Methodology

3. METHODOLOGY

The present study focussed on the antimicrobial and anti-inflammatory properties exhibited by silver and gold nanobioconjugates synthesized from the leaf extracts of *Clitoria ternatea* bearing blue and white flowers. The plan of work and the methodology adopted are presented in this chapter.

The study was conducted in four phases. In Phase I, different parts of *Clitoria ternatea*, namely leaves, seeds and roots, from plants bearing blue and white flowers, were extracted separately into solvents of varying polarity. All the extracts were tested for antioxidant and antibacterial activity, to assess the best plant part and the best extract that exhibited the maximum bioactivity. Having selected the best extract, in the second phase, silver and gold nanobioconjugates were prepared and characterized. In the third phase, the antibacterial and anti-inflammatory activities of the prepared nano-bioconjugates were estimated *in vitro*, in comparison with the respective extracts alone. The biosafety and biocompatibility of the nanobioconjugates synthesized were also analyzed *in vitro*, in this phase. In the fourth phase, the anti-inflammatory activity of the extracts and their nanobioconjugates was tested *in vivo* using experimental mice, in order to validate the results obtained *in vitro*.

The experimental conditions and the procedures used for estimating the various parameters analyzed in each phase of the present study are explained in detail in this chapter. All the chemicals used were of analytical grade and were used as received without further purification.

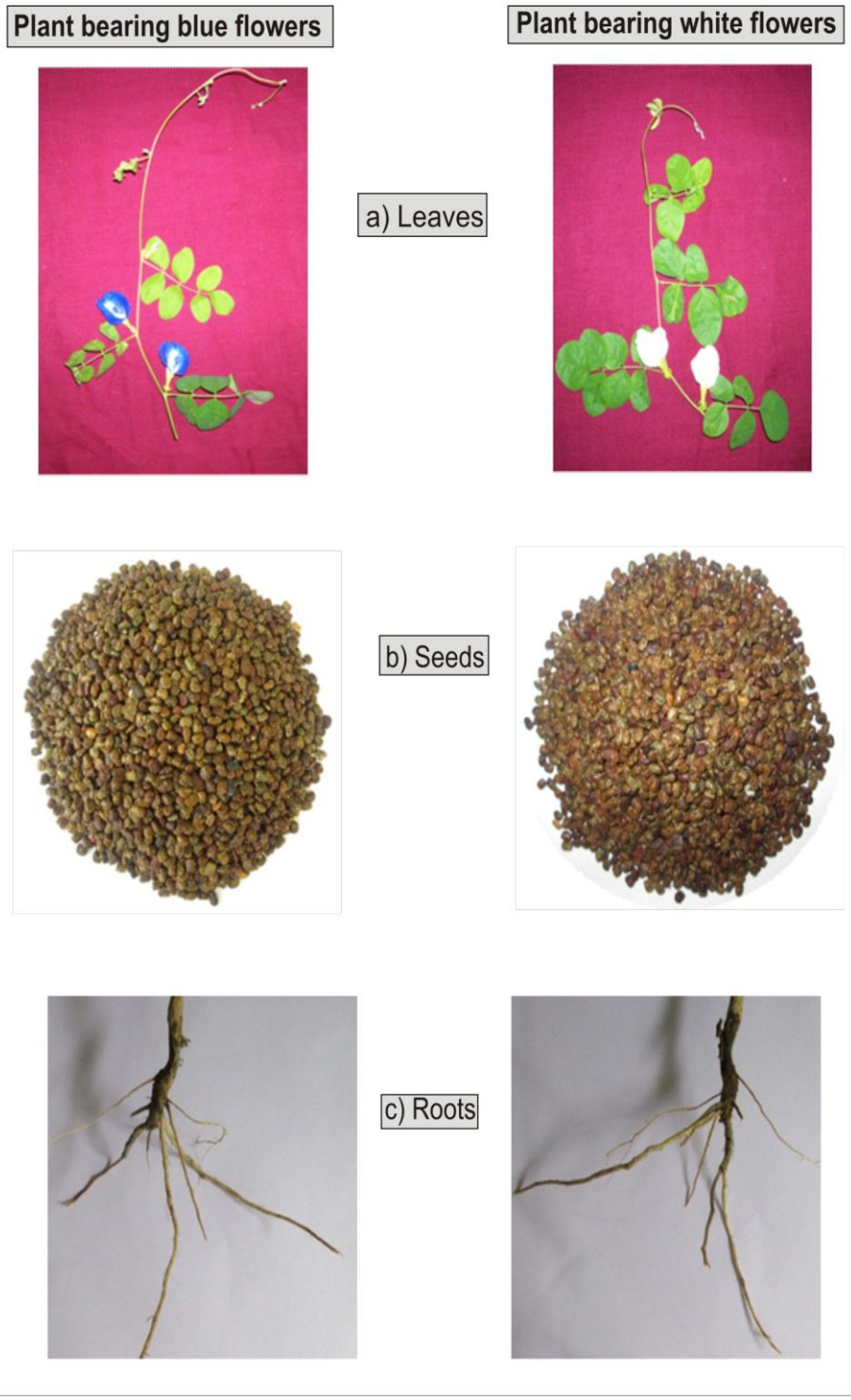
3.1 COLLECTION OF PLANT MATERIAL:

The plants of *Clitoria ternatea*, bearing blue and white flowers (Plate 3.1), were seed-propagated in pesticide-free soil and maintained within the University campus. The plants were authenticated (BSI/SRC/5/23/2014 /Tech./1663) by Botanical Survey of India, Coimbatore. The leaves, seeds and roots were collected fresh from the plants, washed thoroughly in running

tap water in order to remove any dirt or soil particles adhered and blotted gently between folds of tissue paper.

