3. MATERIALS AND METHODS

3.1. Collection of Material

Different plant parts (leaf, stem and root) of *Sesbania sesban* were collected during the month of May and their authenticity was confirmed by the Botanical Survey of India, Coimbatore, Tamil Nadu, India. The Voc No. BSI/SRC/5/23/2012-13/Tech./136.

The plant parts were dried separately under the shade, powdered, stored in airtight containers and used for the studies.

3.2. Pharmacognostic study of *Sesbania sesban*

3.2.1. Organoleptic evaluation

Organoleptic evaluation is a technique of qualitative study of organs of sense of the plant. It provides some specific characteristics of the material which establishes the identity and degree of purity of the plant. In the present study, the colour of the outer and inner surface of the fresh and dried stem was observed. The features like shape, odour, taste, texture and surface characteristics of the stem and the powder were performed.

3.2.2. Anatomical studies of *Sesbania sesban*

The leaf, stem and root of *in vitro* germinated thirty days old plant were taken for the anatomical studies. Care was taken to select healthy plants and normal organs. The required samples of different organs were cut and removed from the plant and fixed in FAA (Formalin-5 ml+ Acetic acid-5 ml+ 70% Ethyl alcohol-90
ml). After 24 hrs of fixing, the specimens were dehydrated with graded series of tertiary butyl alcohol as per the schedule given by Sass, 1940. Infiltration of the specimens was carried by gradual addition of paraffin wax (melting point 58-60°C) until TBA solution attained super saturation. The specimens were cast into paraffin blocks.

**Sectioning**

The paraffin embedded specimens were sectioned with the help of Rotary Microtome. The thickness of the sections was 10-12 µm. Dewaxing of the sections was by customary procedure (Johansen, 1940). The sections were stained with toluidine blue as per the method published by O'Brien et al., (1964). Since toluidine blue is a polychromatic stain. The staining results were remarkably good, and some cytochemical reactions were also obtained. The dye rendered pink colour to the cellulose walls, blue to the lignified cells, dark green to suberin, violet to the mucilage, blue to the protein bodies etc. wherever necessary sections were also stained with saffranin and fast-green and IKI (for starch).

**Photomicrographs**

Microscopic descriptions of tissues were supplemented with micrographs wherever necessary. Photographs of different magnifications were taken with Nikon Lab Photo 2 Microscopic Unit. For normal observations Bright Field was used. For the study of crystals, starch grains and lignified cells, Polarized Light was employed. Since these structures had birefringent property, under polarized light they appeared bright against dark background. Magnifications of the figures were
indicated by the scale-bars. Descriptive terms of the anatomical features were given in the standard Anatomy Book (Esau, 1964).

3.2.3. Scanning electron microscopy (SEM) and energy dispersive X-ray (EDAX) studies

Stem pieces of *Sesbania sesban* were washed in running tap water and carefully rinsed with distilled water. For scanning electron microscopy analysis (SEM), stem sections (3-4 mm) were fixed in 3% glutaraldehyde in 0.05M phosphate buffer for 90 min and dehydrated in an alcohol series. Then the SEM photographs were taken, using SEM model JEOL-JSM-6390 LV attach with energy dispersive X-ray unit, with an accelerating voltage of 20 kV.

3.2.4. Histochemical microscopic study

For the histochemical microscopic study cross section of the stem was taken and stained with reagents as per the standard procedure. The sections were observed under the light microscope Olympus (10X). Powder characteristics were observed under the low power (10X) and subsequently under high power (40X), after stained with saffranin.

3.2.5. Powder characteristics

Powder characteristics were observed under the low power (10X) and subsequently under high power (40X), after staining with saffranin. (Kokate *et al.*, 2007).
3.2.6. Physico-chemical studies

Physico-chemical studies of the stem like the percentage of foreign matter, loss on drying, total ash and acid insoluble ash were determined according to the method described in WHO guidelines on quality control methods for medicinal plants materials.

The percentage of active principles in the plant is determined only in the dry condition. Hence, the moisture lost percentage is very important to decide about the condition of the crude drug. The total ash and the acid insoluble ash indicate the presence of any foreign matter, inorganic composition and purity of the drug. Extractive values are primarily useful for the determination of exhausted or adulterated drugs. Thus, alcohol and water soluble extractive values were determined.

3.2.7. Fluorescence analysis

The fluorescence analysis was done and observed in the day light and UV light in order to find out the presence of any adulterants and specific compounds. The stem powder of *Sesbania sesban* was treated with different chemicals and seen under the normal light and UV radiations at 254 and 365 nm wavelengths as per the standard procedure.

