DISCUSSION
Chapter-V

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A virus inhibitor is an agent that interferes with the pathogenicity of a virus (Ragetli and Weintraub, 1974). According to them pathogenicity may be affected or inhibited \textit{in vitro} or \textit{in vivo}. \textit{In vitro} inhibition takes place when a virus is precipitated, dissociated or complexed. \textit{In vivo} virus is either prevented from establishing or prevented from multiplying. Inhibitors affecting virus integrity are termed as virus inactivators.

Earlier Bawden (1954) divided inhibitors of plant viruses into two categories.

A. Inhibitors of infection which prevent the initiation of infection in plants when inoculated to leaves simultaneously with viruses, and

B. Inhibitors of virus replication or virus increase that retard the rate of virus multiplication when applied to leaves already infected.

The extracts which are capable of reducing the virus infection and multiplication in certain host plants, when applied before the leaves are inoculated and are unable to do so to any extent when applied after virus inoculation have been considered inhibitors of infection and those which affect virus infection after 24 hrs treatment are considered as inhibitors of virus increase.
Subsequent observations by various workers have shown that there are substances that fall into both, the above categories i.e. they can act as inhibitors of infections as well as of virus increase (Ruppel, 1967).


During present investigation the bark extract of Acacia Catechu, Cinamomum Camphora, Citrus aurantium, Ficus elatstica, Ocimum sanctum. Psidium guajava, Temarindus indica. and Terminalia arjuna showed 80 percent or above inhibition of both the viruses (cucumber mosaic virus and cucumber green mottle mosaic virus). Maximum virus inhibitory activity (85.71 per cent) was obtained against cucumber green mottle mosaic virus with bark extract of T. arjuna. Therefore, a detailed study was undertaken with the bark extract of T. arjuna against the activity of CMV.

The bark extract of T. arjuna inhibited infection of CMV in hypersensitive host Chenopodium amaranticolor when applied prior to inoculation. The inhibitory activity was less when virus was inoculated soon after the bark extract application. A gradual increase in the inhibition activity was observed with an increase in time interval (0 to 24 hrs) between bark extract application and virus inoculation and the maximum inhibitory activity
was observed after 24 hrs. The activity then decreased when the virus was inoculated beyond 24 hrs of extract application.

Since the bark extract partially checked the activity of the virus on host leaf at different intervals prior to virus inoculation, it is presumed that this extract inhibits infection of virus particles by altering the susceptibility of host plants rather than affecting the virus particles directly.

Virus infection inhibition in hosts by plant extract when applied prior to inoculation with viruses has been reported by Ragelii (1958), Jones et al. (1959), Loebenstein (1960), Lucas and Wiggs (1963), Gupta and Raychaudhuri (1971 b), Thakur and Sartry (1971), Grohmann and Musumeci (1972) and Fischer and Nienhaus (1973).

Inhibitory activity soon after treatment and persisting for different days has been reported by Mc Keen (1956) Blaszczak et al. (1959), Paliwal and Nariani (1965), Ruppel (1967), Saksena and Mink (1969), Apablaza and Bernier (1972) and Nart (1972). Verma and Awasthi (1979) have however, reported that to bring about a significant decrease in lesion number of 4 viruses with *Boerhaavia diffusa* root extract, the minimum time of treatment required was 6 hrs, but maximum inhibition was observed after 24 hrs of treatment. After 144 hrs of treatment the inhibitory response of the extract was lost.

In another set of experiments when treatment with bark extract was done after virus inoculation, less inhibition was observed when bark extract was applied after 3 hrs of virus inoculation and it further decreased up to 48 hrs. Similar results have been reported by Gupta and Raychaudhuri (1971 b),
Fischer and Nienhaus (1973), Worms and Nienhaus (1975), Verma and Mukerjee (1975) and Verma and Awasthi (1979). El-Kandelgy and Wilcoxson (1966) have reported that an extract from flowers of *Trifolium pratense* inhibited the infection of *Gomphrena globosa* by red clover rein mosaic virus when mixed with the virus or when applied to the leaves before inoculation with the virus.

Application of extract, after the leaves were inoculated, did not interfere with the establishment of virus infection. In a few cases inhibition was observed when leaf extract was applied after 24 hrs of virus inoculation (Weerarante and Rich 1961, Tamura 1969).

