



Effectivity of *Ammi visnaga* (Khella) Methanolic Extract as a Prospective Biocontrol Agent against tomato mosaic virus Infection

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Abstract Tomato mosaic virus, formally designated as *Tobamovirus tomatotessellati* (ToMV) according to Ictv 2025, continues to pose a threat to agriculture due to its rapid spread and resistance to conventional controls. In our study, we evaluated the antiviral efficacy of methanolic *Ammi visnaga* seed extract (MKSE) as a sustainable biocontrol agent. Using *Datura metel* as a susceptible host, phytotoxicity assays confirmed that MKSE safe concentrations are 10, 20 and 30 µg/mL (with <20% phytotoxicity). In phytoantiviral dose–response evaluations using a local lesion assay, MKSE demonstrated a clear and significant dose-dependent effect, with inhibition rates of 66.82% at 10 µg/mL, 78.00% at 20 µg/mL, and 85.64% at 30 µg/mL (p-value <0.05). Specifically, we investigated the MKSE antiviral mechanisms via post-inoculation treatment that demonstrated an 88.82% suppression of viral replication at 30 µg/mL; simultaneous treatments achieved 85.29% virucidal activity, and pre-inoculation gave 81.76%

protection, with all results being statistically significant (p-value <0.05). Mechanistically, MKSE down-regulated PR-2 (β-1,3-glucanase) gene expression by approximately 25-fold, thereby preserving callose deposits at plasmodesmata and indirectly limiting viral movement between host cell. This represents the first-reported antiviral strategy for *A. visnaga* in plant pathology. This dual action, direct viral inhibition coupled with indirect limitation of viral spread, underscores MKSE's potential as an eco-friendly alternative to synthetic virucides. Our findings provide the first evidence of *A. visnaga*'s efficacy against plant viruses and support its integration into sustainable agricultural practices. Future work will focus on isolating bioactive compounds, field validations, and optimizing nanoformulation delivery strategies.

Keywords *Tobamovirus tomatotessellati* · Tomato mosaic virus · *Ammi visnaga* · Antiviral agent · Methanolic extract · PR-2 Gene expression

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Introduction

Tobamovirus tomatotessellati (commonly known as tomato mosaic virus, ToMV) (Ictv, 2025) is among the most contagious plant viruses. It has an extensive host range, infecting plants across approximately 66 families and over 900 species. This includes economically important Solanaceous crops such as tomato (*Solanum lycopersicum* L.), pepper (*Capsicum*

annuum L.), and *Nicotiana benthamiana*. ToMV causes significant damage to crops and ornamental plants, leading to severe infections with devastating consequences (Yang & Klessig, 1996). As a *Tobamovirus* in the *Virgaviridae* family, its rod-shaped particles (~300 nm×18 nm) consist of 5% nucleic acid and 95% protein, with a positive-sense, single-stranded RNA genome. The virus replicates continuously in infected plants, damaging chloroplasts and causing mosaic symptoms. Affected plants display deformed, wrinkled, twisted leaves with narrowed leaflets and stunted growth (Park & Cha, 2002). Fruits turn small, deformed, and exhibit yellow rings with brown necrotic patches, while severe infection delays ripening, reduces yield, and can lead to crop loss (Bazzini et al., 2007). Plant proteins that are activated under pathogenic conditions are known as pathogenesis-related proteins (PRs). Different PRs are effective anti-pathogenic compounds. Plants may produce PRs in response to infection with bacteria, viruses, fungi, or viroids (Elsharkawy et al., 2012). β -1,3 glucanases belong to the PR-2 family and are classified as endonuclease enzymes. Owing to their potential in plant defense, β -1,3 glucanases have been extensively studied, isolated and sequenced. Naturally, β -1,3 glucanase gene expression levels are relatively low, but when a plant–pathogen interaction or elicitors are used, high levels of β -1,3 glucanase can be detected; enzyme accumulation occurs rapidly, and consequently, hydrolytic activity increases (Ali et al., 2022).

It is difficult to control ToMV because it is mechanically transported, spreads easily to new locations, and manifests symptoms in sensitive plants 6–14 days after infection (dai). Traditional chemical treatments fail to protect plants from ToMV infection due to the virus's high genetic variability and the lack of chemical virucides. Plant extracts have long been recognized as rich sources of antimicrobial compounds, making them promising agents for managing plant viral infections. Their application stems from their natural origin, environmental safety, and effectiveness against a wide range of pathogens (Ara et al., 2012). Studies have demonstrated the antiviral potential of various plant-derived compounds. Jin et al., (2014) reported significant inhibition of Tobacco mosaic virus (TMV) using *Eupatorium adenophorum* leaf

extracts, while Petrov, (2016) highlighted *Tanacetum vulgare* extracts as effective against Cucumber mosaic virus (CMV) and Potato virus Y (PVY). Elsharkawy and El-Sawy, (2015) managed Bean common mosaic virus (BCMV) in bean plants, and Prasad et al., (2007) successfully controlled BCMV-BICM infections in cowpea (*Vigna unguiculata* [L.] Walp.). Hamdi et al., (2020) further demonstrated the effectiveness of plant extracts in suppressing Tomato mosaic virus (ToMV), showcasing their potential for sustainable viral management. Foundational studies by Elbadry et al., (2006), Ito et al., (1993), and Kubo et al., (1990) tested plant-based inhibitors, forming a basis for modern approaches. These findings, along with studies on inhibitors like *Plectranthus tenuiflorus* (Othman and Shoman, 2004), highlight the increasing attention to biologically active compounds as safe and sustainable alternatives for plant disease management.