3.2.8. Phytochemical study

Extraction of the plant parts (leaf, stem and root) were done with different solvents based on the polarity of the solvents. The solvents used were hexane, chloroform, methanol, ethanol and water. The extract of the leaf, stem and root were
obtained through the cold percolation method. The powdered plant material was weighed and then soaked in hexane for 72 hrs. Then the extract was taken by filtering the content. The same procedure was repeated again and the extract was collected. The extracts were pooled together and concentrated on a water bath by keeping the temperature below the boiling point of the solvent used. The concentrated extract was kept in the desiccator for further evaporation of the solvent. Then the extract was weighed and the yield was recorded. The same procedure was repeated for all the solvents. The extracts taken were used for further phytochemical analyses of the plant. The standard qualitative phytochemical tests given for the respective compounds were performed.

**Qualitative method of phytochemical analysis** (Sofowara (1993), Trease and Evans (1989) and Harborne (1973))

**Detection of Alkaloids** (Evans, 1997)

About 50 mg of Solvent free extract was stirred with 5 ml of dilute hydrochloric acid, filtered and the filtrate was tested with various alkaloid reagents as follows:

A. Mayer’s test (Evans, 1997)

To 1 ml of filtrate, few drops of Mayer’s reagent was added by the side of the test tube. The colour change was observed.

B. Wagner’s test (Wagner, 1993)

To 1 ml of filtrate, few drops of Wagner’s reagent was added by the side of the test tube and the colour change was observed.
C. Hager’s test (Wagner et al, 1996)

To 1 ml of filtrate, 1 ml of Hager’s reagent was added and the colour change was observed.

D. Dragendorff’s test (Waldi, 1965)

To 1 ml of filtrate, 2 ml of Dragendorff’s reagent was added and the result was observed.

**Detection of Carbohydrates and Glycosides (Ramakrishnan et al., 1994)**

About 50 mg of the extract was dissolved in 5 ml of water and filtered. The filtrate was subjected to the following tests.

A. Molish’s test

To 2 ml of filtrate, two drops of alcoholic solution of α-naphthol was added, the mixture was shaken well and 1 ml of conc. sulphuric acid was added slowly along the side and allowed to stand.

B. Fehling’s test

One ml of extract was boiled on water bath with 1 ml each of Fehling solutions A and B. The colour change was observed.

C. Barfoed’s test

To 1 mL of extract, 1 ml of Barfoed’s reagent was added and heated on a boiling water bath for 2 minutes. The colour change was noted and recorded.
D. Benedict’s test

To 0.5 ml of extract, 0.5 ml of Benedict’s reagent was added. The mixture was heated on a boiling water bath for 2 minutes and the result was observed.

For detection of Glycosides, 50 mg of the extract was hydrolysed with conc. hydrochloric acid for 2 hrs on a water bath, filtered and the hydrolysate was subjected to the following test.

E. Borntrager’s test (Evans, 1997)

To 2 ml of filtered hydrolysate, 3 ml of chloroform was added and shaken, after the separation of the chloroform layer 10% ammonia solution was added.

Detection of Saponins (Kokate, 1999)

50 mg of the extract was diluted with distilled water and made upto 20 ml and the suspension was shaken well for 15 minutes.

Detection of Proteins and Amino acids (Fisher, 1968; Ruthmann, 1970)

The extract was dissolved in 10 ml of distilled water and filtered through Whatman No.1 filter paper and the filtrate was subjected to tests for proteins and amino acids.

A. Millon’s test (Rasch and Swift, 1960)
To 2 ml of filtrate, few drops of Millon’s reagent was added. The result was observed.

B. Biuret test (Gahan, 1984)

An aliquot of 2 ml of filtrate was treated with one drop of 2% copper sulphate solution. To this, 1 ml of ethanol (95%) was added, followed by excess of potassium hydroxide pellets. The colour change was observed.

C. Ninhydrin test (Yasuma and Ichikawa, 1953)

Two drops of ninhydrin solution was added to 2 ml of aqueous filtrate. The colour change was observed.

Detection of Phytosterols (Finar, 1986)

Libermann-Burchard’s test

50 mg of the extract was dissolved in 2 ml acetic anhydride. To this, two drops of conc. sulphuric acid was added slowly along the sides of the test tube. The colour change was observed.

Detection of Fixed Oils and Fats (Kokate, 1999)

A. Spot test

A small quantity of the extract was pressed between two filter papers.
B. Saponification test

To a small quantity of the extract a few drops of 0.5 N alcoholic potassium hydroxide solution was added along with a drop of phenolphthalein. The mixture was heated on water bath for two hrs.

Detection of Phenolic compounds and Tannins

A. Ferric chloride test (Mace, 1963)

About 50 mg of the extract was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution was added.

B. Gelatin test (Evans, 1997)

50 mg of the extract was dissolved in 5 ml of distilled water and 2 ml of 1% solution of gelatin containing 10% sodium chloride was added to it.

