Stress conditions during plant growth increase the anti-herpetic properties of *Lilium candidum* leaf extracts and fractions

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When submitted to stress conditions during growth, plants modify their secondary metabolites production, which could increase therapeutic properties. *Lilium candidum* is a beautiful plant with potent antiviral activity, growing wild in the Middle East. We investigated the effect of biotic and abiotic stress applied on *L. candidum* plants during their growth, on the anti-herpetic efficiency of leaf ethanolic extracts and their fractions against herpes simplex virus (HSV) 1 *in vitro*. Ethanolic leaf extracts were collected from *L. candidum* leaves of healthy plants growing under regular conditions, or submitted to abiotic stresses in the form of drought, heat and salinity or from plants infected with the Lily symptomless virus (LSV). The highest anti-herpetic effect was recorded in extracts from virus-infected plants (biotic stress), followed by extracts from plants submitted to heat stress. Similar antiviral effect was recorded for extracts from plants grown under regular conditions and under salinity. 80% methanolic fraction from heat-shock plants, rich in flavonoid was particularly efficient against HSV-1 infection. We suggest that stress conditions during plant growth increased leaf flavonoid content, inducing anti-herpetic efficiency. The fact that biotic and abiotic stress during plant growth increases anti-herpetic efficiency should be taken into consideration when growing plants for medicinal purposes.

**Key words:** *Lilium candidum*, herpes virus, leaf extract, antiviral activity.

**INTRODUCTION**

Madonna lily, meadow or white lily (*Lilium candidum* L., Liliaceae) is a beautiful bulbous plant, which has been cultivated for centuries in the Middle East. The extraordinary whiteness of its large flowers, combined with golden anthers and delightful fragrance, have led to its adoption as an emblem of purity and sanctity among various cultures. More than 3,000 years ago, *L. candidum* became a symbol of the Jewish civilization and was...
represented on ancient coins (Meshorer, 1982, 2001). Later on, the white lily became associated with the Virgin Mary, especially in the context of the annunciation and has been involved in countless legends (Krymow et al., 1999). L. candidum apparently originates in Lebanon and Israel, where its distribution is restricted to several populations, the North of the country, on Mountain Carmel and Upper Galilee. In Israel, it is considered as an endangered species (Feinbrun-Dothan and Danin, 1991; Zaccai et al., 2009).

Bulbs and flowers of the white lily have traditionally been used for treatment of ulcers, boils, finger ulcers, reddened skin, burns and injuries (Eisenreichova et al., 2000). Antifungal and anti-yeast activities of ethanolic extracts from L. candidum flowers and bulbs, as well as compounds isolated from these extracts, have been reported (Mucaji et al., 2002). In addition, anticancer properties of several spirostanol saponins, jatropham and jatropham glucosides isolated from ethanolic extracts of L. candidum were demonstrated (Vachalkova et al., 2000). Poultice from L. candidum bulbs was also used against the Varicella Herpes Zoster (VZV, shingles or zona) (Pieron, 2000). In a previous study, we reported potent anti-herpetic activity of L. candidum leaf extracts against HSV-1 infection (Yarmolinsky et al., 2009).

Plants have the ability to synthesize a wide variety of metabolites, belonging to two major groups, primary and secondary, according to their direct or indirect involvement in basic plant growth and development, respectively. As static organisms, plants need to defend themselves from a multitude of stress conditions by synthesizing chemical compounds, usually belonging to the secondary metabolite group, triggering the “Immune system” of the plant (Seki et al., 2002). Many of these chemicals have beneficial effects on long-term health when consumed by humans, and can be used to effectively treat human diseases (Rhodes, 1994; Gorelick and Bernstein, 2014).

Approximately eight distinct viruses belonging to the Herpesviridae family of DNA viruses cause serious diseases in humans. The most common are VZV, HSV-1 and HSV-2 (Subak-Sharpe and Dargan, 1998). After first infection, the virus remains in the body for life, inducing occasional outbreaks, mostly when the immune system is weak (Field et al., 2006; Wyrwicz and Rychlewski, 2007). Acyclovir (ACV), a guanosine analogue, is one of the most commonly used antiviral drug, which greatly alleviates the virus symptoms. Nevertheless, the increasing rate of ACV-resistant herpes mutants (Devrim et al., 2008) and undesired side effects, call for the development of alternative medicines.

In the present study we investigated the effect of various stresses applied on L. candidum plants during their growth, on the antiviral activity of their leaf ethanolic extracts against HSV-1, in vitro. We hypothesized that stress exposure of L. candidum plants, which are particularly susceptible to plant viruses and fungi (Proscevićius et al., 2007), would generate an increase in secondary metabolites compounds production, thereby increasing the effectiveness of their anti-viral activity.

