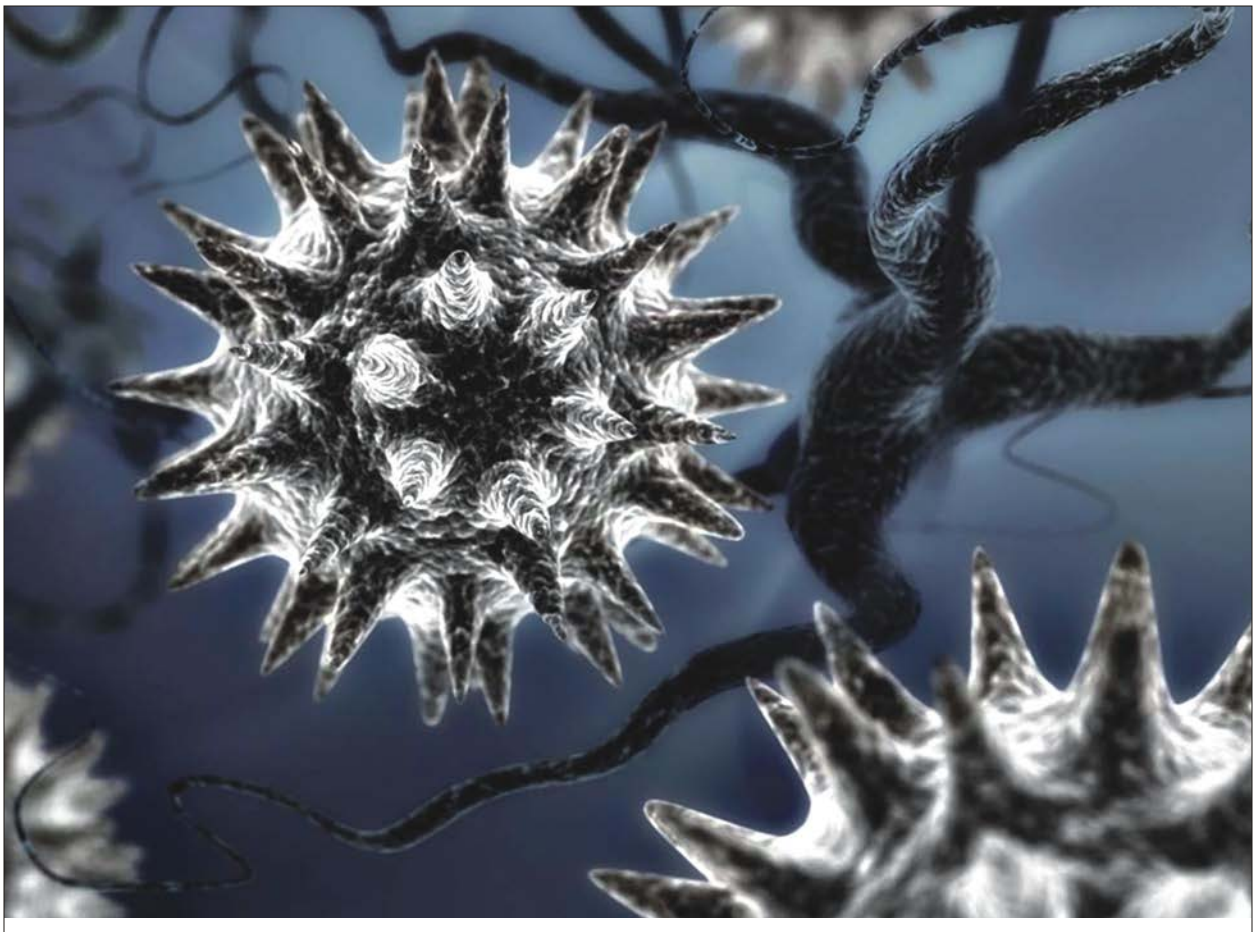

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Effect of Plant Extracts and Acetone Precipitated Proteins from Six Medicinal Plants Against Tobamovirus Infection*