C. Lead acetate test

The extract was dissolved in distilled water and to this 3 ml of 10% lead acetate solution was added. The result was observed.

D. Alkaline reagent test

An aqueous solution of the extract was treated with 10% ammonium hydroxide solution. The colour change was observed.
Detection of Gum and Mucilages (Whistler and BeMiller, 1993)

50 mg of the extract was dissolved in 5 ml of distilled water and to this solution, 12.5 ml of absolute alcohol was added with constant stirring.

Detection of Quinones

One ml of concentrated sulphuric acid was added to 1 ml of the plant extract and the result was observed.

Detection of Cardiac glycosides

Two ml of glacial acetic acid containing few drops of 5% ferric chloride was added to 5 ml of the extract. This was underlayered with 1 ml of concentrated sulphuric acid. The result was noted.

Detection of Terpenoids

Two ml of chloroform and conc. sulphuric acid was added carefully to 0.5 ml of extract and the result was observed.

Detection of Coumarins

10% of 1 ml sodium hydroxide was added to 1 ml of the plant extract. The colour change was observed.

Detection of Acids

0.5 ml of the plant extract was treated with sodium bicarbonate solution.
Detection of Steroids and Phytosteroids

To 0.5 ml of the plant extract equal volume of chloroform was added and subjected with few drops of concentrated sulphuric acid.

Detection of Phlobatannins

Few drops of 10% ammonia solution was added to 0.5 ml of the extract. The result was noted.

Detection of Anthraquinones

Few drops of 2% HCl was added to 0.5 ml of the extract and the result was observed.

Based on the results obtained in the qualitative analysis, the methanol extract of the leaf, stem and root were taken for further analysis.

Quantitative Determination of the Phytochemicals

Quantitative analysis of the methanol extract of the leaf, stem and root were done in triplicates for selective phytochemicals.

Preparation of fat free sample: Two grams of the sample was defatted with 100 ml diethyl ether using a soxhlet apparatus for two hrs.

Determination of total phenols by spectrophotometric method

The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. 5ml of the extract was pipette into a 50 ml flask,
then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amyl alcohol were also added. The samples were made up to the mark and left to react for 30 minutes for colour development. This was measured at 505 nm. Gallic acid was used as the standard.

**Alkaloid determination using Harborne (1973) method**

200 ml of 10% acetic acid in ethanol was added to 5 g of the sample, covered and allowed to stand for four hours. This was filtered and the extract was concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

**Tannin determination by Van-Burden and Robinson (1981) method**

500 mg of the sample was weighed, to this 50 ml of distilled water was added and shaken for one hour in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtrate was pipetted out into a test tube and mixed with 2 ml of 0.1 M ferric chloride in 0.1 N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 min. Tannic acid was used as the standard.

**Flavonoid determination (Boham and Kocipai-Abyazan, 1974)**

10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through
Whatman filter paper No.42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight.

3.3. Characterization of the phytochemicals of *Sesbania sesban*

The identification of phytochemical fingerprint by chromatography may provide effective information about qualitative and quantitative composition of the plants.

3.3.1. Thin layer chromatography

Thin layer chromatography was conducted to study the number of compounds present in the extract. The adsorbent used for thin layer chromatography was silica gel 60 F 254. The pre-coated TLC plate (Merck, Germany) was heated in an oven for activation. Methanol extracts of leaf, stem and root were applied dried and then kept in the developing tank. The chamber was saturated with the solvents for 20 min at room temperature. After the development of the plate, it was air-dried then the numbers of spots were examined under the ordinary light and UV lights and Rf values were calculated. Several solvent combinations were tried to identify a suitable developing solvent system for the separation of compounds. Solvents like chloroform: ethyl acetate in the ratio of 5: 5, ethyl acetate: methanol: ethanol: water 8.1:11:4:8, toluene: acetone 5:5, chloroform: ethyl acetate: acetic acid 6:4:4, chloroform: methanol 15:1 and toluene: chloroform: methanol 1:1:0.8 were tried. After the development of the plate, it was air-dried then the numbers of spots were examined under the ordinary light and UV light at 254 and 365 nm and Rf values were calculated.
The Rf values of the spots were calculated by the formula,

\[
Rf = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent}}
\]

3.3.2. High performance thin layer chromatography (HPTLC)

Instrument: CAMAG Automatic TLC Sampler 4 (ATS4) "ATS4_140608" S/N 140608 (1.02.13) with win CATS software.

Stationary phase: TLC plates silica gel 60 F 254 pre coated layer (15 cm × 10 cm), thickness 0.2 mm.,

No. of tracks: 9, band length: 8 mm.