Ammi visnaga (bisnaga, toothpick weed, or khella) is an herbaceous medicinal plant that grows to approximately 120 cm in height and belongs to the *Apiaceae* Family. The plant is endogenous to Egypt and other regions in the Middle East. Many countries employ *A. visnaga* as a herbal medication for a variety of uses (Hashim et al., 2014). Also, multiple studies have reported the antimicrobial activities of various extracts of *A. visnaga* against bacteria, *Candida* species and aflatoxin formation in toxigenic fungi (Mahmoud, 1999; Semyari et al., 2011). Similarly, two common furanochromones, khellin and visnagin from *A. visnaga*, have been reported to exert antiviral activity against Mammalian viruses, Herpes Simplex Virus-1 (HSV-1) and Vesicular Stomatitis Virus (VSV) (Abou-Mustafa et al., 1990; Ahmed et al., 2021; Hashim et al., 2014).

Although the *A. visnaga* extract has been shown to have antiviral activity against Rotavirus (Harb et al., 2023) and Hepatitis C virus (HCV) (Fahim et al., 2023), its efficacy against plant viruses remains unexplored. As a result, the goal of this study was to: 1) assess the antiviral activity of the methanolic seed extract of *A. visnaga* against ToMV via a local lesion assay on *D. metel*; and 2) illustrate the potential mechanisms underlying this antiviral effect.

Methods

Plants used in the study

Datura metel L. (*D. metel*) and *Nicotiana tabacum* cv. White Burley seeds were sourced from the Virology Laboratory, Department of Microbiology, Faculty of Agriculture, Ain Shams University, Egypt. The plants were grown in an insect-free greenhouse under controlled conditions with natural lighting, 65% humidity, and a temperature of 25 ± 1 °C. The photoperiod was maintained at 16 h of light and 8 h of darkness. They were watered daily to maintain adequate soil moisture levels, and good air circulation was ensured to prevent excess humidity and reduce the risk of fungal infections. *Nicotiana tabacum* cv. White Burley was used as a systemic host for ToMV propagation, while *D. metel* served as a local lesion host for the viral quantification through necrotic leaf lesions.

Source of ToMV isolate

The tomato mosaic virus (ToMV) strain U1 (accession number LC515212.1) was kindly donated by the Virological Lab., Faculty of Agriculture, Ain-Shams University-Egypt, which was isolated previously by Abdelmoamen et al., (2018) from infected tomato plants and maintained on *D. metel* leaves as a local lesion. The purified ToMV inoculum was diluted to 20 µg/mL in 100 mM sodium phosphate buffer (pH 7) before use.

Preparation of ToMV inoculum

Five grams of virus-infected leaves with local lesions were crushed with phosphate buffer (pH 7) (1:2 w/v). The leaky was separated into four layers of muslin and the supernatant was used as the source of inoculum. ToMV was enriched and propagated via mechanical sap inoculation of *N. tabacum* cv. White Burley, and the infection was confirmed serologically by indirect-ELISA.

Indirect ELISA assay

ToMV infection was confirmed by indirect ELISA, which was performed according to Lommel et al., (1982), using polyclonal antibodies provided by the Virological Lab., Faculty of Agriculture, Ain Shams University, Cairo-Egypt.

Source of the methanolic khella seed extract (MKSE)

The crude methanol *A. visnaga* seed extract (MKSE) was obtained as in our previous study (Harb et al., 2023), MKSE was prepared by grounding 50 g of Khella seeds obtained from National Research Center (NRC)-Egypt, after which the resulting powder was treated with 0.5 L of methanol with 3% HCl until a pH of 1.5 was reached. The mixture was stirred for 72 h at room temperature, after which the supernatant was filtered through Whatman No. 1 filter paper. To eliminate residual solvents, the filtrate was concentrated under reduced pressure using a rotary evaporator (40 °C, 200 mbar) and subsequently dried at 60 °C for 24 h. This two-step process ensures the removal of methanol, as confirmed by gravimetric analysis and the subsequent CG-mass analysis. The dried extract was then powdered, weighed, and stored at –20 °C until use. Prior to bioactivity assays, the extract was sterilized by filtration through a 0.22 µm membrane and was reconstituted in sterile to distilled water (X% w/v) (Woo et al., 2009). Chemical profiling of MKSE was performed using a Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) equipped with a TG–5MS capillary column (30 m×0.25 mm×0.25 µm film thickness) at the Regional Centre of Mycology and Biotechnology, Egypt. Chromatographic conditions followed Franchi et al., (1985), with helium as the carrier gas (1.0 mL/min flow rate) and a temperature gradient of 50 °C (2 min hold) to 280 °C at 10 °C/min.

Determination of the non-toxic concentration(s) of MSKE on the virus–host plant

To determine the non-toxic concentrations of MKSE, seeds of *D. metel* L. were sown in 15 cm plastic

pots (5 seeds/pot) containing a mixture of clay and sand, under greenhouse conditions. Then, the leaves of healthy test plants ($n=50$) were sprayed with MKSE in different concentrations (10, 20, 30, 40 and 50 $\mu\text{g/mL}$) while the control plants were sprayed with distilled water and untreated control plants also included. The abnormal morphological changes in shape or color were recorded 3 days after spraying. The toxicity percentage was determined by the following formula: **Toxicity (%)** = (No. of infected sprayed leaves/No. of total sprayed leaves) $\times 100$. Phytotoxicity rates were classified using Nalini and Parthasarathi, (2018)'s rating scale: 0% (no symptoms), 1–10% (slight discoloration), 11–20% (non-lasting damage), 21–30% (moderate), 31–40% (medium), 41–50% (moderately heavy), 51–60% (heavy), 61–70% (very heavy), 71–80% (nearly destroyed), 81–90% (destroyed), and 91–100% (completely destroyed). Concentrations with $\leq 20\%$ phytotoxicity (Ratings 0–2) were deemed non-phytotoxic and used for antiviral assays, ensuring plant health integrity during testing.

