CHAPTER III

# MATERIAL AND METHODS

# 3. Material and Methods

## 3.1. Experimental Plant Material

Experimental plant material selected for the present investigation was *Centella asiatica* (L.) Urban (Fig.3.1). It belongs to the family Apiaceae (Formerly - Umbelliferae). It's systematic position is shown in table-3.1.

It is popularly known as Brahmi in Marathi and Mandookaparni in Hindi. *Centella asiatica* mother plants were obtained from Dhanwantari Udyan (Medicinal Garden) of Mahatma Phule Krishi Vidyapeeth (MPKV) Rahuri, District Ahmednagar, Maharashtra state, India. Experiments were carried out in the experimental fields of Padmshree Vikhe Patil College of Arts, Science and Commerce, Pravaranagar (Loni, MS) during of the years, 2009 to 2012.

## 3.2. Propagation and Cultivation

Several plant cuttings of randomly sampled individual plants of *C. asiatica* were collected from same population from Medicinal Garden (Dhanwantari Udyan) of MPKV, Rahuri, Maharashtra. The cutting of plantlets were more or less uniform size initially planted on four seed beds of 4 X 8 m size containing mixture of fertile soil, black soil and vermicompost in green house (Fig.3.2). The temperature and relative humidity were maintained of 29 ± $10^0$C and 50% respectively in partial shade conditions. They were watered once daily by sprinkler and allowed to multiply for about three months during which period the plants were also acclimatized to the new environment. After 2-3

**Table 3.1. Systematic Position of the *Centella asiatica* (L) Urban**

| | |
|---|---|
| Division | Phenerogams |
| Class | Dicotyledonae |
| Subclass | Polypetalae |
| Order | Umbellales |
| Family | Umbelliferae (Apiaceae) |
| Genus | *Centella* |
| Species | *asiatica* L. Urban. |

Fig.3.1.External Morphology of *Centella asiatica*

months acquiring minimum required number, some plants were transferred to experimental plots in the same green house of Padmashri Vikhe Patil College, Pravaranagar during August, 2009. After such plants served as a field propagation and they were used directly as experimental plant material for the treatment with spindle poisons and in tissue culture experiment.

Although the *Centella asiatica* plants growth is favored by good quality of soil with high water holding capacity and organic matter, black soil was used for cultivation. The land was prepared by plowing to a depth of about 20cm and producing a good friable soil condition. Organic materials such as cow manure or leaf compost were added at planting. Apart from organic manures, fertilizers were rarely applied, except for relatively small amounts of urea, which is periodically added to facilitate leaf growth. The plants were planted at 30 X 25 cm spacing between plant to plant and row to row. All the cultivated plants were watered twice a day during the first week, and later they were watered twice a week.

## 3.3 Methods Employed

The present investigation was aimed at the induction of new genotypes of *Centella asiatica* for the selection of elite lines and that are capable of producing high quantities of saponins. It was used for the plant material production in the

herbal industry. Therefore, two different methods were employed to achieve the objective. They were 1. Induction of autotetraploidy and 2. Inductions of somaclonal variations. Details about methodologies employed are explained below.

Fig.3.2.Field View of *Centella asiatica* (L) Urban.

### 3.3.1. Induction of Autotetraploidy

To obtain autotetraploidy plants of *Centella asiatica*, the plants were subjected to colchicine treatment for different concentration and different time intervals. They are describing in below.

#### 3.3.1.1.Chemical agent for Induction of Autotetraploidy

Nowadays, most common chemical agent used for obtaining polyploid plant is colchicine. Colchicine is an alkaloid extracted from the corm and seeds of *Colchicum autumnale*, an autumn-flowering crocus like plant (Eigsti and Dustin, 1954). It is the best spindle poison widely used for arresting cell division at metaphase in plant and animal cells (Sharma and Sharma, 1980). The present study, colchicine ($C_{22}H_{25}NO_6$, MW.399.45) of Sigma, India make was used. Different concentrations of colchicine viz. 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0% were tried for their efficacy in inducing autotetraploidy in *Centella*. For preparation of different concentrations of colchicine solutions, colchicine powder of appropriate weights were measured on electronic weighing balance and

dissolved in 100 ml distilled water taken in labeled stopper bottles and prepared solution were labeled and stored in a refrigerator until their use.

