3 MATERIALS & METHODS
3. MATERIALS AND METHODS

A. MATERIALS FOR THE STUDY

3.1. SURVEY AND TAXONOMIC STUDIES

3.1.1. Survey, Collection and Identification of Plant material

A detailed survey was conducted about the hepatoprotective plants used by the tribals of Chinnar region of Idukki District, Kerala, India and selected the plant, *Combretum albidum* G.Don, coming under the family Combretaceae. The plants were collected from three different geographical conditions of Kerala *i.e.*, Chinnar, Idukki district, Vavanur, Palakkad district and Kiralur, Thrissur district during the month of January to April and was authenticated at Taxonomy Division, Centre for Medicinal Plants Research (CMPR), Arya Vaidya Sala, Kottakkal, Kerala, India. The voucher specimen (Col. No. 5545) and raw drug (CMPR/RD/211) were deposited at CMPR herbarium and raw drug depository respectively.

3.1.2. Plant parts for the study

The plant parts selected for the study were Stem Bark (CaSB), Heart Wood (CaSHW) and Leaf (CaL) of *C. albidum* and it was collected from three different regions of Kerala, India during the month of February-April (Table 3.1).

**Table 3.1** Plant parts and their code selected for the study

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Place of collection</th>
<th>Code for the useful part</th>
<th>Stem bark</th>
<th>Heart wood</th>
<th>Leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chinnar, Idukki</td>
<td></td>
<td>CaSB</td>
<td>CaSHW</td>
<td>CaL</td>
</tr>
<tr>
<td>2</td>
<td>Kiralur, Thrissur</td>
<td></td>
<td>CaSB</td>
<td>CaSHW</td>
<td>CaL</td>
</tr>
<tr>
<td>3</td>
<td>Vavanur, Palakkad</td>
<td></td>
<td>CaSB</td>
<td>CaSHW</td>
<td>CaL</td>
</tr>
</tbody>
</table>
Hereafter the codes are used to mention the different parts of the plant (C1SB: stem bark of *C. albidum* from Chinnar, Idukki; C2SHW: heart wood of *C. albidum* from Kiralur, Thrissur; C3L: leaf of *C. albidum* from Vavanur, Palakkad etc).

### 3.2. PHARMACOGNOSTIC STUDIES

#### 3.2.1 Plant materials for micro-morphological studies

The selected parts *i.e.*, CaSB, CaSHW and CaL of genuine plant sources were subjected to micro morphological studies to record the identity and variation between plants.

#### 3.2.2. Plant material for histological and histochemical studies

CaSB, CaSHW and CaL were collected in bulk quantity and one portion was preserved for anatomical and histochemical studies and the remaining portions were shade dried, powdered (20mm mesh size) and sealed in an air tight bottle for the estimation of physicho-chemical parameters, preliminary phytochemical investigation, isolation and characterisation of chemical constituents, antioxidant, antimicrobial activities and pharmacological studies (Cell line).

##### 3.2.2.1 Fixatives for anatomical studies

Combination of Formalin, Acetic acid and Alcohol were widely used for fixation in plant microtechnique. This fluid is ideal for anatomical studies because it is stable and has got hardening action, rapid penetration and material can be stored for long time.

**FAA**- standard proportion (Johanson, 1940).

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>70% Ethyl alcohol</td>
<td>- 90 ml</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>- 5 ml</td>
</tr>
<tr>
<td>Formalin</td>
<td>- 5 ml</td>
</tr>
</tbody>
</table>
3.2.2.2 Stains and reagents for anatomical studies

1. Saffranin: dissolved 1gm saffranin in 100 ml distilled water
2. Fast green: dissolved 1gm Fast green in 100 ml clove oil

3.2.2.3 Stains for histochemical studies

• Ferric chloride S: dissolved 5 g of ferric chloride in 100 ml of water.
• Glycerol S: mixed equal amounts of glycerol and water.
• Hydrochloric acid: a suitable commercially available reagent.
• Iodine S: dissolved 2.6 g of iodine and 3 g of potassium iodide in sufficient water to produce 100 ml.
• Nitric acid S: concentrated nitric acid.
• Phloroglucinol: dissolved 1 g of phloroglucinol in 100 ml of ethanol.
• Potassium hydroxide: dissolved 56 g of KOH in 1000 ml of water
• Ruthenium red S: in 10 ml of 10% lead acetate solution dissolved 0.008 g of ruthenium red.
• Sudan red S: dissolved 0.5 g of sudan red in 100 ml of glacial acetic acid AR

3.2.3. Material for the raw drug powder studies

Microscopic studies of the powdered drug of leave, stem bark and heart wood were carried out with proper staining

3.2.4. Material for polarization and fluorescent microscopic studies

Polarization and fluorescent microscopic studies of the useful parts like leaves, stem bark, heart wood were conducted. Leica, Motic and Carl zeiss microscopes were used for the study.
3.2.5. Material for Scanning Electron Microscopic studies

For SEM microscopic studies Hitachi Tabletop Microscope TM-3000 Scanning Electron Microscope was used

3.3. PHYTOCHEMICAL STUDIES

3.3.1. Material for phytochemical studies

3.3.2. Physicochemical characters

1. Moisture content
   Instrument used to determine the moisture content was Moisture Analyser MJ 33 Apparatus.

2. Water soluble extractive
   Materials used to determine the water soluble extractive were powdered drug, conical flask, water bath and oven.

3. Alcohol soluble extractive
   Materials used to determine the alcohol soluble extractive were powdered drug, round bottomed flask, water bath and oven.

4. Ash value
   Materials used to determine the ash value were powdered drug, muffle furnace, gooch crucible and ashless filter paper.

5. Acid- insoluble ash
   Materials used to determine the acid insoluble ash were powdered drug weighing balance, 4N HCl, Gooch crucible, ash less filter paper and desiccators.
3.3.3. Total Phenolics, Flavonoids and Tannin contents

Preparation of extract

10 g of the shade dried powdered drug samples were taken in a soxhlet thimple and extracted with 250 ml methanol for 6 hrs. Filtered, solvent removed by distillation and final traces of solvent under vacuum in a rotavapor. Weight of extract obtained is noted for all the drugs. The extract dissolved in methanol and made up to 100 ml in a volumetric flask (Stock solution).

Total Phenolic Assay

Materials used to determine the total phenolic assay were extracts, Folin-Ciocalteu reagent, Gallic acid, volumetric flask, Na₂CO₃ solution and spectrophotometer.

Total Flavonoid Assay

Materials used to determine the total flavonoid content were extracts, Aluminium chloride colorimetric assay, quercetin, volumetric flask, NaNO₂, AlCl₃, 1M NaOH and spectrophotometer.