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Abstract: The present study was taken up to evaluate the effect of leaf extracts and acetone-precipitated protein of medicinal plants on seed-borne Tobacco Mosaic Virus (TMV) and Tomato Mosaic Virus (ToMV) infection. The antiviral activity was tested on indicator plant *Nicotiana glutinosa*. Acetone precipitated proteins and solvent extracts of six medicinal plants were tested for their effect on tobamovirus infection. The aqueous leaf extracts of Guava, *Phyllanthus* and *Thuja* were effective in reducing the infection by ToMV. The acetone-precipitated fractions of *Tridax*, *Thuja*, Guava and Tulsi were effective in reducing the infection by TMV. The solvent extract of Guava was effective in reducing the ToMV infection. Guava extract was subjected to TLC and the fractions were tested for their antiviral activity. Fraction with R_F value of 0.014 proved to be effective in reducing the ToMV infection. The solvent extract of *Thuja* was effective in reducing TMV infection. TLC fraction of *Thuja* extract with the R_F value of 0.12 reduced the TMV infection. Along with this, effect of aqueous leaf extracts on seed quality parameters of tomato and bell pepper was studied.

Key words: Tomato, bell pepper, tobamoviruses, botanicals

INTRODUCTION

Tomato (*Lycopersicon esculentum* L.) and bell pepper (*Capsicum annuum* L.) are the two important members of family Solanaceae, which are used as vegetables worldwide. Tomato Mosaic Tobamovirus (ToMV) and Tobacco Mosaic Tobamovirus (TMV) are among the important viruses that infect these crops. Yield loss in tomato due to ToMV infection has been reported to be 3 to 95% and in bell pepper, the loss due to TMV infection is between 1 to 90% (Chitra *et al*., 2002). Chitra *et al*. (1999) reported the seed transmission rate of ToMV to be 1-13% and that of TMV to be 1-10%. Control of viral infection by the application of antiviral chemicals has not been successful (Verma *et al*., 1984). Several plant species have been screened as sources of antiviral factors. (McKeen, 1956; Verma *et al*., 1984; Manickam and Rajappan, 1999; He and Liu, 2004).

The botanicals may induce resistance or they themselves may act as inhibitors of viral replication. Ribosome Inactivating Proteins (RIPs) and glycoproteins may block the replication sites. A mobile inducing signal may be produced in treated leaves after the botanical resistance inducers bind with the host plant surface. This signal produces virus-inhibiting agent in the entire plant system. Certain low molecular weight pathogenesis related proteins might also play a role in the induction of systemic acquired resistance (Verma *et al*., 1998).

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Thus, biologically active compounds present in plant products act as elicitors and induce resistance in host plants resulting in reduction of disease development (Verma *et al*., 1998). The present work was carried out with an objective to screen promising botanicals and their fractions against seed-borne tobamoviruses. In this study, six botanicals *viz. Psidium guajava* (Myrtaceae), *Leucas aspera* (Lamiaceae), *Ocimum sanctum* (Lamiaceae), *Tridax procumbens* (Asteraceae), *Phyllanthus niruri* (Euphorbiaceae) and *Thuja occidentalis* (Cupressaceae) were screened for their antiviral activity against TMV and ToMV.

MATERIALS AND METHODS

Maintenance of Viruses, Host Plants and Indicator Plants

Seeds of Tomato PKM-1 (Ashoka Farm Aids) and Capsicum CW (Sultan Seed Farm) were obtained from local seed agencies. *Nicotiana glutinosa* seedlings (two month old) were maintained in an insect-proof screen house condition. TMV was obtained from infected bell pepper plants and ToMV was obtained from infected tomato plants maintained in the insect-proof screen house.

Preparation of Aqueous Plant Extracts and Acetone Precipitation of Proteins

Aqueous extracts were obtained by grinding 100 g fresh leaf of the plant species with 100 mL of phosphate buffer (0.01M, pH 7.2) (1:1 w/v) by using pestle and mortar. The extract was filtered through double-layered cheese cloth. The filtrate was sonicated and centrifuged at the rate of 10,000 rpm for 10 min. The supernatant was used for further studies. Proteins were precipitated from the aqueous extracts by using cold acetone. Four volumes of cold acetone was added to the supernatant. It was kept at 4°C overnight and centrifuged at 10,000 rpm for 15 min. The pellet was washed with acetone, centrifuged and dissolved in minimum amount of phosphate buffer (0.01M, pH 7.2). Protein concentration was estimated by the dye-binding method (Bradford, 1976).

Preparation of Solvent Extracts

Solvent extracts were obtained by grinding 10 g of the leaves in 10 mL of 95:5. Chloroform: Methanol (1:1 w/v). The extract was filtered through double-layered cheese cloth. The solvent was evaporated and the residue was dissolved in a known volume of methanol. This was used for further studies.

