Biochemistry may be defined as a science concerned with the chemical nature and chemical behaviour of the living matter. It takes into account the studies related to the nature of the chemical constituents of living matter, their transformations in biological systems and the energy changes associated with these transformations. The goal of biochemistry is to understand the chemical basis of biological phenomenon. It is the most rapidly developing and innovative subject in medicine.

Nature has been recognized as a rich source of medicinal compounds for thousands of years. It is estimated that about one third of currently marked drugs are related to natural products (Grabley and Thiericke, 1999). Advances in biochemistry have tremendous impact on human welfare and have largely fitted humanity and their living styles. These include the application of biochemistry for the diagnosis of diseases, the products (Insulin, interferon, growth hormones etc.) obtained from genetic engineering and the possible use of gene therapy in the near future.

Biochemistry deals with the chemical properties of important biological molecules, like proteins, and in particular the chemistry of enzyme-catalyzed reactions. Researchers in biochemistry use specific techniques native to biochemistry, but increasingly combine these with techniques and ideas from genetics, molecular biology and biophysics.

Primary metabolites are substances widely distributed in nature, occurring in one form or another in virtually all organisms. They are
of prime importance and essentially required for the growth and development of plants. In plants such compounds are often concentrated in seeds and vegetative storage organs and are needed for physiological development because of their role in basic cell metabolism (Vijayvergia et al., 2009).

Secondary metabolites are chemicals produced by means of secondary reactions resulting from primary carbohydrates, amino acids and lipids (Ting, 1982). These compounds are very important for both humans and plants (Rafael et al., 2008). These molecules are known to play a major role in the adaptation of plants to their environment and also represent an important source of active pharmaceuticals. These secondary metabolites are sought after because they are known to exhibit numerous biological activities that promote positive health effects, such as antibacterial, anticancer, antifungal, antioxidant and antiviral activities that can be used in the food, agriculture and pharmaceutical industries.

Many thousands of secondary metabolites have been isolated from plants, and many of them have powerful physiological effects in humans and are used as medicines. The current interest in secondary metabolites obtained from the plant kingdom was probably stimulated by the hope of obtaining new sources of compounds potentially useful in therapeutic programs. This interest was supported by the rapid development of analytical methods for determining complicated molecular structures. Many studies have been undertaken with the objective of improving the in vitro production of secondary metabolites. There are a number of techniques which are employed in secondary metabolite isolation, purification and characterization
from diverse groups of plants. These techniques include Column chromatography, Thin layer chromatography (TLC), High performance thin layer chromatography (HPTLC), Infrared spectroscopy (IR), Nuclear magnetic resonance (NMR) and Gas chromatography and mass spectrometry (GC-MS). Highly specific in vitro and in vivo bioassay techniques, chromatographic methods and spectroscopic techniques have made easier to screen, isolate and identify bioactive compounds quickly and precisely.

Determination of the physiologically active chemical constituents responsible for the therapeutic action of a plant drug is an appropriate yardstick to evaluate the quality of the raw material. This type of biochemical screening is very much helpful in obtaining high yielding planting material for mass propagation and selection of suitable planting material for large scale cultivation of medicinal plants (Dagade, 2003; Shukry et al., 2007 and Grover, 2012).

Besides this, isolation, identification and quantification of these biologically active plant derived chemicals play a significant role in the commercial development of new products for regulating plant and animal growth and for insect/weed control.

Hence, in the present investigation, biochemical studies were carried out in Cocculus hirsutus under the followings heads:

- GC-MS analysis of the leaves of Cocculus hirsutus.
- Isolation, identification and quantification of β-sitosterol from in vivo and in vitro samples of Cocculus hirsutus.
- Isolation, identification and quantification of gallic acid from in vivo and in vitro samples of Cocculus hirsutus.
4.1 GC-MS ANALYSIS

Plants are a rich source of secondary metabolites with interesting biological activities. In general, these secondary metabolites are an important source with a variety of structural arrangements and properties (De-Fátima et al., 2006). Distinguished examples of these compounds include flavonoids, phenols and phenolic glycosides, saponins and cyanogenic glycosides (Shahidi et al., 2008). Natural products from microbial sources have been the primary source of antibiotics, but with the increasing recognition of herbal medicine as an alternative form of health care, the screening of medicinal plants for active compounds has become very significant because these may serve as talented sources of book antibiotic prototypes (Koduru et al., 2006). It has been shown that in vitro screening methods could provide the needed preliminary observations necessary to select crude plant extracts with potentially useful properties for further chemical and pharmacological investigations (Mathekaga and Meyer, 1998).