Mobile phase: Chloroform: Methanol: Formic acid (9.0: 7.5: 2.5)

Sample : Brown powder

Solubility: Methanol

Sample concentration: Leaf, Stem and Root: 25 mg/ml

Sample application volumes (µl): 2, 5 and 10 µl

Development chamber: Twin trough chamber (20 × 10)

Development mode: Ascending mode

Distance run: 75 mm

Scanning wavelength: 254 nm

Lamp: D2

Slit dimensions 4.00 × 0.30 mm, Micro

Measurement mode: absorbance

Preparation of the plates: The plates used for HPTLC was silica gel 60 F 254 (E.MERCK KGaA). 25 mg/ml of the samples were applied in the form of bands using LINOMAT IV applicator and the volumes of the samples were 2, 5 and 10 µl.
The mobile phase used was Chloroform: Methanol: Formic acid (9.0: 7.5: 2.5). The chromatograph was developed for 5 minutes, dried at room temperature and scanned at 254 nm.

3.3.3. UV-Vis spectrum of Sesbania sesban

The UV-Vis spectrum was recorded (200-600 nm) for the methanol extract of the leaf, stem and root on UV-Vis spectrophotometer (Shimadzu). To compare the yields of extraction of leaf, stem and the root of Sesbania sesban, Extraction Factor (EF) was calculated, considering the absorption values (A $\lambda_{\text{max}}$) recorded for each $\lambda_{\text{max}}$, multiplied with the dilution factor (d). The formula applied was:

$$EF = A (\lambda_{\text{max}}) \times d$$

3.3.4. Fourier Transform Infrared spectrum (FTIR)

Dried powder of the methanol extract of the plant materials (leaf, stem and root) were used for FTIR analysis. 10 mg of the dried extract powder was encapsulated in 100 mg of KBr discs, in order to prepare translucent sample discs. The powdered sample of the plant specimens were loaded in FTIR spectrophotometer 100 (Shimadzu), with a scan range from 400 to 4000 cm$^{-1}$ with a resolution of 4 cm$^{-1}$.

In the above analyses mentioned, the methanol extract of the stem responded better. Hence the methanol extract of the stem was taken for further analysis.
3.3.5. High Performance Liquid Chromatography (HPLC)

HPLC is a chromatographic technique which separates non-volatile mixtures into their individual components. The principle is that the sample components are carried by a mobile phase through a column that contains a stationary phase, where they separate and elute one by one at the end of the column. They immediately enter a detector where they are measured. The recorded peaks are displayed on a chart called a chromatogram. The peak area indicates the quantity of a component, and the retention time, the time it takes the substance to get through the column, can give an indication of the components identity, although this indication should not be trusted completely as several different compounds might have the same or similar retention times on the same HPLC-system.

HPLC-UV was done to determine the number of compounds present in the methanol extract. The analysis was performed using a liquid chromatograph (Shimadzu). The separation was performed by a column of 250×4.6 C18, 5µ, 100 Å. A mobile phase of 60% acetonitrile and 40% water was used. Detection was accomplished using a UV- detector at λ = 386 nm and the mobile phase flow rate was 2mL min⁻¹. The concentration of the sample used was 10 mg/ml and the sample application volumes were 4, 5, 6, 8, 10, 12, 15 and 20 µl.

3.3.6. Gas Chromatography Mass Spectrometry (GC-MS)

For the analysis GCMS - QP 2010 (Shimadzu) instrument was used. To analyze the sample, the column oven temperature and injector temperature was set to 800°C and 200°C respectively. The flow control mode was maintained in linear velocity with a split injection mode of split ratio 20. The column flow was 1.46
ml/min with a helium carrier gas of 99.9995% purity. The column oven temperature program was set as follows: The temperature was set at 80°C with 2 min hold time. By the rate of 10 the temperature was 300°C with 10 minutes hold time. The column DB – 5ms was used with a length of 30 meter and diameter of 0.25 mm and its film thickness is 0.25 μm. The ion source temperature for MS condition was 200°C and interface temperature was 240°C. Starting m/z was 40 and ending m/z was 700.

**Identification of the phytochemical constituents:** To identify the unknown phytochemical components present in the methanol extract of the stem, their individual mass spectral peak value was compared with the database of National Institute of Science and Technology which holds 62,000 patterns. Then the phytochemicals were identified based on the hits returned after comparing the unknown peak value and chromatogram from GCMS against the known chromatogram, peak value from the NIST Library database.

**3.3.7. Liquid Chromatography Mass Spectrometry (LC-MS)**

LC/MS, also called HPLC/MS, is an analytical technique used worldwide, which combines liquid chromatography (separation technique) and mass spectrometry (analytical technique). Very basically put the former technique is highly efficient when it comes to separating non-volatile mixtures into their component substances, without being able to identify them, and the latter, as explained above, can identify the single substances, but have some limitations when it comes to dealing with mixtures. However, some modern MS-instruments can even separate two ions with exactly the same mass from each other based on differences in the shape of the molecules. The combination of these two techniques gives almost
unlimited possibilities when it comes to separating complex mixtures and then identifying the individual substances.

