3. MATERIALS AND METHODS

The experimental material used was *Withania somnifera*, popularly known as “Ashwagadha” one of the important medicinal plant often quoted in the “Siddha and Ayurvedic” literatures. In the present study, the plants were collected from six different locations namely, Tiruchirapalli, Thanjavur, Tiruvarur, Nagapattinam and Cuddalore in Tamilnadu, South India.

**Systematic position**

Division : Dicotyledons  
Order : Tubiflorae  
Family : Solanaceae  
Binomial : *Withania somnifera* (L.) Dunal

**Vernacular names**

<table>
<thead>
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<th>Language</th>
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<tr>
<td>Sanskrit</td>
<td>Ashwagandha</td>
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<td>Hindi</td>
<td>Asgandh</td>
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<td>Kannada</td>
<td>Viremaddinagaddi</td>
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<td>Malayalam</td>
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<td>Tamil</td>
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<td>Telugu</td>
<td>Vajigandha</td>
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<td>English</td>
<td>Winter cherry</td>
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**Geographical distribution:** *Withania somnifera* grows wildly in all drier parts of subtropical regions of India. It occurs in Madhya Pradesh, Uttar Pradesh, Punjab, Tamilnadu and North Western parts of India like Gujarat and Rajasthan. It also found in South Africa, Egypt, Morocco, Jordan, Pakistan, Congo and Afganistan (Saldanha and Nicolson, 1978).

**Botanical description:** An erect branching under shrub reaching about 150 cm in height, usually clothed with minutely stellate tomentum; leaves ovate upto 10 cm long,
densely hairy beneath and sparsely above, flowers greenish or lurid yellow in axillary fascicles, bisexual, pedicel long, fruits globose berries which are orange coloured when mature, enclosed in a persistant calyx. The fleshy roots when dry are cylindrical, gradually tapering down with a brownish white surface and pure white inside when broken.

**Parts used:** Roots, leaves and berry.

**Medicinal uses:** *Withania somnifera* is one of the important herbal components of geriatric tonic mentioned in Indian systems of medicine. In the traditional system of medicine ‘Ayurveda’, this plant is claimed to have potent aphrodisiac, rejuvenative and life prolonging properties. It has the general animating and regenerative qualities and is used among others for the treatment of nervous exhaustion, memory related conditions, insomnia, tiredness potency tissues, skin problems and coughing. It improves learning ability and memory capacity. The traditional use of ‘Ashwagandha’ was to increase energy, youthful vigour, endurance, strength, health, nurture the time elements of the body, increase vital fluids, muscle fat, blood, lymph, semen and cell production. It helps counteract chronic fatigue, weakness, dehydration, bone weakness, loose teeth, thirst, impotency, premature aging amaciation, debility, convalescences and muscle tension. It helps invigorate the body by rejuvenating the reproductive organs, just as a tree is invigorated by feeding the roots (Nadkarni, 1993).

### 3.1. Analysis of physico-chemical properties of soil

The physico-chemical properties of soil samples such as pH, electrical conductivity, nitrogen, phorphorous, potassium and micronutients, organic carbon, zinc, copper, iron and manganese were analysed following the methods of Bernes (1959), Muthuvel and Udhayasooriyan (1999). The soil samples were collected from the five different localities of cauvery delta area *viz*, Tiruchirappalli, Thanjavur, Thiruvarur, Nagapattinam and Cuddalore.
3.2. Morphometry

Morphological parameters of *Withania somnifera* such as habit, plant height, stem, leaves, petiole length, flowers, pedicel length, calyx, corolla, anther, shape of the fruit collected from the 5 different locations (Tiruchirapalli, Thanjavur, Thiruvarur, Nagapattinam and Cuddalore) were determined. In each sample 10 replicates of the particular part from five plants each, from wild populations were taken and mean length and breadth of root were calculated and tabulated.

3.3. Phytochemical Analysis

3.3.1. Plant material

Samples of *W. somnifera* collected from five different locations (Tiruchirapalli, Thanjavur, Thiruvarur, Nagappattinam and Cuddalore) were used for the phytochemical analysis.

3.3.2. Qualitative Analysis

Phytochemical analysis of the plant extracts was undertaken using standard qualitative methods as described by various authors (Kapoor *et al*., 1969; Odebiyi and Sofowora, 1990). The plant extracts were screened for the presence of biologically active compounds such as alkaloids, flavonoids, carbohydrates, phytosterols, proteins, phenolics, tannins and saponins.

3.3.2.1. Preparation of plant extracts

The root and leaves were cleaned and dried in shade for 7 days, then ground well to fine powder. About 500g of dry powder was extracted with methanol (80%) at 70°C by continuous hot percolation using soxhlet apparatus. The extraction was continued for 24 hours. The methanolic extract was then filtered and kept in hot air oven at 40°C for 24 hours to evaporate the methanol from it. A dark brown residue was obtained. The residue was kept separately in air tight containers and stored in a deep freezer.
3.3.2.2. Alkaloids (Salehi Surmaghi et al., 1992)

Dragendroff’s test (Kraut reagent-Potassium bismuth iodide)

8 g of Bi (NO\textsubscript{3})\textsubscript{3} 5H\textsubscript{2}O was dissolved in 20 ml of HNO\textsubscript{3} and 2.72 g of potassium iodide in 50 ml of water. They were mixed and allowed to stand till KNO\textsubscript{3} got crystallised. The supernatant was decanted and made up to 100 ml with distilled water. The alkaloids were regenerated from the precipitate by treating with Na\textsubscript{2}CO\textsubscript{3} followed by extraction of the liberated base with ether.

To 0.5 ml of herbal extract 2 ml of HCl was added. Then 1 ml of reagent was added to this acidic medium. An orange red precipitate was produced immediately, which indicated the presence of alkaloids.

Wagner’s reagent (Iodine-Potassium iodide solution)

1.2 g of iodine and 2.0 g of potassium iodide were dissolved in 5 ml of H\textsubscript{2}SO\textsubscript{4} and the solution was diluted to 100 ml. 10 ml of herbal extract was acidified by adding 1.5% v/v HCl and a few drops of Wagner’s reagent. The formation of a yellowish brown precipitate confirmed the presence of alkaloids.

Meyer’s reagent (Potassium mercuric iodide)

1.36 g of mercuric chloride was dissolved in 60 ml of distilled water, and 5 g of potassium iodide in 10 ml of water. The two solutions were mixed and diluted to 100 ml with distilled water.

A few drops of the reagent were added to 1 ml of the herbal extract. The formation of a pale precipitate showed the presence of alkaloids.

3.3.2.3. Flavonoids (Somolenski et al., 1972)

In a test tube containing 0.5 ml of herbal extract, 5-10 drops of diluted HCl and a small piece of zinc or magnesium were added, and the solution was boiled for a few minutes. In the presence of flavonoids, a reddish pink or dirty brown colour was produced.
3.3.2.4. Carbohydrates

**Fehling’s test (Kokate, 1994)**

Solution A: 34.65 g of copper sulphate was dissolved in water and made up to 500 ml.

Solution B: 125 g of potassium hydroxide and 173 g of Rochelle’s salt (sodium potassium tartarate) were dissolved in water and made up to 500 ml.

To 2 ml of herbal extract, 1 ml of a mixture of equal parts of Fehling’s solution ‘A’ and ‘B’ was added. The contents were boiled for a few minutes. The formation of a red or brick red precipitate was the indication of the presence of carbohydrates.

**Benedict’s test**

173 g of sodium citrate and 100 g of sodium carbonate were dissolved in 500 ml of distilled water. 17.3 g of copper sulphate dissolved in 100 ml of distilled water was added to this solution.

