Chapter III

ISOLATION AND STUDY OF PHYTOCONSTITUENTS
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3.1: INTRODUCTION:-

Phytochemistry, especially of plants used in traditional medicines, received a new impetus with the introduction of a wide spectrum of sophisticated Analytical Techniques. Improved methods of Extraction, Fractionation, Isolation and Characterization of compounds can now work with extremely small quantities as well (Harborne J B, 2011).

Challenge faced by Phytochemistry is that, there are natural variations in quality and quantity of Phytoconstituents due to non-uniformity of raw material. Factors that contribute to variation in Content and Composition of raw material can broadly be grouped into four categories namely Climatic, Nutritional, Collection and Post-Harvest Factors. Climatic factors include Temperature, Rainfall, Humidity, Daylight and Altitude of the Field. Production of Biomass and its Composition are affected by Nutritional Factors. Availability of Micro and Macronutrients and pH are important soil factors for optimal growth of plants and accumulation of Phytoconstituents. Quantity of Phytoconstituents varies with the Age of Plant, Season and Time of Collection and part of the plant collected. Enzymatic and Metabolic processes continue even after collection and hence may affect the quantity. Due to crushing and cutting of material, reactive chemical constituents of plants which were naturally located in intact cells, get liberated and may interact to affect Quantity of Phytoconstituents. The collected material faces oxidation when exposed to air and light. There may be a physical loss of some Phytoconstituents also (Trease and Evans, 2002).

Hence, it is evident that, several parameters are required to be tested to assure quality of plant derived drugs. As a result, precise control is very difficult (Trease and Evans,
2002). To lay standards for herbal drugs is not an easy task and a comprehensive system of standards cannot be laid down for such drugs. But now, under the directions of the World Health Organization, many countries are setting up norms for quality assurance and WHO has been regularly updating the norms required to ensure quality of natural products (WHO global survey, 2005).

Besides the problem of inconsistency of raw material, plant drugs have inherent problems as they are combinations of infinite known and unknown complex molecules. Metabolism in living cells is an assembly of enzyme-controlled chemical reactions. The resultant substances are essential for growth and survival of a plant in different circumstances. Primary Metabolic routes produce Primary Metabolites which are essential for all forms of life. Secondary Metabolites are grouped into different Metabolite classes and they possess a wide variety of structures. They may have a role in providing defense against pest and pathogens, providing protection against UV radiation and biotic and abiotic stresses, or acting as attractive volatile odor compounds or pigments (Ari Tolonen, 2003).

Traditional systems of medicine harness plant Secondary Metabolites as Pharmaceuticals. Opium Alkaloids with medicinal properties were isolated in early nineteenth century which laid the foundation of Modern Pharmacy. Some more examples of common drugs derived from ethnopharma sources are: Atropine from Atropa belladonna, traditionally used in Europe as Hallucinogenic brews; Caffeine from Coffee Coffea Arabica used as Stimulant drink in Ethiopia and from tea Thea sinensis, used in China as drink; Ephedrine from Ephedra sinica used in China for respiratory complaints and such many more (Trease and Evans, 2002).

Phytoconstituents are also used as a template for the synthesis of drugs. Some of the examples are: Sodium cromoglycate, an anti-asthematic drug based on ‘Khellin’ as template, derived from plant Ammi visnaga which was used in Egypt; a muscle relaxant drug during anaesthesia based on ‘Tubocurarin’ as template, derived from Chondodendron tomenosum which was traditionally used in Guyana, north Brazil as paralyzing dart poison; Dextromethorphan, a cough suppressant analgesic is based on ‘Morphin’ derived from Papaver somniferum used traditionally in Mediterranean region as analgesic (Trease and Evans, 2002).

Biotechnological methods are employed to enhance pharmaceutically important compounds in plants. In a few cases, Cell Cultures have been found to produce higher
levels of Secondary Metabolites than the differentiated mother plant itself. Some such examples are, production of anti-inflammatory Naphthoquinone Shikonin from *Lithospermum erythrorhizon* and antiseptic alkaloid Berberine from *Coptis Japonica* (Ari Tolonen, 2003). In recent times, Molecular farming has gained attention as plants can be turned into molecular medicine factories. Plants such as Tobacco, Potato, Tomato, Banana, Legumes and Cereals as well as Alfalfa, are used in Molecular farming and have emerged as promising bio farming systems for production of Pharmaceutical Proteins such as Antibodies, Vaccines, Regulatory Proteins and Enzymes (Stefen Pelzer *et al.*, 2003).

Secondary Metabolites derived from plants can be grouped as:

- Terpenes derived from the C₅ precursor, Isopentenyl diphosphate (IPP)
- Alkaloids derived from Amino Acids
- Shenolics derived from Shikimate pathway or Malonate /Acetate pathway

Main Secondary metabolites containing Nitrogen are:

- Alkaloids- Non Protein Amino Acids, Amines, Cyanogenic Glycosides and Glucosinolates.

Main Secondary metabolites without Nitrogen are:

- Phenolics: Flavonoids, Polyacetylenes, Polyketides and Phenylpropanoids.
- Terpenoids: Monoterpene, Sesquiterpene, Diterpenes, Triterpenes, Steroids and Saponins.

All major classes of Secondary Metabolites have found applications in various systems of traditional medicine as well as in modern medicine. Out of all the Secondary Metabolites, Terpenes and Terpenoids constitute a large class of natural products built up from Isoprene units. Terpenes are technically only hydrocarbons, while Terpenoids are oxygenated. *Isoprene Rule*: The basic molecular formulae of Terpenes are multiples of \((C₅H₈)_n\) where \(n\) is the number of linked Isoprene units.

Classes of Terpenes: Monoterpenes are made from two isoprene units, so are C₁₀. Sesquiterpenes are made from three isoprene units, so are C₁₅. Diterpenes are made from four isoprene units, so are C₂₀. Triterpenes are made from six isoprene units, so are C₃₀. Tetraterpenes are made from eight isoprene units, so are C₄₀. Steroids are Terpene derived, but carbons are rearranged or even removed during biosynthesis, so the isoprene units may not be recognizable (Gold Book, 2012).
Structures of some of the medicinally important plant derived Triterpenoids and Steroids are as shown in Figures 3.1a and 3.1 b.

*Figure 3.1a: Structures of some Plant Derived, Medicinally Important Triterpenoids*
Figure 3.1b: Structures of some Plant Derived, Medicinally Important Triterpenoids
Isolation of pure, pharmacologically active constituents from plants is a long and tedious process. To begin with, process of Extraction of Phytoconstituents is to be standardized. Chemical screening of the extracted Phytoconstituents is then performed to allow localization and targeted isolation of constituents with potential activities. Thin Layer Chromatography (TLC) is the simplest and cheapest method of detecting Phytochemicals. Separation is based on the principle that different constituents have different retention factors on chromatographic plate when subjected to a particular Mobile phase. TLC method is easy to run and perfectly reproducible when all controlling factors are identical (Poole C F et al., 1989).

Raw materials derived from different plant sources may have different profiles of Phytoconstituents. In order to develop a plant derived standard drug, raw material must produce uniform profile on chemical screening. Identity of raw material must be established beyond doubt by employing advance technology. Fingerprinting of Extract of raw material by High Performance Thin Layer Chromatography (HPTLC) is one such important technique. Herbal Extracts can be validated and standardized with HPTLC technique (Biringanine G et al., 2006). Separation and Identification are also achieved with HPTLC method (Mitja M et al., 2009; Yamunadevi M et al., 2011).

