Antiviral activity of olive leaf extract (OLExts) against *tomato yellow leaf curl virus* (TYLCV)

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Abstract: New and safe antiviral agents are needed to prevent and/or overcome severe plant viral infections. We postulated that olive (*Olea europaea*) leaf extracts (OLExts) can offer an effective interference mechanism upon viral infection. To study the effect of pre incubating TYLCV with aqueous and alcoholic OLEXts on viral activity, 200 mg/ml of TYLCV was mixed with OLExts at different concentrations (50 to 500 µg/ml) for two days at 4°C and used to infect healthy tomato plants. The results of minimum inhibitory concentrations (MICs) of both alcoholic and aqueous extracts indicated that both extracts had antiviral activity against TYLCV. The MICs of alcoholic and aqueous extracts were 300 µg/mL and 500 µg/ml, respectively. The incubation of 200 mg/ml TYLCV with 50, 100, 200, 300, 400 and 500 µg/ml OLExts prior to infection reduced the viral infectivity to 12%. DAS-ELISA results of tomato plants infected with OLExts-treated TYLCV, showed drastically decreased in viral titers and viral protein accumulation by >50% (virucidal effect). DNA-PCR confirmed that the MIC of OLExts was 500µg/ml. Healthy tomato plants were pre-treated with OLExts at different concentrations (50 to 500 µg/ml) and then challenged with 200 mg/ml TYLCV. The results of minimum inhibitory concentrations (MICs) of alcoholic and aqueous extracts were 200 µg/ml and 400 µg/ml respectively. DAS-ELISA results of OLExts-treated tomato plants infected with TYLCV, showed drastically decreased viral titers and viral protein accumulation by 46.1%. DNA-PCR confirmed that the MIC of OLExts was 400µg/ml. Pre-treatment of tomato plants with OLExts and then challenging with the TYLCV was more efficient than infection by TYLCV mixed with OLExts in reducing virus concentration as evident by the results of ELISA and PCR.

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1. Introduction

Tomato yellow leaf curl disease (TYLCD) is one of the most devastating viral diseases affecting tomato crops in tropical, subtropical and temperate regions of the world. TYLCVD has emerged in countries around the Mediterranean Basins during the last 20 years (**Fauquet *et al*., 2005**). 100% of the fall- grown tomato plants are infected with TYLCV, with production losses reaching 80% **(Moustafa, 1991)**. The spread of TYLCV in Egypt and in Saudi Arabia appears to have been closely associated with the spread of ***B. tabaci*** (**Mazyad *et al*., 1979; Liu *et al*. 1998; Abdelkader & Rifaat 2009**). Infected tomato plants are stunted or dwarfed, with leaflets rolled upwards and inwards, young leaves are slightly chlorotic in recently infected plants (**Díaz-Pendón *et al*., 2010**), and fruits might not be produced or, if produced, are small and unmarketable.

The treatment in industrial countries includes mainly the use of insecticides against the insect vector and the introduction of more tolerant crop varieties. The implementation of physical barriers and growing tomatoes under greenhouse conditions has also cut the damage to an average 20%, conservatively estimated at more than $300 million, in Europe and the US (**Gianessi *et al.*, 2002, 2003**).

Medicinal plants are known to be self-sustaining chemical chameleons capable of synthesizing different compounds according to their needs. Many plants synthesize antimicrobial compounds as a mechanism of protection against a number of pathogenic agents. Some plants such as Olives are tolerant to various plant viruses, and might produce antiviral compounds that confer this resistance (**Lindbo *et al*., 1993**). Leaves from olive tree, *Olea europaea*, are rich in biophenols (BPs), such as oleuropein (Ole) (**Charrouf and Guillaume, 2007**), verbascoside, ligstroside, tyrosol or hydroxytyrosol. These compounds have shown several biological activities such as antioxidant (**Visioli *et al*., 1998; Benavente-Garcia *et al*., 2000)** antithrombotic, antiviral ((**Fredrickson, 2000**) and even skin photoprotective properties (**Saija and Uccella, 2001**). Furthermore, some of these compounds have demonstrated antimicrobial activity

by inhibiting the growth of a wide variety of bacteria, fungi and viruses (**Renis, 1969; Hirschman, 1972**). The effect of plant extracts, plant seed oils and salicylic acid on tobamoviruses in both indicator and host plant was studied by **Madhusudhan *et al.* (2011)**. The results showed that all the extracts used for screening were effective in reducing the number of local lesions formed by the challenge inoculation of tobamoviruses.