To 0.5 ml of herbal extract, 5 ml of Benedict’s reagent was added and boiled for 5 minutes. The formation of a bluish green colour showed the presence of carbohydrates.

3.3.2.5. Proteins

**Million’s test (Walsh and Farrel, 1961)**

1 part of mercury was digested with 2 parts of concentrated HNO₃ and the resulting solution was diluted with 2 volumes of water.

To a small quantity of herbal extract, 5-6 drops of Million’s reagent was added. A white precipitate which turned red on heat, indicated the presence of proteins.

3.3.2.6. Phenols (Malick and Singh, 1980)

**Ferric chloride test**

To 1 ml of herbal extract, 2 ml of distilled water followed by a few drops of 10% aqueous FeCl₃ solution were added. Formation of a blue or green precipitate indicated the presence of phenols.
Lead acetate test

1 ml of herbal extract was diluted to 5 ml with distilled water and then a few drops of 1% aqueous solution of lead acetate was added. Appearance of yellow precipitate indicated the presence of phenols.

Libermann’s test

A small amount of herbal extract was dissolved in 0.5 ml of 20% sulphuric acid solution followed by the addition of a few drops of aqueous sodium nitrate solution. A red colour was obtained on dilution and it turned blue when made alkaline with aqueous sodium hydroxide solution, which indicated the presence of phenol.

3.3.2.7. Saponins (Malick and Singh, 1980)

In a test tube containing about 5 ml of herbal extract, a drop of sodium bicarbonate was added. The mixture was shaken vigorously and kept for 3 minutes. Formation of a honey comb like froth showed the presence of saponins.

3.3.2.8. Tannins (Segelman et al., 1969)

Ferric chloride test

To 1-2ml of herbal extract, a few drops of 5% aqueous FeCl₃ solution were added. A bluish black colour was formed, which then disappeared on addition of a few ml of dilute H₂SO₄. This was followed by the formation of yellowish brown precipitate.

Lead acetate test

In a test tube containing about 500ml of herbal extract, a few drops of 1% solution of lead acetate was added. Formation of yellow or red precipitate indicated the presence of tannins.

3.3.2.9. Phytosterols (Malick and Singh, 1980)

About 0.5 ml of test solution was mixed with minimum quantity of chloroform and then 3-4 drops of acetic acid and one drop of concentrated H₂SO₄ were added. Formation of a deep blue or green colour showed the presence of steroids.
3.3.3. Quantitative Analysis

3.3.3.1. Estimation of total carbohydrate (Hodege and Hofreiter, 1962)

Carbohydrates are the important components of storage and structural materials in the plants. They exist as free sugars and polysaccharides. Carbohydrates were quantitatively estimated following the method of Hodege and Hofreiter (1962). Carbohydrates were first hydrolyzed into simple sugars using dilute hydrochloric acid. In hot acid medium glucose was dehydrated to hydroxy methyl furfurate. This compound formed a green coloured product on addition of anthrone with an absorption maximum at 630 nm.

100 mg of the sample (leaf and root powder of *W. somnifera*) was weighed into a boiling tube. It was hydrolysed by keeping in a boiling water bath for three hours with 5 ml of 2.5 N HCl and cooled to room temperature. It was neutralized with solid sodium carbonate until the effervescence ceases. The volume was made upto 100 ml and centrifuged. The supernatant was collected and 0.5, 1 ml aliquots were taken for analysis. The volume was made upto 1 ml in all tubes by adding distilled water. Then 4 ml of anthrone reagent was added to each tube and kept in a boiling water bath for 8 minutes. The tubes were cooled rapidly and the green to dark green colour was read at 630 nm. Standard graph was prepared by plotting concentration of the standard on the X-axis versus absorbance on the Y-axis. From the graph, the amount of carbohydrate present in the sample tube was calculated, using the following formula.

\[
\text{Total carbohydrate} = \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100
\]

3.3.3.2. Estimation of Protein (Lowry et al., 1951)

The carbamyl group of protein molecules reacts with copper and potassium of the Lowry’s reagent to give a blue coloured complex, together with tyrosine and phenolic compounds present in the proteins reduces the phosphomolybdic, phosphotungstate compounds in the Folin’s coicalteau reagent.
500 mg of each sample of leaf and root of *W. somnifera* was homogenized thoroughly with 5 ml of distilled water and centrifuged at 5000 rpm for 10 minutes. The supernatant was collected and cooled, to which 5 ml of 10 per cent TCA was added and centrifuged at 3,000 rpm for 5 minutes. The precipitated pellet was dissolved in 3 ml of 0.1N NaOH and the total soluble protein content was determined as follows.

0.2 ml of the extract was taken and made upto 1 ml with distilled water. To this 5 ml of 0.1 per cent CuSO₄ and 2.5 ml of 12.5 per cent Na₂CO₃ was added and allowed to stand for 10 minutes. Then 0.5 ml of Folin-Ciocalteau reagent (25%) was added. Similarly blank and standard were prepared. The intensity of the blue colour developed was colorimetrically read at 660 nm. Protein content was expressed as mg protein per gram fresh weight of the sample. This was calculated by plotting OD values on the graph using BSA (Bovine serum albumin) as standard.

**3.3.3.3. Alkaloids (Harborne, 1973)**

5 gram of sample (leaf and root power of *W. somnifera*) was weighed and taken into a 250 ml beaker. 200 ml of 10 per cent acetic acid in ethanol was added, covered with aluminium foil and allowed to stand for 4 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 dropwise to the extract until the completion of precipitation. The whole solution was allowed to settle and the precipitate was collected and washed with dilute ammonium hydroxide and then filtered. The alkaloid residue was dried and weighed.

**3.3.3.4. Flavonoids (Bohm and Kocipai-Abyazan, 1974)**

10 gm of plant sample (leaf and root of *W. somnifera*) was extracted repeatedly with 100 ml of 80 per cent aqueous methanol at room temperature. The whole solution was filtered through Whatman No.42 filter paper. The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to constant weight.
3.3.3.5. **Tannins (Van-Burden and Robinson, 1981)**

500 mg of the sample (leaf and root of *W. somnifera*) was weighed and transferred to a 100 ml plastic bottle. 50 ml of distilled water was added and shaken for 1 hour in a mechanical shaker. This was filtered into a 50 ml volumetric flask and made upto the mark. Then 5 ml of the filterate was pipetted out into a test tube and mixed with 2 ml of 0.1 M FeCl₃ in 0.1N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 mts.

3.3.3.6. **Phenols (Malick and Singh, 1980)**

The powdered sample was boiled with 50 ml of ether for the extraction of the phenolic components for 15 mints. 5 ml of the extract was pipetted 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 upto the volume (50 ml) mark and left to react for 3 minutes for colour development. This was measured at 505 nm.

3.3.3.7. **Saponins (Obadoni and Ochuko, 2001)**

The samples (leaf and root of *W. somnifera*) were ground and 20 gm of each was put into conical flask and 100 cm³ of 20 per cent aqueous ethanol was added. The samples were heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 200 ml ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethylether was added and shaken vigorously. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 ml of n-butanol was added. The combined n-butanol extracts were washed twice with 10 ml of 5 per cent aqueous sodium chloride. The remaining solution was heated in water bath. After evaporation the samples were dried in the hot air oven to constant weight; the saponin content was calculated and represented in terms of percentage.
3.3.3.8. Phytosterols (Malick and Singh, 1980)

0.5 gm of the dried samples (root and leaf of *W. somnifera*) was hydrolyzed with 10 ml of concentrated HCl. Then samples were extracted three times with 10 ml of chloroform. The chloroform was evaporated to obtain the residue. The residue was weighed and dissolved in glacial acetic acid to obtain a solution of 10 mg/ml. To this 5 ml of acetic anhydride and concentrated sulphuric acid was added and kept in dark for 20 minutes. The colour developed was read on spectrophotometer at 540 nm. The values were compared with the calibration curve of the standard cholesterol (Himedia).

