MATERIALS AND METHODS
Materials and Methods

3.1 GROWTH OF CYANOBACTERIA

3.1.1 Cultures used

Unicellular and filamentous non-heterocystous marine cyanobacteria were obtained from the germplasm of National Facility for Marine Cyanobacteria (NFMC), Bharathidasan University, Tiruchirappalli, Tamilnadu, India.

1. *Gleocapsa* sp. BDU 110711
2. *Chroococcus turgides* BDU 142111
3. *Synechocystis pevalekii* BDU 130051
4. *Spirulina subsalsa* BDU 141021
5. *Oscillatoria salina* BDU 92071
6. *Oscillatoria laetevirens* BDU 20801
7. *Phormidium tenue* BDU 141753
8. *Phormidium valderianum* BDU 20041
9. *Phormidium corium* BDU 60121
10. *Phormidium fragile* BDU 42911
11. *Lyngbya* sp. BDU 90181
12. *Lyngbya* sp. BDU 141961

3.1.2 Media and growth conditions

Marine cyanobacteria were grown and maintained in Artificial Sea Nutrients-III (ASN III) (*Rippka et al., 1979*) medium under white fluorescent light of 13.8 μE m⁻² s⁻¹ at 25 ±2°C with 14/10 hr Light/Dark cycle.
### 3.1.2a Composition of ASN III Medium

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<td>*A5 micronutrients</td>
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<tr>
<td>Distilled water</td>
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<td>pH</td>
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* *A5 Micronutrients*

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<td>Co(NO₃)₂·6H₂O</td>
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<tr>
<td>Distilled water</td>
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3.1.3 Lignocellulosic material

Woody stems of Prospis juliflora were collected from Bharathidasan University campus, Trichirapalli, Tamilnadu. The finely chopped wood was dried under sunlight and ground into powder in a ball mill and passed through a 100 µm-1 mm, 1 mm-2 mm, 2 mm-3 mm mesh sieves.

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<tr>
<td>Genus</td>
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<tr>
<td>Species</td>
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</table>

3.1.4 Screening of cyanobacteria

The listed marine cyanobacteria were grown with the lignocellulosic waste P. juliflora separately at a fresh:dry weight ratio of 0.1:0.1 (cyanobacteria: lignocellulosic) in ASN III media under above mentioned conditions (Ref. 3.1.2) for 15 days. Effective strain was selected based on the growth of cyanobacteria along with the lignocellulose.

3.2 DEGRADATION OF PROSOPIS JULIFLORA BY O. LAETEVIRENS

3.2.1 Optimization of P. juliflora wood particle size and dry weight ratio

The ability of the selected cyanobacterium O. laetevirens to grow in the presence of the different wood particle size (100 µm-1 mm, 1-2 mm, 2-3 mm) and at varying dry weight ratios (0.05, 0.1, 0.2, 0.3 and 0.4) were tested in ASN III media as above mentioned earlier (Ref. 3.1.2) for 15 days. The pellet and supernatants were separated and subjected to biochemical analysis to check the degradability of P. juliflora wood particle by O. laetevirens.
3.2.2 Biochemical estimations of degraded *P. juliflora*

3.2.2a Estimation of Chlorophyll *a* (Mackinney, 1941)

**Principle**

Chlorophyll content provides the physiological status of the cyanobacteria and the estimation of chlorophyll usually involves the extraction of this fat-soluble pigment in methanol.

**Reagents**

- 80% methanol

**Procedure**

- One gram of *O. laetevirens* culture was taken and centrifuged at 5000 rpm for 10 min.
- Pellet was washed twice in distilled water.
- Pellet was resuspended in 4 mL of methanol and vortexed thoroughly.
- Tubes were incubated in a water bath at 60°C for 1 hr, in dark with occasional shaking.
- The suspension was centrifuged at 5000 rpm for 10 min and the supernatant was stored.
- The process was repeated to ensure complete extraction.
- Absorbance of the supernatant was read at 663 nm in JASCO UV-Vis spectrophotometer against 80% methanol as blank.
- The amount of chlorophyll *a* in the sample was calculated using the formula

\[
\text{Chl } a = \frac{A_{663} \times 12.63 \times \text{weight of sample}}{\text{Volume of methanol}} \quad \text{mg/g}
\]

**Calculation**

- \( A_{663} \) - absorbance at 663nm
- 12.63 - correction factor and the amount was expressed as mg/g
3.2.2b Estimation of Reducing Sugar (Miller, 1959)

Principle

3,5-dinitrosalicylic acid (DNSA) reagent appears yellow due to its nitro group. An alkaline solution of DNSA is reduced to 3-amino, 5-nitrosalicylic acid with the reducing sugar to form orange brown coloration which was read at 540 nm.

