3.1 METHODLOGY

The entire work flow is represented in flow chart and is shown in Fig. 3.1.
The present research deals with *Ficus benghalensis* fruits and it’s have been collected from the Western Ghats region of Kanyakumari District and the *Ficus benghalensis* fruits have been analyzed by different laboratory techniques.

The *Ficus benghalensis* fruits were subjected to soxhlet extraction (Methanol, Chloroform and Water) and the extracts were again subjected to phytochemical analysis (both qualitative and quantitative) for the presence of flavonoids, alkaloids, saponins, and steroids. Based on the phytochemical analysis the anti-cancerous compounds have been identified and purified from the *Ficus benghalensis* fruit extract.

Anti-cancerous compound were subjected to chemical informatics studies, including structure prediction, docking and finding the binding affinities of selected ligands and receptors of the proteinaceous and drug compounds present in the extract. The resulting docking studies reveals the percentage of docking score for the selected ligands and receptors from the *Ficus benghalensis* fruit extracts. These results can be used for further drug development and modelling.

Nanomedicine played a vital role in cancer therapeutics. Nano vehicle is necessary to carry the drug to the target. Iron oxide nano particles were synthesized, characterized and used as a nano vehicle. The iron oxide nano particles were bioconjugated with the anti-cancerous compound of the fruit extract for drug delivery.

The effectiveness of drug delivery to the cancer cells carried out by both *in vitro* and *in silico* techniques were compared with the conventional techniques. The *in vitro* approach of drug delivery were carried out in three different cancer cell lines. The partially purified drug molecule and bioconjugated nanomedicine from *Ficus benghalensis* fruit extracts were subjected for in vitro cancer cell lines and the results were compared with standard anticancer drugs.
3.2 **FICUS BENGHALENSIS FRUITS**

3.2.1 Sample Collection

The *Ficus benghalensis* fruit samples were collected from South Western Ghats regions of Kanyakumari District, Tamilnadu, India during the months of August, September and October in the year 2013, for the purpose of soxhlet extraction.

3.2.2 Sample Identification

The *Ficus benghalensis* fruits were validated by an eminent botanist. The authenticated samples were stored and preserved for future extraction.

3.2.3 Preparation of Extract

The *Ficus benghalensis* fruits were washed thoroughly with distilled water and dried. The drying was performed until all the water molecules evaporated and became suitable for grinding. Thereafter, using a mechanical grinder, the fruits were ground well into a fine powder. They were then transferred into an airtight container and tagged for future use. Extraction of the dried and powdered *Ficus benghalensis* fruits was done sequentially with methanol, chloroform, and water employing Soxhlet apparatus.

3.3 **PHYTOCHEMICAL SCREENING**

3.3.1 Extraction of *Ficus benghalensis* Fruits by Continuous Hot Percolation Method

500 g of dried fig fruit powder was weighed and successively extracted with 2.5 litres of solvents like methanol (60 °- 80 °C), chloroform, and water by soxhlation for a period of 72 hrs were shown in Fig. 3.2. To concentrate the extract, the solvent was subjected to distillation and further concentrated with a rotary evaporator under reduced pressure and dried.
Fig. 3.2. *Ficus benghalensis* fruits were extracted using Soxhlet apparatus

### 3.3.2 Qualitative Phytochemical screening

Screening was performed on the methanol, chloroform and water fruit extracts for identifying the following phytochemical constituents: Protein, Carbohydrates, Vitamin C, Reducing sugars, Terpenoids, Steroid, Flavonoids, Saponins, Amino acids, Alkaloids, Tannins, Phenol, Glycosides, Phlobatanins, Anthroquinones and Chloride.

#### 3.3.2.1 Phytochemical Qualitative Screening Tests for Extracts

The secondary metabolites were analysed using the following methods:

*Test for Carbohydrates Using Benedict's Test*

The crude extract (2 ml) was mixed with Benedict’s reagent (2 ml) and then boiled. Formation of a reddish brown precipitate confirmed the presence of carbohydrates.

*Test for Reducing Sugars*

The extracts were mixed well with distilled water and filtered. Then a few drops of Fehling’s solution A and B were added to the filtrate and boiled for a few minutes. Formation of an orange red precipitate showed the presence of reducing sugars.
Test for Amino Acids Using Ninhydrin Test

In a water bath, 3 ml of crude extract was heated with 3 drops of Ninhydrin solution (5%) for 10 minutes. A purple or bluish colour indicated the presence of amino acids.

Test for Proteins Using Biuret Test

3 ml of crude extract was added to 3 ml of 4% NaOH, followed by addition of a few drops of CuSO₄ (1%) solution. The appearance of violet or pink colour indicated the presence of proteins.

Test for Vitamin C

The crude extract (1 ml) was diluted with distilled water (5 ml). To this, 1 drop of freshly prepared sodium nitroprusside solution (5%) and diluted sodium hydroxide solution (2 ml) was added. Further, hydrochloric acid was added (about 0.6 ml) drop wise while stirring. Presence of vitamin C was indicated by a colour change from yellow to blue.

Test for Chloride

3 ml test solution was prepared in HNO₃ and a few drops of AgNO₃ solution (10%) were added to it. A white precipitate of AgCl₂ implied the presence of chloride.

Test for Tannins

FeCl₃ solution (5%) was added to 2-3 ml of the crude extract. Deep blue-black colour indicated tannin.

