MATERIALS AND METHODS

1. PHYTOCHEMICAL STUDIES

The samples for the phytochemical studies were collected from fifteen years old mango trees growing in the Central Fruit Nursery of Baroda, Gujarat State. Healthy and infected inflorescences and leaves were collected from the same plant. The fungal pathogens were isolated from the infected samples and cultured on PDA medium. Pathogenicity tests were performed to confirm the Koch's postulates The pathogenic fungus was stained with lactophenol-cotton blue and identified under microscope.

Samples of both the healthy and infected inflorescences and leaves were analysed for qualitative changes in flavonoids, phenolic acids, tannins, saponins, proanthocyanidins, iridoids, alkaloids and steroids.

To prove the antimicrobial activity of certain compounds bioassay tests of mycelial growth, spore germination and chromatogram technique were carried out

1.1 PREPARATION OF MEDIUM

Formula for potato dextrose agar (PDA).

Potato - 200g

Dextrose - 20g

Agar - 15g

Distilled water - 1000ml

Two hundred grams of peeled potatoes were sliced into small pieces in 500ml of distilled water and boiled for abount 25 minutes. The broth obtained was then filtered through cheese cloth and the solution was made up to volume of 500ml. To this 20gm of dextrose was added. Simultaneously 15gm of agar was melted in 500ml of distilled water by heating on a hot plate. The potato broth

was poured into agar solution and restored to 1000ml with distilled water. The medium was then sterilized after adjusting the pH to 6.0, because the pH of the medium has been known to influence the germination of fungal spores. Although, in general, spores germinate with in 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 - 20ml

Lactic acid - 20ml

Glycerol - 40ml

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

1.3 ISOLATION AND CULTURE OF PATHOGEN

The samples collected from infected leaves inflorescences, roots, fruits and stem were washed thoroughly with distilled water and surface sterilized with ethyl alcohol. The fungus was cultured and maintained on PDA slants. The fungal cultures were identified in the lab itself. However for the sake of authenticity identification of the fungus was done at IARI, New Delhi and Agharkar Research Institute, Pune.

1.4 PATHOGENICITY TESTS

Healthy inflorescences, leaves, roots, fruits and stems of the plants were inoculated with the spore suspension or the fungal culture of the test fungus (Pathogen) after surface sterilising these organs with ethyl alcohol and kept them in plastic bags In the susceptible plants, the symptoms of disease

appeared after a few days of inoculation. Pathogenicity was then confirmed according to Koch's postulates (Koch (1882))

1.5 CHEMICAL ANALYSIS

Leaves and inflorescences were thoroughly washed in tap water followed by distilled water and dried in an oven at 60°C. The dried samples were powdered and stored in air tight glass bottles or plastic bags. The samples were then analysed for pre-infectional and post-infectional compounds. The various methods followed for the extraction and characterisation of compounds are presented below.

a. Flavonoids

For extraction, isolation and identification of flavonoids the following procedure was followed:

10gm of leaves/inflorescences powder was extracted in a soxhlet extractor with methanol for 48hrs till the plant material became colourless. The methanolic extract was concentrated to dryness in a waterbath 50ml of water was added to dry residue and the water soluble phenolic glycosides were filtered out. The filtrate was hydrolysed in a waterbath 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 (Mabry *et al.*, 1970, Markham, 1982; Harborne, 1984) 67 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 under 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 additon of various reagents were studied. The reagents using for such studies and their preparation are given below:

(I) Sodium methoxide (NaOMe)

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

(II) Aluminium Chloride ($AlCl_3$)

5gm of fresh anhydrous AR grade $AlCl_3$ (which appeared yellow green and reacted violently when mixed with water) was added cautiously to spectorscopic methanol (100ml).

(III) Hydrochloric acid (HCl)

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

(IV) Sodium acetate (NaOAc)

Anhydrous powdered AR grade NaOAc was used.

(V) Boric acid (H_3BO_3)

Anhydrous powdered AR grade H_3BO_3 was used.