Today natural products derived from plants are being tested for the presence of new drugs with new modes of pharmacological action. A special feature of higher plants is their capacity to produce a large number of secondary metabolites (Castello et al., 2002). Recent studies are involved in the identification and isolation of new therapeutic compounds of medicinal importance from higher plants for specific diseases (Kumar et al., 2007). Knowledge of the chemical constituents of plant is helpful in the discovery of therapeutic agent as well as new sources of economic materials like oil and gums. The
most important bioactive constituents of the plants are alkaloids, tannins, flavonoids and phenolic compounds. In India a large number of plant species have been screened for their pharmacological properties but still a vast wealth of endangered species are unexplored. Medicinal plants are at interest to the field of biotechnology, as most of the drug industries depend in part on plants for the production of pharmaceutical compounds (Velmurugan et al., 2010; Banu and Nagaranjan, 2013).

In recent years, interest for the study of organic compounds from plants and their activity has increased. A knowledge of the chemical constituents of plants is desirable not only for the discovery of therapeutic agents, but also helps in disclosing new sources of economic phytocompounds for the synthesis of complex chemical substances and for discovering the actual significance of folkloric remedies (Milne, 1993). There is an increasing interest in the phytochemical compounds, which could be relevant to their nutritional incidence and their role in health and disease (Steinmetz and Potter, 1996). The combination of an ideal separation technique (GC) with the best identification technique (MS) made GC-MS an ideal technique for qualitative and quantitative analysis of volatile and semi volatile compounds. This technique has proved to be a valuable method for the analysis of non polar components and volatile essential oil, fatty acids, lipids and alkaloids (Betz et al., 1997). There is growing awareness in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity (Prachayasittikul et al., 2008). Screening active compounds from plants has lead to the
invention of new medicinal drugs which have efficient protection and treatment roles against various diseases, including cancer (Sheeja and Kuttan, 2007) and Alzheimer’s disease (Mukherjee et al., 2007). Various bioactive compounds have been detected by several workers using GC-MS (Hema et al., 2011; Maruthupandian and Mohan, 2011; Ghannadi et al., 2012; Ananthi and Ranjitha Kumari, 2013; Banu and Nagaranjan, 2013).

In the present study, methanolic extract of *Cocculus hirsutus* leaf was analyzed by GC-MS technique to study the major and minor phytoconstituents of the plant.

### 4.1A MATERIALS AND METHODS

(i) **Collection of plant material**

The leaves of *Cocculus hirsutus* were collected from the Kulish Smriti Van, Jaipur, India. They were identified and authenticated by the herbarium of Department of Botany, University of Rajasthan, Jaipur.

(ii) **Preparation of powder and extract**

Leaves were shade dried, powdered and extracted with methanol for 6-8 hours using soxhlet apparatus. The extract was then filtered through muslin, evaporated under reduced pressure and vacuum dried to get the viscous residue. The methanolic extract of the plant was used for preliminary phytochemical and GC-MS analysis. 1 µl of the methanolic extract of *C. hirsutus* was employed for GC-MS analysis.
(iii) GC-MS analysis of the leaves of *Cocculus hirsutus*

GC-MS analysis was carried out on a GC clarus 500 Perkin Elmer system comprising a AOC-20i autosampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions: column Elite-1 fused silica capillary column (30 x 0.25 mm ID x 1µM df, composed of 100% Dimethyl poly dimethoxane), operating in electron impact mode at 70eV; helium (99.999%) was used as carrier gas at a constant flow of 1 ml /min and an injection volume of 0.5 µl was employed (split ratio of 10:1) injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 10°C/min. to 200°C, then 5°C/min to 280°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds and fragments from 40 to 450 Da. Total GC running time was 45 minutes.

