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Determination of Inhibition Effect of Propolis Extract on *Watermelon mosaic virus* in Edible Seed Squash

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ABSTRACT

Edible seed squash (*Cucurbita pepo* L, ESS), which is grown for its seeds to consume as a snack, has a very important place in vegetable production in Türkiye. Viruses are one of the most problems in the cultivation of ESS in the country. One of these diseases is *Watermelon mosaic virus* (WMV, *Potyvirus*, Potyviridae). Also, it's very common and devastating on other cucurbits worldwide. Propolis, which is composed mainly by the plant resins and exudates that honey bees gather, showed antiviral activity against plant and human viruses. In this study, the potential of propolis to control WMV was investigated by taking advantage of these antimicrobial properties of propolis. For this purpose, propolis extracted with 95% ethanol and diluted in distilled water to obtain different concentrations as 2, 4, 6, 8 and 10% were used. Effects of propolis against WMV were determined by *in vitro* and *in vivo* studies. Except of 2, and 4%, all concentrations caused to symptoms reductions of the infection, in all studies. In the result of *in vitro* studies, the ratios of healthy plants were calculated as 10, 20, 40% and 20, 30, 50% after one and two hours, respectively. *In vivo*, by spraying concentrations of 6, 8 and 10% before inoculation obtained healthy plants as 20, 40 and 60% after one-hour period, and as 20, 30 and 50% after two-hour period, while the extracts sprayed after inoculation, ratios of healthy plants as 10, 20 and 30% after one-hour period, and as 10, 20 and 20% after two-hour period. According to the results of the study, it was determined that applications of different propolis concentrations had the potential to reduce WMV infection, and these results should be supported by field trials.

1. Introduction

Propolis is a resinous mixture that bees produce from a mixture of various flower nectars and their own secretions to prevent other organisms (such as insects and microorganisms) from entering the hive through the hive entrance and cracks in the hive. The word propolis is derived from two Greek words, pro (in front of) and polis (city or community). This substance has been used in traditional medicine all over the world since ancient times. Recent research has shown that propolis has a wide range of pharmacological properties, including antibacterial, antioxidant, anti-inflammatory and anti-tumor activities (Marcucci, 1995; Bankova et al., 1996; Kujumgiev et al., 1999; Abd El Hady and Hegazi, 2002). The activity and components of propolis vary according to its geographical origin (Kujumgiev et al., 1999). Many different studies have been conducted on the antifungal and antibacterial activities of propolis, but there is also abundant information on the antiviral activity of this substance (Marcucci, 1995; Takemura et al., 2012). The antiviral activity of propolis has been reported for different plant and animal viruses such as Influenza A and B viruses, Human immunodeficiency virus (HIV), Hepatitis virus, Infectious bursal disease virus (IBDV), Herpes simplex virus (HSV), Vaccinia virus, Poliovirus and reovirus, *Broad bean mottle virus* (BBMV), various viruses in potato (Abd El Hady and Hegazi, 2002; Fahmy and Omar, 1989; Mohamed and Owayss, 2005; Bufalo et al. , 2009; Schnitzler et al., 2010; Doğan and Hayoğlu, 2012; Coelho et al., 2014).

Squashes or pumpkins are produced in many parts of the world and have an important commercial importance in different economies. Total cucurbit production worldwide is higher than that of tomatoes or citrus fruits and is about half the size of potatoes (Gaba et al., 2004). According to the data of 2021, ESS was planted in 866.682 da agricultural area in Türkiye and 64.861 tons of squash seeds were produced (TÜİK, 2022). As in many parts of the world, squash is produced in Türkiye for its seeds and flowers as well as for its fruits (Vural et al., 2000).

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Squash plant has a special importance among cucurbits since its fruits are consumed as vegetable and its seeds are consumed as snacks. Seeds of squash or pumpkin (*Cucurbita pepo* L., *C. moschata* Duchesne) are one of the most consumed snack foods in the Türkiye. In recent years, with the decrease in rainfall and the increase in irrigation costs, there has been an increased interest in edible seed squash (ESS) cultivation in Türkiye, since it can be grown well in arid environments (Yeşil, 2021a).

Watermelon mosaic virus (WMV) is a disease agent of the genus *Potyvirus* in the family Potyviridae with a single-stranded RNA genome and 780x12 nm curved rod-shaped virions. This virus is non-persistently transmitted by more than 20 aphid species (*Aphis gossypii, A. craccivora, A. spiraecola, Myzus persicae* and *Macrosiphum euphorbiae*). It can also be easily mechanically transferred from infected plants to healthy plants by plant sap. In warm regions, it persists throughout the year in wild cucurbits (*Melothria pendula, Momordica* spp. and other cucurbits) and cultivated cucurbits. WMV causes apparent leaf symptoms and severe stunting in susceptible cucurbit species. Symptoms such as mosaic, blister mosaic, vein banding, curling and narrowing of the leaf area occur on the leaves of cucurbit crops. Some varieties show mottling on the leaves. In fruits, deformation usually occurs and fruit color changes in some varieties (Anonymous, 2022). WMV can infect more than 170 plants, including cucurbits, some legumes and orchids (Sharifi et al., 2008). Many studies have reported that WMV is widespread in cucurbit production areas of Türkiye and causes economically important yield and quality losses in these plants (Yılmaz et al., 1992; Kaya and Erkan 2011; Topkaya and Ertunç 2012; Yılmaz 2014; Şevik and Balkaya 2015; Korkmaz et al., 2018; Yeşil: 2019a, b; 2020, 2021a).