MATERIALS AND METHODS

Plant material, biotic and abiotic stresses

L. candidum bulbs propagated from plant material collected from Israeli wild populations of Mount Carmel (Zaccai et al., 2009) were grown in a controlled greenhouse at the Ben-Gurion University, Beer-Sheva, Israel.

Biotic stress

Plants showing viral disease symptoms such as curly leaves, and yellow stripes and plants with no visible viral symptoms were tested for virus mottle (LMoV) lily, cucumber mosaic virus I & II (CMV, CMVII) and lily symptomless virus (LSV) in Prof. Abed Gera’s laboratory, at the Agricultural Research Organization (ARO, Israel), using electron microscopy (EM), biological tests, serological and molecular techniques. Only LSV virus was detected in plants showing viral symptoms, while no virus was detected in symptom less plants. Plants which tested positive for LSV virus were considered as being in a state of “biotic stress”. Four different treatments of abiotic stress were applied:

(1) Heat shock: incubation of the plants at 40°C for 24 h.
(2) Drought stress: withholding irrigation for 4 weeks.
(3 and 4) Salinity: irrigation with 200 or 500 mM NaCl solution for 3 weeks.

Preparation of plant ethanolic extracts

Ethanolic extracts were prepared from L. candidum leaves as previously described (Yarmolinsky et al., 2009).

Fractionation of ethanolic plant extracts

The obtained plant ethanolic extracts were separated into different fractions by reverse phase chromatography (RP-C18 Sepack) as previously described (Yarmolinsky et al., 2009).

Cells and viruses

African green monkey kidney (Vero) cells were purchased from the American Type Culture Collection (ATCC), Rockville, MD, USA. Cells were grown as described (Yarmolinsky et al., 2009). HSV-1 was obtained from the ATCC (VR-735). All of these viruses are susceptible to ACV. The viruses were propagated to > 108 plaque forming units (PFU) per ml in Vero cells and their concentration was estimated by a standard plaque assay (Huleihel et al., 2001).

Cytotoxicity examination

Vero cells were treated with various concentrations of plant extracts
Antiviral activity

The antiviral activity of the tested plant extracts was evaluated by plaque assay and cytopathic effect (CPE) development as described in Yarmolinsky et al. (2009). Vero cells were seeded at 0.15 × 10^6 cells/well in 24-well culture plates, in RPMI medium in 10% fetal calf serum (FCS) containing antibiotics. Following overnight incubation, medium was removed and each well was infected at a multiplicity of infection (m.o.i.) of 1 PFU/cell, with or without the tested extract at 37°C for 2 h.

The virus that was not absorbed at this stage was removed. Cells were coated with a layer of either carboxymethyl cellulose (CMC) (for plaque assay) or RPMI containing 2% FCS or antibiotics and increased concentrations of the tested extract was added, while no extract was added to control plates. Two days post-infection (p.i.) the CMC coat was removed, monolayers of cells were fixed in 10% formalin in saline and subsequently stained with crystal violet. Plaques were counted. CPE development was evaluated daily by microscopic observation and expressed as the percentage of damaged cells. IC50 were calculated as the extract concentration needed to cause plaque inhibition of 50% in virus-infected cell monolayers.

Selectivity index (SI)

SI was calculated for each extract as the CC50/IC50 ratio, where CC50 is the extract concentration inducing 50% cytotoxicity and IC50 is the extract concentration providing 50% prevention of PFU, measured for HSV-1.

Antiviral mechanism of plant extracts

Infected cells were treated with increasing concentrations of the extracts for several periods of time before, during or after infection. To examine possible interactions between the cells and the plant extract, Vero cells were incubated in medium supplemented with plant extract for two hours at 37°C, washed two times with saline, after which cells were infected with the virus, without further treatment. To assess possible direct effects of the plant extracts on viral infectivity, 10^7 PFU of the appropriate virus particles were pre-incubated with the extract at 37°C for either 30 min or 1 h. These mixtures were then diluted 10^6 times with fresh medium in order to minimize the extract concentration at the time of infection, and cell monolayers were infected with the diluted mixture.

Endogenous virus test

To examine possible effects of the plant extracts on the replication of the virus inside the host cell, vero cells were infected with 0.1 m.o.i. of HSV-1. After 2 h of infection the cells medium was removed and replaced with fresh medium containing 500 µg/ml plant extracts. Twenty hours post-infection; the cells were collected, washed 3 times with saline and broken by three freeze-thaw cycles. The supernatant, containing the infectious virus particles (endogenous virus) was then used for infecting fresh cell mono-layers and evaluated by the plaque assay.

Statistical analyses

Statistical analyses were performed by analysis of variance (ANOVA) and t-test (Microsoft Excel 2013).