Phytoantiviral Activity of MKSE (dose-dependent inhibition)

The antiviral efficacy of MKSE was evaluated using *D. metel* as a local lesion host for ToMV according to Hamdi et al., (2020) with modifications detailed below, and using final MKSE concentrations of 10, 20, and 30 $\mu\text{g/mL}$ based on prior phytotoxicity tests confirming their safety (see Sect. "Phytotoxicity assay of the MKSE"). A ToMV stock suspension (2 mg/mL in phosphate buffer, pH 7.0) was mixed 1:1 (v/v) with MKSE stocks (20, 40, or 60 $\mu\text{g/mL}$) to yield a 50 μL inoculum containing 1 mg/mL ToMV and MKSE at final concentrations of 10, 20, or 30 $\mu\text{g/mL}$. For dose-dependent inhibition, this entire 50 μL mixture was immediately applied to carborundum-dusted leaves by gently rubbing the upper surface. The positive control consisted of leaves inoculated with 50 μL of a 1:1 (v/v) mixture of ToMV stock and sterile water (final ToMV concentration of 1 mg/mL), establishing the baseline infectivity. In addition, MKSE-only controls (50 μL of MKSE at 10, 20, or 30 $\mu\text{g/mL}$ without ToMV) confirmed that MKSE does not independently induce lesions, in agreement with

our phytotoxicity results. A mock control was set up by dusting leaves with carborundum and rubbing them with 50 μL of sterile phosphate buffer only, and untreated leaves (with no virus, MKSE, or mechanical damage) were included to confirm baseline lesion formation in the absence of experimental variables. Each treatment was replicated on 50 leaves (distributed over 5 plants with 10 leaves per plant), and local lesions were quantified 5 days post-inoculation. **Virus infectivity (%)** was calculated as (Number of local lesion-sprayed leaves/Number of local lesion-infected positive control leaves) $\times 100$, and the percentage of inhibition was determined using Smookler, (1971) formula: **Inhibition (%)** = $100 - (A/B \times 100)$, where A represents the number of local lesions on treated leaves and B represents the number of local lesions on the positive control leaves.

Studying the mechanism of antiviral action of MKSE

These experiments were performed following the protocols described by Rabiee, (2020), with modifications to comprehensively evaluate MKSE's antiviral mode of action.

Pre-inoculation treatment (Protective activity): In this assay, *D. metel* leaves were first dusted with carborundum and subsequently sprayed with 50 μL of MKSE solution at final concentrations of 10, 20, or 30 $\mu\text{g/mL}$, 24 h prior to inoculation. Following this pre-treatment, each leaf was inoculated with 50 μL of a ToMV suspension (1 mg/mL in phosphate buffer, pH 7.0). The positive control for this assay comprised leaves that were directly inoculated with ToMV suspension mixed 1:1 (v/v) with sterile water, with no MKSE pre-treatment.

Post-inoculation treatment (Viral replication inhibition): For the post-inoculation assay, leaves were first inoculated with 50 μL of ToMV suspension (1 mg/mL). Twenty-four hours later, the inoculated leaves were sprayed with 50 μL of MKSE solution at final concentrations of 10, 20, or 30 $\mu\text{g/mL}$. The positive control in this assay involved leaves treated with a 1:1 (v/v) mixture of ToMV suspension and sterile water, omitting any subsequent MKSE application.

Simultaneous inoculation treatment (Direct inactivation/virucidal activity): To assess virucidal activity, equal volumes of MKSE and ToMV suspension were mixed in a 1:1 (v/v) ratio and incubated at room temperature for 1 h. This process yielded a 50 µL inoculum containing 1 mg/mL ToMV and 30 µg/mL MKSE, which was then applied to carborundum-dusted leaves. The corresponding positive control consisted of ToMV suspension mixed 1:1 (v/v) with sterile water and incubated under identical conditions (1 h at room temperature) to ensure viral stability and to confirm MKSE's virucidal effect.

Data analysis and controls: In all assays, inhibition (%) was calculated as $100 - (A/B \times 100)$, where A is the number of lesions on treated leaves and B is the number on control leaves. In addition to the positive controls, both mock controls (buffer-treated) and untreated controls (unexposed) were included to validate the results. Each treatment was replicated on 50 leaves (10 leaves per plant across 5 plants), ensuring robust statistical analysis and a comprehensive evaluation of MKSE's protective, inhibitory, and virucidal activities against ToMV.

Analysis of defense-related gene expression levels

Extraction of total RNA

One hundred milligrams (100 mg) of fresh-weight *D. metel* leaves (treated and untreated) were added to each Eppendorf tube and frozen immediately in liquid nitrogen for 5 min. The tissues were homogenized in a mortar for 4 min. One millimeter (1 ml) of TRIZOL was added to the homogenized tissues and incubated at room temperature for 10 min. Then, the samples were centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatants were transferred to new tubes and 0.2 ml of chloroform per 1 ml of TRIZOL was added, and the mixture was shaken vigorously by hand for 15 s and incubated at room temperature for 3 min. Then, the samples were centrifuged

at 13,000 rpm for 15 min at 4 °C for phase separation. The upper phase of the aqueous solution was transferred to fresh tubes. RNA was precipitated by combining 0.5 mL of isopropanol with 1 mL of TRIZOL and incubating at room temperature for 10 min. The samples were subsequently centrifuged at 13,000 rpm for 10 min at 4 °C to obtain pellets. After that, the supernatants were removed, and the pellets were washed with 1 ml of 75% ethanol and then centrifuged at $7500 \times g$ for 5 min at 4 °C. The supernatant was discarded, and the pellets were dried for 5 min at room temperature. The pellets were dissolved in sterilized dsH₂O and incubated at 55 °C for 10 min. Then, they were stored at –80 °C until use (Chomczynski & Sacchi, 2006).

cDNA synthesis

DNase-treated RNA (1 µg/sample) was used to synthesize cDNA in a reverse transcription reaction via a Thermo Scientific™ Maxima™ Reverse Transcriptase Kit (Thermo Fisher Scientific Inc., USA) according to the manufacturer's instructions. The optimal reaction temperature was 50–55 °C, and the inactivation temperature was 85 °C for 10 min. The reaction mixture was preserved in the freezer at –20 °C until utilization.