Estimation of Total Tannins

Materials used to determine the total tannin content were powdered drug extracts, tannic acid, volumetric flask, Folin-Denis reagent, Na₂CO₃ solution and spectrophotometer.

3.3.4. Assay/Analytical Methods

1. TLC Identity test

Thin Layer Chromatographic (TLC) studies conducted using pre-coated plates of silica gel 60 F₂₅₄ (E. Merck) of uniform thickness of 0.2mm.
2. High Pressure Liquid Chromatography (HPLC) system

Shimadzu prominence LC-20AD UFLC system equipped with online degasser, autosampler and diode array detector. Waters Spherisorb ODS (2) 5µm, 250 mm x 4.6 mm column was used.

3. High Pressure Thin Layer Chromatography (HPTLC)

HPTLC studies were done using aluminium plates precoated with silica gel GF$_{254}$ 0.2mm (E.Merck), Camag automatic sampler V, Camag automatic developing chamber with humidity controller ADC-2, Camag TLC Densitometric scanner 3, Camag TLC visualizer for photo-documentation and with WINCATS operating software were used.

4. GC/MS analysis

Gas chromatography-mass spectrometry was carried out on an Agilent GC-MS 6850 under electron impact ionization (70 eV). The interface temperature was 230°C, and the MS scan range was 50-800 atomic mass units (AMU). The chromatographic column for the analysis was done by HP5 - MS capillary column (30 m x 0.25 mm internal diameter). The carrier gas used was helium at a flow rate of 1 ml/min. The oven temperature was 60°C to 250°C with a constant increase of 10°C. The injection was performed in split mode at 250°C.

**Mass Spectrum of β- sitosterol**

**System:** Agilent 5975 MSD

**Injection:** 1 µL at 270°C with split ratio 10

**Column:** 5 m length, 0.25 mm ID, 0.25µ film DB-5MS UI

**Helium column flow rate:** 5 ml/min for 9 minutes followed by flow program of 8 ml/min up to 30 ml/min.
GC Oven: 50ºC followed by 25ºC/min to 330ºC and 3.8 min hold for total of 14 min.

El Source: 12 mA emission, 70 eV electron energy, 56 ml/min He makeup flow.

SMB transfer-line temperature: 270ºC for 5 min followed by temperature program of 10 ºC/min to 330ºC

Mass range: 50-950 amu at about 1.6 Hz scan frequency.

3.4. BIOLOGICAL ACTIVITIES

3.4.1. ANTIOXIDANT STUDIES

Plant extracts, 2, 2-diphenyl-1-picrylhydrazil (DPPH), Quercetin, 96 microwell plates, microplate reader (Thermo, Multiskan Ex).

3.4.2. ANTIMICROBIAL STUDIES

- Materials and Instruments
  - Culture media: The following media were used, Cysteine Lactose Electrolyte Deficient medium (CLED, HiMedia M1146), Mac Conkey Agar (Merk 105465), 5% sheep Blood agar, Muller Hinton agar (HiMedia, M1657), Citrate agar (HiMedia, M728), Triple Sugar Iron Agar (HiMedia, M021I), Bile Esculin Agar Base (HiMedia, M340),
  - Antibiotics used: Nitrofuraturoin (HiMedia SD090), Co-trimoxazole (HiMedia SD010), Cefotaxime (HiMedia,SD040), Gentamicin 40mg/ml (HiMedia SD016), Chloramphenicol (HiMedia SD006), For MIC gentamicin (HiMedia, CMS461), Clotrimazole 1% W/V
  - Autoclave, Incubator
Microorganisms

- **Gram positive Bacteria** - *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 3160) and *Enterococcus faecalis* (ATCC 29212).

- **Gram negative Bacteria** - *Escherichia coli* (MTCC 40), *Klebsiella pneumoniae* (MTCC 3384) and isolated pathogenic *Salmonella typhi*.

- **Fungi** - *Candida albicans* (MTCC 183), *Aspergillus niger* (MTCC 281), *Aspergillus flavus* (MTCC 2799), *Pencillium chrysogenum* (MTCC 161) and isolated Rhizopus species.

3.4.3. CYTOTOXICITY STUDIES

Materials and Instruments

- HEK (Human embryonic kidney) cell line was purchased from American Type Culture Collection (ATCC), Manassas, USA. Cells were maintained in DMEM containing HEPES and Sodium bicarbonate supplemented with 10% fetal bovine serum (FBS) and IX antibiotic antimycotic mix solution.

- Growth Heat inactivated fetal calf serum (FCS), Ficoll-Histopaque- 1017, thiobarbituric acid (TBA), phenozine methosulphate (PMS), nitroblue tetrazolium (NBT), 5,5-dithiobis 2-nitrobenzoic acid (DTNB), 3-(4,5-dimethyl-2-thiaozoly)-2,5-diphenyl-2H tetrazolium bromide (MTT), nicotinamide adenine dinucleotide (NAD), 2-deoxy-D-ribose, FeCl2, FeCl3, EDTA, H2O2 and ascorbic acid were purchased from Sigma chemicals Co., St. Louis, USA. Other chemicals for blood lymphocyte cultures (RPMI-1640, penicillin, streptomycin, L-glutamine), low melting point agarose (LMPA), normal melting point agarose (NMPA) Ethidium bromide, acridine orange and phosphate buffer saline (PBS) were purchased from Himedia, Mumbai. All other chemicals and solvents of
analytical grade were obtained from SD Fine chemicals, Mumbai and Fisher Inorganic and Aromatic Limited, Chennai.

- Haemocytometer, CO₂ incubator, Thermoscientific multiskan EX

3.4.4. ANTICANCEROUS STUDIES

- Plant extracts, Cell lines: K562 (Human chronic myelogenous leukemia).

3.4.5. IN VITRO STUDIES ON HEP3B CELL LINES

Human Hepatocellular Carcinoma (HCC) cell lines, Hep3B, were purchased from American Type Culture Collection (ATCC), Manassas, USA. Cells were maintained in DMEM containing HEPES and Sodium bicarbonate supplemented with 10% fetal bovine serum (FBS) and IX antibiotic antymycotic mix solution. Cells were incubated at 37°C in a humidified 5% CO₂ atmosphere (Hera cell 150, Heraeus, Langenese, Germany).

