MATERIALS AND METHODS
Chapter-III

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STUDY SITE:

The present investigation was carried out in the Post Graduate Department of Botany, M.L.K. College, Balrampur. The Department is provided by all infrastructure facilities to conduct this type of work. The insect proof chamber is regularly fumigated by insecticide. The experiments on fractional analysis and chromatography were conducted in the Department. The chemical nature of the inhibitor was studied at NBRI and CIMAP Lucknow.

VIRUS INOCULUM:

The virus inoculum i.e., cucumber mosaic virus (CMV) and cucumber green mottle mosaic virus (CGMMV) were collected from the naturally infected Cucubita maxima and Legenaria siceraria from the fields. On the basis of host range, physical properties, mode of transmission, cross protection and serological relationship, these viruses have been identified as Tobamo virus. The culture of cucumber mosaic virus (CMV) and cucumber green mottle mosaic virus (CGMMV) were maintained on Cucurbita pepo by repeated mechanical inoculation and were put in an insect proof chamber.

HOST (TEST PLANT):

The Chenopodum amaranticolor Coste and Reyn, a local lesion host for both the viruses was used as test plant throughout the present investigation.

Raising of test plants:
Seeds of *Chenopodium amaranticolor* were sown in compost soil. Seedlings in two to three leaf stage were transplanted to 1” to 6” earthen pots filled with sterilized soil. After transplantation the pots were kept into insect proof glass house. Plants of the same age, height and vigour raised under similar conditions of manuring, watering and illumination were selected for experimental work.

**INOCULUM PREPARATION:**

For virus inoculum preparation, the young infected leaves showing severe symptoms were washed thoroughly with distilled water and excess of moisture was soaked with the help of filter paper. These leaves were macerated with distilled water (1ml/gm of leaf) in a sterilized postle and mortar. The extract was strained through two folds of muslin cloth and centrifuged at 5000 r.p.m for 10 minutes. The sediment was discarded and the clear supernatant fluid was suitably diluted with distilled water and used for inoculation.

**INOCULATION:**

For inoculation, the forefinger dipped in inoculum was applied gently and uniformly on the upper surface of the leaves in one direction along both sides of the midrib form the leaf base to the apex of the leaf of the test plant. Carborundum powder of 600 mesh was used as an abrasive, whenever required. It, was either sprinkled over the leaf surface or mixed with inocula prior to virus inoculation. After inoculation the leaves were washed with
distilled water. The amount of inoculum and abrasive applied were kept constant in each set of experiment. The plants inoculated with distilled water alone served as control. All the experiments were conducted in an insect free glass house under natural light conditions.

**VIRUS ASSAY:**

During present investigation local lesion method of Holmes (1929) was used for active virus assay. The treatments were arranged according to Randomized Latin Square System (Youden and Beale, 1937). To avoid plant to plant and leaf to leaf variation, half leaf inoculation technique was used. In this method one side of the midrib was rubbed with the treated sample where as the other half of the leaf was rubbed with the control sample. Care was taken so as to distributed the treatments and controls on right and left half, respectively.

The bark of different plants were washed, dried, weighed and grinded in a pestle and mortar with an equal amount of distilled water (w/v). The pulp thus obtained, was squeezed through cheese cloth and the clarified sap was inoculated on local lesion host *Chenopodium amaranticolor*. By comparing the local lesions which developed by treated sample with the number of local lesions that developed by control sample, the results were evaluated.

The results expressed in terms of per cent inhibition which was calculated according to the following formula.

\[
\text{Per cent Inhibition} = \frac{C-T \times 100}{C}
\]
Where,

C= number of local lesions on control half leaf.
T= number of local lesions on treated half leaf.
In= Per cent inhibition.

The experiments were repeated thrice whenever necessary to confirm the results.

**PLANTS SCREENED:**

1. *Acacia catechu* wild
2. *Albizzia lebbeck* Benth
3. *Artocarpus heterophyllus* Lamk
4. *Azadirachta indica* L.
5. *Aegle marmelos* (L.)
6. *Annona squamosa* (L.)
8. *Bauhinia varigata* L.
9. *Butea monosperma* (Lamk)
10. *Caesalpinea coriara* wild
11. *Cassia fistula*
12. *Cordia dicholoma* Forst. F.
13. *Collistemon citrunus* DC
14. *Casuarina equisetifolia* Forst
15. *Cinamomum Camphora* Nees & Ebrm.
16. *Citrus aurantium* L.
17. C. sinensis L.
18. Croton roxburghii Bal
19. Ficus benghalensis L.
20. F. elastico Roxb.
21. F. hispida L.
22. F. racemosa L.
23. F. religiosa L.
24. F. vinens Ait.
25. Jatropha curcas L.
26. Lawsonia inermis L.
27. Mangifera indica L.
28. Morus alba L.
29. Madhurca longifolia (Koen) Mac-Br.
30. Melia azedarach L.
31. Moringa olefera Lam
32. Nerium indicum L.
33. Nyctanthes arbor-trists
34. Ocimum sanctum L.
35. Pongamia pinnata L.
36. Polyalthia longifolia Benth.
37. Prunus persica (L.) Benth
38. Psidium guajava L.
39. Punica granatum L.
40. *Saraca indica* L.