### 3.3.1.2. Method for Induction of Autotetraploidy

The experiments were conducted in order to inducing autotetraploidy in *Centella asiatica*. Six months old, field grown and acclimatized plants of *Centella* were used for the experiments These plants were treated with various concentrations of colchicine solution from 0.1% to 1.0% with 0.1 % interval and various time intervals from 1hr. to 8hr with 1hr interval at an environmental condition. Ten experimental plants running shoots were selected for the colchicine treatment. Before treatment running shoots were washed with water to remove dirt. Later they were treated with different concentrations of the test solutions (spindle poisons), for varying time intervals, as mentioned above. This was repeated with plants containing running tips at various stages of growth and lengths. Uniform watering was done to all treated and control experimental plants Following methods were used in the induction of autotetraploids for selected running tips, the methodology suggested by Dalve, (2007) was followed.

1. Immersion of running shoot tips, directly in the test solutions (of different concentrations) for varying time intervals,

2. Injection of varying concentrations of colchicine solution, directly into the stolons of the plant,

3. Treatment of artificially injured shoot tips with colchicine,

All above methods were implemented with all the concentrations of colchicine for different time intervals. All 10 test solutions with different time intervals were repeated for several times, until plants showed change in their morphological characters. After treatment, the running shoots were thoroughly washed with distilled water, to remove traces of the test solution. All the treated plants were labeled properly, indicating the concentration of the test solution and duration of treatment. As soon as the nodal region, proximal to the treated running bud, produced new roots, the bud along with the rooted node was excised from the mother plant to serve as treated plants. Similar untreated running buds with

proximal nodes containing roots were also excised from the same mother plant to serve as controls. Such separated treated and untreated plants were cultivated separately in experimental plots, with proper labeling. Treated and control plants were maintained in the same greenhouse and allowed to grow in uniform light, air, water, soil and manure.

Fig.3.3. *Centella* plants showing running shoot tips (arrows) which were treated with colchicine, following the method of Immersion.

### 3.3.2. Induction of Somaclonal Variations

The leaf and nodal explants of *Centella asiatica* were used for induction of somaclonal variation. Earlier work as per the recommendations of Karthikeyan *et al*., (2009), Raghu *et al*., (2007), Aziz *et al*., (2007), Mukundan *et al*., (2004), Nath and Buragohain (2005), Tiwari *et al*., (2000) and Banerjee *al*., (1999) was reported in this species. Murashige and Skooge (1962) medium, supplemented with different concentrations and combinations of auxins and cytokinins were used as a nutrient medium.

#### 3.3.2.1. Preparation of Nutrient Medium

The nutrient medium used was essentially the MS basal medium as suggested by Murashige and Skooge (1962), supplemented with auxins and

cytokinins. The composition of the basal medium is given in table 3.2. nutrient media preparation was referred by Dalve, (2007). The nutrient media preparation is briefly explained below.

This medium was prepared by dissolving appropriate volumes of stock solutions to make 1 liter with double distilled water. For preparation of stock solutions of MS basal medium, all the essential components were weighed on electronic weighing balance. All the chemical compounds were grouped as per the requirement and named as macro and micronutrients. Macronutrients were divided into two stocks of 5X concentration; Stock-A and Stock-B. Similarly micronutrients were divided in to two stocks, stock-C of 20 X concentration and stock-D of 100X concentration. In addition, a 10X concentrated stock-E of vitamin source was also prepared. All the stock solutions were of 100 ml in volume (Table. 3.2). The basal medium was supplemented with different combinations and concentrations of auxins and cytokinins. Steps involved in preparation of medium and supplementation of components of growth hormones are explained below.

### 3.3.2.2. Plant Growth Hormones

Initially response of the plant material was studied for the different concentrations of the different growth hormones (Auxins and Cytokinins) were prepared for supplementation to the MS basal nutrient medium. The present study, six growth hormones, viz., Indol 3-butyric acid (IBA), Indol- 3-acetic acid (IAA), α-napthalene acetic acid (NAA), 2-4 D (2-4 dichlorophenoxy acetic acid), BAP (N-benzylaminopurine) and Kintein (6-furfurylamino purine) were used.