Materials and Instruments

- Plant extracts, Dulbecco’s Modified Eagle Medium, Ethylene Diamine Tetra Acetate, Fetal Bovine Serum, N-2-Hydroxyethylpipерagine-N’-2-ethanesulphonic acid, Methyl Thiazole blue Tetrazolium bromide, Phosphate Buffer Solution, 96 well plates (Greiner, Frickenhausen, Germany), Hoechst staining, Cryo-baby, dimethyl sulfoxide, Diethylpyrocarbonate (DEPC), Trypsin, disc Durapore Syringe filters (Millipore, Bedford, USA), Silymarin, IX antibiotic-antimycotic

- Micoplate reader (BIO-RAD, Hercules, CA, USA), PCR BIO-RAD I cycler, Leica DFC 280 CCD Camera (Leica Microsystems Digital Imaging, Cambridge, UK), BIO-RAD gel documentation apparatus, Bench top Centrifuge, Deep freezer 80°C, Filtration system for making cell culture media, six well culture plate (NUMEC), Durapore Syringe filters (Millipore, Bedford, USA), Magnetic stirrer
(SPINOT), glass wares (Scott-Duran, USA), Cell scaper (Co-star, Mexico), Haemocytometer, CO₂ incubator

Software

- Fast PCR
- Quantity one (1D analysis software)
- BLAST
- Leica application Suit

B. METHOD OF STUDY

3.1. TAXONOMIC STUDIES

3.1.1. Taxonomical profiling

For the study, the genuine source plant collected from the natural habitat was subjected to taxonomic identification and herbarium preparation. The authenticated samples of herbarium of each drug and useful part were kept in the Herbarium and raw drug museum of AVS- CMPR for further use. The useful part was fixed in Formalin, Acetic acid and Alcohol mixture for further study. The plant material was shade dried and powdered for the Phytochemical, Antioxidant, Antimicrobial and cell line studies.

3.2. PHARMACOGNOSTIC STUDIES

3.2.1. Micro morphological studies

The genuine plant material subjected to micro-morphological studies and recorded the most identifying features of dermal morphology, texture, colour and cut surface. Organoleptic characters like smell, taste and fracture were also studied (Khandelwal, 2008).
3.2.2. Histological studies

Histological studies of the useful part were carried out to find out the type of cells, shape of stem in transection, nature of epidermal hairs, epidermal cell shape, nature of cortex, type of phloem fibres, crystals and cambium, nature of xylem vessel, secondary wood, pith, type of anatomical growth- normal or abnormalities if any. TS & LS were taken using sledge and rotary microtome and double stained with appropriate staining procedure for the preparation of permanent slide. All the anatomical characters were observed under Carl Zeiss Axiostar plus microscope with a G3 Canon camera attached to a computer system.

3.2.2.1. Stains and reagents for Anatomical studies

1. Safranin: stained lignified, cutinized and suberised structures

2. Fast green: the stains act on non-lignified tissues. It is a good counter stain for safranin.

3.2.3. Histochemical studies

Histochemical characterization of raw drugs were carried out to find the presence of starch, tannin; presence, nature and position of laticifers, resin ducts/ oil ducts; identification of depositions like cutin, lignin, suberin, wax; inclusions like calcium oxalate, calcium phosphate, cystolith etc. using appropriate histochemical techniques.

- Starch

For examine the presence of starch the specimen was stained with Iodine. The blue colour indicated the presence of starch content.
• **Aleurone grains**
  For examining the presence of aleurone grains prepared a specimen in iodine; aleurone grains get stained yellow.

• **Fixed oil**
  For examining the presence of fixed oil, prepared a specimen in a solution of sudan red; droplet of fixed oil get coloured orange pink.

• **Tannin**
  For examining the presence of tannins, prepared a specimen in ferric chloride; bluish black or grayish black colouration indicated the presence of tannins.

• **Anthraquinone derivatives**
  For examining the anthraquinone derivatives, prepared a specimen in potassium hydroxide; anthraquinone give blood red colour.

• **Lignified cells**
  For examining the lignified cells or cell walls, the specimen was stained in phloroglucinol and allowed to drying. Added one or two drops of hydrochloric acid. The presence of lignin indicated by the pink or cherry red colour.

3.2.4. Microscopic studies
All the anatomical characters were observed under Carl Zeis Axiostar plus microscope with a G3 Canon camera attached to a computer system.

3.2.5. Raw drug powder studies
For examining the cell structure in powder form, material were powdered and sieved and mounted under glycerol, chloral hydrate and safranin to study the nature and identification of particles.
3.2.6. Polarization microscopic studies

To locate and distinguish the types of crystals and minerals present in the useful part polarization microscopy was used. The characters were observed under Motic BA 400 polarization microscope.

3.2.7. Fluorescent microscopic studies

Fluorescent microscopic studies of the useful part were done with the help of UV light. Observations were done under Leica DM 1000 LED fluorescent microscope and photographs were taken with the help of a digital camera.

3.2.8. Scanning Electron Microscopic studies

The Scanning Electron Micrographs of leaf were taken with the help of Hitachi TM 3000 Tabletop Scanning Electron Microscope. The materials were kept directly on a diaphragm and kept inside the SEM Chamber and figures were captured in the computer using inbuilt software.

3.3. PHYTOCHEMICAL STUDIES

3.3.1. Physicochemical characters

3.3.1.1. Determination of Moisture content

Air dried drug (5g) placed in an Aluminium plate of the moisture analyser and heated to 110° till constant weight is obtained. Difference between the initial and final weight given the moisture content of the drug.

3.3.1.2. Determination of Water soluble extract

Macerated 5 g of the air dried drug, coarsely powdered, with 100 ml of alcohol in a closed flask for twenty-four hours, shaken frequently during six hours and allowed to stand for eighteen hours. Filtered rapidly, taking precautions against loss of solvent, evaporated 25 ml of the filtrate to dryness in a tarred flat bottomed shallow dish, and
dried at 105°, to constant weight. Calculated the percentage of alcohol-soluble extractive with reference to the air-dried drug.

**3.3.1.3. Determination of Alcohol soluble extractive**

Macerated 5 g of the air-dried drug, coarsely powdered, with 100 ml of alcohol in a closed flask for twenty-four hours, shaken frequently during six hours and allowing to stand for eighteen hours. Filtered rapidly, taking precautions against loss of solvent, evaporated 25 ml of the filtrate to dryness in a tarred flat bottomed shallow dish, and dried at 105°, to constant weight. Calculated the percentage of alcohol-soluble extractive with reference to the air-dried drug.

**3.3.1.4. Determination of Ash value**

Weighed accurately 2 g of the air-dried powdered drug in a Gooch crucible and incinerated at a temperature not exceeding 450° until free from carbon, cooled and weighed. Calculated the percentage of ash with reference to the air-dried drug.

