Materials and Methods

3. MATERIALS AND METHODS

Explants source and Asymbiotic seed germination

The immature capsules of *Pholidota pallida* and *Arundina graminifolia* (naturally pollinated) obtained from the Tropical botanical garden and research institute, palode, Trivandrum were used for Asymbiotic seed germination.

Callus and multiple shoot induction

The 80 days old protocorms (before the emergence of first leaves) of *P.pallida* and *A.graminifolia* were used as explants for callus and multiple shoot induction.

Pseudobulb culture

The well-developed green pseudobulb segments of about 0.5 to 1.0 cm excised from one year old *in vitro* raised pseudobulb was sliced and the segments were used for further studies.

Sterilization

The freshly collected capsules were surface sterilized in sodium hypochlorite solution (NaOCl, 0.6 % w/v) for 10 minutes, rinsed thrice with sterile distilled water, dipped in 70 % (v/v) ethanol for 30 seconds and flamed. Seeds from the surface sterilized capsules were extracted by splitting the capsule longitudinally with a sharp sterilized surgical blade. The seeds were then spread as thin film in the test tube containing 10-15 ml of culture media.

Culture media and initiation of culture

Five different basal media such as Murashige and Skoog (1962) medium, Vacin & went Lindemann orchid medium (1970), BM-1 Terrestrial Orchid Medium and Knudson C medium (1946) were tested to select a suitable medium for Asymbiotic seed germination of *P.pallida* and *A.graminifolia*. KC medium and VW medium with growth regulators such as 2,4-D (2.26, 4.52 and 9.03 µM), IAA (2.9, 5.7 and 11.4 µM) either individually or in combinations with BA (2.22, 4.44 and 8.88 µM) and Kinetin (2.32, 4.64 and 9.29 µM) were used to assess the effect on callus induction, multiple shoots

development and pseudobulb culture. Half strength basal medium without growth regulators was used for the differentiation of callus into complete plantlets.

All the media were supplemented with 3 % (w/v) sucrose and solidified with 0.8% (w/v) agar (Hi media-India). The pH of the media was adjusted to 5.6–5.8 with 1 N NaOH or HCl. Around 10-15 ml of the medium was dispensed into 250 mm X 150 mm culture tubes (Borosil). The mouth of the tubes was covered with aluminium foil and was autoclaved at 1.06 kg pressure for about 20 minutes at 121°C. The autoclaved medium in the culture tubes was cooled and allowed to solidify as slants. The inoculations were done after four days to ensure that the media were free from contamination. Five replicates were used for each treatment and maintained at 25 ± 2°C in culture room under a 12 hrs photoperiod of 50 µmol m^{-2} s^{-1} irradiance provided by white fluorescent tubes and with relative humidity of 70 %. All the experiments were repeated three times with 5 replicates per treatment.

Rooting

For root development, individual shoots were inoculated on half strength liquid basal medium supplemented with 0.044 M sucrose and auxins such as IAA (1.45, 2.9, 5.7, and 11.4 µM), IBA (1.23, 2.46, 4.92, and 9.84 µM) and NAA (1.34, 2.69, 5.38 and 10.76 µM) individually. The micro shoots were supported on filter paper bridges (Whatman's No. 1).

Table 3.1: Chemical composition of the media used in the present study (mg/l)

S. No.	Components	MS	VW	LOM	BM	KC
1.	Aluminium chloride.6H2O	-	-	0.56	-	-
2.	Ammonium nitrate	1650	1650	-	-	-
3.	Ammonium phosphate mono basic	-	-	-	300	-
4.	Ammonium sulphate	-	-	1000	-	500
5.	Boric acid	6.20	6.20	1.01	5.00	-
6.	Calcium chloride.2H2O	440	440	-	200	-

S. No.	Components	MS	VW	LOM	BM	KC
7.	Calcium nitrate	-	-	347.20	-	694.85
8.	Cobalt chloride.6H_2O	0.025	0.025	-	0.10	-
9.	Copper sulphate.5H_2O	0.025	0.025	0.02	0.20	-
10.	Ferric citrate	-	-	4.40	-	-
11.	Ferrous sulphate.7H_2O	27.80	27.80	-	15.00	25.00
12.	Glycine (free base)	2.00	-	2.00	-	-
13.	Magnesium sulphate	180.69	180.69	58.62	195.33	122.09
14.	Manganese sulphate.H_2O	22.30	16.90	0.05	10.00	5.68
15.	Molybdic acid(sodium salt).2H_2O	0.25	0.25	-	0.10	-
16.	Myo-inositol	100	100	100	1000	-
17.	Na_2-EDTA	37.30	37.30	-	20	-
18.	Nickel chloride.6H_2O	-	-	0.03	-	-
19.	Nicotinic acid (free acid)	0.50	-	1.00	5.00	-
20.	Potassium chloride	-	-	1050	-	-
21.	Potassium iodide	0.83	0.83	0.10	1.00	-
22.	Potassium nitrate	1900	1900	-	2500	-
23.	Potassium phosphate monobasic	-	170	135	-	250
24.	Pyridoxine hydrochloride	0.50	-	1.00	0.50	-
25.	Sucrose	30000	30000	30000	30000	30000
26.	Thiamine hydrochloride	0.10	0.40	10	5	-
27.	Zinc sulphate.7H_2O	8.60	8.60	0.57	1	-

MS - Murashige and Skoog (1962) medium, VW – Vacin and went, LOM - Lindemann orchid medium (1970), BM- BM1 Terrestrial Orchid Medium and KC - Knudson C medium (1946).