Plate 3.1

Clitoria ternatea plant bearing blue and white flowers



3.2 PREPARATION OF EXTRACTS:

Seeds, roots and fresh leaves from two varieties of *Clitoria ternatea* were collected and 10g of them was homogenized in 100ml of the solvents (water, ethanol, methanol, chloroform, benzene, ethyl acetate and petroleum ether) and extracted at 4ºC for 72 hours with occasional shaking, protected from light. The organic extracts were filtered using Whatman No.1 filter paper. The filtrate was dried at 60°C protected from light, the residue was weighed and dissolved in dimethyl sulfoxide (DMSO) to obtain a final concentration of 2mg/5µl.

PHASE I:

3.3 RADICAL SCAVENGING EFFECTS:

A lot of medicinal plants, traditionally used for thousands of years, are present in a group of herbal preparations of the Indian traditional healthcare system (Ayurveda) named Rasayana proposed for their interesting antioxidant activities. Among the medicinal plants used in ayurvedic Rasayana for their therapeutic action, only some have been thoroughly investigated (Saxena *et al*., 2011). Several anti-inflammatory and antimicrobial activities of the plants have been shown to have an antioxidant and/or radical scavenging mechanism as part of their activity (Chandrappa *et al*., 2013; El-Rafie and Hamed, 2014).

In the current study, the radical quenching potential of the leaf, seed and root extracts were tested *in vitro* against a battery of radicals namely DPPH (a stable radical), ABTS (a cation radical), non-radical oxidant H_2O_2 and hydroxyl radicals.

3.3.1 DPPH scavenging effects:

The method proposed by Mensor *et al*. (2001) was adopted to test the DPPH scavenging ability of extract of the leaves, seeds and root of the

candidate plant using different solvents namely water, ethanol, methanol, chloroform, benzene, ethyl acetate and petroleum ether.

Principle:

DPPH (2,2-diphenyl-1-picrylhydrazyl), when reacted by the antioxidants, is converted into 1,1'-diphenyl-2-picryl hydrazine, a yellow coloured derivative, by donating its hydroxyl (OH) group. This change is proportional to the antioxidant activity and can be quantified spectrophotometrically at 518 nm.

Requirements:

1. DPPH (0.3mM in methanol)
2. Methanol

Procedure:

Different solvent extracts of leaves, seeds and roots (5µl) were added with 500µl of methanol solution of DPPH and 495µl of methanol. The mixture was then allowed to stand at room temperature for 30 minutes. DPPH solution was used as a positive control and methanol alone acted as blank. After incubation, the conversion of purple colour to yellow colour was read at 518nm in a spectrophotometer. The per cent inhibition was calculated using the following formula

$$\% \text{ Inhibition } = \frac{A_{Control} - A_{Sample}}{A_{Control}} \text{x } 100$$

3.3.2 ABTS scavenging effects:

The method proposed by Shirwaikar *et al*. (2006) was used to assess the ABTS radical scavenging effect of different solvent extracts of the leaves, seeds and roots by the radical cation de-colourisation assay.

Principle:

ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid), a chromogen, changes into $ABTS^+$, a coloured mono-cation radical form in the presence of an oxidative agent, and has absorption peak at 750nm. Antioxidants can reduce $ABTS^+$ into its colourless form and the extent of decolourisation corresponds to the per cent reduction of $ABTS^+$. This can be measured spectrophotometrically.

Requirements:

1. Ethanol
2. ABTS solution (7mM ABTS with 2.45mM ammonium persulfate - incubated at room temperature for 12-16 hours before use).

Procedure:

Different solvent extracts of leaf, seed and root extracts were added to ABTS solution (300 µl) and the final volume of each was made up to 1ml with ethanol. ABTS solution with ethanol serves as control. The absorbance was read at 745nm and the percentage inhibition was calculated using the formula

$$\% \text{ Scavenging} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \text{ x } 100$$

3.3.3 Hydroxyl radical scavenging effects:

The effect of leaf, seed and root extracts on oxidant induced damage to deoxyribose *in vitro* was quantified as the amount of thiobarbituric acid reactive substances (TBARS) formed, according to the procedure explained by Elizabeth and Rao (1990).

Principle:

Hydroxyl radicals are generated from a Fe^{2+}/ascorbate/EDTA/H_2O_2 system, which attacks deoxyribose and eventually produces TBARS. The ability of the plant extract or compounds to inhibit TBARS formation is measured spectrophotometrically at 532nm.