Olive leaf extract (OLExt) showed *in vivo* capacity to inhibit viral infectivity in a dose dependent manner when pre-inoculated with the virus before infecting tomato plants and also when administered during infection. Since it has been previously reported that OLExt interacts with phospholipid bilayers (**Saija *et al.*, 1998; Paiva-Mrtins et al. 2004)** leaf extract contains phenolics such as oleuropein, and appears to have highly protective effects and antiviral activity against viral infection. In the current study, the effect of olive leaves plant extracts (OLExts) on TYLCV was examined. This study may lead to more effective, natural, selective, safe control strategies.

2. Material and methods

Reagents

Olive leaf of *Olea europaea L.* was obtained from Tabouk, (KSA) in the period of winter 2013. Leaves were washed to remove impurities such as dust and then dried in an air for 5-7 days in the dark. The leaves were ground to fine powder and kept at -20ºC until use. TYLCV was isolated and purified from *Lycopersicon esculentum* Castle rock and Condor Atlas tomato plants collected from Al-Hada and El-shefa, Taif, KSA.

Preparation of extracts from Olive Leaves (OLExts)

For obtaining alcoholic extract, 20 gm of powdered olive leaves were added to 200 ml aqueous alcohol solution (70%) and kept in the dark for at least 4 hours and is then drained. This process is repeated at least two more times, and the drained extracts (600ml) are combined, concentrated and dried by distillation under vacuum. The steps of the extraction are conducted at a temperature of about 20ºC to 85ºC according to **Nashman (1998)** (Patent No.5,714,150). This powder contains 30-40% by weight oleuropein (Fig.1).

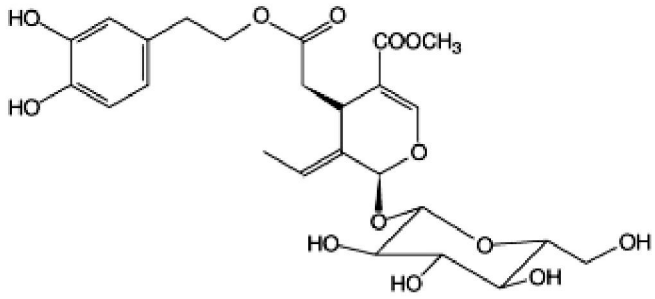

Fig. (1). Chemical structure of oleuropein, the major component of the olive leaf extract (Nashman, 1998).

To obtain an aqueous extract, 50 gm of powdered Olive leaves were soaked in 500 ml distilled sterilized water for at least 1h at room temperature to make stock solution. The extract was filtered through four layers of moisture muslin cloth. The filtrate was centrifuged at 10,000 rpm for 15 minutes at 4 ºC. The supernatant was sterilized by bacterial filter (0.45 µm) before use.

Viral infectivity assays

Pre-incubation of TYLCV (200mg/ml) with either alcoholic and/or aqueous extracts with concentrations (50 to 500 µg/ml) of OLExts, for 2 h at 4ºC in dilution buffer (5 g/l Tween 20, 1% PBS pH 6.8) was carried out in order to test the influence of OLExts on viral infectivity. After incubation, the mixtures containing TYLCV were injected by syringe into healthy tomato plants (cv. Castle rock), grown in an isolated greenhouse. Healthy tomato plants were injected with dilution buffer only under the same conditions as negative control. Alternatively, OLExts-tomato plants were challenged with TYLCV (200 mg/ml). The infected tomato plants were left for symptom development. The concentration of OLExts at which viral infectivity was 50% inhibited (IC50) was determined. in both cases. The 50% inhibition concentration (IC50), was defined as the concentration of OLExts which reduced the percentage of TYLCV-induced leaf curling symptoms by 50%, with respect to untreated plants and expressed in µg /ml.