Hardening of plantlets and transferring to community potting mix

About 2–4 cm long rooted plantlets (with 2–3 roots) were placed in perforated plastic cups containing charcoal pieces (≈2-4 cm), brick pieces (≈2-4 cm), coconut husks, and sand in the ratio of 1:1:1:1 and covered with holed transparent poly bag. The plantlets were maintained for 3–4 weeks in normal laboratory condition and irrigated at regular intervals. The potted plants were exposed to normal day light for about 1 hr in a day for the first week and subsequently the exposure period was increased by 2 hrs and finally after 1 month the plantlets were placed in full day light condition.

Data analysis

Asymbiotic seed germination

Bursting of the seed coat and emergence of the enlarged embryo *i.e*. the protocorm was considered as germination. The germination of seeds was recorded and percentage of seed germination was calculated. Five samples of the seeds were taken out after 15 days at random and the slides were prepared for observation by placing a drop of glycerin and covering it with a cover-slip. The seeds were scooped out and scrutinized randomly and observed under the microscope. The seeds were classified as germinated/non-geminated and the germination frequency was calculated by counting the total number of seeds germinated with that of total number of seeds observed.

$$\text{Total number of seeds germinated: \% Of germination} = \frac{\qquad\qquad}{\qquad\qquad} \times 100$$

The induction period, frequency of callus induction/multiple shoot formation, survival rate and conversion frequencies on various hormonal treatments were tabulated.

Phytochemical analysis and biological evaluation

Plant material

P.pallida and *A.graminifolia* leaves were collected in the month of November, from the Tropical Botanical Garden and Research Institute, Palode, Trivandrum, Kerala , India.

Phytochemical screening

Solvent extraction

The *P.pallida* and *A.graminifolia* leaves were washed and shade dried, then ground into fine powder. The extraction was carried by soxhlet extraction technique. Different solvents were used successively with gradient polarity (petroleum ether, chloroform, Acetone, Methanol and aqueous). The colour and percentage yield were noted. The extracts were completely evaporated by vacuum distillation and stored.

Preliminary phytochemical screening

Preliminary phytochemical screening was performed to analyze the secondary metabolites present in the plant extracts. The phytochemical screening of the plant extracts was carried out based on the methods described by Trease and Evans (1978), Harborne (1984) and Raaman (2006). The different qualitative chemical tests were carried out for establishing profile of plant extract for its chemical composition. The following tests may be performed on orchid leaf extracts to detect various phytoconstituents present in the extract.

Detection of alkaloids

Solvent free extract, 50 mg was stirred with few ml of dilute hydrochloric acid and filtered. The filtrate is tested carefully with alkaloid reagents as follows

Mayer's test

To a few ml of filtrate, one or two drop of Mayer's reagent was added by the side of the test tube. A white or creamy precipitate indicates the test as positive.

Mayer's Reagent

Mercuric chloride (1.358 g) was dissolved in 60 ml of water and potassium iodide (5.0 g) was dissolved in 10 ml of water. The two solutions are mixed and made up to 100 ml with distilled water.

Wagner's test

1 ml of HCl was added to 3 ml of extract in a test tube. The mixture was heated for 20 minutes, cooled and filtered. 2 drops of Wagner's reagent was added to 1 ml of the filtrate and observed for reddish brown precipitate.

Wagner's reagent

Iodine (1.27 g) and potassium iodide (2 g) was dissolved in 5 ml of water and made up to 100 ml with distilled water.

Detection of Carbohydrates

The extract (100 mg) was dissolved in 5 ml of water and filtrated. The filtrate is subjected to the following test.

Molish's test

Two drops of alcoholic solution of alpha-napthol was added to 2 ml of filtrate. The mixture was shaken well and 1 ml of concentrated sulphuric acid was added slowly along the sides of the test tube and allowed to stand. A violet ring indicates the presence of carbohydrates.

Fehling's test

1 ml of filtrate was boiled on water bath with 1 ml each of Fehling solution A and B. A red precipitate indicates the presence of sugar.

Fehling solution A

Copper sulphate (34.66 g) was dissolved in distilled water and made up to 500 ml using distilled water.

Fehling solution B

Potassium sodium tartarate (173 g) and sodium hydroxide (50 g) was dissolved in water and made up to 500 ml.

Detection of glycosides

50 mg of extract was hydrolysed with concentrated hydrochloric acid for 2 hrs on a water bath, filtered and the hydrolysate was subjected to the following tests.

Borntrager's test

3 ml of chloroform was added with 2 ml of filtered hydrolysate and shaken; when the chloroform layer was separated, 10 % ammonia solution was added. Pink colour indicates the presence of glycosides.

Legal's test

50 mg of the extract was dissolved in pyridine; sodium nitroprusside solution was added and made alkaline using 10 % sodium hydroxide. Presence of glycoside was indicated by pink colour.

Test for saponins

a. Frothing test

About 1 ml of extract was diluted separately with 20 ml of distilled water and shaken in a graduated cylinder for 15 minutes. 2 cm layer of foam indicates the presence of saponins.

Detection of Proteins and Amino acids

The extract (100 mg) was dissolved in 10 ml of distilled water and filtered through whatmann No.1 filtrate paper and the filtrate was subjected to tests for proteins and amino acids.

Millons test

To 2 ml of filtrate, few drops of Millon's reagent are added. A white precipitates indicates the presence of proteins.

Millon's reagent

Mercury (1 g) was dissolved in 9 ml of fuming nitric acid when the reaction was completed. Equal volume of distilled water was added.

Biuret test

An aliquot of 2 ml of filtrate was treated with one drop of 2 % copper sulphate solution. To this 1 ml of ethanol (95 %) was added, followed by excess of potassium hydroxide pellets. Pink colour in the ethanolic layer indicates the presence of proteins.