Requirements:

1. Deoxyribose (28mM)
2. $FeCl_3$ (1mM)
3. EDTA (1mM)
4. H_2O_2 (10mM)
5. Ascorbic acid (1mM)
6. KH_2PO_4 KOH buffer (pH 7.4) (20mM)
7. Thiobarbituric acid (TBA) (1%)
8. HCl (25%)

Procedure:

The reaction mixture contained 0.1ml deoxyribose, 0.1ml $FeCl_3$, 0.1ml H_2O_2, 0.1ml ascorbate, 0.1ml buffer and 5µl of different solvent extracts of leaves, seeds and roots. The total volume was made up to 1ml with water. The tubes were capped tightly and incubated in a water bath at 37ºC for one hour. The reaction was terminated by the addition of TBA (0.5 ml) and HCl (0.5 ml). The tubes were heated in a boiling water bath for 20 minutes for colour development. The intensity of the pink colour formed, as the indication of TBARS formation, was measured at 532nm. The per cent TBARS produced for positive control (H_2O_2) was fixed as 100% and the relative per cent TBARS was calculated for the extract treated groups.

$$\% \text{ TBARS formation} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \text{ x } 100$$

3.3.4 Hydrogen peroxide scavenging effects:

The ability of the leaves, seeds and roots extracts to scavenge H_2O_2 was determined by the method proposed by Ruch *et al*. (1989).

Principle:

H_2O_2 scavenging activity was measured in terms of a decrease in the absorbance spectrophotometrically at 230nm.

Requirements:

1. H_2O_2 (40mM in 0.1M phosphate buffer)

2. Phosphate buffer (0.1M, pH 7.4)

Procedure:

Different solvent extracts of leaves, seeds and roots were added to 0.6ml of H_2O_2 solution and the final volume was made up to 3ml with the same buffer. After 10 minutes, the absorbance values at 230nm of the reaction mixtures were recorded against a blank containing phosphate buffer without H_2O_2 for each sample. The per cent inhibition was calculated using the formula

$$\% \text{ Inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \text{ x } 100$$

3.4 ASSESSMENT OF ANTIMICROBIAL ACTIVITY:

The antimicrobial activity of the different solvent extracts of the leaves, seeds and root were determined by turbidity method (Devienne and Raddi, 2002) with minor modifications, against clinical isolates of *Escherichia coli* and *Staphylococcus aureus,* as a representation of Gram negative and Gram

positive bacteria respectively. The organisms were collected from Kovai Medical Center and Hospital, Coimbatore and maintained in our laboratory.

3.4.1 Turbidity method:

Principle:

Bacterial suspension has turbidity due to the scattering of light passing through the suspension. This scattering occurs mainly at the cell surface because of the high refractive index gradient between the medium and the cell surface. The inhibition of the growth of microorganisms upon addition of an antibacterial agent is directly proportional to the reduction in the turbidity.

Procedure:

Clinical isolates of *E. coli* and *S. aureus* were grown for 24 hours and the cells at their logarithmic growth phase was inoculated with and without leaf extracts in a 96-well plate. These were incubated for 18 hours at 37ºC. Standard antibiotic ampicillin (50µg/well) was used as a positive control and the absorbance was measured spectrophometrically at 540nm.

In the assays described above, the methanolic extracts of the leaves showed the maximum scavenging activity, as well as antimicrobial activity, as evidenced by the results presented in the next chapter. Therefore, methanol extracts of the leaves were used for the further study. Leaves from both blue flower bearing and white flower bearing plants were taken for analysis.

PHASE II:

3.5 PREPARATION OF NANOPARTICLES:

In this phase, silver and gold nanobioconjugates were prepared from the methonolic extracts of the leaves of the two varieties of *Clitoria ternatea*. For the preparation of silver nanobioconjugates, 10ml of methanolic extracts of the leaves was mixed with 90ml of 1mM silver nitrate solution. For the gold nanobioconjugates, 10ml of methanolic extracts of the leaves was mixed with

90ml of 1mM auric chloride solution. In order to optimize the conditions for the extract-driven green synthesis of the nanobioconjugates, these mixtures were subjected to different conditions as given below.

3.5.1 Heating in the water bath:

The extract and the precursors, silver nitrate and auric chloride solutions ($AgNO_3$ and $HAuCl_4$) respectively, were heated in a water bath at 60ºC for different durations namely 5, 10, 15 and 20 minutes.

3.5.2 Direct exposure to the sunlight:

Recently, sunlight mediated synthesis of NPs using plant extract is gaining more interest over the existing methods, since it is renewable, non-toxic and cost effective. In the present study, the extract and the precursors were exposed to direct sunlight for different durations namely 5, 10, 15 and 20 minutes.

3.5.3 Heating in microwave:

The mixtures of extract and the precursors were heated in the microwave oven for different durations namely 10, 20, 30 and 40 seconds.

3.5.4 Separation of nanobioconjugates:

In all the above methods, the synthesis of the nanobioconjugates was monitored by a rapid change in the colour of the solution. The mixtures were incubated at room temperature for 24 hours and the nanoparticles synthesized were collected by centrifugation at 2000g for 45 minutes at 4ºC. The pellet was washed thrice with deionized water, transferred to a pre-weighed container and dried at 50ºC. The yield was calculated as the difference in weight and the residue was dissolved in DMSO at a concentration of 0.2mg/5µl for further analysis.