(iv) Identification of Components

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

4.1B OBSERVATIONS AND RESULTS

GC-MS chromatogram of the methanolic extract of the leaves of *C. hirsutus* (Plate-34, Fig. A) showed 32 peaks indicating the presence of thirty two compounds. The chemical compounds identified
in the methanolic extract of the leaves of *C. hirsutus* are presented in Table-28. The active principles with their retention time (RT), molecular formula, area %, compound name and RI are presented in Table-28. The total ion chromatograph (TIC) showing the peak identities of the compounds identified have been given in Plate-34, Fig. A. GC-MS analysis revealed the presence of palmitic acid, oleic acid, phthalic acid, stearic acid, linolenic acid etc. Quinic acid was present in maximum amount (36.29%), followed by 2,3,4,5-Tetrahydroxypentanal (28.07%) in the methanolic extract of leaves of *C. hirsutus*. Steroids like beta-sitosterol, campesterol, squalene were also detected. Vitamin-E was also present in considerable amount i.e. 10.27%. GC-MS analysis revealed the minimum presence of 5,5-Dibutylmononane, Dimethyl aminoethylacrylate, Tridecane, 1-Butoxy ethoxy-2-propanol and Gamma tocopherol. It revealed that the methanolic extract is mainly composed of esters, phenolics and steroids. The gas chromatogram shows the relative concentrations of various compounds eluted as a function of retention time. The heights of the peak indicate the relative concentrations of the components present in *C. hirsutus*. The mass spectrometer analyzes the compounds eluted at different times to identify the nature and structure of the compounds. The large compound fragments into small compounds giving rise to appearance of peaks at different m/z ratios. These mass spectra are fingerprint of that compound which can be identified from the data library.
4.1C CONCLUSIONS

The results concerning the present investigation revealed the following conclusions:-

- GC-MS analysis of methanolic extract of leaves showed the presence of carbohydrates, steroids, alkaloids, glycosides, flavonoids, tannins and saponins.
- The presence of various bioactive compounds confirms the application of *Cocculus hirsutus* for various ailments by traditional practitioners.
4.2 ISOLATION, IDENTIFICATION AND QUANTIFICATION OF $\beta$-SITOSTEROL IN VIVO AND IN VITRO

Phytosterols (also called plant sterols) are a group of steroid alcohol phytochemicals naturally occurring in plants. Steroids are synthesized in most groups of organisms and are critically important to plants and animal life. They include the adrenal cortical hormones, the sex hormones, some of the vitamins, plant sterols such as ergasterol, animal sterols such as cholesterol, sapogenins, steroidal alkaloids and cardiac glycosides. They have many applications as food additives, and in medicine and cosmetics.

Sterols are a structurally unique type of secondary metabolite, which are basically triterpenoids and made up of terpene units containing 30 carbon atoms. It is assumed that the sterols stabilize cellular membranes and control their permeability. Phytosterols (plant sterols) are members of the “triterpene” family of natural products and more than hundred different plant sterols have been identified. Phytosterols abundant in nature are $\beta$-sitosterol, campesterol and stigmasterol (Belitz and Grosch, 1999).

$\beta$-sitosterol is the most abundant phytosterol, which is found in plants alone or in combination with similar plant sterols. It has been effective in the reduction of cholesterol level of blood as well as reduction of benign prostrate hyperplasia in human beings (Berges et al., 1995; Klippel et al., 1997; Wilt, 2000; Mohanapriya and Vijaiyan Siva, 2013). It has antifungal, antibacterial, anti-inflammatory and anticancerous activities (Kiprono et al., 2000; Award et al., 2000).
β-sitosterol has been reported in many plants by several workers like Jirage et al., (2010); Miser et al., (2010); Iyer et al., (2011) and Akshada and Magdum, (2012). Plant tissue culture technique serves as an ideal system for studying the fundamental aspects of biosynthesis of steroids. β-sitosterol was isolated from many plants i.e. Pluchea lanceolata (Arya, 2007); Cissus quadrangularis (Sharma et al., 2011); Citrullus colocynthis (Meena et al., 2011); Clerodendrum infortunatum and Alternanthera sessilis (Gupta and Singh, 2012) and Svensonia hyderobadensis (Lingarao and Savithramma, 2013).

Realising the medicinal importance of β-sitosterol, the present study was undertaken for isolation and identification of β-sitosterol from in vivo (leaf) and in vitro (callus) cultures of Cocculus hirsutus.

4.2A MATERIALS AND METHODS

Chemicals

All the chemicals used in the experiments were of analytical grade procured from Merck. Reference standard β-sitosterol was purchased from Sigma Chemicals.