Test for Alkaloids Using Wagner's Test

To 2-3 ml of filtrate, a few drops of Wagner's reagent was added. A reddish brown precipitate showed the presence of alkaloids.

Detection of flavonoids

Flavonoids were detected by the lead acetate test. A few drops of lead acetate solution (10%) was added to the extract. Appearance of a yellow precipitate confirmed the presence of flavonoids.
**Test for Phlobatannins**

The extract was boiled with aqueous hydrochloric acid (1%) and observed for the appearance of a red precipitate.

**Test for Steroids**

2 ml of acetic anhydride and 2 ml of sulphuric acid were added to 2 ml of extract. A colour change from violet to blue or green confirmed the presence of steroids.

**Test for Terpenoids Using Salkowski Reaction**

2 ml chloroform and 2 ml concentrated sulphuric acid were added to 2 ml of extract. The chloroform and acid layers were observed for red colour and fluorescence respectively. A reddish brown colouration on the boundary between the layers indicated the presence of terpenoids.

**Test for Phenolic Compounds Using Ferric Chloride Test**

To 5 ml of diluted extract, a few drops of neutral ferric chloride solution (5%) were added. Appearance of a dark green colour indicated presence of ferric chloride.

**Test for Saponins**

The extract was made up to 20 ml by adding distilled water. The solution was then mixed well in a graduated cylinder for 15 minutes. Formation of a 2 cm foam layer confirmed the presence of saponin.

**Test for Glycosides**

0.5 ml HCL was added to 2 ml of the crude extract. Then, it was neutralized with 0.5 ml of NaOH solution. To this, a few drops of Fehling’s solution A and B were added. The solution was observed for the formation of a red precipitate.

**Test for Anthroquinones**

In a water bath, 0.5 g of the extract was dissolved in HCL (10%) and boiled for a few minutes. Then it was strained and left to chill. To the filtrate, an equal amount of chloroform was added. Further, a few drops of ammonia (10%) were added to the solution and heated. The presence of anthroquinones was indicated by a rose-pink colour formation.
3.3.3 Quantitative Phytochemical Screening

Since the qualitative phytochemical screening proved that maximum number of secondary metabolites were present in the methanolic extract of *Ficus benghalensis* fruits, further quantitative analysis was done on this extract. Flavonoids were also found only in the methanolic fruit extract.

3.3.3.1 Phytochemical Quantitative Screening Tests for Extracts

The methanolic fruit extract was screened for three major phytochemical constituents: total protein, total flavonoid content and ascorbic acid. The methods employed to analyse the phytochemical components are described below.

*Estimation of Protein*

The total protein content was assessed as proposed by Lowry *et al* (1951). A bovine serum albumin stock solution (1mg/ml) was prepared in sodium hydroxide (1N). Five different concentrations (0.2, 0.4, 0.6, 0.8, 1 ml) of the prepared stock were taken in different test tubes. 0.1 and 0.2 ml of the extract were taken in another set of test tubes. The volume was made up to 1 ml in each test tube, followed by addition of the prepared alkaline solution (5 ml) at room temperature. The solutions were left undisturbed for about 10 minutes. Then, rapid addition of 0.5 ml Folin-Ciocalteu reagent was done. The solutions were incubated for 30 minutes at room temperature until a blue colour appeared. Before observing the readings of the standard and the samples, the spectronic colorimeter was set at a wavelength of 750 nm and transmittance of 100% using blank. A regression curve of concentrations of standard solutions vs. their respective absorbance was plotted. It was found to follow Beer’s law.

*Estimation of Flavonoid*

To determine the total flavonoid content, aluminium chloride colorimetric technique was employed. For the assay, the sample (1 ml) was diluted with distilled water (4 ml) followed by addition of 0.3 ml of 5% NaNO₂. To this, 0.3 ml of AlCl₃ (10%) was added after 5 minutes. 2 ml of NaOH (1M) was added after a minute. Then the solution was diluted to 10 ml by adding 2.4 ml distilled water and thoroughly stirred. The absorbance was measured at 510 nm. The total flavonoid content was denoted in terms of mg catechin equivalents (CE) per gram of sample.
Estimation of Ascorbic Acid

The extract (50 μg) was homogenized in 4% oxalic acid (10 ml) and further centrifuged for a period of 15 minutes at 5000 rpm. The supernatant was collected. Next, drop wise addition of bromine water was done under constant stirring until a yellow colour was formed. Excess bromine was removed by a pipette. The volume of the resultant solution was increased to 3ml with distilled water. This was then reacted with 1 ml of DNPH (2%) and filtered by a filter paper. Then, a few drops of thiourea (10%) was added.

To prepare the blank, the same procedure was followed. Instead of the extract, distilled water was used. The solutions were incubated at 37 °C for a period of 3 hours. Osazone crystals were dissolved in 7 ml of H₂SO₄ (80%). Measurement of absorbance was done at 540 nm using a UV-Vis spectrophotometer. The results were denoted in terms of milligrams of ascorbic acid equivalent per gram of dry weight.

3.3.4 Characterization of Ficus benghalensis Fruit Extracts

Ficus benghalensis methanolic, chloroform and aqueous crude fruit extracts were subjected to basic preliminary characterization techniques viz., UV-vis Spectroscopy and Fourier Transforms Infrared (FTIR) spectroscopy.

3.3.4.1 UV-vis Spectroscopy

The UV-Visible spectrum was recorded on Systronic double beam spectrophotometer: 2202. The progress of the reaction among different ions of Ficus benghalensis fruits extracts was monitored by the spectra. The measurements were taken in a wavelength ranging from 200-1100 nm.

