MATERIALS AND METHODS

3.1 Plant Material

*Cordia dichotoma* is a species of flowering tree in the *borage* family, *Boraginaceae*, that is native to the Indomalaya ecozone, northern Australia, and western Melanesia. For the present study the ten genotypes was used. The plant material was collected from different geographical region of Nanded district. The ten genotypes was selected on the basis of morphological variations. Few of the genotypes was selected from forest area and few of them was selected from farmers field.

3.1.1 Collection and authentication of plant material

*Cordia dichotoma* were collected from Nanded district of Maharashtra region (India) in the month of June-Jully. Plant was authenticated by Dr. Mulani R.M. Associate Professor, S.R.T.M.University, School of Life Sciences, Botanical Laboratory Nanded, India. The specimen sample of the plant was preserved in department.

The present study was carried out by using three parts of plant i.e Leaves, Bark, and Fruit.

The leaves of the plants are properly washed in tap water and then rinsed in distilled water. The rinsed leaves are dried in an oven at 35°C for 4 days. The dried leaves of each plant was crushed to obtain powder. These powdered samples are then stored in airtight polythene bags protected from sunlight until use. The ripened fruit material was collected in June to July month. The fruit material was dried in shadow. After 25 to 30 days the fruit material get dried and powdered was made in electric blender which were used to prepare powder of ayruvedic preparations. The bark was collected by removing upper layer of trunk of trees. Bark of tree was cut into small pieces and dried in shadow for few days. The bark was dried after 20 to 25 days then powder was made by using electric blender. Powdered material of leaves, fruit and bark was extracted with methanol in soxhlet apparatus. The prepared plant extract was concentrated by rotatory evaporator at optimum temperature and pressure.

3.2 Cultures for Antimicrobial activity.
In present study two bacterial strain and one fungal strain was used. Bacterial strains were gram +ve *Staphylococcus aureus*, gram –ve *Escherichia coli* and fungal strain *Aspergillus niger* was used. Pure culture of *E.coli, S.aures* and *A.niger* was procured from Department Of Microbiology S.R.T.M.University, Nanded. and also from Royal Bioresearch Centre, Velachery, Chennai. Bacterial cultures was further recultured on Muller Hinton Agar and fungal culture was recultered on PotatoDextrose Agar. Inoculum of bacteria and fungi was used for practical purpose.

**3.3 Preparation of inoculums of Cultures.**
According to Jayaraman *et al.*, (2008) stock cultures was maintained at 4°C on nutrient agar slants for bacteria and potato dextrose agar for fungi. Active cultures for experiments were prepared by transferring a loopful of culture to 5 ml of Muller Hinton broth and potato dextrose broth and incubated at 37 °C and 30 °C for 24 hours respectively.

**3.4 Chemicals and Media**

**3.4.1 Screening of Phytochemicals**
Methanol, Ferric chloride, Wagner’s reagent, Mayer’s reagent, Ammonium hydroxide, sodium hydroxide, sodium carbonate, HCl, potassium iodide, mercuric chloride. The chemicals were from Himedia and Qualigen

**3.4.2 Phytochemical Estimations** - Ammonium hydroxide, aluminium chloride, quercetin, potassium acetate, Folin-Ciocalteu reagent, Gallic acid, Folin-Denis reagent, diethyl ether, tannic acid etc.

**3.4.3 Antimicrobial Activity** - Muller Hinton agar and broth, Potatodextose agar and broth, ampicilin, Fluconazole, DMSO, used from of himedia

**3.4.4 Minimum Inhibitory Concentration** - Muller Hinton broth, Potatodextose broth used from of himedia

**3.4.5 Antioxidant Activity and Ic50 value** - 2,2-diphenyl-1-picryl hydrazine, potassium ferricyanide, of sigma and fischer scientific.