Reagents

a) Dinitrosalicylic acid (DNSA): 1g DNSA was dissolved in 20 mL 2 N NaOH (Sodium hydroxide) which was made upto 100 mL with distilled water

b) 30 g of Sodium potassium tartrate

Procedure

➢ To 1 mL of sample, 1 mL of reagent was added.
➢ The tubes were kept in boiling water bath for 10 min.
➢ After cooling it was made up to 10 mL by adding distilled water.
➢ Absorbance was measured spectrophotometrically at 540 nm.
➢ Concentration of sugar was determined by plotting optical density against the standard curve, prepared using sugar (glucose) concentration from 10-100 µg/mL.

3.2.2c Estimation of Phenol (Bray and Thorpe, 1954)

Principle

Phenols are aromatic compounds that possess one (or) more hydroxyl substituent bonded on to an aromatic ring. Total phenol estimation can be carried out with the Folin-Ciocalteu’s agent.

Phenols react with phosphomolybdic acid in Folin-Ciocalteu’s reagent in alkaline medium to produce blue coloured molybdenum complex.

Reagents

a) 12% Sodium carbonate solution

b) Folin’s phenol: Distilled water (1:2)
Procedure

- To 1 mL of culture filtrate 2.5 mL of 12% sodium carbonate solution was added and shaken well.
- Then 0.75 mL of Folin's phenol was added and mixed thoroughly.
- The tubes were incubated for 1 hr at room temperature.
- After incubation, optical density was measured spectrophotometrically at 725 nm.
- Concentration of phenol was determined by computing optical density against the standard curve, prepared using standard phenol concentration from 10-100 µg/mL.

3.2.2d Estimation of Lignin (Modified Klason Lignin Assay) (Tappi, 1992)

Principle

Sulfuric acid (H₂SO₄) hydrolysis is adopted to solubilize cellulose, hemicellulose and protein leaving the lignin as residue.

Reagents

Concentrated sulfuric acid

Procedure

- 100 mg of lignocellulosic residue was taken into a boiling tube.
- To this 2 mL of concentrated sulfuric acid was added and incubated for one hour with occasional shaking.
- After incubation, 56 mL of distilled water was added.
- The suspension was autoclaved at 121°C and allowed to cool.
- The acid insoluble material (Klason Lignin) was collected on a pre-weighed filter paper (Whatman No.1) (A) and washed several times with distilled water.
- The filter paper was dried at 70-80°C for 48-72 hr and weighed (B).
- The lignin content was calculated by using initial and final weight of the filter paper (B-A).

The final percentage of lignin present in the sample was calculated by

\[
\text{Lignin (\%) = \frac{\text{Initial weight}}{\text{final weight}} \times 100}
\]
3.2.3 Biodegradation with using optimized conditions

- Based on the biochemical analysis the optimum ratio and particle size was selected as 0.1:0.3 (fresh:dry weight ratio) and 1-2 mm for better degradation of *P. juliflora*.

- Hence, further experiments were carried out with the optimized ratio in which the biodegradation experiment was performed with cyanobacterial biomass (*O. laetevirens*) treated with lignocellulosic waste of *P. juliflora* in ratios of 0.1:0.3.

- Respective control was also maintained with culture (*O. laetevirens*) alone and with waste (*P. juliflora*) alone.

- The experimental setup was incubated for 30 days under white fluorescent light of 1500 lux at 25±2°C with 14/10 hr dark/light cycle.

- After the incubation period, pellet and supernatant were separated and subjected to biochemical estimations to confirm the degradative ability of the selected cyanobacterial strain and the rate of degradation.

3.2.4 Microscopic observation of *P. juliflora* degradation

Microscopic observation at different stages (1st, 5th, 15th and 30th) of *P. juliflora* degradation by *O. laetevirens* was observed under light microscope at 10X magnification.

3.2.5 Estimation of growth parameters

3.2.5a Estimation of Chlorophyll *a*

The protocol was followed as mentioned elsewhere in the thesis (See section 3.2.2a).

3.2.6 Colorimetric enzyme assay

Lignolytic enzyme profile of *O. laetevirens* was studied colorimetrically by estimating the activity of laccase, polyphenol oxidase and manganese independent peroxidase. The respective enzymes activity was also studied at varying pH (4, 5, 6, 7, 8 and 9) and temperatures (25 and 35°C).
3.2.6a Laccase (Caramelo et al., 1999)

Reagents
Sodium acetate buffer
Guaiacol

Procedure
- 0.1 mL of enzyme sample was added to 0.9 mL of sodium acetate buffer containing 10 mM guaiacol.
- Optical density was immediately taken at 470 nm

3.2.6b Polyphenol oxidase (Caramelo et al., 1999)

Reagents
Sodium tartrate buffer
O-catechol.
Sulfuric acid (H₂SO₄)

Procedure
- 0.1 mL of enzyme sample was added to 2 mL sodium tartrate buffer containing 0.15 M O-catechol.
- OD was taken at 420 nm for 2 min. and the reaction was stopped by 0.5 mL of 5% sulfuric acid (H₂SO₄).