The combinations of the MS media supplemented with hormones were MS + different concentrations of Auxins, MS + Different concentrations of Cytokinins and MS + Auxins + Cytokinins. Auxin includes IAA, IBA, NAA, 2-4D, whereas the Cytokinins BAP and Kinetin. The concentrations of stock solutions of the

| Table. 3.2. Nutritional components of MS medium | | | | |
|---|---|---|---|---|
| **Sr. No.** | **Constituents** | **Formula** | **Stock mg/100ml** | **Stock taken** |
| **1.** | **Macronutrients (5x concentration )** | | | |
| a) | Ammonium nitrate | $NH_4NO_3$ | 8250 mg | 20 ml |
| b) | Potassium nitrate | $KNO_3$ | 9500 mg | |
| c) | Magnesium sulphate | $MgSO_4 7H_2O$ | 1850 mg | |
| d) | Potassium dihydrogen ortho phosphate | $KH_2PO_4$ | 850 mg | |
| **2.** | **Macronutrients (5x concentration )** | | | |
| a) | Calcium chloride | $Cacl_2, H_2O$ | 2200 mg | 20 ml |
| **3.** | **Micro nutrients (20 x concentration )** | | | |
| a) | Sodium EDTA | $Na_2EDTA$ | 752 mg | 5ml |
| b) | Ferrous sulphate | $FeSO_4 7H_2O$ | 556 mg | |
| **4.** | **Micro nutrients (100 x concentration)** | | | |
| a) | Boric acid | $H_3BO_3$ | 620 mg | 1ml |
| b) | Manganese sulphate | $MNSO_4 7H_2O$ | 2230 mg | |
| c) | Zinc sulphate | $ZNSO_4 7H_2O$ | 860 mg | |
| d) | Sodium Molydate | $NA_2MOO_4 2H_2O$ | 25 mg | |
| e) | Cupric sulphate | $CuSO_4 2H_2O$ | 2.5 mg | |
| f) | Cobalt Chloride | $CoCl_2 6H_2O$ | 2.5 mg | |
| g) | Potassium iodide | KI | 83 mg | |
| **5.** | **Vitamin (10 x concentration )** | | | |
| a) | Glycine | | 20 mg | 10 ml |
| b) | Nicotinic acid | | 5mg | |
| c) | Pyridoxine HCL | | 5 mg | |
| d) | Thiamine HCL | | 2 mg | |
| e) | Myomositol | | 1000 mg | |

auxins and cytokinins were prepared by using weighed on an electronic weighing balance. Then, they used in the preparation of the media and stored under refrigeration. Auxins were normally dissolved in 1 N KOH and cytokinins in 0.1N HCL before making up the final volume of 100 ml with distilled water.

### 3.3.2.3. Supplementation of Nutrient Medium with Different Growth Hormones

The MS basal medium was supplemented with various concentrations of plant growth hormones, auxins and cytokinins individually or in combination, were used for *in vitro* culture *Centella*. For this, appropriate quantity of growth hormone was added to 1 liter basal medium before autoclaving. The following media, labeled as CM1 to CM2, SM1 to SM3 and RM1 to RM2 consisting of MS basal medium supplemented with different concentrations and combinations of auxins and cytokinins were used as nutrient media to induce somaclonal variations in *Centella*.

CM1: MS + 0.5-3mg/l 2-4 D + 1-6mg/l BAP.

CM2: MS + 0.5 - 3mg/l IAA + 1 - 6mg/l BAP.

SM1: MS + 0.5mg/l IBA to 2mg/l IBA.

SM2: MS + 0.5mg/l KIN + 2.0mg/ l KIN.

SM3: MS + 1mg/l IBA + 1-4mg/l KIN.

RM1: MS + 0.5 mg/l NAA to 2mg/l NAA

RM2: MS + 0.5 mg/l IBA + 3.0 mg/l IBA.

### 3.3.2.4. Other Supplementations and Steps Involved in Preparation of Medium

The above combinations of growth hormones, medium were further supplemented with concentration of 0.8% (w/v) agar and sucrose 4.0% (w/v). The agar is used as a solidifying agent and sucrose as carbon source were added to the MS basal medium. After preparations of all stock solution of MS medium, growth hormones then medium was prepared. The following steps are involved in preparation of medium.