The main objective of this study was to determine the effect of different propolis concentrations on WMV infection and to demonstrate an environmentally friendly approach in the control of WMV, one of the most common viral diseases in cucurbits including vegetable species grown in large areas in Türkiye.

2. Materials and Methods

2.1. Virus inoculum

Within the scope of the study, WMV isolate coded W-26 (Yeşil, 2013), which was previously isolated from zucchini and diagnosed both serologically and molecularly, and at the same time, the coat protein gene (cp) was subjected to partial sequence analysis and uploaded to the gene bank (NCBI) with the accession number KF021299, was used as the virus inoculum source. WMV-infected leaf samples stored in the deep freezer were crushed in mortars containing PBS (Phosphate-Buffered Saline, phosphate buffer, 0.01M, pH:7) buffer using a mortar and pestle on ice. In order to confirm the purity of the WMV-contaminated plant extract, serial inoculations were carried out consecutively on *Chenepodium quinoa* Willd. plants, the local lesion host of the virus. Subsequently, the virus was inoculated with cotton by applying previously carborundum powder to the leaves of the squash for replication. These plants were kept under climate chamber conditions (25 ± 2 ℃, 14 s. light / 10 s. dark) and were observed 14 days after inoculation and were found to show WMV symptoms. WMV infections in samples taken from these plants were confirmed by DAS-ELISA test before the virus was used as an inoculum source.

2.2. Preparation of propolis extracts

Pure propolis samples obtained from honey bee breeders were extracted with 95% pure ethanol, filtered through two layers of muslin and the ethanol was removed by evaporation (Mohamed and Owayss, 2005). Dilutions of 2, 4, 6, 8 and 10 % concentrations were prepared with distilled water to be used in the experiments. The effects of different propolis concentrations on WMV infection were investigated in in vitro and in vivo experiments.

2.3. In vitro effect of propolis extract on WMV

In this phase of the study, different concentrations (2, 4, 6, 8 and 10 %) of propolis dilutions were mixed with WMV-infected plant extract and the effect of this mixture on the activity of the virus was tried to be revealed. For this purpose, 500 µl each of WMV-infected plant extract and different concentrations of propolis dilution were mixed. *In vitro* experiments were carried out by mechanically infecting 1 ml of the mixture (WMV-infected plant extract + propolis dilution) on the leaves of edible seed squash immediately, after 1 and 2 hours of waiting. In each assay, 10 edible seed squash plants that had developed true leaves were inoculated and treatments were evaluated 14 days later according to the presence of symptoms. Mechanically inoculated plants were tested by DAS-ELISA to confirm the presence or absence of WMV. In addition, only WMV-inoculated plants were used as positive controls and only propolis dilution-inoculated plants were used as negative controls.

2.4. In vivo effect of propolis extract on WMV

In this phase of the study, propolis dilutions of different concentrations (2, 4, 6, 8 and 10 %) were applied directly to the leaves before (immediately before, 1 and 2 hours before) and after (immediately after, 1 and 2 hours after) WMV infection. Spraying was carried out until the edible seed squash leaves infected or to be infected with WMV were completely wetted with the dilution. In the *in vivo* stage, all plants were mechanically infected with WMV. In each experiment, 10 edible seed squash plants that had developed true leaves were infected with the virus and kept under climate chamber conditions (25 ± 2 ℃, 14 s. light/10 s. dark photoperiod) for symptom development. After 14 days, treatments were evaluated according to the presence of symptoms. Both symptomatic and healthy-looking plants were tested by DAS ELISA test to confirm the presence and

absence of WMV, respectively. In addition, only WMV-infected plants were used as positive controls and only propolis dilution-infected plants were used as negative controls.

3. Results and Discussion

3.1. Symptoms observed in WMV-infected ESS plants

The WMV isolate coded W-26 used during the study was obtained from the leaves of the edible seed squash (*Cucurbita pepo* L.), which was shown to be naturally infected by different methods in a previous study. When the isolate was inoculated on *C. quinoa* Willd. plants, local lesions were observed and the isolate was purified by repeated inoculations. Subsequently, the isolate was inoculated on the edible seed squash plants for propagation.

Mechanical inoculations of the W-26 isolate on the edible seed squash plants caused the formation of mosaic symptoms approximately 1 week after inoculation. However, observations performed on the 10th day of inoculation showed that in addition to the mosaic symptom, deformations such as blistering and roughening also developed on the leaves.