Ninhydrin test

2 drops of Ninhydrin solution (10 mg of Ninhydrin in 200 ml of acetone) are added to 2 ml of aqueous filtrate. A characteristic purple colour indicates the presence of amino acids.

Detection of phytosterols

a. Libermann-Burchard's test

The extract (50 mg) was dissolved in 2 ml of acetic anhydride. To this, one or two drops of concentrated sulphuric acid are added slowly along the sides of the test tube. An array of colour changes shows the presence of phytosterols.

Detection of fixed oils and fats Spot test

A small quantity of extract was pressed between two filter papers. Oil stain on the paper indicates the presence of fixed oil.

Saponification test

A few drops of 0.5 N alcoholic potassium hydroxide solutions was added to a small quantity of extract along with a drop of phenolphthalein. The mixture was heated on water bath for 2 hrs. Formation of soap or partial neutralization of alkali indicates the presence of fixed oils and fats.

Detection of phenolic compounds Ferric chloride test

The extract (50 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5 % ferric chloride solution was added. A dark green colour indicates the presence of phenolic compounds.

Lead acetate test

The extract (50 mg) was dissolved in distilled water and to this; 3 ml of 10 % lead acetate solution was added. A bulky white precipitate indicates the presence of phenolic compounds.

Detection of gum and mucilages

The extract (50 mg) was dissolved in 10 ml of distilled water and to this; 25 ml of absolute alcohol was added with constant stirring. White or cloudy precipitate indicates the presence of gums and mucilages.

Detection of phlobatannins

10 ml of aqueous extract of each plant sample was boiled with 1 % HCL in a test tube. If the sample contains phlobatannins, a deposition of a red precipitate will occur and indicates the presence of phlobatannins.

Detection of flavonoids

A few drop of 1 % ammonia solution was added to the extract in a test tube. A yellow colouration was observed if flavonoid compound was present.

Detection of anthraquinones

The extract was boiled with 10 % HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate.

Few drops of 10 % NH_3 solution were added to the mixture and heated. Formation of rose-pink colour indicates the presence of anthraquinones.

Detection of carotenoids

About 50 mg of plant extract was extracted with 10 ml of chloroform in a test tube with vigorous shaking. The resulting mixture was filtered and 85 % sulphuric acid was added. A blue colour at the interface showed the presence of carotenoids.

Detection of steroids

2 ml of acetic anhydride was added to the 50 mg plant extract along with 2 ml of concentrated H_2SO_4 the colour changes from violet to blue or green shows the presence of steroids. Detection of terpenoids 2 ml of chloroform was added to 20 mg plant extract. 3 ml of concentrated H2SO4 was carefully added to the sides of the test tube to form a layer. A reddish brown colour interface formed indicates the presence of terpenoids.

Detection of tannins

Small quantity of extract mixed with water and heated on water bath 60°C. The mixture was filtered and added with ferric chloride solution; dark green colour indicates the presence of tannins.

Estimation of total phenol content (Singleton and Rossi, 1965)

Principle

Phenols react with phosphomolybdic acid of Folin-Ciocalteau reagent in alkaline medium and produce a blue coloured complex (molybdenum blue) that can be estimated colourimetrically at 650 nm.

Reagents

20 % Sodium carbonate Folin-Ciocalteau reagent.

Standard Gallic acid: 10 mg of Gallic acid / 10 ml of distilled water

Procedure

An aliquot of the sample extract (0.1 ml) was mixed with distilled water (3 ml) and 0.5 ml of Folin- Ciocalteau reagent was added. After 3 minutes, 2 ml of 20 % sodium

carbonate was added and mixed thoroughly. The tubes were incubated in a boiling water bath exactly 1 minute. It was then cooled and absorbance was measured at 650 nm using spectrophotometer against the reagent blank. The total phenolic content was determined as gallic acid equivalents (GAE)/mg DW.

Estimation of total flavonoid (Jia *et al*., 1999)

Principle

Flavonoids reacts with aluminum chloride in ethanolic solution forms a yellow colour which was read colourimetrically at 420 nm.

Reagents

5 % Sodium nitrite

10 % Aluminum chloride 1 M Sodium hydroxide

Stock standard: 10 mg of Quercetin /10 ml of distilled water.

Procedure

0.5 ml of the sample in a test tube makes up with1.25 ml of distilled water. Then 0.075 ml of 5 % sodium nitrite solution was added and allowed to stand for 5 min. Then the mixture was added with 0.15 ml of 10 % aluminum chloride, after 6 min 0.5 ml of 1.0 M sodium hydroxide solution was added and the mixture were diluted with another 0.275 ml of distilled water. The absorbance of the mixture at 510 nm was measured immediately. The results were expressed as Quercetin equivalents/ mg DW

Fourier Transform Infrared Spectroscopy (FT-IR) spectrum analysis

Based on the preliminary phytochemical screening, the Methanol and aqueous extracts of *P.pallida* and the Chloroform and aqueous extracts of *A.graminifolia* were screened for FTIR analysis. The extract was mixed with KBr salt, using a mortar and pestle, and compressed into a thin pellet. Infrared spectra were recorded on a Shimadzu FTIR Spectrometer 8000 series, between 4,000–400 cm^{-1}.