Isolation of Bioactives

Silica plates were prepared by preparing slurry of silica powder in water in the ratio 1:2 (w/v). This slurry was poured onto the plates with the help of an applicator. The slurry was coated onto the glass plates. The plates were allowed to dry for 15-30 min. These plates were then heated in an oven at 100-120°C for 1-2 h to remove the moisture and to activate the adsorbent (silica gel) on the plate (Sadasivam and Manickam, 1991).

Fractions were collected from the crude solvent extracts that were effective in reducing the virus concentration on the indicator plants of *N. glutinosa*. The solvent extracts were passed through a silica column (60-100 mesh) and 4 mL fractions were collected. The fractions were spotted onto a silica plate of 0.3 mm thickness. Presence of a single UV fluorescent band confirmed the purity of the fraction. Retardation factor (R_F) of each fraction was calculated using:

$$R_{F=}\frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

Treatment of the Plants

Precipitated Protein

Equal amount of protein (0.5 mg) was sprayed onto the leaves of *N. glutinosa*. Challenge inoculation with TMV or ToMV was done after 1 h.

Crude Extract

The methanol extract was sprayed onto the leaves of *N. glutinosa*. After one hour, challenge inoculation with the viruses was done. A control was maintained.

The silica gel fractions were tested for their antiviral activity. The fractions were dissolved in methanol and sprayed onto the leaves of *N. glutinosa*. After one hour, challenge inoculation with the viruses was done.

Challenge Inoculation of Viruses

The leaves of tomato and bell pepper infected with ToMV and TMV were ground well in pre-chilled mortar and pestle by using 10 mM Phosphate buffer (pH 7.2) along with carborandum powder (240-1000 mesh) as abrasive. The *N. glutinosa* leaves treated with the acetone precipitated proteins and solvent extracts of the plants were inoculated with the viruses by using a cotton swab dipped in the inoculum. The inoculum was then swabbed onto the leaves of the treated plants. The leaves were washed with water after 10 min of inoculation of viruses.

The leaves were inoculated and after three days the inoculated leaves were observed for the formation of necrotic local lesions. The number of local lesions on inoculated leaf/100 cm^2 was calculated using the formula:

$$\text{No. of local lesions/100 cm}^2 = \frac{\text{No. of local lesions}}{\text{Area of inoculated leaf}} \times 100$$

Effect of Aqueous Leaf Extracts on Seed Quality

Four hundred seeds of tomato and bell pepper were placed in conical flasks containing the plant leaf extracts (10% w/v). The flasks were kept on a rotary shaker for 24 h. Distilled water was used as control.

The treated seeds were subjected to germination test using the between paper method (ISTA, 2003). Vigour index was calculated using the formula.

$$VI = (MSL + MRL) \times \% \text{ germination}$$

Statistical Analysis

The data generated was average of three independent experiments having three replicates each. Data was subjected to Analysis of Variance (ANOVA) and the means were compared for significance using Duncan's Multiple Range Test (DMRT; $p = 0.05$).

RESULTS

Effect of Acetone Precipitate of Botanicals on Tobamoviruses

The antiviral activity was assessed based on the number of local lesions formed on control and treated *N. glutinosa* leaves. The leaves treated with acetone precipitate of Guava (100), *Tridax* (49) and *Thuja* (39) showed reduced number of local lesions due to TMV infection when compared to control (385) and other acetone precipitated fractions (Table 1). Guava and *Thuja* acetone precipitate also reduced the number of local lesions by ToMV. Tulsi fraction was also effective against ToMV.

Table 1: Antiviral activity of acetone precipitated proteins against TMV and ToMV

	No. of local lesions/100 cm^2±SE	
Treatments	TMV	ToMV
Control	385±4.0^b	108±2.2^c
P. guajava	100±3.3^d	32±1.4^e
L. aspera	380±3.7^b	176±2.8^b
P. niruri	345±2.7^c	215±3.6^a
T. procumbens	49±1.8^e	99±3.1^d
T. occidentalis	36±2.0^f	12±1.6^f
O. sanctum	411±3.0^a	16±2.0^f

Every value represents the mean of three experiments with Standard Error (SE). The values with different letter are significantly different according to DMRT (p = 0.05)