3.3.4.2 Fourier Transforms Infrared (FTIR) spectroscopy

Dried powder of Ficus benghalensis methanolic, chloroform and aqueous crude fruit extracts were subjected to FT-IR instrumental analysis (Bruker optics, Alpha-T, Germany). The dried powder of fruit extracts (10 mg) was mixed with 100 mg of KBr to prepare translucent sample pellets. The pellet discs were evaluated by KBr pellet mode of FTIR spectroscopy.
3.4 IN VITRO ANTIOXIDANT ACTIVITY

Antioxidants are molecules that slow or prevent the oxidation of molecules by eliminating free radical intermediates. They can also oxidize themselves, thus inhibiting other oxidation reactions. Antioxidants may be hydrophobic or hydrophilic. In general, the former reacts with oxidants in the cell cytosol and blood plasma, while the latter defend cell membranes from lipid peroxidation. The antioxidant potential of fruit extracts of *Ficus benghalensis* were evaluated by analysing free radical scavenging activity on DPPH (1,1 diphenyl-2-picryl hydrazyl), reducing power assay method, and nitric oxide radical scavenging activity.

3.4.1 Sample Preparation

The extracts of *Ficus benghalensis* fruits were dissolved in methanol at a concentration of 100 mg/ml to prepare the stock solution.

3.4.2 Free Radical Scavenging Activity on DPPH

The method proposed by Blois (1958) was employed to determine the antioxidant potential of the samples. It was indicated in terms of hydrogen donating or radical scavenging ability. For the analysis, the stable radical DPPH (1, 1 diphenyl-2-picryl hydrazyl) was used. Various concentrations of the sample extracts were made (200 – 1000 µg). Using methanol, the volume was made up to 100 µl. Then, about 5 ml of 0.1 mM methanolic solution of DPPH was added and the solution was left undisturbed at 27 °C for 20 min. The absorbance was measured at a wavelength of 517 nm and results were calculated using Eq:3.1.

Percentage radical scavenging activity of the sample was determined as follows:

\[
\% \text{ DPPH radical scavenging activity} = \left( \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} \right) \times 100 \quad \text{Eq:3.1}
\]

The analysis was repeated thrice. A graph of inhibition percentage versus sample concentration was plotted. From the graph, the sample concentration providing a 50% inhibition (IC\(_{50}\)) under the analysis condition was evaluated.

3.4.3 Nitric Oxide Radical Scavenging Activity

The nitric oxide scavenging activity of the extract was evaluated as per the Sreejayan and Rao (1997) procedure. 10 mM sodium nitroprusside (3 ml) was dissolved in 0.2
M PBS solution (phosphate buffered saline - pH 7.4). This was further mixed with solvent extracts taken at different concentrations and then incubated at room temperature for 150 minutes. Incubation was followed by addition of 0.5 ml Griess reagent (1% sulphanilamide and 0.1% naphthylethylene diamine dihydrochloride dissolved in 2% H₃PO₄). Measurement of absorbance was done at a wavelength of 546 nm and calculated using Eq 3:2.

Percentage of radical scavenging activity of the extract was determined as follows:

% NO radical scavenging activity = \[ \frac{(\text{control OD} - \text{sample OD})}{\text{control OD}} \times 100 \] Eq 3:2

The analysis was repeated thrice. A graph of inhibition percentage versus sample concentration was plotted. From the graph, the sample concentration providing 50% inhibition (IC₅₀) under the analysis condition was evaluated.

### 3.4.4 Reducing Power Assay

500 µl of phosphate buffer (2M, pH 6.6) and 500 µl of potassium ferricyanide (1%) were added to 100 µl of extract. The solution was incubated at 50 °C for a period of 20 minutes. After incubation, 500 µl of trichloroacetic acid (10%) was added to the mixture. This solution was then centrifuged at 3000 rpm for 10 min. The supernatant was collected and its volume was estimated (around 500 µl). To it, an equal volume of distilled water and 100 µl of ferric chloride solution (0.1%) were added. The absorbance was measured at 700 nm. To prepare the blank, 200 µl of ethanol was added instead of the extract. The reducing power of the extract was denoted in terms of ascorbic acid equivalent.

### 3.5 ANTI-MICROBIOLOGICAL EVALUATION

Microbial assay is a measure of the activity of antibiotics (inhibit microbial growth) or vitamins and amino acids (support microbial growth), whereas chemical assays of such substances estimate only their concentration or amount. Vitamins can be assayed by turbidimetry, whereas antibiotics can be assayed by both turbidimetry and diffusion assay methods.

#### 3.5.1 Culture and Media Preparation

Antimicrobial activity was assessed by agar well diffusion method. The fruit extracts of *Ficus benghalensis* were tested for antimicrobial activity against the following
bacterial pathogens: *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*. The microorganisms were collected from the Microbial Type Culture Collection (MTCC), Chandigarh, India and preserved in the laboratory by periodic subculture.

### 3.5.2 Muller Hington (MH) Agar Medium Preparation

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>3 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15-20 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.4 ± 0.2</td>
</tr>
</tbody>
</table>

All the ingredients of culture medium were weighed and dissolved in water. The solution was heated with proper agitation. Then, agar was added with constant stirring for proper mixing of constituents.

