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Research Article

Antiviral activity of aqueous extract of alligator plant, *Bryophyllum daigremontianum* L., against RNA and DNA plant viruses

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Abstract: Here we showed that aqueous extract of alligator plant, *Bryophyllum daigremontianum* L., is able to inhibit systemic and local infection of *Tobacco mosaic virus* (TMV: family Virgaviridae) in broad bean, *Vicia faba* L., nettle-leaved goosefoot, *Chenopodium murale* L., and tobacco, *Nicotiana tabacum* var. Turkish, *N. tabacum* var. Xanthi and *N. glutinosa* L., hosts. Antiviral activity of the extract was retained for a period of 8 days on treated broad bean and tobacco leaves. This activity was negatively correlated with the extract concentration, and it was completely lost by washing the treated leaves of tobacco plants 2 h post application, and was ineffective when applied 24 h post inoculation. No inhibitory effect was found against agro-inoculated strain of *Beet curly top virus* (BCTV: family *Geminiviridae*) on sugarbeet, *Beta vulgaris* L., seedlings. To determine the antiviral agent, *Bryophyllum* bulk protein designated BBP was isolated from the extract. BBP exhibited RNase activity against total RNA of TMV-infected tobacco tissues and genomic RNA of TMV while it failed to degrade genomic DNA of BCTV. Additionally, BBP completely inhibited TMV on *N. glutinosa* leaves at concentration of 40 µg/ml. These results suggest that a ribonuclease is mainly responsible for antiviral activity of alligator plant extract. To our knowledge, this is the first report on inhibitory effect of alligator plant extract on a plant virus. This plant species can be considered as a promising source for antiviral proteins in order to develop plant-derived compounds for effective control of plant mosaic diseases caused by TMV.

Keywords: *Tobamovirus*, *Curtovirus*, RNase activity, virus inhibition, antiviral protein

Introduction

Plant viruses cause a large number of diseases in different crops, trees and ornamentals around the world and reduce the yield significantly. Due to various ways of transmission, rapid dissemination and lack of curative practices, the effective control of viral diseases is troublesome. Two major methods including the use of host genetic resistance and vector-targeted chemicals have been used to reduce the losses. However, the large expenses of production of resistant cultivars and environmental aftermaths of chemicals restrict these methods. Moreover, the emergence of

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novel viruses and chemical-resistant vectors would negatively affect the aforementioned methods. It is therefore required that effective and environment-friendly methods are developed to control the viral diseases.

Diverse plant-derived proteins have been shown to have antiviral effects against human and animal viruses which include peptides, ribonucleases, pathogenesis-related (PR) proteins, and ribosome-inactivating proteins (RIPs) as well as natural proteins (Pushpa *et al*., 2013; Ilinskaya and Shah Mahmud, 2014; Wong *et al*., 2014; Schrot *et al*., 2015). However, there are only a few studies on the antiviral effects of plant-derived compounds against plant viruses a majority of which has focused on the non-protein compounds rather than investigation of active antiviral proteins (Dayan *et al*., 2009; Kwon *et al*., 2011; Verma and Baranwal, 2011; Bezic *et al*., 2013; Iftikhar *et al*., 2013; Milad *et al.*, 2014).

Alligator plant, *Bryophyllum daigremontianum* L., is a perennial succulent plant from the family Crassulaceae, originated from Madagascar and introduced to tropical and subtropical areas such as USA, Puerto Rico, Hawaii, Venezuela, Spain, Australia, New Caledonia and Canary Islands (Herrera *et al.*, 2016). The members of *Bryophyllum* include a large number of species which are used as ornamental or medicinal plants (Wächter, 2011; Milad *et al.*, 2014). *Bryophyllum* extract has been frequently used to treat many human diseases such as periodontal diseases, external and internal infections, skin diseases, bruises, wounds, boils, insect bites, inflammatory diseases, coughs, cytotoxic activity, headaches, arthritis, gastric ulcers and rheumatism (Siddiqui *et al*., 1989; Liu *et al*., 1989; Rossi-Bergmann *et al*., 1994; Mourao *et al*., 1999; Akinpelu, 2000; Kuo *et al*., 2008). The methanolic extract of the leaves of *B. daigremontiana* were demonstrated to have insecticidal effects (Supratman *et al*., 2001). Additionally, the crude extract of these plants has antiviral effects as eight species have shown inhibitory effects against human viruses including Human alpha herpesvirus (HHV) and vaccinia virus (VACV) (Cryer *et al*., 2017), Epstein-Barr virus (Greer *et al*., 2010), hepatitis C virus (Aoki *et al*., 2014), Entrovirus and coxsackievirus (Shirobokov *et al.*, 1981; Wang *et al*, 2012). The first antiviral study on *Bryophyllum* extract revealed that the antiviral effecter mains stable under different treatments including ether, alcohol and potassium periodate while it was suppressed by adding cattle serum or purified proteins to the juice (Shirobokov *et al.*, 1981). Another antiviral research showed that *Bryophyllum* leaf extract inhibits *in vitro* replication of Enterovirus 71 and coxsackievirus A16, however the exact compound responsible for the antiviral activity was not determined (Wang *et al.*, 2012). No study has been carried out on antiviral activity of *Bryophyllum* species against plant viruses. This study was aimed to identify any antiviral activity of aqueous extract of *B. daigremontiana* against two economically important plant viruses i.e. *Tobacco mosaic virus* (TMV) and *Beet curly top virus* (BCTV). Attempt was made to find out the main mechanism by which alligator plant extract inhibits TMV infection within plant host.