3.6 CHARACTERIZATION OF NANOBIOCONJUGATES:

Among the different methods experimented for the synthesis of nanobioconjugates and the varying time periods of exposure tried, 20 minute exposure to direct sunlight gave the maximum yield, as presented in the next chapter. Hence, this method was adopted for the synthesis of NPs and the synthesized NPs were characterized and evaluated for their bioactivity in comparison with their unconjugated leaf extract.

3.6.1 UV-Visible Absorption Spectroscopy:

The nanobioconjugates synthesized using different methods with varying time of exposure were subjected to spectral analysis using UV-Visible Nanophotometer (Optizen, Korea) in a range of 220nm to 800nm.

3.6.2 Transmission electron microscopy (TEM):

TEM is almost always the first method used to determine the size and size distribution of nanoparticle samples. It is a vital characterization tool for directly imaging nanomaterials to obtain quantitative measures of particle and/or grain size, size distribution, and morphology (Gomaa *et al*., 2014). The nanoparticles were observed under TEM (TECNAI G2, FEI TECNAI software) at an accelerating voltage of 120V. The samples were prepared by placing a drop of aliquot on carbon-coated copper grids and were estimated using high resolution.

3.6.3 Field Emission Scanning Electron Microscopy (SEM) with energy dispersive X-ray (EDX):

The samples were mounted on a grid with a thin carbon-film support (~10 nm thick). The nanoparticles were observed under FESEM (∑igma Field Emission Scanning Electron Microscope with x flash detector 5030 - Zeiss, SmartSEM v05.05. software) at an accelerating voltage of 10kV and EDX

(Bruker) analyses was also executed to identify the composition of the nanoparticles.

3.6.4 X-Ray Diffraction (XRD):

XRD is used to find out average particle size for a bulk sample and can identify individual crystals. The synthesized nanoparticle were analyzed to obtain their X-Ray diffraction pattern by placing the nanoparticle suspension on a microscopic glass slide, which was dried in a hot air oven at 50ºC, repeated until a layer was formed on the slide. The samples were dried and analyzed with the help of an XRD (PANalytical, XPERT-PRO diffractrometer) with a Cu source at 1.5406 Å wavelength as X-ray source in thin film.

3.6.5 Fourier Transform Infrared Spectroscopy (FTIR) Analysis:

FTIR is one of the most widely used tools for the detection of functional groups in pure compounds and mixtures and for compound comparison. To identify the functional groups involved in the synthesis of nanoparticle FTIR analysis was carried out using FTIR Spectroscopy (Sigma, Infinity). FTIR analysis was carried out to determine the nature of the capping agents in each of these leaf extracts. The FTIR spectrum was recorded for nanobioconjugates as well as the plant extracts for the functional groups identification.

3.6.6 Zeta potential:

Zeta potential is an important tool for understanding the state of the nanoparticle surface and predicting the long term stability of the nanoparticle. To be acquainted with the stability of synthesized nanoparticles, its zeta potential was measured with Zetasizer Nano ZS (Malvern Instruments) in a disposable cell at 25ºC, the results were analyzed using Zetasizer 7.01 software.

PHASE III

Bioactivity, biocompacatibility and drug release play an important role in exhibiting the toxicity and availability of any drug formulation. Phase III of the present study focused on evaluating the potential of the leaf extracts and their nanobioconjugates as effective drugs.

3.7 ASSESSMENT OF THE BIOACTIVITY OF THE NANOPARTICLES:

3.7.1 Antibacterial activity of the nanobioconjugates:

The antibacterial activity of the nanobioconjugates and their respective unconjugated plant extracts were determined by agar-well diffusion method (Chauhan *et al.,* 2010) against clinical isolates of *Escherichia coli* and *Staphylococcus aureus,* as a representation of Gram negative and Gram positive bacteria respectively, which were collected from Kovai Medical Center and Hospital, Coimbatore and maintained in our laboratory.

3.7.1.1 Agar well diffusion method:

Principle:

The antimicrobials present in the sample are allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition are uniformly circular in a confluent lawn of growth. The diameter of the zone of inhibition can be measred in millimeters.

Requirements:

1. Muller Hinton Agar Medium (1L): The medium was prepared by dissolving 33.9g of the commercially available Muller Hinton Agar Medium (HiMedia) in 1L of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121ºC for 15 minutes. The sterilized medium was mixed well, cooled to ~45ºC and poured onto sterile 100mm petri plates (25-30ml/plate) while still molten.

2. Nutrient broth (1L): Nutrient broth was prepared by dissolving 13g of commercially available nutrient broth (HiMedia) in 1L of distilled water and boiled to dissolve completely. The medium was dispensed into 10ml aliquots and sterilized by autoclaving at 15 lbs pressure at 121ºC for 15 minutes.