3.4. Pharmacology – Nephrotoxicity

**Animals:** Male albino rats weighing about 180-220 g were obtained from the Indian Institute of Science, Bangalore, India. The animals were housed in polypropylene cages and maintained in the controlled temperature with 12 hours period of light and dark and fed with standard rat feed food and water was provided *ad libitum*.

**Chemicals**

TAB, DTNB, GSH was purchased from Sigma chemical and all other reagents and chemicals used in this study were of analytical grade with high purity.

**Plant materials and drug preparation**

*W. somnifera* roots and leaves were collected from Papanasam in Thanjavur district were used.

3.4.1. Ethanolic extracts of *W. somnifera* leaf and root

The ethanolic extracts of the roots and leaves of *W. somnifera* were separately prepared according to the method of Hossain *et al.* (1992). 500 g of fresh roots and leaves of *W. somnifera* were dried, powdered and then soaked in 1500 ml of 95 per cent ethanol overnight. After filtration, the residue obtained was again resuspended in equal volume of 95 per cent ethanol for 48 h and filtered again. The above two filtrates were mixed and the solvent was made to evaporate in a rotavapour at 40-50°C under
reduced pressure. An 11% semisolid light yellow material of *W. somnifera* roots and 12% semisolid light greenish yellow materials of leaves obtained were stored at 0-4°C until used.

Known amount of the residual extracts were suspended in distilled water and was orally administered to the animals by gastric incubation using force-feeding needle during the experimental period.

**Induction of kidney damage**

Kidney damage was induced in rats by administering Gentamicin (GM) intraperitoneally at the dose of 100 mg/kg body weight.

**Experimental design**

The animals were grouped as follows and each group contains 6 rats.

**Group I**

Normal animals received standard feed and water *ad libitum*.

**Group II**

Group II received gentamicin (100 mg/kg body weight, ip) for 6 consecutive days along with standard feed and water *ad libitum*.

**Group III**

Group III received GM as group II for 6 days followed by the oral administration of ethanolic extract *W. somnifera* roots (500 mg/kg wt per day for 10 days).

**Group IV**

Group IV received GM as group II for 6 days followed by the oral administration of ethanolic extracts of *W. somnifera* leaves (300 mg/kg wt per day for 10 days).

**Group V**

Group V received only ethanolic extract of *W. somnifera* roots orally (500 mg/kg b.wt per day for 10 days).
Group VI

Group VI received only ethanolic extract of the leaves of *W. somnifera* orally (300 mg/kg b.wt per day for 10 days).

**Collection of Blood and preparation of serum sample**

After the completion of the experimental period the animals were kept under fasting overnight and were sacrificed by cervical dislocation under light ether anesthesia. Blood was collected by cardiac puncture into serum separator tubes. The blood was allowed to clot by standing at room temperature for 30 minutes and the refrigerated for another 30 minutes. The resultant clear part was centrifuged at 3,000 rpm for 10 minutes, then the serum (supernatant) was isolated and stored in refrigerator until required for analysis. The kidney was excised immediately and washed with ice cold physiological saline. A portion of the kidney was then homogenized in 0.1 M Tris HCl buffer (pH 7.4) and the homogenate was used for assaying the activities of SOD, CAT, GPx and also estimate MDA, vitamin E and GSH.

3.4.2. **Estimation of protein**

The serum protein content was determined by the method of Lowery *et al.* (1951) as described in the section (3.3.3.2).

3.4.3. **Estimation of Urea**

Serum urea was estimated by DAM - thiosemicarbazide method (Natelson *et al.*, 1957). 0.1 ml of blood, 3.5 ml of distilled water, 0.2 ml of 10 per cent sodium tungstate and 0.2 ml of 2/3 N sulphuric acid were added. This was centrifuged for 5 minutes. To 2 ml of the supernatant, 3 ml of DAM (1 gram of DAM, 200 mg of thiosemicarbazide and 9 g of sodium chloride were dissolved in 500 ml of distilled water and made upto 1 litre) thiosemicarbazide reagent was added. Blank was set without the sample, adding only the reagents. For standard, 1 ml of standard urea was treated with 3 ml of DAM thiosemicarbazide and 3 ml of acid reagent (10 ml of orthophosphoric acid, 60 ml of sulphuric acid and 10 per cent of aqueous ferric
chloride were added to 800 ml of distilled water and made upto 1 litre). Urea reacted with DAM and thiosemicarbazide ion and developed pink colour. The tubes were kept in a boiling water bath for 15 minutes. Cooled and then the pink colour was read at 250 nm. The blood urea was calculated using the following formula:

\[
\text{Concentration of Urea (mg/dl)} = \frac{\text{O.D of test} - \text{O.D of control}}{\text{O.D of std - O.D of blank}} \times 40
\]

The values are expressed as mg/ml.

### 3.4.4. Estimation of creatinine

The creatinine was measured by the method of alkaline picrate method (Boneses and Jaussky, 1945). 1 ml of the serum was diluted with 3 ml of distilled water and precipitated the protein by adding 2 ml of 5 per cent sodium tungstate (5 gms of sodium tungstate. 2H₂O was dissolved in water and made upto 100 ml) followed by adding 2 ml of 2/3 N H₂SO₄ (1.8 ml of concentrated H₂SO₄ (36 N) was added top to 98.2 ml of distilled water) drop by drop with constant shaking and allowed to stand for 20 minutes and filtered it. 3 ml of the supernatant was taken for the experiment.

1.0 ml of 0.75N Sodium hydroxide (30 gm of sodium hydroxide was dissolved in distilled water and made upto one litre) was added. All the tubes are left for 15 minutes. The tubes were shaken well and the colour developed acid filtrate of creatinine develops orange red colour when treated with picric acid in the presence of strong alkali, the colour is due to the formation of creatinine picrate. The colour depends upon the amount of creatinine present, which was read at 500 nm. The creatinine was quantitatively determined following the formula:

\[
\text{Concentration of creatinine (mg/dl)} = \frac{\text{O.D of test} - \text{O.D of control}}{\text{O.D of std- O.D of blank}} \times 3
\]

The values were expressed as mg/ml.
3.4.5. Estimation of serum alkaline phosphatase

The serum alkaline phosphatase was estimated by King and King’s method (1965). 2 ml of each of the buffered substance was taken in two test tubes and placed in a water bath at 37°C for a few minutes. Then 0.1 ml of the serum was added to the first tube and both the tubes were incubated for fifteen minutes. Then the tubes were removed from the water bath and 0.8 ml of 0.5N sodium hydroxide was added and then 0.1 ml of serum to the second tube alone.

To both of the tubes 1 ml of potassium ferric cyanide and amino antipyrine were added. For the standard 1.1 ml of buffer was taken and to this 1 ml of phenol standard containing 0.01 mg of phenol was added. For the blank, 1.1 ml of buffer and 1 ml of water were added. To both of the tubes, sodium hydroxide, aminoantipyrine (0.6 g of 4-aminoantipyrine was dissolved in 100 ml of distilled water) and ferric cyanide (2.4 of potassium ferrocyanide was dissolved in 100 ml of distilled water) were added as above. In the presence of alkaline oxidizing agents (Potassium Ferricyanide) 4-amino antipyrine gives a red or purple colour with compounds containing a phenolic group. The reaction has been used to determine the phenol produced by the action of phosphatases of disodium phenylphosphate. Colour development is rapid and the colour is stable for atleast an hour of a bright light. The intensity of colour was read at 520 nm. The phenol was quantitatively determined following the formula.

\[
\text{Concentration of ALP (mg/dl)} = \frac{O.D \text{ of test} - O.D \text{ of control}}{O.D \text{ of std} - O.D \text{ of blank}} \times 10
\]

The values are expressed as micromoles of phenol liberate/μg/mg protein.