Plant material

The leaves of C. hirsutus used in the present investigation were collected fresh from their natural habitats and cut into small pieces, dried, powdered and used for the estimation of β-sitosterol contents. Unorganized cultures were raised and established as described in chapter 3. These cultures were harvested at their maximum growth. Tissue samples were dried at 100°C for 15 min. to inactivate enzymes followed by 60°C till constant weight was achieved. The dried leaves
were then powdered and analyzed separately for their β-sitosterol contents. Five replicates were taken in each case.

**Extraction procedure**

Each of the powdered tissue samples *viz.* *in vivo* (leaf) and *in vitro* (callus) was taken in a soxhlet apparatus and extracted with methanol for 24 hrs. The solvent was recovered by distillation. The residue was concentrated, dried and stored in the desiccators for further experiment and analysis. The dried residue was taken up in methanol for further analysis. Isolation, identification and quantification of β- sitosterol was carried out by TLC, HPTLC and IR spectral studies in the plant species.

**HPTLC Chromatographic conditions**

**Stationary Phase**: Precoated silica gel plates Merck 60 F$\text{ }^{254}$ (10x10, 0.2 mm thickness)

**Mobile Phase**: Toluene: methanol (9:1 v/v)

**Spotting Device**: Linomat V Automatic sample spotter, CAMAG (Switzerland).

**Development Mode**: CAMAG twin trough chamber, CAMAG Densitometer: TLC Scanner III, CATS software, CAMAG.

**Preparation of standard solution**

The stock solution of β-sitosterol (20 µg/ml) was prepared by transferring 2 mg of beta-sitosterol, accurately weighed, into a 100 ml volumetric flask, dissolving in 50 ml methanol. It was then sonicated for 10 minutes and the final volume of the solution was made up to
100 ml with methanol to get a solution of appropriate range of β-
sitosterol.

**Instrumentation and chromatographic conditions**

HPTLC was performed on 20 cm×10 cm aluminium packed
plates coated with silica gel 60 F254 (Merck, Mumbai, India).
Standard solution of beta-sitosterol and sample solution were applied
to the plates as bands 8.0 mm wide, 30.0 mm apart and 10.0 mm from
the bottom edge of the same chromatographic plate by use of a Camag
(Muttenz, Switzerland) Linomat V sample applicator equipped with a
100 µL Hamilton (USA) syringe. Ascending development to a
distance of 80 mm was performed at room temperature (28±2°C), with
Toluene: methanol (9:1 v/v) as mobile phase in a Camag glass twin-
trough chamber previously saturated with mobile phase vapour for 20
minutes. After development, the plates were dried in air and sprayed
by using anisaldehyde sulphuric acid reagent solution and subsequently
heated at 120°C for derivatization. These plates were scanned and
visualized under visible light at 525 nm and UV light at 254 nm and
366 nm absorbance/reflection mode using reflection mode by
CAMAG Scanner III and CATS software and deuterium lamp was
used to analyze the plates.

**Calibration curve of the standard**

A stock solution of standard β-sitosterol (20 µg/ml) was
prepared in methanol. Different volumes of stock solution 2,4,6,8 and
10 µl, were spotted on to TLC plate to obtain concentration 20, 40, 80,
120, 160, 180 and 200 µg/spot of beta-sitosterol respectively. The
working standard was applied on precoated silica gel F$_{254}$ HPTLC plates and the plates were developed as described earlier. The peak areas were recorded. The calibration curve of the standard concentration (X-axis) over the average peak height/area (Y-axis) was prepared to get a regression equation by Win Cats software, which was used for the estimation of β-sitosterol.

**HPTLC Quantification of the extracts**

The β-sitosterol content of various extracts was determined by comparing the area of chromatogram with the calibration curve of concentration of standards. The R$_f$ value of standard β-sitosterol (0.45) was compared with the R$_f$ value of the extracts. Quantitative estimation of the plate was performed in the remission/absorption mode at 254 nm, with the following conditions slit width 6.00x0.30 mm, micro scanning speed 20 mm/s and data resolution 100 µm step. Calibration parameters were as follows: calibration mode- single level, statistics mode-cv, evolution mode- peak height. The average content of β-sitosterol in different extracts was expressed in percentage.