Table 2: Antiviral activity of solvent extract (crude) against TMV and ToMV

	No. of local lesions/100 cm^2±SE	
Treatments	TMV	ToMV
Control	193±1.0^f	100±1.8^f
P. guajava	431±0.9^c	66±0.8^g
L. aspera	365±0.8^d	220±1.0^b
P. niruri	1107±0.9^a	266±1.0^a
T. procumbens	329±1.3^e	200±1.9^d
T. occidentalis	92±1.0^g	181±1.6^e
O. sanctum	522±1.4^b	209±1.6^c

Every value represents the mean of three experiments with Standard Error (SE). The values with different letter are significantly different according to DMRT (p = 0.05)

Table 3: Antiviral activity of the silica gel fractions from *Thuja* leaves against TMV

R_F value	No. of local lesions/ 100 cm^2±SE
0.13	48±0.7^f
0.136	40±1.3^h
0.14	131±0.9^d
0.17	45±0.7^g
0.171	214±0.8^a
0.178	62±1.0^e
0.205	47±0.9^g
0.52	167±1.0^c
Control	200±1.3^b

Every value represents the mean of three experiments with standard error (SE). The values with different letter are significantly different according to DMRT (p = 0.05)

Effect of Solvent Extracts on Tobamoviruses

The solvent extracts were less effective in inhibiting the viruses. Only *Thuja* extract was effective in inhibiting TMV. The number of local lesions formed on *N. glutinosa* leaves treated with *Thuja* extract was 92 as compared to 193 in control (Table 2). Guava leaf extract was found to be effective in reducing the ToMV infection. The number of local lesions reduced to 66 in comparison to 100 in control (Table 2).

The fractions eluted from the silica gel column from the extracts of *Thuja* and Guava leaf extract were separated on TLC plates. The fractions having different R_F value were used in the study. In all the fractions, a single band was seen under UV, which confirmed the purity of the fractions.

The fractions of *Thuja* leaf extract having R_F value of 0.130, 0.136, 0.170, 0.178 and 0.205 showed reduced the number of local lesions to 40 to 62 in comparison to control (200) upon challenge inoculation with TMV (Table 3).

The fractions of Guava leaf extract having R_F value of 0.046, 0.120 and 0.420 showed reduced the number of local lesions to 115, 63 and 77 when compared to control (364) upon challenge inoculation with ToMV (Table 4).

Table 4: Antiviral activity of the silica gel fractions from guava leaves against ToMV

R_F value	No. of local lesions/ 100 cm^2±SE
0.04	198±0.9^{h}
0.046	115±1.0^{j}
0.08	326±0.4^{f}
0.093	370±0.8^{c}
0.12	63±0.9^{l}
0.178	339±0.4^{e}
0.38	707±0.8^{a}
0.42	77±0.6^{k}
0.43	601±0.8^{b}
0.52	225±0.5^{g}
0.89	166±1.0^{i}
Control	364±0.6^{d}

Every value represents the mean of three experiments with Standard Error (SE). The values with different letter are significantly different according to DMRT (p = 0.05)

Table 5: Effect of aqueous leaf extracts on seed quality

	Germination%±SE		MSL±SE		MRL±SE		Vigor index±SE	
Extract	Tomato seeds	Bell pepper seeds	Tomato seeds	Bell pepper seeds	Tomato seeds	Bell pepper seeds	Tomato seeds	Bell pepper seeds
Control	85±1.1^{b}	54±1.2^{a}	6.91±0.05^{c}	3.02±0.04^{d}	6.29±0.1^{bc}	4.16±0.06^{ab}	1122.76±2.4^{b}	392.93±6.1^{c}
P. guajava	56±0.8^{d}	47±0.8^{c}	6.63±0.1^{d}	3.00±0.07^{d}	6.61±0.1^{a}	2.87±0.05^{d}	748.17±9.9^{f}	276.58±3.3^{e}
L. aspera	84±0.5^{b}	53±0.8^{a}	7.25±0.04^{b}	3.86±0.04^{b}	5.63±0.03^{d}	4.24±0.05^{ab}	1082.45±5.7^{c}	421.08±11.8^{b}
P. niruri	71±0.8^{c}	56±0.8^{a}	6.61±0.06^{d}	5.19±0.02^{a}	5.76±0.1^{d}	4.30±0.02^{a}	886.52±2.0^{e}	534.91±6.3^{a}
T. procumbens	82±0.6^{b}	48±0.8^{c}	7.51±0.07^{a}	3.10±0.05^{d}	6.10±0.8^{c}	3.72±0.08^{c}	1125.86±7.4^{b}	332.43±4.9^{d}
T. occidentalis	72±0.8^{c}	52±1.1^{b}	6.69±0.1^{cd}	3.32±0.04^{c}	6.43±0.04^{ab}	4.01±0.1^{b}	936.47±11.2^{d}	381.31±3.2^{c}
O. sanctum	93±0.8^{a}	25±1.2^{d}	6.00±0.07^{e}	2.24±0.06^{e}	6.49±0.02^{ab}	2.95±0.1^{d}	1166.22±4.0^{a}	131.08±2.5^{f}