3.4.6. Estimation of acid phosphatase

The serum acid phosphatase was estimated by King and King’s Method (1965). 2 ml each of buffered substance in two test tubes were placed in a water bath at 37°C for a few minutes. 0.01 ml of serum was added to the first tube and the two tubes were incubated for fifteen minutes. Then the tubes were removed from the bath,
and 0.8 ml of 0.5N sodium hydroxide (4.2 g of sodium bicarbonate was dissolved in 100 ml of distilled water) was added.

To both the tubes 1 ml of aminoantipyrine reagent and 1 ml of potassium ferricyanide were added. For the standard 1.1 ml of buffer was taken and to this 1 ml of phenol standard containing 0.01 mg of phenol was added. For blank, 1.1 ml of buffer and 1 ml water was added. To both the tubes sodium hydroxide, aminoantipyrine and ferricyanide were added. The presence of oxidizing agent potassium ferricyanide and forms an orange-red coloured complex which was measured colorimetrically. The colour intensity was read at 520 nm.

\[
\text{Concentration of ACP (mg/dl)} = \frac{\text{O.D of test - O.D of control}}{\text{O.D of std - O.D of blank}} \times 5
\]

3.4.7. Estimation of serum lactate dehydrogenase

Serum LDH was determined by the following method of King and King’s (1965) by measuring pyruvate formation with 2, 4 dinitrophenyl hydrazine reagent. For each sample 2 tubes were labeled as ‘Test’ and ‘Control’. In to both tubes 1.0 ml each of buffered substance was taken and 0.1 ml diluted serum was pipetted out and taken in to the ‘Test’ tube and 0.2 ml of distilled water was added to the ‘control’ tube and both the tubes were placed in the water bath at 37°C for 3 minutes. Then 0.2 ml NAD solution was added to the ‘Test’ tube and both the tubes were incubated at 37°C for 15 minutes. Then 1.0 ml of 2, 4-DNPN was added to each tube, mixed and incubated for 15 minutes at 37°C. Tubes were removed from the water bath and 10 ml of 0.4 N NaOH was added to each tube, mixed and read the absorbance at 510 nm after 10 minutes. For standard, 1.0 ml of 2, 4-DNPH was added to all tubes and incubated for 15 minutes at 37°C. 10 ml of 0.4N sodium hydroxide was added to the ‘Test’ and ‘Control’. 2, 4-DNPH produced with pyruvate gives a brown colour in alkaline solution which is proportional to the concentration of pyruvate. The absorbance was read at 510 nm.

\[
\text{Concentration of LDH (mg/dl)} = \frac{\text{O.D of test - O.D of control}}{\text{O.D of std - O.D of blank}} \times 3
\]

The values are expressed as micromoles of pyruvate liberated min / mg protein.
3.4.8. **Estimation of tissue TBARS**

Lipid peroxidation (TBARS) in tissue was estimated by the method of Ohkawa et al. (1979). 0.2 ml of tissue homogenate, 0.2 ml of 8.1 per cent sodium dodecyl sulfate and 1.5 ml of 20 per cent acetic acid were added. The pH was adjusted to 3.5 with sodium hydroxide. Then 1.5 ml of 0.8 per cent aqueous solution of TBA was added to the mixture and the volume was made up to 4 ml with distilled water. The reaction mixture was heated in an oil bath at 95°C for 60 minutes. After cooling by tap water, 1 ml of distilled water and 5 ml of n-butanol pyridine mixture (n-butanol and pyridine (5:1 v/v) were added and shaken vigorously. After centrifugation at 4000 rpm for 10 minutes, the organic layer was removed and absorbance was read at 535 nm.

\[
\text{Concentration of TBARS (mg/dl) = } \frac{\text{O.D of test} - \text{O.D of control}}{\text{O.D of std} - \text{O.D of blank}} \times 5
\]

The values are expressed as µ mol/mg protein.

3.4.9. **Estimation of superoxide dismutase (SOD)**

Superoxide dismutase activity was assayed by the method of Kakkar et al. (1984). 0.5 ml of the tissue homogenate was diluted to 1.0 ml with ice-cold water, followed by 2.5 ml ethanol and 1.5 ml chloroform (chilled reagents). This mixture was shaken for 60 seconds at 4°C and then centrifuged. The enzyme activity in the supernatant was determined as described below.

The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of PMS and 0.3 ml of NBT and appropriately diluted enzyme preparation in a total volume of 3 ml. The reaction was started by the addition of 0.2 ml NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and the butanol layer was separated. The colour intensity of the chromogen in butanol layer was measured in a colorimeter at 520 nm. A system devoid of enzyme served as
control. One unit of enzyme activity is defined as the enzyme concentration, which gives 50 per cent inhibition of NBT reduction in one minute under assay conditions. Superoxide dismutase activity was expressed as U/mg protein for tissues. The amount of enzyme required to inhibit 50 per cent nitroblue tetrazolium (NBT) reduction).

3.4.10. Estimation of catalase (CAT)

The activity of catalase was assayed by the method of Sinha (1972). To 0.9 ml phosphate buffer, 0.1 ml of tissue homogenate and 0.4 ml H₂O₂ were added. The reaction was arrested after 15, 30, 45 and 60 seconds by adding 2.0 ml of dichromate-acetic acid mixture (Dichromate-acetic acid reagent: Potassium dichromate and glacial acetic acid were mixed in the ratio of 1:3. From this 1 ml was diluted again with 4 ml of acetic acid). The tubes were kept in a boiling water bath for 10 minutes, cooled and the colour developed was read at 530 nm. Standards in the concentration range of 201-100 μmoles were processed. The values are expressed as micromoles of H₂O₂ consumed min / mg protein.

3.4.11. Estimation of Glutathione Peroxidase (GPx)

The activity of glutathione peroxidase was determined by the method of Beutler and Kelley (1963) with modifications. The reaction mixture in a total volume of 1 ml-contained 0.2 ml of phosphate buffer, 0.2 ml EDTA, 0.1 ml of sodium azide and 0.5 ml of the enzyme preparation (tissue homogenate). 0.2 ml of glutathione and 0.1 ml H₂O₂ were added, to this mixture and incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5 ml of 10 per cent TCA. The tubes were centrifuged and the supernatant was assayed for GSH by the method of Beutler and Kelley (1963). A blank was treated similarly to which 0.2 ml of the enzyme was added after the incubation. The values are expressed as micromoles of GSH consumed / min / mg protein.

3.4.12. Estimation of reduced glutathione (GSH)

The reduced glutathione level was determined by the method of Beutler and Kelley (1963). 0.2 ml of the sample (tissue homogenate) was mixed with 1.8 ml of
EDTA solution. To this 3.0 ml of precipitating reagent was added, mixed thoroughly and kept for 5 minutes before centrifugation. To 2.0 ml of the filtrate, 4.0 ml of 0.3 M disodium hydrogen phosphate solution and 1.0 ml of DTNB reagent (5, 5′-Dithio (bis)-2-nitrobenzoic acid (DTNB) reagent: 40 mg of DTNB in 100 ml of 1% sodium citrate) were added, development of yellow colour when 5, 5′-dithio-bis-2-nitrobenzoic acid (DTNB) is added to compound containing sulphhydryl groups and it was read at 412 nm. A set of standard solutions containing 20-100 µg of reduced glutathione was treated similarly. The values are expressed as micro moles/mg protein.