Materials and Methods

Plant materials

Alligator plant, *B. daigremontiana*, was collected from the botanic greenhouse of Horticulture Department, Agricultural Sciences Natural Resources University of Khuzestan, Khuzestan province, Iran. Experimental and natural hosts of TMV including: tobacco, *Nicotiana tabacum* var. Turkish, *N. tabacum* var. Xanthi and *N. glutinosa* L., broad bean, *Vicia faba* L., and nettle-leaved goosefoot, *Chenopodium murale* L., were used. Also, sugar beet, *Beta vulgaris* L. var. Zarghan, was chosen in this study as it is a systemic susceptible host of BCTV (Ghodoum Parizipour *et al*., 2013). Seeds of these plantswere sown in pots containing cocopeat and perlite (1:1) and

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irrigated daily. The emerged seedlings were transferred into new pots containing peat moss and soil (1: 2) and kept at greenhouse conditions: 25 °C, 16: 8 h photoperiod, and 40% relative humidity.

Preparation of aqueous extract of *B. daigremontiana*

Fresh leaf tissue of *B. daigremontiana* was homogenized in 100 mM potassium phosphate buffer [pH 7.0] at 4 °C and the homogentae was passed through 0.45 µm filter. The filtrate was then lyophilized using a freeze dryer machine (IWAKI FDR-50P) and the resulting powder of leaf aqueous extract was transferred to sterile tubes and stored at -20 °C.

Virus source

An Iranian isolate of TMV collected from symptomatic plants in tomato fields of Shush, Khuzestan province, Iran, was used in this study. The virus was first purified through serial inoculation of *N. glutinosa* plants with local lesions developed on their leaves and then propagated and maintained on *N. tabacum* var. Turkish plants. The source of viral inoculum was an infectious clone of the Iranian isolate of BCTV (BCTV-Svr): the *Sph*I/*Eco*RI head-to-tail 1.7 mer construct in the binary transformation vector pBin20 (pBin-1.7BCTV-Svr) (Ebadzad Sahrai *et al*., 2008).

Antiviral bioassay

Tobacco mosaic virus

TMV hosts which had been shown to develop local lesion, *N. tabacum* var. Xanthi, *N. glutinosa* and *V. faba*, and systemic mosaic, *Nicotiana tabacum* var. Turkish and *C. murale,* due to the viral infection, were used for bioassay of antiviral activity. The aqueous extract (200 mg/ml) of alligator plant and TMV inoculum (85 mg/ml) were prepared by fresh tissue extraction in 100 mM potassium phosphate buffer [pH 7.0] (1:5 and 1: 13, w: v, respectively). The homogenates were clarified by centrifuging at 5000 rpm for 10 min and mixed equally before inoculation. The mixture was mechanically inoculated on leaves of *V. faba*, *N. glutinosa*, or half leaf of *N. tabacum* var. Xanthi. One leaf of each plant was inoculated with buffer as control (Rasoulpour *et al*., 2017). Six replicates were considered for each treatment. The inoculated plants were kept at greenhouse conditions and inspected every 24 h for symptom development. The plants which did not show any visible symptom were subjected to indirect enzyme-linked immunosorbent assay (Indirect-ELISA) (see below). The percentage of virus inhibition was determined using the equation: $I = [(C–T)/C] \times 100$, in which I is inhibition percent, C is the number of infected plants (for hosts showing systemic mosaic) or local lesions (for hosts showing local lesion) produced among control plants and T is the number of infected plants or local lesions of treated plants (Verma *et al*., 1996).

Beet severe curly top virus

Agrobacterium tumefaciens (strain C58) cultures harboring the infectious construct pBin-1.7BCTV-Svr were grown at 28 ºC for 24 hand diluted to a concentration of optical density of 1.0 at 600 nm (4.5×10^7 cell/ml). Sugar beet seedlings at 4-6-leaf stage grown under greenhouse conditions were inoculated by injection of 50 µl of diluted culture into the crown of each plant (Ghodoum Parizipour *et al*., 2013). The bacterial suspension was mixed with an equal volume of aqueous extract (200 mg/ml) of alligator plant and agro-inoculated into the sugar beet plants. Bacterial culture mixed with an equal volume of LB medium instead of aqueous extract of alligator plant was used as control. Four plants were used for each treatment. The agro-inoculated plants were kept in the greenhouse for symptom development. Newly emerging leaves were sampled 21 days post inoculation and subjected to DNA extraction and polymerase chain reaction (PCR) using two specific BCTV-Svr primers, $p358^V$ (5′-GTGGATCAATTTCCAGACAATTA TC-3′) and $p853^C$ (5′-CCCCATAAGAGCC ATATCAAACTTC-3′), designed to amplify a 519 bp fragment of coat protein (CP) of BCTV-Svr (Eini *et al*.,2016). The aforementioned method was used to determine the percentage of virus inhibition.

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Characterization of antiviral activity of alligator plant extract against TMV

Different dilutions of 20, 15, 10, 5, 2.5,1, 0.5, 0.25 and 0.1% were prepared from alligator plant extract and used for superficial treatment of tobacco, *N. tabacum* var. Xanthi and *N. glutinosa*, and broad bean, *V. faba,* leaves. After two hours, the treated plants were mechanically inoculated with TMV and the number of local lesions were counted 48 hours post inoculation. Inoculation buffer (100 mM potassium phosphate) was used to treat the plant leaves as control.