Detection of TYLCV- protein in treated tomato plants by DAS-ELISA

All saps from OLExts-treated tomato plants were extracted and diluted in 0.05 M Tris/HCI buffer, pH 8.0, 2% polyvinylpyrrolidone (PVP), 0.5% Tween 20 (1 g tissue in 10 ml buffer) containing 0.005 M EDTA and incubated over night at 4°C. Polyclonal antibodies

(PAbs) were used in DAS-ELISA as described in **Muniyappa *et al.* (1991)**. Microtiter plates were coated with 1:1000 diluted TYLCV polyclonal antibodies in coating buffer and incubated in microtiter wells for 3 h at room temperature. Alkaline phosphatase conjugated PAbs diluted 1:l0000 in PBST containing 1% BSA and 2% PVP (2 h at room temperature) following by the substrate p-nitrophenyl phosphate was used to detect captured antigen (2 h at room temperature) readings were taken after incubation with substrate for 30 min or 1 h at room temperature and again after overnight incubation at 4°C.

Detection of TYLCV-DNA in treated tomato plants by PCR

TYLCV-infected tomato plants were collected on day 14 post-infection, homogenized directly in DNA extraction buffer (2% CTAB; Hexadecyl trimethyl-ammonium bromide) as described by **Cullings, (1992)**. Integrity and quantity of the extracted DNA were estimated spectrophotometrically and verified visually using 1% agarose gel. PCR kit Cat.No. 201203 was purchashed from Qiagen, GmbH, Germany was used in PCR reactions. PCR reactions were optimized for 25 µl and the final concentrations of reaction components were: 25 µM deoxynucleotide triphosphate (dNTPs), 2.5µl of 10x PCR buffer, 2.5 mM $MgCl_2$, 5 units Taq DNA polymerase, 0.5µl of 25pm each complementary and viral-sense primers were selected in the C1 & IR region of viral DNA as shown in Fig. 2. (TYc138) 5'-AAGTGGGTCCCACATATTGCAAGAC-3' and (TYv2337) 5'-ACGTAGGTCTTGACATCTGTTGAGCTC-3', 2.5 µl of 10^{-2} diluted DNAs (60 µg/ml) extracted from tomato plants treated with both alcoholic and aqueous OLExts was used as PCR templates. PCR cycling parameters were as follows: one cycle at 94°C for 2 min; 35 cycles at 94˚C for 1 min, 60˚C for 1.5 min, followed by one cycle at 72˚C for 5 min. All PCR reactions were performed in a programmable thermal cycler (PXE 0.5 Thermal cycler; Thermo Electron Corporation, USA).

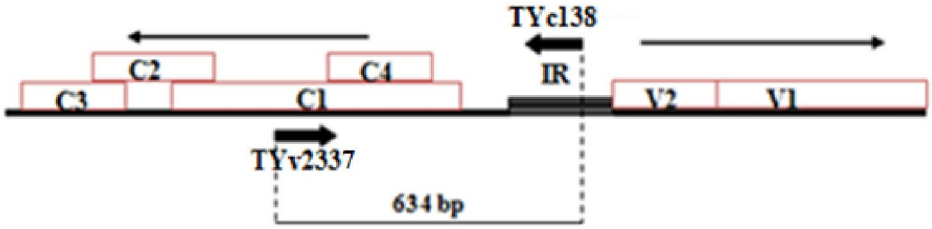

Fig. 2. Genome organization of (TYLCV). Open reading frames (ORFs) are indicated by black arrows. The V1 and V2 are designated as ORFs for plus-strand. C1 to C4 are designated as ORFs for minus strand. IR: the intergenic region. The position of the PCR primers and the size of the PCR product is indicated.

3. Results

The inhibitory effect of alcoholic and or aqueous OLExts on TYLCV infectivity was measured by the development of leaf curling symptoms formed on tomato plants after infection, ELISA, and PCR.