Besides affecting local protection (inhibition) at treated site in different hosts against the virus, bark extracts induced systemic resistance also. Since the susceptibility of the upper untreated leaves of plants whose basal leaves have been treated with inhibitor was reduced, it would show that this type of protection was systemic in nature. It seems plausible that some substance (s) was/ were sytheised after the application of the extract which could be responsible for the development of resistance at the site of application and also at the remote site.

The development of systemic acquired resistance (Loebenstein, 1972a) or systemic induced resistance (Ross, 1961 a,b) in untreated tissues or parts of hypersensitive hosts after other parts have been infected with a virus has been observed by different workers (Gilpatrick and Weintraub 1952, Ross 1961 a,b, 1966, Loebenstein 1963, Kimmins 1969 and Nagaich and Singh 1970).
Systemic resistance induction by plant extracts and its persistence, however, is not common (Srivastava et al., 1976). The induction of such type of resistance by different plant extracts against a few viruses has largely been reported in hypersensitive hosts (Verma and Mukerjee, 1975, 1977, Srivastava et al., 1976 Verma and Awasthi 1979 and Verma et al., 1979 a,b,c). This type of resistance has now been shown in non hypersensitive (systemic) host also (Verma et al., 1979 b).

The pathogens other than viruses, such as bacteria and fungi are also reported to induce resistance in the plants (Mandryk, 1963, Hecht and Bateman 1964, Gill 1965, Klement et al., 1966 Hodgesen and Munro 1966, Albouy et al., 1969, Wheeler and Pircne 1969, Gupta et al., 1974 and Hanusova 1974). In addition, some of the products of microbial and synthetic origin also induced resistance against certain viruses. Native TMV protein (Loebenstein 1962). Phytic acid-Insitol-hexaphosphoric acid (Maia and Morel, 1965), Yeast RNA (Gicherman and Loebenstein 1968) mutistranded synthetic polynucleotide complex (Stein and Loebenstein, 1970) and Polyacrylic acid (Gianinazzi and Kassanis 1974) have been shown to have such a property.

Apablaza and Bernier (1972) have also reported inhibition of lesion development on primary leaves opposite to the treated leaves of bean plant by *Palargonium, Capsicum and Datura stramonium* leaf extracts and showed systemic resistance induced by these extracts.

During decapitation experiment, when test plant (*C. amaranticolor*) were decapitated and lower basal leaves were treated with bark extract 24 hrs
before, being inoculated with cucumber mosaic virus, it was observed that
inhibition of the virus in the upper leaves of respective hosts was much reduced
as compared with non-capitated plants showing the retarding effect of
decapitation on the movement of the extract. Holmes, (1929) also reported
similar findings.

The results of the decapitation experiment suggest that the inhibitive
agent as such or some protective substance formed after bark extract
application, may be transported from treated to untreated areas affording
protection in a systemic manner. Similar results were reported by Verma and
Awasthi (1979) who presumed that inhibition in Borhaavia diffusa root extract,
when applied before virus inoculation may induce synthesis of translocable
virus inhibitory or protective substance(s) in host plants. These findings are in
support with the results reported by Singh and Singh (1975), Singh (1984).

Reduced susceptibility of upper untreated leaves of plants whose lower
leaves had been treated with bark extract, showed that this type of protection
was systemic. If actinomycin D was applied simultaneously with bark extract,
inhibition was completely checked. No effect of actinomycin D was observed
when it was applied after 24 hrs of bark treatment. Similar observations have
also been reported by Singh, (1994).

The induced resistance reversal by simultaneous application of
actinomycin D with plant extracts on basal leaves was also observed by Verma
and Awasthi (1979) and Verma et al., (1979 aandb). The systemic resistance
induced in plants by yeast RNA, poly I.C. and poly carboxylates was also

Loebenstein (1972 a) Ross (1974) and Verma et al., (1979 b) reported that the induced resistance can be explained by the production of resistance inducing substance (s) in the extract treated plants. The actinomycin D experiments indicate that the development of induced resistance depends on the transcription mechanism of the cell from DNA to RNA thereby producing a resistance inducing substance.


Verma et al., (1979 a and b) suggested that the host genome presumably, in the hypersensitive and nonhypersensitive hosts, carries the necessary information for the development of resistance inducing substance (s) in a repressed state. Following treatment with certain viral or non viral agents the resistance mechanism may be activated perhaps by a de repression process. This could be because of presence of certain repressors in the system. The inhibitors may act as co-repressors which temporarily bind with these repressors and the non-functional part of genome function to produce substances, which often have been called antiviral factor or induced interfering agent or even new proteins. New proteins or products could include antiviral substances.

