MATERIALS AND METHODS

CHAPTER - II

MATERIALS AND METHODS

The plant materials for the present work were collected from Pavagadh hills and Baroda, Gujarat State, India. Healthy and infected leaves were always collected from the same plant. The infected leaves were collected from the diseased trees and the fungal pathogen was isolated and cultured on PDA medium. Pathogenicity tests were performed and thus the Koch's postulates were confirmed. The pathogenic fungus was stained with lactophenol-cotton blue and identified.

Healthy and infected leaves of the trees were analysed for qualitative changes in flavonoids, quinones, phenolic acids, tannins, saponins, proanthocyanidins, iridoids, alkaloids, steroids, sugars, aminoacids and volatile oils.

Phytoalexin response was tested by using the drop diffusate technique and facilitated diffusion technique. Bioassay tests of mycelial growth, spore germination and germ tube length were performed to prove the antimicrobial activity of certain compounds.

1.1 PREPARATION OF MEDIUM

Formula for potato dextrose agar (PDA)

Potato : 200 g

Dextrose : 20 g

Agar : 15 g

Water : 1000 ml

Two hundred grams of peeled potato were sliced into small pieces in 500 ml of water and boiled for about 25 minutes. The broth was filtered through a cheese cloth and made upto 500 ml. 20 gm of Dextrose was added to this. At the same time 15 gm of Agar was melted in 500 ml of water by heating on a hot plate. The potato broth was poured into the agar solution and restored to 1000 ml with distilled water. The medium was sterilised after adjusting the pH to 6 because the pH of the medium has been known to influence the germination of fungal spores. Although, in general, spores germinate within a wide range of pH,an acidic medium is favourable for germination of most of the fungi.(Lilly and Barnett, 1951).

1.2 LACTOPHENOL-COTTON BLUE STAIN FOR FUNGI

Phenol : 20 ml

Lactic acid : 20 ml

Glycerol : 40 ml

The above mentioned ingredients were mixed and heated at 70°C and then 5 ml of 1% aqueous cotton blue solution was added.

1.3 ISOLATION AND CULTURE

The infected leaves of the plant were collected and washed thoroughly with distilled water. The infected portions of the leaves were surface sterilised with ethyl alcohol. The

fungus was cultured and maintained on PDA slants. The fungal cultures were identified in the lab itself. But the final identification is done at CMI, Kew, England.

1.4 PATHOGENICITY TESTS

Healthy leaves of the plant were inoculated with the spore suspension or the fungal cultures of the test fungus (pathogen) after surface sterilising them with ethyl alcohol and kept in plastic bags. If the plants are susceptible to the pathogen, then the symptoms appear after a few days. Pathogenicity was then confirmed according to Koch's postulates (1882).

1.5 CHEMICAL ANALYSIS

The healthy and infected leaves were washed well and dried in an oven at 60°C. The dried leaves were powdered and stored in air tight glass bottles or plastic bags. The leaves were analysed for pre-infectional and post-infectional compounds. The various methods followed for their extraction and characterisation are presented below:

a. Flavonoids

Flavonoids are a group of polyphenols which include all the C_6-C_3-C_6 compounds related to a flavone skeleton, which may be considered as consisting of (i) a C_6-C_3 fragment (phenyl propane unit) that contains the 'B' ring and (ii) a C_6 fragment,

the 'A' ring, both being of different biosynthetic origin. The flavonoids are subdivided based on the oxidation level of C_3 fragment of the phenyl propane unit, as flavones, flavonols, chalcones, aurones etc. (Geissman, 1962).