RESULTS

Cytotoxicity of L. candidum leaf extracts

Cytotoxicity of leaf extracts from L. candidum plants grown under regular and stress conditions were examined at different concentrations (Figure 1). Among all extracts, extracts from plants under biotic stress showed the most toxicity, both at 500 and 1000 µg/ml, inducing 40% and almost 100% cell death, respectively. Extracts of plants submitted to heat shock showed some toxicity at 500 µg/ml (20% cell death) while the other extracts were not toxic at this concentration. All extracts were highly toxic at a concentration of 1000 µg/ml (Figure 1). No significant differences (ANOVA, p < 0.05) were observed among treatments at 1, 10 and 1000 µg/ml. At 100 and 500 µg/ml, toxicity of extracts from plants submitted to biotic stress was significantly higher (p < 0.05) than in all other extracts. At 500 µg/ml, toxicity of extracts from plants submitted to heat shock was significantly higher (p < 0.05) than extracts from plants submitted to drought and salinity.

Antiviral activity of plant extracts

In a previous study performed on another L. candidum ecotype, we showed that the antiviral activity of leaf ethanol extracts from plants grown under regular conditions against HSV-1, was positively correlated with the extract concentration (Yarmolinsky et al., 2009). In the same research, the highest antiviral activity was recorded against HSV-1 and HSV-2. Similar preliminary results were obtained in the present study and further experiments were conducted with the HSV-1 virus only. We examined the antiviral activity of extracts from plants grown under stress, or regular conditions, by treating Vero cell monolayers with 100 µg/ml extracts at the time of infection with 1 m.o.i. of HSV-1. Extracts from plants exposed to stress during their development had higher antiviral activity against HSV-1 than extracts from plants grown under regular conditions (Figure 2). These differences were significant (p < 0.05) in the case of heat shock, drought and biotic stress, but not for salinity. The highest effect was recorded in extracts from virus infected plants (biotic stress), followed by extracts from plants submitted to heat stress (Figure 2).
Figure 1. Effect of the environmental conditions during *L. candidum* plant growth on the cytotoxicity of their leaf extracts.

Figure 2. Antiviral effect of leaf extracts (100 µg/ml) from *L. candidum* plants grown under regular or stress conditions. Different letters above bars indicate significant differences (p<0.05).
Antiviral activities of *L. candidum* methanol extract fractions

As a further step towards isolation of active compounds, leaf extracts from plants grown under regular conditions, heat shock and drought were separated into a gradient of methanol (MeOH) fractions. Selectivity index (SI) was calculated for the extracts and the fractions, based on their antiviral efficiency against HSV-1 (Figure 3). SI was in most cases higher for extracts and fractions originating from plants which underwent stress during their growth compared to plants which grew under regular conditions (Figure 3). For all plant treatments, the highest antiviral efficiency was obtained in the 80% MeOH fraction, followed by the 60% MeOH fraction (Figure 3). In these two fractions, the SI of plants submitted to heat shock was the highest. It was interesting to note that the SI of plants grown under regular conditions was remarkably increased in the 80 and 60% fractions. The difference in antiviral efficiency of extracts from plants grown under regular conditions and plants submitted to heat shock was somewhat maintained in the fractions (Figure 3).

Effect of time of extract addition on herpes virus infection of vero cells

Vero cells were infected with 1 m.o.i. of HSV-1 virus and treated with 250 µg/ml leaf extracts at various time points before, during or after infection. No significant difference was found in viral inhibition when cells were incubated with the virus and before infection (p < 0.05). Statistically significant differences were obtained between these and all other treatments. The strongest inhibition of HSV-1 infection (about 60%) was obtained when the cells were treated with leaf extracts during the time of infection or before.
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Figure 4. Effect of time of extract addition to infected cells on plaque formation. Vero cells were infected with 1 m.o.i. of HSV-1 virus. 250 µg/ml leaf extract from L. candidum plants grown under regular conditions was added either after being incubated with the virus, before infection, during infection, after infection, or both during and after infection. Different letters above bars indicate significant differences (p<0.05).

both during the time and afterwards (Figure 4). When the cells were treated with the extract after infection only, infection was inhibited by about 25%, while extract addition before infection only, did not reduce the infection. Incubation of the extract for 30 min with the virus prior to its addition to infected cells had no effect on infection intensity (Figure 4). Supporting evidence for the interference of the plant extract with the viral replication inside the host cells came from the experiments examining the effect of the extract on one cycle of the virus replication (as detailed in the methods section). Our results clearly showed that addition of any L. candidum extract totally inhibited virus replication (Figure 5).