To test the durability of antiviral activity of alligator plant extract, tobacco, *N. glutinosa* and *N. tabacum* var. Xanthi, and broad bean leaves were pre-treated with 200 mg/ml of alligator plant extract and mechanically inoculated with TMV at 2, 4, 6 and 8 days post treatment. Tobacco and broad bean leaves of similar sizes were mock-inoculated with buffer at the same time intervals and were used as control. The local lesions emerged on inoculated leaves were recorded and statistically analyzed.

In another experiment, the TMV-inoculated *N. tabacum* var. Turkish and *C. murale* plants were treated with aqueous extract (200 mg/ml) of alligator plant 24 h post inoculation. The inhibition percent was calculated as described before.

Indirect-ELISA

Systemic infection of *N. tabacum* var. Turkish and *C. murale* plants with TMV was assessed by Indirect-ELISA with polyclonal antibody against TMV coat protein (BIOREBA, Switzerland) using the method described by Clark and Adams (1977). Samples with an optical density (OD) greater than $M + 3SD$ (M = mean OD value of negative controls, SD = mean standard deviation value of negative controls) were considered positive.

Virus purification

TMV was purified according to the method described by Bateman and Chant (1979) with some modifications. Approximately 40 g of infected tissuewas homogenized in 200 ml of 0.1 M potassium phosphate buffer [pH 7] containing 0.01% sodium metabisulphide. The extract was clarified by passing through four layers of cheese cloth. Approximately 20 ml of n-butanol was added to 200 ml of the clarified extract while vigorously stirring, and then shaked for 45 min. The mixture was centrifuged at 9500 rpm (rotor type SS-34) for 30 min and the pell et was discarded. Then 8 g of polyethylene glycol (PEG) 6000 (Sigma-Aldrich, Germany) was gradually added while stirring at 4 °C. After PEG was dissolved completely, the mixture was centrifuged as above. This time, the supernatant was discarded and the pellet was resuspended in 43 ml of 0.05 M potassium phosphate buffer [pH 7] and incubated at 4 °C for 1 h. Then the mixture was centrifuged for 15 min at 10000 *g*. Virus particles were isolated by adding 1.5 g of NaCl and PEG (1:1) and centrifuging at 10000 *g* for 15 min. The pell et was dissolved in 4.3 ml of 0.05 M potassium phosphate buffer [pH 7] and then centrifuged at 10000 *g* for 5 min. Finally, the supernatant containing partially purified TMV was stored at -20 °C. The quality and quantity of the virus preparation was assessed using NANODROP spectrophotometer (Thermo Scientific, USA).

Isolation of viral nucleic acid and protein

The viral nucleic acid and protein were isolated according the method described by Fraenkel-Conrat (1957). To assay the quality and quantity of each preparation, OD was measured using spectrophotometry.

Protein extraction

Around 20 g of apical fresh leaves of alligator plant were homogenized in the extraction buffer (0.2 M KH_2PO_4 [pH 7.2], 150 mM NaCl, 8 M urea, 1% [w/v], 3-(3-cholamidylpropyl)-dim ethylammonio-1-propane sulfonate [CHAPS], 10% [v/v] glycerol and 2 mM ethylene diamine tetra acetic acid [EDTA]). The homogenate was then centrifuged at 14000 rpm for 25 min at 18 °C and the supernatant was isolated and stored

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at 4°C for further experiments (Sharma *et al*., 2014). This protein suspension was designated *Bryophyllum* bulk protein (BBP).

RNase and DNase activity

A total RNA extraction kit (DENAzist Asia, Iran) was used to extract total RNA from TMV-infected leaf tissue of *N. tabacum* var. Turkish according to the protocol provided by the supplier. Reactions were made with 4 mg of total RNA in sterile distilled water and 250 ng of BBP in 10 mM potassium phosphate buffer [pH 7], in total volume of 10 ml and incubated at 37 °C for 1.5 h. Total RNA samples treated with water and boiled BBP were used as negative control. The viral RNA isolated from partially purified TMV preparation (see above) was also treated with BBP as described before.

To test DNase activity of BBP, one PCR-positive sample was subjected to rolling circle amplification (RCA) using Phi29 DNA polymerase from Illustra™ TempliPhi 100 Amplification kit (GE Life Sciences, USA) as described by Haible *et al.* (2006). The amplification was followed by emzymatic restriction to amplify and release the complete genomic DNA of BCTV-Svr. The enzyme *Aat*II whose restriction site is single-located on the genome was used to release complete gemonic DNA of the virus with the size of ~3.0 kb from the RCA-amplified DNA molecules. The viral DNA was sequently treated with BBP as described before. DNase I (Thermo Scientific, USA) was used as positive control in this experiment.

Bioassay of BBP against TMV

The antiviral activity of BBP was assayed against TMV in tobacco, *N. glutinosa*, plants. Approximately 50 µg/ml of BBP was applied onto plant leaves and then they were mechanically inoculated with TMV as mentioned above. Each tobacco half leaf served as one experimental unit. Virus inhibition was calculated as described before. As second treatment, BBP-treated leaves were washed using distilled deionized water (DDH_2O) 2 h post treatment. Three replicates were considered per treatment the experiment was repeated for two times.

Statistical analysis

The data were statistically analyzed using the paired Student's T-test in SPSS software (ver. 24.2). The data were shown as mean ± standard error.

Results

Antiviral activity of alligator plant extract against TMV and BCTV

Application of crude extract of alligator plant significantly inhibited TMV on tobacco, *N. tabacum* var. Turkish, *N. tabacum* var. Xanthi and *N. glutinosa*, broad bean and nettle-leaved goosefoot plants (Fig. 1). The details of virus inhibition in the inoculated plant host was summarized in Table 1. Accordingly, the highest level of antiviral activity was achieved in nettle-leaved goosefoot host where 86.08% of TMV inhibition was found. In contrast, the lowest level of TMV inhibition (51.45%) was observed inroad bean plants (Table 1).