Flavonoids are present in almost all vascular plants but some classes of these compounds, such as flavones and flavonols are more widely distributed than isoflavones and biflovones which are found to have a restricted occurrence. Most of the flavonoids occur as water soluble glycosides in plants. They are extracted with 70% ethanol or methanol and remain in the aqueous layer following partition of this extract with solvent ether. Due to the phenolic nature of flavonoids they change in colour when treated with bases or with ammonia and thus are easily detected in chromatograms or in solutions. Flavonoids contain conjugated aromatic systems and thus show intense absorption bands in UV and in the visible regions of the spectrum. A single flavonoid aglycone may occur in a plant, in several glycosidic combinations and for this reason it is considered better to examine the aglycones present in hydrolysed plant extracts. (Harborne,1984). Normally the flavonoids are linked to sugar by an O-glycosidic bond, which are easily hydrolysed by mineral acids. But there is another type of bonding in which sugars are linked to aglycones by C-C bonds. The latter group of compounds, known as C-glycosides (glycoflavones) are generally observed among flavones. They are resistant to normal methods of hydrolysis and will remain

in the aqueous layer when the hydrolysed extract is fractionated with ether to remove aglycones.

The procedures followed for the extraction, isolation and identification of flavonoids are described below:

Ten grams of leaf powder was extracted in a soxhlet extractor with methanol for 48 hrs till the plant material became colourless. The methanolic extract was concentrated to dryness in a water bath. 50 ml of water was added to the dry residue and the water soluble phenolic glycosides were filtered out. The filtrate was hydrolysed in a water bath for one hour using 7 % HCl. This hydrolysate was extracted with diethyl ether/ solvent ether, whereby the aglycones got separated into ether fraction (Fraction A). The remaining aqueous fraction was further hydrolysed for ten hours to ensure the complete hydrolysis of all the O-glycosides. Aglycones were once again extracted into diethyl ether (Fraction B) and residual aqueous fraction was neutralised and evaporated for the analysis of glycoflavones.

Ether fraction A and B were combined and analysed for aglycones using standard procedures (Harborne,1967; 1984; Mabry et al., 1970 ; Markham, 1982). The combined concentrated extract was banded on Whatman No.1 paper. The solvent system employed was Forestal (Con: HCl:Acetic acid: Water 3:30:10) or 30% glacial acetic acid. The developed chromatograms were dried in air and the visibly coloured compounds were marked

out. The papers were observed in ultraviolet light (360 nm) and the bands were noted. Duplicate chromatograms were then sprayed with 10% aqueous Na_2CO_3 and 1% $FeCl_3$ and the colour changes were recorded. The bands of compounds were cut out from unsprayed chromatograms and were eluted with spectroscopic grade methanol. The UV absorption spectra of these compounds were recorded in methanol using 'Shimadzu UV 240' spectrophotometer. The bathochromic and hypsochromic shifts induced by the addition of various reagents were studied. The reagents used for such studied and their preparations are given below:

Sodium methoxide (NaOMe)

Freshly cut metallic sodium (2.5 gm) was added continuously in small portions to dry spectroscopic methanol (100 ml). TThe solution was stored in a tightly closed glass bottle.

Aluminium Chloride ($AlCl_3$):

Five gm of fresh anhydrous AR grade $AlCl_3$ (which appeared yellow green and reacted violently when mixed with water) was added cautiously to spectroscopic methanol (100 ml).

Hydrochloric acid (HCl):

Concentrated AR grade HCl (50 ml) was mixed with distilled water (100 ml) and the solution was stored in a glass toppered bottle.

Sodium acetate (NaOAc): Anhydrous powdered AR grade NaOAc was used.

Boric acid (H_3BO_3) : Anhydrous powdered AR grade H_3BO_3 was used.