**3.3.1.5. Determination of Acid-insoluble ash**

To the crucible containing total ash, added 25 ml of *dilute hydrochloric acid*. Collected the insoluble matter on an ashless filter paper (Whatman 41) and washed with hot water until the filtrated is neutral. Transferred the filter paper containing the insoluble matter to the original crucible, dried on a hot-plate and ignited to constant weight. Allowed the residue to cool in a suitable desiccator for 30 minutes and weighed without delay. Calculated the percentage.

**3.3.2. Total Phenolic assay**

The total phenolics content were determined by using the Folin-Ciocalteu assay. (Singleton *et al.*, 1965). An aliquot (1 ml) of extracts or standard solution of Gallic acid (20, 40, 60, 80 and 100µg/ml) was added to 25 ml of volumetric flask,
containing 9 ml of distilled water. A reagent blank using distilled water was prepared. 1 ml of Folin-Ciocalteu phenol reagent was added to the mixture and shaken. After 5 minutes 10 ml of 7% Na₂CO₃ solution was added to the mixture. The volume was then made up to the mark. After incubation for 90 minutes at room temperature, the absorbance against the reagent blank was determined at 550 nm with an UV-Visible spectrophotometer (Shimadzu 1700, Japan). Total phenolics content was expressed as mg Gallic acid Equivalents (GAE)

3.3.3. Total Flavonoid Assay

Total flavonoid content was measured by the aluminium chloride colorimetric assay. (Zhishen et al., 1999). An aliquot (1ml) of extracts or standard solutions of quercetin (20, 40, 60, 80 and 100µg/ml) were added to 10 ml volumetric flask containing 4 ml of distilled water. To the flask added 0.30 ml 5% NaNO₂, after five minutes 0.3 ml 10 % AlCl₃ was added. After five minutes, 2 ml IM NaOH was added and the volume was made up to 10 ml with distilled water. The solution was mixed and absorbance was measured against the blank at 510 nm with an UV-Visible spectrophotometer (Shimadzu 1700, Japan). The total flavonoid content was expressed as mg quercetin equivalents (QE).

3.3.4. Total Tannins Assay

Preparation of standard

100 mg of tannic acid was dissolved in 100 ml of distilled water. 1 ml of this solution was diluted into 100 ml with distilled water to give 10 µg/ml tannic acid solution.

Method

A series of calibrated 10 ml volumetric flask were taken and working standards of 5-45 µg solutions were taken. To each flask 0.5 ml Folin-Denis reagent and 1 ml
sodium carbonate solution were added, the volume was made up to 10 ml with distilled water. The solution without tannic acid was used as blank. The blue colored complex thus produced was measured at 775 nm with an UV-Visible spectrophotometer (Shimadzu 1700, Japan).

1 ml of sample was made up to 10 ml in similar manner. From the calibration curve the corresponding concentration of tannin was calculated (Burns & Cope, 1974). It was expressed as mg Tannic acid Equivalents.

3.3.5. Assay/Analytical methods to Identify Chemical constituents

3.3.5.1. Preliminary phytochemical investigation

a) Tests for carbohydrates

Fehling’s Test

1 ml Fehling’s A solution and 1 ml of Fehling’s B solution were mixed and boiled for one minute. Now the equal volume of test solution was added to the above mixture. The solution was heated in boiling water bath for 5-10 minutes. First a yellow, then brick red precipitate was observed.

Benedict’s test

Equal volumes of Benedict’s reagent and test solution were mixed in a test tube. The mixture was heated in boiling water bath for 5 minutes. Solution appeared green showing the presence of reducing sugar.

Molisch’s test

Equal volumes of Molisch’s reagent and test solution were mixed in a test tube. The mixture was heated in boiling water bath for 5 minutes. Appearance of violet or purple colour ring showing the presence of reducing sugar.
b) Tests for Anthraquinone glycosides

**Borntrager’s Test**

To the 3ml of extract, dil. H$_2$SO$_4$ was added. The solution was then boiled and filtered. The filtrate was cooled and to it equal volume of benzene was added. The solution was shaken well and the organic layer was separated. Equal volume of dilute ammonia solution was added to the organic layer. The ammonia layer turned pink showing the presence of glycosides.

c) Tests for Coumarins

To the 2ml of extract, 10% NaOH was added and shaken well for 5 min showed yellow colour.

d) Tests for Quinone

To the 2ml of extract, conc. H$_2$SO$_4$ was added and shaken well for 5 min showed Red colour.

e) Test for steroids

**Salkowski Test**

To 2 ml of extract, 2 ml of chloroform and 2 ml of conc. H$_2$SO$_4$ was added. The solution was shaken well. As a result chloroform layer turned red and acid layer showed greenish yellow fluorescence.

f) Tests for alkaloids

**Hager’s Test**

To the 2-3 ml of filtrate, 1ml of dil. HCl and Hager’s reagent was added and shaken well. Yellow precipitate was formed showing the presence of alkaloids.
**Mayer’s Test**

To the 2-3 ml of filtrate, 1ml of dil. HCl and Mayer’s reagent was added and shaken well. Formation of yellow precipitate showed the presence of alkaloids.

**Dragendroff’s Test**

To the 2-3 ml of filtrate, 1ml of dil. HCl and Dragendroff’s reagent was added and shaken well. Formation of orange-brown precipitate showed the presence of alkaloids.

**Wagner’s reagent test**

To the 2-3 ml of filtrate, 1ml of dil. HCl and Wagner’s reagent was added and shaken well. Formation of redish-brown precipitate showed the presence of alkaloids.

g) **Tests for flavonoids**

With Lead Acetate; to the small quantity of extract, lead acetate solution was added. Formation of yellow precipitate showed the presence of flavonoids.

**h) Tests for Tannins and Phenolic compounds**

**FeCl₃ Solution Test**

On addition of 5% FeCl₃ solution to the extract, deep blue black colour appeared.

**Lead Acetate Test**

On addition of lead acetate solution to the extract, white precipitate appeared.

i) **Test for Saponins**

**Foam Test**

To 1ml extract 20ml distilled water was added and shaken well in a measuring cylinder for 15 min. Then 1cm layer of foam was formed.
j) Test for triterpenes
Methanol extract was treated with concentrated sulphuric acid (H₂SO₄). Appearance of reddish brown ring indicated the presence of triterpenes.

Above phytochemicals analysis were carried out using standard procedure (Kokate, 1988; Harborne, 1998, 1973 and Sadasivam & Manickam 2005).

3.3.6. Extraction of the material

1. General method of extraction
In phytochemical evaluation, the first step is extraction of plant material. The selection of extraction method depends on the nature and compounds to be isolated from the plant material. The dried material usually powdered before extraction. Extraction was carried out by cold maceration or by methods involving heating of the drug with suitable solvent.