41. *Shorea robusta* Gaerth.

42. *Strychnos nux vomica* L.

43. *Streblus asper* Lour

44. *Tamarindus indica* L.

45. *Tectona grandis* L.


47. *T. tomentosa* W.A.


49. *T. chebula* Retz.

50. *Zizyphus mauritiana* Juss.
INHIBITORY BARK EXTRACT PREPARATION:

Extracts of higher plants to be tested for inhibitors was obtained from barks which were washed with distilled water dried with blotting paper and crushed with equal amount of distilled water (w/v 1:1) in a pestle and mortar. The pulp thus obtained was squeezed through double fold of muslin cloth. The filtrate was centrifuged at 5,000 r.p.m. for 20 minutes to remove the cell debris and clear solution decanted. This supernatant was used for experimental purposes.

MODE OF TREATMENTS:

(a) In vitro combination:

The crude infective sap (viral inoculum) was mixed 1:1 with bark extract (test solution), shaken vigorously and kept for 30 minutes at room temperature before inoculation. Virus inoculum 1:1 with double distilled water served as control. The activity of the treated and controlled samples were tested by inoculating them on test plants.

(b) In vivo combination:

In this combination the upper surface of the half leaves of the test plants were treated with the bark extract to be tested by forefinger dipped in the solution, the other half of leaves rubbed with distilled water served as control. In pre inoculation treatments the infective virus inoculum was applied on the whole leaf surface at various time intervals after the application of bark extract while in post inoculation treatments, the whole leaf was first treated with the
virus inoculum before the application of bark extract on half leaf and distilled water on other half (control) at various time intervals.

For induced resistance studies, leaves of test plants were treated with bark extract and also distilled water which served as control. These were then washed with distilled water and inoculated with virus after 24 hrs. In several experiment, actinomycin D solution was rubbed on the leaves of test plants simultaneously and after 24 hrs following bark extract treatment. The control leaves were rubbed second time with distilled water. As the bark extract was found to act in a systemic manner, experiments in these studies were done with full leaves.

**ISOLATION OF INHIBITOR:**

Isolation and purification of the virus inhibitor was done as follows and as presented in.

**Solvent fractionation:**

For isolating the inhibitory active principle from the bark of *T. arjuna* one Kg of the dried and coarsely powdered bark material was filled in a percolator and sufficient ethanol was poured in it to cover the dried barks completely. After 24 hours at room temperature the alcoholic extract was taken out and concentrated in a distillation appellants to recover the excess alcohol. The bark material was again treated with this recovered alcohol and this process was repeated several times till the alcoholic extract was colourless. The alcoholic extract obtained in this cold extraction method was then concentrated in vacuum to get the residue (F1).
The brown viscous mass thus obtained was mixed with n hexane. The mixture was shaken vigorously in this flask and allowed to settle. The hexane soluble portion was separated and concentrated in vacuum (F 11). The insoluble mass was extracted with solvent ether and the ether soluble portion was collected separately (F III). The residue after extraction with ether was treated with chloroform and the chloroform soluble fraction (F IV) was separated out. The insoluble residue mass thus obtained was treated with chloroform methanol solution (CM 53:1). The organic layer soluble in CMS was separated out (F.V.). The insoluble residue mass thus obtained was treated with chloroform methanol solution was separated out (F.V.). The insoluble mass left after this treatment was extracted with n-butanol. The butanol soluble fraction (FVI) and insoluble mass left over fraction was collected separately.

Hexane, ether, chloroform, chloroform methanol soluble fractions were separately washed with water, dried with anhydrous sodium sulphate and solvents were removed in vacuum. The water washing in each treatment were collected, mixed and extract with butanol (FVII).

Fractions (FVI) and (FVII) were then mixed and solvent removed leaving a residue (FVIII).

All the fractions obtained were concentrated in vacuum at low temperature, till the solvent had been completely removed. The dried materials were dissolved in distilled water separately and tested for their inhibitory effect on the infectivity of the virus.
Chromatography:

From the different fractions collected, two fractions (FII and F III) showing maximum antiviral activity were examined by thin layer chromatography (TLC) and preparative thin layer chromatography (PTLC).

Silica gel GF= 204 was used for this purpose PTLC was carried out over plates (20 × 20cm) coated with 9-10g of silica gel. The spots on TLC/PTLC were visualized by spraying either with 1 per cent acidic potassium permagnate solution or 2 per cent ceric sulphate solution in 2N sulphuric acid.

The active fraction which was found to be a complex mixture of many compounds by TLC/PTLC was carefully chromatographed on silica gel column.