Tomato plants treated with 200mg/ml TYLCV mixed with 50 µg/ml, 100µg/ml, 200µg/ml, 300 µg/ml, 400 µg/ml and 500µg/ml of OLExts on each individual plant, showed inhibition of leaf curling formation of TYLCV in a dose dependent manner. When TYLCV was mixed with OLExts for 2 days at 4°C before infection, the OLExts reduced TYLCV infectivity to about 12 % compared to the untreated TYLCV controls (**Table 1**).

The percentage of viral infectivity decreased steadily as the OLExts concentrations were increased up to 500 µg/ml. This reduction of the viral infectivity is clearly observed in **Fig. 3** where the leaf curling symptoms were drastically reduced at 300 µg/ml OLExts, and almost completely abolished at 500 µg/ml when compared to the untreated tomato plants. The results showed that, the OLExts inhibited leaf curling formation of TYLCV in a dose dependent manner after 14 days of TYLCV post infection at greenhouse conditions. However, the pretreatment of tomato plants with 50-500µg/ml OLExts prior challenging with 200mg/ml TYLCV, OLExts reduced TYLCV infectivity to about 46.1% compared to the untreated TYLCV controls (**Table 2**). The percentage of viral infectivity decreased steadily as the OLExts concentrations were increased up to 500 µg/ml. This reduction of the viral infectivity is clearly observed in **Fig. 4** where the leaf curling symptoms were drastically reduced at 200 µg/ml OLExts, and almost completely abolished at 300 µg/ml when compared to the untreated tomato plants. The results showed that, the OLExts inhibited leaf curling symptoms of TYLCV in a dose dependent manner when tomato plants were pre-treated with OLExts prior challenging with TYLCV at greenhouse conditions.

The minimum inhibitory concentration (MIC) of OLExts on TYLCV detected by DAS-ELISA after 14 days post infection by TYLCV mixed with different concentrations of OLExts was 300 µg/ml as shown in (Table 1 & Fig 5A). However, the minimum inhibitory concentration (MIC) of OLExts-treated tomato plants measured by DAS-ELISA 14 days post TYLCV challenge was 200 µg/ml as shown in **(Table 2 &Fig. 5B)**.

The primers successfully amplified a DNA fragment of 634 bp from TYLCV infected tomato plants (+ve control) and from OLExts-treated tomato plants (50, 100, 200, 300 µg/ml OLExts) at 2 days prior viral challenge. There are no amplifed products were observed in agarose gel when 500 µg/ml OLExts was applied two days before TYLCV infection as well as in untreated tomato plants (-ve control) (**Table 3**). Therefore, the MICs of OLExts which inhibit viral DNA replication is 500 µg/ml. This reduction of the viral infectivity is clearly detected by DNA-PCR. However, PCR detection after 14 days post infection with TYLCV mixed with OLExts) showed that the MIC was 400 µg/ml as shown in **Table 4**.

Fig. 3. Detection of TYLCV infectivity by observed symptoms on tomato plants after infection with (200mg/ml) TYLCV mixed with 50 µg/ml OLExt; 100 µg/ml OLExt; 200 µg/ml OLExt; 300 µg/ml OLExt; 400 µg/ml OLExt and 500 µg/ml OLExt). H: Untreated tomato plants injected with dilution buffer only (5 g/l Tween 20, 1% PBS pH 6.8) as negative control. I: Leaf curling symptoms developed on tomato plants infected with 200mg/ml TYLCV as positive control.

Fig. 4. Detection of TYLCV infectivity by observed symptoms on OLExts-treated tomato plants (50 µg/ml, 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, and 500 µg/ml OLExts) and challenged with 200mg/ml TYLCV. H: Untreated tomato plants injected with dilution buffer only (5 g/l Tween 20, 1% PBS pH 6.8) as negative control. I: Leaf curling symptoms developed on tomato plants infected with 200mg/ml TYLCV as positive control.

Table (1): Detection of inhibitory concentration of OLExts on TYLCV as measured by DAS-ELISA after 14 days post infection with TYLCV mixed with different concentrations of OLExts.