The concentration of the sample solution prepared by eluting chromatograms strips were adjusted so that the optical density (OD) fell in the region of 0.6 to 0.8. The methanol spectrum was taken using 2-3 ml of this stock solution. The NaOMe spectrum was measured immediately after the addition of three drops of NaOMe stock solution to the flavonoid solution used for methanol spectrum. The solution was then discarded. The $AlCl_3$ spectrum was measured immediately after the addition of 6 drops of $AlCl_3$ stock solution to 2-3 ml of fresh stock solution of the flavonoids. The $AlCl_3$/HCl spectrum was recorded next, after the addition of 3 drops of the HCl stock solution to the cuvette containing $AlCl_3$. The solution was then discarded. For NaOAc spectrum, excess coarsely powdered anhydrous AR grade NaOAc was added by shaking the cuvette containing 2-3 ml of fresh solution of the flavonoids, till about a 2 mm layer of NaOAc remained at the bottom of the cuvette. The spectrum was recorded 2 minutes after the addition of NaOAc. NaOAc/H_3BO_3 spectrum was taken after sufficient H_3BO_3 was added to give a saturated solution. The solution was discarded after recording the spectrum.

The structure was established with the help of R_f values absorption maxima, shape of the curves, shifts (both

bathochromic and hypsochromic) with different reagnets, colour reactions and co-chromatography with authentic samples.

The aqueous fraction remaining after the separation of aglycones was neutralized by the addition of anhydrous Na_2Co_3 and concentrated to dryness. To the dry residue, methanol was added and filtered. The methanolic extract was banded on Whatman No.1 paper and the chromatogram was developed with water as the solvent system. Glycoflavones were visualized by their colour in UV, and with 10% Na_2Co_3 spray. Further analysis and identification were done using spectroscopic methods as explained before.

b. Quinones

Quinones include all the aromatic diketones. They are also extracted with other flavonoids in the methanol extract. Aglycones as well as the C-glycosides are isolated in papers or thin layers in the same way as the flavonoids are separated. The presence of quinones in a chromatogram is indicated by the persistant pink or red colour when the chromatogram is sprayed with 10% sodium carbonate solution. Spectral analysis was followed further to identify the quinones.

c. Phenolic acids

Phenolic acids are simple phenols, having a functional acid group and varying number of hydroxyl groups at different

positions. Acid hydrolysis of plant tissues releases a number of ether soluble phenolic acids. These acids occur either associated with lignin or bound to the glycosides. They are also seen as depsides or as esters in hydrolysable tannins. The phenolic acids are extracted in ether along with flavonoid aglycones from the hydrolysed extract (fraction A and B) of plant materials.

Analysis of phenolic acids in the combined ether fraction (A and B) was carried out by two-dimensional ascending paper chromatography. Benzene : acetic acid : water (6:7:3, upper organic layer) in the first direction, and sodium formate : formic acid : water (10:1:200), in the second direction, were used as irrigating solvents. The sprays used to locate the compounds on the chromatogram were diazotised p-nitra-aniline or diazotised sulphanilic acid, followed by an over spray of 10% Na_2Co_3 (Ibrahim and Towers, 1960). Diazotization : 0.7 gm of p-nitra-aniline/sulphanilic acid was dissolved in 9 ml of HCl and the volume made up to 100 ml. Five ml of 1% $NaNO_2$ was taken in a volumetric flask and kept in ice till the temperature was below 4°C. The diazotised sprays were prepared by adding 4 ml of p-nitra-aniline/sulphanilic acid stock solution to the cooled $NaNO_2$ solution. The volume was made upto 100 ml with ice cold water.

The various phenolic acids present in the extract were identified based on the specific colour reactions they produce

with the spray reagents, relative R_f values in different solvent systems, UV absorption spectra and co-chromatography with standard samples.

d. Tannins:

Tannins are polyphenolic compounds which combine with protein, forming water insoluble and non-putrescible leather. There are two main types of tannins; the condensed tannins and the hydrolysable tannins. The condensed tannins (proanthocyanidins) universally occur in ferns and gymmosperns and are widespread among the woody angiosperms. In contrast, hydrolysable tannins are limited to dicotyledonous plants and are only found in a relatively few families.