The LC-MS system consisted of a liquid chromatograph (Shimadzu), micro vacuum degasser, system controller, MS detector (LC-MS-QP 8000 Shimadzu) and injector. The column used was Hypersil Hypurity C18 (50mm x 4.6mm i.d., 5 µ particle size) analytical column. The flow rate of the mobile phase was kept at 0.5 ml/min. The concentration of the sample used was 10 mg/ml and the injection volume was 3µl. Column oven temperature was set at 40°C. The mobile phase consisted of 0.1 % formic acid and methanol in the ratio of 20:80 (v/v). The interface between HPLC and mass spectrometer was atmospheric pressure ionization source with the electrospray inlet operated in the positive mode. LC-MS solution (Shimadzu) software was used to control the LC-MS system and for data processing.

3.3.8. Preparative Thin layer chromatography of methanol extract of *Sesbania sesban* stem

Preparative Thin Layer Chromatography was conducted to get fractions of the methanol extract. The solvent system consisting of toluene: ethyl acetate: formic acid: methanol in the ratio of 5.5:3:1:0.5 was used as the developing system. The TLC plates were examined under the ordinary light and UV light. Separated constituents were recovered by scraping off the adsorbent at the appropriate places on the developed plate and further eluted with the solvents chloroform: methanol in the ratio of 12:1. The eluted fractions were centrifuged, dried and tested for their purity. Then they were subjected to crystallization separately and identified with the
help of UV, FTIR, GC-MS and LC-MS methods. The melting points of the fractions were measured with that of the laboratory instrument.

3.3.9. Quantification of Quercetin

HPTLC method

HPTLC method has been done again for quantification of quercetin in the methanol extract of the stem.

Instrument: CAMAG Automatic TLC Sampler 4 (ATS4) "ATS4_140608" S/N 140608 (1.02.13) with win CATS software.

No. of tracks: 16, band length: 8 mm.

Solubility: Methanol

Development chamber: Twin trough chamber (20 X 10)

Development mode: Ascending mode

Distance run: 75 mm

Lamp: D2

Slit dimensions 4.00 x 0.30 mm, Micro

Measurement mode: absorbance.

TLC silica gel 60 F 254 (E.MERCK KGaA) pre coated layer (20 cm X 10 cm), thickness 0.2 mm plate was used as stationary phase and the solvent system toluene: ethyl acetate: formic acid: methanol (5.5:3:1:0.5) as the mobile phase. 50 µg/ml of the standard was applied in the form of bands using LINOMAT IV applicator. The volumes applied were 1, 2, 4, 6, 8, 10, 12, 14, 16 and 18 µl. The sample concentration was 10 mg/ml and the different volumes were 1, 2, 4, 8, 12 and 16 µl. The chromatograph was developed for 15 minutes, dried at room
temperature and scanned at 386 nm. Average peak area of the standard was calculated. The calibration curve of the standard drug concentration (X-axis) over the average peak height / area (Y-axis) was prepared to get a regression equation by Win Cats software.

**Estimation of quercetin in methanol extract of *Sesbania sesban* stem**

The mean peak height / area of the sample were calculated and the content of quercetin was quantified using the regression equation obtained from the standard curve.

**Limits of Detection and Limit of Quantification**

The limit of detection (LOD) was the lowest amount of the analyte in the sample which can be detected. The limit of quantification was the lowest amount of the analyte in the sample which can be quantitatively determined. The signal-to-noise ratios were 3:1 and 10:1 respectively.

**3.4. Biological activities of the methanol extract of *Sesbania sesban* stem**

**3.4.1. Antibacterial activity**

The antibacterial assay was performed by agar well diffusion method (Murray *et al.*, 1995). The bacterial species used were *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Salmonella typhi* (MTCC 733), *Enterococcus faecalis* (ATCC 29212), *Erwinia amylovora* (MTCC 2760), *Proteus vulgaris* (MTCC 1771), *Pseudomonas aeruginosa* (MTCC 424), *Klebsiella pneumoniae* (ATCC 15380), *Shigella dysenteriae* (MTCC 5151) and *Bacillus*
*Bacillus subtilis* (ATCC 441). The nutrient agar was inoculated with 100 μl of the inoculum (106 CFU/ml) and poured into the petri plate. A well was prepared in the plates with the help of a cork-borer (6 mm). About 50 μl of the extract (100, 250 and 500 μg/ml) was dispensed into the well. The flavonoid quercetin 100 μg/ml was used as the standard. The plates were incubated overnight at 37°C. For each bacterial strain, bacitracin 100 μg/ml as positive control and pure solvent (methanol) as the negative control were maintained. The diameters of the inhibition zones were measured in mm.