Tomato plants infected with OLExts /TYLCV mix	dilutions of OLExt / TYLCV (µg /ml)							
	50 T1	100 T2	200 T3	300 T4	400 T5	500 T6	+ve Ctrl	IC µg/ml
R1	1.185 (80%)	1.152 (78.4%)	0.482 (32.8%)	0.191 (13%)	0.010 (0.68%)	0.011 (0.06%)	1.468	
R2	1.164 (73%)	1.109 (69.6%)	0.478 (30%)	0.189 (11.8%)	0.012 (0.75%)	0.012 (0.12%)	1.593	
R3	1.175 (79%)	0.958 (64.6%)	0.450 (30.3%)	0.184 (12.4%)	0.011 (0.74%)	0.011 (0.06%)	1.481	
Mean average (%)	1.174 (77.5%)	1.073 (70.8%)	1.11 (73.3%)	0.188 (12.4%)	0.011 (0.72%)	0.011 (0.72%)	1.514 (100%)	

T1 to T6: Tomato plants infected with different concentrations of TYLCV OLExts / mixture.
+ve Ctrl: Positive control (TYLCV infected tomato plants)
IC: Inhibitory concentrations of OLExts. R1, R2 and R3: Each experiment repeated three times

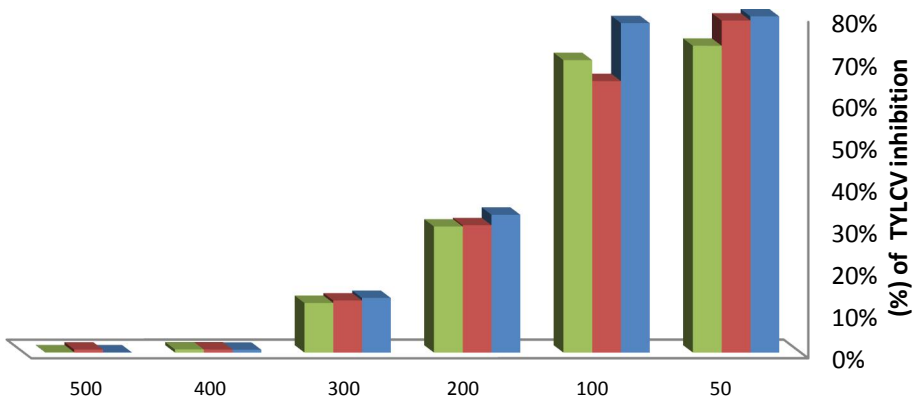

Fig. (5A). Percentage (%) of viral inhibition produced by different concentrations of olive leaf extracts (OLExts) mixed with 200mg/ml TYLCV. O.Ds were measured after 14 days post viral infection *in vivo.*

Table (2): Inhibitory concentration of OLExts-treated tomato plants by DAS-ELISA 14 days post TYLCV challenge

Pre-injected Tomato plants with OLExt	dilutions of OLExts (µg /ml)							
	50 T1	100 T2	200 T3	300 T4	400 T5	500 T6	+ve Ctrl	IC µg/ml
R1	1.080 (73.5%)	1.125 (76.6%)	0.148 (10.0%)	0.015 (1.02%)	0.011 (0.74%)	0.012 (0.81%)	1.468	
R2	1.317 (82.6%)	1.194 (74.9%)	0.324 (20.3%)	0.014 (0.87%)	0.010 (0.62%)	0.011 (0.69%)	1.593	
R3	1.423 (94.9%)	1.121 (74.8%)	0.229 (15.2%)	0.022 (1.46%)	0.013 (0.86%)	0.010 (0.66%)	1.498	
Mean average (%)	83.8%	75.4%	46.1%	1.11%	0.74%	0.72%	1.519 (100%)	

T1 to T6: Tomato plants pre-treated with different concentrations of OLExt 2 days before challenge with 200mg/ml TYLCV.
+ve Ctrl: Positive control (TYLCV infected tomato plants) IC: Inhibitory concentrations of OLExts.
R1, R2 and R3: Each experiment repeated three times