The concentration of the sample solution prepared by eluting chromatogram 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 2-3ml of fresh stock solution of 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 2-3ml of fresh solution of the flavonoids, till about a 2mm layer of NaOAc remained at the bottom of the cuvette. The spectrum was recorded 2 minutes after the additon 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 curve, shifts (both bathochromic and hypsochromic) with different reagents, 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 then filtered The methanolic extract was banded on Whatman No 1 paper and the chromatogram was developed with water as the solvent system. Gycoflavones were visualized by their colour in UV and with 10% Na_2CO_3 spray. Further analysis and identification were done using spectroscopic method as explained before.

b. Phenolic acids

The phenolic acids are extracted in ether along with flavonoid aglycones from the hydrolysed extract (fraction A and B) of the sample

Analysis of phenolic acids in the combined ether fraction (A and B) was carried out by two-dimensional ascending paper chromatography method. Benzen: 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 diazotized suphanilic acid, followed by an over spray of 10% Na_2CO_3 (Ibrahim and Towers, 1960)

Diazotization: 0 7gm of p-nitra-aniline/suphanilic acid was dissolved in 9ml of HCl and the volume made up to 100ml. 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 4ml of p-nitra-aniline/sulphanilic acid stock solution to the cooled $NaNO_2$ solution The volume was made up 100ml 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.

c. Tannins

Tannins were extracted in water by boiling 5gm plant material in 50 ml water. To this 2% freshly prepared gelation solution was added. The formation of a white (or milky) precipitation showed the presence of tannins in the material (Hungund *et al.*, 1971).

d. Saponins

About 5gm of the powdered leaves/inflorescences material was boiled in 50ml water for half an hour and then the 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 persistent froth of 1cm length in test-tube showed the presence of saponins (Hungund *et al.*, 1971). Foam formation may also occur even during aqueous extraction if the concentration of the saponins are more in the plant materials (Harborne, 1984)

e. Proanthocyanins

For testing proanthocyanins, about 5gm of finely chopped (fresh) plant material or 2gm dry powdered material was taken in a test-tube with approximately 5ml of 2M HCl. Hydrolysis was carried out by placing the test-tubes in boiling waterbath 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 proanthcyanins. An olive yellow colour represented a negative reaction (Gibbs, 1974).

f. Iridoids

The samples were tested for iridoids by a simple procedure based on the Trim-Hill colour test (Trim and Hill, 1951). Fresh or dry powdered leaves/inflorescence material (1g) was placed in a test tube with 5ml of 1% aqueous HCl After 3-6 hours, 0 1ml of macerate was decanted into another tube containing 1ml of Trim-Hill reagent (made up from 10ml acetic acid, 1ml of 0 2% $CuSO_4$ $5H_20$ in water and 0.5ml Conc HCl). When the tube was heated for a short time on a flame, a colour was produced if iridoids are present

g. Alkaloids

Five grams of powdered sample was extracted with 50ml of 5% ammonical ethanol for 48hrs. 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 denotes 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 60ml of distilled water and 5gms of KI in 10ml of water. The two solutions were mixed and diluted to 100ml with distilled water A few drops of this reagent was added

Wagner's reagent: (Potassium iodide) 1 27 grams of I_2 and 2grams of KI were dissolved in 5ml of water and the solution diluted to 100ml It gave brown flocculent precipitate with most of the alkaloids.

Dragendorff 's reagent: (Potassium bismuth iodide) 8grams of Bi $(NO_3)_3$ 5 H_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 supernatant was decanted off and made up to 100ml with distilled water

h. Steroids

The ethyl acetate fraction of the plant extract was analysed by thin layer chromatography using chloroform: carbon tetrachloride· 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-Burchard reagent was prepared by mixing 1ml of Conc. H_2SO_4, 20ml acetic anhydride and 50ml of chloroform. Steroids give characteristic colour which indicates their substitution

1.6 PREPARATION OF SPORE SUSPENSION

The spore suspension from 7-10 days old fungal culture was prepared in sterile distilled water (spore concentration 10^4/ml, Muller, 1958) To ensure the maximum transfer of spores from the mycelial mat proper care was taken under sterile conditions