Gas Chromatography - Mass Spectrometry (GC-MS) in phyto chemical analysis

Based on the preliminary phytochemical screening, the Aqueous extract of *P.pallida* and the Aqueous extract of *A.graminifolia* were screened for GC- MS analysis. The GC-MS analysis was carried out on a GC clarus 500 Perkin Elmer system comprising an AOC- 20i auto sampler and gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument employing the following conditions. Column Elite-5MS fused Silica capillary column (30 mm X 0.25 mm X 0.25 µmdf, composed of 5 % Diphenyl / 95 % Dimethyl polySiloxane), operating in electron impact mode at 7oev; Helium (99.999 %) was used as carrier gas at a constant flow of 0.1 ml/minute and an injection volume of 2 l was employed (split ratio of 10:1) injector temperature 250°C; lon- source temperature 280°C. The over temperature was programmed from 110°C (isothermal for 2 minutes), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 minutes isothermal at 280°C mass spectra were taken at 70ev; a scan interval of 0.2 seconds and fragments from 40 to 450 Da. Total GC running time is 22 minutes. Identification of the components in the extract was assigned by comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library and also with published literatures. NIST08s.LIB and WILEY8. LIB (Stein, 1990) library sources were used for matching the identified components from leaf extracts of orchids.

Antibacterial activity

All the extracts of *P.pallida* and *A.graminifolia* leaves were screened for antibacterial activity. Test Microorganisms The organisms included *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*), *Enterococcus faecalis* ATCC 29212 (*E. faecalis*), *Escherichia coli* MTCC 40 (*E. coli*) *Staphylococcus aureus* ATCC 25923 (*S. aureus*), *Bacillus subtilis* MTCC 2393 (*B. subtilis*), *Salmonella enterica* MTCC 98 (*S. enterica*) and *Corynebacterium diphtheriae* MTCC 3080 (*C. diphtheriae*) *Aspergillus flavus* and *Fusarium species.* All the strains were collected from American Type Culture Collection, Manassas, USA and Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. The microorganisms were grown in the nutrient broth at 37°C and maintained on nutrient agar slants at 4°C.

Disc diffusion method

All the extracts of *P. pallida* and *A.graminifolia* leaves were screened for antimicrobial activity using disc diffusion method (Doughari, 2008). 18 hrs old cultures of microorganisms maintained in Mueller hinton agar medium were used. Sterilized discs (Hi media, 6 mm), soaked in a known concentration of the crude extracts of orchid leaves (500 µg/ml DMSO of per disc). Soaked discs were applied over each of the culture plates previously seeded with the 0.5 McFarland. Antibiotic discs of cefotaxime (30 mcg/disc) were used as positive control and paper discs with DMSO were used as negative control and incubated at 37°C for 24 - 48 hrs. Zones of inhibition were measured, and the mean diameter was recorded.

Potato dextrose agar was prepared by dissolving 39gm in 1000ml of distilled water and sterilised this was used for antifungal activity, *Aspergillus flavus* and *Fusarium Spp*. were swabbed and using above method sample added and incubated at 30^0C for 5-7 days and zone of inhibition was measured, fluconazole were used as a positive control.

Determination of minimum inhibitory concentration (MIC)

The determination of the minimum inhibitory concentration was carried out according to the methods of (Bauer *et al*., 1966) with little modification. A stock concentration 1 mg/ml of the extract was prepared. From the stock, 50, 100, 150, 250, 500, 750 and 1000 µg of each concentration were added to each 9 ml of nutrient broth containing 0.1 ml of standardized test organisms. The tubes were incubated at 37°C for 24 hrs. A positive control was equally set up by using DMSO and test organisms without extract. The tube with least concentration of extract without growth after incubation was taken and recorded as the minimum inhibitory concentration.

Antioxidant activity

All the extracts of *P.pallida* and *A.graminifolia* leaves were screened for antioxidant activity.

DPPH radical scavenging activity (Shimada *et al*., 1992)

Principle

The molecule of 1,1-diphenyl-2-picrylhydrazyl (α,α-diphenyl-β- picrylhydrazyl; DPPH) is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalisation also gives rise to the deep violet colour, characterised by an absorption band in ethanol solution centered at about 517 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present).

Reagents

0.2 mM DPPH 80 % Methanol Ascorbic acid (Standard)

Procedure

Various concentrations of plant extracts (4.0 ml) were mixed with 1.0 ml of methanolic solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.1 mM. The mixture were shaken vigorously and left to stand for 30 minutes and the absorbance was measured at 517 nm. Ascorbic acid was used as standard. The percentage of DPPH decolorization of the sample was calculated according to the equation:

$$\% \text{ decolorization} = [1-(\text{ABS sample}/\text{ABS control}] \times 100$$

The % decolorization was plotted against the sample extract concentration, and a logarithmic regression curve was established in order to calculate the IC_{50} (inhibitory concentration 50 μg/ml) which is the amount of sample necessary to decrease by 50 % the absorbance of DPPH.

Total antioxidant activity

Using phospho molybdenum method total antioxidant activity was confirmed by the protocol of Jesteena et al., (2018) with slight modification. 0.5ml of the sample was mixed with the 0.5ml of reaction mixture of 0.6 M H_2SO_4, 28mM sodium phosphate and

4mM ammonium molybdate reagent solution and the tube was incubated at 45^0C for 90minutes with blank solution. After incubation the tubes were normalised to room temperature and the absorbance was read at 695nm. Ascorbic acid was used a standard to calculate the mg/gm of the total antioxidant activity. Triplicate value was used to finalise the total antioxidant activity.

Reducing power activity (Oyaizu, 1986)

Principle

Reducing power was measured by direct electron donation in the reduction of $Fe^{3+}(CN\text{-})_6$ to $Fe^{2+}(CN\text{-})_6$. The product was visualized by the addition of free Fe^{3+} ions after the reduction reaction, by forming the intense Prussian blue colour complex, Fe_4^{3+} $[Fe^{2+}(CN\text{-})_6]_3$ and quantified by absorbance at 700 nm.