### 3.5.3 Antibacterial Assay

The antibacterial activity of the extracts was studied by well diffusion method. In the present investigation, four bacterial strains namely *Bacillus cereus*, *Escherichia coli*, *Klebsiella pneumonia*, and *Staphylococcus aureus* were used. Sterilized discs were soaked overnight in fruit extracts (methanol, chloroform, and water) at room temperature. Then the discs were dried aseptically to ensure evaporation of solvents. The media used for the test was nutrient broth. The bacterial strains were inoculated into nutrient broth and incubated at 37 °C for 24 hours. The culture tubes were then matched with the turbidity standard.

On the nutrient agar plate, fresh bacterial culture (0.1 ml, 108 CFU) was spread with a swab. 6 mm diameter wells were perforated in the medium with an aseptic cork borer. The wells were filled with 50 μl of *Ficus benghalensis* fruit extracts by using a sterile micropipette. The plates were then refrigerated for 30 minutes to allow the extracts to pre-diffuse. Further, the plates were incubated at 37 °C for 24 hrs. The activity was evaluated by measuring the zone of inhibition.
3.6 PRELIMINARY ANTICANCER ACTIVITIES

The preliminary tests were done on the three different *Ficus benghalensis* fruit extracts to evaluate their activity. The preliminary results confirmed that methanolic fruit extract shows better antioxidant activity. Moreover phytochemical screening too confirmed that methanolic fruit extracts have more secondary metabolites than other two extracts.

In the preliminary studies the methanolic extract of *Ficus benghalensis* fruit was subjected to cytotoxic activity. Anticancer effect of *Ficus benghalensis* methanolic fruit extracts was determined on A431 melanoma cell lines.

3.6.1 In vitro Studies of Anti-cancer Activity against A431 Melanoma Cell Lines

A431 melanoma cell lines were purchased from NCCS Pune and maintained in Dulbecco’s modified eagles media (HIMEDIA) enriched with 10% FBS (Invitrogen). Confluency was achieved by incubation in a CO₂ incubator (5%) at 37 °C in a humidified atmosphere (NBS, EPPENDORF, Germany). The cells were trypsinized (500 µl of 0.025% Trypsin in PBS/ 0.5 mM EDTA solution (Himedia)) for 2 minutes and transferred to T flasks in highly sterile conditions. 6.2 µl, 12.5 µl, 25 µl, 50 µl and 100 µl of sample was added to grown cells and incubated for 24 hours. The percentage difference in viability was estimated by standard MTT assay.

3.6.2 MTT Cell Viability Assay

MTT, a colorimetric assay technique, involves measuring the reduction of yellow 3-(4, 5dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. It goes into the cells and to the mitochondria where it is reduced to an insoluble, coloured (dark purple) product, namely formazan. The cells are then solubilised with dimethyl sulfoxide. The released, solubilized formazan product was measured at 540 nm. MTT reduction takes place only in metabolically active cells; hence the extent of activity is a measure of the viability of the cells.

The cells were washed with PBS. 30 µl of MTT solution (MTT -5 mg/ml in PBS) was added to the culture. It was then incubated at 37 °C for 3 hours. MTT was removed by washing with 1x PBS. This was followed by addition of 200 µl dimethyl sulfoxide to the culture. Then it was incubated for 30 minutes at room temperature until the cells got lysed and colour was attained. Next, the solution was centrifuged at
high speed for 2 minutes. This was done for cell debris precipitation. Optical density was noted at 540 nm using dimethyl sulfoxide as blank in a microplate reader (ELISASCAN, ERBA) and calculated using the Equation 3.3.

Percentage viability of anti-cancer activity of the given sample was calculated as follows:

\[
\text{% Percentage viability} = \frac{OD_{\text{Test}}}{OD_{\text{Control}}} \times 100 \quad \text{Equation 3:3}
\]

3.7 CHROMATOGRAPHIC TECHNIQUES

Chromatography involves breaking up a sample in a versatile stage (which may be a gas, a fluid or a supercritical liquid). The movable stage is then constrained through a fixed, immiscible stationary stage. The stages are picked in such a way that parts of the specimen have varying solubilities in every stage. A part which is entirely soluble in the stationary stage will take more time to go through it than a segment which is not exceptionally dissolvable in the stationary stage but rather extremely solvent in the portable stage. As an after effect of these distinctions in mobilities, test parts will get to be isolated from each different as they go through the stationary stage.

3.7.1 Thin Layer Chromatography

The preliminary analysers revealed that methanolic extract show better anticancer activity. Thus the major aim of the chromatographic technique is to purify the anticancer flavonoids from methanolic *Ficus benghalensis* fruit extracts. Flavonoids were derived and separated from crude *Ficus benghalensis* methanolic fruit extracts by using standard methods.

The flavonoids are all basically obtained from the guardian substance flavone, which happens as a white coarse farina on Primula plants, and all share various properties in a like manner. Flavonoids are principally water dissolvable mixes. They can be extricated with 70 % ethanol and stay in the fluid layer, taking after parcel of this concentrate with petroleum ether. Flavonoids are phenolic and consequently change in their colour when treated with base or with alkali; therefore they are effortlessly distinguished on chromatograms or in arrangement.
3.7.1.1 TLC Slurry Preparation

Silica gel and a little amount of calcium sulfate (gypsum) were blended with distilled water. This mixture was spread as a thick slurry on a perfect glass plate. Drying and activation of the resultant plate by heating it at 80 °C for 3 hours. The slurry of silica gel was then prepared with a coating thickness of 500 µ in the glass slide.