Condensed tannins or flavolans can be regarded as being formed by the condensation of catechin or gallocatechin molecules and flavan-3,4-diols to form dimers and higher oligomers; with carbon-carbon bonds linking one flavan unit to the next by a 4-8 or 6-8 linkage. The name proanthocyanidins is used alternatively for condensed tannins because on treatment with hot acids, some of the carbon-carbon bonds are broken and anthocyanidins are released. Hydrolysable tannins are mostly gallotannins and ellagitannins depending on whether gallic acid or ellagic acid is present esterified with glucose. They yield the corresponding phenolic acid and glucose on hydrolysis.

Tannins are extracted in water. To the water extract prepared by boiling 5 gm plant material in 50 ml water, 2%

freshly prepared gelation solution was added. The formation of a white (or milky) precipitation showed the presence of tannins in the plant material. (Hungund et al., 1971).

e. Saponins

Saponins are glycosides which form emulsion with water and possess marked haemolytic properties. They possess steroidal or triterpenoid aglycones. The steroid saponins are common in monocots, while the triterpenoid saponins are found in dicots.

About 5 gm of the powdered leaf material was boiled with 50 ml water for half an hour. This extract was filtered, the filtrate was taken in a test-tube after cooling and shaken vigorously (to froth) for a minute or two. The formation of a persistent froth of 1 cm length showed the presence of saponins. (Hungund et al.,1971). Foam formation takes place even during aqueous extraction if the concentration of the saponins are more in the plant materials. (Harborne, 1984).

f. Proanthocyanidins

For testing the proanthocyanidins, about 5 gm of finely chopped (fresh) leaf-material or 2 gm dry powdered material was taken in a 20 ml test tube and covered with approximately 5 ml of 2 M HCl. Hydrolysis was carried out by placing the test tube in a boiling water bath for half an hour. The extract was decanted after cooling and shaken with amyl alcohol. Presence

of a red or near carmine colour in the upper alcohol layer denoted a positive reaction for proanthocyanidins. An olive yellow colour represented a negative reaction (Gibbs, 1974).

g. Iridoids

Iridoids are a group of monoterpenoid glycosides present in a number of Dicotyledons (Hegnauer, 1966, 1969,1971; Kubitzki,1969; Meuse,1970; Bate-smith,1972; Jensen et al.,1975). The plants were tested for iridoids by a simple procedure based on the Trim-Hill colour test (Trim and Hill, 1951). Fresh or dry powdered leaf material (1 gm) was placed in a test tube with 5 ml of 1% aqueous HCl. After 3-6 hours, 0.1 ml of the macerate was decanted into another tube containing 1 ml of Trim-Hill reagent (made up from 10 ml acetic acid, 1 ml of 0.2% $CuSO_4.5H_2O$ in water and 0.5 ml Con : HCl) when the tube was heated for a short time in a flame, a colour was produced, if iridoids are present.

h. Alkaloids

Alkaloids comprise the largest single class of secondary metabolites. They are basic plant products having a nitrogen containing heterocyclic ring system and high pharmacological activity. Alkaloids are insoluble in water but soluble in organic solvents. Alkaloids are normally extracted from plants into weak acid solution (1 M HCl or 10% acetic acid) and are then

precipitated with concentrated ammonia. They are also extracted into any organic solvent after treating the plant material with a base. The base frees the alkaloids and makes them soluble in organic solvents. From the organic solvents, the alkaloids are extracted into acidic solutions and tested with specific reagents.

Five grams of powdered leaf material was extracted with 50 ml of 5 % ammonical ethanol for 48 hrs. The extract was concentrated and the residue was treated with 10 ml of 0.1 N H_2SO_4. The acid soluble fraction was tested with Mayer's, Wagner's or Dragendorff's reagents. (Peach and Tracey, 1955). The formation of a precipitate denoted the presence of Alkaloids. The preparation of these reagents was as follows:

Mayer's reagent: (Potassium mercuric iodide) 1.36 gms of $HgCl_2$ was dissolved in 60 ml of distilled water and 5 gms of KI in 10 ml of water. The two solutions were mixed and diluted to 100 ml with distilled water. A few drops only of this reagent were added.