**Minimum Inhibitory Concentration (MIC)**

Minimum Inhibitory Concentration (MIC) is a technique which is used to determine the lowest concentration of different antibacterial agents necessary to inhibit visible growth of microorganisms after overnight incubation. MIC tests are used to confirm resistance, and are used as a tool to determine the activity of new antibacterial agents (Mims and Goering, 2008, Andrews, 2001).

Minimum inhibitory concentration (MIC) was determined based on micro-well dilution method. The test was done only for selected strains which include *Bacillus subtilis, Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. 95 μl of nutrient broth and 5 μl of bacterial suspension were dispensed in 35 (5x7) wells of a 96-well plate. The first column of the plate was maintained as negative control. The second column was added with 100 μg/ml of bacitracin and was kept as positive control. A volume of 100 μl extract at concentrations 60, 70, 80, 90 and 100 μg/ml were added into the other five consecutive wells. After 24 hours incubation, microbial growth was determined. The
lowest dilution of the extract that retained its inhibitory effect resulting in no growth (absence of turbidity) of the bacteria was recorded as the MIC value of the extract.

**Minimum Bactericidal Concentration (MBC)**

Minimum Bactericidal Concentration (MBC) was defined as no microbial growth after plotting samples on the nutrient agar plates. The activity was screened only on selected organisms which include *Bacillus subtilis*, *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia*.

The MBC was determined by on a fresh, drug-free, solid medium in a plate divided into five sectors with five different bacterial strains. The extract concentration taken was 100 μg/ml. The plates were incubated for 24 h. Triplicates were maintained.

### 3.4.2. Antifungal activity of methanol extract of *Sesbania sesban* stem

The antifungal activity was studied by Poison plate method (Murray *et al.*, 1995). Fungal species taken for the study were *Aspergillus fumigatus*, *Colletotrichum gloeosporioides*, *Curvularia lunata*, *Fusarium oxysporum* and *Verticillium glaucum*. Different concentrations (100, 250, 500 and 1000 μg/ml) of the methanol extract was added to the potato dextrose agar and poured into the petriplate. Carbendazim 100 μg/ml was used as positive control and pure solvent (methanol) as the negative control. The flavonoid quercetin 100 μg/ml was used as the standard. A disc (6 mm in diameter) of actively growing mycelium of the test fungi was obtained using a sterile cork borer. These fungal discs were placed on the potato dextrose agar which was mixed with the extract. The plates were maintained
at a temperature of 28±2°C. After 48 hrs, the plates were observed and the diameter of the fungal growth was measured. The zone of inhibition was measured for pathogenicity of the extract.

3.4.3. Antioxidant activity of methanol extract of *Sesbania sesban* stem

In majority of the plants the phenols and flavonoids are found to possess the anti-oxidative properties. The phytochemical analyses of the plant revealed the presence of phenols and flavonoids. Hence, the antioxidant study of the plant was done. The following activities were conducted with the methanol extract of the stem.

**DPPH free radical scavenging activity**

DPPH (1,1-diphenyl-2-picryl hydrazyl) is usually used as a reagent to evaluate antioxidative activity of the extracts/antioxidant compounds. DPPH is a stable free radical and accepts an electron or hydrogen molecule to become a stable diamagnetic molecule. The antioxidants are able to reduce the stable radical DPPH to the yellow-coloured diphenyl picryl hydrazine.

The effect of the methanol extract of *Sesbania sesban* stem on DPPH radical was estimated using the method of Liyana-Pathirana and Shahidi (2005). The extract at various concentrations (1 μg/ml to 100 μg/ml) were added to a 0.1mM solution of DPPH in methanol and the reaction mixture was shaken vigorously. The amount of DPPH remaining was determined at 517 nm.

The radical scavenging activity was obtained from the following equation:
Radical scavenging activity (%) = \{\text{OD control} - \text{OD sample}\}/\text{OD control}*100.

The antioxidant activity of plant extracts was expressed as EC₅₀, which was defined as the effective concentration (in μg/ml) of extract required to scavenge the DPPH radicals by 50%. Results were compared with the known antioxidant caffeic acid (EC₅₀-1μM/ml).

**Nitric oxide Assay**

Nitric oxide radical activity was determined according to the method (Garret, 1964). RAW 264.7 cells were plated at 5x10⁵ cells/well in 24-well plates and incubated with or without LPS (1μg/ml) and with various concentrations (5, 10, 25, 50 and 100 μg) of methanol stem extract for 24h.

Nitrite levels were determined using the Griess reaction. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent (1 % sulfanilamide and 0.1 % NEDD in 2.5 % ortho phosphoric acid) and incubated at room temperature for 10 min.