3.4.13. **Estimation of Vitamin E**

The concentration of total Vitamin E in tissues was estimated by the method of Klein *et al.* (1991). The lipid extracts were dried under liquid nitrogen gas and the residues were suspended in 66 per cent ethanol and then 4 ml of hexane and 0.6 ml of 60% H₂SO₄ were added. The tubes were vortexed and centrifuged. The upper hexane phase was removed and its fluorescence intensity was measured at an excitation of 295 nm and emission of 320 nm. The standard α-tocopherol was used to prepare the standard graph. The values are expressed as microgram / mg protein.

3.4.14. **Statistical analysis**

The data are expressed as Mean ± SD. The statistical comparisons were performed by Two-way Analysis of Variance (ANOVA) followed by student’s *t*-test. The null hypothesis was rejected for *p*<0.05.

3.5. **Histopathology**

Staining of tissue sections was made by using hematoxylin and eosin. The staining of tissue sections was made following by the method of Oche and Kolhatkar (2000). Hematoxylin and eosin are the principle stains used for the demonstration of nucleus and the cytoplasmic inclusions. Alum acts as a modrant and hematoxylin containing alum strains the nucleus light blue which turns red in the presence of acid. The cell differentiation was achieved by treating the tissue with acid solution. The counter staining was performed by using eosin solution which imparts pink colour to cytoplasm.
Reagents

1. Harriss Hematoxylin Stain
   a) 1 g of Hematoxylin was dissolved in about 10 ml 95% (v/v) ethanol in a mortar with a pestle.
   b) 20 g of ammonium (or) potassium alum was dissolved in 200 ml of hot distilled water.
   c) Solutions ‘a’ and ‘b’ were mixed while hot and bring quickly to boil with constant stirring.
   d) Then 0.5 g of mercuric oxide was added the flame was removed and cooled as rapidly as possible with use of running tap water.
   e) It was then filtered and stored in an amber coloured bottle at room temperature for further use.

2. Eosin Solution
   a) 1.0 g of yellow eosin was dissolved in about 80 ml of distilled water.
   b) Then 320 ml of 98% (v/v) ethanol was added.
   c) 0.4 ml of glacial acetic acid was added. This solution was labelled and stored at room temperature for further use.
   d) 0.5 % (v/v) hydrochloric acid.
   e) Dilute ammonia water.
      1.5 ml of 28% (v/v) strong ammonia solution was added in distilled water and made up to final volume of 1500 ml with distilled water.

3.5.1. Staining procedure
1. Deparaffinize the section
   The slide was flamed on a burner and then placed in xylene for 2 to 4 minutes. The xylene treatment was repeated with agitation.

2. Take the section of water (Hydration)
   The section was hydrated by passing it through decreasing concentration of alcohol baths and water. The alcohol solutions used were 100% (absolute alcohol),
90%, 80% and 70% and the sections were placed for 30 to 60 seconds in each alcohol solution. Washed in tap water and rinsed with distilled water. The section was drained well before staining.

3) Staining

The section was stained with hematoxylin solution for 3 to 5 minutes and washed in running water. Then the slide was quickly dipped in 0.5% (v/v) hydrochloric acid (differentiation was checked by using a microscope - The nuclei appeared dark purple and the rest of the tissue appear pale). The slide was quickly rinsed in tap water for 30 to 60 minutes. Then dipped several times in dilute ammonia water (section became blue coloured ones). Then it was washed in tap water and rinsed in 95% alcohol. The slide was agitated in eosin solution for 10 to 60 seconds, and the staining solution was drained off.

4. Dehydration

Dehydration of the slide was made by passing it over the couplin jars containing progressively increasing concentration of alcohol viz., 70, 90% and absolute alcohol, and keeping the slide in each concentration for 30 – 60 seconds. Two changes of absolute alcohol were made at an interval of 30 – 60 seconds.

5. Clearing

It is the process of removal of alcohol, which was made by placing the slide twice in xylene for 30 to 60 seconds each.

6. Mounting

The excess xylene was drained and mounted with DPX or Canada balsam mountant with a coverslip.

3.6. Antibacterial activity studies

Leaves and roots of the W. somnifera were collected wild from grown plants in Papanasam, Thanjavur district were used.

3.6.1. Preparation of extract (Prakash et al., 1975)

The powdered plant materials were successively extracted (10 g each) with methanol, chloroform and ethanol (60°C) using Soxhlet apparatus. The extraction was continued for 24 hours and filtered through Whatman No.1 filter paper and kept in hot
air oven at 40°C for 24 hours to evaporate the solvents from it. Aqueous extract was centrifuged at 5,000 rpm and then the supernatant was taken and dried.

### 3.6.2. Microorganisms

Gram negative bacterial strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis* and gram positive bacteria *Staphylococcus aureus* and *Streptococcus mutans* were used as test organisms. The bacterial strains were obtained from MTCC, Chandigarh, India.

<table>
<thead>
<tr>
<th>Bacterial species</th>
<th>Strain Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>MTCC – 433</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>MTCC – 109</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>MTCC – 733</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>MTCC – 425</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>MTCC – 096</td>
</tr>
<tr>
<td><em>Streptococcus mutans</em></td>
<td>MTCC – 899</td>
</tr>
</tbody>
</table>

### 3.6.3. Culture medium

The media used for antibacterial test was nutrient agar / broth.

#### 3.6.3.1. Composition of Nutrient Agar Medium

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.0 gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 gm</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

After mixing the ingredients into the distilled water it was melted in the water bath and sterilized by autoclaving at 15 lbs of 121°C for 15 minutes.
3.6.4 **Inoculum preparation**

Bacteria were inoculated into nutrient broth (liquid medium) and incubated at 37°C for 24 hours and the propagule density was checked for approximately $10^5$ CFU/ml by turbidometric method (Senior *et al.*, 1995).

3.6.5. **Determination of antibacterial activity (Bauer *et al.*, 1966)**

The antibacterial activity of the plant extract was determined by disc diffusion method. The 24 hours bacterial culture was diluted ($10^5$ CFU/ml) and 0.2 ml was spread over nutrient agar plates using sterile glass L-rod.

Sterile Whatman No.1 filter paper discs (6.0 mm in diameter) were used for the experiment. The discs were impregnated with plant extracts and stored at 5°C prior to use. Extract impregnated discs were placed on agar medium in the laminar flow and incubated at 37°C for 48 hours. Antibacterial activities were determined by measuring the clear zones of inhibition (MIC) formed around the discs.

Standard antibiotic chloramphenicol (30 µg/disc) (Span Diagnostics Ltd., Surat, India) was used as reference or positive control. The antibacterial activity of plant extracts was compared with standard antibiotic chloromphenicol.

3.7. **UV-Visible spectroscopy**

Ultraviolet-Visible spectroscopy was an analytical technique used to identify some functional groups in molecules, was used in the structural elucidation in biomolecules present in the leaf and root extracts of *W. somnifera*.

Plant samples (leaf and root powder) were used in solution (ethanol) and were placed in a small silica cell. Two lamps were used (hydrogen or deuterium lamp for the ultraviolet region and a halogen lamp for the visible region). In this way radiation across the whole range was scanned by the spectrometer. A reference cell containing only solvent (ethanol) was used and light was passed simultaneously through the sample cell and reference cell. The spectrometer compared the light passing through
the sample with that passing through the reference cell. The transmitted radiation was
detected and the spectrometer recorded the absorption spectrum by scanning the wave
length (254nm) of the light passing through the cell.