HPTLC is a form of TLC that provides superior separation power. TLC plates, coated with fine micro particles of silica (as used in Columns of HPLC), are used here. Layer conditioning as well as improved and automated sample application makes HPTLC more efficient. HPTLC ensures Higher Separation Efficiencies, Shorter Analysis Time, Lower Amounts of Mobile Phase, and Efficient Data Acquisition and Processing. Major parameters that influence separation of constituents within a mixture are Partition Coefficients, Retention Factors (Rf) of the individual constituents and Selectivity of Mobile and Stationary phases with respect to Solutes. Separation efficiency as well as Resolution of the individual constituents within a mixture is also decided by plate height (Sherma J et al., 2003; Srivastava M M, 2011).
Once plant constituents are isolated and purified using some technique, they are to be identified. To identify the compound completely, many parameters like Melting Point, Boiling Point, Optical Rotation and Rf on a TLC plate may be obtained and compared with the data of known compounds. Ultraviolet (UV), Infrared (IR), Nuclear Magnetic Resonance (NMR) and Mass Spectral (MS) measurements and other such hyphenated characterization techniques are of great importance. If a new fraction is obtained in crystalline form in sufficiently large quantity, X-ray Crystallography technique is the most suited method. When observed readings match with library data, compound may get identified. In other methods to identify a new compound, the compound is either put through Chemical Degradation to obtain indirect proof of structure or an identical Synthetic Compound is produced and compared with the compound under study (Harborne J B, 2011).

Photon energy, associated with frequencies ranging from γ- rays to radio waves is used in the field of research to understand life. Infrared Spectroscopy forms a part of such “biophotonics”, a field that merges Biology and Optics. Infrared Spectroscopy may be broadly defined as the study of absorption characteristics, arising from Molecular Vibrations in materials. Certain functional groups (groups of atoms) in molecules interact and absorb IR at Characteristic Frequencies when exposed to a broadband Infrared spectrum. Valuable clues to the structure of molecule can be obtained by identifying some of the functional groups that are present. Absorptions are classified as strong, moderate and weak. Fourier-transform Infrared (FTIR) spectroscopy represents an advanced version of more traditional infrared (IR) spectroscopy. FTIR is used primarily for structural determination of Organic Molecules. Computer carries out a mathematical operation, called Fourier transform on the data collected. FTIR instrument performs measurements in a time-based domain. This allows an FTIR to collect data for entire frequency range simultaneously. The Fourier transform converts time-based data to conventional frequency-based output. Thus, the output from FTIR and non-FTIR instruments appears identical (John Coates, 2000).
Primary advantage of FTIR lies in the time saving as it typically takes under one minute to complete a scan. Fourier Transform Infrared Spectroscopy is exceptionally suitable for obtaining spectra in energy limited situations (small quantities of samples, trace impurities in mixtures, weakly absorbing samples etc.) and conditions under which conventional dispersive instruments fail to produce the desired spectra. FTIR technique is a potent tool in the hands of Organic Chemists and is widely employed to get valuable data for Structure Determination. The use of FTIR in research, analytical and quality control laboratories has brought new and extended capabilities to all users (Harborne J B, 2011).

In order to fulfill the quest to study Chemical Profile of leaves of Costus pictus D.Don, following tasks were undertaken during the course of the present study:

- Establishing optimum procedure for Extraction and finding the most suitable Solvent for Extraction
- Fingerprinting of Methanolic Extract of leaves, with respect to four major groups of Phytochemicals, by employing HPTLC technique.
- Isolation of Triterpenoids fractions by employing optimum Mobile Phase discovered during Fingerprinting studies and using Analytical and Preparative TLC plates.
- FTIR analysis for identification of Functional groups and associated bonds in isolated fractions.

3.2: MATERIALS AND METHODS:

The Phytochemical study of a plant involves the following steps:

1. Extraction of Secondary Metabolites
2. Fingerprinting of Major groups of Phytoconstituents
3. Isolation and Analysis of Phytoconstituents of interest
4. Quantitative evaluation.
3.2.1: Extraction of Secondary Metabolites:

Extraction of Secondary Metabolites from medicinal plant is the first step in production of an herbal drug. It involves separation of medicinally active portions of a plant from inactive or inert components by using Selective Solvents known as menstruum and by employing Standard Extraction Procedures to obtain therapeutically desired portions and to eliminate unwanted material. Profile of Secondary Metabolites extracted depends mainly on part of the plant and the solvent used for extraction. Extracts contain complex mixtures of many metabolites, such as Alkaloids, Glycosides, Terpenoids, Flavonoids and Lignans (Handa S S et al., 2008). Standardization of extraction procedure contributes significantly to the final quality of herbal drug. Standardized extract as a whole, can be used as medicinal agent. Extract may be subjected to further fractionation in order to isolate individual chemical entities. Some examples of isolated fractions being used as modern drugs are: Vincristine, Vinblastine, Hyoscyamine, Hyoscine, Pilocarpine, Forskolin and Codeine (Handa S S et al., 2006).

Costus pictus belongs to the Zingiberaceae family. From pharmaceutical point of view, importance of the Zingiberaceae family is known from earlier times, as many plants with proven medicinal applications belong to the family (Trease and Evans, 1987). The plants from Zingiberaceae family are reported to contain Volatile Oils, Pungent Principles, Curcuminoids, Tannins, Phenolic Acids, Leucoanthocynins, Flavonoids, Ketones and Terpenoids. Only a few isolated examples of Alkaloids have been reported. Costus speciosus is reported to contain Diosgenin and Steroidal Sapogenins (Trease and Evans, 1987). Some plants from Zingiberaceae family possessing well-known medicinal usages are: Curcuma longa, Zingiber officinale, Elettaria cardamomum, Aframomum melegueta etc. Other plants from the family also are subjects of research and are now being developed into drugs effective in treatment of various diseases (Trease and Evans, 1987).
3.2.1a: Technique for extraction:

With increasing demand for herbal medicinal products all over the world, it has become necessary to use the most appropriate extraction technology in order to produce extracts of defined quality with the least variations from batch to batch. General techniques of medicinal plant extraction include Maceration, Infusion, Percolation, Digestion, Decoction, Hot Continuous Extraction (Soxhlet), Aqueous-Alcoholic Extraction by Fermentation, Counter-current Extraction, Microwave-assisted Extraction, Ultrasound Extraction (Sonication) and Supercritical Fluid Extraction.

Quality and therapeutic effectiveness of extract depends on the part of the plant as well as the solvent used. The Methanolic Extract of Leaves of Costus pictus was reported to contain most of the classes of Phytoconstituents (Nandhakumar Jothivel et al., 2007). Earlier studies on effectiveness of Costus pictus as a potential drug, have reported use of Methanolic Extract of leaves as test substance (Shilpa K et al., 2009; Majumdar M et al., 2012).

In the present study, protocol to obtain standard Methanolic Extract of dried leaves using Soxhlet Extractor was established.

Fresh green leaves of Costus pictus were collected from Medicinal and Aromatic Gardens of S.H.Kelkar and Company, Mulund Mumbai. Any foreign matter was completely eliminated by washing the leaves with mild soap water. The leaves were always collected in the mornings and during the months of October to April. The following sequential steps were followed during extraction: drying of plant material; size reduction; extraction; filtration; concentration and drying of extract.

In the present study, the leaves were naturally dried under shed for 20 days. Electrical Grinder was used to make powder with due care so as not to generate heat. Powdered plant material was passed through sieve no 38 to get small particles of uniform size.
i) **Choice of Solvent:**
Choice of solvent depends on many factors: A high selectivity is required for solvent to extract all the desired components; boiling point of solvent should be as low as possible in order to facilitate removal of solvent from the product; solvent should not react chemically with extract; solvent should not decompose during extraction; it should be nonflammable and non-corrosive, and should not present a toxic hazard; solvent should be readily available at low cost; solvent has to be separated easily from extract to produce a solvent-free extract.