2. Hot Continuous Extraction (Soxhlet)
In this study, all the extractions were carried out using following method. The finely ground crude drug is placed in a porous bag or “thimble” made of strong filter paper, which is placed in chamber of the Soxhlet apparatus. The extracting solvent in tarred flask is heated, and its vapors condensed in a condenser. The condensed extractant drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in chamber rises to the top of siphon tube, the liquid contents of chamber siphon into flask A. This process was continuous and carried out until a drop of solvent from the siphon tube does not leave residue when evaporated. After the completion of extraction, the solvent distilled off in a water bath and final traces of solvent removed under vacuum at 90°C. Weight of extract obtained was noted.
3. Isolation of volatile oil/essential oil using Clevenger’s apparatus

In this method, the material was completely immersed in water, which was boiled by applying heat i.e., direct fire. The main characteristic of this process is that there is direct contact between boiling water and plant material. When the still is heated by direct fire, adequate precautions are necessary to prevent the charge from overheating. The plant material in the still were agitated as the water boils, otherwise agglomerations of dense material will settle on the bottom and become thermally degraded. From this laboratory trial, the yield of oil from a known weight of the plant material can be determined. During water distillation, all parts of the plant charge must be kept in motion by boiling water; this is possible when the distillation material is charged loosely and remains loose in the boiling water. For this reason only, water distillation possesses one distinct advantage, i.e. it permits processing of finely powdered material or plant parts that, by contact with live steam, would otherwise form lumps through which the steam cannot penetrate.

The hydro distillation usually takes 1-1/2 hours to 7 or more hours, depending up on the plant material and nature of the oil. In general, the oil formed along with the water in the trap is drained at ½ hour, replacing the water after each such removal, until no more oil distils. The resulting oil water mixture is saturated with sodium choride (to salt out oil dissolved in the water, if any) and is collected as pure oil with the use of separating funnel or extracted with pet ether (60° - 80°) and filtered to a weighed flask, Na₂SO₄ (3 to 4 times, the combined either extract, dried over anh. Na₂SO₄), washed with pet ether and pet ether is stripped off, using 15-20 cms vigreaux column (to minimize loss of traces of oil due to its slight co-distillation with pet ether). The last traces of solvent (pet ether) are removed by heating on a water
bath (~90°) under reduced pressure (~100mm) for 10 mins and finally briefly (~2-3 mins) at 60° - 80°, 4-6 mm and the flask is weighed. From the weight of fresh raw material and that of the essential oil obtained, the % yield (W/W fresh weight basis) can be evaluated (Clevenger, 1928).

3.3.7. Isolation of Phytochemical constituents

1. Thin layer Chromatography (TLC) studies

Thin-layer chromatography is a technique in which a solute undergoes distribution between two phases, a stationary phase acting through adsorption and a mobile phase in the form of a liquid. The adsorbent is relatively thin, uniform layer of dry finely powdered material applied to a glass, plastic or metal sheet or plate. Glass plates are most commonly used. Separation may also be achieved on the basis of partition or a combination of partition and adsorption, depending on the particular type of support, its preparation and its use with different solvent. Identification can be effected by observation of spots of identical R<sub>f</sub> value and about equal magnitude obtained, respectively, with an unknown and a reference sample chromatographed on the same plate. A visual comparison of the size and intensity of the spots usually serves for semi-quantitative estimation. In this study all the TLC studies were conducted using MERCK aluminium backed pre-coated plates of silica gel 60 F<sub>254</sub> of uniform thickness of 0.2mm.

Preparation of spray reagents for derivatisation of TLC

1. Anisaldehyde sulphuric acid reagent

Mixed 0.5 ml anisaldehyde with 10 ml glacial acetic acid, then added 85 ml methanol and 5 ml conc. Sulphuric acid. Sprayed the plate and heated at 100° for 5-10 min. Used freshly prepared reagent for good result.
2. **1% Alcoholic ferric chloride reagent**

Dissolved 1g of ferric chloride in 100ml of alcohol.

2. **Column chromatography**

This is the most common method used to purify individual chemical compounds from mixtures of compounds. The classical preparative chromatography column, is a glass tube with a diameter from 5 mm to 50 mm and a height of 5 cm to 1 m with a tap and some kind of a filter (a glass frit or glass wool plug – to prevent the loss of the stationary phase) at the bottom. A slurry was prepared of the eluent with the stationary phase powder and then carefully poured into the column. Carefully carried out to avoid air bubbles. A solution of the organic material was pipetted on top of the stationary phase. This layer was topped with a small layer of cotton to protect the shape of the organic layer from the velocity of newly added eluent. Eluent was slowly passed through the column to advance the organic material. An eluent-filled and stoppered separating funnel was put on top of the column. The individual components were retained by the stationary phase differently and separated from each other while they are running at different speeds through the column with the eluent. At the end of the column eluted one at a time. During the entire chromatography process the eluent was collected in a series of fractions. The composition of the eluent flow was monitored and each fraction was analyzed for dissolved compounds, e.g. by analytical chromatography, UV absorption, or fluorescence. Colored compounds (or fluorescent compounds with the aid of an UV lamp) were observed through the glass wall as moving bands. The stationary phase or adsorbent in column chromatography was a solid material. The most common stationary phase used was silica gel. The mobile phase or eluent was either
a pure solvent or a mixture of different solvents. It was chosen so that the retention factor value of the compound of interest was roughly around 0.2 - 0.3 in order to minimize the time and the amount of eluent to run the chromatography. The eluent has also been chosen so that the different compounds can be separated effectively.

3. High Pressure Liquid Chromatography (HPLC) system

Simadzu Prominenece UFLC system equipped with online degasser, automatic sampler and DAD detector. Waters SPHERISORB ODS (2) C18 column of dimensions 250mmx4.6mmx5µm and mobile phase methanol:phosphate buffer (0.0.3M, pH 3) 90:10 with a flow rate of 0.5 ml per minute.

4. Liquid chromatography-Mass Spectrometry (LC/MS)

Coupling of MS to chromatographic techniques has always been desirable due to the sensitive and highly specific nature of MS compared to other chromatographic detectors. Agilent 1260 Infinity series LC equipped with Mass detector 6120B, Agilent C_{18} column of dimensions 250 x 4.6 x 5µ and mobile phase methanol: water (95:5 v/v) delivered at a flow rate of 1 ml per min. Atmospheric pressure chemical ionization was operated in negative ion mode.