3.8. **Infrared spectroscopy (IR)**

Infrared spectroscopy is a well developed technique to identify chemical
compounds. The plant substance was measured in an automatic recording IR
spectrophotometer (Perkin Elemer – FT-IR-Spectrophotometer) as a mull with nujol
oil or in the solid state, mixed with potassium bromide (KBr). In the later case, a thin
disc was prepared under anhydrous conditions from a powder containing about 1 mg
of material and 10-100 mg potassium bromide, using a mould and press. The range of
measurement was from 4000 to 667cm⁻¹ (2.5 to 15 µm) and the spectrum was recorded
within three minutes.

3.9. **Gas Chromatography – Mass Spectroscopy (GC-MS) analysis**

*(Ivanova et al., 2002)*

**Sample preparation**

The powdered sample (20 g) was soaked and dissolved in 75 ml of ethanol for
24 hrs. Then the filtrates were collected and evaporated under liquid nitrogen.

The GC-MS analysis was carried out using a Clarus 500 Perkin-Elmer (Auto
system XL) Gas Chromatograph equipped and coupled to a Mass detector Turbo mass
gold-Perking Elmer Turbomas 5.1spectrometer with an Elite-1 (100% Dimethyl1 ply
siloxane), 30 m x 0.25 mm ID x 1 µm df capillary column. The instrument was set to
an initial temperature of 110°C, and maintained at this temperature for 2 min. At the
end of this period, the oven temperature was raised up to 280°C, at the rate of an
increase of 5°C/min, and maintained for 9 min. Injection port temperature was ensured
as 250°C and Helium flow rate as 1 ml/min. The ionization voltage was 70eV. The
samples were injected in split mode as 10:1. Mass spectral scan range was set at
25-400 mhz.
The chemical constituents were identified by GC-MS. The fragmentation patterns of mass spectra were compared with those stored in the spectrometer database using National Institute of Standards and Technology - Mass Spectral database (NIST-MS). The percentage of each component was calculated from the relative peak area of each component in the chromatogram.

3.10. Tissue culture

3.10.1. Collection of plant materials

Berries of *W. somnifera*, grown in wild were collected from Thanjavur, Tiruchirapalli, Thiruvarur, Nagapattinam and Cuddalore districts. Collected berries were sun dried and the seeds were packed and maintained at room temperature 31°C. Then the seeds were used to raise seedlings in the Botanical Research Garden, Department of Botany, A.V.V.M. Sri Pushpam College, Poondi, Thanjavur district, Tamil Nadu, India.

3.10.2. Seed germination and seedling development

**Sterilization of seeds (Kulkarni et al., 1999)**

Seeds of *W. somnifera* were collected from the natural populations of study sites. The healthy and uniform seeds were washed thoroughly in running tap water to remove the dust and other particulate matter adhering on the surface of the seeds. They were then washed with distilled water, and soaked in teepol solution 2% for 10 minutes. Teepol was poured off and the seeds were washed 3-4 times in distilled water. The washed seeds were transferred to inoculation chamber and surface sterilized with 0.1% mercuric chloride solution for 5-7 minutes. Then the solution was decanted and the seeds were washed thoroughly in sterilized distilled water to ensure that the last traces of mercuric chloride were removed. The excess of water was removed by using sterilized filter paper. Then the sterilized seeds were used for inoculation.
3.10.3. Effect of presoaking seed treatment on germination

(Sen and Sharma, 1991)

Seeds of W. somnifera were collected from wild grown plants and then dried and stored in the polythene bags at room temperature (31°C). Screening of 100 seeds showed 80 per cent of healthy and 20 per cent of shrunken and damaged seeds. Healthy seeds were used for further treatments. For germination tests, air dried seeds were disinfected with 0.1 per cent HgCl₂ (5-7 minutes) and washed thoroughly and soaked in sterile distilled water for 24 hours and then used for various pretreatments.

Pre-treatment solutions

1. Sulphuric acid 2 and 5% solutions – 2 minutes
2. GA₃ 25 μg/l – 15 minutes.
4. Control was maintained using sterile distilled water.

The treated seeds were washed at least for 3 times in sterile distilled water and were placed in boiling tubes containing sterile moist cotton and sealed. The cotton in the tubes was moistened daily using sterile distilled water. Seeds were considered germinated only when there was the emergence of radicle. The first germination was observed after 3 weeks. Germination was obtained up to nine weeks. The mean germination time was calculated by using the formula: where ‘X’ is the number of newly germinated seeds on each day and ‘f’ is the number of days after seeds was collected to germinate. The germinated seedlings were also incubated under laboratory condition (Nichols and Heydecker, 1968).

3.10.4  *In vitro* culture of *Withania somnifera* (L.) Dunal

3.10.4.1. Selection and sterilization of the explants

The explants such as leaf, internode, shoot tip, axillary bud and node were excised from the well grown plants and washed with running tap water for 30 minutes followed by treatment with an aqueous solution of 2% (v/v) Teepol (Reckitt Benckiser, India) and 70% (v/v) ethanol for 15 seconds and washed with sterile...
distilled water for three to five times. The explants were then disinfected with 0.1% (w/v) aqueous mercuric chloride (HgCl₂) solution for a period of 5 minutes and finally rinsed with autoclaved distilled water (five to seven changes). The disinfected plant segments were then trimmed at both ends prior to inoculation on culture media.

3.10.4.2. Culture medium

Murashige and Skoog (1962) (MS) medium consisting of agar (0.8%) and sucrose (3%) was used (Table-1). Various growth regulators viz., BAP, 2, 4-D, NAA, IAA, IBA, KN, GA₃ were fortified according to the nature of ensuing experiments. The pH of the medium was adjusted to 5.8 with the help of 0.1 N NaOH and 0.1 N HCl before autoclaving at 121°C for 15 minutes.

3.10.4.3 Preparation of stock for growth regulators

Auxins such as NAA (50 mg), IAA (50 mg) and IBA (50 mg) were dissolved separately in 0.1 N NaOH, diluted with distilled water and made up to 200 ml of stock solution. Similarly 2, 4-D (50 mg) was dissolved in ethanol and diluted with distilled water and made up to 200 ml of stock solution. Cytokinins such as Kinetin (50 mg), BAP (50 mg) and GA₃ (50 mg) were dissolved separately in 0.1 N NaOH, diluted with distilled water and made up to 200 ml of stock solution. All these growth regulators were stored in the refrigerator. From this every 4 ml has 1.0 mg/l of hormone in the stock solution.

3.10.4.4. Preparation of stock solutions for nutrients

The macronutrients were dissolved in 500 ml of double distilled water. Micronutrients were dissolved in 250 ml of double distilled water KI was dissolved in 250 ml of double distilled water. Minor nutrients were dissolved in 500 ml of double distilled water. Na₂ EDTA and FeSO₄ were dissolved separately in 100 ml of double distilled water. Na₂ EDTA solution was boiled separately and then slowly added with the FeSO₄ solution and made up to 250 ml with double distilled water. Vitamins were dissolved separately in 100 ml of double distilled water.
Table 1 Composition of MS medium and B5 vitamins