**IR Spectral studies**

Each of the fluorescent spots coinciding with those of standard reference compound of β-sitosterol were marked, scrapped and collected separately with the adsorbant from plates. The bands were then eluted with methanol, elutes dried in vacuo and crystallized separately with acetone and methanol. Each of the crystallized isolates from all the samples tested were subjected to Infra-red spectrophotometric (Perkin-Elmer 337 Grating, Infra-red spectrophotometer using nujol or
potassium bromide pellets) studies along with respective standard compound of β-sitosterol.

4.2B OBSERVATIONS AND RESULTS

The TLC procedure was optimized with a view to quantify the samples extract. Initially toluene: methanol in varying ratios was tried. The mobile phase toluene: methanol (9:1, v/v) gave good resolution with \( R_f = 0.45 \). Well defined spots were obtained when the chamber was saturated with mobile phase for 20 min. at room temperature. The TLC plates were visualized under UV light at 254 nm after derivatization. A photograph of a TLC plate after chromatography of β-sitosterol standard and a methanolic extract of the samples of *C. hirsutus* are shown in Plate-35 Figs. A&B. The identity of the β-sitosterol bands in the sample chromatogram was confirmed by the chromatogram obtained from the sample with that obtained from the reference standard solution. The \( R_f \)'s obtained for the said plant extracts closely replicate the \( R_f \)’s found for standard β-sitosterol, thus making it a significant fingerprint parameter. The chromatogram of standard β-sitosterol is shown in Plate-36, Fig. A and that of β-sitosterol identified in *C. hirsutus* leaf and callus samples are shown in Plate-36, Figs. B&C. The respective \( R_f \)'s obtained for each sample are shown in Table-29. The peak corresponding to beta-sitosterol (0.44, 0.46) from the sample solution had almost same retention factor as that of standard β-sitosterol (0.45). The characteristic IR spectral peaks were found to be superimposable with those of their respective standard reference of β-sitosterol [Plate-37, Fig. A]. The 3D spectra of all tracks scanned at 580 nm are shown in Plate-37, Fig. B. A three dimensional view enhances the visible similarities amongst all spectral
tracks at a selected wavelength which in the present study was 580 nm at different vertices thus bringing out the desired fingerprints. The 3D spectra obtained from the present study has brought out the spectra for all tracks viewed together and are suggestive of similarities between the test tracks and the standard tracks also elucidating strong presence of the biomarker in the plant extracts. The linearity regression for the calibration showed correlation coefficient of 0.99 with respect to height and area in the range of 2.0-10.0 µl and the content of β-sitosterol in methanolic extract of leaf and callus was found to be 0.25% and 0.097% respectively.

4.2C CONCLUSIONS

The results concerning the present investigation revealed the following conclusions:-

- A rapid, simple, accurate and specific HPTLC method for quantitative estimation of β-sitosterol present in the dried leaf and callus of C. hirsutus has been developed.
- The method used in this work resulted in good peak shape and enabled good resolution of β-sitosterol from C. hirsutus samples.
- β-sitosterol was identified in in vivo (leaf) and in vitro (callus) tissues. Presence of isolated β-sitosterol was further confirmed by superimposable IR spectra of isolated and authentic samples of β-sitosterol.
- Variation in β-sitosterol content in in vivo and in vitro samples in C. hirsutus was observed. In vivo leaf had maximum amount of β-sitosterol (0.25%) while minimum amount was found in in vitro callus (0.097%).
4.3 ISOLATION, IDENTIFICATION AND QUANTIFICATION OF GALLIC ACID IN VIVO AND IN VITRO

Tannins are natural compounds widely distributed in the plant kingdom. The function of tannins is the defense system of plants against microbial and animal attacks due to their astringent capacity and the ability to form complexes with proteins and polysaccharides (Swain and Bate-Smith, 1962). Tannins are secondary metabolites of plants. It is generally accepted that considering their sugar content, polymerization and esterification degrees, they are divided into three groups: condensed tannins, hydrolysable tannins and complex tannins.