3. Ampicillin (standard)

Procedure:

Petri plates containing ~20ml Muller Hinton medium were swabbed with 24 hours culture of clinical isolates evenly onto the single plates. Wells were cut and 20µl of the methanol leaf extracts (blue and white varieties), nanobioconjugates (silver and gold) were added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well (NCCLS, 1997). Standard antibiotic ampicillin (50mg/ well) was used as a positive control and DMSO as negative control.

3.7.1.2 Determination of minimum inhibitory concentration (MIC):

MIC of the methanolic extracts of the leaves and nanobioconjugates were determined by a modified micro dilution method (Jorgensen and Ferraro, 2009).

Principle:

The level of bacterial growth can be determined by measuring the turbidity. Growth of bacteria increases the turbidity of the media, which can be measured spectrophotometrically. The samples are diluted in the nutrient broth and inoculated with the test organisms, upon incubation, the level of reduction in the turbidity gives the level of the antimicrobial activity exhibited by the samples.

Requirements:

- Nurtient broth
- Standard (Aspirin)

Procedure:

Plant extracts, as well as the nanobioconjugates synthesized from the respective leaf extracts, were serially diluted in nutrient medium to final concentrations ranging from 2.5µg-800µg/well in a 96-well plate. Exponentially growing *E. coli* and *S. aureus* suspensions were added to the broth dilutions to a final concentration of ~$5x10^5$ CFU/ml respectively for determining MIC of the leaf extracts. Ampicillin (50µg/ well) was used as standard antibiotic drug. Medium inoculum free from plant extracts or antibiotic served as positive control and medium alone without inoculum was used as negative control. These were incubated for 12 hours at 37ºC. The absorbance was measured spectrophometrically at 540nm. MIC was defined as the concentration at which there was a sharp decline in the absorbance value.

3.7.2 Assessment of *in vitro* anti-inflammatory activity:

Inflammation is a local response of living mammalian tissues to injury. It is a body defense reaction to eliminate or limit the spread of injurious agents. The intensive search for alternative drugs with analgesic, anti-inflammatory and chondro-protective properties from natural sources such as herbs is an important research effort.

As the methanolic leaf extracts and their nanobioconjugates exhibited antioxidant and antibacterial activity (results of phase-I presented in the next chapter) their anti-inflammatory activity was studied both in *in virto* and *in vivo*. The *in vitro* anti-inflammatory activity of the extracts and their silver and gold nanobioconjugates was evaluated by Human Red Blood Cell (HRBC) membrane stabilization method, heat induced hemolysis, protein denaturation and proteinase inhibition activity, as given below.

3.7.2.1 Hypotonic solution-induced haemolysis or membrane stabilizing activity:

Requirements:

1. Hypotonic solution (phosphate buffer): 50mM NaCl in 10mM sodium phosphate buffer saline (pH 7.4)
2. Isotonic phosphate buffer: 154mM NaCl in 10mM sodium phosphate buffer (pH 7.4)
3. Standard drug: Aspirin

Human Blood: Blood was collected from a healthy human volunteer who had not taken any drug for 2 weeks prior to the experiment into a heparinzed vacutainer. The blood was washed three times with saline by centrifuged at 3000 rpm for 10 minutes and a 40% v/v suspension was made using isotonic phosphate buffer to use as stock erythrocyte or RBC suspension.

Procedure:

This test was done according to the method described by Shinde *et al.* (1999) with minor modifications. Stock erythrocyte (RBC) suspension was mixed with hypotonic buffer solution in a ratio of 1:10. Samples were prepared in isotonic buffer solution (50µg/50µl) and taken for the analysis. To aliquots of 150µl of RBC suspension mixed with hypotonic buffered solution, respective samples were added. RBC suspension mixed with hypotonic buffered solution alone served as control. The standard drug aspirin was treated similar to test. The experiment was carried out in triplicate. The mixtures were incubated for 10 minutes at room temperature, centrifuged for 10 minutes at 3000 rpm and the absorbance of the supernatant was measured spectrophotometrically at 540nm. The percentage inhibition of haemolysis was calculated as a measure of membrane stabilization by the following equation.

$$\% \text{ Inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \text{ x } 100$$

3.7.2.2 Heat Induced Hemolysis:

Principle:

Stabilization of human red blood cell membrane can be measured by the protection exhibited by the extracts/ nanoparticles against heat induced membrane lysis.

Requirements:

1. 0.15M Phosphate buffer (pH 7.4): 40.5ml of 0.2M dibasic sodium phosphate and 9.5ml of 0.2M monobasic sodium phosphate and made up to 100ml with distilled water.
2. Hypo saline (0.36 %)
3. HRBC suspension (10 % v/v): Fresh whole human blood was collected and mixed with equal volume of sterile buffer solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride in water). The blood was centrifuged at 3000 rpm for 10 minutes and the packed cells were washed three times with isosaline (0.85%, pH 7.2). The volume of the blood was measured and reconstituted as 10% v/v suspension with isosaline and used for the analysis.
4. Standard drug: Aspirin

Procedure:

The inhibition of heat induced HRBC membrane lysis, i.e., stabilization of HRBC membrane was taken as a measure of the anti-inflammatory activity. The percentage of membrane stabilization for methanol extracts and their silver and gold nanobioconjugates and aspirin were done.