Out of 5 sugar be et seedlings agro-inoculated with infectious clone of BCTV-Svr, 4 plants from treatment showed typical curly top disease symptoms, including upward leaf roll, enations on the lower side of the newly emerged leaves 30 days post inoculation (Fig. 2A). PCR assay showed that nearly all treated plants together with control plants were virus-positive, while one treated plant did not exhibit any viral infection (Fig. 2B). These results showed that application of alligator plant extract is not able to inhibit BCTV-Svr on sugar be et plant.

Characterization of antiviral activity of alligator plant extract against TMV

Alligator plant extract maintained its antiviral activity against TMV ata concentrationof 0.25% on pre-treated *Nicotiana tabacum* var. Xanthiand and broad bean plants (Fig. 3A). However, this activity was retained at a

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dilution of 0.1% on treated *N. glutinosa* leaves. This activity was negatively correlated with the concentration of the alligator plant extract as decreased concentration resulted in deteriorated anti-TMV activity. The lowest 50% inhibition concentration (IC_{50}) (10 mg/ml) was observed in *N. glutinosa* and the highest (200 mg/ml) in broad bean plants. The IC_{50} of 54 mg/ml was determined for inhibition of TMV in *N. tabacum* var. Xanthi plants (Fig. 3A). TMV inhibitionin of all tested hosts treated with 200 mg/m of aligator plant extract retained up to 8 days post treatment (Fig. 2B). However, this character was decreased in the course of time.

Furthermore, treatment of *N. tabacum* var. Turkish and *C. murale* plants with alligator plant extract 24 h post inoculation did not show any TMV inhibition (data not shown).

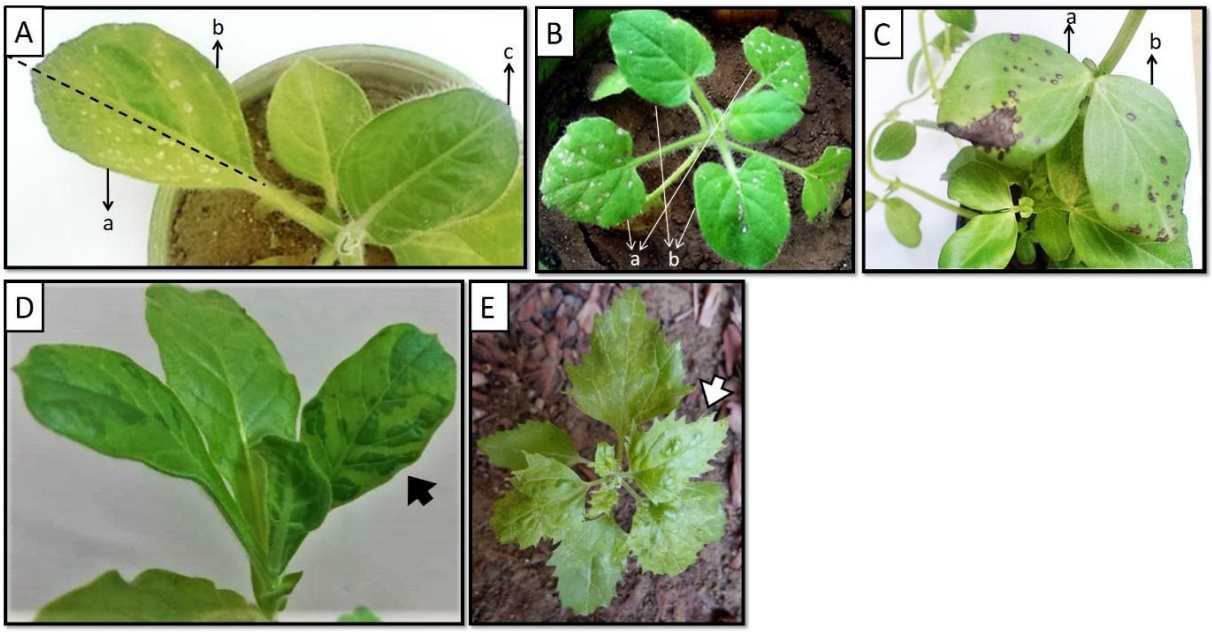

Figure 1 Inhibition of TMV activity by application of alligator plant extract (200 mg/m) on (A) *Nicotiana tabacum* var. Xanthi, (B) *Nicotiana glutinosa*, and (C) broad bean leaves. (a) control and (b) treated. Systemic mosaic caused by TMV on (D) *Nicotiana tabacum* var. Turkish and (E) nettle-leaved goosefoot plants.

Table 1 Antiviral activity of aqueous extract of alligator plant (200 mg/mL) against TMV.

Host	Disease parameter	Control[1]	Treatment[2]	Virus inhibition (%)	Significance
Nicotiana tabacum var. Xanthi	Local lesion	29.33 ± 0.88	6.66 ± 0.76	77.29	$P < 0.01$
Nicotiana glutinosa	Local lesion	37.50 ± 2.09	7.33 ± 0.76	80.45	$P < 0.01$
Vicia faba	Local lesion	33.33 ± 0.98	16.18 ± 0.47	51.45	$P < 0.01$
Nicotiana tabacum var. Turkish	Systemic mosaic	86.49 ± 1.11	12.88 ± 2.72	85.10	$P < 0.01$
Chenopodium murale	Systemic mosaic	81.54 ± 1.39	11.35 ± 2.16	86.08	$P < 0.01$

[1] Inoculated with TMV.
[2] Treatment with TMV and aqueous extract of alligator plant.