Every value represents the mean of three experiments with Standard Error (SE). The values with different letter are significantly different according to DMRT (p = 0.05)

Effect of Aqueous Leaf Extracts on Seed Quality

The seeds of tomato and bell pepper treated with aqueous leaf extracts had influenced the seed quality parameters. In tomato seeds treated with botanicals, the maximum germination was found when seeds were treated with the aqueous extract of Tulsi (93%) in comparison with control (85%) and other treatments. Seeds treated with aqueous extracts of *Leucas* and *Tridax* showed 84 and 82% germination respectively. But seed treatment with Guava leaf extract reduced germination to 56%. In tomato seeds treated with Tulsi, vigour index increased to 1166 whereas in Guava, it reduced to 748 from 1122. Maximum mean shoot length was observed in tomato seeds treated with *Tridax* (7.51) along with *Leucas* (7.25) in comparison to control (6.91) and other treatments. Tomato seeds treated with Guava (6.61), *Thuja* (6.43) and Tulsi (6.49) leaf extracts showed increased mean root length when compared to control (6.29) and other treatments (Table 5).

In bell pepper seeds treated with botanicals, increase in germination was noticed in seeds treated with extract of *Phyllanthus* (56%) in comparison to control (54%) and other treatments. Seed treatment with *Leucas* and *Thuja* extracts showed 53 and 52% germination, respectively. But seed treatment with Tulsi extract reduced germination 54%. Vigour was more in seeds treated with *Leucas* (421) and *Phyllanthus* (534) extracts when compared to control (392). In seeds treated with Tulsi extract, vigour reduced to 131. Maximum mean shoot length was observed in seeds treated with extract of *Phyllanthus* (5.19) in comparison to control (3.02) and other treatments. The mean shoot length increased in seeds treated with extracts of *Leucas* (3.86), *Tridax* (3.10) and *Thuja* (3.32). The mean shoot length was minimum in seeds treated with Tulsi extract (2.24) when compared with control and other seed treatments. The mean root length was maximum in seeds treated with extract of *Phyllanthus* (4.30) when compared to control (3.02) and other treatments. Seeds treated with *Leucas* extract showed a

mean root length of 4.24. The mean root length was minimum (2.87) when seeds were treated with Guava leaf extract. The mean root length decreased in seeds treated with *Tridax* (3.72), *Thuja* (4.01) and Tulsi (2.95) extracts in comparison to control (Table 5).

DISCUSSION

In the present study, an attempt was made to control TMV and ToMV by using certain plant extracts. The effect of these extracts on germination and vigour was also evaluated. Plant extracts have gained importance in the recent days because of their safety and target specificity. The plant constituents are easily bio-degradable, less phytotoxic and more systemic. Six different plant species viz, *Psidium guajava*, *Leucas aspera*, *Phyllanthus niruri*, *Tridax procumbens*, *Thuja occidentalis* and *Ocimum sanctum* were evaluated for their antiviral activity against ToMV and TMV. In a similar way, many workers have reported the use of some of these plants for the control of viruses (Mandal and Sigh, 2001; Jayashree *et al*., 1999).

Mandal and Sigh (2001) reported that Turnip Mosaic Virus was effectively inhibited by guava leaf extract. In our experiments also, the acetone precipitated fraction of guava inhibited both TMV and ToMV whereas the solvent fraction inhibited ToMV. Jayashree *et al*. (1999) reported that a plant derivative from *Thuja*, called Thuja 30 inhibited the pumpkin yellow vein mosaic virus. In our experiments, the solvent extract of *Thuja* inhibited only TMV whereas the acetone-precipitated fraction inhibited both TMV and To MV.