This test was done according to the method described by Labu *et al*. (2015), with minor modifications. Isotonic buffer solution containing 2mg/ml of samples were put into two duplicates of centrifuging tube. Two sets of control tubes contained 1ml of saline and 1ml of aspirin (0.1mg/ml) respectively. Erythrocyte suspension (10µl) was added to each tube and mixed gently by

inversion. One pair of tubes was incubated at 54ºC for 20 minutes in a water bath. The other pair was maintained in ice bath. The reaction mixture was centrifuged at 3000rpm for 10 minutes and the absorbance of the haemoglobin content in the supernatant was measured at 540nm. The per cent inhibition or acceleration of hemolysis was calculated according to the following equation

$$\% \text{ hemolysis} = \frac{A_{Control} - A_{heated}}{A_{Control} - A_{unheated}} \text{x } 100$$

3.7.2.3 Protein denaturation assay:

Protein denaturation is implicated in inflammation. To assess the potential of anti-inflammatory activity of the candidate plant extract and its nanoparticles, protein denaturation assay was performed as described by Elias and Rao (1998) with some modifications.

Requirements:

1. Egg albumin solution (1mM)
2. Standard drug: Aspirin

Procedure:

The test solution consisting of 100µl of leaf extracts or its nanobioconjugates 50µg/ml or standard drug aspirin 50µg/ml was mixed with 100µl of egg albumin solution (1mM) and incubated at 27±1ºC for 15 minutes. Denaturation was induced by keeping the reaction mixture in a water bath at 70ºC for 10 minutes. After cooling, the turbidity was measured spectrophotometrically at 660 nm. Percentage inhibition of denaturation was calculated from control where no drug was added as per the following formula. Each experiment was done in triplicate and the average was taken.

$$\% \text{ Inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \text{x } 100$$

3.7.2.4 Proteinase inhibitory activity:

Neutrophils are the most important source for proteinases, which are carried in their lysosomal granules and are involved in arthritic reactions. Proteinases have been implicated in arthritic reactions. It was already reported that leukocytes proteinase play important role in the development of tissue damage during inflammatory reactions and significant level of protection can be provided by proteinase inhibitors (Chandrappa *et al*., 2013). The proteinase inhibitory activity was estimated using modified method of Oyedepo and Femurewa (1995).

Requirements:

1. Tris HCl buffer (20mM) (pH 7.4)
2. Casein (0.8% w/v)
3. Perchloric acid (70%)
4. Trypsin (0.25%)
5. Standard drug: Aspirin

Procedure:

The reaction mixture contained 1ml of different test samples (50µg), 1ml of 20mM Tris HCl buffer (pH 7.4) containing 0.06mg trypsin were mixed and incubated at 37ºC for 5 minutes. Then 1ml of 0.8% (w/v) casein was added and incubated for an additional 20 minutes. The reaction was terminated by adding 2ml of 70% perchloric acid. The cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210nm against buffer as blank. Aspirin was used as the standard drug. The experiment was performed in triplicate. The percentage of proteinase inhibition was determined as follows.

$$\% \text{ Inhibition} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \text{ x } 100$$

3.8 ASSESSMENT OF BIOCOMPATIBILITY:

In recent years, NPs have increasingly found practical applications in technology, research, and medicine. The small particle size coupled with their unique chemical and physical properties is thought to underline their exploitable biomedical activities. Its form may be latex body, polymer, ceramic particle, metal particles, and the carbon particles (Feng *et al*., 2015). Due to their small size and physical resemblance to physiological molecules such as proteins, NPs possess the capacity to revolutionize medical imaging, diagnostics, therapeutics, as well as carry out functional biological processes. But these features may also underline their toxicity. Indeed, a detailed assessment of the factors that influence the biocompatibility and toxicity of NPs is crucial for the safe and sustainable development of the emerging NPs (Li *et al*., 2012). Biocompactability of any drug can be measured by analyzing its effect on blood. For this, red blood cell lysis, blood clotting time and morphological changes of RBC and blood lymphocytes were studied.

3.8.1 Red blood cell lysis:

Principle:

Test samples incubated with erythrocytes may induce lysis and release hemoglobin, which could be collected in the supernatant by centrifugation and measured at 542nm. The hemolysis is directly proportional to the absorbance value.

Procedure:

The nanobioconjugates synthesized were dissolved in saline to a final concentration of 2mg/ml. HRBC were obtained by centrifuging (2000g for 20minutes). Erythrocytes were washed thrice with saline discarding the supernatant to remove serum proteins. Erythrocyte stock dispersion (ESD) was prepared by adding centrifuged erythrocytes and saline in the ratio of 1:5. ESD (100ml) was taken and added with 1ml of sample, shaken and incubated at 37ºC for 1 hour. After centrifugation at 3000 g for 5 minutes to remove the

intact erythrocytes and debris, the optical density of the supernatant was measured at 542nm against blank samples. Results were determined in relation to control samples of 0% hemolysis (in saline solution) and 100% hemolysis (in distilled water).