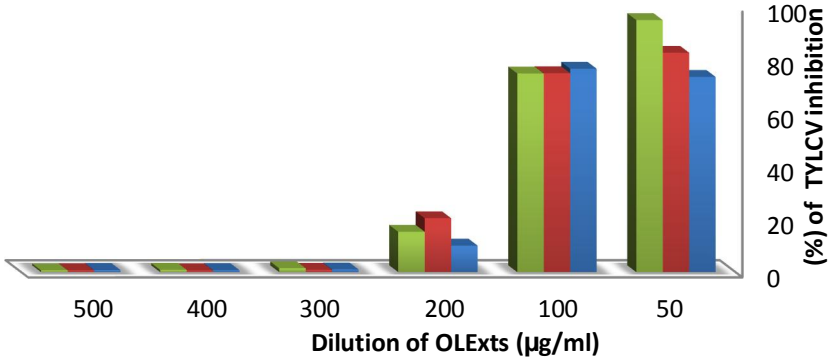

Fig. (5B). Percentages (%) of viral inhibition by diluted OLExts pre-injected to tomato plants two days before TYLCV infection. O.Ds were measured after fourteen days post virus infection *in vivo.*

Table 3. TYLCV detection by PCR in OLExts-treated tomato plants 2 days before virus challenge.

TYLCV (+) control	50µg/ml OLExt	100µg/ml OLExt	200µg/ml OLExt	300µg/ml OLExt	400µg/ml OLExt	500µg/ml OLExt	Untreated (buffer only)
+	+	+	+	+	+	-	-

Table 4. TYLCV detection by PCR after 14 days in tomato plants infected with TYLCV and OLExts.

TYLCV (+) control	200mg/ml TYLCV+ 50µg/ml OLExt	200mg/ml TYLCV+ 100µg/ml OLExt	200mg/ml TYLCV+ 200µg/ml OLExt	200mg/ml TYLCV+ 300µg/ml OLExt	200mg/ml TYLCV+ 400µg/ml OLExt	200mg/ml TYLCV+ 500µg/ml OLExt	Untreated (buffer only)
+	+	+	+	+	-	-	-

4. Discussion

In this study, alcoholic and water OLExts were screened for the antiviral activity against TYLCV pre and during viral infection. The results showed that viral concentration was reduced in all treated plants when compared to Untreated ones.

Medicinal plants have been traditionally used for the treatment of different health conditions, including infectious diseases (**Severson *et al*., 2008; Li *et al*., 2009)**. There is no doubt that traditional medicinal plants may serve as potential sources for the development of new antiviral agents in the future.

The pretreatment of tomato plants with OLExts prior challenging with TYLCV, was more effective as antiviral agents as compared to OLExts used during viral infection.

Higher plants possess endogenous virus inhibitors, of which proteinaceous antiviral substances are of particular interest (**Balasubramanyam *et al*., 2000**). Induction of resistance against virus infection by plant extracts has not been fully understood. It is possible that plant extracts themselves may not act directly on the virus.

Antiviral activity by plant extracts could be due to one of the following mechanisms, de novo synthesis of antiviral compounds, production of virus inhibiting proteins and production of mobile signal that bind to host plant surface, which produce virus inhibiting agents (**Verma *et al*., 1988**). In the current study, the plant extract such as Olive leaves extract (OLExs) (400 - 500µg/ml) showed lower virus concentration in treated tomato plants when compared to control. Similar results noticed by **Deepthi *et al*. (2007)** when used different plant extracts and acetone precipitated proteins from six medicinal plants against tobamovirus infection. **Ramesh *et al*. (2009)** studied the effect of latex of *Euphorbia Tirucalli* against Tobamoviruses and suggested that, the extracts reduces the concentration of Tobamoviruses.

The results of the present study revealed that treatment of tomato plants with alcoholic or aqueous OLExts before challenging with TYLCV was more efficient in inducing resistance against TYLCV. The ELISA and PCR results showed lower concentration of the virus in both OLExts treated tomato plants compared to control plants. This study demonstrated that OLExts might be used as highly effective antiviral agent.

Conclusion

Olea europaea leaf extracts (OLExts) were found to be effective antiviral in reducing virus concentration. Both alcoholic and/or water OLExts were effective in reducing concentration of TYLCV in all tomato plants under study. It is concluded that alcoholic or aqueous OLExts might be useful as natural and safe antiviral products, for the prevention of TYLCV infections.

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