Quantitative real-time PCR (qPCR) assay

The effect of MKSE extract on ToMV-induced β–1,3-glucanase accumulation was assessed in *D. metel* leaves using a qRT-PCR kit (Thermo Fisher Scientific, USA) per the manufacturer's instructions. Primer sequences for the target gene and the housekeeping gene (actin), utilized for normalization, are listed in Table 1 (Beffa and Meins Jr, 1996). The qRT-PCR conditions were 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min; followed by a final cycle at 95 °C for 15 s

Table 1 Nucleotide sequences of qPCR primers used for β–1,3 glucanase gene

Primers		Single nucleotide sequence (5'–3')	Size (bp)	Related Pathway
B–1,3glucanases	F	ATGCGCTAAGAGGATCAAACA	107	Pathogenesis-related proteins
	R	GTTCTTCTGTACCCACCATCTC		
β-Actin	F	ACAATGAGCTCCGAGTTGCT	900	Housekeeping
	R	TTGATCTTCATGCTGCTTGG		

and 60 °C for 15 s. Expression levels were quantified using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001) across five groups: 1) healthy control; 2) MKSE alone; 3) ToMV alone; 4) MKSE pre-inoculation (48 h), and 5) MKSE post-inoculation (48 h). Experiments were conducted in triplicate. PCR products for β -1,3-glucanase were separated on a 2% agarose gel at 75 V, visualized under UV, and sized against a 100 bp ladder (BioRoN, Germany).

Statistical analysis

In the present study, quantitative data were statistically analyzed as the means ($\pm$ SD) and percentages. The analyzed data were statistically evaluated via one-way ANOVA, where P values < 0.05 were considered statistically significant. Duncan's test was used to determine significant differences in the mean at a $p \leq 0.05$ level of probability, and the standard deviation ($\pm$ SD) is displayed as a column bar. Columns with the same letter do not significantly differ. All the statistical calculations were performed via SPSS version 23 (SPSS Inc., USA), and the graphs were generated via Microsoft Excel version 2019.

Results

Active compounds of the methanolic khella seed extract (MKSE)

We analyzed MKSE via a GC–MS assay in our previous study (Harb et al., 2023). Our findings revealed that

the most active compounds of our MKSE were Visnagin (**MF**, C₁₃H₁₀O₄, MW.230); Benzopyran (**MF**, C₁₃H₁₀O₄, MW.246); P Camphorene (**MF**, C₂₂H₃₆O, MW.332); Khellin (**MF**, C₁₄H₁₂O₅, MW.260); DITRIETHYLSILYL ETHER (**MF**, C₁₆H₁₁N, MW.217); Z-Cnidimine (**MF**, C₂₁H₂₂O₇, MW.386); Benzene-propanoic acid (**MF**, C₃₅H₆₂O₃, MW.530); and Proscillaridie (**MF**, C₃₅H₆₂O₃, MW.530).

Phytotoxicity assay of the MKSE

A plant cytotoxicity test was conducted to ensure the safety of MKSE for use with plant cells and to determine the non-toxic concentrations (NTCs) suitable for subsequent antiviral experiments. Based on Nalini and Parthasarathi (2018) scale, concentrations of 10, 20, and 30 μ g/mL showed minimal phytotoxicity ($\leq 20\%$), with ratings ranging from 0 to 2 (no symptoms to transient discoloration), and were considered safe for use. Statistical analysis revealed no significant phytotoxicity differences among these concentrations ($p > 0.05$). However, at a concentration of 40 μ g/mL, significant phytotoxicity (58%) was observed ($p < 0.05$), with a rating of 6 (heavy damage), indicated by chlorosis and leaf curling. At 50 μ g/mL, severe phytotoxic effects (72%) were recorded ($p < 0.05$), with a rating of 8 (nearly destroyed), including symptoms such as chlorosis, mottling, curling, wilting, necrosis, and plant collapse occurring within 4–6 days after spraying (Table 2; Fig. 1).

Antiviral activity of MKSE against the ToMV

Only the non-toxic concentrations of MKSE (10, 20, and 30 μ g/mL) were tested for antiviral

Table 2 The phytotoxicity test of MKSE on *D. metel* leaves

MKSE Concentration	Symptoms Observed	No. of infected Leaves ^a	Phytotoxicity (%) ^b	Verbal Description
Untreated Control	—	0 $\pm$ 0 ^A	—	—
Control (Distilled Water)	—	0 $\pm$ 0 ^A	—	—
MKSE 10 μ g/mL	No morphological changes	0 $\pm$ 0 ^A	0	No symptoms
MKSE 20 μ g/mL	No morphological changes	0 $\pm$ 0 ^A	0	No symptoms
MKSE 30 μ g/mL	No morphological changes	0 $\pm$ 0 ^A	0	No symptoms
MKSE 40 μ g/mL	Chlorosis, mottling, curling, wilting, necrosis	29 $\pm$ 0.71 ^B	58	Heavy
MKSE 50 μ g/mL	Chlorosis, mottling, curling, wilting, necrosis, dying	36 $\pm$ 0.28 ^C	72	Nearly destroyed

Total sprayed leaves = 50 leaves with each MKSE concentration

^a Mean No. of infected leaves $\pm$ SD

^b Phytotoxicity (%) = (No. of infected sprayed leaves/No. of total sprayed leaves) X100

^{A–C} Different superscript letters (A–C) indicate significant differences in phytotoxicity (%) (ANOVA, $p < 0.05$; Duncan's test)