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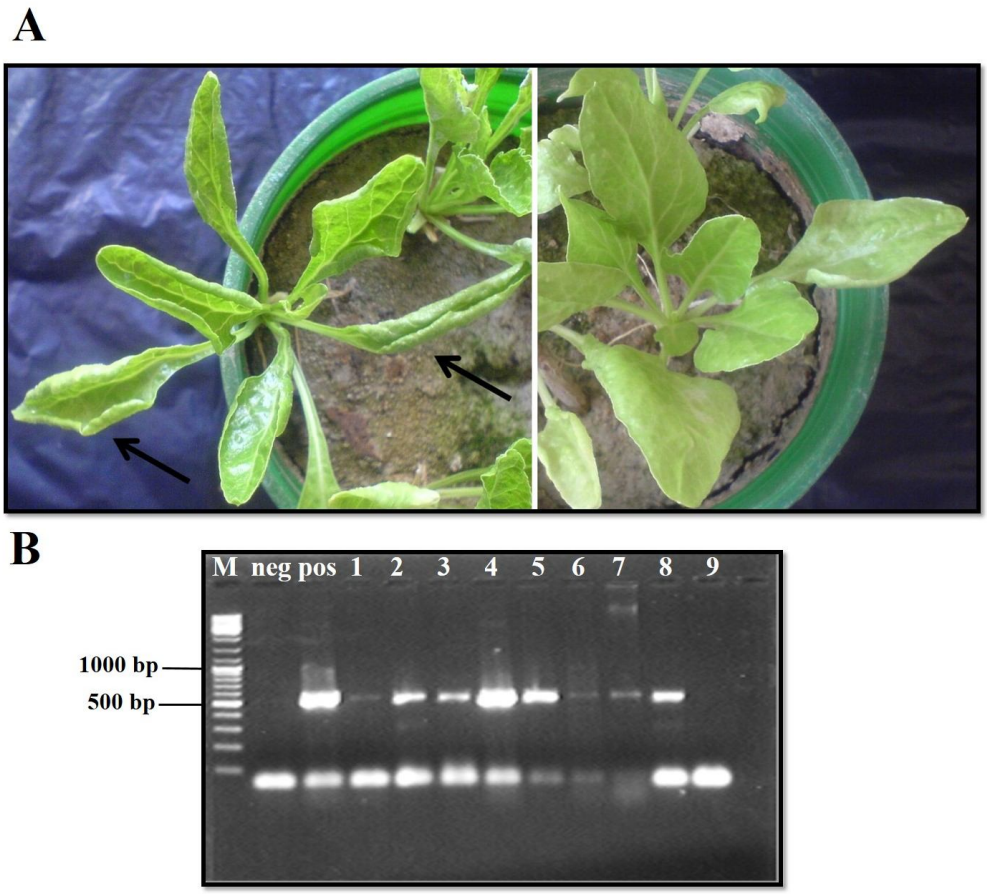

Figure 2 (A) Upward leaf role and enations (arrows) developed on the lower side of theleaves of sugar be et agroinoculated with infectious clone of BCTV-Svr, 4 weeks post inoculation (left). Sugar be et co-inoculated with infectious clone of BCTV-Svr and aqueous extract of alligator plant which did not exhhibit any clear symptoms untill 35 days post inoculation (right). (B) PCR detection of BCTV-Svr specific 500 bp DNA fragment in thesugar beet plants agro-inoculated with BCTV-Svr (lanes1-4) and BCTV-Svr mixed with aligator plant extract (lanes 5-9). pos: positive control, neg: neative control, M: DNA ladder (Thermo Scientific, USA).

Ribonucleic acid degradation properties of BBP

BBP was able to degrade total RNA that was extracted from TMV-infected tissues of *C. murale* and *N. tabacum* var. Xanthi; 4 mg total RNA of TMV with 250 ng BBP per reaction during a 1.5 h incubation time tested (Fig. 4A, lanes 3 and 6). DDH_2O-treated total RNAs (Fig. 4A, lanes 1 and 4) and total RNA samples treated with a boiled BBP that was used as control (Fig. 4A, lanes 2 and 5) did not show any degradation.

Degradation of TMV genomic RNA was observed when the antiviral protein was used in the reaction (Fig. 4B, lane 2). However, treatment of BCTV-Svr DNA with BBP did not result in DNA degradation (Fig. 4C, lane 3) compared to control samples.

Anti-TMV activity of BBP

Treatment of BBP (50 µg/ml) on inoculated half leaf of *N. glutinosa* resulted in a 100% inhibition of TMV so that no local lesion was developed till 3 days post inoculation (Fig. 5A). The antiviral activity was completely lost by washing the BBP-treated half leaf with DDH_2O 2 h post treatment as the number of local lesions developed on TMV-inoculated half leaves did not show any significant difference compared to control (Fig. 5B).