While choosing the solvent for the present study, these important factors were taken into consideration. Methanol was the solvent of choice.

ii) **Procedure for Extraction:**
Hot Continuous Extraction (Soxhlet) procedure was adapted for extraction. Advantage of Soxhlet extraction is the displacement of transfer equilibrium by repeatedly bringing fresh solvent into contact with solid matrix. No filtration of the extract is required as the plant material is held in filter paper.

Soxhlet apparatus consists of a flask, a Soxhlet extractor and a reflux condenser. Advantage of this method, compared to other methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent by this procedure. Finely ground powder of dried leaves was placed in a thimble made of strong filter paper and wrapped with muslin cloth. It was placed in wide central chamber of the Soxhlet apparatus. Extracting solvent was placed in the flask and heated up to boiling point. Vapors of solvent passed through larger right hand tube into the upper part of extractor and then to condenser where vapors condensed and dropped back onto the raw material. During this period, soluble constituents were extracted in solvent. When level of extract reached top of siphon tube, entire volume of extract siphoned over into flask. Cycles of extraction were continued till tissue debris was completely free of green colour as it can be assumed that at such point, all the low molecular weight compounds have been extracted (Harborne, 1984). This procedure is thus a series of short macerations. Suitable precaution was taken to avoid higher than required temperature. Insufficient extraction time means incomplete extraction and with longer
extraction time, unwanted constituents may also be extracted. Extract in the flask was processed further.

In the present study, the optimum time for extraction was found to be 36 hours spread over three days when 15 grams of powder was extracted with about 250 ml of Methanol. It yielded about 3 grams of dried extract. Concentration and drying was performed under normal room temperature and pressure conditions, to ensure stability of active constituents. It required about 5 days for complete drying of extract.

3.2.2: Fingerprinting With Respect To Four Groups of Secondary Metabolites:-

Thin Layer Chromatography (TLC), is a widely accepted and extensively used separation technique as it is simple, cost effective and versatile. It can be employed in any given situation of qualitative, quantitative or preparative separation. There is no substitute for TLC technique for qualitative analyses of plant extracts. Multiple sample analysis and low cost per sample are main advantages of TLC. Two prominent uses of TLC in standardization of plant materials include fingerprint profiling for assessment of Phytoconstituents of extract and quantitative analysis thereof. (Stahl Egon, 2007).

In the present study, HPTLC technique, which is an advance version of TLC, is extensively used to obtain Fingerprints of 4 classes of Secondary Metabolites present in Methanolic Extract of leaves. Fingerprint analysis gives idea about $R_f$ of separated Phytoconstituents and their proportion in extract.

i) Sample Preparation:
Crude Methanolic Extract of dry leaves was obtained as described earlier. Solvent was completely removed from Extract by natural evaporation. Stock solution was prepared by dissolving 1 gram dried extract in 20 ml Methanol and was homogenized using vortex. Such stock solution was used to prepare solutions of different concentrations in order to find optimum concentration for fingerprinting.

ii) Selection of Chromatographic Plate:
For the present study, precoated plates made of aluminum base coated with normal phase silica gel (20 x 20 cm) were used. Normal phase silica gel is more suited for non-polar components. Specifications of plates used are: TLC Silica gel 60 F$_{254}$ by MERCK Germany (Product Code 1.05554.0007).
iii) Selection of Mobile Phase:
Infinite combinations of wide choice of solvents are available for TLC development. Mobile phase with 1 to 3 components is preferred over a multicomponent mobile phase. Polarity of the compounds of interest is the key to selection of a mobile phase. In the present study, trial and error method was used to select the composition of mobile phase for all the four classes of phytoconstituents. Details of Mobile Phases which resulted in best separation and the derivatizing agents used for visualization are given in Table 3.1.

<table>
<thead>
<tr>
<th>Solvent System Code No</th>
<th>Group of Secondary Metabolites</th>
<th>Solvent System as Mobile Phase</th>
<th>Derivatizing Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>Alkaloids</td>
<td>Toluene: ethyl acetate : diethyl amine (7 : 2 : 1)</td>
<td>Dragendorff Reagent Plate heated at 110°C for about 10 minutes</td>
</tr>
<tr>
<td>A2</td>
<td>Alkaloids</td>
<td>Cyclohexene : diethyl amine (7 : 3)</td>
<td>Dragendorff Reagent Plate heated at 110°C for about 10 minutes</td>
</tr>
<tr>
<td>F1</td>
<td>Flavonoids</td>
<td>Ethyl acetate: formic acid : glacial acetic acid: water (10: 0.5: 0.5: 1.3)</td>
<td>Anisaldehyde+H₂SO₄ Plate heated at 110°C for about 10 minutes</td>
</tr>
<tr>
<td>S1</td>
<td>Saponins</td>
<td>Chloroform: glacial acetic acid: methanol: water (6.4 : 3.2 : 1.2 : 0.8)</td>
<td>Anisaldehyde+H₂SO₄ Plate heated at 110°C for about 10 minutes</td>
</tr>
<tr>
<td>T1</td>
<td>Triterpenoids</td>
<td>Petroleum ether (40-60) : isopropanol (12 : 1)</td>
<td>Anisaldehyde+H₂SO₄ Plate heated at 110°C for about 10 minutes</td>
</tr>
<tr>
<td>T2</td>
<td>Triterpenoids</td>
<td>Toluene: ethyl acetate: methanol (7 : 1 : 0.5)</td>
<td>Anisaldehyde+H₂SO₄ Plate heated at 110°C for about 10 minutes</td>
</tr>
<tr>
<td>T3</td>
<td>Triterpenoids</td>
<td>n-hexane: ethyl acetate (1 : 1)</td>
<td>Anisaldehyde+H₂SO₄ Plate heated at 110°C for about 10 minutes</td>
</tr>
</tbody>
</table>

Table 3.1: List of Mobile Phases and Derivatizing agents used for Fingerprinting.
iv) Application of Sample:

Pilot study was performed to find best suited Mobile Phases for Alkaloids, Flavonoids, Saponins and Triterpenoids. Narrow strips of size 3cm X 10cm were cut from TLC plates and used in pilot study. Pencil lines were marked at 1.5 cm from bottom for loading of sample and at 2 cm from the top to indicate the maximum running of the solvent front. To achieve optimum concentration of sample in solvent, various concentrations were tried. Manual application of sample was executed by the technique of touch and deliver, using a graduated micropipette of 10 μl capacity and with beveled tips. 1, 2 or 5 μl volumes were tried for quantitative purposes. Precision and accuracy was fairly high. Experiment was carried out on TLC Silica gel 60 F$_{254}$ plates.