**3.4.6 HPTLC**
Ethyl acetate, butanol, acetic acid,
3.4.7 RAPD
Cetyl-trimethyl ammonium bromide, polyvinyl pyridine, isoamylalcohol phenol, tris-HCl, EDTA, NaCl, β-mercaptopethanol, isopropanol, ethanol, agarose, EtBr, TE-buffer, Taq-polymerase, primers-OPBE-01, OPBE-02, OPBE-03, OPBE-08, OPBE-09, MgCl₂, dNTP's, template DNA,

3.5 Methodology:

3.5.1 Morphological Characterisation

Morphological character is the phenotypic character which we could observed and determined by external appearance. For the study of variation in different plants or different genotypes of same species. Morphological character is the strong base. According to data available if there is variation in morphological character there is change in biochemical and molecular level of individual species. For the study of morphology of *Cordia dichotoma* the number of character were enlisted as follows

Ten accessions of 10-15 years old tree were selected for morphological characterization. From each accession three trees were selected for replication. leven morphological characters including leaf, immature and mature fruit, pulp and seed ratio were recorded as per the method described by earlier researchers (Vashishtha *et al.*, 1985, Nagar and Fageria 2006, Samadita 2007).

3.5.2 Preparation of Plant Extracts

**Methanol Extract**
50 g of air-dried powder of *Cordia dichotoma* leaves, bark and fruit was taken separately in 500 ml of 96% methanol for 8 hours in Soxhlet apparatus for extraction. The extract was filtered and allowed to evaporate in oven at 45 °C. The dried extract is dissolved in methanol and stored in refrigerator for further use (Shihabudeen, 2010).

3.6 Qualititative Analysis of Phytochemicals
In present study five secondary metabolites was analysed qualitatively and Quantitatively. The extracts of the dry powdered of leaves, fruit and bark of *Cordia dichotoma* was analyzed for the presence of various phytoconstituents like alkaloid, phenol, flavonoid, saponin and tannin.

### 3.6.1 Test for alkaloids:

A) Wagner’s Test: 3-4 drops of Wagners reagent was added to 2 mg of methanolic extract acidified with 1.5%v/v hydrochloric acid. Alkaloid presence was confirmed after the formation of yellow or brown precipitate. (Sofowara 1993, Trease and Evans 1989, Harborne 1973).

(Wagners reagent for alkaloids- To 100ml of distilled water 6 g of potassium iodide and 2g of iodine was dissolved

B) Mayer’s Test: To 2mg of methanolic extracts 3-4 drops of the Mayer’s reagent was added. Alkaloids presence was confirmed after the formation of white or pale yellow precipitate. (Mayer’s reagent –To 60 ml of distilled water 1.35g mercuric chloride was dissolved and pours into 10ml of distilled water contained 5g potassium iodide. Total volume was made 100ml)

### 3.6.2 Test for Flavonoids:

A) Shinoda’s Test: To 5 ml of ethanol 2 mg of methanolic extract was dissolved to this 10 drops of dilute hydrochloric acid was added, it was followed by adition of small piece of magnesium. Flavonoids presence was confirmed after the formation of brown reddish or pink color. (Sofowara 1993, Trease and Evans 1989, Harborne 1973).

B) Alkaline Reagent Test: Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

### 3.6.3 Test for Phenols:
A) Ferric chloride Test: The extract (50 mg) is dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution is added. A dark green colour indicates the presence of phenolic compounds. (Sofowara 1993, Trease and Evans 1989, Harborne 1973).

3.6.4 Test for Tannin:

A) Ferric chloride Test: About 1 ml of the mehanol extract was added in 2 ml of water in a test tube. 2 to 3 drops of diluted ferric chloride solution was added and observed for green to blue-green (cathechic tannins) or a blue-black (gallic tannins) coloration (Sabri et al., 2012)

B) Lead acetate Test: 1ml of the different filtrate was added with three drops of lead sub acetate solution. A cream gelatinous precipitation indicates positive test for Tannins. (Singh et al., 2012)

3.6.5 Test for Saponins:

A) Foam Test: A drop of sodium bicarbonate was added in 5ml of methanolic extract in the test tube. Later on test tube was shaken vigorously and kept test tube stand for 3 minute. Saponin presence was confirmed after the formation of honeycomb like froth.