The nitric oxide concentration was estimated using standard curve plotted against known quantity of sodium nitrite. Results were expressed in μM obtained from the mean OD of triplicate wells. The standard curve was plotted using different concentrations of sodium nitrite (1 to 100 μM) with absorbance of 570 nm.
Determination of Total Phenols (Heo et al., 2005)

About 1 ml of stem extract, 1 ml of 95 % ethanol, 5 ml of distilled water and 0.5 ml of 50 % folin-ciocalteu reagent were mixed. The mixture was allowed to react for 5 minutes, and then 1 ml of 5 % sodium carbonate was added. This was thoroughly mixed and placed in dark for one hour. Absorbance was measured at 725 nm and gallic acid standard was used.

\[
\% \text{ of inhibition} = \left( \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \right) \times 100.
\]

Fe\textsuperscript{2+} chelation assay

The ability of the sample extracts to chelate Fe\textsuperscript{2+} was determined using a modified method of Minotti and Aust (1987) with a slight modification (Puntel et al., 2005). Briefly 150 μl of freshly prepared 500 μM FeSO\textsubscript{4} was added to a reaction mixture containing 168 μl of 0.1 M Tris-HCl (pH 7.4), 218 μl saline and the methanol stem extracts (100 - 500 μl). The reaction mixture was incubated for 5 min, before the addition of 13 μl of 0.25 % 1, 10-phenanthroline (w/v). The absorbance was subsequently measured at 510 nm in the spectrophotometer.

\[
\% \text{ of inhibition} = \left( \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \right) \times 100.
\]

Reducing power assay

The reducing power was determined according to the method previously described by Oyaizu (Oyaizu, 1986). Different concentrations of the methanol stem extract (100-500 μg) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K\textsubscript{3}Fe(CN)\textsubscript{6}] (2.5 ml, 1 %). The
mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloro acetic acid (10 %) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml 0.1 %) and the absorbance was measured at 700 nm. Ascorbic acid was used as the standard.

\[
\text{% of inhibition} = \left( \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \right) \times 100.
\]

**Assay of superoxide radical scavenging activity**

The method used by Martinez *et al.*, (2001) for determination of the superoxide dismutase was followed with modification (Dasgupta *et al.*, 2004) in the riboflavin-light-nitroblue tetrazolium (NBT) system (Charles Beauchamp *et al.*, 1971). Each 3 ml of reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 ml of 1 M riboflavin, 100 ml of 1 M EDTA, NBT (75 1 M) and 1 ml of sample solution. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min of illumination from a fluorescent lamp.

\[
\text{% of inhibition} = \left( \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \right) \times 100.
\]

**3.4.4. Anti-proliferation activity**

For the discovery of new anticancer agents, the herbal extracts were evaluated using several cancer cell lines. In the present study, the anti-proliferative test was conducted with three cell lines - A-431 human skin cancer epidermoid carcinoma, MCF-7 human adenocarcinoma of breast and HT-29 human colorectal
adenocarcinoma. The cell lines were purchased from National Centre for Cell Science (NCCS), Pune.

**Media preparation**

The DMEM medium (Sigma) was prepared as per the procedure. The quality of the medium was checked by incubating 5 ML of filtered medium in the CO$_2$ incubator for two days. The antibiotics and serum was added before it was used for cell culture.

**Cell culture and MTT assay**

The cells were grown in a DMEM medium supplemented with 10% foetal bovine serum and antibiotics as mentioned earlier. Cell proliferation (MTT) assay was performed following the method described by Carmichael *et al.*, (1987) and percentage of cell viability was determined by spectrophotometric determination of accumulated formazan derivative in treated cells at 570 nm in comparison with the untreated ones. For the MTT assay, the cells were grown in 25 cm $\times$ 25 cm $\times$25 cm tissue culture flasks. When a cell density in a culture flask reached 70-80% confluence, they were trypsinized and seeded in 96-well plates at varying cell number according to the size and shape of the and incubated for 24 hours at CO$_2$ incubator.

The cells were treated with increasing concentrations (100, 10, 1.0, 0.1 and 0.01 μg/ml) of the test samples. The plates were further incubated for 24, 48 and 72 hours in the CO$_2$ incubator.
MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution was dispensed into each well to achieve 1 mg/ml as final concentration. The plate was further incubated for 2.30. The formazan crystals formed were air dried in dark place and dissolved in DMSO and the plates were shaken gently at room temperature and the OD was measured using Synergy H4 microplate reader at 570 nm.

From the optical density the percentage growth were calculated using the following formula:

\[
\text{Percentage of growth} = 100 \times \frac{(T - T_0)}{(C - T_0)}
\]

\(T\) - optical density of test sample, \(C\) - optical density of control, \(T_0\) - optical density of test sample at time zero.

