**Cocculus hirsutus** which is an important medicinal plant of Rajasthan, India has been selected for the present study.

### 2.1 PLANT MATERIAL

Plant material of *Cocculus hirsutus* was collected from the Kulish Smriti Van, Jaipur (Rajasthan). Explants such as nodal stem segments, shoot tips, leaves and seeds were used for experimental work and chemical analysis.

### 2.2 MICROPROPAGATION

#### (a) Glassware and Instruments

Measuring cylinders, Petri dishes, Borosil glassware, refrigerator, distillation unit, pipettes, test tubes, conical flask (100-1000 ml capacity), forceps, scalpel, non-absorbent cotton, pH meter, electronic balance, autoclave, laminar air flow bench etc. were used for all experiments. All the glassware were sterilized by keeping in an oven at 200°C for 4 hours, prior to use. All dissection instruments like forceps, scalpels were also sterilized by autoclaving at 15 pounds per square inch (15 psi) pressure for 20 minutes.

#### (b) Chemicals

Chemicals used for the preparation of the basal media contained inorganic salts, vitamins, growth regulators, carbon source and organic supplements (Table-2). All the chemicals used were pure grade laboratory chemicals. B.D.H. bacteriological grade agar (0.8 %) was used to solidify the media throughout the course of the experiments. Stock solution of major salts, minor salts, vitamins and growth
regulators were prepared aseptically in double distilled water and stored in a refrigerator in brown bottles. Auxins and cytokinins were initially dissolved in a few drops of 1N NaOH/ ethanol and 1N HCl respectively. Thereafter, final volume of each solution was prepared by adding sterilized distilled water.

(c) Media Preparation

The nutritional media has essential and optional components. The essential nutrients consist of inorganic salts, carbon energy sources and vitamins – a medium with these constituents will be referred to as basal medium. During the present research endeavour, various media were tried, such as, MS medium (Murashige and Skoog, 1962), B\textsubscript{5} medium (Gamborg et al., 1968) and MSB\textsubscript{5} medium (Modified MS). Different growth regulators such as auxins and cytokinins were added to the basal medium either singly or in various concentrations and combinations. All media compositions were used as such or with slight modification in their basal components as per requirement of the experiment.

For the sake of convenience, different stock solutions of mineral salts and other constituents were prepared. They were mixed in required volumes and the final volume of the medium was made up with distilled water after adding sucrose. Approximately 40 ml medium was dispensed in each flask and 20 ml in a test tube. pH of the medium was adjusted to 5.8 by adding 1N HCl or 1N NaOH. 0.8% agar (Bacteriological grade) was used as solidifying agent. For rooting purpose, highly pure phytagel (Sigma/Aldrich) 0.2% was used. The media were then heated to homogenize the agar and dispensed into
presterilized culture vessels. The culture vessels were then plugged using non-absorbent cotton. For sterilization, the medium was autoclaved at 15 psi for 15-20 minutes. It was then taken out from the autoclave and left to solidify.

(d) Selection of explant

Selection of proper explant is very important. The stem explant from plant had to be collected at the right stage, right age and during the right season. The idea behind this kind of selection was to raise maximum number of clonal propagules under *in vitro* conditions in a short time which otherwise takes a long time to propagate an equal number of plants by conventional methods.

(e) Sterilization

Shoot tips, nodal stem segments and leaves were collected and kept under running tap water for about half an hour. Subsequently these explants were cut into appropriate size. They were washed with a 2% detergent solution (Extran, E. Merck – a commercial grade detergent) followed by several rinses in sterile distilled water. The disinfected explants were surface sterilized by 0.05% mercuric chloride for 1-3 minutes followed by several rinses with distilled water to remove every trace of mercuric chloride. The surface disinfected explants were now ready for inoculation.

(f) Inoculation of the explant/aseptic manipulation

All the inoculations were carried out in a laminar air flow cabinet (Thermadyne/Kirloskar). The working bench of laminar air flow was swabbed with cotton dabbed in rectified spirit and then
irradiated with ultra violet light for 15-20 minutes before use. The stainless steel instruments and other items such as forceps, scalpel, scissor, coupling jar etc. were autoclaved prior to use. Petri plates, flasks containing distilled water were also autoclaved prior to use. After keeping all the required material for inoculation except the explant, in the laminar air flow cabinet, ultraviolet irradiation was given for 30-40 minutes. The surface disinfected explants were then inoculated into the culture vessels containing medium.

(g) Incubation

After inoculation, cultures were incubated in the growth room, which was provided with two air conditioners (Voltas split unit, 1.5 ton capacity) and temperature controller (Saveer, India) to regulate temperature and humidity of growth room at 26±2°C and 55±5 % respectively. Fluorescent tubes and incandescent bulbs were fitted in culture shelves to render constant high intensity of 2000-3000 lux. A photoperiod of 16 hours light and 8 hours of darkness was regulated with the help of timer (Sequential Timer Model R.).