3.2.6c Manganese independent peroxidase (Caramelo et al., 1999)

Reagents
Sodium tartrate buffer
2,6-dimethoxy phenol
Hydrogen peroxide

Procedure
- 0.1 mL of enzyme sample was added to 2 mL sodium tartrate buffer containing 0.1 mM 2, 6-dimethoxy phenol.
4 mM H$_2$O$_2$ was added and the optical density was immediately taken at 469 nm.

3.2.6d Hydrogen peroxide (Green and Hill, 1984)

Reagents

A. Phenol
B. 4-aminoantipyrine
C. Potassium phosphate buffer pH 6.9 (0.1 M)
D. Horseradish peroxidase
E. Hydrogen peroxide

The reagent solution (100 mL) was prepared using 0.234 g reagent A, 0.10 g reagent B and 1 mL of reagent C and contains 2x10$^{-8}$ M reagent D.

Procedure

- The reaction mixture (4 mL) was mixed with the peroxide sample (control *O. laetevirens* and *O. laetevirens* exposed to *P. juliflora*) and made up to 10 mL with double distilled water.
- The change in absorbance at 505 nm was measured until a constant reading was obtained (approximately 5 min. at ambient temperature).
- A 4 mL aliquot of the reagent solution made up to 10 mL with double distilled water served as reference.
- The amount of hydrogen peroxide released by *O. laetevirens* was calculated from a standard curve prepared with varying amounts (1-10 μM) of standard hydrogen peroxide.
- The results are expressed as μmol hydrogen peroxide per gram dry weight.

3.2.7 Analysis of biochemical parameters

3.2.7a Estimation of reducing sugar

The protocol was followed as mentioned elsewhere in the thesis (See section 3.2.2b).
3.2.7b Estimation of Phenol

The protocol was followed as mentioned elsewhere in the thesis (See section 3.2.2c).

3.2.7c Spectrum analysis (Viswajith, 2008)

The ability of the cyanobacterium Oscillatoria laetevirens to grow in the presence of P. juliflora at varying dry weight ratios were tested in ASN III medium under previously mentioned condition for 40 days. The supernatants were centrifuged and subjected to spectral analysis using a Jasco UV-550 spectrophotometer (Japan) in the wavelength range from 200-800 nm.

3.2.7d Estimation of nitrate (Jenkins and Medsken, 1964)

Principle

2, 4 phenoldisulphonic acid produces yellow coloured 6-nitro-1, 2, 4 phenoldisulphonic acid, (an alkaline salt) with nitrate which can be spectrophotometrically read at 410 nm.

Reagents

Standard nitrate solution

13.7 mg sodium nitrate (NaNO₃) was dissolved in 100 mL distilled water

[Concentration of nitrate (NO₃) 100 μM L⁻¹].

Brucine reagent

In a beaker 50 mL of distilled water and 3 mL of concentrated HCl was taken and heated to boil.

1 g of brucine and 0.1 g sulphanilic acid was added and stirred.

The solution was allowed to cool and made up to 100 mL.

Sulphuric acid

500 mL of concentrated sulphuric acid was carefully mixed with 100 mL distilled water.
Procedure
- 2 mL of sample was taken into a 100 mL beaker.
- To this, 1 mL of brucine sulphanilic acid reagent and 10 mL sulphuric acid was added.
- The content was stirred gently for 5 minutes.
- Then the beaker was covered with watch glass and kept in dark for 10 minutes.
- After the development of yellow colour, 10 mL of distilled water was added and incubated in dark for 30 minutes.
- Absorbance of solution was measured at 410 nm.
- The concentration of nitrate present in the sample was determined by extrapolating the optical density in standard curve (10-100 µg) and expressed in µg/mL.

3.2.7e Estimation of ammonia (Emmet, 1968)

Principle
Ammonia reacts with phenol and alkaline hypochlorite to form indophenols blue. The reactions are catalyzed by the nitroprusside or ferrocyanide. The resulting absorbance is proportional to the concentration of ammonia and is measured spectrophotometrically at 640 nm.

Reagents
Standard ammonia solution
Standard solution of ammonium chloride (conc.10 µg/mL) was prepared by dissolving 3.1 mg of ammonium chloride in 100 mL distilled water.

Hypochlorite stock (1.6 N)
5.5% chlorine solution.