Gallic acid (GA) is a phenolic compound. Structurally gallic acid has phenolic groups that serve as a source of readily available hydrogen atoms such that the subsequent radicals produced can be delocalized over the phenolic structure (Robards et al., 1999; Nikolic, 2006). The interest in these compounds is due to their pharmacological activity as radical scavengers (Kaur et al., 2005). It has been proved to have potential preventive and therapeutic effects in many diseases, where the oxidative stress has been implicated, including cardiovascular diseases, cancer, neurodegenerative disorders and in aging. Phenolics are also of interest in food, cosmetic and pharmaceutical industries, as substitutes for synthetic antioxidants (Soong and Barlow, 2004). Several chromatographic methods have been documented for determination of gallic acid in plant extracts (Rizzo et al., 2006) but due to the complex nature and inherent variability of the chemical constituents of the plant based drugs, it is difficult to establish quality control parameters and hence modern
analytical techniques are expected to help in circumvention of this problem. Gallic acid has been reported in many plants by several workers (Leela et al., 2010; Borde et al., 2011; Mali et al., 2011; Vazirian et al., 2011; Arumugam et al., 2012; Hussain et al., 2012; Kardani et al., 2013 and Sampath, 2013).

The objective of the present investigation was to establish and validate the fast and sensitive high performance liquid chromatography (HPTLC) method for determination of gallic acid in methanolic extract of Cocculus hirsutus.

4.3A MATERIALS AND METHODS

Chemicals

All chemicals used in the experiments were of analytical grade procured from Merck. Reference standard gallic acid was purchased from Sigma Chemicals.

Plant material

The leaves of C. hirsutus used in the present investigation were collected fresh from their natural habitats and cut into small pieces, dried, powdered and used for the estimation of gallic acid contents. Unorganized cultures were raised and established as described in chapter 3. These cultures were harvested at their maximum growth. Tissue samples were dried at 100°C for 15 min. to inactivate enzymes followed by 60°C till constant weight was achieved. The dried leaves were then powdered and analyzed separately for their gallic acid contents. Five replicates were taken in each case.
Extraction procedure

Each of the powdered tissue samples viz. *in vivo* (leaf) and *in vitro* (callus) was taken in a soxhlet apparatus and extracted with methanol for 24 hrs. The solvent was recovered by distillation. The residue was concentrated, dried and stored in the desiccators for further experiment and analysis. The dried residue was taken up in methanol for further analysis. Isolation, identification and quantification of gallic acid was carried out by TLC, HPTLC and IR spectral studies in the plant species.

HPTLC Chromatographic conditions

**Stationary Phase:** Precoated silica gel plates Merck 60 F_{254} (10 x 10, 0.2 mm thickness)

**Mobile Phase:** Toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2 v/v)

**Spotting Device:** Linomat V Automatic sample spotter, CAMAG (Switzerland).

**Development Mode:** CAMAG twin trough chamber, CAMAG Densitometer: TLC Scanner III, CATS software, CAMAG.

**Preparation of standard solution**

The stock solution of gallic acid (20 µg/ml) was prepared by transferring 2 mg of gallic acid, accurately weighed, into a 100 ml volumetric flask and dissolving in 50 ml methanol. It was then sonicated for 10 minutes and the final volume of the solution was made up to 100 ml with methanol to get a solution of appropriate range of gallic acid.
Instrumentation and chromatographic conditions

HPTLC was performed on 20 cm×10 cm aluminium packed plates coated with silica gel 60 F254 (Merck, Mumbai, India). Standard solution of gallic acid and sample solution were applied to the plates as bands 8.0 mm wide, 30.0 mm apart and 10.0 mm from the bottom edge of the same chromatographic plate by use of a Camag (Muttenz, Switzerland) Linomat V sample applicator equipped with a 100 µL Hamilton (USA) syringe. Ascending development to a distance of 80 mm was performed at room temperature (28±2°C), with Toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2 v/v) as mobile phase in a Camag glass twin-trough chamber previously saturated with mobile phase vapour for 20 minutes. After development, the plates were dried in air and was sprayed with 5% methanolic FeCl₃ and subsequently heated at 120°C for derivatization. These plates were scanned and visualized under visible light at 525 nm and UV light at 254 nm and 366 nm absorbance/reflection mode using reflection mode by CAMAG Scanner III and CATS software and deuterium lamp was used to analyze the plates.

Calibration curve of the standard

A stock solution of standard gallic acid (20 µg/ml) was prepared in methanol. Different volumes of stock solution 2, 4, 6, 8 and 10 µl, were spotted on to TLC plate to obtain concentration 20, 40, 80, 120, 160, 180 and 200 µg/spot of gallic acid respectively. The working standard was applied on precoated silica gel F₂₅₄, HPTLC plates and the plates were developed as described earlier. The peak areas were recorded. The calibration curve of the standard concentration (X-axis)
over the average peak height/area (Y-axis) was prepared to get a regression equation by Win Cats software, which was used for the estimation of gallic acid.