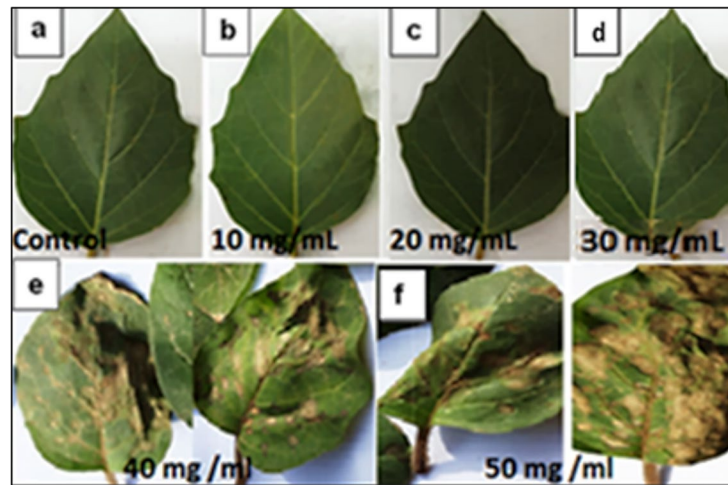


Fig. 1 Phytotoxicity evaluation of MKSE on *D. metel* leaves. **a)** Untreated control (no MKSE) showing healthy leaf morphology; **b-f)** Leaves treated with MKSE at concentrations of 10 $\mu\text{g/mL}$; **c)** 20 $\mu\text{g/mL}$; **d)** 30 $\mu\text{g/mL}$, showing no phytotoxic effects (e.g., necrosis, chlorosis, or lesions), confirming safety for antiviral assays; **e)** 40 $\mu\text{g/mL}$, caused significant phytotoxicity (58% severity; Rating 6: heavy damage), characterized by chlorosis and leaf curling; **f)** MKSE at concentration 50 $\mu\text{g/mL}$, induced severe phytotoxicity (72% severity; Rating 8: near-total destruction), with symptoms including chlorosis, mottling, curling, wilting, necrosis, and plant collapse, justifying the exclusion of these two doses from subsequent experiments

activity against ToMV on *D. metel* leaves using a local lesion inhibition assay. The results demonstrated that all three concentrations significantly inhibited ToMV infection compared to untreated controls ($p < 0.05$), with inhibition percentages

increasing in direct proportion to the MKSE concentration. Specifically, 30 $\mu\text{g/mL}$ achieved an inhibition rate of 85.64%, which was significantly higher than the 66.82% observed at 10 $\mu\text{g/mL}$ ($p < 0.05$; Table 3). Figure 2 further illustrates

Table 3 Antiviral activity of MKSE against ToMV based on the local lesion inhibition assay

MKSE Concentration	Variability of L.L. Morphologically	No. of L.L. ^a	Virus Infectivity (%) ^b	Virus Inhibition (%) ^c
Untreated Control	No lesions observed	0 $\pm$ 0 ^A	0.0	0.0
Mock Control	No lesions observed	0 $\pm$ 0 ^A	0.0	0.0
Control (MKSE)	No lesions observed	0 $\pm$ 0 ^A	0.0	0.0
Control (ToMV)	Small necrotic L.L. with chlorotic center surrounded by chlorotic halo	110 $\pm$ 0.83 ^B	100.0	0.0
MKSE 10 $\mu\text{g/mL}$	Large irregular chlorotic L.L. surrounded by a chlorotic halo	36.5 $\pm$ 0.32 ^C	33.18	66.82
MKSE 20 $\mu\text{g/mL}$	Medium irregular chlorotic L.L. surrounded by necrotic circular brown and chlorotic halo	22.6 $\pm$ 0.97 ^D	20.54	79.46
MKSE 30 $\mu\text{g/mL}$	Medium irregular chlorotic L.L. surrounded by necrotic circular brown and chlorotic halo	15.8 $\pm$ 0.63 ^E	14.36	85.64

Total sprayed leaves = 50 leaves with each MKSE concentration

L.L.: Local Lesion

^a Mean No. of Local Lesions $\pm$ SD/cm²

^b Virus infectivity (%) = No. of L.L. sprayed leaves / No. of L.L. infected control leaves $\times$ 100

^c Virus inhibition (%) = % of virus infectivity (infected control) - % of virus infectivity (sprayed leaves)

^{A-E} Different superscript letters (A–E) indicate significant differences in local lesion counts (ANOVA, $p < 0.05$; Duncan's test)

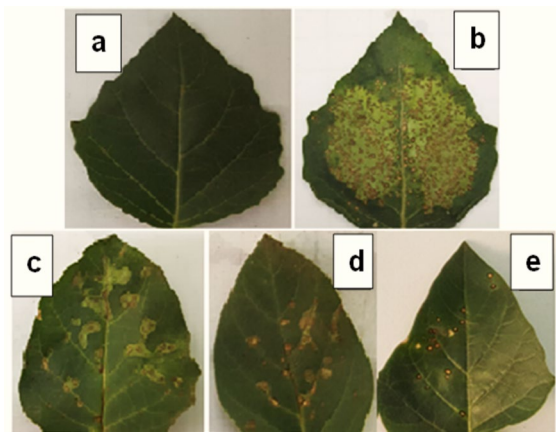


Fig. 2 Dose-dependent inhibition of ToMV infection by MKSE in *D. metel* leaves. **a)** MKSE-only control (30 µg/mL) showing no lesions, confirming absence of phytotoxicity; **b)** Positive control (ToMV only, 1 mg/mL) with abundant local lesions, establishing baseline infectivity; **c)** ToMV-infected leaves treated with MKSE at 10 µg/mL; **d)** 20 µg/mL; **e)** and 30 µg/mL, demonstrating progressive reduction in lesion formation with increasing MKSE concentration

the extent of viral lesion inhibition across the tested concentrations, highlighting the proportional relationship between MKSE concentration and inhibition percentages. The morphological variability of the local lesions corresponding to the different MKSE concentrations is presented in Table 3. The MKSE-only control confirmed MKSE's non-toxic nature and absence of lesion

induction, aligning with the phytotoxicity data. Mock controls and untreated leaves ensured that mechanical damage or lack of treatment did not contribute to lesion formation. Together, these controls, alongside the data presented in Table 3 and visualized in Fig. 2, reinforced the specificity and reliability of MKSE's antiviral effects.