From the percentage growth a dose response curve was generated and GI\(_{50}\) values were interpolated from the growth curves.

**3.5. Phylogenetic analysis of Sesbania sesban**

Two nucleotide sequences namely

- *Sesbania sesban* 5.8S ribosomal RNA gene sequence (gi:336245365, SS58rRNA)

- *Sesbania sesban* chloroplast rbcL gene sequence (gi:3114994, SSrbcL)

were collected from NCBI Nucleotide Database in FASTA format.
Nucleotide Database is a collection of nucleotide sequences from several sources, including GenBank, RefSeq, the Third Party Annotation (TPA) database, and PDB. Searching the Nucleotide Database will yield available results from each of its component databases (Altschul et al., 1990).

Related or similar sequences for both SS58rRNA and SSrbcl were retrieved using NCBI BLAST (Basic Local Alignment Search Tool). Based on the E-value and species of interest, 20 sequences for SS58rrna and 30 sequences for SSrbcl were selected from the BLAST output.

**BLAST:** The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families. BLAST returns a table of the best matches (“hits”) from the database. The hit table includes several useful pieces of information, including the similarity score, query coverage (percent of the query sequence that overlaps the subject sequence), E-value and maximum identity. Hits were selected based on the e-value. Smaller the E-value, higher the probability that the homology reflects a true evolutionary relationship (Altschul et al., 1990).

Multiple Sequence Alignments were performed with 20 sequences of SS58rRNA and 30 sequences of SSrbcl (FASTA format) using Clustal Omega Software. The aligned output sequences were saved in PHYLIP format.
Clustal Omega is a new multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques to generate alignments. Multiple Sequence Alignment (MSA) is generally the alignment of three or more biological sequences (protein or nucleic acid) of similar length. From the output, homology can be inferred and the evolutionary relationships between the sequences studied. By contrast, pairwise sequence alignment tools are used to identify regions of similarity that may indicate functional, structural and/or evolutionary relationships between two biological sequences (Sievers et al., 2011).

Then, phylogenetic tree for the two set of sequences namely 20 sequences of SS58rRNA and 30 sequences of SSrbcl were generated using PHYLIP 3.695 version software on a Linux platform.

PHYLIP is a program package used to generate phylogenetic trees from MSAs. It is a comprehensive phylogenetic analysis package created by Joseph Felsenstein at the University of Washington. Each program carries out a specific task, and they are designed to be used sequentially to produce the best possible phylogenetic tree. The programs are used in a sequential way. The output from the first program is used as an input in the next program (Felsenstein et al., 2005).

From now onwards the two set of sequences 20 sequences of 5.8S rRNA and 30 sequences of ribulose- 1,5-bisphosphate carboxylase / oxygenase chloroplast gene including Sesbania sesban will be represented as SS58rRNA_20 and SSrbcl_30 respectively.
The following programs were used for DNA sequence analysis:

SEQBOOT, DNADIST, NEIGHBOR, CONSENSE

SEQBOOT : Generates random samples by bootstrapping or jack-knifing. This program reads in a sequence alignment, and generates a specified number of random samples into a n output file. These random samples are usually used in subsequent analysis as a sequence alignment file.

DNADIST: DNA distance matrix calculation

First a distance matrix is calculated by DNADIST program from the multiple sequence alignment. The matrix is then transformed into a tree by Fitch, Kitsch or Neighbor program.

NEIGHBOR: Neighbor-Joining and UPGMA tree drawing method

CONSENSE: Draws consensus trees from multiple trees. This program constructs a consensus tree from multiple trees. DNADIST can produce multiple trees, which can be summarized by the program CONSENSE. Also the results of bootstrapping are summarized by the program CONSENSE as a majority rule tree.

The MSA files of SS58rRNA_20 and SSbcl_30 were bootstrapped using SEQBOOT, with block size = 1, replicates = 1000, and input sequences interleaved. The program multiplied the input file to 1000 times (Multiple data set). The resulting output file was used to calculate 1000 distant matrices for DNADIST. The NEIGHBOR program created 1000 trees from these matrices. The CONSENSE program reduced the 1000 trees to a single one. It indicates the bootstrap values as numbers on the branches.
The topology of the CONSENSE tree was viewed by a text editor and tree file was viewed by TREEVIEW Software and a graphical output was generated (Page et al. 1996).

Bootstrapping is a resampling tree evaluation method. The result of bootstrapping is a number associated with a particular branch in the phylogenetic tree. Bootstrap value is a measure of repeatability and in more recent interpretations, it is considered as a measure of accuracy - a biologically more relevant parameter that gives the probability that the tree phylogeny has been associated (Jill Harrison et al., 2006, Felsenstein et al., 2005).