1. The medium was prepared by adding appropriate quantities of the stock solution and correct volume was made up with distilled water,
2. Sucrose were added in above appropriate volume medium,
3. Then, pH in all cases was set to 5.8 by using either 1 N KOH or 1N HCL,
4. After, pH maintain agar were used for the semisolid medium for the initiation, multiplication and rooting of the microshoots,
5. Before autoclaving, the medium was poured into washed and dried test tube (up to 15-20ml) or glass bottles (250ml),
6. They were then autoclaved at $121^0$C for 20 minutes at 15-psi pressure and transferred to the inoculation room where they were stored under aseptic conditions till their further use.

### 3.3.2.5. Explant Selection and Sterilization

The disease free, young and healthy leaves explants were selected, for *in vitro* culture of *Centella asiatica*. These plantlets were maintained in the greenhouse of the college. Following are important steps involved in preparation of the explants and surface sterilization.

1. The young leaves and fresh leaves were removed from the explants and they were trimmed to approximately 3-4cm,
2. The explants were then washed under running tap water for 30 minutes to wash off the external contaminants,
3. Explants were again washed twice washed twice with double distilled water for 5 minutes each.,
4. Then, the explants were soaked in an aqueous solution containing 1-2% (v/v) liquid detergent solution (Labolene; Qualigens, India) for 15 min,
5. They were washed again in sterile distilled water. Then the explants were taken to laminar air flow under sterile condition,

6. Further surface sterilization was done using 0.1% Mercuric chloride (High Media, India) aqueous solutions for 3-5 minutes in the laminar air flow chamber,

7. Explants were removed from the sterilizing solution and rinsed completely for 4-5 times using sterilized double distilled water to remove traces of the sterilant.

### 3.3.2.6. Initiation of Explant Cultures

In the above important explants sterilization process, then sterilized explants were used in the initiation of cultures. Following are the important steps involved in laminar air flow for preparation of the explants initiation of cultures.

1. The sterilized explants were transferred aseptically to the sterilized glass plates under the laminar air flow and they were further trimmed to small pieces of 1cm x 2cm size,

2. Then, a cut was given on both as well as the top portion of the explants to remove undesirable or dead portions after surface sterilization,

3. The forceps were rinsed in the 70% ethanol and were flamed and then kept for some time to get cool. Then the lid from one test tube was removed and test tube mouth was flamed to avoid further chances of cross contamination,

4. Prepared explants were inoculated into test tube (15-20ml) and glass bottles (250ml) with long forceps without touching the rim of the test tube and glass bottles containing different media combinations. The lid was finally replaced carefully onto the test tube and glass bottles, flamed lightly and sealed with parafilm or cotton ball. Above all steps are involved under a laminar air flow,

5. After inoculation, all the cultures were finally placed in the incubation room with temperature condition 25 ± $2^0$ C , with a photoperiod of 16 hours daylight and 8 hours night break under cool white fluorescent light of average 2,500 lux,

6. After inoculation, daily observations were made regarding callus, embryoids, shoot and root initiation of the culture.

### 3.3.2.7. Transplantation and Acclimatization of the plantlets

The transfer of plants from the culture vessel to the soil requires a careful, stepwise procedure. The roots of the plants are gently washed to remove the agar sticking to them. The most essential requirement for the successful transplantation is to maintain the plants under a very high humidity (90 - 100%). For the first 10-15 days by keeping them under moist or covering them with clear plastic. Some small holes may be poked in the plastic for air circulation. Inside the culture vessel the humidity is high and thus, the natural protective covering of cuticle is not fully developed. During this time plant attain ability to synthesize more food and develop cuticular covering. Plants are maintained under shade and are then ready to be used in open nursery. Following are the steps involved in preparation of transplantation and acclimatization of the plantlets.

1. After 10-14 days of culture on rooting media, the rooted plantlets were transplanted to pots or trays for hardening prior to their final transfer to soil,

2. Rooted plantlets were taken out of the culture bottles with the help of forceps and washed thoroughly with water to remove any remaining of the medium. 0.1% Bavistin treatment was given to the plants in order to protect them from the fungal attack in the near future,

3. After this the plants are carefully planted in the plastic trays containing coco peat and allowed to harden in the growth room for a week,

4. After planting, plantlets are thoroughly watered and kept in polyhouse under humidity range of approximately 80%. These plantlets should be sprinkled with water time to time as per the requirement,

5. After hardening and acclimatization, these plantlets were cultivated on seed beds containing a mixture of loamy and black cotton soil.

Later two weeks, high survival rate was observed when the plants were transferred to the field. Such somaclonal variants propagated on large scale in the polyhouse and screened for morphological variations.