Studying the mechanism of the antiviral action of MKSE

As demonstrated in our previous experiment, the antiviral efficacy of MKSE against ToMV is clearly concentration dependent. Our current results further reveal that MKSE deploys multiple antiviral mechanisms (Table 4). In the pre-inoculation treatment, MKSE at 10, 20, and 30 mg/mL achieved inhibition rates of 56.70%, 65.52%, and 81.76%, respectively, suggesting an effective priming of plant defenses that limits viral entry. In the post-inoculation treatment, when MKSE was applied 24 h after infection, inhibition improved to 70.35%, 78.00%, and 88.82%, indicating interference with viral replication or spread. Similarly, the virucidal assay resulted in inhibition rates of 61.88%, 71.17%, and 85.29% at the same concentrations, implying that MKSE can directly inactivate the virus. Controls confirmed these outcomes: untreated plants exhibited high lesion counts, while MKSE-only and mock (buffer-treated) controls produced no lesions; the positive control consistently registered around 85 lesions, affirming viral stability. All differences were statistically

Table 4 Effect of MKSE Pre-, Post-, and Simultaneous Inoculation (10–30 mg/mL) on ToMV Lesion Development in *D. metel* Leaves

Treatments	Pre-inoculation treatment		Post-inoculation treatment		Simultaneous inoculation treatment (Virucidal)	
MKSE conc	No. of L.L. ^a	Virus inhibition (%) ^c	No. of L.L. ^a	Virus inhibition (%) ^c	No. of L.L. ^a	Virus inhibition (%) ^c
Untreated control	0 ± 0 ^A	0.0	0 ± 0 ^A	0.0	0 ± 0 ^A	0.0
Mock control	0 ± 0 ^A	0.0	0 ± 0 ^A	0.0	0 ± 0 ^A	0.0
Positive Control (ToMV) ^b	85 ± 0.47 ^B	0.0	85 ± 0.91 ^B	0.0	85 ± 0.64 ^B	0.0
MKSE 10 µg/mL	36.8 ± 0.28 ^C	56.70	25.2 ± 0.52 ^D	70.35	32.4 ± 0.82 ^D	61.88
MKSE 20 µg/mL	29.3 ± 0.87 ^E	65.52	18.7 ± 0.69 ^F	78.00	24.5 ± 0.41 ^F	71.17
MKSE 30 µg/mL	15.5 ± 0.36 ^G	81.76	9.5 ± 0.22 ^H	88.82	12.5 ± 0.75 ^H	85.29

L.L.: Local Lesion

^a Mean No. of Local Lesions ± SD/cm²

^b Positive Control (Pre/Post-Inoculation): Non-incubated ToMV + sterile water

^b Positive Control (Virucidal): ToMV + sterile water incubated for 1 h at room temperature (85 lesions confirm viral stability)

^c Inhibition (%): 100 – (A/B × 100), where A = L.L. on treated leaves, B = L.L. on control leaves

^{A-H} Different superscript letters (A-H) within columns indicate significant differences (ANOVA, $p < 0.05$; Duncan's test)

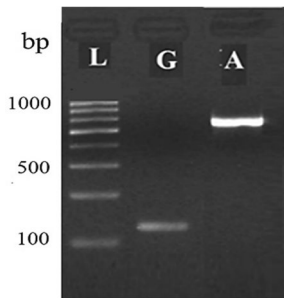


Fig. 3 Photo of agarose gel showing amplification of PR-2 (β -1,3-glucanase) gene. **Lane L:** 1000 bp DNA Ladder; **Lane G:** amplicons of β -1,3-glucanase gene (107 bp) and **Lane A:** amplicons of β -Actin gene (900 bp)

significant ($p < 0.05$), underscoring the robust, multi-faceted antiviral activity of MKSE through protective, curative, and direct virucidal mechanisms against ToMV.

Transcriptional levels of the PR-2 (β -1,3-glucanases) gene

The PR-2 (β -1,3-glucanase) gene was amplified at a size of 107 bp via qRT-PCR (Fig. 3). With respect to the PR-2 transcript levels, the results revealed a significant difference in the PR-2 expression levels between the treatment groups studied (p -value=0.01). Compared with those in the control and other treatment groups, the PR-2 expression level was significantly

upregulated, and the highest expression level was detected in the plants inoculated with ToMV (59.10-fold). In contrast, compared with those in the ToMV group, the PR-2 expression in the two groups of MKSE-treated plants pre- or post-inoculation with ToMV clearly decreased; however, the post-inoculation treatment group presented a slightly lower expression level (24.76-fold) than did the pre-inoculation treatment group (26.17-fold), with no significant difference between them. Interestingly, there was no significant difference in the PR-2 expression level between the independent MKSE treatment group (10.02-fold) and the healthy control group (Fig. 4).

Discussion

Viral disease management via the use of useful plant extracts has gained much attention since it is effective, safe, and environmentally friendly. However, the use of plants for the biocontrol of viral plant diseases is still limited, and their potential actions against viral diseases are not understood. Because *A. visnaga* is widely distributed in Egypt as an endogenous plant, considerable effort has been made to identify new and innovative methods for its integrative management. The chemical composition and comprehensive

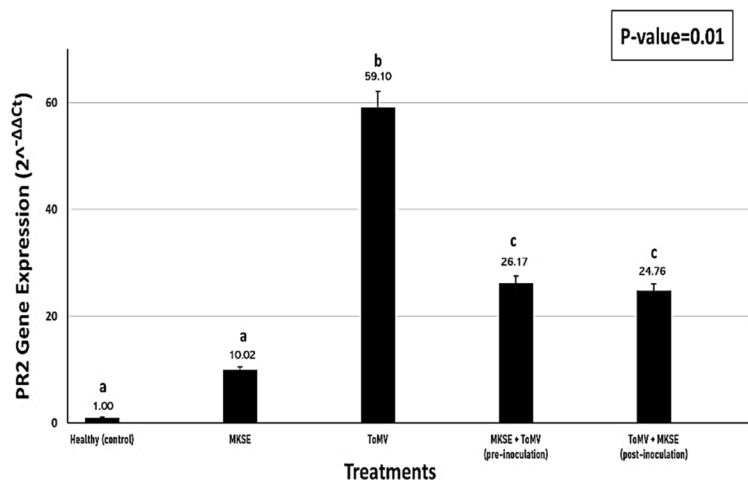


Fig. 4 The quantitative gene expression levels of PR-2 gene in healthy control *D. metel* plants; plants treated with MKSE only; plants inoculated with ToMV; plants treated with MKSE 48 h before ToMV, and plants treated with MKSE 48 h after ToMV using RT-PCR analysis. The columns represent the average value of three replicates, while the bars represent the standard deviation (SD). Different letters (a, b, and c) indicate significant differences, according to Duncan's test ($p = 0.05$)