![TLC plate for flavonoid separation](image)

**Fig. 3.3. TLC plate for flavonoid separation**

*Ficus benghalensis* methanolic fruit extract 0.05 ml was stacked on the TLC plates simply above 2 cm from the base utilizing a capillary tube and the plates were set in a developing jar containing the versatile stage solvents blend. The versatile stage contained n-butanol, acetic acid and water (2: 2: 6). The plates were evacuated and permitted at room temperature for 30 minutes and results were pictured specifically in UV light illuminator.

### 3.7.2 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatographic (HPLC) analysis was performed on a HPLC system (Waters) using 4.6 x 250 mm C18 column with 2484 UV-Vis detector at 400 nm and 210 nm. The solvent system Acetonitrile and Water were used with the ratio of 65: 35. The mixture was remixed and sonicated in the sonicator for 15 minutes. 25 µl of the sample from each sample was injected on HPLC using C18 with an isocratic
elution with a 1 ml/min flow rate. Area (V* sec), retention time (min), and height (V* sec) of the peaks were noted.

The high performance chromatography system was performed by Breeze 2 HPLC instrument. The Breeze 2 HPLC System conveys innovation and execution in a moderate, smaller, and easy to understand framework stage. Complete with programming, pump, identifier and injector, the framework comes pre-designed for various levels of HPLC operational needs. The Breeze 2 HPLC System incorporates effortlessness, affectability, precision, and unwavering quality.

3.7.3 Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS examination was completed by using Perkin Elmer Turbo Mass Spectrophotometer (GCMS-5975C, AGILENT, USA) outfitted with an auto sampler XLGC. The column utilized was Perkin Elmer Elite-5 capillary column (dimethyl polysiloxane, 30m × 0.25 mm) with a film thickness of 0.25 mm. The bearer gas utilized was Helium at a 1.5 ml/min flow rate. The sample infusion volume used was 1µl. The injector temperature was maintained at 250 °C. The oven temperature was altered, initially at 70 °C for 3 minutes and afterward customized to increment to 300 °C in steps of 10 °C. The overall run time was 35 minutes. The MS exchange line was kept at a temperature of 240 °C. It was recorded utilizing electron splash ionization at 70 eV and information was assessed utilizing all out particle number (TIC) for compound distinguishing proof and evaluation. The spectra of the parts were contrasted and otherworldly database of known segments in the GC-MS library (NIST-11). Estimation of crest zones and information handling were done by Turbo-Mass-OCPTVS-Demo SPL programming.

3.7.4 Partial Purification of Flavonoid Compounds

Development of the plates was performed using the upper phase of an ethyl acetate/formic acid/water in the ratio 10:2:3 (v/v/v). The desired bands were scraped from the TLC plates and soaked in 50 mL spectral grade methanol for 30 minutes. This was followed by filtration and evaporation to near dryness in a rotary evaporator to near dryness at 45 °C under vacuum. The residue was re-dissolved in 1 mL spectral grade methanol and filtered through glass wool to eliminate any residual silica gel. The constituents were further separated by streaking on silica gel TLC plates (0.5
mL/plate). The partially purified flavonoids were send for GC-MS analysis to identify the similar compounds present in the NIST-11 library.

3.8 NANOPARTICLE SYNTHESIS

Fundamentally there are two methodologies for nanoparticle synthesis; in particular the Bottom up methodology and the Top down methodology. Some of the regularly utilized chemical and natural (biological) techniques are co-precipitation, solvothermal, chemical reduction, gel combustion, and sol-gel method.

3.8.1 Synthesis of Zinc Oxide Nanoparticles by Green Method

3.8.1.1 Preparation of Fresh Fruit Extract

*Ficus benghalensis* fruits were gathered from the surroundings of Kumaracoil. The fruits were washed a few times with distilled water to remove unwanted particles and properly dried. The dried fruits were cut and ground to a fine powder. The extract utilized for the decrease of zinc particles (Zn$^{2+}$) to zinc oxide nanoparticles (ZnO) was made by setting 5 g of washed, dried, finely powdered fruits in 250 ml glass beaker together with 100 ml of double distilled water. The solution was then stirred for an hour by a magnetic stirrer until the shade of the mixture changes from watery to light yellow. The concentrate was cooled to room temperature and filtered. It was put away in an icebox with a specific aim of further characterization.

3.8.1.2 Preparation of Zinc Oxide Nanoparticles

50 ml of *Ficus benghalensis* fruits extract was taken and heated to 60-80 °C in a stirrer-heater. As the temperature reached 60 °C, 5 g of Zinc Nitrate was added to it. This mixture was then heated until it changed to a deep yellow paste. The paste was then transferred to a ceramic crucible and heated at 400 °C in a hot air oven 2 hours. A light yellow coloured powder was obtained. This was collected and crushed well in a mortar-pestle. The resultant powder was stored for characterization purposes.

3.8.2 Synthesis of Zinc Oxide Nanoparticles by Chemical Method

Zinc oxide nanoparticles were synthesized by gel-combustion method. Zinc nitrate was used as the zinc source and citric acid as the fuel. The fuel to zinc nitrate ratio was 0.5. The precursors were weighed, dissolved in distilled water, and well-stirred. After proper mixing, the solution was heated to carry out the combustion process. Black
coloured powder was formed. This was ground in a mortar and pestle and further calcined at a temperature of 450 °C, light yellow coloured zinc oxide nanoparticles were formed. The synthesized particles were properly stored for further characterization.