Reagents

0.2 M Phosphate buffer, pH 6.6 1 % Potassium ferricyanide 10 % Trichloroacetic acid 0.1 % Ferric chloride, Ascorbic acid (Standard)

Procedure

The reaction mixture contained 2.5 ml of various concentrations of plant extracts, 2.5 ml of 1 % potassium ferricyanide and 2.5 ml of 0.2 M sodium phosphate buffer. The control contained all the reagents except the sample. The mixture was incubated at 50°C for 20 min and were terminated by the addition of 2.5 ml of 10 % (w/v) of trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 minutes. 5.0 ml of the supernatant upper layer was mixed with 5.0 ml of deionized water and 1.0 ml of 0.1 % ferric chloride. The absorbance was measured at 700 nm against blank that contained distilled water and phosphate buffer. Increased absorbance indicates increased reducing power of the sample.

FRAP assay (Benzie and Strain, 1996)

Principle

The total antioxidant potential of sample was determined using ferric reducing ability of plasma FRAP assay as a measure of antioxidant power. FRAP assay measures

the change in absorbance at 593 nm owing to the formation of a blue-coloured Fe II tripyridyl triazine compound from colorless oxidized Fe III form by the action of electron donating antioxidants.

Reagents

10 mM 2,4,6 tripyridyl-s-triazine (TPTZ) 40 mM HCl 20 mm Ferric chloride 0.3 M Acetate buffer, pH 3.6, FRAP reagent: It contains 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer. It were freshly prepared and warmed to 37°C., Ascorbic acid (Standard)

Procedure

900 µl FRAP reagent were mixed with 90 µl water and 30 µl test sample/ methanol/distilled water/standard antioxidant solution. The reaction mixture was then incubated at 37°C for 30 min and the absorbance was recorded at 595 nm. An intense blue colour complex were formed when ferric tripyridyl triazine (Fe^{3+}- TPTZ) complex were reduced to ferrous (Fe^{2+}) form. The absorption at 540 nm was recorded. Calculations were made for the conversion of (TPTZ)-Fe (III) complex to TPTZ-Fe (II) compared with those of ascorbic acid. The results were expressed in µM ascorbic acid equivalence (AAE) /g dry weight of extract using standard calibration curve.

Anticancer activity

Based on the screening of antioxidant potential, the aqueous extract of both the *P. pallida* and *A.graminifolia* leaves were chosen for anticancer activity.

In vitro cytotoxicity

Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay (Mosmann, 1983) with little modification. The human breast cancer cell line (MCF- 7) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Eagles Minimum Essential Medium (EMEM) containing 10 % fetal bovine serum (FBS). All cells were maintained at 370°C, 5 % CO_2, 95 % air and 100 % relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

The monolayer cells were detached with trypsin- ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5 % FBS to give final density of $1x10^5$ cells/ml. 100 µl per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 370°C, 5 % CO_2, 95 % air and 100 % relative humidity. After 24 hrs the cells were treated with serial concentrations of the test samples. They were initially dissolved in neat dimethyl sulfoxide (DMSO) and diluted to twice the desired final maximum test concentration with serum free medium. Additional four, 2fold serial dilutions were made to provide a total of five sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulted the required final sample concentrations. Following drug addition the plates were incubated for an additional 48 hrs at 370°C, 5% CO_2, 95 % air and 100 % relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

MTT assay

MTT is a yellow water-soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate- dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 hrs of incubation, 15 µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 370°C for 4 hrs. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using the following formula.

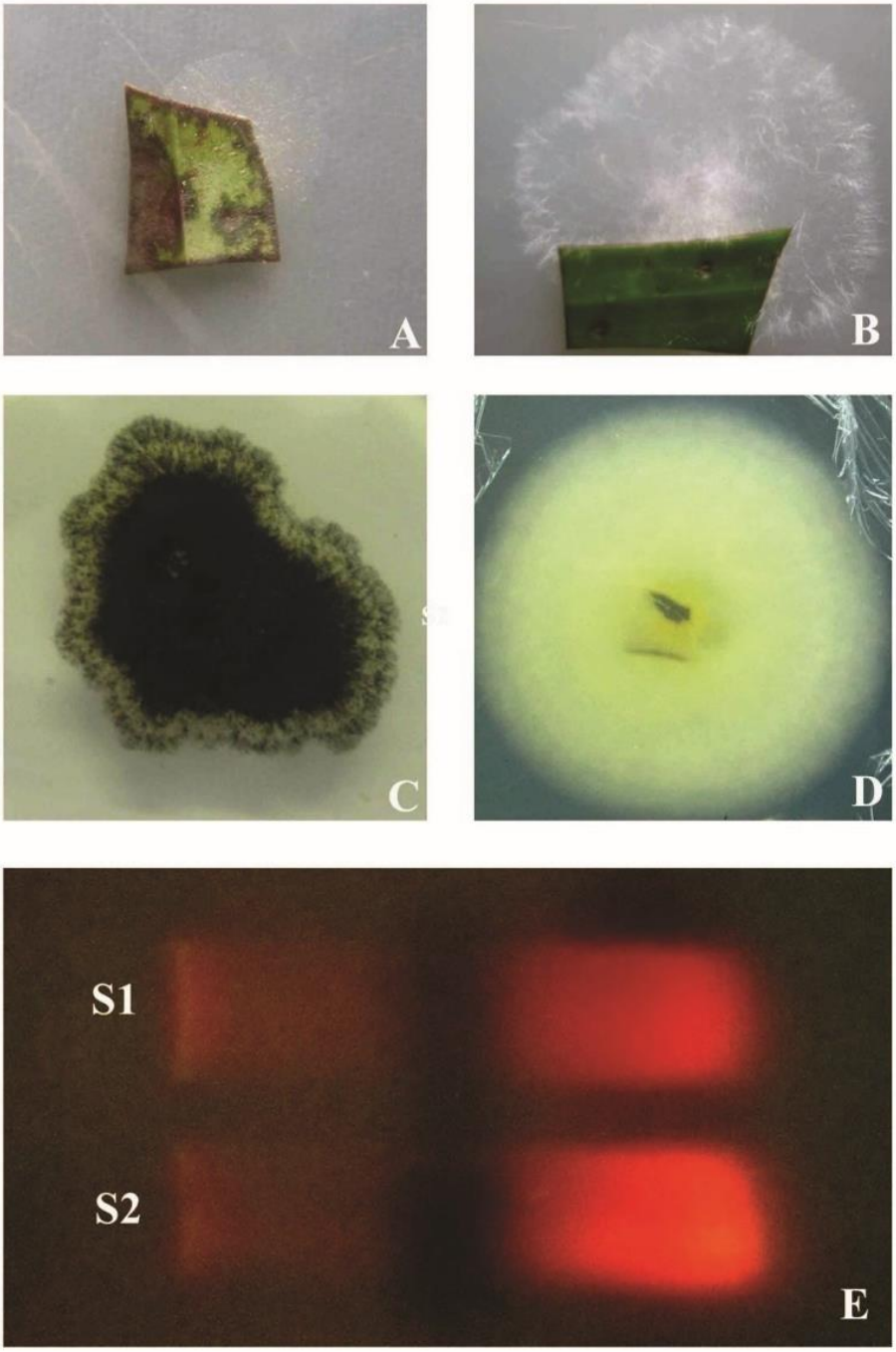
$$\% \text{ Cell Inhibition} = 100 - \text{Abs (sample)}/\text{Abs (control)} \times 100.$$