Alkaline stock
100g tri-sodium citrate and 5 g of sodium hydroxide was dissolved in 300 mL of distilled water and made up to 500 mL.
Nitroprusside reagent

1 g of sodium nitroprusside was dissolved in 50 mL distilled water and made up to 200 mL.

Oxidizing reagent

Alkaline stock and hypochlorite solutions were mixed in a 4:1 ratio. This solution was prepared freshly.

Phenol reagent

100 g phenol was dissolved in 50 mL of 95% ethyl alcohol and made up to 1000 mL with distilled water.

Procedure

- To 1 mL of sample, 0.4 mL of phenol reagent and 0.4 mL of nitroprusside reagent was added and mixed well.
- Then 1 mL of the oxidizing reagent was added and tubes are stoppered immediately.
- The content was vortexed and incubated for 1 hr at room temperature in the dark.
- The absorbance was measured at 640 nm in a spectrophotometer.
- Standard graph was prepared using different concentrations of ammonia (1 to 10 μg ammonium chloride).
- Ammonia concentration was determined by plotting the optical density in a standard graph.

3.2.7f Estimation of protein (Lowry et al., 1951)

Principle

Protein reacts with Folin-Ciocalteu’s reagent to give a coloured complex. The colour formation is due to the reaction of the alkaline copper with the protein at the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein. The intensity of the colour depends on the amount of these aromatic acids present and this may vary for different proteins.
Materials and Methods

Reagents

Alkaline sodium carbonate solution

2 g of sodium carbonate was dissolved in 0.1 N sodium hydroxide.

5% Copper sulphate

10% Sodium potassium tartrate

Copper sulphate - Sodium potassium tartrate solution

One part of copper sulphate solution was mixed with one part of sodium potassium tartrate solution and eight parts of distilled water was added.

Alkaline reagent

Prepared freshly by mixing 50 mL of alkaline sodium carbonate solution and 1 mL of copper sulphate - sodium potassium tartrate solution.

Folin-Ciocalteu’s reagent

The reagent was diluted with equal amount of distilled water.

Trichloro acetic acid

10% (w/v) in distilled water.

Standard protein

Bovine serum albumin (BSA) (100 μg/mL)

Procedure

- 100 mg of sample was ground with water in mortar and pestle.
- The contents were centrifuged at 5000 rpm for 5 min.
- 0.1 mL of this solution was made up to 1 mL using distilled water.
- 4.5 mL of alkaline reagent was added and incubated for 3 min.
- Then 0.5 mL of Folin - Ciocalteu’s phenol reagent was added and allowed to stand for 30 min.
- Absorbance was read at 750 nm in JASCO UV-Vis spectrophotometer.
- The amount of protein in each sample was calculated using a standard graph and expressed as μg/mL.
3.2.7g Estimation of lignin

The protocol was followed as mentioned elsewhere in the thesis (See section 3.2.2d).

3.2.7h Estimation of holocellulose (Tappi, 1992)

Principle

Holocellulose fractions were obtained after acidic hydrolysis of polysaccharides or delignification with sodium chlorite and glacial acetic acid.

Reagents

Sodium chlorite
Glacial acetic acid

Procedure

- Extractive free dust 5 gm (oven dry) was taken in Erlenmeyer flask (500 mL) containing distilled water (160 mL).
- The dust was then treated with sodium chlorite (1.5g) and glacial acetic acid (0.5 mL) was added.
- The process was repeated at least 5 times till the dust became white.
- Dust thus obtained was filtered through G2 crucible, washed with distilled water followed by acetone washing.
- The crucible was dried to constant weight in an oven at 105±3 °C.

The holocellulose content was calculated as follows:

\[
\text{Holocellulose (\%) } = \frac{A \times 100}{W}
\]

A = oven dried weight of holocellulose
W = oven dried weight of sample
3.2.8  Phytochemical analysis (Edeoga et al., 2005)

3.2.8a Alkaloids (Dragendorff’s Test)

To 1 mL of extract, 1 mL of dragendorff’s reagent (Potassium bismuth iodide solution) was added. Formation of an orange red precipitate indicates the presence of alkaloids.

3.2.8b Flavonoids

5 ml of diluted ammonia solution was added to the extract followed by addition of concentrated sulfuric acid. A yellow coloration observed in each extract indicates the presence of flavonoids.

3.2.8c Terpenoids (Puncal D test)

A few mL of puncal D reagent (Ammonium Heptamolybdate + Ceric sulphate in concentrated sulphuric acids) solution was added to the extract and heated. Formation of blue colour indicates the presence of terpenoids.

3.2.8d Saponin

A few mL of water was added to the extract and formation of frothing indicates the presence of saponin.

3.2.8e Steroids

A few mL of concentrated sulphuric acid was added to the extract and formation of green colour indicates the presence of steroids.