application of *A. visnaga* have been extensively studied, with its use as a biological medicine and antimicrobial agent, whereas its antiviral activity against plant viruses remains unexplored. Thus, this is the first study to assess the antiviral activity of methanol khella seed extract (MKSE) against ToMV.

We previously performed GC/MS analysis of MKSE to assess its antiviral activity against human rotavirus (Harb et al., 2023), but in this study, we reused it in a completely different manner to assess its antiviral activity against a plant virus (ToMV), which has not yet been explored. Our findings from the GC/MS analysis of MKSE revealed that the most active gradient compounds were Visnagin, Benzopyran, P Camphorene, Khellin, Ditriethylsilyl Ether, Z-Cnidimine, Benzene-propanoic acid and Proscillaridie, which contain pyrimidine and/or purine cycles that can be used to inhibit viruses; therefore, in this study, we aimed to determine the effects of MKSE on a plant virus (ToMV). Other studies on the phytochemistry of *A. visnaga* have shown the existence of many classes of chemical ingredients, including flavonoids, pyrones, saponins and essential oils (Talaat et al., 2014), and the seeds of *A. visnaga* also include twenty-one chemicals, which are classified as follows: 97.3% essential oil with 2,2-dimethylbutanoic acid (30.1%), isobutyrate (14.0%), croweacin (12.2%), linalool (12.1%), bornyl acetate (7.3%) and thymol (6.0%) by GC/MS analysis (Sellami et al., 2013).

Our findings highlight the antiviral potential of *A. visnaga* seed extracts, consistent with methodologies widely adopted in phytochemical research (Al-Kaf et al., 2019; Jin et al., 2014; Petrov, 2016; Šukele et al., 2023; Terfa et al., 2021; Woo et al., 2009). While our methodology rigorously minimizes solvent residues through rotary evaporation and sterile filtration, future studies may incorporate solvent only controls (e.g., solvent processed identically without plant material) to further isolate procedural artifacts such as residual solvent or reagent interactions. Such controls, though not yet standard in natural product research, could enhance interpretability of bioactivity findings and refine attribution to plant-derived compounds.

Our results of the phytotoxicity test indicated that MKSE at concentrations of 10, 20 and 30 µg/mL was safe and did not cause any signs of morphological changes in *D. metel*; therefore, only these non-toxic concentrations were used in the following antiviral activity assessment experiments. The antiviral activity of

MKSE against ToMV on *D. metel* leaves was assessed via a local lesion inhibition assay. Our results revealed that all three concentrations (10, 20 and 30 µg/mL) of MKSE have great potential to inhibit ToMV infection, with percentages ranging from 66.82% to 85.64%. This inhibition was directly proportional to the concentration of the extract used, so a concentration of 30 µg/mL was the most effective at inhibiting ToMV.

Many studies have assessed the antiviral activity of different plant extracts against ToMV infection in different plants. Our findings were consistent with those of Elhelaly and El-Shennawy, (2022), in which four different plant species, namely, *Psidium guajava*, *Solanum nigrum*, *Spinacea oleracea* and *Thymus vulgaris*, were evaluated for their antiviral activity against ToMV on *N. glutinosa* leaves. Their results indicated that concentration of 10% of all the plant extracts was the most effective; the *Solanum nigrum* extract had the highest inhibitory rate, whereas the lowest inhibitory rate was obtained when the *Thymus vulgaris* leaf extract was used. Furthermore, Imran et al., (2012) applied aqueous extracts of three plants, *Azadirachta indica*, *Withania somnifera* and *Achyranthus aspera*, on tomato plants to control ToMV disease and revealed that *Azadirachta indica* was most likely effective against the disease. Similarly, Abbas et al., (2022) reported that *Azadirachta indica* was the most effective at managing ToMV disease, whereas *Allium cepa* was the least effective at managing ToMV disease. Moreover, a report by Deepthi, (2007) revealed that the crude extracts of *Psidium guajava* and *Thuja spp.* lowered the number of local lesions developed on *N. glutinosa* infected with ToMV. Additionally, Narusaka et al., (2020) reported that *Alpinia* plant extract exhibited strong antiviral activity against ToMV and TMV viruses when it was applied to tomato and *N. benthamiana*. Additionally, Thymus oil (5% concentrate) was found to have antiviral properties against TMV and ToMV (Madhusudhan et al., 2005).

The high efficacy of the antiviral activity of MKSE against ToMV led us to study further details concerning its mechanism of action. Our findings indicate that the time of application of MKSE significantly influences its activity against ToMV infection. The best inhibitory effect was obtained through the post-inoculation treatment, in which the MKSE had the greatest ability to inhibit the ToMV replication

process in the host cell, with a percentage ranging from 70.35% to 88.82%; followed by the simultaneous inoculation treatment, MSKE was found to be an effective virucidal agent, as the MKSE was able to interact with the ToMV particles in the primary stage of the infection and block the freeing of its nucleic acid, which eventually led to virus inactivation and prevented viral replication (Radwan et al., 2008), with ToMV inhibition percentages ranging from 56.70% to 85.29%; and the least inhibitory effect was obtained by pre-inoculation treatment, in which the inhibitory effect was obtained through protective treatment (before virus inoculation), with inhibition percentages ranging from 61.88% to 81.76%. These findings indicate that the antiviral effect of MKSE involves direct inhibition of ToMV replication or indirect inhibition through the limitation of ToMV movement between the host plant cells (Beffa and Meins Jr, 1996; Hashim et al., 2014). Our findings were consistent with those of Hamdi et al., (2020) and Rabiee, (2020) concepts.