After optimum concentration and volume was arrived at, semi-automatic application device CAMAG Linomat 5 was used for application of sample on HPTLC plates. The device had following application parameters: Spray gas: Inert gas; Sample solvent type: Methanol; Dosage speed: 150 nl / s; Pre dosage volume: 0.2 μl and Syringe size: 100 μl. Syringe was manually cleaned with Methanol and was filled with required volume of sample. The sample was uniformly dissolved in Methanol so as to avoid blocking of the fine needle. Bubbles were removed by gentle tapping to ensure uniform application. Syringe was inserted in the slot. A 10cm X 10cm plate was marked at 2 cm from top to indicate the maximum run of solvent front. Plate was placed on the board and was properly secured. Application of sample was by spray-on technique and was automated through computer commands. Y position was at 8.0 mm and band length was 8.0 mm with predetermined gaps in between. Predetermined volumes i.e. 5 μl, 10 μl and 20 μl were sprayed as separate bands onto the plate. Bands gave better resolution and results than spots, because a narrow band was better suited to the optics of the TLC scanner. The experiment was carried out personally.

v) Development of the plate to obtain Chromatograms:

Mobile phase was freshly prepared for each run, by mixing constituting solvents in a conical flask with stopper. Twin trough developing chamber was used for ascending development of the loaded plate. 20 ml of mobile phase was transferred to developing
chamber and levels in both troughs were equalized by tilting the chamber. Chamber was closed and was allowed to stand at room temperature for 15 minutes for saturation. Saturation of the chamber was quickened by lining one wall from inside with filter paper and pouring Mobile Phase over it. Side distortion of solvent front was corrected by saturation of chamber before development. Chamber saturation resulted into decreased R_F values. Loaded plate was placed as nearly vertical as possible in the chamber, ensuring that points of application were above the level of Mobile Phase and sides of plate did not touch container walls. TLC results are sensitive to temperature and humidity variations because R_F values vary with temperature, degree of saturation, activity of adsorbent layer and composition of Mobile phase. To avoid such variations, plates while not in use, were stored in desiccators. Development of plates was performed under controlled temperature of 20°-30° C so as to avoid oblique running of solvent resulting from difference in temperatures. Y position of application was 8.0 mm. Mobile phase was allowed to run up to 80 mm mark and time for run was noted down.

*Improvement in quality of development of plates was achieved by using precoated commercially available HPTLC plates, selecting correct concentrations of sample and by using correct instrument parameters.*

vi) *Drying of Developed Plate:*
After development, the plate was dried by blowing hot-air over surface of the plate. Mobile phase was completely removed before proceeding to derivatization or scanning of Chromatograms.

vii) *Derivatization:*
Derivatization involves treatment of developed Chromatograms with suitable spray reagents to locate positions of constituents which are neither detected under UV 254 nm, 366 nm and 580 nm nor under visible light. Qualitative evaluation and quantifying Ultraviolet-insensitive markers can be achieved for such constituents. Dipping of plate produces uniform wetting as against spraying of reagent and results into better performance. Hence derivatization of plates was executed by dipping of
plate in a specially built chamber filled with appropriate derivatizing reagent (Table 3.1). After derivatization, plate was dried to remove reagent. The plate was heated on special purpose heating plate to achieve uniform heating, at 110°C for about 10 min or until clear spots appeared.

viii) **Visualization and Evaluation of Chromatograms:**
As a result of good resolution, separated fractions can be distinctly identified and a Chromatogram can be evaluated. But all separated Phytoconstituents may not be visible under white light. So as to “see” all separated fractions, following method was employed.

a. Developed HPTLC plate was observed under short-wave and long-wave Ultraviolet light (254 nm, 366 nm and 580 nm) for visualizing Fluorescent Compounds.

b. As the HPTLC plate was coated with fluorescent silica gel, on exposure to UV short wavelength 254 nm, some constituents absorbed this region of frequency and stood out distinctly as dark bands on the green fluorescent layer as background.

c. Plates were derivatized with suitable reagents and heated to give colored and/or fluorescent products. Such plates were again visualized under UV short and long wavelengths.

d. Plates were also visualized under visible light, both before and after derivatization.

All the steps listed until now under section 3.2.2 are followed to obtain Fingerprints and Chromatograms of

- Alkaloids with Solvent Systems A1 and A2
- Flavonoids with Solvent System F1
- Saponins with Solvent System S1
- Triterpenoids with three Solvent Systems T1, T2 and T3 (Table 3.1).

Quantitative evaluation was achieved by scanning the plates with HPTLC scanner. Ultraviolet, Fluorescence and visible light absorption modes or Chromatograms were recorded with scanner. Data acquisition and analysis was done with the help of standard PC-based software. Scanning and recording of Chromatograms was
performed on CAMAG TLC Scanner "Scanner_170422" S/N 170422 for UV 254 nm, 366 nm and 580 nm. On providing the data like distance of point of application and distance run by solvent front, \( R_F \) values for various bands were calculated automatically. Relative concentrations of individual Phytoconstituents in the sample were obtained automatically from area under curve with the help of winCATS Planar Chromatography Manager. Scanning of plates was performed before as well as after derivatization.

Only a few isolated examples of Alkaloids have been reported from Zingiberaceae family (Trease and Evans 1987). But earlier study has reported presence of Alkaloids in Methanolic Extract of the leaves (Nandhakumar Jothivel et al., 2007). So as to test presence of Alkaloids in Methanolic Extract, Fingerprinting of Alkaloids was undertaken by using two Solvent Systems A1 and A2 as Mobile Phases (Table 3.1).

The following pages contain Fingerprints and the analysis thereof for four major Groups of Secondary Metabolites under study. The Fingerprints, Chromatograms and tables are bearing the numbers as shown below:

- Figure numbers 3.2 a to c indicate Alkaloids Fingerprint with A1 Mobile phase, under UV 254 nm, 366 nm and visible light before derivatization and Figure 3.2d under visible light after derivatization. Figure numbers 3.2 e to g indicate Chromatograms under UV 254 nm, 366 nm and 580 nm before derivatization. Table numbers 3.2e to g list \( R_F \) values and areas under the curves for various fractions of Alkaloids

The number patterns for other Fingerprints are similar to the above mentioned pattern.

- Figures 3.3a to g and Tables 3.3 e to g are for Alkaloids (Mobile Phase A2).
- Figures 3.4a to g and Tables 3.4 e to g are for Flavonoids (Mobile Phase F1)
- Figures 3.5a to g and Tables 3.5e to g are for Saponins (Mobile Phase S1).
- Figures 3.6a to g and Tables 3.6e to g are for Triterpenoids (Mobile Phase T1)
- Figures 3.7a to g and Tables 3.7e to g are for Triterpenoids(Mobile Phase T 2)
- Figures 3.8a to g and Tables 3.8e to g are for Triterpenoids (Mobile Phase T3)
Finger Printing of Alkaloids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent system A1)

**Figures 3.2 a, b and c:** Fingerprints of Alkaloids with Solvent system A1 under UV 254 nm, 366 nm and Visible light respectively, before derivatization.

**Figure 3.2 d:** Fingerprints of Alkaloids under Visible light, after derivatization.
Chromatograms of Alkaloids present in Methanolic Extract of leaves of 

Costus pictus D. Don (Solvent System A1)

![Chromatogram](image)

**Figure 3.2e:** Chromatogram for Alkaloids under UV 254 nm

<table>
<thead>
<tr>
<th>Peak</th>
<th>Start Rf</th>
<th>Start Height</th>
<th>Max Rf</th>
<th>Max Height</th>
<th>Max %</th>
<th>End Rf</th>
<th>End Height</th>
<th>Area</th>
<th>Area %</th>
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<td>0.15</td>
<td>38.9</td>
<td>8.74</td>
<td>0.20</td>
<td>0.3</td>
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<td>1.2</td>
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<td>9.0</td>
<td>165.8</td>
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</tr>
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**Table 3.2e:** Analysis of Chromatogram for Alkaloids under UV 254 nm

![Chromatogram](image)

**Figure 3.2f:** Chromatogram for Alkaloids under UV 366 nm
Chromatograms of Alkaloids present in Methanolic Extract of leaves of

*Costus pictus D. Don* (Solvent System A1)

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*Table 3.2f: Analysis of Chromatogram for Alkaloids under UV 366 nm*

![Chromatogram for Alkaloids under UV 366 nm](image)

*Figure 3.2g: Chromatogram for Alkaloids under UV 580 nm*

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*Table 3.2g: Analysis of Chromatogram for Alkaloids under UV 580 nm*
**Finger Printing of Alkaloids present in Methanolic Extract of leaves of *Costus pictus* D. Don** (Solvent system A2)

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<tr>
<td>![Image of before derivatization]</td>
<td>![Image of after derivatization]</td>
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</table>

**Figures 3.3a, 3.3b and 3.3c**: Fingerprints of Alkaloids with Solvent system A2 under UV 254 nm, 366 nm and Visible light respectively, before derivatization.