3.3. COMPOUND IDENTIFICATION

3.3.1 Preparation of extract (Sirmah, 2009)

- The degraded *P. juliflora* by *O. laetevirens* was ground to fine powder, passed through a 115-mesh sieve and dried at 60 °C before extraction.
- Soxhlet extraction was done using ethanol in which 10 g of sample powder was extracted with 180 mL of the solvent for 15 hours at a rate of 10 to 12 cycles per hour.
After extraction, the solvent was evaporated under reduced pressure in a Buchi rotavapor and the crude extract dried under vacuum in a desiccator over P$_2$O$_5$.

The mass of the remaining extract was measured and used for phytochemical analysis, compound identification, animal experiments and antioxidant testing.

3.3.1a Spectrum analysis

The protocol was followed as mentioned elsewhere in the thesis (See section 3.2.7e).

3.3.1b Thin Layer Chromatography (TLC) (Touchstone, 1992)

Principle

In thin layer chromatography, the mobile phase is a liquid and the stationary phase is a solid absorbent. The solid phase, adsorbent was coated onto a solid support as a thin layer (about 0.25 mm thick). The mixture to be separated was dissolved in a solvent and the resulting solution is spotted onto the thin layer plate near the bottom. A solvent or mixture of solvents called eluant is allowed to flow up the plate capillary action.

Requirements

- Precoated Silica gel TLC plates
- TLC solvent chamber
- Capillary tubes
- Iodine chamber
- Iodine
- Ethyl acetate
- Hexane

Loading Sample

- The precoated silica gel aluminium plates (Merck, Germany) were cut into 8 cm x 7 cm size.
- A line was drawn lightly with a pencil about 1.5 cm from the bottom and 1.0 cm from the top.
Materials and Methods

- The capillary tubes (1 mm in diameter) were washed with chloroform for three times to avoid contamination on each time of spotting.
- The extracts to be separated were applied as a small spot (1 to 2 mm diameter) using capillary tubes.

Development of Thin Layer Plates
- The chamber used for development of the chromatogram (20 cm x 10 cm) was covered with a glass plate.
- The developing solvent, Ethyl acetate : Hexane (4:6) poured into the chamber was saturated by lining with filter paper for 30 minutes prior to development.
- The spotted plate was then placed in the chamber with the spotted end down and the solvent level should be below the spots.
- The solvent was then slowly raised in the adsorbent by capillary action.

Visualization
- When the solvent front was moved to within about 1 cm of the top end of the plate (after 30 minutes), the plate was removed from the developing chamber.
- The position of the solvent front marked and the solvent was allowed to evaporate.
- Then the plate was kept in iodine chamber and visualized.

Calculation
The relationship between the distance travelled by the solvent front and the substance is usually expressed as Rf value.

\[
R_f \text{ Value} = \frac{\text{distance travelled by solute}}{\text{distance travelled by solvent}}
\]

The Rf values are strongly depend upon the nature of the adsorbent and solvent.

3.3.1c High Performance Thin Layer Chromatography (HPTLC) - (Khakhpour et al., 2005)

Principle
High performance thin layer chromatography is a sophisticated and automated form of TLC which utilizes the conventional technique of TLC in a more optimized
way which includes an optimized coating material with separation power, with a new method of feeding the mobile phase with competent data acquisition and processing system.

**Steps Involved In HPTLC**

1. Selection of chromatographic layer.
2. Sample and standard preparation.
3. Layer pre-washing.
4. Layer pre-conditioning.
5. Application of sample and standard.
6. Chromatographic development.
7. Detection of spots.
8. Scanning.

20 µl of samples was applied on precoated silica gel aluminium plates (Merk, Germany). HPTLC was performed using a CAMAG HPTLC Spectrometer provided with a scanner II densitometer and Linomat IV applicator.

**Analysis of the Sample**

The samples were applied on an HPTLC plate using an automatic applicator. The following parameters were used.

**Scanning Parameters**

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<tr>
<th>Parameter</th>
<th>Value</th>
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<td>Tungsten</td>
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<td>Reflectance/Transmission</td>
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<tr>
<td>Monochromator band width</td>
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</tbody>
</table>
Materials and Methods

Detection and visualization

- Spots of fluorescent compounds can be seen at 254 nm (short wave length) or at 366 nm (long wave length).
- Non UV absorbing compounds were observed by dipping the plates in 0.1% iodine solution.

3.3.1d High Performance Liquid Chromatography (HPLC) (Sirmah, 2009)

Principle

HPLC is used to separate components of a mixture by using a variety of chemical interactions between the substance being analysed and the column. The principle is to force the analyte through a column of the stationary phase by pumping a liquid (mobile phase) at high pressure through the column.