3.8.3 Synthesis of Iron Oxide Nanoparticle by Green Method

3.8.3.1 Preparation of Fresh Fig Fruit Extracts

*Ficus benghalensis* fruits were gathered from the surroundings of Kumaracoil. The fruits were washed a few times with distilled water to remove the unwanted dust particles and further dried. The dried fruits were again thoroughly washed with distilled water and properly dried. They were then crushed well to make a juice. The *Ficus benghalensis* fruit juice was transferred for filtration. Whatmann No.1 filter paper was used to filter the crude fig juice to remove the impurities present in the juice.

3.8.3.2 Preparation of Iron Oxide Nanoparticles

FeCl₂ (Iron (II) chloride) solution was prepared by adding 4 g FeCl₂. 4H₂O in 10 ml distilled water. FeCl₃ (Iron (III) chloride) solution was prepared by adding 4 g FeCl₃ in 25 ml distilled water. After that 8 ml concentrated hydrochloric acid (HCl) was added to the solution. 5 ml of FeCl₂ (Iron (II) chloride) solution was added in a 600 ml beaker on a stir plate. In addition to that 25 ml of FeCl₃ (Iron (III) chloride) solution was added to the beaker in the same way. After adding both the solutions, *Ficus benghalensis* fruit juice were slowly added to the beaker using a glass pipette approximately at the rate of 50 ml per minute. The brown colour of the solution turned into black which indicated the synthesis of iron oxide nanoparticles.

Once the last drops of *Ficus benghalensis* fruit juice have been added, the stir plate was turned off, and the iron nanoparticles were separated from the solution by attracting the magnetic particles to the side of the glass beaker with a solid magnet. Cleaning and separation process was done twice or thrice by removing the magnet and re-dispersing the black solid in the solution. Using the magnet the black solid particles were drawn to one side and excess solution was decanted. Once the particles were perfect and a large portion of the water was expelled with the partition strategy, a last wash and detachment was processed. The settled black solid particles were collected and kept for drying. Once all the water molecules were completely expelled, the
material was ground in a mortar and pestle in order to get finer particles for characterization.

3.8.4 Synthesis of Iron Oxide Nanoparticles by Chemical Method

3.8.4.1 Preparation of Iron Oxide Nanoparticles

FeCl₂ (Iron (II) chloride) solution was prepared by adding 4 g FeCl₂, 4H₂O in 10 ml distilled water. FeCl₃ (Iron (III) chloride) solution was prepared by adding 4 g FeCl₃ in 25 ml distilled water. Then, 8 mL concentrated hydrochloric acid (HCL) was added to the solution. Add 5 ml of FeCl₂ (Iron (II) chloride) solution in a 600 mL beaker on a stir plate. In addition to that add 25 mL of FeCl₃ (Iron (III) chloride) solution to the beaker. After adding both the solutions, 250 mL of 1M NH₃ (Ammonia) solution was slowly added to the beaker using a glass pipette approximately at the rate of 50 mL per minute. The brown colour of the solution turns into black indicating the synthesis of iron oxide nanoparticles.

Once the last drops of ammonia have been added, the stir plate was turned off, and the iron nanoparticles were separated from the solution by attracting the magnetic particles to the side of the glass beaker with a solid magnet. Cleaning and separation process was done twice or thrice by removing the magnet and re-dispersing the black solid in the solution. Using the magnet the black solid particles were drawn to one side and excess solution was decanted. Once the particles were perfect and a large portion of the water was expelled with the partition strategy, a last wash and detachment was performed. The settled black solid particles were collected and kept for drying. Once all the water molecules were completely expelled, the material was ground in a mortar and pestle in order to get finer particles for characterization.

3.9 CHARACTERIZATION OF NANOPARTICLES

Characterization of nanomaterials post synthesis is a very important process. Since the nanoparticles possess varied properties as compared to their bulk, properly evaluating and analysing them becomes necessary so as to utilize them for various applications. Characterization mainly included structure analysis and property measurements. Several critical parameters about the particles such as size, shape, chemical structure, functional groups, crystallinity, thermal stability, and much more can be obtained by different characterization techniques.
3.9.1 Characterization of Zinc Oxide Nanoparticles

The zinc oxide nanoparticles synthesized by chemical and biological (green) methods were characterized by UV-Visible and FT-IR spectroscopy.

3.9.1.1 UV-Vis Spectroscopy

The UV-Vis spectrum of the synthesized zinc oxide nanoparticles was obtained by analysing it on Systronic double beam spectrophotometer: 2202. The measurements were done in a wavelength ranging from 200-1100 nm.

3.9.1.2 Fourier Transforms Infrared (FT-IR) spectroscopy

The synthesised zinc oxide nanoparticles were subjected to FT-IR spectroscopic analysis (Bruker optics, Alpha-T, Germany). For analysis, about 10 mg of the powder was mixed with 100 mg of KBr in order to prepare translucent pellets. The sample pellets were further analysed by the FT-IR in the KBr pellet mode.

3.9.2 Characterization of Iron Oxide Nanoparticles

Iron oxide nanoparticles were synthesized by chemical and green methods. Both of these were characterized by UV-Visible spectroscopy, Fourier Transform Infrared (FTIR) spectroscopy, Thermo-Gravimetric/Differential Thermal Analysis (TG/DTA), Raman Spectroscopy, Field Emission Scanning Electron Microscope (FESEM) and X-ray powder diffraction (XRD).