## 3.4. Morphological Analysis

The morphological features of diploid and autotetraploidy plants were visually examined and some changes were recorded due to colchiploidization in aspects of the plant growth habit. These morphological features were vegetative and flower characteristics. Therefore, the promising plants of *C. asiatica*, produced through autotetraploidy induction, and induction of somaclonal variations were grown in the experimental plots of polyhouse and allowed to multiply for several generations. Similarly control plants were also grown simultaneously in adjacent plots to enable comparison. The vegetative characteristics data were used as morphological markers viz., Plant height, internodal distance of the main stem, leaf area, visual leaf color, thickness of leaf, petiole length, fresh weight of 100 leaves, dry weight of 100 leaves, weight of 100 seed and weight of 100 fresh bud, were recorded at every generation for comparison with those of the control plants. The results obtained for several generations, confirmed the stability of the new phenotypes over the control plants.

### 3.4.1. Quantitative Estimation of Morphological Parameters

1. **Plant survival:** Survival of plants in each treatment and their respective controls were recorded at the time of maturity in the field. Rate of survival at maturity was expressed as percentage over the control.

2. **Plant Height:** Height of the treated, selected and control plants were measured only after flowering of the plants. Height of the plants was secured using a thread and a meter scale.

3. **Leaf Area:** Leaf area of the control, treated and selected plant populations were measured with Leaf area meter, AM-100 (ADC Bioscientific Ltd.).

4. **Leaf Thickness:** The leaf thickness was measured in 10 replications. Leaf thickness was measured with a digital vernier caliper.

5. **Determination of Fresh Weight:** Hundred leaves from each of the colchicine treated plants, somaclonal variants and control plants were collected from mature plants and washed under running tap water. They were blotted between folds of blotting paper in order to remove water and weighed on an electronic balance to determine the fresh weight of hundred leaves.

6. **Determination of Dry Weight:** After fresh weight determination, same leaves were allowed to dry in shade, in a well-ventilated place for 4-5 days. Such dry leaves were weighed on an electronic balance and dry weight was determined. Oven drying of the leaves was avoided as it results in evaporation of volatile compounds of medicinal importance. After determination of dry weight, the dried leaves were directly used for estimation of therapeutically active compounds.

7. **Hundred Seed and Flower Bud Weight:** Hundred seed and flower bud from each of the colchicine treated plants, somaclonal variants and control plants were collected from mature plants. Then, the weighed on an electronic balance to determine the fresh weight of hundred seed and flower bud.

## 3.5. Stomatal Analysis

Promising plants from colchicine treated and untreated similar sizes leaves from 3-4 months old plants were taken for the study of stomatal characteristics, such as stomatal width, stomatal length, number of stomata and size of stomata. The stomatal studies were done by mechanical peelings off or scraping off the epidermis from fresh leaves (Topho and Ghosh,1997). Leaves peels were observed under 40x a binocular compound microscope.

Stomata length, width and size were measured employing 40x ocular micrometer. The values obtained were calculated into micrometer using stage micrometer. Similarly Stomatal numbers were also observed in 40x ocular

micrometer. The treated and control leaves are major 100 times. The measurements were undertaken from both sides of each leaves.

## 3.6. Cytological Studies

Promising plants from colchicine treated populations, showing variations in morphological and stomatal markers were processed for cytological studies to determine the chromosome number and establish their ploidy level.

### 3.6.1. Selection and Fixation of Root Tips

After such plants served as a field propagation and they were used directly as cytological investigations. The young developing root tips (2-3mm), from actively growing roots were excised and collected in the early hours (6 to 9 am). Then, the root tips material was fixed in immediately a freshly prepared of carnoy's (1886) solution (glacial acetic acid: 95% ethanol, 1:3) for 1 day. After fixation, such root tips were washed in distilled water and preserved in 70% alcohol in a refrigerator until use.