Nonlinear regression graph was plotted between % Cell inhibition and Log_{10} concentration and IC_{50} was determined using GraphPad Prism software.

Isolation of Endophytes

Fresh and healthy *ex situ* leaves of *P. pallida* and *A.graminifolia* were collected from the, Tropical Botanical garden and Research Institute, palode, Trivandrum, India.

Figure 3.1. Endophyte isolation from orchid leaves



These leaves were packed in air-tight polyethylene bags and transported to the laboratory within 24 hrs, then processed and plated on media for isolation of endophytic fungi. The leaves were carefully rinsed under gently running tap water and air dried. The leaves were then surface sterilized by immersing them in 70 % ethanol for 30 seconds and 0.1 % $HgCl_2$ for 4 min and rinsed thoroughly with sterile distilled water. The excess water was dried under laminar airflow chamber. Then, with a sterile scalpel, the leaves were cut about 1.0 cm size and were carefully placed on petri-plates containing Potato Dextrose Agar (PDA) (Himedia). The media were supplemented with streptomycin sulphate (100 mg/L) to suppress bacterial growth.

The plates were sealed with Parafilm to avoid desiccation then incubated at 25±2°C until fungal growth appeared. The plant segments were observed every day for fungal growth. Hyphal tips emerging from the plated leaf segments were immediately transferred into PDA slant and maintained at 4°C.

Fungal cultivation

The fungal endophytes were cultivated on Potato Dextrose Broth (PDB) (Himedia) by placing agar blocks of actively growing pure culture (3 mm in diameter) in 250 ml conical flasks containing 100 ml of the medium. The flasks were incubated at 25±2°C for 3 weeks with periodical shaking at 80 rpm. After the incubation period, only the cultures actively growing in Potato Dextrose Broth were taken out and filtered through sterile cheesecloth to remove the mycelia mats which were used for DNA isolation. For mass culturing of the fungus the actively grown pure culture was inoculated in 2000 ml conical flask with 500 ml of Potato Dextrose Broth and incubated at 25±2°C for 2 months in dark.

Genomic DNA isolation and visualization.

Four-day-old mycelial mats were washed with sterile distilled water and ground in liquid nitrogen. Genomic DNA was extracted from grown cultures of the selected fungal isolates using DNA isolation kit (Qiagen) with the following procedure.

About 0.5 g of powdered mycelium was taken in a 1.5 ml microcentifuge tube. The powdered culture was added with 200 µl of GT buffer and mixed by vortexing. Incubated at room temperature for 5 minutes.

200 µl of GB buffer was added to the tube and mixed by vortexing for 5 seconds

Incubated at 70°C for 10 minutes until the sample lysate was clear. During incubation, tubes were inverted every 5 minutes.

200 µl of ethanol (99.9 %) were added to the sample lysate and mixed immediately by vortexing for 10 seconds. The precipitate was broken up by pipetting.

Placed a GD column on a 2 ml collection tube.

Transfered all the mixture (including any precipitate) from previous step to the GD column. Centrifuged at 8000 rpm for 2 minutes.

500 µl of wash buffer was added into the column. Centrifuged at 8000 rpm for 30 seconds.

Discard the flow- through, place the GD column back in the collection tube. Repeated the wash step

500 µl of wash buffer was added into the column. Centrifuged at 8000 rpm for 30 seconds

Discarded the flow-through and placed the GD column back in the collection tube. Centrifuged at full speed for 3 minutes to dry the column matrix.

Transferred the dried GD column into a clean 1.5 ml microcentrifuge tube. Added 50 µl of preheated elution buffer into the center of the column matrix. Allowed to stand for 2 minutes until elution buffer absorbed by the matrix.

Centrifuged at 8000 rpm for 30 seconds to elute purified DNA.

The presence of DNA was confirmed by 1 % Agarose gel electrophoresis as described below (Sambrook*et al*., 1989).

The gel was prepared by fitting the dried and cleaned plastic tray in the casting apparatus along with suitable comb.