Standard

(+-)Catechin (98% assay) was used as reference control and purchased from Sigma Alderich

Requirements

- HPLC instrument (UFLC)
- Sonicator, with temperature control (BRAUN)
- Volumetric flasks, appropriate sizes
- Syringes, 3-cc disposable with Luer-lok tip
- Filters, 0.45-μm
- Pipettes

Chemicals

- Water (deionized) HPLC grade
- 0.05% of trifluoroacetic acid
- Methanol (HPLC grade)
Running conditions

<table>
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<th>Parameter</th>
<th>Description</th>
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<td>Column temperature</td>
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<tr>
<td>Mobile phase</td>
<td>Solvent A (water containing 0.05% of TFA)</td>
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<tr>
<td></td>
<td>Solvent B (methanol (HPLC grade) containing 0.05% of TFA)</td>
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<tr>
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<tr>
<td>Injection volume</td>
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<tr>
<td>Detection wavelength</td>
<td>210 nm to 400 nm</td>
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<tr>
<td>Running time</td>
<td>30 minutes</td>
</tr>
<tr>
<td>Post running time</td>
<td>10 minutes</td>
</tr>
<tr>
<td>Detector</td>
<td>Waters 2996 photo diode array (PDA) detector</td>
</tr>
</tbody>
</table>

3.3.1e Gas Chromatography Mass Spectroscopy (GC-MS) (Sirmah, 2009)

Principle

GC-MS composed of two major systems, a gas chromatograph and a mass spectrometer. In gas chromatography the chemical properties between different molecules in a mixture separate the molecule as the sample travels the column and the mass spectrometer breaks each molecule into an ionized fragment and detects them using their mass to charge ratio.

Requirements

Instrument: JEOL GCMATE II GC-MS

Chemicals

Anhydrous acetonitrile

N,O-bis-trimethylsilyl

Trimethylchlorosilane

Sample Preparation

Test samples were analyzed as trimethyl derivatives using the following procedure.
Materials and Methods

- In a screw-capped vial, 1 mg of dry sample was dissolved in 0.5 mL of anhydrous acetonitrile (Acros Organics).
- 0.4 mL of \( N,O \)-bis-trimethylsilyl (trifluoroacetamide) containing 1% of trimethylchlorosilane (Acros Organics) was added.
- The solution was sonicated for about 1 min and heated at 60°C for 60 min.
- After evaporation of the solvent in a stream of dry nitrogen, the residue was diluted in 1 mL of anhydrous acetonitrile.

**Running conditions**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>5% diphenyl / 95% dimethyl polysiloxane fused-silica capillary column (Elite-5ms, 60 m x 0.25 mm, 0.25 mm film thickness, Perkin Elmer Inc,)</td>
</tr>
<tr>
<td>Injector Mode</td>
<td>split</td>
</tr>
<tr>
<td>Injection volume</td>
<td>1 ( \mu )l</td>
</tr>
<tr>
<td>GC Inlet Temperature</td>
<td>250°C</td>
</tr>
<tr>
<td>Total Flow</td>
<td>20 mL/min</td>
</tr>
<tr>
<td>Septum Purge</td>
<td>1.2 mL/min</td>
</tr>
<tr>
<td>GC Oven Ramp</td>
<td>200°C constant for 4 min, 200°C to 330°C at a rate of 5°C/min and then constant for 330°C</td>
</tr>
<tr>
<td>Ion source</td>
<td>electron impact</td>
</tr>
<tr>
<td>Ionization voltage</td>
<td>70 eV</td>
</tr>
<tr>
<td>Scan Range</td>
<td>35 to m/z 700 a.m.u</td>
</tr>
<tr>
<td>Solvent Delay</td>
<td>10 min</td>
</tr>
</tbody>
</table>

3.3.1f Fourier Transform Infrared Spectroscopy (FTIR) (Sirmah, 2009)

**Principle**

The FTIR spectrum is a plot of infrared light absorbed by the sample as a function of wavelength or frequency. It is perhaps the most powerful tool for identifying types of chemical bonds (functional groups). Thus, by interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined.
**Running conditions**

<table>
<thead>
<tr>
<th>Model:</th>
<th>PERKIN ELMER Spectrum one FTIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scan Range</td>
<td>MIR 450-4000 cm</td>
</tr>
<tr>
<td>Resolution</td>
<td>1.0 cm⁻¹</td>
</tr>
<tr>
<td>Sampling Technique</td>
<td>KBr</td>
</tr>
</tbody>
</table>

**3.3.1g Nuclear Magnetic Resonance Spectroscopy (NMR) (Sirmah, 2009)**

**Principle**

NMR spectroscopy is the name given to a technique which exploits the magnetic properties of certain nuclei. It is one of the principal techniques used to obtain physical, chemical, electronic and structural information about molecules due to either the chemical shift, Zeeman effect or the Knight shift effect or a combination of both on the resonant frequencies of the nuclei present in the sample. It is a powerful technique that can provide detailed information on the topology, dynamics and three-dimensional structure of molecules in liquid or solid state.