3.9.2.1 UV-Visible Spectroscopy

The UV-Vis spectra were recorded on Systronic double beam spectrophotometer: 2202. Measurements were taken in the wavelength range of 200-1100 nm.

3.9.2.2 Fourier Transform Infrared (FTIR) spectroscopy

Both the chemical and green synthesised iron oxide nanoparticles were subjected to Fourier Transforms Infrared (FT-IR) spectra analysis (Bruker optics, Alpha-T, Germany). For analysis, the dried powders of both chemical and green synthesised iron nanoparticles were (10 mg) separately mixed with KBr (100 mg) in order to prepare translucent sample pellets. The pellets were further analysed by the spectroscope.
3.9.2.3 Thermo Gravimetric / Differential Thermal Analysis (TG/DTA)

Thermal Analysis (TA) techniques study the properties of materials as they change with temperature. It provides information on properties like thermal capacity, mass changes, enthalpy, coefficient of heat expansion etc. It is widely used in solid state chemistry to study solid state reactions, thermal degradation reactions, and phase diagrams. The weight loss curve indicates changes in sample structure, heat stability, and other parameters.

Differential Thermal Analysis (DTA) involves exposing the material under study and an inactive reference to similar heat cycles. Any temperature distinction amongst test and reference is recorded. In this procedure the heat flow to the specimen and reference is kept constant instead of the temperature.

3.9.2.4 Raman Spectroscopy

The Raman spectra of the samples were analyzed at room temperature by a Raman spectrometer (Renishaw, in Via Raman; 632.8 nm excitation wavelength; He-Ne laser).

3.9.2.5 FE SEM

The samples were analysed by Feld Emission Scanning Electron Microscope (FE SEM, model JSM-7000F, JEOL Ltd.). FESEM was linked to an EDS/INCA 350 (Energy dispersive X-ray analyser, Oxford Instruments Ltd.). Samples were not coated with a conductive layer. Analysis was done at low accelerating voltage.

3.9.2.6 X-ray Powder Diffraction (XRD)

The iron oxide nanoparticles synthesised by chemical and green methods were characterized by X-ray powder diffraction (XRD) [Philips X 'Pert MPD] at 40 kV and 40 mA. The angular range was set at 5 ° to 75 ° at steps of 0.05 ° and counting time was 3 seconds per step.

3.10 BIOCONJUGATION

Bioconjugation is a surface modification procedure which appends natural elements to manufactured materials. To biofunctionalize attractive nanoparticles, various natural substances like proteins, ligands, antibodies, compounds, and others can be utilized.
3.10.1 Modification of Iron Oxide Nanoparticles by APTES

The acquired magnetite nanoparticles powder (1 g) was dissolved in 150 mL ethanol/water (1:1 v/v) mixture by sonication for 30 min. (3-aminopropyl) triethoxysilane (APTES) (99%, 3 ml) was then added to it. At that point, mixture was stirred under argon environment at 40 °C for 24 hours. Finally, the carrier was isolated from the mixture and washed 5 times with water, acetone and ethanol. The precipitated item (APTES and iron oxide) was dried at room temperature under vacuum.

3.10.2 Adsorption of Purified Fruit Extract on APTES- Iron Oxide Nano Carrier Surface

2 gm of APTES acquainted with 100 ml of hexane arrangement containing purified fruit extract (10 mg/ml). The adsorption was done at room temperature for 24 hours. After attractive partition, drug nano-transporters were consummately washed by hexane arrangement and dried at room temperature for 24 hours. The purified fruit extracts drug loading efficiency were calculated using Eq3.4.

\[
\text{Drug loading efficiency(\%)} = \frac{(\text{UV absorbance of drug} - \text{UV absorbance of sample})}{\text{UV absorbance of drug}} \times 100
\]

Eq 3:4

3.11 MOLECULAR DOCKING

Molecular docking study was performed to explore the coupling or binding affinities and cooperation modes between the inhibitors and the objective protein, and also to relate DHFR restraint activities of the integrated mixes to their chemical structures. Docking process requires a three dimensional (3D) structure of both protein and ligand. Structure-data file (MDL SD file) document design (*.sdf or *.sd) was utilized to store the three dimensional (3D) structures of all atoms that were produced, cleaned and geometrically improved by GOLD programming.

3.11.1 GOLD - Protein-Ligand Docking

GOLD, a collaborative project of University of Sheffield, GlaxoSmithKline plc and CCDC, is a popular program utilized for calculating the docking modes of small molecules in protein binding sites. It is a component of the GOLD suite which includes Hermes (for visualizing structure and manipulation), GOLD (for protein-ligand
docking), and GoldMine (for processing and visualizing docking results). It is highly accurate and reliable.

GOLD has been authenticated against 305 varied and widely tested protein-ligand complexes from the PDB. 72% of its solutions were found to be highly accurate as per strict success criteria. Validation of another set of 85 various, finest drug-like complexes indicated that for 81% of structures, GOLD reproduces the binding mode within 2Å. Its cross-docking performance has also been analysed using Astex Diverse Set. It is optimised for parallel processing and virtual screening application.

3.11.2 ACD/ChemSketch Tool

ACD/ChemSketch is an advanced chemical drawing tool. It is an accepted interface for the industries best NMR and molecular property predictions, nomenclature, and analytical data handling software.

ACD/ChemSketch is available as a freeware, with functionalities that are highly competitive with other popular commercial software packages. The freeware contains tools for 2D structure cleaning, 3D optimization and viewing, In ChI generation and conversion, drawing of polymers, organometallics, and Markush structures. It also has an IUPAC systematic naming capability for molecules with fewer than 50 atoms and 3 rings. The capabilities of ACD/ChemSketch can be further extended and customized by programming.