(h) Observations

Explants were inoculated on the basal medium containing various levels of growth regulators or other growth adjuvants. Initially cultures were examined every day up to a week of inoculation and later weekly up to 4 weeks or more, photographed and the observations were recorded at various stages of responses.

(i) Sub-Culturing procedure

- **Callus**: Callus (with or without shoot buds) obtained during primary culture was transferred to fresh medium after 3-4 weeks
of culture in order to evaluate whether the callus showed any morphogenic response or continued to proliferate as such. For maintenance of stock callus rhizogenic and old callus (brown) were discarded in every passage and only regenerable callus pieces were used for further subculture on fresh medium at intervals of 3-4 weeks. The callus developed in the experiment was maintained upto six weeks and used for biochemical analyses. Regular data were recorded usually after 4 weeks of sub culturing.

➢ **Shoot buds:** Directly formed shoot buds were also subcultured on fresh medium containing various concentration of auxins and cytokinins as well as on the same medium on which they proliferated. Sub culturing was done to obtain normal, healthy and elongated shoots.

(j) **Methods used for rooting of plantlets**

The *in vitro* developed shoots were transferred to a medium containing various concentrations of auxin for development of proper root system. Light, temperature and humidity conditions were standardized.

(k) **Hardening and pot transfer**

All the regenerated plantlets were taken out carefully from the culture vessels. The roots were thoroughly washed with sterile distilled water to remove the adhering media traces and treated with dilute fungicide solution (Bavistin) for a few seconds. These plantlets were then transferred to a pot containing a sterile mixture of
vermiculite and soil (1:3) and covered with inverted glass beakers for 2 weeks to retain high humidity. After the removal of glass beakers, plantlets were brought to direct sunlight for their acclimatization under natural conditions. The plantlets were watered daily with few drops of half strength MS inorganic solution and later on alternating with distilled water. They were then transplanted in pots containing garden soil.

(I) **Histological procedure**

Shoot tip and nodal segment with multiple shoot buds and pieces of callus with shoot buds were kept in FAA overnight for killing of microorganism and fixation of materials and then preserved in 70% alcohol. The fixed material was passed through dehydration series and in TBA-xylol series (Johansan, 1940). Infiltration and embedding was done in paraffin wax. Finally, blocks were prepared in pure paraffin wax. Serial sections (10-12 µm) were cut with the help of rotatory microtome.

Sections were fixed on the slide with the help of 4% formalin and gum water. These slides were dipped in pure xylene for dissolution of wax and were passed through a down/up series of alcohol. Staining of the slides was done in 1% safranin and fast green. Finally, the sections were mounted in DPX. Serial sections were studied under the microscope in order to understand the origin of shoot buds and their successive development. Photographs were taken with the help of a Nikon stereo microscope with photographic attachments.
2.3 BIOCHEMICAL STUDIES

Biochemical studies were carried out under the following heads viz. preliminary phytochemical analysis using GC-MS and secondary metabolites *in vivo* (leaf) and *in vitro* (non-differentiating callus). Callus that was raised from nodal explants on MS medium supplemented with IAA (0.5 mg/l) in tissue culture experiments were used for all biochemical analyses.

The preliminary phytochemical studies included GC-MS analysis of dried powder of leaf by using standard methods.

Isolation, identification and quantification of bioactive ingredient i.e. β–sitosterol and gallic acid was carried out by IR, TLC and HPTLC *in vivo* and *in vitro*.

Details of the experimental materials and methods for the estimation and extraction of various metabolites are mentioned in the respective chapters.

2.4 ANTIOXIDANT ASSAY

Fresh plant samples (*C. hirsutus*: leaf and stem) were collected and washed individually under running tap water to remove soil particles and other dirt. Furthermore, *in vitro* callus obtained on MS medium fortified with IAA (0.5 mg/l) was also taken for the present study. The *in vivo* leaf and stem were dried in the laboratory at room temperature for 7 days while the callus was dried at 60°C for 2 days in an oven. All dried samples were ground well into a fine powder in a mixer grinder.
Preparation of Extracts

The above powered materials were soxhlet extracted with methanol for 72 hours. At the end of extraction each extract was passed through Whatman No.1 filter paper and evaporated under vacuum. All extracts were stored at 4°C in a refrigerator until used for further analysis.

Evaluation of Antioxidant activity

DPPH assay

The antioxidant activity of the different plant parts was determined on the basis of their scavenging activity of the stable 2, 2-diphenyl-2-picryl hydrazyl (DPPH) free radical. DPPH is a stable free radical containing an odd electron in its structure and usually utilized for detection of the radical scavenging activity in chemical analysis. 1ml of each solution of different concentrations (10-1000 µg/ml) of the extracts was added to 2 ml of 0.002% methanolic DPPH free radical solution. After 30 minutes the absorbance of the preparations were taken at 517 nm by a UV spectrophotometer which was compared with the corresponding absorbance of standard ascorbic acid concentrations (10-1000 µg/ml). The method described by Hatano et al., (1988) was used to measure the absorbance with some modifications. Then the % inhibition was calculated by the following equation:

\[
% \text{radical scavenging activity} = \left( \frac{\text{absorbance of blank} - \text{absorbance of sample}}{\text{absorbance of blank}} \right) \times 100
\]
From calibration curves, obtained from different concentrations of the extract, the IC$_{50}$ (Inhibitory concentration 50%) was determined. IC$_{50}$ value is inversely proportional to the antioxidant potential. IC$_{50}$ value denotes the concentration of sample required to scavenge 50% of the DPPH free radicals (Gupta et al., 2003).