**Figure 3.3d**: Fingerprints of Alkaloids under Visible light, after derivatization.
Chromatograms of Alkaloids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent System A2)

**Figure 3.3e:** Chromatogram for Alkaloids under UV 254 nm

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**Table 3.3e:** Analysis of Chromatogram for Alkaloids under UV 254 nm

**Figure 3.3f:** Chromatogram for Alkaloids under UV 366 nm
Chromatograms of Alkaloids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent System A2)

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*Table 3.3f*: Analysis of Chromatogram for Alkaloids under UV 366 nm

![Chromatogram for Alkaloids under UV 366 nm](image)

*Figure 3.3g*: Chromatogram for Alkaloids under UV 580 nm

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*Table 3.3g*: Analysis of Chromatogram for Alkaloids under UV 580 nm
Finger Printing of Flavonoids present in Methanolic Extract of leaves of *Costus pictus D. Don* (Solvent system F1)

Before Derivatization

After Derivatization

**Figures 3.4a**  
Under 254 nm

**3.4b**  
366 nm

**3.4c**  
Visible light

**3.4d**  
Visible light

*Figures 3.4 a, b and c:* Fingerprints of Flavonoids with Solvent system F1 under UV 254 nm, 366 nm and Visible light respectively, before derivatization.

*Figure 3.4d:* Fingerprints of Flavonoids under Visible light, after derivatization.
Chromatograms of Flavonoids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent System F1)

**Figure 3.4e:** Chromatogram for Flavonoids under UV 254 nm

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**Table 3.4e:** Analysis of Chromatogram for Flavonoids under UV 254 nm

**Figure 3.4f:** Chromatogram for Flavonoids under UV 366 nm
Chromatograms of Flavonoids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent system F1)

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*Table 3.4f: Analysis of Chromatogram for Flavonoids under UV 366 nm*

![Chromatogram for Flavonoids under UV 366 nm]

*Figure 3.4g: Chromatogram for Flavonoids under UV 580 nm*

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*Table 3.4g: Analysis of Chromatogram for Flavonoids under UV 580 nm*
Finger Printing of Saponins present in Methanolic Extract of leaves of *Costus pictus D. Don* (Solvent system S1)

**Figures 3.5a, 3.5b, 3.5c and 3.5d:**

- **Figures 3.5a and 3.5d:** Fingerprinting results before derivatization.
- **Figures 3.5b and 3.5c:** Fingerprinting results after derivatization.

**Figures 3.5a, 3.5b and 3.5c:** Fingerprints of Saponins with Solvent system S1 under UV 254 nm, 366 nm and Visible light respectively, before derivatization.

**Figure 3.5d:** Fingerprints of Saponins under Visible light, after derivatization.
Chromatograms of Saponins present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent System S1)

![Graph showing chromatogram for Saponins under UV 254 nm](image)

*Figure 3.5e* Chromatogram for Saponins under UV 254 nm

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*Table 3.5e* : Analysis of Chromatogram for Saponins under UV 254 nm

![Graph showing chromatogram for Saponins under UV 366nm](image)

*Figure 3.5f* : Chromatogram for Saponins under UV 366nm
Chromatograms of Saponins present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent System S1)

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*Table 3.5f*: Analysis of Chromatogram for Saponins under UV 366 nm

![Chromatogram](image)

*Figure 3.5g*: Chromatogram for Saponins under UV 366nm

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*Table 3.5g*: Analysis of Chromatogram for Saponins under UV 580 nm
Finger Printing of Triterpenoids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent system T1)

**Figures 3.6a, b and c:** Fingerprints of Triterpenoids with Solvent system T1 under UV 254 nm, 366 nm and Visible light respectively, before derivatization.

**Figure 3.6d:** Fingerprints of Triterpenoids under Visible light, after derivatization.
Chromatograms of Triterpenoids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent System T1)

![Graph](image1)

**Figure 3.6e:** Chromatogram for Triterpenoids under UV 254 nm

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**Table 3.6e:** Analysis of Chromatogram for Triterpenoids under UV 254 nm

![Graph](image2)

**Figure 3.6f:** Chromatogram for Triterpenoids under UV 366 nm
Chromatograms of Triterpenoids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent System T1)

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*Table 3.6f: Analysis of Chromatogram for Triterpenoids under UV 366 nm*

![Chromatogram under UV 366 nm](image)

*Figure 3.6g: Chromatogram for Triterpenoids under UV 580 nm*

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*Table 3.6g: Analysis of Chromatogram for Triterpenoids under UV 580 nm*
Finger Printing of Triterpenoids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent System T2)

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*Figures 3.7 a, b and c:* Fingerprints of Triterpenoids with Solvent system T2 under UV 254 nm, 366 nm and visible light respectively, before derivatization.

*Figure 3.7 d:* Fingerprints of Triterpenoids under visible light, after derivatization.
Chromatogram of Triterpenoids present in Methanolic Extract of leaves of Costus pictus D. Don (Solvent System T2)

Figure 3.7e: Chromatogram for Triterpenoids under UV 254 nm

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Table 3.7e: Analysis of Chromatogram for Triterpenoids under UV 254 nm

Figure 3.7f: Chromatogram for Triterpenoids under UV 366 nm
Chromatogram of Triterpenoids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent System T2)

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*Table 3.7f: Analysis of Chromatogram for Triterpenoids under UV 366 nm*

![Graph showing UV 366 nm analysis](image)

*Figure 3.7g: Chromatogram for Triterpenoids under UV 580 nm*

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*Table 3.7g: Analysis of Chromatogram for Triterpenoids under UV 580 nm*
Finger Printing of Triterpenoids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent system T3)

![Before Derivatization](image1)
![After Derivatization](image2)

**Figures 3.8a, b and c:** Fingerprints of Triterpenoids with Solvent system T3 under UV 254 nm, 366 nm and Visible light respectively, before derivatization.

**Figure 3.8d:** Fingerprints of Triterpenoids under Visible light, after derivatization.
Chromatograms of Triterpenoids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent System T3)

**Figure 3.8e:** Chromatogram for Triterpenoids under UV 254 nm

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**Table 3.8e:** Analysis of Chromatogram for Triterpenoids under UV 254 nm

**Figure 3.8f:** Chromatogram for Triterpenoids under UV 366 nm
Chromatograms of Triterpenoids present in Methanolic Extract of leaves of *Costus pictus* D. Don (Solvent System T3)

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*Table 3.8f: Analysis of Chromatogram for Triterpenoids under UV 366 nm*

![Figure 3.8g: Chromatogram for Triterpenoids under UV 580 nm](image)

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*Table 3.8g: Analysis of Chromatogram for Triterpenoids under UV 580 nm*
3.2.3: Isolation and Analysis of Fractions:

Knowledge about active components of any plant is ever expanding and always incomplete. For analysis of Phytoconstituents with modern techniques, sufficient quantity of pure fraction is required. Isolation of Phytoconstituents from crude extract is generally carried out by employing any one of the Chromatographic Techniques.

3.2.3a: Isolation of Fractions:

The suitability of chromatographic method depends upon the class of compounds to be separated. These methods overlap as far as their applications are concerned. A combination of techniques may be the best approach to separate a particular class of compounds. When fractions are to be further analyzed, quantities in milligrams or grams are to be collected. Due to low thickness of silica layer, there is an upper limit on initial loading of sample on Analytical HPTLC plates. It is found that separations were often less good if loading was increased. Solution to this problem is to increase thickness of Silica gel layer and the size of plate. These features are present in Preparative TLC (PTLC) plates. The Mobile Phase developed with Analytical TLC plates can be successfully used for PTLC plates as well (Kowalska et al., 2009).