Wagner's reagent : (Potassium iodide) 1.27 grams of I_2 and 2 grams of KI were dissolved in 5 ml of water and the solution diluted to 100 ml. It gave brown flocculent precipitates with most of the alkaloids.

Dragendorff's reagent : (Potassium bismuth iodide) 8 grams of $Bi(NO_3)_3.5H_2O$ (Sp.gr.1.18) and 27.2 grams of KI in 50 ml of

water. The two solutions were mixed and allowed to stand when kNO_3 crystallized out. The supernantant was decanted off and made upto 100 ml with distilled water.

i. Steroids

Steroids are triterpenes based on the cyclopentanoperhydrophenanthrene ring system.

The ethyl acetate fraction of the plant extract was analysed by thin layer chromatography using chloroform: Carbontetrachloride: acetone (2:2:1) as the solvent system. The plate was sprayed with Liebermann-Burchard reagent or 50% sulphuric acid and heated at 85-90°C for 15 minutes. The Liebermann-Buchard reagent was prepared by mixing 1 ml of Conc. H_2SO_4, 20 ml acetic anhydride and 50 ml of chloroform. Steroids give characteristic colors which indicate their substitutions.

j. Sugars

The major free sugars in plants are the monosaccharides, glucose and fructose and the disaccharide surcose, together with traces of xylose, rhamnose and galactose. Sugars are colourless substances and when present in micro amounts have to be detected by reaction with a suitable chromogenic reagent.

The water fraction of the plant extract was hydrolysed for 10 hours with 7 per cent conc. HCl. After hydrolysis, it was separated with ethyl acetate in a separating funnel. The

water fraction was neutralised with sodium carbonate and concentrated to dryness. To the dry residue methanol was added. This extract was then chromatographed in paper using different sugars as standards with n-butanol : acetic acid : water (4:1:5) as the solvent system. The dried chromatogram was sprayed with aniline hydrogen phthalate and the paper is heated at 110°C for 10 min. when sugars appear as colored spots.

Aniline hydrogen phthalate was prepared by adding 0.9 ml of Aniline and 1.6 gms of phthalic acid to 100 ml of water saturated n-butanol.

k. Amino acids

Amino acids are colourless ionic compounds, their solubility properties and high melting points being due to the fact that they are zwitterions. They are all water soluble, although the degree of solubility varies. Since they are basic, they form hydrochlorides with conc: HCl and being acids they can be esterified.

The water fraction of the plant extract or the methanol extract was analysed on paper chromatogram with n-butanol : acetic acid : water (4:1:5). After the paper was dried, 1% Ninhydrin was sprayed and heated over a hot plate.

l. Volatile oils

Volatile oils have a characteristic scent, odour or smell and are found in a few plant families like the composite,

Myrtaceae. Rutaceae etc.

Fresh leaves collected were macerated and oils were extracted by keeping the tissue in solvent ether for one hour. The ether solubles were filtered and spotted on TLC plates. Chloroform : Benzene (1:1) was used as the solvent system. The terpenes were visualised by spraying the TLC plates with Vanillin-Sulphuric acid. The plate was then heated over a hot plate for 10 minutes when the different monoterpenes gave characteristic colours. Vanillin-H_2SO_4 reagent was prepared by adding 0.5 gm of vanillin and 2 ml of H_2SO_4 to 8 ml of cold ethanol.

1.6 PREPARATION OF SPORE SUSPENSION

The spore suspension was prepared in sterile distilled water from 10-15 day old fungal cultures. Under sterile conditions proper care was taken to ensure that maximum number of spores were transferred from the mycelial mat.