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Stock solution weight in g/vol.</th>
<th>Volume of stock solution taken for one litre medium</th>
<th>mg/litre of medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>3.700</td>
<td></td>
<td>370</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>4.400</td>
<td></td>
<td>440</td>
</tr>
<tr>
<td>KNO₃</td>
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<td>1900</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>16.500</td>
<td></td>
<td>1650</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.700</td>
<td></td>
<td>170</td>
</tr>
<tr>
<td><strong>Micronutrients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MnSO₄.7H₂O</td>
<td>2.230</td>
<td></td>
<td>22.3</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>0.860</td>
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<tr>
<td>H₃BO₃</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Minornutrients</strong></td>
<td></td>
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<td></td>
</tr>
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<td>Na₂MoO₄.7H₂O</td>
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<td></td>
<td>0.25</td>
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<tr>
<td>CuSO₄.5H₂O</td>
<td>0.012</td>
<td>1.0 ml</td>
<td>0.025</td>
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<tr>
<td>CaCl₂</td>
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<td></td>
<td>0.025</td>
</tr>
<tr>
<td>KI</td>
<td>0.083</td>
<td>2.5 ml</td>
<td>0.83</td>
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<tr>
<td><strong>Iron</strong></td>
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<tr>
<td>Na₂EDTA</td>
<td>1.8625</td>
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<tr>
<td>FeSO₄</td>
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</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiamine -HCl</td>
<td>10 mg</td>
<td></td>
<td>0.1</td>
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<tr>
<td>Pyridoxine-HCl</td>
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<td>0.5</td>
</tr>
<tr>
<td>Nicotinic acid</td>
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<td>0.5</td>
</tr>
<tr>
<td>Glycine</td>
<td>200 mg</td>
<td></td>
<td>2.0</td>
</tr>
<tr>
<td>Meso-inositol</td>
<td></td>
<td></td>
<td>100 mg</td>
</tr>
<tr>
<td>Sucrose</td>
<td></td>
<td></td>
<td>30 g</td>
</tr>
<tr>
<td>Agar</td>
<td></td>
<td></td>
<td>8 g</td>
</tr>
</tbody>
</table>
Steps involved in preparation of MS medium

i) Stock solution of macro, micro and minor elements including iron and vitamins were prepared by dissolving adequate quantities of each element (Murashige and Skoog, 1962). Analytical grade of chemicals and double distilled water were used in all the preparation.

ii) Required quantities of agar was weighed and melted in double distilled water with the help of water bath.

iii) Appropriate quantities of various stock solutions sucrose, meso-inositol and growth regulators were added and mixed thoroughly. Final volume was made upto 1000 ml using double distilled water.

iv) The pH was adjusted to 5-8 using 0.1 N NaOH and 0.1 N HCl, before the agar was added.

v) The medium was poured into culture tubes (about 20 ml of the medium in each culture tube) and plugged in the sterile non absorbant cotton plugs wrapped with cheese cloth and sterilized by autoclaving at 121°C and 15 psi for 15 minutes.

3.10.4.5. Inoculation of explants

Shoot tip, axillary bud and nodal explant (1 cm size) were inoculated in different culture tubes in vertical position on the medium while other explants like internode were placed in horizontal position. The leaf explants (1 x 1 cm) were cultured with their adaxial and abaxial surface touching the medium.

3.10.4.6 Cultural conditions

Single disinfected cotyledonary nodal segments were cultured on MS basal medium supplemented with 3% (w/v) sucrose (Hi Media, India) and 0.8% (w/v) agar for culture initiation and the served as sources of explants for subsequent experiments. The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.8 with 1 N NaOH or 1 N HCl before the addition of 0.8% (w/v) agar (Himedia, India). In all the experiments, the chemicals used were analytical grade (Himedia, Kelco, Merk and Sigma). The medium was dispensed into culture vessels (Borosil,
India) and autoclaved at 105 Kpa (121°C) for 15 minutes. The surface disinfected explants were implanted vertically on the culture medium (test tubes (150 x 25 mm) containing 15 ml medium) and plugged tightly with non-absorbent cotton. All the inoculated tubes were incubated at 25 ± 2°C with 16 hr photoperiod of 45-50 μmol/m² s⁻¹ irradiance, as provided by cool white fluorescent tubes (1500 lux, Philips, India) and with 55-60% relative humidity (RH). All the subsequent subcultures were made at four weeks intervals.

3.10.5 Micropropagation

3.10.5.1. Direct organogenesis

Based on the preliminary experiments the culture medium was supplemented with appropriate growth regulators for different growth response. Leaf, internode, nodal, shoot tip and axillary bud explants were cultured on MS basal medium containing sucrose 3% (w/v) agar 0.8% supplemented with different cytokinins (0.5 to 3.0 mg/l) such as BAP and KN, were used either individually or in combination with NAA (0.5 mg/l).

3.10.5.1.1. Shoot multiplication

The tender leaves were taken from 2 months old seedlings and the mature leaves were excluded. Young leaves with optimum size of 15 to 30 mm length and 20 to 25 mm width were selected and cut length wise into 1 x 1 cm size with a portion of midrib. They were inoculated into the culture tubes containing MS Medium supplemented with different combinations BAP, KN and NAA and sucrose (3% w/v) for shoot multiplication. Care was taken to seen that the explant was in contact with medium either by the adaxial or abaxial side. Subsequent subcultures were made at periodic intervals of 25 to 30 days. Subculture media were formulated corresponding to organogenesis.

Shoot tip, internode, axillary bud and nodal segments were also inoculated in the same manner. The cultures were incubated at 25 ± 2°C with a photoperiod of 16
hours and 2000 lux light intensity. Number of multiple shoot were excised in different explants inoculated.

3.10.5.2. **Indirect organogenesis**

Explants *viz.*, leaf, node, internode, shoot tip and axillary buds were excised from *in vitro* germinated young 2 months old plants and inoculated on MS medium supplemented with various growth regulators such as BAP, KN, 2,4-D and NAA (0.5 mg/l) for indirect organogenesis.

3.10.5.2.1. **Callus induction**

For the induction of callus, *in vitro* raised different explants were used. These explants were inoculated in MS basal medium with B₅ vitamins and supplemented with different concentrations and combinations of BAP (0.5 mg to 2.5 mg/l), 2, 4-D (0.5 to 2.5 mg/l), KN (0.5 mg to 2 mg/l) and NAA (0.5 mg/l) for callus induction.

3.10.5.2.2. **Proliferation of shootbud and shoot multiplication**

MS basal medium and 3 per cent sucrose, supplemented with BAP (0.5 to 3 mg/l) and NAA (0.5 mg/l) were used for initiation of shoot buds. 3 weeks grown calli were used for proliferation and shoot induction. The regenerated buds were excised from the calli and subcultured on shoot multiplication medium with same concentration of growth regulators.

3.10.5.3. **Shoot elongation**

The multiple shoots (2 to 3 cm) were excised and sub cultured on shoot elongation medium supplemented with different concentrations of BAP (2.5 mg/l), KN (1.5 mg/l) and GA₃ (0.5 to 3.0 mg/l) under cultured conditions.

3.10.5.4. **Rooting and Hardening (Gita Rani et al., 2003)**

Multiple shoots of 6 to 7 cm in length after three weeks were excised and transferred to the rooting medium containing both IAA and IBA (0.5 to 2.5 mg/l) in different concentrations. After 30 days of culture the well rooted plantlets were
removed from culture tubes and washed thoroughly with running tap water to remove the traces of agar medium and treated with 0.1 per cent bavistin (fungicide) by immersion and planted in small plastic cups containing sterilized vermiculite and nourished with half strength MS basal liquid medium. The plastic cups were covered with transparent polythene bags with punch holes to maintain humidity.

Fifty per cent of the plantlets were maintained under culture room. The remaining plantlets were placed under shade with short exposure (2-3 hr/day) to sunlight every day for acclimatization. The well grown plants from both conditions were used for further studies.

3.10.5.5. **Hardening with bioinoculants (Senthilkumar et al., 2005)**

The well grown plants from both conditions were transferred to larger plastic pots filled with a mixture of garden soil, sand and farmyard manure at 2:1:1 ratio.