2.5 ANTIMICROBIAL ASSAY

Fresh plant materials of *Cocculus hirsutus* were collected from the wild regions of Jaipur (Rajasthan). The leaves and stem were then separated and carefully washed with tap water, rinsed with distilled water, and air-dried for 1 hr. Then the leaves and stem were shade dried in room temperature for one week. Similarly the callus was shade dried at room temperature for one week. Then they were ground into powder and subjected to extraction with different solvents.

**Preparation of plant extract**

The finely ground leaves, stem and callus were extracted with methanol following the method of Eloff, (1998). The extracts were collected in different conical flasks and the same was repeated thrice to attain maximum extraction. Then the solvents were evaporated and condensed to concentrate the extracts obtained. The concentrated residues were weighed and re-dissolved in respective solvents to yield 10 mg/ml solutions for further analysis.

**Antibacterial activity**

The crude extracts were subjected to antibacterial screening against (1) *Zymomonas mobilis* (MTCC 88), (2) *Staphylococcus epidermidis* (MTCC 3615), (3) *Staphylococcus aureus* (MTCC 3160),
(4) Micromonospora sp. (MTCC 3296) and antifungal screening against (1) Alternaria solani (MTCC 2101), Fusarium culmorum (MTCC 349), Phanerochaete chrysosporium (MTCC 787) and Penicillium chrysogenum (MTCC 161).

**Well diffusion assay**

Nutrient agar was prepared and poured in the petridish. 24 hrs old-broth cultures of respective bacteria and fungi were swabbed on it. The wells (6 mm diameter) were made on the agar surface by using cork borer and different concentrations of the crude extract were loaded in the wells. The plates were then incubated at 37ºC for 24 hrs. The inhibition diameter was then measured (Fazeli *et al.*, 2007) and MIC value was calculated.

**2.6 RAPD ANALYSIS**

**DNA extraction and PCR amplification**

To test the clonal fidelity, the *in vitro* raised plantlets at various stages of subculture were chosen randomly. These *in vitro* grown plants were compared with the mother plant from which explants were taken. Total genomic DNA of the mother plant and *in vitro* raised plants was extracted from young leaf tissue by using modified Cetyl Trimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1990). Twenty RAPD primers (Genei, Bangalore, India) were used for initial screening. PCR amplifications were carried out in total volume of 25 µl containing 2 µl (20 to 25 ng) of genomic DNA. The reaction buffer for RAPD consisted of 2.5 µl Taq buffer, 0.5 µl MgCl₂, 0.2 µl dNTPs, 1.5 µl primer, 0.17 µl Taq polymerase and 18.13 µl water.
PCR amplification consisted of initial denaturation at 94°C for 5 min, followed by 45 cycles of 1 min denaturation at 94°C, 1 min annealing at 37°C and 2 min extension at 72°C, with a final extension at 72°C for 7 min. The amplified products were resolved by electrophoresis on 1.8% agarose gel in tris-borate EDTA (TBE) buffer stained with ethidium bromide. The fragment sizes were estimated with Lambda DNA/ Eco RI + Hind III marker (Bangalore Genei Pvt. Ltd, Bangalore, India).

Only the most intense and reproducible DNA bands were considered for analysis. They were scored as 1 (for presence) and 0 (for absence). The genetic associations were evaluated by calculating the Jaccard’s similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to the cluster analysis of unweighted pair group method with arithmetic averages (UPGMA) and a dendrogram was generated by using NTSYS-pc version 2.1 software (Rohlf, 2000).

2.7 DATA ANALYSIS

All the experiments were repeated thrice and five replicates per experiment were taken. Suitable control was maintained with each experiment. The cultures were regularly subcultured on a fresh medium usually at 4 weeks interval and observations were recorded after 5, 10, 20 and 30 days of inoculation. In certain specific cases, the period of observation was further extended for 8-10 weeks. The data represent the mean ± S.E. All data were subjected to the following statistical analysis.
2.8 STANDARD DEVIATION (S.D.)

This measure of dispersion was calculated by squaring the deviation of each observation from the mean, adding the square, dividing by number of observation and extracting the square root according to the formula.

\[ \sigma = \sqrt{\frac{\sum d^2}{N}} \]

Here,

\( \sigma \) = Standard deviation  
\( N \) = Number of observations  
\( \sum d^2 \) = Summation of square of deviation of each observation from the mean.

2.9 STANDARD ERROR (S.E.)

S.E. was calculated by dividing the S.D. by square root of the number of observations according to formula.

\[ S.E. = \frac{\sigma}{\sqrt{N}} \]