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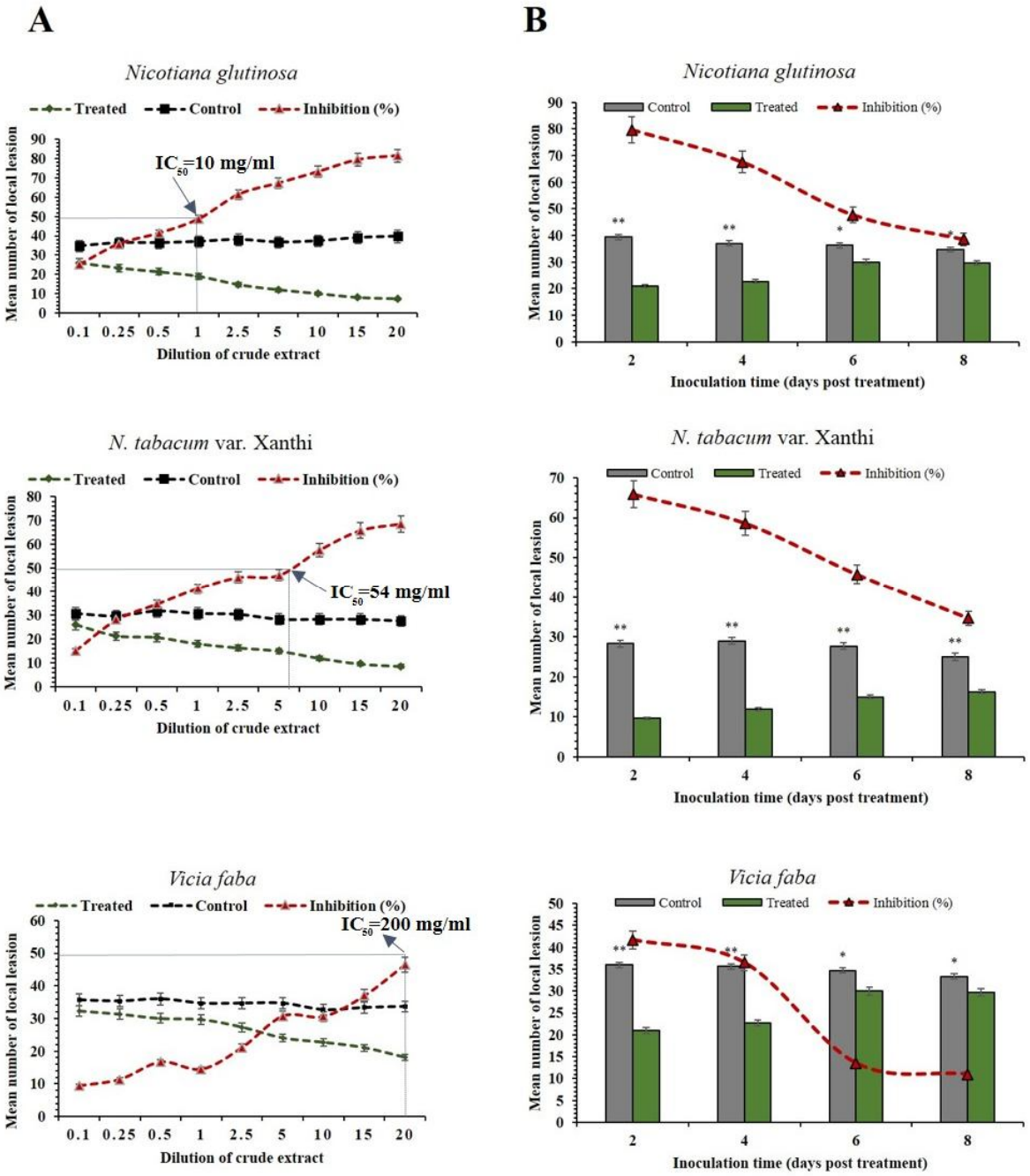

Figure 3 (A) Effect of different concentrations of aligator plant extract against TMV activity on tobacco, *Nicotiana tabacum* var. Xanthi & *N. glutinosa,* and broad bean, *Vicia faba*, leaves. The leaves were pre-treated with different concentrations of the extract and mechanically inoculated with TMV 2 h post treatment. (B) Durability of antiviral activity of the extract on the aforementioned plants. Plants were pre-treated with 200 mg/ml of alligator plant extract and mechanically inoculated with TMV at 2, 4, 6 and 8 days post treatment.
*: significant at the $p < 0.01$;
**: highly significant.
IC_{50} shows the concentration of the extract which caused a 50% inhibition of TMV.

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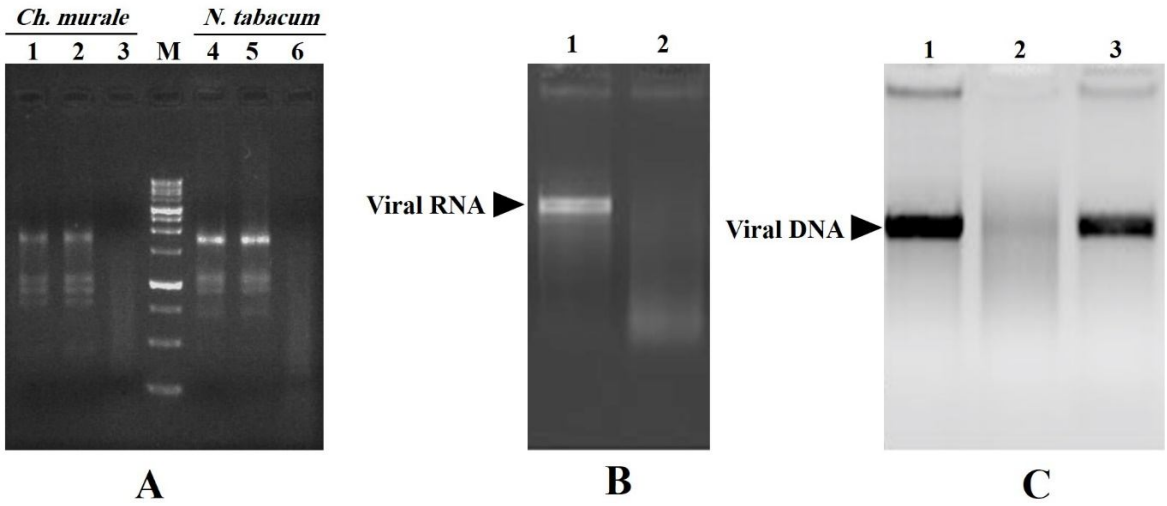