**Requirements**

Instrument: Bruker AVIII 500 MHz NMR spectrometer

**Chemicals**

methanol-D4

**Running conditions**

<table>
<thead>
<tr>
<th>Magnetic field</th>
<th>11.7 Tesla, Wide bore (89mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probes</td>
<td>5mm Broad Band inverse probe</td>
</tr>
<tr>
<td>Solvent</td>
<td>methanol-D4</td>
</tr>
<tr>
<td>Magnetic nuclei</td>
<td>H</td>
</tr>
</tbody>
</table>

**3.4. PHARMACOLOGICAL STUDIES**

**3.4.1 Acute toxicity study (Ecobichon, 1997)**

- Acute toxicity studies were performed according to Organisation for Economic Cooperation and Development guidelines (OECD).
Male albino rats (*Rattus norvegicus*) (150-180g) were selected by random sampling technique and employed in this study.

Before experimentation the animals were fasted for four hours with free access to water only.

Twenty five male rats were divided into five groups, containing five animals each were tested for this study up to a period of 3 days.

The first group served as control and received 3 ml/kg normal saline orally. Group II, III and IV received graded doses (50, 100, 200, and 500 mg/kg) of *P. juliflora* wood extract to observe the mortality.

### 3.4.2 Subacute toxicity study (Ecobichon, 1997)

**Experimental animals**

- Twenty (20-month old) albino rats (140-180 g body weight) were used.
- Animals were housed in tarsons poly propylene cages (8”x12”x8”) with metal grill tops.
- The cages were kept in a well ventilated room with 28±2°C temperature, humidity of 50-60% and regular 12 hours light/dark cycle.

**Experimental group**

- The animals were separated into four groups.
- Group I served as control received 3 ml/kg normal saline orally.
- Group II was orally administered with *O. laetevirens* extract (200 mg/kg body weight).
- Group III received *Prosopis juliflora* extract (200 mg/kg body weight).
- Group IV was administered with degraded *Prosopis juliflora* with similar dose.
- Extract were given orally using oral gavage needle for 30 days without anaesthesia using hand restraint.

**Food and water consumption**

All animals were allowed to free access of water and fed with commercially available pellet feed (M/s. Hindustan Lever Ltd., Mumbai).
3.4.3  Morphological observation
3.4.3a  Body weight

The change in their body weight was measured at an initial and final stage of the experimental period using an electronic balance (Mettler Toledo).

3.4.4  Haematological and biochemical analysis by auto analyzer

Anaesthesia (William, 1965)

The rats were anaesthetised using the solvent diethyl ether.

Blood Collection

- Blood was collected from the orbital sinus (retro orbital puncture technique) of rats.
- Bleeding requires that the capillary tube was inserted with gentle rotation while directing the tube caudally and towards the midline.
- 1-1.5 mL of blood was collected in EDTA coated tubes for haematological analysis.
- Serum was obtained by centrifugation of fresh blood samples without EDTA at 3000 rpm for 10 minutes and used for biochemical analysis.

Analysis

The following biochemical and haematological analysis were measured using a fully automated analyzer (Erba Mannheim EM 360 clinical chemistry analyser, Mannheim, Germany). Commercial Erba kits (Erba Diagnostics Mannheim, Germany) were used for this analysis.

Haematology

- Haemoglobin
- Erythrocyte Sedimentation Rate (ESR)
- Packed Cell Volume (PCV)
- Red Blood Cells (RBC)
- White Blood Cells (WBC)
- Neutrophil
- Lymphocyte
- Eosinophil
Materials and Methods

Biochemical studies
- Glucose
- Protein
- Albumin
- Globulin

Lipid analysis
- Cholesterol
- Triglycerides (TGL)

Renal function tests
- Creatinine
- Urea
- Uric acid

Liver function tests
- Serum Glutamic Pyruvic Transaminase (SGPT)
- Serum Glutamic Oxaloacetic Transaminase (SGOT)
- Total bilirubin
- Indirect bilirubin
- Alkaline Phosphatase (ALP)