3.11.3 Receptor - Bcl-2 Receptor (1G5M)

B-cell lymphoma-2 (Bcl-2) is an important member of the regulator protein family BCL-2 responsible for regulating apoptosis (cell death). It is encoded in humans by the BCL2 gene. Bcl-2 occurs in two isoforms: 1G5M and 1G5O/1GJH. The Bcl-2 cancerous receptor/protein molecule were shown in Fig. 3.4.

The discovery of the anti-death gene BCL-2 had broad ramifications in tumour biology. Numerous members of the human Bcl-2 family have been recognized, including 6 antiapoptotic, and several proapoptotic interacting proteins out of which 3 are structurally similar. These proteins are controlled via various post-translational modifications with other proteins.
Bcl-2 proteins act as chief nodes at convergence of numerous pathways relevant to oncology, as it is responsible for regulation of major types of cell death like apoptosis, necrosis, and autophagy. Current clinical tests focus on targeting Bcl-2-family mRNAs or proteins, indicating potential and availability of a novel class of anticancer drugs. The Bcl-2 gene has been identified as a cause of a number of cancers, including melanoma, breast, prostate, chronic lymphocytic leukemia, and lung cancer, and a possible cause of schizophrenia and autoimmunity.

Fig. 3.4. Bcl-2 Receptor (1G5M)

3.11.3.1 MetaPocket 2.0 for Ligand Binding Sites

MetaPocket 2.0 is a meta server to identify ligand binding sites on protein surface. Metapocket is a consensus method, in which the predicted binding sites from eight methods:

- LIGSITEcs
- PASS
- Q-SiteFinder
- SURFNET
- Fpocket
These eight methods are used for combining together to improve the prediction success rate. MetaPocket 2.0 is both maintained at two web-sites:

- http://projects.biotec.tu-dresden.de/metapocket
- http://sysbio.zju.edu.cn/metapocket

### 3.11.4 Ligand – Eicosane (LFA)

As observed by GC-MS analysis the active anticancer agent present in the fig fruit extract was confirmed to be eicosane. This compound was used as the ligand for the docking studies with the Bcl-2 receptor protein. Eicosane or Icosane (C₂₀H₄₂), is a dreary, non-polar, idle alkane. It appears lifeless except when it smoulders. However its size, idleness or state does not deprive it of the qualities of its smaller alkane partners. It is less thick and water-insoluble. Its non-polarity enables it to perform feeble intermolecular bonding. The ball and stick model of Eicosane molecules are shown in Fig. 3.5. They are not affected by aqueous solutions of acids, alkalis, oxidizing agents, and most reducing agents. When heated sufficiently or when ignited in the presence of air, oxygen or strong oxidizing agents, they burn exothermically to produce carbon dioxide and water.

![Eicosane LFA](image)

Fig. 3.5. Eicosane LFA
3.12 In vitro Anticancer Activity

The methanolic extract of *Ficus benghalensis* fruits were subjected to cytotoxicity test. The pharmacological effect of *Ficus benghalensis* methanolic fruit extracts may be due to its rich content of phytochemicals including flavonoids, which possess rich antioxidant properties.

3.12.1 In vitro Anticancer Activity against Different Cell Lines

The partially purified eicosane and bioconjugated nano-formulated eicosane modified APTES + iron oxide molecules were subjected to anti-cancer activity against three different cell lines viz., A431 melanoma cell lines (skin cancer), A549 cell lines (lung cancer) and MCF-7 cell lines (breast cancer). A-431 melanoma, A-549 lung, and MCF-7 breast cancer cell lines were purchased from NCCS Pune and maintained in Dulbecco’s modified eagles media (HIMEDIA) supplemented with 10% FBS (Invitrogen) and grown to confluence at 37 °C in 5 % CO₂ in a humidified atmosphere in a CO₂ incubator (NBS, EPPENDORF, GERMANY). The cells were trypsinized (500 µl of 0.025% Trypsin in PBS/ 0.5 mM EDTA solution (Himedia) for 2 minutes and passaged to T flasks in complete aseptic conditions. 5 µl, 50 µl, 100 µl, 500 µl and 1000 µl of samples were added to grown cells and incubated for 24 hours. The percentage difference in viability was determined by standard MTT assay after 24 hours of incubation.

3.12.2 MTT Cell Viability Assay

MTT is a colorimetric assay that measures the reduction of yellow 3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilised with an organic solvent Dimethyl sulfoxide (Himedia) and the released, solubilized formazan product was measured at 540 nm. Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells.

The cells were washed with 1x PBS and then added 30 µl of MTT solution to the culture (MTT -5mg/ml dissolved in PBS). It was then incubated at 37 °C for 3 hours. MTT was removed by washing with 1x PBS and 200 µl of DMSO was added
to the culture. Incubation was done at room temperature for 30 minutes until the cell got lysed and colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 minutes to precipitate cell debris. Optical density (OD) was read at 540 nm using DMSO as blank in a micro plate reader (ELISASCAN, ERBA) and the results were calculated using the Eq3.5.

Percentage viability of anti-cancer activity of the given sample was calculated as follows:

\[
\text{% Percentage viability} = \frac{\text{OD of Test}}{\text{OD of Control}} \times 100 \quad \text{Eq 3:5}
\]