In the present study, Preparative TLC plates with silica gel 60 F_{254} of 1 mm thickness on Glass Base from Merck Germany (product code 1.13895.0001) were used. Analytical TLC plates with silica gel thickness of 0.25 mm on Aluminum Base also were employed. Isolation of 10 fractions of Triterpenoids was accomplished.

Mobile Phase was developed for Triterpenoids class of compounds on analytical TLC plates as described above. Out of the three Solvent Systems, T2 (Toluene: Ethyl acetate: Methanol (7:1:0.5)) was used as Mobile Phase for both categories of plates. Size of plate, Amount of Mobile Phase, Chamber Saturation Time, Amount of Sample to be loaded, length of Applied Bands, Run of Solvent Front, Mechanical Adjustments for Height of Applicator and Syringe were optimized for both the plates (Table 3.19).
3.2.3b: Collection of fractions:

After development of the plates, visible bands were marked with pencil. Plates were kept under UV and fluorescent bands as well as dark quenching bands were marked. Derivatization alters the original compound but is a necessity to visualize colorless compounds. To overcome this difficulty, a small band of 8 mm was laid along with 140 mm main experimental band. A gap of 8 mm was left between the two bands as insulation band. As both bands were on the same plate, they developed identically. Only the 8 mm band was derivatized and larger potion of plate was insulated. Colored bands as well as quenching bands developed in the derivatized portion. Bands were marked on the portion which was not derivatized by drawing parallel lines with pencil by keeping bands on derivatized portion as reference. Pencil marks were labeled appropriately. The derivatized portion was immediately discarded in order to avoid any alteration of main portion. The Analytical TLC plates as well as Preparative TLC plates were used during the course of study.

*Figure 3.7h:* Fractions marked under UV 254 nm

*Figure 3.7i:* Fractions marked under visible light after Derivatization
i. Analytical TLC plates:

All the developed ATLC plates with Aluminum base were cut in parallel strips with scissors and strips containing same fraction were collected separately. The strips were further cut in smaller pieces. The pieces holding separate fractions were kept in different flasks with stoppers. Mobile Phase originally used for ATLC was added in the flasks so that the pieces submerged completely. The flasks were kept on Sonicator for 5 cycles so as to shake and agitate the pieces and facilitate elution of fraction back in Mobile Phase. The flasks were allowed to stand overnight so as to ensure complete elution of fractions adsorbed on the silica layer into the solvent system. The Solvent was filtered with Whatman filter paper to separate the residual adsorbent. The filtrate was collected in porcelain dish and the solvent was allowed to evaporate naturally. The residues were isolated fractions.

ii. Preparative TLC plates:

When PTLC plates were used, procedure followed for loading, development, derivatization, marking of strips and elution of fraction in mobile phase was identical to that of Analytical TLC plates except the differences in quantities collected and the time required for various stages. Fraction located in the scribed area of the plate was recovered by scratching and removing the portion of adsorb zone from the Glass Base. The outlined areas of the layer containing Phytoconstituents of interest were scraped off cleanly down to the glass backing on the plate with the help of scalpel or razor blade. The layer was dampened little bit with the mobile phase before scratching so as to avoid loss of fine adsorbent particles by flaking and blowing during the scraping and transfer steps. Area about 10% larger than visualized sample area was scraped to compensate for three dimensional development of the zone in the layer due to greater thickness of adsorbent layer. Loosened adsorbent was transferred to a flask fitted with solvent resistance cap and mobile phase was added to it to elute the fraction. Absolute Methanol as a single solvent was not used at this stage because silica gel and its common impurities are soluble in Methanol. After filtration, Mobile phase was allowed to evaporate naturally leaving behind the fractions in solid form (Kowalska and Sherma (ed.), 2009). The Fractions in solid form so collected were subjected to FTIR analysis.
3.2.3c: Analysis of Fractions with FTIR Technique:

Infrared Spectroscopy gives information about functional groups present in an organic molecule and also identifies the vibrational and rotational modes of motion in a molecule. IR provides a unique fingerprint of a compound, readily distinguishable from the absorption patterns of other compounds. Hence FTIR is an important technique for identification and characterization of a substance. Fractions isolated during the course of present study were subjected to FTIR analysis.

Fourier Transform Infrared Spectrometer was used to obtain FTIR transmittance graphs. The Instrument Details are as follows:
Make: Nicolet Instruments Corporation, USA;
Model: MAGNA 550;
Specification: Range of wave numbers: 4000 cm\(^{-1}\) to 500 cm\(^{-1}\).

All 10 samples were in solid form. A small quantity of every sample was ground finely with a purified salt, KBr. Fine grinding removes scattering effects from large crystals. Such powder mixture was then pressed in a mechanical press to form a translucent pellet. The pellet was exposed to IR radiation over the range of wave numbers from 4000 cm\(^{-1}\) to 400 cm\(^{-1}\) and computerized spectra were obtained and analyzed. Fractions 1 to 5 were marked under UV 254 nm. Fractions A to E were marked under visible light, corresponding to color bands developed on derivatization, in derivatized portion of the TLC plate. FTIR spectra for Fractions 1 to 5 and A to E are represented by Figures 3.9 to 3.18 respectively. Analysis of FTIR spectra for Fractions 1 to 5 and A to E is as per Tables 3.9 to 3.18 respectively.
Figure 3.9: FTIR for Triterpenoid Fraction 1 isolated from Methanolic Extract of Leaves of Costus pictus

<table>
<thead>
<tr>
<th>Wavenumber cm(^{-1})</th>
<th>Function Groups</th>
<th>Bonds</th>
</tr>
</thead>
<tbody>
<tr>
<td>3424.28</td>
<td>alcohol, phenols</td>
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</tr>
<tr>
<td>2927.33</td>
<td>alkanes</td>
<td>C-H stretch</td>
</tr>
<tr>
<td>2856.59</td>
<td>alkanes carboxylic acids</td>
<td>C-H stretch</td>
</tr>
<tr>
<td></td>
<td>carboxylic acids</td>
<td>O-H stretch</td>
</tr>
<tr>
<td>1737.70</td>
<td>carboxyls(general) carboxylic acids esters, saturated</td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td>aliphatic aldehydes, saturated aliphatic</td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C=O stretch</td>
</tr>
<tr>
<td>1673.51</td>
<td>carboxyls(general)</td>
<td>C=O stretch</td>
</tr>
<tr>
<td>1569.24</td>
<td>aromatic amines, amides Nitr</td>
<td>C=C stretch</td>
</tr>
<tr>
<td></td>
<td>Nitro</td>
<td>N-H bend</td>
</tr>
<tr>
<td>1417.24</td>
<td>aromatic</td>
<td>C-C stretch(in ring)</td>
</tr>
<tr>
<td>1108.49</td>
<td>aliphatic amines</td>
<td>C-N stretch</td>
</tr>
<tr>
<td>797.38</td>
<td>alkyl halide</td>
<td>C-Cl stretch</td>
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<tr>
<td>619.68</td>
<td>alkyl halide</td>
<td>C-Br stretch</td>
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</table>

Table 3.9: Functional Groups and Bonds in Fraction 1 responsible for IR absorption
Figure 3.10: FTIR for Triterpenoid Fraction 2 isolated from Methanolic Extract of Leaves of Costus pictus