For column chromatography, a column of 90 cm length with 5 cm diameter was taken. A small plug of cotton-wool was inserted within the narrow neck of the tube. The column was then clamped in a vertical position.
Terminalia arjuna

Dried bark (1kg)
  ↓ Percolated with ethanol
  ↓ Ethanolic fraction (FI)
  ↓ Concentrated in vacuum
  ↓ Green viscous mass
  ↓ Extracted with hexane

Hexane extract washed with H₂O dried (anhyd, Na₂SO₄) & Solvent removed in vacuum Residue (A) (Tested) (FII)

Ether extract
  ↓ Washed with H₂O
  ↓ Dried (anhyd. Na₂SO₄) & solvent removed in Vacuum Residue (B) (Tested) (FIII)

CHCl₃ extracted washed with H₂O Dried (anhyd. Na₂SO₄) Solvent removed in vacuum Residue (c) (tested) (FIV)

Water washings obtained from Hexane, ether CHCl₃ & CHCl₃: MeOH extraction were mixed and then extracted with n-Butanol

Butanol mixed Extract (FVII)
  ↓ Butanol extract (FVI) solvent removed
  ↓ Residue (E) (tested)/(FVIII) water soluble

Insoluble mass
Extracted with ether

Insoluble mass
Extracted with CHCl₃

Insoluble mass
Extracted with CHCl₂ MCOH (3:1)

Insoluble mass extracted with n-butanol

Isolation of inhibitor from T. arjuna
Subsequently, a thick slurry of suitable consistency was prepared by mixing silica gel powder and hexane. It was gently heated with constant stirring. Finally the slurry was deaerated with the help of a vacuum pump. This slurry was poured in the vacuum tube in aliquotes with constant taping of the wall to ensure homogenous packing. Under no circumstances the column was allowed to dry. It was filled up to 3/4th length and enough n-hexane was allowed to percolate through it for 36 hours so as to cover the entire column. This resulted to a uniform column bed. The active fractions was mixed with appropriate amount of silica gel and acetone. The mixture was homogenised and acetone was allowed to evaporate. The dry residue thus obtained was added to the top of the column bed the remained residue adhering to the flask was washed out in the column adding n-hexane. It was subsequently eluted with hexane, benzene, ethyl, acetate a mixture of ethyl acetate and methanol and finally with methanol. The fractions collected were each of 70 ml. Progress of elution was checked by TLC examination of elvates. The fractions obtained were concentrated and tested for their inhibitory effect on the infectivity of the virus. Later on, the compounds present in these fractions were separated by PTLC.

**Spectral analysis:**

After the purification of the compounds, their spectral studies such as IR, UV, NMR and Mass spectrometry were done to identify the chemical nature of the compounds.
The UV spectra were recorded on Beck man model and Parki n-E lmer 202 automatic recording spectrometers, where as IR studies were carried out on Peckin- Elmer Infra- cord Instrument Model 157 and 177. The NMR Spectra were recorded on Parkin-Elmer R-32 Spectrometer (90 MHZ) instrument in CCl4 with TMS as internal standard. The mass spectra were taken on Hitachi RMU-SE and Jeol D-300 instruments.

The melting points were taken on sulphuric acid bath and Richart Thermover and are uncorrected.

**QUALITATIVE TESTS:**

**Test for alkaloids:**

To test the presence of alkaloids two ml of aqueous solution was mixed with dil. H2SO4. A few drops of the more sensitive mayer's reagent when added in the presence of an alkaloid produce a distant precipitate or turbidity. To prepare mayer's reagent 1.36 g of Hg Cl2 were dissolved in 60 ml of distilled water and 5 g of K1 in 10 ml of water. The two solutions were mixed and diluted to 100ml with distilled water (Cromwell, In peach and Tracy 1955).

**Test for phenols:**

The neutral aqueous solution of a sample gives violet red colourations with ferric chloride in the presence of phenol. In carrying out this test one drop of ferric chloride in the presence of phenol. In carrying out this test one drop of solution was added to the neutral solution to be tested. The ferrichloride color
formation at the interface was them observed (Clarke and Nord, In peach and Tracy, 1955).

**Test for proteins:**

The protein determinations was done by Biurette reaction. In this reaction one ml of sample and one ml of 10 per cent NaOH were mixed well. To this, one to two drops of 0.1 per cent CuSO₄ were added. Violet to pink coloration was produced in the presence of proteins (Mann and Sounders, 1969).

**Liebermann and Burchard test for sterols:**

Five ml of the sample was dissolved in 0.2 ml to 0.3 ml of chloroform to which 0.15 ml acetic acid anhydride was added. A positive test gave a greenish color (Mann and Sounders, 1969).

**Test for Carbohydrate (Molisch’s test):**

0.1 gm. of the substance was dissolved in 2 ml of water and mixed with 2 to 3 drops of 1 per cent alcohal solution of 1- naphthol (ignoring traces of the latter precipitated by the water). In this mixture 2 ml of cone. H₂SO₄ was carefully Poured down the side of the test tube so that it formed a heavy layer at the bottom. In the presence of carbohydrate a deep violet colouration was produced where the liquid met the acid (Mann and Saurders, 1969).