The sufficient electrophoresis buffer (1X TAE) was prepared from 50X TAE stock (contains 242 g/l Tris- base; 57.1 ml/l glacial acetic acid; 100 ml/l 0.5M EDTA, pH 8.0) to fill electrophoresis tank enough to immerse the gel.

Agarose solution was prepared in electrophoresis buffer (1X TAE) by adding known quantity of agarose (1 %) in a glass bottle and heat until the agarose to dissolve.

Ethidium bromide was added to a final concentration of 0.5 μg/ml and the agarose solution was poured into the casting tray. Made sure that there are no air bubbles in the gel or trapped between the wells.

The gel was allowed to set at room temperature then small amount of buffer was poured on the top of the gel and the comb was carefully removed then mounted on the electrophoresis tank. Additional buffer was added as needed.

Sample was prepared by adding 1 volume of 6X loading dye (contains 0.25 % bromophenol blue; 0.25 % xylene cyanol FF; 30 % glycerol in water) to 6 volumes DNA sample and mixed.

The agarose gel electrophoresis was run by applying the samples in gel loading buffer to the wells of the gel. Prior to sample loading, air bubbles were removed from the wells by rinsing them with electrophoresis buffer. The entire gel was submerged in the electrophoresis buffer.

The sample was loaded by inserting the pipet tip deep into the well expeling the liquid slowly. The electrodes were connected.

The power supply was turned on and the gel was run at 1-10 V/cm until the dyes have migrated an appropriate distance.

Visualization of the DNA band was carried out by UV transilluminator and by using gel documentation system. Ethidium bromide-DNA complexes display increased fluorescence compared to the dye in solution. This means that illumination of a stained

gel under UV light (254-366 nm) allows bands of DNA to be visualized against a background of unbound dye.

PCR analysis

The DNA was suspended in 100 µl of elution buffer (10 mM/L Tris-HCl, pH 8.5) and quantified by measuring OD at 260 nm. The PCR amplification reactions were performed with a total 25 µl of reaction that comprised 20 ng of genomic DNA, 2.5 µl of 10X PCR buffer with 25 Mm $MgCl_2$, 0.5 µl of 10 mM dNTPs, 1.5 µl of 25 mM $MgCl_2$, 1.25 µl of 5% (v/v) DMSO, 1 unit of Taq polymerase, and 10 pmol of each primer. The sequences of ITS primers used were as follows.

ITS1: 5′- TCCGTAGGTGAACCTGCGG -3′ ITS4: 5′ - TCCTCCGCTTATTGATATGC -3′

Amplification was carried out with an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 56°C for 45 sec, extension at 72°C for 1 min and final extension at 72°C for 5 min using a thermocycle (iCycler; Bio-Rad Laboratories, CA). PCR products were analyzed on 1 % agarose gel for amplicons in 1X TBE buffer at 100 V.

ITS region sequencing and phylogenetic analysis

The gel section with desired band was carefully excised under UV light and subjected to extraction using the QIA quick gel extraction kit (Qiagen, Valencia, CA) from the agarose gel as per the following manufacturer's instructions.

The DNA fragment was excised from the agarose gel with a clean and sharp scalpel. The gel slice was weighed in a colourless tube. 3 volume of Buffer QG to 1 volume gel.

Incubated at 50°C for 10 minutes (or until the gel slice has completely dissolved). Vortexed the tube every 2-3 minutes to help dissolve gel.

After the gel slice has dissolved completely, the colour of the mixture was yellow (similar to buffer QG without dissolved agarose).

1 gel volume of isopropanol was added to the sample and mixed. QIA quick spin column placed in a provided 2 ml collection tube.

To bind DNA, the sample was applied to the QIA quick spin column and centrifuged for 1 minute. Flow-through was discarded and the QIA quick column was placed back into the same tube.

0.5 ml Buffer QG was added to the QIA quick column and centrifuged for 1 minute.

The flow-through was discarded and the QIA quick column was placed back into the same tube. To wash, 0.75 ml of Buffer PE was added to QIA quick column and centrifuge for 1 minute.

The flow-through was discarded and the QIA quick column was placed back into the same tube and the column was let to stand 2-5 minutes after addition of Buffer PE.

The QIA quick column was centrifuged once more in the provided 2 ml collection tube for 1 minute at 13,000 rpm to remove residual wash buffer.

Placed QIA quick column into a clean 1.5 ml microfuge tube.

To elute DNA, 50 µl EB Buffer (10 mM Tris Cl, pH 8.5) was added to the center of the QIA quick membrane and centrifuged the column for 1 minute.

For increased DNA concentration 30 µl EB Buffer was added to the center of the QIA quick membrane, the column stand1 minute and then centrifuged for 1 minute.

Then the purified DNA was sequenced using automated DNA sequencer (Model 3100, Applied Biosystems, USA). The sequences were analysed using the Basic Local Alignment Search Tool (BLAST) software (http://www.ncbi.nlm.nih.gov/blast) against the nucleotide sequence database.

Phylogenetic analysis

The sequences of these ITS region were compared against the sequences available from GenBank using the BLASTN program (Altschul *et al.*, 1990) and were aligned

using CLUSTAL W software (Thompson *et al.*, 1994). Distances were calculated according to Kimura's two-parameter correction (Kimura, 1980). Phylogenetic trees were constructed using the neighbour-joining method (Saitou and Nei, 1987). Bootstrap analysis was done based on 1000 replications. The MEGA5 package (Kumar *et al.*, 2008) was used for all analyses.