3.4.5 Sperm count (Gopalakrishnan et al., 1990)

Reagents
Trypan Blue
Phosphate buffer saline

Procedure
- The cauda epididymal duct on one side was exposed and incised.
- The connective tissue capsule around the cauda epididymus was teased out and the duct was uncoiled.
The semen that oozed out into the cavity block was quickly sucked into a
capillary tube up to the 0.05 µl mark and transferred to an eppendorf tube.
The sample was diluted 200-250 times in physiological saline.
After thorough mixing by blowing air throw a blowpipe, the sperm
suspension was used for analysis of count.
Then a drop of the diluted semen was transferred to an improved neubauer
counting chamber and a cover glass was overlaid.
The counting chamber was observed under a research microscope at 400X
magnification and sperm in the central core were counted.
The central square which has 25 large squares and volume of each of the 25
squares was 0.1 mL.
The sperm counts are counted using the following formula

\[
\text{Sperm count} = \frac{\text{Number of sperm in 25 squares}}{25} \times 10 \times \text{dilution factor} \times 1000
\]

3.4.6 Histopathology
For the study of the structure and function of the organ, the histological studies
are used in which tissue sections were made and observed under microscope. The steps
involved are as follows.

3.4.6a Kidney (Mark et al., 2008)
Step 1: Fixation
The fixation of the tissue was done with Bouin’s fluid.

Composition of Bouin’s fluid:
Saturated Picric acid-70 mL
40% formaldehyde-25 mL
Glacial acetic acid-5 mL
The duration of fixation was 24 hrs. After fixation the tissues were washed with
tap water to remove picric acid.
Step 2: Dehydration
- The dehydration was performed with the series of increasing concentrations of ethanol, i.e. 30%, 50%, 70%, 90% and 100%.

Step 3: Clearing
- Clearing was done with xylene and the time period was 5 to 10 min.
- The process was repeated thrice followed by infiltration with paraffin.

Step 4: Impregnation
- Tissues were then taken out of xylene and kept in molten embedding bath.
- The bath temperature is 58-60°C and the process was repeated three times with 20 minutes gap.

Step 5: Embedding
- Tissues are embedded in fresh paraffin wax and the optimum melting point of which is 58-60°C.
- The glass plate used for embedding was smeared with glycerine.

Step 6: Section cutting
Section cutting was done with a rotary microtome.
- Excess paraffin must be removed by trimming.
- 0.5 cm of the tissue was used.
- Block was attached to a heated object holder.
- Additional support by extra wax was given along with the length of the block.
- Before sectioning the screws were tightened.
- Uniform section microtome knife was adjusted in a proper angle.
- Care must be taken so that the paraffin block was contacted by knife alone but not the knife holder.
- The thickness of the section was 5-10 µm.

Step 7: Flattening and spreading
- The tissue was floated in warm water bath.
- The section spread was detached from the knife by hair line brush.
- The slides are coated with egg albumin and then they are kept at room temperature.
Step 8: Staining

- Deparaffinization with xylene was carried out for 2 times with 10 min interval.
- After rehydration with descending grades of alcohol the sample was stained with haemotoxylin for 15 min followed by washing in tap water and blowing for 10 min.
- Again the destained sample was rinsed with distilled water and stained with eosin dye.
- After staining, the sample was dehydrated with ascending grades of alcohol followed by clearing with xylene for two times in 5 min interval.

Step 9: Mounting

- The slide was mounted with DPX and a micro cover slip was placed and observed under microscope.

3.4.6b Liver

The protocol was followed as mentioned elsewhere in the thesis (See section 3.4.6a)

3.4.7 Antioxidant properties (Rao et al., 2008)

Principle

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols or polyphenols.

The free radical scavenging activity of the extract was analyzed by free radicals like 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical. The various methods used to measure antioxidant activity of food products can give varying results depending on the specific free radical being used as a reactant.
Materials and Methods

Reagents

1, 1-diphenyl-2-picrylhydrazyl (DPPH)
Methanol

Methodology

- The initial OD value of 0.1 ml of DPPH solution (0.1 mM DPPH in methanol) and 0.8 mL of methanol was taken at 517 nm in UV Spectrometer.
- 10 mg of the test sample was weighed and dissolved in 1 mL of 99% methanol with a final concentration of 10 mg/ml.
- In this assay, 100 μl of test sample was mixed with DPPH solution and incubated for half an hour.
- The absorbance of the solution was then measured at 517 nm by a UV Spectrometer.
- The radical scavenging activity was represented as percentage inhibition of DPPH radicals.

DPPH inhibition ratio was expressed as a percentage after being calculated from the following equation:

% inhibition = \( 100 \times \frac{a_c - a_e}{a_c} \)

where \( a_c \) is the absorbance of the control and \( a_e \) the absorbance of the test extract.

Data and Statistical Analysis

Only significant data were included in the tables and were presented with mean and standard error (±) of three replicates per treatment and repeated three times. Two factor analysis of variance (ANOVA) was used to assess the significance (\( p = 0.05 \)) of the mean values of treatments and the differences were compared using Duncan’s Multiple Range Test.