Figure 4 (A) Ribonucleic acid degradation activity test of BBP on total RNA extracted from plant tissues infected with TMV. Four mg of total RNAwas treated with DDH_2O (lanes 1 and 4), 250 ng of boiled (lanes 2 and 5) and native (lanes 3 and 6) antiviral protein 1.5 h post incubation. The host species are shown above the gel lanes. M: 100 bp DNA ladder (Thermo Scientific, USA). (B) Ribonuclease activity of BBP on TMV genomic RNA (lane 2) compared with DDH_2O-treated viral RNA (lane 1). (C) BBP did not exhibit any DNase activity on BCTV-Svr DNA (lane 1) compared to as negative control (a DDH_2O-treated [lane 3]) and positive control (a DNase I-treated [lane 2]).

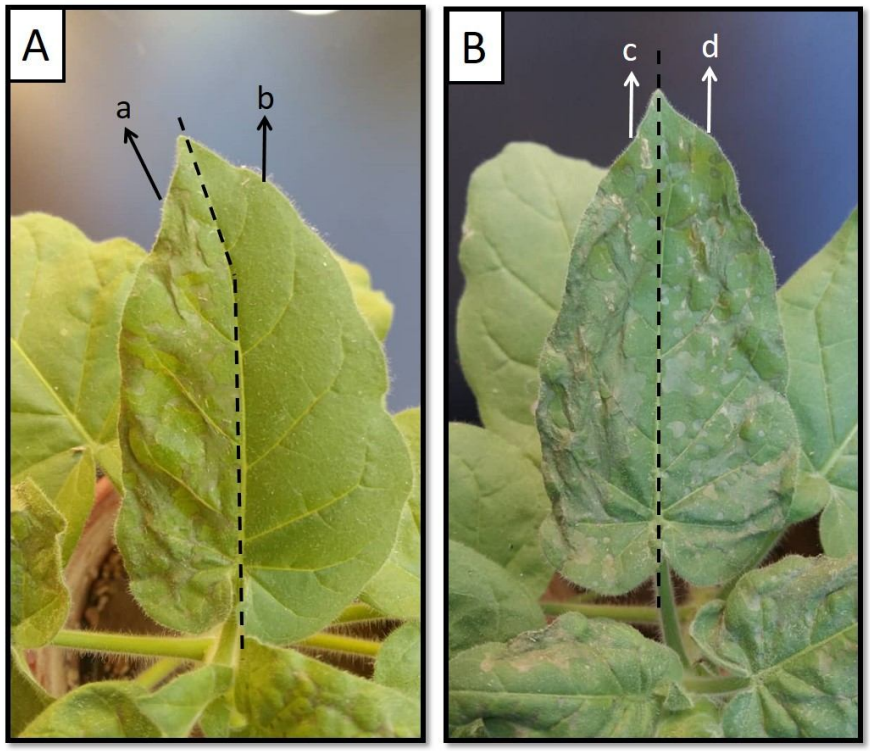

Figure 5 (A) Anti-TMV activity of BBP (50 µg/ml) in *Nicotiana glutinosa* plants 3 days after inoculation (b) compared to control (a). (B) The loss of antiviral activity by washing BBP-treated half leaf 2 h post treatment (d) compared to control (c).

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Discussion

Antiviral effects of plant extracts and plant-derived compounds against a number of plant viruses have been reported (Kwon *et al*., 2011; Verma and Baranwal, 2011; Bezic *et al*., 2013; Iftikhar *et al*., 2013; Milad *et al*., 2014; Rasoulpour *et al*., 2017;). Here we investigated the antiviral activity of alligator plant extract against RNA and DNA plant viruses, which have different genome organizations and genome expression strategies. Our results showed that application of alligator plant extract can significantly inhibit TMV activity in five plant species. Similar to the previous studies emphasizing the investigation of antiviral effects in both systemic and local lesion hosts (Rasoulpour *et al*., 2017; Rasoulpour *et al*., 2018), antiviral effects were assesed using two viruses. Results indicate infection rate in *Nicotiana tabacum* var. Turkish and *C. murale* and number of local lesions in *N. tabacum* var. Xanthi, *N. glutinosa* and *V. faba* were significantly reduced when the virus was treated with aqueous extract.

Alligator extract showed anti-TMV activity when applied as a pre-treatment or co-treatment with TMV on plants. However, post-treatment of broad bean and tobacco plants by alligator plant extract 24 h after virus inoculation did not inhibit the virus. These results demonstrate that the antiviral agent of alligator plant might enter epidermal cells and inhibit primary phases of virus propagation, as has been proposed for RIPs (Vandenbussche *et al*., 2004).

Reduced TMV inhibition was associated with reduced concentration of alligator plant extract which has been previously reported about other antiviral extracts (Rasoulpour *et al*., 2017; Rasoulpour *et al*., 2018). The virus inhibition, however, was found to be different among the plant hosts which might be due to variations in host response to the viral infection.