1.7 DROP DIFFUSATE TECHNIQUE (Harborne, 1982):

This is the procedure adopted for testing the phytoalexin response and isolating them from plants. In this technique leaves were floated on water and droplets of spore suspension of a pathogenic or non-pathogenic fungus were placed on the upper leaf surface.

A surface active agent such as Tween-20 was added to prevent the droplets from spreading over the leaf surface.

A second batch of leaves were set up at the same time with aqueous tween-20 droplets were collected. In the case of a positive response, the droplets are found to contain high levels of phytoalexin largely uncontaminated with other plant constituents. Thus in a very simple way, it is possible to obtain a relatively pure phytoalexin sample, which can be used for further investigation. In the leaf-droplet system, the fungal spores start germinating in the droplets within 1-2 hr and the resulting germ tubes then penetrate the host cells. As a consequence of this "trigger" the plant immediately responds by de novo synthesis of phytoalexins. Such compounds which are often detectable after several hours reach maximum production at 48 to 72 hours. The phytoalexins are themselves synthesized within the leaf but much material is 'pushed out' into the leaf surface where the fungal invasion has taken place; hence the accumulation of phytoalexin in the droplets. It has been argued that the drop diffusate technique does not provide a complete picture of phytoalexin production since certain substances may not diffuse from the leaf into the overlying droplets. However, tests have shown that for many species the same compounds are present in both the leaf and in the droplets although there may be quantitative differences if more than one compound is produced. The diffusate (control and treated) were analysed on TLC using ether: Hexane (3:1) or Toluene : Ethyl formate : acetic acid (5:4:1) as the solvent system and on PC using 15 % or 50% acetic acid as the solvent system. The TLC plates and paper chromatograms were observed

in ultraviolet light (360 nm) and the bands were eluted with methanol and the absorption spectrum was recorded using a UV spectrophotometer. The leaves (control and treated) used in the drop diffusate experiment was analysed like the healthy and infected leaves for post infectional changes (see 1.5).

1.8 FACILITATED DIFFUSION TECHNIQUE

Since the drop diffusate technique was not easily applied to large amount of tissue and the utility of the technique being diminished further by the relatively slow solubility of many phytoalexins in pure water, the facilitated diffusion technique was proposed by Keen (1978). It allows relatively efficient extraction of phytoalexins with little removal of pigments and other interfering leaf compounds. This was first tested with the soybean-*pseudomonas glycinea* host parasite system. (Keen and Kennedy, 1974).

Leaves inoculated with a pathogenic or nonpathogenic fungus were placed in Erlenmeyer flasks with approximately 15 ml/g (fresh tissue weight) of 40% aqueous ethanol. The flasks containing the plant tissue immersed in the ethanol solution were placed on a rotary shaker operating at approximately 110 cycles per minute at 25°C. The flasks were kept for shaking for a period of 4-6 hours depending on the thickness of the leaves. After the agitation by shaking, the leaves were removed by filtration. The filtrate were then concentrated

to approximately one-half volume at 45°C. The concentrated solution was then extracted twice with ethyl acetate. The fractions were then analysed by TLC using Toluene : ethyl formate: acetic acid (5:4:1) or ether : Hexane (3:1) as the solvent system and PC by using 15% or 30% acetic acid as the solvent system.

1.9 TLC BIOASSAY

Lactose-mineral salt medium (Frank and Somkuti, 1979): This liquid medium was used for spraying on TLC plates before an overspray of spore suspension in TLC bioassay tests:

Lactose	: 10 g
$KHPO_4$	: 5 g
NH_4Cl	: 2 g
NaCl	: 1 g
$MgSO_4.7H_2O$	: 0.25 g
Distilled water	: 1000 ml
pH	: 7.6

In order to confirm phytoalexin synthesis the droplet extracts collected from the drop diffusates were tested for fungitoxicity. This was done by TLC bioassay in which the two extracts (phytoalexin solution and water control) were developed by TLC in a suitable solvent like Ether-Hexane (3:1). After drying, the plate was sprayed with the Lactose-mineral salts medium or potato Dextrose medium. Then the plate was further sprayed with a fungal spore suspension prepared from 10-14 day old

cultures and was incubated at about 25-27°C. During this time the fungus grew over the whole place except where fungitoxic zones were present. The duplicate chromatograms were sprayed with a range of diagnostic reagents (eg: diazotised p-nitraniline solution-test for phenolic acids) in order to determine to which chemical class the phytoalexin belongs.