Evaluation of antibacterial and antioxidant activity of the isolated endophytes

Solvent extraction

From the mass cultured isolated S1 and S2 endophytes the mycelial mats were removed and washed with sterile distilled water to remove the medium present at the surface of the mats. Then the mycelial mats were carefully transferred to sterile mortar and pestle, powdered along with liquid nitrogen. The powder was weighed and about 6 gram of powder was packed in filter paper and the extraction was carried by soxhlet extraction technique. The solvents used in the soxlet extraction was Methanol and water for the endophyte S1 isolated from *P.pallida* leaves, and Acetone and water forth endophyte S2 isolated from the leaves of *A.graminifolia.* The solvents used were based on the results obtained in chapter 2.

After the removal of mycelial mats, the spent culture broth was filtered to obtain the culture exudates, the broth was extracted with four times the volume of Methanol for S1 isolate and Acetone for S2 isolate upto 24 hrs and further concentrated in vacuum to remove organic solvent (Wang *et al*., 2006b). The concentrate was volatilized later on 60°C water bath to obtain dried residues prior to antimicrobial assays. Methanol extract of S1 and Acetone extract of S2 was diluted with sterile distilled water at the concentration of 10 mg/ml and was sterilized by filtration through a 0.22 µm Millipore filter.

Antibacterial activity

Test Microorganisms

The organisms included *Pseudomonas aeruginosa* ATCC 27853 (*P. aeruginosa*), *Enterococcus faecalis* ATCC 29212 (*E. faecalis*), *Escherichia coli* MTCC 40 (*E. coli*)

Staphylococcus aureus ATCC 25923 (*S. aureus*), *Bacillus subtilis* MTCC 2393 (*B. subtilis*), *Salmonella enterica* MTCC 98 (*S. enterica*) and *Corynebacterium diphtheriae* MTCC 3080 (*C. diphtheriae*). All the strains were collected from American Type Culture Collection, Manassas, USA and Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. The microorganisms were grown in the nutrient broth at 37°C and maintained on nutrient agar slants at 4°C.

Disc diffusion method

18 hrs old cultures of microorganisms maintained in mueller hinton agar medium were used. Sterilized discs (Hi media, 6 mm), soaked in a known concentration of endophyte extracts (500 µg/ml DMSO of per disc). Soaked discs were applied over each of the culture plates previously seeded with the 0.5 McFarland. Antibiotic discs of cefotaxime (30 mcg/disc) were used as positive control and paper discs with DMSO were used as negative control and incubated at 37°C for 24 - 48 hrs. Zones of inhibition were measured, and the mean diameter was recorded.

Antifungal activity

Potato dextrose agar was prepared by dissolving 39gm in 1000ml of distilled water and sterilised this was used for antifungal activity, *Aspergillus flavus* and *Fusarium Spp*. were swabbed and using above method sample added and incubated at 30^0C for 5 -7 days and zone of inhibition was measured , fluconazole were used as a positive control.

Antioxidant activity

DPPH radical scavenging activity (Shimada *et al*., 1992)

Principle

The molecule of 1,1-diphenyl-2-picrylhydrazyl (α, α-diphenyl-β- picrylhydrazyl; DPPH) is characterised as a stable free radical by virtue of the de localisation of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalisation also gives rise to the deep violet

colour, characterised by an absorption band in ethanol solution centered at about 517 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet colour (although there would be expected to be a residual pale-yellow colour from the picryl group still present).

Reagents

0.2 mM DPPH 80 % Methanol, Ascorbic acid (Standard)

Procedure

Various concentrations of plant extracts (4.0 ml) were mixed with 1.0 ml of methanolic solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.1 mM. The mixture were shaken vigorously and left to stand for 30 minutes and the absorbance was measured at 517 nm. Ascorbic acid was used as standard. The percentage of DPPH decolorization of the sample was calculated according to the equation:

$$\% \text{ decolorization} = [1-(\text{ABS sample}/\text{ABS control}] \times 100$$

The % decolorization was plotted against the sample extract concentration, and a logarithmic regression curve was established in order to calculate the IC_{50} (inhibitory concentration 50 μg/ml) which is the amount of sample necessary to decrease by 50 % the absorbance of DPPH.

Synthesis of Nanoparticles

Preparation of nanoparticle

The aqueous extract was used for the synthesising of silver nanoparticles. 10ml of the aqueous extract was mixed with equal amount of the 1mM $AgNO_3$ solution (prepared by standard formula, MW of $AgNO_3$- 169.87) and incubated for 24 to 48hrs in dark room temperature. After incubation the sample changes were observed and characterised to confirm the nanoparticle.

Nanoparticle synthesis were analysed on Antioxidant and antimicrobial assays

Characterisation of nanoparticles

UV-Visible spectroscopy

The synthesised nanoparticle was primarily characterised by UV-Visible spectroscopy. The absorption of AgNPs depends on the particle size, dielectric medium, and chemical surroundings, UV Visible analysis was done from the nanometer of 300 to 600nm after setting the baseline in UV-Visible spectrophotometer of Labtronics LT 291.

FTIR

The synthesised sample was further characterised by Fourier-transform infrared spectroscopy. FTIR analysis of the sample was done from the range of 4000cm-1 to 500 cm-1 using Shimadzu instrument to identify the functional group which is in the synthesised nanoparticle.

SEM

SEM is a surface imaging method, totally capable of determining diverse particle sizes, nanomaterial shapes, size distributions and the surface morphology of the synthesized silver particles at the micro and nanoscales. The synthesised sample was powdered and the fine powder was used for the SEM analysis to characterise the morphological analysis.

XRD

The crystalline nature of AgNPs was further confirmed by Xray Diffraction (XRD) analysis. Operated at 35 kV and 28 mA in flat plane geometry mode with each scan taking 2 seconds. The respective diffraction patterns were collected over a 2θ range of 20° to 90°.