After three weeks, the hardened and acclimatized plantlets were transferred to larger pots with the application of different bioinoculants such as *Azospirillum*, Phosphobacteria and arbuscular mycorrhizal fungi. The biofertilizers as carrier based inocula were procured from the Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Pot culture experiment was conducted at the Botanical Garden, A.V.V.M. Sri Pushpam College, Poondi, Thanjavur to study the effect of combined inoculation of rhizomicroorganisms on the growth, yield and quality of *W. somnifera*. The rhizobacterial isolates viz., *Azospirillum brasilense*, Phosphobacteria (*Bacillus subtilis* and *Pseudomonas striata*) and AM fungi (*Glomus fasciculatum* and *G. mosseae*) were used as carrier based inoculants. The pots were filled with potting mixture (soil + sand and FYM; 2:1:1 ratio) and the bioinoculants treated with basal dose *Azospirillum* (2 gm/pot), Phosphobacteria (2 gm/pot) and VAM (2.5 gm/pot)).
In the pot culture experiment, effect of individual and combined inoculation of different plant growth promoting rhizomicroorganisms (PGPR) on growth, yield and quality of *W. somnifera* was analysed.

**Treatments**

T<sub>1</sub> – *Azospirillum*

T<sub>2</sub> – Phosphobacteria

T<sub>3</sub> – Arbuscular mycorrhizal fungi

T<sub>4</sub> – *Azospirillum* + Phosphobacteria

T<sub>5</sub> – *Azospirillum* + Phosphobacteria + Arbuscular mycorrhizal fungi

T<sub>6</sub> – Uninoculated (control).

The biofertilizers were inoculated in the pots containing garden soil, farm yard manure and sand in 2:1:1 ratio.

3.11. **Agrobacterium** mediated genetic transformations

3.11.1. Plant material and explant preparation

Leaf, shoot tip and nodal segments from 60 days old *in vitro* germinated seedling were used as the source of explant. The explants were excised and used for transformation studies.

3.11.2. Sensitivity of explants to Kanamycin

The sensitivity tests of different selection agents were carried out in order to find out the inhibitory concentration which arrest the growth of the explants. The sensitivity of leaf, nodal and shoot tip explants to kanamycin was determined by culturing the explants in shoot induction medium [MS basal + B<sub>5</sub> Vitamins + BAP (2.0 mg/l) + KN (1.5 mg/l) + NAA (0.5 mg/l)] along with kanamycin (Sigma, USA) at concentrations ranging from 50 mg/l to 300 mg/l. The minimum inhibitory concentration of the selection marker was used throughout the selection procedure of transformed shoots from explants. A positive control without selection agent was also maintained.
3.11.3. **Bacterial strains and plasmid vector**

Agrobacterium tumefaciens strain EHA105 and Agrobacterium rhizogenes LBA 9402 were used for transformation. The strain EHA 105 containing the binary vector PGA492GL carrying nptII gene regulated by nos promoter and ‘bar’ and ‘gus’ (uid A) genes regulated by CaMV355 promoter. The ‘gus’ has an intron in the N-terminal region of the coding sequence. The ‘bar’ gene confers resistance to phosphinothricin and the npt II gene has resistance to aminoglycosidic antibiotics such as kanamycin and gentamycin. A. rhizogenes a wild-type agropine strain LBA 9402 (PR 1855), was used for the induction of hairy root.

3.11.3.1. **Growth and maintenance of microbial cultures**

Short-term storage of pure culture was achieved by maintaining in the form of single colonies on plates of respective solid medium kept at 4°C. When needed for experimentation, the strain was grown routinely from single colonies on respective liquid medium, in dark and at 200 rpm in an incubator shaker at 28°C. The culture in the log phase of growth (usually grown for 24-48 hours) was used for all the experiments (OD = 1.0 at 600 nm) (Senior *et al*., 1995). Long term storage was achieved by making glycerol stocks of the pure strain and storing it at a temperature of -70°C (Maniatis *et al*., 1982).

3.11.3.2. **Agrobacterium culture**

Single colony of Agrobacterium tumefaciens strain EHA105 and A. rhizogenes strain LBA 9402 suspended in 50 ml of Luria-Bertani (LB) broth containing 50 mg/l kanamycin (Sigma, USA) and 20 mg/l tetracycline (Sigma, USA) (Jouanin, 1984) and incubated at 28°C on a shaker (Orbitek, India) at 200 rpm for 24 hrs. The suspension of the Agrobacterium strain was diluted with half strength MS liquid medium to obtain 1.0 OD (600 nm) concentration (5×10⁸ cells/ml).

3.11.4. **Tissue culture and Transformation**

3.11.4.1. **Washing of co-cultivated explants**

After co-cultivation, the infected explants were washed three times with sterile distilled water containing 300 mg/l cefotaxime followed by washing with hormone
free MS liquid medium containing 300 mg/l cefotaxime for three times with vigorous stirring using sterile forceps, and then blotted dry with sterile filler paper. After washing the co-cultivated explants were subjected to selection. The cultures were maintained under 16 hours photoperiod (30 μmol m^{-2}s^{-1}) at 25 ± 2°C. Then the explants were transferred to MS medium containing BAP (2.5 mg/l), KN (1.5 mg/l), NAA (0.5 mg/l), cefotaxime (300 mg/l) and kanamycin (300 mg/l) for shoot bud induction.

### 3.11.4.2. Selection of transformed cultures

After 3 weeks, the explants with emerging shoots were transferred to MS medium containing BAP (2.5 mg/l), KN (1.5 mg/l), NAA (0.5 mg/l), kanamycin (300 mg/l) and cefotaxime (300 mg/l) for shoot proliferation. Two sub cultures were made on the same medium at 4 weeks interval. The regenerated shoots were excised from the explants and transferred to MS medium supplemented with BAP (2.5 mg/l) KN (1.5 mg/l), NAA (0.5 mg/l), GA₃ (1.5 mg/l), kanamycin (300 mg/l) and cefotaxime (300 mg/l) for shoot elongation. The precultured co-cultivated explants were randomly selected and subjected to the _gus_ assay after 30 days of co-cultivation.

### 3.11.4.3. GUS assay

Transformed leaves, nodal segments and shoots were assayed for the expression of _gus A int_ gene following the histochemical procedure of Jefferson _et al._ (1987). After 30 days, the transformed plant parts like shoot bud, leaf and nodal segments were washed three times using distilled water and incubated for 10 minutes in phosphate buffer (0.5 mM NaH₂PO₄ and 0.5 mM Na₂HPO₄) at pH 7.0 containing 0.5 mM potassium ferric ferro cyanide and 10 mM Na₂EDTA. The buffer was removed and fresh phosphate buffer containing 1 per cent (v/v) Triton X-100 was added to the plant tissues and incubated for 1 hour at 37°C. After draining the solution, fresh phosphate buffer containing 1.0 mM X-glue (5-bromo-4-chloro-3-indolyl β-D glucorinide) and 20 per cent of 95 per cent methanol were added. The reaction mixture was placed under a mild vacuum for 5 minutes and incubated over night at 37°C and then they were examined visually. Following the incubation, the
chlorophyll was removed and fixed in 95 per cent (v/v) ethanol: 1 per cent (v/v) glacial acetic acid.

3.11.4.4. **Confirmation *A. rhizogenes* transformation**

The *A. rhizogenes* co-cultivated explants were confirmed by the formation of hairy root from the leaf, shoot, and nodal segments.

3.11.4.5. **Statistical analysis**

Each treatment consisted of a total of 50 explants and each experiment was repeated three times. The percentage of GUS expression was calculated by the number of explants showing GUS positive expression divided by the number of explants subjected to co-cultivation and multiplied by 100 (Cao *et al.*, 1998).