2.0 BIOASSAY OF ANTIFUNGAL ACTIVITY BY THE SPORE GERMINATION METHOD :

The ability of antifungal substances to inhibit the germination of spores of the test fungus is taken as a criterion to measure its concentration. The method described by Müller (1958) is routinely used in phytoalexin studies. Spore suspension was prepared from 7-10 days old cultures of the test fungus, grown on potato dextrose agar.

Care was taken to prepare the spore suspension in cold to prevent premature germination. 3% Water agar, 1% Formalin and Lactophenol-cotton blue solution were used as the reagents. The water agar mixture is enriched with traces of yeast extract when required to enhance the visibility of spores under a microscope. 10 ml of double sterilised water agar is poured into test tubes. The temperature of the agar medium is maintained at 40°C by placing the test tubes in a water bath. 1 ml of the spore suspension is added to each tube and the contents were mixed well. The seeded agar is poured into flat bottomed sterile 9 cm petri plates. The petriplates are stored in cold prior to use.

Suitable dilutions of the test solution was prepared. An appropriate control with either water or the solvent used for solubilising the test substance was maintained. 5 ml of the test solution was added into each watch glass arranged on enamel trays lined with moist filter paper. The seeded agar was cut into 5 x 5 mm blocks with a sterile stainless steel scalpal or disc cutter. One agar block was placed into each test solution. The tray was kept covered. The tray was incubated at room temperature or at 25°C in the incubator for 6-8 hours. After the incubation period, the spores are killed by adding 1% formalin or lactophenol-cotton blue solution. With the help of the microscope the spores are classified into germinated or non-germinated. The spores are said to be germinated if the germ tube is longer than the diameter of the spore. The percentage of inhibition of spores were calculated and the length of the germ tube was measured.

2.1 BIOASSAY TESTS

(A) Preparation of test solutions : 10 gm of healthy leaves were extracted with petroleum ether. The petroleum ether extract was fractionated with water to yield a petroleum ether fraction (A) and a water fraction (B). Both the fractions were made upto 100 ml by adding petroleum ether and water respectively and maintained as stock solutions. 2,5 and 10 ml of fraction A was added to three different petri plates containing PDA. The same was done with fraction B. For control,

5 ml of petroleum ether was added to the medium in the case of fraction A, while 5 ml of sterile water was added in the case of fraction B. The plates were kept in a sterile chamber for a few hours until petroleum ether evaporated. Three replicates were maintained for each test solution and control. The above procedure for the preparation of test solution was followed in the case of chloroform extract containing chloroform fraction (C) and water fraction (D) and Methanol extract containing methanol fraction (E) and water fraction (F) and water extract (G).

The centre of each petriplate was inoculated with an agar plug of the test fungus cut from a parent colony growing on PDA. The assay plates were incubated at 25° ± 2°C for a period of 6 days. Mycelial growth was calculated by measuring perpendcular diameter of three replicate colonies and subtracting the diameter of the mycelial plug used to inoculate the plates. The assay plates of the treated were compared with that of the control.

(B) Preparation of ppm solutions: Different ppm concentration (100,300,500 and 1000) of the test compound was prepared in sterile water. 4 ml of each test solution was added to the different petri plates containing PDA medium. In the case of control 4 ml of sterile water was added. Three replicates were maintained in each case. The rest of the procedure followed for the inoculation of the test fungus, incubation and measurement of mycelial growth are the same as mentioned above.