The loss of antiviral activity of alligator plant through washing suggests that viral inhibitor was not absorbed into plant leaves at a proper amount for virus inhibition.

No inhibition was observed for alligator plant extract against an agro-inoculated infectious clone of BCTV-Svr. This might be due to specificity of viral inhibitor of alligator plant RNA viruses. Furthermore, infectious clone and agro-inoculation system may not be suitable to test antiviral activity against a DNA virus which will require further investigations.

Antiviral proteins have been characterized in many plant species (Pushpa *et al*., 2013; Ilinskaya and Shah Mahmud, 2014; Wong *et al*., 2014; Schrot *et al*., 2015). Ribonucleases are an important group of antiviral proteins which can degrade the viral genome and therefore prevent the infection of host cell by the virus (Ilinskaya and Shah Mahmud, 2014).

When total RNA of TMV-infected tobacco was treated with BBP, the ribonuclease activity was detected. Similarly, RNA degradation was observed when viral RNA was treated with BBP. These results indicate the nuclease ability of alligator plant antiviral agent. However, BBP was not able to degrade viral DNA as judged by its inability to inhibit BCTV-Svr infection. Exogenous RNases are responsible for viral inhibition through three pathways: (1) separate penetration of virus particle and RNase; (2) simultaneous entrance of virus particle and RNase and subsequent inhibition of the viral infection by RNA degradation after virus disassembly; (3) virion inactivation (Ilinskaya and Shah Mahmud, 2014). Similar putative ways have been mentioned for virus inhibition by RIPs (Vandenbussche *et al*., 2004). BBP might apply mechanisms 1 and 2 for virus inhibition, as it was effective when used with virus inoculum, or by leaf pre-treatment 24 h before virus inoculation. Therefore, it may remain on leaf surface and enter epidermal cells along with virus particles at the time of infection, preventing virus replication and movement into adjacent cells by RNA degradation.

Ribonucleases obtained from living organisms such as animals and plants include RNase L, RNase A, quinqueginsin and panaxagin which are recognized as the main groups of proteins with antiviral effects against

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certain human viruses such as human immunodeficiency virus-1 and influenza viruses (Wang and Ng, 2000; NG and Wang, 2001; Ilinskaya and Shah Mahmud, 2014). Also, plant-derived ribonucleases, including the members of PR-10 family have been reported to be involved in defense mechanisms against viral infection in plants and can be used as promising source for generation of virus-resistant plants through transgenic approaches (Huh and Paek, 2013; Lee *et al*., 2013). Antiviral and ribonuclease activity of BBP investigated in the present study agrees with results of previous researches. Similarly, pre-treatment of tobacco leaves with BBP protected them from TMV infection highlighting the role of antiviral protein in alligator plant extract. This is the first report of TMV inhibition using alligator plant extract.

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فعالیت ضدویروسی عصاره گیاه آشک تمساح، *Bryophyllum daigremontianum* L.، در برابر ویروس‌های گیاهی با ژنوم RNA و DNA

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چکیده: در این تحقیق نشان داده شد که عصاره گیاه آشک تمساح، *Bryophyllum daigremontianum* L.، قادر به ممانعت از آلودگی سیستمیک و موضعیِ ویروس موزاییک توتون (TMV) در میزبان‌های باقلا، *Vicia faba* L.، سلمه، *Chenopodium murale* L.، و توتون، *Nicotiana tabacum* var. Turkish و *N. tabacum* var. Xanthi و *N. glutinosa* L.، است. فعالیت ضدویروسی این عصاره در گیاهان تیمار شده باقلا و توتون، تا هشت روز قابل ردیابی بود. این فعالیت با غلظت عصاره ارتباط معکوسی داشت و در اثر شستشوی برگ‌های تیمار شده توتون دو ساعت قبل از کاربرد آن، کاملاً از بین رفت. هم‌چنین کاربرد عصاره ۲۴ ساعت پس از مایه‌زنی، اثر ضدویروسی معنی‌داری را نشان نداد. این عصاره هیچ‌گونه فعالیت ممانعت‌کنندگی را در برابر گیاهان چغندرقند، *Beta vulgaris* L.، مایه‌زنی شده با ویروس پیچیدگی بوته چغندرقند (BCTV)، نشان نداد. به‌منظور تعیین عامل ضدویروسی، پروتئین محتوای بریوفیلوم (BBP) از عصاره جداسازی شد. BBP در برابر RNA کُلِ استخراج شده از گیاهان توتونِ آلوده به TMV و هم‌چنین RNA ژنومی TMV، فعالیت RNase نشان داد، اما قادر به تخریب DNA ژنومی BCTV نبود. به‌علاوه، BBP با غلظت ۴۰ میکروگرم در میلی‌لیتر، به‌طور کامل از فعالیت TMV در برگ‌های توتون جلوگیری کرد. این نتایج پیشنهاد می‌دهند که یک ریبونوکلئاز مسئول فعالیت ضدویروسی عصاره گیاه آشک تمساح است. این اولین گزارش از تأثیر ممانعت‌کنندگیِ عصاره گیاه آشک تمساح در یک ویروس گیاهی است. این گونه گیاهی می‌تواند به‌عنوان منبع امیدبخشی برای پروتئین‌های ضدویروسی و توسعه ترکیبات گیاهی برای کنترل مؤثرِ بیماری‌های گیاهیِ ناشی از TMV باشد.

واژگان کلیدی: توباموویروس، کورتوویروس، فعالیت RNase، ممانعت از ویروس، پروتئین ضدویروسی

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