In this study, we analyzed the effect of MKSE on PR-2 gene expression, which encodes β -1,3-glucanases, enzymes crucial for the cleavage and hydrolysis of β -1,3-glucans, a primary component of plant cell walls (Sinha et al., 2014). Our findings revealed a significant upregulation of PR-2 in ToMV-treated plants, with the highest expression level (59.10-fold) compared to the control and other treatment groups. This is consistent with previous studies, including Aseel et al., (2022), who reported a 36.05-fold increase in PR-2 expression in virus-treated plants, and Oide et al., (2013), who documented PR-2 induction during viral infections in Arabidopsis.

Conversely, MKSE treatment significantly downregulated PR-2 expression in plants, both pre- and post-inoculation with ToMV (26.17-fold and 24.76-fold decreases, respectively) compared to the ToMV-treated group. This suggests that MKSE application may limit ToMV movement between cells by reducing PR-2 activity, leading to indirect inhibition of viral infection. PR-2's substrate, callose, forms during viral infection as a physical barrier to prevent viral spread. Viral upregulation of PR-2 degrades callose, facilitating cell-to-cell viral movement and systemic propagation (Beffa and Meins Jr, 1996). Moreover, Iglesias and Meins, (2000) demonstrated that β -1,3-glucanase deficiency delayed viral spread by enhancing callose

deposition and reducing the plasmodesmatal size exclusion limit. The suppression of PR-2 by MKSE likely helps maintain these callose deposits, counteracting the viral strategy.

Additionally, β -1,3-glucanases are implicated in cell-to-cell communication during viral infections. Beffa et al., (1996) demonstrated that reduced class I β -1,3-glucanase levels in tobacco mutants increased susceptibility to viral infection, whereas cloning β -1,3-glucanase coding sequences into TMV accelerated viral movement through cells. These findings align with Aseel et al., (2022), who reported that nano-clay application limited PVY movement by decreasing PR-2 activity. Additionally, Mauch-Mani and Mauch, (2005) and (Fernandez-Crespo et al., 2017) highlighted that abscisic acid inhibits β -1,3-glucanase transcription, significantly enhancing plant resistance to viruses.

The dual functionality of β -1,3-glucanase (PR-2) in plant-pathogen interactions is highlighted by its critical role in both viral pathogenesis and fungal defense. Although PR-2 has historically been linked to antifungal activity by breaking down β -1,3-glucans, it also affects callose deposition at plasmodesmata, a mechanism that viruses like Tobacco Mosaic Virus (ToMV) use to encourage cell-to-cell migration and systemic infection. ToMV and other viruses take advantage of PR-2 to break down callose and promote systemic infection (Zavaliev et al., 2013). According to recent research by (Aseel et al., 2022), PR-2 suppression increases resistance to Potato Virus Y (PVY) by maintaining callose deposits. In a similar vein, our findings show that MKSE's downregulation of PR-2 inhibits the spread of ToMV, offering a novel antiviral tactic that blocks viral hijacking mechanisms.

While our focus on PR-2 provides valuable mechanistic insights into its role in plant-virus interactions, we recognize the limitation of analyzing a single gene. Including additional defense-related genes such as PR-1, PAL, and other pathogenesis-related markers would offer a broader perspective on resistance pathways and enable a more comprehensive characterization of induced defense mechanisms. Future studies will expand the analysis to include a wider range of genes, facilitating a deeper understanding of the molecular pathways involved. Moreover, integrating transcriptomic approaches could reveal novel regulatory networks and genes influenced by our extract, further strengthening the findings. These steps will address current limitations and build upon the foundation established in this work.

Noteworthy, no significant difference was observed in PR-2 expression levels between the MKSE-treated group (10.02-fold) and the healthy control group. This confirms the safety of MKSE application, with no harmful side effects on plants. These findings are particularly valuable given the lack of natural-origin antiviral treatments with minimal phytotoxicity. MKSE offers a promising eco-friendly solution to mitigate viral infections and minimize losses in plant production, addressing a critical gap in sustainable agriculture.

Conclusion

In conclusion, this study establishes methanolic *Ammi visnaga* (khella) seed extract (MKSE) as an innovative and sustainable antiviral agent against *Tobamovirus tomatotessellati* (ToMV), addressing critical agricultural challenges through dual mechanisms. MKSE directly inhibits viral replication (88.82%) and inactivates viral particles (85.29% virucidal efficacy), while indirectly modulating host defenses via PR-2 (β -1,3-glucanase) downregulation (24.76–26.17-fold). This mechanism preserves callose deposits at plasmodesmata, effectively restricting viral cell-to-cell movement which is a novel strategy for plant viruses and the first reported mechanism for *A. visnaga*. With negligible phytotoxicity ($\leq 20\%$ at 10–30 $\mu\text{g/mL}$), MKSE presents a sustainable alternative to synthetic virucides, aligning with EU directives on pesticide reduction and marking the first evidence of *A. visnaga*'s efficacy against plant viruses. Future research must isolate bioactive compounds (e.g., visnagin, khellin) to clarify their antiviral roles, validate field efficacy in diverse agroecosystems, and decode systemic resistance pathways (e.g., PR-1, PAL) through transcriptomic analyses. Nanoformulation strategies to enhance foliar absorption and stability, alongside synergies with biocontrol agents, will optimize MKSE's integration into sustainable pest management systems. This work advances plant-derived solutions for viral disease control, supporting global food security and ecological resilience while addressing the urgent need for reduced chemical dependency in agriculture.

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Data Availability All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval consent This study did not involve human participants or animals, and therefore ethical approval was not required.

Conflict of interest The authors have neither conflict of interests nor competing interests to declare.

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