporated to get dried whitish/yellow crystals and then again redissolved in 1 ml methanol. After mixing and centrifugation, the supernatant was used as test solution for screening. For assay in the linear working range, the samples were diluted as necessary. The sample procedure was followed for the sample harvested from growth kinetics experiment i.e. 0th, 7th, 14th, 21th, and 28th days. The only difference was the amount taken for extraction was dry weight of sample harvested of each day.

3.6.3.3 Reference solutions and derivatizing reagent:

Two standard solutions were prepared. For screening, 0.12 mg of artemisinin (Sigma, USA) was dissolved in 1 ml of methanol (standard solution I of 120 ng/μl). This solution was diluted with methanol 1:10 (standard solution II of 10 ng/μl) to cover the linear working range. All solutions were stored at 6°C. Anisaldehyde reagent was prepared by adding 20 ml of acetic acid, 4 ml of sulfuric acid, and 2 ml of anisaldehyde to a mixture of 100 ml of ethanol with 80 ml of water.

3.6.3.4 Chromatography and Evaluation

The general SOP for HPTLC published by CAMAG (2007) were followed. Sample volumes of 2–10 μl were applied as 8 mm bands using the sprayon technique. HPTLC plates silica gel 60 F254 (Merck, Germany) were developed by using mobile phase cyclohexane, ethylacetate, acetic acid (20:10:1) over a distance of 70 mm from the...
lower edge of plate using a twin trough chamber, saturated for 20 min with 10 ml of mobile phase per trough. The developed plates were then dried with a hairdryer (cold air) for 5 min. For derivatization, the plate was immersed in the reagent for 1 min. After waiting for 1 min to allow complete absorption of the reagent, the plate was heated at 100°C for 12 min. The photograph of plate was captured manually with Nikkon camera. The quantitation of artemisinin was performed by densitometric evaluation in fluorescence mode at 520 nm with cut-off filter at 540 nm using a tungsten lamp. The size of the scanning slit was adjusted to 4.00 - 0.20 mm and the scanning speed to 20 mm/s at a data resolution of 100 mm/step.

Artemisinin standard were purchased from Sigma- Aldrich (USA). Chemicals were purchased from HiMedia (Mumbai, India) and Merck (Darmstadt, Germany). HPTLC plates Silica gel 60 F254 were manufactured by Merck. Solvents of practical grade were purchased from Qualigens and HiMedia and Merck. Chromatographic equipment (twin trough chamber 20-10 cm, immersion device, Automatic TLC Sampler 4, digital documentation system, TLC Scanner 3, winCATS 1.4.2 software, VideoScan 1.02 software) were made by CAMAG (Muttenz, Switzerland).

3.7 Testing of Biological Activity of *A. annua* Cell Culture

3.7.1 Determination of Antiproliferative Activity

Artemisinin is also reported to have antiproliferative activity against cancer cell lines. The crude extract derived from root organ culture, shoot culture as well as callus culture was tested for presence of anti-proliferative compounds using MTT assay against
Studies on Production of Antimalarial Compound Artemisinin Through Root Organ Culture of Artemisia annua.

breast cancer cell lines MCF-7 and HeLa. MCF-7 and HeLa cells were procured from National Centre for Cell Science, Pune and were maintained at 5% CO₂ at 37°C in CO₂ incubator using RPMI 1940 using 10% Fetal Bovine Serum (FBS).

3.7.1. MTT Assay Protocol

To carry out this assay MCF 7 and HeLa cell lines were used. 100 µl of cell suspension (7000-10000 cells/well) were added in each well of 96 well plate and it was incubated at 37°C, 5% CO₂ for 24 hrs. On next day 20 µl of methanol extract of each sample with concentration ranging from 0.2-1 mg/ml in DMSO were added and again incubated for 24 hrs at same incubation conditions. After incubation, 20 µl of MTT(5 mg/ml of distilled water) dye was added in each well and incubated for 3-4 hrs. Then 100 µl of DMSO was added and incubated for 1-2 hr to dissolve precipitate of formazan produced by viable cells. After complete dissolution of formazan blue, the absorbance was measured at 492 nm and 630 nm on Microplate Plate Reader (Robonik). The percentage of cell viability was calculated according to the equation described by Moongkarndi et al. (2004).

\[
\text{% of cell viability} = \left( \frac{\text{OD of treated cells}}{\text{OD of control cells}} \right) \times 100
\]

Anticancerous compound Trycolumus was used as standard for comparison and DMSO as negative control. The concentrations required for inhibition of 50 % of cell viability (IC₅₀) were calculated.
3.7.2 Evaluation of Antioxidant Potential of Root, Shoot and Callus Extract by DPPH Assay.

DPPH radical scavenging activity of *Artemisia* extract was carried out by the method developed by Brand-Williams *et al.* (1995). The Sample stock solution (1mg/ml) was prepared and diluted to final concentration of 500, 250, 100, 50µg/ml in methanol. Now 500 µl sample of each concentration in separate microcentrifuge tubes was taken and 1 ml of freshly prepared DPPH solution (20 mg/L) was added in each tube and allowed to react at room temperature in dark condition. After 30 min on incubation, the absorbance was measured at 517 nm and converted into antioxidant activity using the following formula:

\[
\text{DPPH radical scavenging activity (\%)} = \left[\frac{(A_{\text{control}} - A_{\text{extract}})}{A_{\text{control}}}\right] \times 100
\]

Methanol and plant extract was used as a blank while DPPH solutions and methanol was used as negative control. The positive control was DPPH solution plus Glutathion and β carotene samples. The IC\textsubscript{50} values were calculated by using nonlinear regression analysis of dose dependent curves. IC\textsubscript{50} value is the sample concentration required to scavenge 50 % of the DPPH free radicals (Mosquira *et al.*, 2007). The assay was performed in three sets.

3.7.3 Antimicrobial Screening

Disc diffusion method (Tepe *et al.*, 2004) was carried out to study the combined effect of crude extract of *A. annua* (stem, callus, root) against pathogenic bacteria: *Salmonella typhi, Pseudomonas sp, Klebsiella pneumoniae* and *E. coli* on Mullar and
Hinton (MH) agar plate. The overnight grown bacterial cultures were inoculated on the top of the solidified MH agar medium. The standard antibiotic discs (Std. disc containing specific concentration of antibiotics i.e. 30 µg) viz. Gentamycin, and Amoxicillin purchased from HiMedia and disc having only methanol (control) were used for comparison. To determine the effect crude extract of stem, root and callus of *A. annua* plant (60µg/disc) and Standard antibiotics) disc was placed on MH agar plates inoculated with test bacteria. 20µl of ethanol extract of each sample were used. The plates were incubated at 37°C for 24 hours. After incubation the zones of inhibition were measured and antibacterial activities were calculated from increased in the fold area of the zone. The assays were performed in triplicate.

### 3.8 Statistical Analysis

All the experiments were carried out in triplicates and data were expressed as mean ± SD. Statistical analysis was performed by using Students’s t-test. The 50% inhibitory concentration (IC₅₀) was calculated from the Prism dose-Response curve (Statistical program) obtained by plotting the percentage of inhibition versus the concentrations. Differences were considered significant at *P*≤0.001. All the statistical analysis was performed by using the software Graphpad Prizm 5 and Microsoft Office Excel 2007.