<table>
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<tr>
<td>2959.63</td>
<td>alkane</td>
<td>C-H stretch</td>
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<td>2929.56</td>
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</tr>
<tr>
<td>2857.65</td>
<td>alkane</td>
<td>C-H stretch</td>
</tr>
<tr>
<td>1737.24</td>
<td>carbonyls(general) carboxylic acids esters, saturated aliphatic aldehydes, saturated aliphatic</td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C=O stretch</td>
</tr>
<tr>
<td>1578.74</td>
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<td>N-H bend</td>
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<td>Nitro</td>
<td>N=O stretch</td>
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<tr>
<td>1460.91</td>
<td>alkane</td>
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<td></td>
<td>aromatic</td>
<td>C-C stretch(in ring)</td>
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<td>1412.56</td>
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<td>C-C stretch(in ring)</td>
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<td>1100.26</td>
<td>aliphatic amines carboxylic acids alcohols, carboxylic acids esters, ethers</td>
<td>C-N stretch</td>
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<td></td>
<td>C-O stretch</td>
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<td></td>
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<td>C-O stretch</td>
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</tbody>
</table>

Table 3.10: Functional Groups and Bonds in Fraction 2 responsible for IR absorption
**Figure 3.11:** FTIR for Triterpenoid Fraction 3 isolated from Methanolic Extract of Leaves of *Costus pictus*

<table>
<thead>
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<th>Bonds</th>
</tr>
</thead>
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<td>2958.32</td>
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<td></td>
<td>carboxylic acids</td>
<td>O-H stretch</td>
</tr>
<tr>
<td>2927.76</td>
<td>alkanes</td>
<td>C-H stretch</td>
</tr>
<tr>
<td></td>
<td>carboxylic acids</td>
<td>O-H stretch</td>
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<tr>
<td>2855.41</td>
<td>alkanes</td>
<td>C-H stretch</td>
</tr>
<tr>
<td></td>
<td>carbonyls (general)</td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td>carboxylic acids</td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td>esters, saturated aliphatic aldehydes</td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td>saturated aliphatic</td>
<td></td>
</tr>
<tr>
<td>1737.40</td>
<td>amines, amides</td>
<td>N-H bend</td>
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<tr>
<td></td>
<td>Nitro</td>
<td>N=O stretch</td>
</tr>
<tr>
<td>1420.79</td>
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<td>C-C stretch (in ring)</td>
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<td></td>
<td>aliphatic amines</td>
<td>C-N stretch</td>
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<td>1105.68</td>
<td>alcohols, carboxylic acids</td>
<td>C-O stretch</td>
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<td>esters, ethers</td>
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<tr>
<td>799.52</td>
<td>alkyl halide</td>
<td>C-Cl stretch</td>
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**Table 3.11:** Functional Groups and Bonds in Fraction 3 responsible for IR absorption
Figure 3.12: FTIR for Triterpenoid Fraction 4 isolated from Methanolic Extract of Leaves of Costus pictus

<table>
<thead>
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<td>2926.38</td>
<td>alkanes, carboxylic acids</td>
<td>C-H stretch</td>
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<tr>
<td>2859.31</td>
<td>alkanes, carboxylic acids</td>
<td>O-H stretch</td>
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<tr>
<td>1730.01</td>
<td>carbonyls(general) carboxylic acids aldehydes, saturated aliphatic</td>
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<td>1644.85</td>
<td>alkenes 1° amines</td>
<td>-C=C-stretch, N-H bend</td>
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<td>1570.70</td>
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<td>N-H bend</td>
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<td>1452.05</td>
<td>alkanes</td>
<td>C-H bend</td>
</tr>
<tr>
<td>1233.54</td>
<td>aliphatic amines alkyl halides</td>
<td>C-N stretch, C-H wag (-CH₂X)</td>
</tr>
<tr>
<td>1160.37</td>
<td>aliphatic amines alkyl halides</td>
<td>C-N stretch, C-H wag (-CH₂X)</td>
</tr>
<tr>
<td>1029.07</td>
<td>aliphatic amines</td>
<td>C-N stretch</td>
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Table 3.12: Functional Groups and Bonds of Fraction 4 responsible for IR absorption
Figure 3.13: FTIR for Triterpenoid Fraction 5 isolated from Methanolic Extract of Leaves of Costus pictus

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<tr>
<td>1718.54</td>
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<td>C=O stretch</td>
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<tr>
<td></td>
<td>saturated aliphatic</td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td>α, β unsaturated esters</td>
<td>C=O stretch</td>
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<tr>
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<td>carboxylic acid</td>
<td>C=O stretch</td>
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<tr>
<td>1662.72</td>
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<td>-C=C-stretch</td>
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<td>aromatic amine</td>
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<td>alcohols, esters, ethers</td>
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<td></td>
<td>aliphatic amines</td>
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Table 3.13: Functional Groups and Bonds of Fraction 5 responsible for IR absorption
**Figure 3.14:** FTIR for Triterpenoid Fraction A isolated from Methanolic Extract of Leaves of *Costus pictus*

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<td>2925.78</td>
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<td>O-H stretch</td>
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<tr>
<td>2857.26</td>
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<td>C-H stretch</td>
</tr>
<tr>
<td>1731.77</td>
<td>carboxyls(general) carboxylic acids</td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td>aldehydes, saturated aliphatic</td>
<td>C=O stretch</td>
</tr>
<tr>
<td>1656.76</td>
<td>alkenes</td>
<td>-C=C- stretch</td>
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<tr>
<td>1566.67</td>
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<tr>
<td>1451.97</td>
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<tr>
<td>1417.82</td>
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</tr>
<tr>
<td>1379.57</td>
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<td>---</td>
</tr>
<tr>
<td>1262.28</td>
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<td>C-H wag (-CH₂X)</td>
</tr>
<tr>
<td>1119.40</td>
<td>alcohols, esters, carboxyl acids, ethers</td>
<td>C-O stretch</td>
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</table>

**Table 3.14:** Functional Groups and Bonds of Fraction A responsible for IR absorption
Figure 3.15: FTIR for Triterpenoid Fraction B isolated from Methanolic Extract of Leaves of Costus pictus

<table>
<thead>
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<th>Wavenumber cm(^{-1})</th>
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<td>2856.00</td>
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<td>O-H stretch, C-H stretch</td>
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<td>1731.83</td>
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<td>carboxylic acids</td>
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</tr>
<tr>
<td></td>
<td>aldehydes, saturated aliphatic</td>
<td></td>
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<tr>
<td>1620.65</td>
<td>1(^0) amines</td>
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<td>1556.49</td>
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<td>1446.50</td>
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Table 3.15: Functional Groups and Bonds of Fraction B responsible for IR absorption
Figure 3.16: FTIR for Triterpenoid Fraction C isolated from Methanolic Extract of Leaves of Costus pictus

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<td>O-H stretch</td>
</tr>
<tr>
<td>2856.67</td>
<td>carboxylic acid alkanes</td>
<td>C-H stretch</td>
</tr>
<tr>
<td>1730.98</td>
<td>carbonyls (general) carboxylic acids, aldehydes, saturated aliphatic</td>
<td>C=O stretch</td>
</tr>
<tr>
<td>1570.51</td>
<td>aromatic alkenes Nitro amines, amides</td>
<td>C-C stretch, C-H bend N=O stretch N-H bend</td>
</tr>
<tr>
<td>1430.27</td>
<td>aromatic</td>
<td>C-C stretch (in ring)</td>
</tr>
<tr>
<td>1376.98</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>1249.82</td>
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<tr>
<td>1163.47</td>
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Table 3.16: Functional Groups and Bonds of Fraction C responsible for IR absorption
Figure 3.17: FTIR for **Triterpenoid Fraction D** isolated from Methanolic Extract of Leaves of *Costus pictus*