5. High Pressure Thin Layer Chromatography (HPTLC) Analysis

Chromatographic analyses were performed on silica gel 60 F_{254} TLC plates (20x10 cm; Merck, Darmstadt, Germany). Samples were applied to the plates by means of CAMAG automatic sampler V. Detection and quantification of the developed plate was performed with a CAMAG TLC Scanner 3 at 559 nm. TLC images were documented using CAMAG TLC visualizer. Wincats as an integrated Software was used for the detection as well as for the evaluation of data.
3.3.8. Isolation of Individual compounds

1. Densitometric estimation of Ursolic acid

**Test solution**
Extracted about 3 g of bark of *C. albidum* was mixed with 50 ml methanol and kept for overnight. This extract was filtered and the solvent was evaporated off using rotary evaporator till dryness. The residue was redissolved in ethanol in order to obtain sample solution containing 100 µg/ml for analysis.

**Standard solution**
Dissolved 1.8 mg of standard ursolic acid in 10 ml of methanol in a volumetric flask

**HPTLC conditions**
Procedure and Estimation of Ursolic acid

For calibration, standard ursolic acid solutions were prepared at concentrations of 1, 2, 3 … 20µg/ml in ethanol. The standard solutions were injected in triplicate and the average detector responses measured. Plant samples were assayed in triplicate and detection was done at 265 nm.

2. Densitometric estimation of Gallic acid

**TLC plates**
Precoated plates of silica gel 60 F\textsubscript{254} (E. Merck) of uniform thickness of 0.2 mm

**Solvent system**
Hexane: Ethyl acetate (8:2)

**Scanning**
254 & 365nm

**Test solution**
Extracted about 3 g of stem bark of *C. albidum* was mixed with 50 ml ethanol and
kept for overnight. The extract was filtered and the solvent was evaporated off using rotary evaporator till dryness. The residue was redissolved in ethanol in order to obtain the sample solution containing 100µg/ml for analysis.

**Standard solution**

Dissolved 10 mg of standard Gallic acid in 10 ml of methanol in a volumetric flask

**Calibration curve**

Applied 0.1 to 0.5 µl of standard solutions corresponding to 0.26 µg to 1.56 µg of Gallic acid on a precoated silica gel 60 F_{254} TLC plate. Developed the plate using the solvent system in a twin trough chamber to a distance of 8 cm and scanned densitometrically at visible, 254nm & 366 nm. Recorded the peak area and prepared the calibration curve by plotting peak area vs concentration of Gallic acid applied.

**Estimation of gallic acid in the drug**

Applied 3, 5µl of the test solution on a precoated silica gel 60 F_{254} TLC plate (E. Merk). Developed the plate using the solvent system and recorded the chromatogram. Calculated the amount of Gallic acid present in the sample from the calibration curve.

3. **Densitometric estimation of β-sitosterol**

**TLC plates**

Precoated plates of silica gel 60 F_{254} (E. Merck) with uniform thickness of 0.2 mm

**Solvent system**

Toluene: Ethyl acetate (7:3)

**Scanning**

500 nm
Test solution
CaSB (5 g) was extracted with 100 ml methanol in a Soxhlet extractor at 60°C for 2 hrs. The extract was filtered and the solvent was evaporated using rotary evaporator till dryness. The residue was redissolved in methanol and made up to 10 ml. This solution was used for HPTLC profile.

Standard solution
Dissolved 1 mg of standard β-sitosterol in 25 ml of methanol in a volumetric flask

Calibration curve
Applied 2 to 25 µl of standard solutions on a precoated silica gel 60 F254 TLC plate. Developed the plate using the solvent system in twin trough chamber to a distance of 8 cm and scanned densitometrically at 500nm. Recorded the peak area and prepared the calibration curve by plotting peak area vs concentration of β-sitosterol applied.

Estimation of β-sitosterol in the drug
Applied 2 to 4 µl of the test solution on a precoated silica gel 60 F254 TLC plate (E. Merk). Developed the plate using the solvent system and recorded the chromatogram. The amount of β-sitosterol present in the sample was calculated from the calibration curve.

3.3.9. GC/MS analysis of essential oil
Gas Chromatography-Mass Spectrometry (GC/MS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample. The GC/MS is composed of two major building blocks: the gas chromatograph and the mass spectrometer.

Fresh leaves hydro distilled in a Clevenger-type apparatus for 4 hours. At the end of distillation the oils were measured, collected, dried with anhydrous Na2SO4, and
transferred to 5 ml glass ampules that filled to the top and kept at a temperature of
−10°C for analysis. GC-MS analysis was performed on Agilent 5975C VL MSD
with triple axis detector, using a HP-5-MS (Agilent) fused silica capillary column (30
m × 0.25 mm; 0.25 μm film thickness composed of 5% phenylmethylpolysiloxane)
and temperature programmed as above. The carrier gas was He at a flow rate of 1.0
mL/min and the split mode at a ratio of 1:20. The injection port was set at 220°C.
Quadrupole MS operating parameters were at interface temperature 240°C; electron
impact ionization at 70 eV with scan mass range of 50-500 m/z at a sampling rate of
1.0 scan/s. Compounds were identified by computer search using digital libraries of
mass spectral data [National Institute of Standards and Technology, 2011] and by
comparison of their retention indices and authentic mass spectra [Adams, 2007].

3.4. BIOLOGICAL ACTIVITIES

3.4.1. ANTIOXIDANT STUDIES

DPPH free radical scavenging assay, the potential antioxidant activity of the studied
plant materials were assessed on the basis of the free radical scavenging activity of
the C1SB methanol extracts against the stable 2, 2-diphenyl-1-picrylhydrazil (DPPH)
free radical. The assay was performed according to the method described by Brand-
Williams et al., (1995) with slight modification. A total 250μl of a reaction mixture
was prepared in 96 microwell plates. Firstly, the reaction mixture consisted of 50μl
of extracts and positive control (quercetin) (after dilution to a final concentration of
100μg/ml for both extract and quercetin) and 150 μl of 0.3 mM DPPH ethanolic
solution was incubated at 37 °C for 30 min. Then, the decrease in absorbance value
was measured at 515 nm using microplate reader (Thermo, Multiskan Ex). Obtained
absorbance value was then converted into the percentage of radical scavenging
activity using the following equation: Radical scavenging activity 
(\%) = 100 - [(AS/AC) × 100] where AS: absorbance of the sample; AC: absorbance of 
the negative control (ethanol). For IC\textsubscript{50} determination, the extracts were serially 
diluted to six different concentrations (from 100 to 3.125\(\mu\)g/ml). IC50 values were 
obtained from the graph of radical scavenging percentage against log concentration, 
at 50\% of radical scavenging.