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 was taken as a criterion to measure its concentration. The method described by Muller (1958) was routinely used in phytoalexin studies Spore suspension was prepared from 7-10 days old cultures of the test fungus, grown on potato dextrose agar medium Care was taken to prepare spore

suspension in cold to prevent premature germination. 3% water agar, 1% formalin and lactophenol-cotton blue solution were used as the reagent. The water agar mixture was enriched with traces of yeast extract when required to enhanced the visibility of spores under microscope 10ml of double sterilised water agar was taken into test tubes and maintained the temperature of the medium at 40°C. Then 1ml of the spore suspension was mixed well with the medium in each test tube. The seeded agar was poured into flat bottomed sterile petriplates

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. 5ml 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 5×5mm blocks with a sterile stainless steel scalpal or disc cutter After placing one agar block in each test solution, the tray was kept covered and incubated at room temperature or at 25°C in the incubator for 6-8 hours. After the incubation period the spores were killed by adding 1% formalin or lactophenol-cotton blue solution. With the help of microscope, germinated spore having germtube longer than the diameter of the spore were measured. The percentage inhibition of spore germination was calculated and the length of the germ tube was measured.

2.1 BIOASSAY TESTS

(A) Different concentrations (2ml, 4ml, 6ml, 8ml, and 10ml) of leachates obtained from drop diffusate technique was added in different petriplates containing PDA medium for mycelial growth assay (Kulshrestha and Chauhan, 1988). Three petriplates were maintained in each case. 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°c ± 2°c for a period of 6days. Mycelial growth was calculated by measuring perpendicular diameter of the mycelial plug used to inoculate the plates. The assay plates of the treated were compared with that of the control. For control 5ml of sterile water was added to petriplate.

(B) Preparation of ppm solutions: Different concentrations (200, 400, 600, 800 and 1000 ppm or 250, 500, 750 and 1000 ppm) of the test compound was prepared in sterile water. 4ml of each test solution was added to the different petriplates containg PDA In case of control 4ml of sterile water was added Three petriplates were maintained in each case and followed the procedures explained above

(C) By using chromatograms: Glass frame with fiber-glass and cheesecloth layer was kept on one of the baking dish with moist filter paper Other dish was kept inverted on top of the first one. Chamber was sealed with masking tape to maintain humidity in the chamber. Chamber was sterilized in an autoclave Under aseptic condition a chromatogram containing test substance was placed on the frame Chamber was tilted approximately 15° and spore suspension of the test organism was sprayed on the chromatogram. 50ml of warm water (60°C) was poured at the bottom of the chamber. Chamber was sealed and incubated for 36-48hrs After 48 hrs inhibitory zone on the chromatogram appeared as white zone on a dark back ground (area showing growth)

3.0 BIOCHEMICAL STUDIES

Healthy, treated and infected leaves, inflorescences and fruits were brought to the laboratory and washed thoroughly under running tap water For estimation of ascorbic acid, peroxidase activity, mangiferin and total phenol collected samples were analysed immediately.

Washed leaves, inflorescences and fruits were oven dried at 70°C for 48 hrs. Then they were powdered and sieved to obtain a fine powder. These samples were used for the estimation of carbohydrates (soluble sugars).

3.1 Ascorbic acid (AA)

Samples (100mg) were extracted in 5% metaphosphoric acid and 100% glacial acetic acid solution and centrifuged. Suitable aliquots of the supernatant were taken in a test tube containing known amount of metaphosphoric acid, di-nitrophenyl hydrazine and thiourea solutions The test tubes were kept for incubation for 3 hrs at 37°C on waterbath, after which the reaction was terminated by adding 85% sulphuric acid. Absorbance was read at 540 nm (Roe, 1964) Standard graph was prepared using pure ascorbic acid. Level of ascorbic acid was estimated and expressed in mg g^{-1} fr.wt.