B) Hemolytic Test: Crude extract was mixed with 5 ml of distilled water in a test tube and it was shaken vigorously. Add some drops of olive oil. The formation of stable foam was taken as an indication for the presence of saponins. (Sofowara 1993, Trease and Evans 1989, Harborne 1973)

3.7 Quantitative phytochemical analysis

3.7.1 Determination of alkaloids
1. 5 g of the samples were weighed into 250ml beaker
2. 200ml of 20% acetic acid was added and covered to stand for 4hr.
3. This was filtered and the extract was concentrated using a water bath to one quarter of the original volume.
4. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitate was complete.
5. The whole solution was allowed to settle and the precipitate
6. The precipitate was collected by filtration and weighed (Harborne, 1973, Obadoni and Ochuko, 2001).

### 3.7.2 Total flavonoid content

1. The total flavonoid in the crude extracts was measured using the Aluminum Chloride Colorimetric Method (Chang et al., 2002, Boyer 2003)
2. To 1 ml of plant extract or standard of different concentrations 3 ml methanol was added
3. Addition of 2 ml of 10% aluminum chloride.
4. Addition of 0.2 ml potassium acetate (1M)
5. 5.6 ml of distilled water were added.
6. Then the solution was incubated for 30 minutes at room temperature.
The absorbance was measured at 510 nm using UV spectrophotometer against a blank.
Standard curve was prepared using quercetin by dissolving it in methanol followed by serial dilution to 100 to 1000 μg/ml. Flavonoid content of extracts were determine using standard graph.

### 3.7.3 Total phenolic content

1. The amount of phenol in the aqueous extract was determined by Folin-Ciocalteu reagent method with some modifications. (Sadasivan and Manickam 1999)
2. 2.5ml of 10% Folin-Ciocalteu reagent was added.
3. 2ml of 2% solution of Na₂CO₃ were added to 1ml of plant extract.
4. The resulting mixture was incubated for 15 minutes at room temperature.
5. The absorbance of the sample was measured at 750nm.
6. Gallic acid was used as standard (1mg/ml).
The results were determined from the standard curve and were expressed as gallic acid equivalent (mg/ml of extracted compound) determined from the standard curve and were expressed. (Aiyegboro and Okoh 2010, Thimmaiah 2004)
3.7.4 Total tannin content
1. Accurately weighed 0.5g of the powdered material was transferred to a 250mL conical flask.
2. Add 75mL water.
3. Heat the flask gently and boil for 30 min.
4. Centrifuged at 2,000rpm for 20 min and collect the supernatant in 100mL volumetric flask and make up the volume.
5. Transfer 1mL of the sample extract to a 100mL volumetric flask containing 75mL water.
5. Add 5mL of Folin-Denis reagent.
6. 10mL of sodium carbonate solution and dilute to 100mL with water and Shaken well.
7. Read the absorbance at 700nm after 30 min. (Saxena et al., 2013).
(Folin-Dennis reagent-)

3.7.5 Total saponin content
1. For the saponins determination, 5 g of each plant samples was weighed and was dispersed in 100 ml of 20% ethanol.
2. The suspension was heated over a hot water bath for 4 h with continuous stirring at about 55°C.
3. The filtrate and the residue were re-extracted with another 100 ml of 20% ethanol.
4. The combined extracts were reduced to 40 ml over water bath at about 90°C.
5. The concentrate was transfered into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorously.
6. The aqueous layer was recovered while the ether layer was discarded.
7. The purification process was repeated and about 30 ml of n-butanol was added.
8. The combined n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride.
9. The remaining solution was heated in a water bath.
10. After evaporation, the samples were dried in the oven to a constant weight.
11. The saponin content was calculated in percentage (Obadoni and Ochuko, 2001).