3.4.2. ANTIMICROBIAL STUDIES

**Preparation of plant extract:** The CaSB were thoroughly washed, dried in shade 
and powered. 100g of the dried stem bark was successfully extracted using Soxhlet 
apparatus with methanol as solvent. After two days of extraction the solvent was 
evaporated off using rotary evaporator till dryness and the residue obtained was used 
for the studies. The extract was dissolved in Dimethyl sulfoxide (DMSO) and used 
antibacterial screening at the concentration of 1g/5ml. The water extract was 
prepared by weighing 100mg powder of stem bark by boil with 300ml of distilled 
water in a water bath for 24 hours, filtered and evaporated. The extract obtained was 
dissolved in sterile distilled water at a concentration of 1g/5ml and used for 
antibacterial screening.

**Preparation of inocula:** Several colonies were transferred to sterile peptone water (5 
ml) from the sub cultured organism. The suspensions were mixed for 15 seconds to 
ensure homogeneity and subsequently diluted to match the turbidity of a 0.5 
McFarland standard (\textit{i.e.} OD = 0.12–0.15 at \(k = 530 \text{ nm, corresponding to } 1–5 \times 10^6 \text{ CFU/ml).} 

**Antimicrobial screening:** The antimicrobial assay was performed by two methods 
viz. agar disc diffusion method (Bauer \textit{et al.}, 1966) and agar well diffusion method
(Perez et al., 1990). Mueller Hinton Agar (MHA) was prepared in plates as the media for test bacteria. The bacterial inoculum (0.5 MacFarlands standard) was spread evenly on the surface of the MHA plates using a sterilized cotton swab. For agar disc diffusion method, sterile filter paper discs (6mm) were saturated with different concentrations of the test compound, allowed to dry and introduced on the upper layer of the seeded agar plate. For agar well diffusion method, a well was prepared in the plates with the help of a cork-borer (0.6 cm). 100 µl of the test compound was introduced into the well. The plates were incubated overnight at 37 °C. For each bacterial strain controls were maintained where pure solvents were used instead of the extract. Sterile distilled water served as negative control. The result was obtained by measuring the zone diameter. The experiment was done thrice and the mean values are presented. The results were compared with the standard antibiotics nitrofurantoin (300µg/disc), chloram-phenicol (30µg/disc), cephalxin (30µg/disc) and gentamicin (10µg/disc).

**Agar disc diffusion method**

Sterile cotton swab on to the standardized suspension and then rotated and compressed against the wall of the test tube so as to expel the excess fluid. Inoculated the surface of the MHA plate with the swab to ensure that the growth is uniform and confluent. Whatman No.1 filter paper discs of 6mm in diameter were prepared, sterilized at 121°C and 15 lbs pressure. Sterile paper discs were placed equidistantly on the surface of MHA plates to screen antibacterial activity. Each paper discs were impregnated with 5mg, 10mg, 20mg, and 30mg of the plant extract. A cotrimoxazole (25µg), gentamicin (10µg) discs were used as positive control and 10% dimethyl sulfoxide as negative control. The plates were incubated at 37°C for 24
hours in an incubator. After incubation, the plates were examined for zone of growth inhibition which are expressed in millimeter.

**Agar well diffusion method**

Sterile cotton swab on to the standardized suspension and then rotated and compressed against the wall of the test tube so as to expel the excess fluid. Inoculated the surface of the MHA plate with the swab to ensure that the growth is uniform and confluent. Using a sterile cork borer, wells of 6mm diameter were made on agar surfaces. Wells are made equidistantly on each plate, wells are loaded with 5mg, 10mg, 20mg, 30mg, 40mg, 60mg, 80mg and 100mg (corresponding to 3g/5ml) of the plant extract and cotrimoxazole and gentamicin antibiotic discs used as positive control and 10% dimethyl sulfoxide as negative control. Plates were incubated at $37^\circ$C for 24 hours in an incubator and examined for zone of growth inhibition expressed in millimeter.

**3.4.3. CYTOTOXICITY STUDIES**

The cytotoxicity of the extracts was done on HEK293 cell lines under *in vitro* condition.

**3.4.4. ANTICANCEROUS STUDIES**

**Preparation of Plant extract**

The shade dried CaSB was powdered. The sieved powder was then extracted with methanol (5g in 200 ml). The organic solvent extract was dried in a rotary evaporator.

Isolation of lymphocytes Blood samples were aseptically collected in heparinized sterile tubes (14–17 U/mL) from median cubital vein of non-smoking healthy individuals (22–25 years). Lymphocytes were isolated using ficoll–histopaque
(Sigma, USA) by the method (Boyum, 1968). Blood was diluted 1:1 with phosphate-buffered saline (PBS) and layered onto histopaque with the ratio of blood and PBS: Histopaque maintained at 4:3. The blood was centrifuged at 400g for 35 min at room temperature. The lymphocyte layer was removed and washed twice in PBS at 250g for 10 min each, and then washed with (RPMI-1640) media. Isolated lymphocytes were divided into six groups; in each group six samples were processed.

Group A : CaSB (10 µg/mL)
Group B : CaSB (25 µg/mL)
Group C : CaSB (50 µg/mL)
Group D : CaSB (75 µg/mL)
Group E : CaSB (100 µg/mL)
Group F : CaSB (150 µg/mL)
Group G : DMSO Control
Group H : Normal Control

**Treatment of the cells**

Thirty min. prior to irradiation, six test-doses (10, 25, 50, 75, 100, 150 µg/mL) of methanol extract of CaSB were added to the grouped normal lymphocytes. Before added the extract, the cells were washed twice with (RPMI-1640) media.

**MTT assay**

The MTT test is a colorimetric non-radioactive assay for measuring cell viability through increased metabolisation of tetrazolium salt (Moshmann, 1983). Isolated lymphocytes in concentration of 1x10^6 cells/ml were taken 200 µL into different eppendorf tubes. Then the cells were pretreated with different concentration (10, 25, 50, 75, 100, 150 µg/mL) of methanol extract of CaSB and added 1 mL of RPMI-
1640 medium for 30 min. After irradiation cells were incubated in the presence of 5% CO2 and 95% O2 at 37°C for 24 h. The incubated cells were added MTT (0.5 mg/mL), further incubated for 4 h. After incubation, all the tubes were centrifuged for 10 min. Under standard conditions the medium with MTT was removed and 200 µL of DMSO were added into each tubes. Absorbance was measured to colorimetry at 570 nm. The OD values are plotted to calculate percentage cell death.