The per cent hemolysis was calculated as follows, where OD of test sample, negative and positive control are the absorbance of the test samples, solution of 0% hemolysis and a solution of 100% hemolysis, respectively.

$$\% \text{ Hemolysis} = \frac{A_{\text{Sample}} - A_{\text{Negative Control}}}{A_{\text{Positive Control}} - A_{\text{Negative Control}}} \text{x } 100$$

3.8.2 Blood clotting time:

Principle:

Test samples incubated with Human Red Blood Cells (HRBC). The addition of clotting agent, $CaCl_2$ induces clotting, which can be measured at 542nm. The size of the clot is inversely proportional to the absorbance value.

Procedure:

Blood was drawn from healthy adult volunteers by venipuncture into anticoagulant vacutainer tubes. Samples (20µl of each of plant extracts and nanoparticles of 50µg) were added to saline in individual 24-well plates (positive control). Saline without erythrocytes served as a negative control. The clotting reaction was activated by the addition of 2.5ml of 0.1M $CaCl_2$ to a 25ml blood. A 100µl volume of activated blood was carefully added to each nanoparticle sample and incubated for 5minutes. At the end of each time point, the samples were incubated with 3ml of distilled water for 5 minutes. Each well was sampled in triplicates and 100µl was transferred to a 96-well plate. The red blood cells that were not trapped in a clot were lysed by the addition of distilled water for subsequent measurement. The concentration of hemoglobin in the solution was assessed by measuring the absorbance at 542 nm using a microtitre plate reader (Bio-rad, USA).

3.8.3 Blood cell morphology:

Principle:

Samples incubated with whole blood may induce toxic morphological changes in the blood cells, if they are toxic/allergic to the cells, which can be visualized under the microscope.

Procedure:

Anti-coagulated human blood (50 µl) was incubated for 20 minutes at 37ºC with samples in saline at final concentration of 50µg/well. Whole blood incubated with saline alone acted as negative control, incubated with water alone acts as positive control. After incubation, the RBC were examined by inverted microscopy (Metzer, India). The images were captured under 400x magnification.

3.8.4 Plasma re-calcification profile:

Principle:

$CaCl_2$ induces clotting in platelet poor plasma (PPP). The kinetics of the clotting process due to re-calicification can be monitored by measuring the absorbance at 405 nm at different time points.

Procedure:

Plasma re-calcification profile was studied using a modified method of Chippada *et al*. (2011). PPP was obtained by centrifuging the anti-coagulated human blood at 2000g for 10 minutes. Samples and saline (negative control) in 10µl aliquots were placed as triplicates in 96-well plate. To this, 100µl of prepared PPP was added in each well. Then 100µl of 0.025M $CaCl_2$ was added excluding the well with saline alone. The absorbance was read immediately at 405 nm (every 30 seconds for 45 minutes) in the plate reader (Bio-rad, USA). Kinetics of clotting process of re-calicification can be

monitored by this assay. The clotting time to reach half maximal absorbance was calculated and analyzed (Motlagh *et al*., 2006).

3.8.5 Assessment of drug release:

For medical and therapeutic purposes, a range of less than 10-100 nm (in at least one dimension) appears to be generally accepted, with a few exceptions where sizes greater than 100nm may be applicable (Cruz *et al*., 2013). Due to their small size, nano-sized dosage forms possess an unusually large surface-to-volume ratio that alters the chemical, physical, and biological properties of the dosage form allowing them to cross cell and tissue barriers, thereby altering the pharmacokinetics and pharmacodynamics of the therapeutic agent. This unique aspect of nano-particulate preparations has been exploited to deliver therapeutics to specific cells, organs, and other challenging *in vivo* targets. Another consequence of enhanced delivery to the target site is an increase in potency of the drug, including the potential for elevated toxicity due to the carrier material, possibly leading to reduced safety. Therefore, determination of product quality and performance becomes a crucial aspect during nano-particulate dosage form development (D'Souza, 2014).

Principle:

When the nanoparticles are kept in dialysis bag/tube (semipermeable membrane), they diffuse into the buffer surrounding the bag, solute concentration of which is lower than that of the dialysate (nanoparticles). Small solutes (nanoparticles) diffuse from a high concentration solution (dialysate) to a low concentration solution (buffer) across a semipermeable membrane until equilibrium is reached.

Requirements:

1. Dialysis bag/tube (cut off of MW=12KDa)
2. PBS (pH 7.4)

Procedure:

Nanobioconjugates were placed in a dialysis bag. The bag was suspended in phosphate buffered saline (30ml, pH 7.4). A continuous release of silver and gold NPs was measured by reading the absorbance at 420nm and 540nm respectively for every hour over a period of 48 hours. The release rate and order of kinetics were calculated. The percentage of silver and gold nanoparticle released was calculated using the equation,

$$\%\ \text{Cumulative}\ \frac{\text{Au}}{\text{Ag}}\ \text{released} = \frac{\text{Au/Ag content in the PBS medium at a time}}{\text{Total Au/Ag content in the dialysis bag}}\ \text{x}\ 100$$

PHASE IV:

3.9 Assessment of the *in vivo* anti-inflammatory activity of methanolic leaf extracts in animal model:

The results of *in vitro* analysis of antimicrobial and anti-inflammatory activity were evaluated for *in vivo* anti-inflammatory activity against acute and chronic inflammation in animal model.