3.8 Isolation of mucilage from Fruits:
3.8.1 Extraction of mucilage
1. Fruits of *Cordia dichotoma* were used for the extraction of mucilage.
2. The fruit were added in 1000ml beaker containing 500ml of distilled water,
3. Allowed it to boil for at least 3-4 h with continuous stirring.
4. Heating at 60°C for sufficient release of mucilage in water.
5. Concentrated solution was then filtered through muslin cloth in order to separate marc from the filtrate and refrigerated for cooling (3-4°C).( Kumar et al., 2012,Guo et al.,1998)

3.8.2 Isolation of mucilage:-
1. To the extract, equal quantity of ethyl alcohol was added for precipitation of mucilage to occur.
2. The precipitated mucilage was washed with ethyl alcohol and then collected through filtration by muslin cloth.
3. Mucilage was further dried in hot air oven at a temperature less than 40°C.
4. The obtained dried mucilage was grinded and passed through sieve #22 and finally stored in air tight container. ( Kumar et al., 2012,Guo et al., 1998)

3.9 Antimicrobial Assay

3.9.1 Paper Disk Diffusion Assay
1. The antimicrobial assay was performed by agar disc diffusion method (Bauer et al.,1966, NCCLS 2003, Parekh and Chanda 2007,Pepeljnjak et.al.,2005)
2. The molten Mueller Hinton Agar (HiMedia) was inoculated with 100μl of the inoculum . (10^8cfu/ml)
3. The disc (5-7 mm in diameter) was saturated with the extract (100 and 200mg/ml) and the discs were introduced on the upper layer of the agar plate.
5. Paper discs loaded with ethanol served as negative control.
6. Ampicilin as standard antibiotics and Fluconazole as standard antifungal were used as positive controls.
7. The plates were incubated at 37°C for all the bacterial strains while that of fungal strains were incubated at 28°C for 48 h.
8. The experiment was carried out three times and the mean values are presented. The antimicrobial activity was evaluated by measuring the diameter of zone of inhibition in mm. (Aneja 2001)

3.9.2 Minimum Inhibitory concentration

1. MIC was determined by micro-dilution method using serially diluted (2 folds) plant extracts according to the National Committee for Clinical Laboratory Standards (NCCLS) (National Committee for Clinical Laboratory Standards, 2000).
2. MIC of the extracts was determined by dilution of extract 100 to 1000μg/ml of *Cordia dichotoma*.
3. Equal volume of each extract and nutrient broth were mixed in a test tube.
4. Specifically 0.1 ml of standardized inoculums (10⁷ cfu/ml) was added in each tube.
5. The tubes were incubated aerobically at 37°C for 18-24 h.
6. Two control tubes were maintained for each test batch.
7. These included antibiotic control (tube containing extract and growth media without inoculum) and organism control (tube containing the growth medium, saline and the inoculum).
8. The lowest concentration (highest dilution) of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tubes were regarded as MIC. (Hassan, and Rahman, 2009)

3.10. Antioxidant Activity and IC₅₀ value

3.10.1: DPPH radical scavenging activity of selected genotypes and calculation of IC₅₀ value.

Preparation of DPPH Solution
1. Solution of DPPH (1mM) in methanol was prepared by dissolving 2 mg of DPPH in methanol and volume was made up to 100ml with methanol.
2. The solution was kept in darkness for 30 minutes to complete the reaction.
3. The stable 1-1 diphenyl 2 picryl hydrazyl radical (DPPH) was used for determination of free radical scavenging activity of the extracts (Koleva et al., 2002).
4. Different extracts (1mg/ml) were added at an equal volume to methanolic solution of DPPH.
5. After 15min at room temperature, the absorbance was recorded at 517nm.
6. The experiment was repeated for two times.
7. IC50 values denote the concentration of sample, which required to scavenge 50% of DPPH free radical (Pourmorad and Hosseinimehr 2006).
8. The %DPPH radical scavenging activity was calculated as follows. Ethanol (2.0 ml) plus the plant extract solution (0.5ml) was used as the blank, while the DPPH solution plus ethanol was used as the control. The inhibition percentage was calculated using the following formula

\[
\% \text{DPPH scavenging activity} = 1 - \frac{O.D. \text{of Test}}{O.D. \text{of control}} \times 100
\]

9. IC50 value was determined using linear regression equation.

3.10.2 Determination of Reducing power and calculation of IC50 value.

1. The reducing power of selected extracts was tested by the virtue of conversion of ferric to ferrous ions.
2. In this case 0.5 ml of 1mg/ml of test extract was added to 3 ml of 1 mM potassium ferricyanide solution.
3. The mixture was shaken thoroughly and incubated for 10 min at room temperature.
4. Finally the reducing capacity of the compound was tested spectrophotometrically at 420nm using an appropriate blank.
5. 3.5 ml potassium ferricyanide solution after every 10 min interval (Crespo et al., 2008).
6. This reducing power activity was calculated as follows. The inhibition percentage was calculated using the following formula.
3.11.HiHigh Performance Thin Layer Chromatography

HPTLC studies were carried out using following the method.