3.4.5. IN VITRO STUDIES ON HEP3B CELL LINES

Preparation of Plant extract

The shade dried bark was powdered. The sieved powder was then extracted with water and organic solvents with different polarity like alcohol and hexane (5g in 200 ml). The organic solvent extract was dried in a rotary evaporator. 100 mg of extracts were suspended in 1 ml dimethyl sulfoxide (DMSO) and sterilized using 0.22 µm disc Durapore Syringe filters (Millipore, Bedford, USA). The prepared stock was maintained at 4°C. Test solutions were prepared freshly by diluting the stock solution in DMSO to give different concentrations (100, 50 and 25 µg/ml), with DMEM containing 10% (v/v) FBS and IX antibiotic-antimycotic for cell culture experiments. Similarly the drug silymarin (50µg/ml) was diluted. Maximum concentration of DMSO was maintained as =0.1% (v/v).

Cell revival (from Cryo-stock)

Cryo vials containing Hep3B and HEK cell line were taken from liquid nitrogen or from Cryo baby (-80°C) and thawed immediately at 37°C in water bath. It was then transferred to a sterile eppendorff and centrifuged at 4000 rpm for 10 min. at room temperature. 1 ml complete medium (10% FBS) was added to the pellet. This
mixture was transferred to a culture flask and 5 ml complete medium with 10% FBS was added and kept in CO₂ incubator at 37°C and 5% CO₂.

**Trypsinization and cell splitting**

When the cells became monolayer the medium was discarded and washed with 1-2 ml of PBS for 15 seconds. Discard the PBS solution, added Trypsin EDTA (0.25% Trypsin in PBS-EDTA) along the floor of the plate. Replaced 2/3rd of Trypsin spread it on the surface. The flask was incubated in CO₂ incubator for 3 min. 1 ml of complete media was added so that the glass get suspended in the medium and are pippetted to detach out the cells to make a fine suspension of cells in the complete medium. The media along with cells was transferred to an eppendorff, centrifuged at 4000 rpm for 10 min. at room temperature. Resuspended the pellet in 1 ml complete media and 500µl of the cell suspension was transferred to a new flask. 5 ml of complete medium and 500 µl of cell suspension was added to the parent flask and incubated in CO₂ incubator. The trypsinized cells were counted using a haemocytometer.

**Heat-inactivating Serum**

The serum bottles were placed in a 56°C water bath and incubated for 30 min., swirled the bottles every 3-5 min to ensure uniform heating of the serum. After 30 min (25 min for equine serum), removed the serum bottles and cooled slowly to room temperature. Heat-inactivated serum stored at -20°C to avoid the amount of accumulated precipitate.

**DEPC water preparation:** Added 1 ml of 0.1% Diethylpyrocarbonate (DEPC) to 1000ml distilled water, Mixed well with a magnetic stirrer and allowed to set at room temperature for 1 hour. After autoclaving, cooled to room temperature prior to use.
Cell Counting using Haemocytometer

Cells were trypsinized and suspended in fresh 1 ml complete medium. 20 µl the cell suspension was taken and loaded to the haemocytometer to determine the cell number.

The total count from 4 sets of 16 corners =

\[
\text{Cells / ml x 10^4} \times \text{4 squares from one Haemocytometer grid}
\]

Cell freezing and Cryo-stock preparation

Cells were trypsinised and suspended in complete medium, centrifuged at 4000 rpm for 10 min. at room temperature. Removed the supernatant and added 2 ml freezing mixture (DMSO and FBS 1:9). 1 ml of the cell suspension was transferred to the Cryo-vial and edge of the cryo-vial was sealed with paraffin film. Placed in the Cryo-baby and transferred to -80°C for 24 hours and then transferred to liquid nitrogen (-196°C).

Seedling of cells to culture plates (96 well plate, for MTT Assay)

The cells were trypsinized and suspended in 1 ml complete media with 10% FBS. The number of cells/ml of the complete medium was counted using haemocytometer. Each square should contain approximately 100 cells i.e. 5000 cells in 1 ml (1000µl). 5000 cells per well were required for MTT assay. The capacity per well is 300 µl. Took 1 ml of cell suspension to 29 ml DMEM in a sterile test tube. The plate was incubated in CO₂ incubator for 2 days for the formation of monolayer.

MTT assay

Cytotoxicity of *C. albidum* extract was tested in Hep3B cells as well as non-hepatocyte cell line HEK, using the MTT assay as described (Wills and Asha, 2009). The cells were seeded in 96 well plates (Greiner, Frickenhausen, Germany) with 5 X
10^3 cells/100 µl and incubated for 24 h at 37°C. The cells were treated with three different concentrations of the extract (100, 50 and 25 µg/ml), Silymarin (50 µg/ml) and DMSO 0.1% (v/v) and incubated for different time periods (24, 48 and 72 h). After incubation, 50 µl of (5mg/ml) MTT in PBS was added and incubated for 4-5 hours. 150 µl of 10% SDS in DMSO was added to stop the reaction and incubated overnight at 37°C. Absorbance was read at 570 nm using a 96-well micoplate reader (BIO-RAD, Hercules, CA, USA).

The percentage growth inhibition and percentage viability of the culture were calculated according to the following equation

**Percentage of growth inhibition**

\[ \text{Percentage of growth inhibition} = 100 - \frac{\text{Mean OD of individual test group} \times 100}{\text{Mean OD of the control}} \]

**Percentage of viability** = 100 – percentage of inhibition

- Value is 0 : No inhibition
- Value is below 0 (-ve) : Cell proliferation
- Value is above 0 (+ve) : Cell inhibition

**Detection of apoptosis**

**Morphological changes of cells**

Cells were seeded in 6 well plates at 1 x 10^5 cells/wells and treated with 100µg/ml concentrations of extracts at 24 and 48 hours time intervals. The morphological changes in Hep3 B cells were observed with a phase contrast microscope [Leica DFC 280 CCD Camera (Leica Microsystems Digital Imaging, Cambridge, UK)].

**Hoechst staining**

The changes in DNA chromatin morphological features were detected by Hoechst 33342 staining (Diaz-Ruiz et al., 2001). Cells were seeded in 6 well plates and
treated with the extract for desired time period and concentration. After drug
treatment, remove the media; washed the cell with 1 ml PBS to remove the debris.
Add Hoechst stain (1 µg/ml) and incubate for 30 min. in dark, removed the staining
solution. Apoptotic cells were quantified using fluorescent microscope [Leica DFC
280 CCD Camera (Leica Microsystems Digital Imaging, Cambridge, UK)].

**Statistical Analysis:** The data were statistically analysed for accuracy

**PREPARATION OF THESIS**

Compilation of all data available from the studies carried out with necessary
photographs for the preparation of final report.