The bark extract of *T. arjuna* showed maximum activity at 1:1 dilution but it retained slightly lower activity at 1:5 dilution and there after the activity gradually decreased as the dilution increased. Persistance of inhibition at much greater dilution (10-3 –10-4) has been reported by Kuntz and Walker (1947) and Simons *et al.*, (1963) in *Spinach* and succulent plant extracts. Balevezak *et al.*, (1959) have also reported similarly that many plants extracts tried against potato virus x were only inhibitory at lower dilutions, Saxena and Mink (1969) observed a gradual decrease in the activity of apple chlorotic leaf spot virus on dilution by the leaf extract of *C. quinoa* and the *T. arjuna* bark extract retained its inhibitory activity for 36 hrs at room temperature, Weintraub and Willison (1953) found that inhibitory property of cucurbit extract was lost within 4-8 hours. Other plant extracts have been reported to be resistant to aging in vitro.

The inhibitor present in *T. arjuna* bark extracts was found to be nondialysable like inhibitor from pepper, cucumber and *chenopodium* (Blaszczak et al., 1959, Apablaza and Bernier, 1972).

The inhibitor(s) present in the non dialysable fraction of bark extract could not be precipitated by 95% ammonium sulphate and was readily soluble in hexane and solvent ether but not in chloroform and butanol. This suggested the non polar nature of the inhibitor in bark extract and it was confirmed by Liebermann and Berchard test.

The solvent fractionation of bark extract indicated that antiviral activity was concentrated in hexane and ether soluble fractions of ethanolic extract but some sort of antiviral activity was also observed in chloroform methanol, butanol and water soluble fractions showing thereby that such phenols or carbohydrate as may be present in bark extract contribute very little to the inhibitory activity.

During present investigation the non protein nature of the inhibitor in the hexane and ether fraction was indicated by its nonprecipitation by ammonium sulphate. Similar results were obtained when it was tested for coumarin. The positive results with Liebermann and Burchard test indicated its sterol nature. The test for substances likes alkaloids and carotenes which non polar in nature were negative.
The bark extract of *T. arjuna* yield five compounds A, B, C, D, and E which were terpenoid in nature, compound A was identified as B-sitosterol on the basis of spectroscopic data and chemical reaction, comparison of M.D., Co T.L.C. and superimposable IR with an authentic sample. The compound B was identified as an unsaturated triterpenoid as it responded to Liebermann Burchard test and gave a yellow colouration with tetrinitromethane. It was assigned the molecular formula C$_{30}$H$_{40}$O on the basis of its analytical data and mass pecturm (M+424). The compound C was identified as pentacyclic triterpenes, as it gave positive colouration in Liebermann Burchard test and no colour with tetrinitromethane for unsaturation. It was assigned the molecular formula C$_{30}$H$_{40}$O on the basis of its analytical data and mass spectrum. Compound D was assigned the molecular formula C$_{20}$H$_{20}$O$_{0}$ on the basis of its analytical dat and mass spectrum. It gave deep blue colour with Liebermann Burchard test showing it to be an unsaturated sterol. Compound E was assigned the molecular formula C$_{30}$H$_{56}$O$_{2}$ on the bais of its micro analysis and mass specturm (M+448). It again gave a positive test for unsaturated sterols. Thus, all the purified compounds A, B, C, D and E from *T. arjuna* bark extract were terpenoid in nature.

Among the five purified compounds, compound C and E showed maximum inhibition against cucumber mosaic virus in *C. amaranticolor* when applied 24 hrs before virus inoculation. This property indicates a correlation of activity between crude extracts and purified compound D and E and that it
involves an effect on the host plant rather than a reaction between the virus and inhibitory substance.

Compound C and E showed maximum inhibition at 1000 ppm concentration and it decreased with an increase in dilution. Similar results were obtained with crude bark extract by which an increase in dilution decreased the inhibitory percentage of cucumber mosaic virus *C. amaranticolor*.

The maximum inhibition with compound C and E was observed when the compound was unheated. There was a loss of inhibitory activity with increase in heating temperature. The compound also induced resistance in the upper untreated leaves when the lower leaves were treated with purified compounds showing their systemic nature. The resistance induced by compound E was more as compared to compound C.

During the present investigation, it appears that the mechanism of inhibition by bark extract involved the interaction of terpenoids with the host plants. The development of resistance in nontreated Parts showed the systemic nature of bark extract and the reversal of resistance by treatment with actinomycin D indicated the formation of a resistance inducing substance (s) and may have involved the transcription mechanism of the cell.