3.2 Peroxidase (POD)

Samples (50mg) were extracted in 0.9% chilled potassium chloride and then centrifuged. Suitable aliquots were taken and added to test tubes containing acetate buffer (pH 5 5, 0.1M), o-diansidine (0 5%) and H_2O_2 (3%), enzyme reaction was allowed to run for 30s at room temperature. The reaction was terminated with addition of 5 N-H_2SO_4 The absorbance was read at 400nm against a blank (Shanon *et al.*, 1966) and enzyme activity was expressed in

units. One unit was defined as a change in absorbance by 1 0 occured in a minute. It was expressed as units $min^{-1}mg^{-1}$ fr wt

3.3 Carbohydrates (Total soluble sugars)

Soluble sugars were estimated by using anthrone (Mc Cready *et al*, 1968) Known amount of sample (50mg) were taken and extracted 4 times in 90% alcohol. The supernatants were collected in a petriplate and dried at 60°C These were used for the estimation of soluble sugars. Dried extracts in the petridish were dissolved in distilled water. They were filtered through glass wool Suitable aliquots were taken and anthrone reagent (Anthrone dissolved in 100% sulphuric acid) was added. These test tubes were kept in boiling water for 7.0 minutes. Later they were cooled and the absorbance was taken at 620nm Standard graph was plotted using glucose and values expressed in mg g^{-1} dr. wt

3.4 Total phenols

Samples (1g) were extracted in boiling ethyl alcohol for 5-7 minutes. Reextracted in 80% alcohol for 3minutes and filtered. Suitable aliquots were taken and folin-ciocalteu reagent followed by Na_2CO_3 solution were added. After thorough shaking the test tubes were left on boiling waterbath for exactly 1 minute. Then the test tubes were cooled under a running tap water and the blue solution obtained was diluted with 25ml distilled water. absorbance was read at 650 nm (Bray and Thorpe, 1954) Standard graph was prepared with pyrogallol. Values were expressed in mg g^{-1} fr. wt.

3.5 Mangiferin

Samples (100g) were macerated in acetone and filtered Filtrates were evaporated on boiling waterbath. Distilled water was added to the residue to make a suspension The suspension was then extracted with chloroform in a separating funnel At the interface of the aqueous and organic layer mangiferin was separated and collected by filtration and washed with chloroform followed by petrolium ether and ethyl acetate. The residue was dried in a oven and dry weight was taken (Ghosal *et al.*, 1978).

4. ANATOMICAL STUDY

4.1 Collection and Fixation of Samples

Infected and healthy leaves and wood from the main trunk were excised from the trees and immediately fixed in formalin propionic acid - Alcohol, FPA (Dring, 1971). The sample were then aspirated to remove air from the tissues After 24 hrs of fixation, the leaf samples were cut into small pieces and preserved in 70% alcohol till further processing

4.2 Microtomy and Staining

The fixed samples of leaves were embeded in paraffin and sectioned at 8-10 μm thickness in transverse plane using Leica rotary microtome. The sections were stained with periodic acid-schiff's reagent (PAS reagent) / toluidine blue 'o' and mounted in DPX following usual procedure (Berlyn and Miksche, 1976)

Small blocks of wood samples were microtomed at 15-20 μm thickness in transverse, tangential and radial longitudinal planes using Leitz sliding microtome The sections were arranged serially on a slide flooded with 70% ethanol and then tied with a fine cotton thread The sections were then stained

in saffranin-fast green combination and saffrain alone After staining, the sections were dehydrated through ethanol xylene series and mounted in DPX after removing the thread

4.3 HISTOCHEMICAL TECHNIQUES

Transections of leaves and radial longitudinal sections of wood were used for the histochemical localization of following metabolites: Starch with I_2KI (Johansen, 1940), Proteins with Coomassie Brilliant Blue R 250 (Ekalavya, 1979) and Phenolics by nitroso reaction (Reeve, 1951)

4.4 MEASUREMENTS

Vessel lumen diameter, gum-resin duct lumen diameter and thickness of cuticle were measured with an occular micrometer scale mounted in Carl Zeiss Ampliwal reasearch microscope The number of vessels per 1 square mm in transverse section of leaves and wood was counted on Metzer projection microscope For each parameter one hundred random measurements were recorded from healthy and infected tissues The readings were stastically analysed to determine the mean and standard deviation.

4.5 PHOTOGRAPHY

Photographs were taken from stained sections on Zeiss research microscope using ORWO NP 55 negative film. Black and white positive prints were taken using sterling special grade bromide paper. Coloured photographs were taken using Kodak gold 200 negative film