**HPTLC Quantification of the extracts**

The gallic acid content of various extracts was determined by comparing the area of chromatogram with the calibration curve of concentration of standards. The $R_f$ value of standard gallic acid (0.41) was compared with the $R_f$ value of the extracts. Quantitative estimation of the plate was performed in the remission/absorption mode at 254 nm, with the following conditions slit width 6.00x0.30 mm, micro scanning speed 20 mm/s and data resolution 100 µm step. Calibration parameters were as follows: calibration mode- single level, statistics mode-cv, evolution mode- peak height. The average content of gallic acid in different extracts was expressed in percentage.

**IR Spectral studies**

Each of the fluorescent spots coinciding with those of standard reference compound of gallic acid were marked, scrapped and collected separately with the adsorbent from plates. The bands were then eluted with methanol, elutes dried in vacuo and crystallized separately with acetone and methanol. Each of the crystallized isolates from all the samples tested were subjected to Infra-red spectrophotometric (Perkin-Elmer 337 Grating, Infra-red spectrophotometer using nujol or potassium bromide pellets) studies along with respective standard compound of gallic acid.
4.3B OBSERVATIONS AND RESULTS

The TLC procedure was optimized with a view to quantify the samples extract. Initially toluene: ethyl acetate: formic acid: methanol in varying ratios was tried. The mobile phase toluene: ethyl acetate: formic acid: methanol (3:3:0.8:0.2 v/v) gave good resolution with \( R_f = 0.41 \). Well defined spots were obtained when the chamber was saturated with mobile phase for 20 min. at room temperature. The TLC plates were visualized under UV light at 254 nm after derivatization. A photograph of a TLC plate after chromatography of gallic acid standard and a methanolic extract of the samples of \( C. hirsutus \) are shown in Plate-38, Figs. A & B. The identity of the gallic acid bands in the sample chromatogram was confirmed by the chromatogram obtained from the sample with that obtained from the reference standard solution. The \( R_f \)'s obtained for the said plant extracts closely replicate the \( R_f \)'s found for standard gallic acid, thus making it a significant fingerprint parameter. The chromatogram of standard gallic acid is shown in Plate-39, Fig. A and that of gallic acid identified in \( C. hirsutus \) leaf and callus samples are shown in Plate-39, Figs. B&C. The respective \( R_f \)'s obtained for each sample are shown in Table-30. The peak corresponding to gallic acid (0.40, 0.42) from the sample solution had same retention factor as that of standard gallic acid (0.41). The characteristics IR spectral peaks were found to be superimposable with those of their respective standard reference of gallic acid [Plate-40, Fig. A]. The 3D spectra of all tracks scanned at 980 nm are shown in Plate-40, Fig. B. A three dimensional view enhances the visible similarities amongst all spectral tracks at a
selected wavelength which in the present study was 980 nm at different vertices thus bringing out the desired fingerprints. The 3D spectra obtained from the present study has brought out the spectra for all tracks viewed together and are suggestive of similarities between the test tracks and the standard tracks also elucidating strong presence of the biomarker in the plant extracts. The linearity regression for the calibration showed correlation coefficient of 0.99 with respect to height and area in the range of 2.0-10.0 µl and the content of gallic acid in methanolic extract of leaf and callus was found to be 0.44 % and 0.10% respectively.

4.3C CONCLUSIONS

The results concerning the present investigation revealed the following conclusions:-

- A rapid, simple, accurate and specific HPTLC method for quantitative estimation of gallic acid present in the dried leaf and callus of *C. hirsutus* has been developed.
- The method used in this work resulted in good peak shape and enabled good resolution of gallic acid from *C. hirsutus* samples.
- Gallic acid was identified in *in vivo* (leaf) and *in vitro* (callus) tissues. Presence of isolated gallic acid was further confirmed by superimposable IR spectra of isolated and authentic samples of gallic acid.
- Variation in gallic acid content in *in vivo* and *in vitro* samples in *C. hirsutus* was observed. *In vivo* leaf had maximum amount of gallic acid (0.44%) while minimum amount was found in *in vitro* callus (0.10 %).