In our experiments, four of the six plants showed antiviral activity. The acetone-precipitated fractions of Guava, *Thuja* and Tulsi inhibited only TMV. *Leucas* and *Phyllanthus* did not show any inhibitory activity. Baranwal and Verma (1997) have reported the inhibition of TMV by leaf extract of C. cristata. Madhusudhan *et al*. (2005) have reported the antiviral activity of neem oil. At a concentration of 5%, neem oil reduced the concentration of TMV and ToMV. The leaf extract of *Celosia cristata* was found to prevent lesion production by sunnhemp rosette virus, TMV and PVX in several hosts (Baranwal and Verma, 1992). Wang and Tumer (1999) and He and Liu (2004) confirmed the RNAse activities of highly purified pokeweed antiviral protein and a recombinant cinnamomin A chain from *Cinnamomum camphora*. McKeen (1956) showed the inhibition of cucumber mosaic virus infection in untreated opposite primary leaf of cowpea whose other primary leaf was treated with extracts of *C. frutescens*.

The untreated upper leaves of *Cyamopsis tetragonoloba* were disease-free whose basal leaves were treated with leaf extract of different species of *Clerodendrum* a day before virus inoculation (Verma *et al*., 1984). Green gram leaf curl disease, which is caused by Tomato Spotted Wilt Virus (TSWV) was effectively controlled by using plant extracts and chemicals. Among 11 botanicals used, *Cocos nucifera*, *Sorghum vulgare*, *Euphorbia thyifolia*, *Prosopis chilensis*, *Croton sparsiflorus* were found to be effective in controlling Tomato spotted wilt virus when compared to control (Manickam *et al*., 1999). Almost all the virus inhibiting substances from higher plants are recognized as basic proteins. The roots, leaves and stem of *Mirabilis jalapa* show high inhibitory activity against plant viruses. Verma and Kumar (1980) showed that *M. jalapa* leaf extract suppressed the disease symptoms on a few systemic hosts when the extract was used as a foliar spray 24 h prior to virus inoculation. In our experiments, the leaf extracts of Guava, Tulsi, *Thuja* and *Tridax* reduced the concentration of the tobamoviruses effectively.

The antiviral activity was assayed by the number of lesions on the indicator leaf. The reduction in the number of lesions indicated the resistance of the plant to the virus. Verma *et al*. (1996) reported the antiviral activity of *C. aculeatum* against mechanically and white fly transmitted viruses by this method. Praveen *et al*. (2001) have reported the induction of resistance to be maximum when the inducer molecule was applied on plants before 40-60 min of virus inoculation. In our experiment, challenge inoculation with the virus was done 60 min after the treatment.

Methanol, chloroform and hexane extracts of *Maesa lanceolata* were found to effective against fungal plant pathogens (Okemo *et al*., 2004). In our experiment, Chloroform:Methanol (95:5) extracts of six test plants were screened against TMV and ToMV. Guava and *Thuja* crude extracts showed maximum reduction in local lesions formation in *N. glutinosa* challenge inoculated with ToMV and TMV respectively. The fractions of *Thuja* and Guava having R_F value of 0.12 and 0.014 showed maximum reduction in local lesions formed with TMV and ToMV, respectively when compared to control leaf and other fractions.

The seeds of tomato and bell pepper were treated with the plant extracts for 24 h at 10% concentration. The germination percentage and vigour index were evaluated. In some extracts, the germination and vigour index reduced. This discrepancy might be due to the phytotoxicity of the extract on seed quality. Few extracts did not effect of the treatment on the seed germination and growth, which may be due to the non-penetration of the active principle through seed coat.

The results obtained by this research supports that use of botanicals can be useful strategy to reduce the incidence of viruses. The botanicals may induce resistance or they themselves may act as inhibitors of viral replication. Thus, biologically active compounds present in plant products act as elicitors and induce resistance in host plants resulting in reduction of disease development.

Based on the results obtained in our studies, acetone precipitated proteins from leaves of Guava, *Tridax* and *Thuja* were effective against TMV where as *Tridax*, *Thuja*, Guava and Tulsi were effective against ToMV. The silica gel fractions isolated from *Thuja* and Guava having R_F value of 0.136 and 0.12 showed maximum reduction in local lesions formed on *N. glutinosa* challenge inoculated with TMV and ToMV, respectively.

The extracts of four plants and the silica gel fractions from two plants have proved effective in reducing the tobamovirus concentration on *N. glutinosa* leaves. Further studies like characterization of the metabolite, structure elucidation, formulations that can be used to treat the host plants etc. needs to be carried out.

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