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</tr>
</thead>
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<td>carboxylic acid alkanes</td>
<td>O-H stretch</td>
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<td>2855.62</td>
<td>carboxylic acid alkanes</td>
<td>C-H stretch</td>
</tr>
<tr>
<td>1732.86</td>
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<td>1570.54</td>
<td>alkenes Nitro amines, amides</td>
<td>C-H bend, N=O stretch, N-H bend</td>
</tr>
<tr>
<td>1426.71</td>
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<td>C-C stretch (in ring)</td>
</tr>
<tr>
<td>1246.85</td>
<td>aliphatic amines</td>
<td>C-N stretch</td>
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</table>

Table 3.17: Functional Groups and Bonds of **Fraction 9** responsible for IR absorption
Figure 3.18: FTIR for Triterpenoid Fraction E isolated from Methanolic Extract of Leaves of Costus pictus

<table>
<thead>
<tr>
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<th>Bonds</th>
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<tbody>
<tr>
<td>3428.40</td>
<td>alcohol, phenols</td>
<td>O-H stretch, H-bonded</td>
</tr>
<tr>
<td>2925.69</td>
<td>carboxylic acid</td>
<td>O-H stretch</td>
</tr>
<tr>
<td></td>
<td>alkanes</td>
<td>C-H stretch</td>
</tr>
<tr>
<td>2857.52</td>
<td>carboxylic acid</td>
<td>O-H stretch</td>
</tr>
<tr>
<td></td>
<td>alkanes</td>
<td>C-H stretch</td>
</tr>
<tr>
<td>1722.64</td>
<td>carbonyls(general)</td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td>carboxylic acids</td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td>aldehydes,</td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td>saturated aliphatic</td>
<td>C=O stretch</td>
</tr>
<tr>
<td></td>
<td>(\alpha, \beta) unsaturated esters</td>
<td>C=O stretch</td>
</tr>
<tr>
<td>1640.39</td>
<td>alkenes</td>
<td>-C=C-stretch</td>
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<tr>
<td>1566.85</td>
<td>aromatic</td>
<td>C=C stretch</td>
</tr>
<tr>
<td></td>
<td>amines, amides</td>
<td>N-H bend</td>
</tr>
<tr>
<td>1412.28</td>
<td>aromatic</td>
<td>C-C stretch (in ring)</td>
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<tr>
<td>1024.03</td>
<td>alcohols, carboxylic acids</td>
<td>C-O stretch</td>
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<tr>
<td></td>
<td>esters, ethers</td>
<td>C-O stretch</td>
</tr>
<tr>
<td>787.91</td>
<td>alkenes</td>
<td>=C-H bend</td>
</tr>
<tr>
<td></td>
<td>aromatic</td>
<td>C-H “oop”</td>
</tr>
</tbody>
</table>

Table 3.18: Functional Groups and Bonds of Fraction E responsible for IR absorption
3.3: RESULTS AND DISCUSSIONS:

Standard and efficient protocol for Extraction of Phytoconstituents was established.

- It required about 15 to 20 days for the leaves of *Costus pictus* to dry naturally in shed. Ratio of weights of fresh leaves to dried leaves was about 40:1.
- On an average, 1.6 grams of Crude Methanolic Extract was obtained from 15 grams of fine powder of leaves with Soxhlet extractor.
- For complete extraction of Secondary Metabolites from 15 grams of leaf powder, approximately 30 hours running of Soxhlet extractor spread over 3 days was required.
- Extract was odorless, thick, sticky and deep olive green in color.
- It required four days for complete evaporation of solvent from extract under natural conditions.
- Extract was soluble in water.

Protocol for Chemical Finger printing of Methanolic Extract of dried leaves of *Costus pictus* was successfully established and it is a maiden effort.

- Mobile Phases were developed for 4 major Classes of Secondary Metabolites and optimum separation of constituents was achieved.
- Methanol Extract contained Alkaloids, Flavonoids, Saponins and Triterpenoids
- Important information on Retention Factor of individual Constituent was obtained. *R*<sub>f</sub> values are often compared while identifying an unknown compound.
- From Analysis of Chromatograms, information about relative concentration of individual constituents within Extract was derived.
- With the help of Chemical Fingerprinting, authenticity of plant material is successfully established.
For Isolation of various fractions from Methanolic extract of dried leaves of *Costus pictus*, two methods were successfully developed.

- In one method, Analytical TLC plates were used and for the other, Preparative TLC plates were used.
- Sample collection from ATLC plate was by cutting the aluminum plate into small strips and further cutting the strips into pieces.
- Sample collection from PTLC plate was by scratching the layer of silica in horizontal strips, marked with pencil from glass base.
- Both techniques were optimized and produced same results.
- 10 fractions from all plates were separately collected in different containers and the separated Phytoconstituents were dissolved back into Mobile Phase.

Comparison of parameters for two methods for collection of fractions is as given below (Table 3.19).

<table>
<thead>
<tr>
<th>S No</th>
<th>Parameter</th>
<th>Analytical TLC Technique</th>
<th>Preparative TLC Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Description and Product Code of TLC plate from MERCK Germany</td>
<td>Silica gel 60 F&lt;sub&gt;254&lt;/sub&gt; of 0.25 mm thickness on aluminum base Product Code 1.05554.0007</td>
<td>Silica gel 60 F&lt;sub&gt;254&lt;/sub&gt; of 1 mm thickness on glass base Product Code 1.13895.0001</td>
</tr>
<tr>
<td>2</td>
<td>Size of TLC plate used</td>
<td>10 cm X 20 cm</td>
<td>20 cm X 20 cm</td>
</tr>
<tr>
<td>3</td>
<td>Point of loading from bottom</td>
<td>2 cm</td>
<td>4 cm</td>
</tr>
<tr>
<td>4</td>
<td>Solvent front from point of loading</td>
<td>16 cm</td>
<td>14 cm</td>
</tr>
<tr>
<td>5</td>
<td>Time for sample loading</td>
<td>25 minutes</td>
<td>60 minutes</td>
</tr>
<tr>
<td>6</td>
<td>Lengths of applied bands</td>
<td>14 cm and 0.8 cm</td>
<td>14 cm and 0.8 cm</td>
</tr>
<tr>
<td>8</td>
<td>Quantity of Mobile Phase</td>
<td>20 ml</td>
<td>60 ml</td>
</tr>
<tr>
<td>9</td>
<td>Saturation time</td>
<td>15 minutes</td>
<td>15 minutes</td>
</tr>
<tr>
<td>10</td>
<td>Time for development</td>
<td>20 minutes</td>
<td>55 minutes</td>
</tr>
</tbody>
</table>

*Table 3.19:* Comparison of parameters for Analytical and Preparative TLC techniques
After 10 fractions, each in sufficient quantity, were separately collected, fractions were analyzed further with FTIR technique.

- FTIR spectrum of each fraction was different from others.
- It indicates that all 10 fractions were distinct from each other and it can be concluded that separation technique by using ATLC and PTLC plates was successful.
- Use of Preparative or Analytical TLC plates to separate Phytoconstituents from Extract is a very different approach than usual Column Chromatography.
- This method can be successfully employed especially when the fractions in natural form are colorless and hence difficult to visualize separation in Column Chromatography.
- FTIR analysis indicated Functional groups and the corresponding bonds responsible for IR absorption.

It can thus be concluded that

- Methanol Extract of leaves of *Costus pictus* contains very less number of Alkaloids, Flavonoids and Saponins are moderate in number. Triterpenoids fractions are largest in number.
- Both Analytical and Preparative TLC plates can be employed to Isolate Phytoconstituents.
- Use of Preparative TLC plates for separation of fractions from Extract was a maiden effort. By employing PTLC plates instead of ATLC plates, at least 5 times more quantity of a compound can be collected per plate.
- FTIR analysis proved that Isolation of 10 fractions was successful and it is a maiden effort.