### 3.6.2. Staining Method

Staining is necessary for visualization of chromosomes under microscope. The aceto-orcein stain was recommended by La Cour in 1941 for chromosomal staining. The fixed root tips were washed thoroughly with distilled water. Then, take a watch glass and add approximately 1-2 ml of 2% Aceto-Orcein-HCL reagent in it. Add 5-6 *Centella* root tips in the watch glass. After that, heat the watch glass until few vapours start appearing from the watch glass and cool it for about 1 to 2 minute. Then, take out a clean glass slide and place a drop of 2% Aceto-Orcein-HCL reagent on it. Take out a orcein stained root tip from the watch glass and place it in the middle of aceto-orcein drop present on the glass slide. The root tips were squashed in a drop of 2% aceto-orcein and the root tips debris were removed. A cover glass of appropriate size was placed on the drop of orcein taking care that no stain goes out of the cover glass. The microscopic preparation is viewed under a trinoccular microscope (Olympus) to which a photomicrographic camera is attached. Meiocytes at diplotene were observed under oil immersion (1000X).

The number of bivalents present in such cells was counted and photographed immediately.

## 3.7. Biochemical Characterization

The autotetraploids isolated from the colchicine treated populations and their respective controls were analyzed for major saponins content as follows.

### 3.7.1. Preparation of Samples for Biochemical Analysis

The first and foremost step in biochemical analysis is the sample preparation. Samples for biochemical analysis were prepared from dry leaves. Shade dried mature leaves of autotetraploid and control plants were reduced to a moderately coarse powder with the help of a grinder. The grinded meal was sieved through a 56-mesh sieve to facilitate effective contact of the ruptured tissues and cells of the powder with the solvent used for extraction of biochemical compounds. The powdered material was used for the extraction of individual triterpenoids viz., asiaticoside and madecassoside. Extraction of crude drugs was possible only with the fine powder, because the solvents can extract drugs from individual cells and tissues exposed to solvent (Shah and Quadry, 2002).

### 3.7.2. HPLC Analysis of Major Saponins Contents

Major saponins and sapogenins contents were estimated employing HPLC analysis method (Tiwari *et al*., 2010). Following are the important steps involved in saponins and sapogenins analysis was determined using HPLC.

#### 3.7.2.1. Preparation of Samples for HPLC

For the preparation of samples for HPLC analysis from the dried leaves of *Centella asiatica* was as kindly provided by Dr. Murli, Natural Remedies Pvt. Ltd., Veerasandra Industrial Area, Bangalore, Karnataka, India. The following method is as follows.

100 mg of samples were weighed for Batch no.01.lcb and dissolved in methanol with 100ml volumetric flask for extract. For raw material 7grams of samples were weighed and transferred to a 250 ml of beaker. The solution was

extracted with 50ml of methanol by warming on water bath for about 20 min and then transfers the extract to a 250 ml beaker. The procedure was repeated for 4-5 times till the raw material is completely extracted or till the extract is colorless.

### 3.7.2.2. Preparation of Standard Solution

The standard mix dilution was prepared by weighing accurately 10.37 mg of madecassoside, 10.27 mg of asiaticoside and 10.35 mg of asiatic acid in the 10ml volumetric flask and make up the volume 10 ml by using methanol.

### 3.7.2.3. Chemicals and Reagents of HPLC Analysis

Acetonitrite HPLC grade and Methanol was procured from Qualigens. Orthophosphoric acid (AR grade) wase procured from Rankem. Water ultra-pure (18 MOhm resistance HPLC grade) was obtained from a sartorious water purification system. Reference standards of madecassoside, asiaticoside and asiatic acid were procured from R&D centre, NRPL, Bangalore.

### 3.7.2.4. Instrumentation and Chromatographic Conditions

High Performance Liquid Chromatographic system equipped with LC8A pump, SPD-M 10Avp Photo Array Director in combination with Class LC 10A software.

**Mobile Phase:** The mobile phase components were filtered through 0.45 µm membrane filter before use. Gradient elution was performed using, a mixture of Acetonitrile and buffer (Orthophosphoric acid in 1000ml of HPLC grade water).

**Column:** Lichrospher®100; RP-18 e (5µm), Size: 250 x 4.60 mm

**Detector:** SPD-M 10A*vp* Photo diode array detector/UV Detector

**Wave length:** 210 nm

**Flow rate:** 1.8 mL / min.

**Injection volume:** 20 Ml

HPLC analysis of the standard and the samples was carried out

using above mentioned protocol and the chromatograms were recorded. The retention time and peak area of the standard and sample were noted. Amount of asiaticoside, madecassoside + asiaticoside B, madecassic acid + terminolic acid and asiatic acid present in the sample was calculated by comparing the sample peak area with that of the standard.