DISCUSSION

In a previous publication, we reported on the antiviral activity of L. candidum ethanolic leaf extracts (Yarmolinsky et al., 2009). In the present study, we demonstrated that stress conditions applied to L. candidum plants during their growth increased the antiviral efficiency of their extracts and fractions. When submitted to biotic or abiotic stress, plants react by modifying the production of secondary metabolites and in the case of medicinal plants, this feature is likely to increase the plant's therapeutic properties (Setyawans, 2011; Gorelick and Bernstein, 2014). In this study, both biotic (LSV virus infection) and abiotic (heat shock and drought) stresses enhanced the anti-herpetic properties of L. candidum ethanolic extracts, with the best results obtained from the virus-infected plants. These plants also showed the highest cytotoxicity, suggesting that viral infection during plant development induced important changes in the composition of secondary metabolites. Unfortunately, the difficulty to mechanically infect plants with viruses prevented further research on the effects of biotic stress in L. candidum extracts and we focused the study on abiotic stresses. Although basically different, biotic and abiotic stresses apparently share major regulators (Fujita et al., 2006), which could induce similar modifications. Among the abiotic stresses, heat shock induced the highest increase in anti-viral efficiency, followed by drought, while salinity treatment was comparable to regular growing conditions. These results may be due to the major difference in the application of the heat shock as opposed to the other treatments. Drought and salinity were applied to the plants over an
Figure 5. Effect of extracts from *L. candidum* plants grown under regular or stress conditions on endogenous virus production. Cells were first infected with 1 m.o.i. HVS-1 without extract. After infection, the medium was replaced by 500 µg/ml of the tested extracts. The cells were then lysed and the liquid phase, containing the endogenous virus was used for infecting fresh cell monolayers. For details, see Methods section.

extended period, during which acclimation to stress was likely to occur (Xiong et al., 2002; Yamaguchi-Shinozaki and Shinozaki, 2006), possibly lessening the changes in secondary products when the extracts were collected. This was particularly true for plants grown under salinity, whose leaf extracts showed a similar SI as the extracts from plants grown under regular conditions (not shown). On the other hand, heat shock was applied for 24 h and leaf extracts were sampled immediately afterwards, thereby exposing rapid changes in the plant extract's composition, which occurred during the treatment.

In contrast with the extract from plants undergoing biotic stress, extracts from heat- shocked plants did not show higher cytotoxicity. Therefore, the anti-herpetic efficiency of these extracts was much higher than the one from the extract of plants grown under regular conditions, as expressed by the SI values (Figure 3). It was also interesting to note that the highest efficiency of the extracts from the heat shock treatment was conserved in the methanolic fractions. The 80%-MeOH was the most active fraction for all treatments, in agreement with previous results obtained from another *L. candidum* genotype (Yarmolinsky, 2014). The 80%-MeOH fraction is abundant in flavonoids, which are known to have anti-herpetic activity (Yarmolinsky et al., 2012; Yarmolinsky, 2014). As flavonoid concentration increases in plants submitted to stress (Bartwal et al., 2013), it is reasonable to suggest that the higher anti-herpetic efficiency of the 80% methanolic fraction from stressed plants was mediated by a higher flavonoid concentration.

We also observed variation in the antiviral effect of *L. candidum* leaf extracts towards the tested viruses, as the extracts mostly inhibited HSV-1 and to a lower extend HSV-2, but did not had any effect on VZV (results on HSV-2 and VZV are not shown). These results were in line with previous findings from experiments with another
L. candidum ecotype (Yarmolinsky et al., 2009). While HSV and VZV belong to the same subfamily (Alpha-herpesvirinae) and share similar characteristics, they have different clinical manifestations, as well as different biochemical and serological properties (Field et al., 2006; Wyrwicz and Rychlewski, 2007), which may explain the discrepancy in the leaf extracts action.

The mechanism underlying the antiviral effect of the extracts may involve various steps during the life cycle of the virus. Preventing early steps of the viral infection such as attachment and penetration of the virus particles into the host cells, as suggested by the strong inhibition of the HSV-1 virus when the extract was added during the infection, and the inhibitory effect of the extract on the production of new infective viruses during one cycle of replication (Figure 5) may be part of the extract multifunctional effects. Furthermore, since incubation of the cells with the extract only prior infection did not induce viral inhibition (Figure 3), it could be inferred that only weak or reversible interactions, if any, exist between the extract and the cell membrane (Yarmolinsky, 2014).

Conclusion

In the present study we have demonstrated increased antiviral activity in L. candidum plants that were exposed to biotic, abiotic stress. This feature should be taken into consideration when growing plants for medicinal production. We also showed that differences in antiviral efficiency of extracts and fractions could be revealed by the simple in vitro test, which can be readily used in such studies.

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Conflict of interest

Authors have none to declare.

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