3.11.1 Sample Preparation
Methanolic extracts obtained were evaporated under reduced pressure using rotovac evaporator. Each extract residue was re-dissolved in 1ml of chromatographic grade methanol, which was used for sample application on pre-coated silica gel 60F254 aluminum sheets.

3.11.2 Developing Solvent System
A number of solvent systems were tried, for extracts, but the satisfactory resolution was obtained in the solvent Ethylacetate:(10):formic acid(1.1):Gl.acetic acid(1.1):water(2.6) for methanolic extracts.

3.11.3 Sample Application
Application of bands of each extract was carried out (4 mm in length and 1μl in concentration for Extract) using spray technique. Sample were applied in duplicate on pre-coated silica gel 60F254 Aluminum sheets (5 x 10 cm) with the help of Linomat 5 applicator attached to CAMAG HPTLC system, which was programmed through WIN CATS software.

3.11.4 Development of Chromatogram
After the application of sample, the chromatogram was developed in Twin trough glass chamber 10 x 10 cm saturated with solvent Ethyl acetate(10):formic acid(1.10):Gluacetic acid(1.1):water(2.6) for 15 minutes.

3.11.5 Detection of Spots

\[
\% FRAP scavenging activity = \frac{1 - \text{O.D of Test} \times 100}{\text{O.D of control}}
\]
The air-dried plates were viewed in ultraviolet radiation to mid-day light (Plate XVI). The chromatograms were scanned by densitometer at 254 and 366nm for methanolic extract. The Rf values and finger print data were recorded by WINCATS software. (Wagner 1995, Harborne (1973, Banu and Nagranjan 2014)

3.12 Molecular Diversity
Randomly Amplified Polymorphic DNA (RAPD)
3.12.1 Genomic DNA extraction

1. 1g of leaf tissue was weighed and incubated with extraction buffer (2% Cetyl trimethyl ammonium bromide (CTAB)).
2. 0.5% β-mercaptoethanol to lyse the cell wall and membrane.
3. The lysate was then treated with Phenol:Chloroform:iso-amyl alcohol mixture to eliminate complex organic intracellular contents.
4. Finally, DNA was precipitated using chilled 70% Alcohol and eluted in 40μl of TE-RNase, which was visualized using Agarose gel Electrophoresis, stained with Ethidium bromide.(Doyle, 1990)

3.12.2 DNA purification

3.12.3 PCR Analysis

1. The genomic DNA obtained using the above procedure was amplified using RAPD primers of the BE series: OPBE-01, 02, 03, 08, 09.
2. The PCR cocktail containing 10pM of primers, 25μM of each dNTP, 1.5mM MgCl₂ and 1IU of Taq polymerase, along with ~100ng of template, was found to be optimal for the required reaction.
3. The amplification was carried out using 30 cycles of a thermal profile containing 94°C for 30s, 32°C for 1 minute to allow the annealing of the primers, and 72°C for 5min, followed by final extension for 2min 50s.
4. The resultant amplicons were resolved using 1% agarose gel electrophoresis and visualized with ethidium bromide staining.
5. The band profiles were used to calculate by using Jaccard’s similarity coefficients for all samples and a similarity matrix was constructed.

6. The matrix was used as input to generate a phylogenetic tree using the UPGMA method (Lamboy1994,Garcia-Vallvé l. 1999)

3.12.4 Agarose gel electrophoresis

3.12.5 Data analysis

1. The obtained image showed a series of bands varying from sample-to-sample.

2. The number of bands, exclusive and shared between the samples were used to calculate Jaccard’s coefficient values, and a distance matrix was generated.

3. Percentage Polymorphism was computed by counting the number of samples which showed polymorphic bands and expressing them as a percentage of the total number of samples.

5. Distance matrix were generated from calculated Jaccard’s coefficient values.