Animals:

The experiments were carried out in Swiss albino mice of female animals five per group weighing 35-40g. The study was conducted in accordance with CPCSEA (Committee for the Purpose of Control and Supervision of Experiment on Animals) norms. The study was approved by the Institutional Animal Ethical Committee (315/2015/IAEC). The animals were acclimatized under laboratory conditions. They were fed with standard diet and were provided with water *ad libitum*.

Experimental Design:

Group 1: Control

Group 2: Standard (Aspirin 100mg/kg)

Group 3: Methanolic leaf extract of *Clitoria ternatea* bearing blue flowers at a dose of 200mg/kg (BL-200mg/kg)

Group 4: Methanolic leaf extract of *Clitoria ternatea* bearing white flowers at a dose of 200mg/kg (WL-200mg/kg)

Group 5: Silver nanoparticle synthesized from methanolic leaf extract of *Clitoria ternatea* bearing blue flowers at a dose of 200mg/kg (AgB -200mg/kg)

Group 6: Silver nanoparticle synthesized from methanolic leaf extract of *Clitoria ternatea* bearing white flowers at a dose of 200mg/kg (AgW - 200mg/kg)

Group 7: Gold nanoparticle synthesized from methanolic leaf extract of *Clitoria ternatea* bearing blue flowers at a dose of 200mg/kg (AuB -200mg/kg)

Group 8: Gold nanoparticle synthesized from methanolic leaf extract of *Clitoria ternatea* bearing white flowers at a dose of 200mg/kg (AuW - 200mg/kg)

Acute toxicity studies:

Shyamkumar and Ishwar (2012) studied the acute toxicity of *Clitoria ternaea* L. and found the extract is safe even at the dose of 2000mg/kg body weight. So 200mg/kg body weight was chosen as an arbitrary dose in the present study, to determine the anti-inflammatory activity.

3.9.1 Assessment of *in vivo* anti-inflammatory activity against acute inflammation:

Principle:

Carrageenan-induced release of various inflammatory mediators induces edema at the site of injection. Change in the edema size can be measured and taken as an indicator of anti-inflammation.

Procedure:

The anti-inflammatory activity of the methanolic extracts of leaves and the silver and gold nanobioconjugates against acute inflammation was tested by carrageenan induced paw edema method with minor modifications (Ramachandran and Nair, 2011). Acute inflammation was induced by sub-plantar injection of 0.01ml of freshly prepared 1% carrageenan suspension in normal saline in the right hind paw of mice in each group. The animals were orally administered with leaf extracts, silver, gold nanobioconjugates or aspirin in the respective groups, 1 hour before carrageenan injection. The group of animals that received carrageenan alone served as negative control and which received aspirin before carrageenan injection served as positive control. The paw thickness was measured using Vernier callipers just before carrageenan injection, that is, at 0 hour and then at one hour intervals up to 6^{th} hour followed by 24^{th} hour and 48^{th} hour. The percentage inhibition of the rat paw edema was calculated after each hour of carrageenan injection up to 48 hours by the formula described by Agus (2005).

$$\% \text{ Inhibition} = \frac{\text{Mean}_{\text{Control}} - \text{Mean}_{\text{Treated}}}{\text{Mean}_{\text{Control}}} \text{ x } 100$$

3.9.2 Assessment of *in vivo* anti-inflammatory activity against chronic inflammation:

The anti-inflammatory activity of methanolic leaf extracts and their silver and gold nanobioconjugates against chronic inflammation was tested by adjuvant induced arthritis method in mice (Vogel, 2002). On day 1, the animals were injected into the sub-plantar region of the right hind paw with 0.01ml of complete Freund's adjuvant. Dosing with the test compounds or the standards to the respective groups was started on the same day and continued for 12 days. The paw thickness of both sides and the body weights were recorded on the day of injection as in the paw edema test. On day 5, the thickness of the injected paw was measured again, indicating the primary lesion and the influence of the therapeutic agent on this phase. The severity of the induced adjuvant disease was followed by the measurement of the non-injected paw (secondary lesions) with Vernier caliper. Purposely, from days 13 to 21, the animals were not dosed with the test compound or the standard. On day 21, the non-injected paw thickness and the body weight were determined again and the poly arthritis severity was graded on a scale of 4 as indicated below.

0 = no swelling;
1 = isolated phalanx joint involvement;
2 = involvement of phalanx joint and digits;
3= involvement of the entire region down to the ankle;
4 = involvement of entire paw, including ankle.

For primary lesions: The percentage inhibition of paw thickness of the injected right paw over control was measured at day 5.

For secondary lesions: The percentage inhibition of paw thickness of non-injected left paw over control was measured at day 21.

Arthritis index: It was calculated as the sum of the scores as indicated above for each animal.

3.10 Statistical Analysis:

The parameters studied were analyzed statistically using Sigma Stat statistical package (Version 3.1). One way ANOVA with $P<0.05$ was considered significant. The results obtained for the various parameters analyzed during the different phases of the study are presented and discussed in the next chapter.