3.2. In vitro effect of propolis extract on WMV

The results of mixing different concentrations of propolis dilutions (2, 4, 6, 8 and 10 %) with WMV-infected plant extracts and the effect of this mixture on the activity of the virus immediately or after 1 and 2 hours are summarized in Table 1. After inoculation of the propolis+WMV mixture on edible seed squash plants, the plants were observed on the 14th day and symptoms were recorded. In the treatments carried out at this stage of the study, all of the propolis dilutions mixed with WMV-infected plant extract were found to be ineffective against WMV infections when applied to the leaves without waiting, while all other propolis concentrations except 2% and 4% concentrations were found to be effective in suppressing WMV infection when applied after waiting for 1 or 2 hours. The percentages of healthy plants were calculated as 10, 20, 40 and 20, 30, 50 %, respectively, after 6, 8 and 10 % propolis dilutions were applied to the leaves of the edible seed squash after one and two hours of waiting. As a result of *in vitro* treatments, propolis extract at 10% concentration was found to be the most successful concentration in inhibiting WMV infections.

In addition, the plants were subjected to DAS-ELISA test to confirm that the presence or absence of WMV infection was the cause of the presence or absence of symptoms in all inoculated squash plants in the treatments. Therefore, the presence of WMV was detected in symptomatic plants, while WMV infections were not detected in healthy plants.

Table 1
Inhibition effects of different concentrations of propolis extract on WMV infections according to different periods of storage

Treatment	Nr. of plants	K+[1]	K-[2]	Propolis concentration (%)				
				2	4	6	8	10
Immediately	Inoculated	10	10	10	10	10	10	10
	Healthy	0	10	0	0	0	0	0
	Effect (%)	0	100	0	0	0	0	0
After 1 hour	Inoculated	10	10	10	10	10	10	10
	Healthy	0	10	0	0	1	2	4
	Effect (%)	0	100	0	0	10	20	40
After 2 hours	Inoculated	10	10	10	10	10	10	10
	Healthy	0	10	0	0	2	3	5
	Effect (%)	0	100	0	0	20	30	50

[1]K+: Only WMV-inoculated positive control, [2]K-: only propolis-inoculated negative control.

3.3. In vivo effect of propolis extract on WMV

The results of the inhibition of WMV infection by spraying the leaves with propolis extract prepared at concentrations of 2, 4, 6, 8 and 10 % before and after WMV infection are given in Table 2. At this stage of the study, propolis concentrations were sprayed on the leaves of the edible seed squash at different times: immediately before WMV infection, 1 and 2 hours before WMV infection, immediately after WMV infection and 1 and 2 hours after WMV infection. Again, 14 days after WMV inoculation, the plants were evaluated according to the presence or absence of symptoms. When the *in vivo* treatments results were evaluated, it was observed that, as in the *in vitro* treatments, propolis applications made immediately before or immediately after WMV inoculation did not show success at any concentration. However, it was observed that spraying the leaves with 2% and 4% propolis dilution was ineffective in terms of preventing WMV infection for all application times. In *in vivo* treatments, the ratios of healthy plants obtained by spraying 6, 8 and 10% dilutions of propolis before inoculation were calculated as 20, 40 and 60 % when applied one hour before inoculation and 20, 30 and 50 % when applied 2 hours before inoculation. Again, in propolis applications performed after WMV inoculation, healthy plant ratios were determined as 10, 20 and 30 % after one hour and 10, 20 and 20 % after two hours.

In addition, plants were subjected to DAS-ELISA test in order to confirm that the presence or absence of WMV infection was the cause of the presence or absence of symptoms in all inoculated squash plants in the treatments. Thus, the presence of WMV was detected in

symptomatic plants, while WMV infections were not detected in healthy plants.

As a result of the *in vivo* treatments, the effect of different concentrations of propolis extract on reducing WMV-induced symptoms in the edible seed squash plant was highest when applied before WMV inoculation. When the results were analyzed, it was observed that when propolis extract was applied to the leaves of the squash plant 1 hour before WMV inoculation, the concentrations of 6, 8 and 10% prevented WMV infection by 20, 40 and 60%, respectively. These results are promising especially in the control of WMV, which is a virus that has a large number of host plants and weed species belonging to different families and can be transmitted very effectively by about 20 different aphid species. In this context, it would be useful to confirm the data obtained in this study by testing them in field conditions.

Table 2
Inhibition effects of different concentrations of propolis extract applied before and after WMV inoculation on WMV infection in edible seed squash plants

Treatment	Nr. of plants	K+[1]	K-[2]	Propolis concentration (%) (Before / After inoculation)				
				2	4	6	8	10
Immediately	Inoculated	10	10	10/10	10/10	10/10	10/10	10/10
	Healthy	0	10	0/0	0/0	0/0	0/0	0/0
	Effect (%)	0	10	0/0	0/0	0/0	0/0	0/0
1 hour	Inoculated	10	10	10/10	10/10	10/10	10/10	10/10
	Healthy	0	10	0/0	0/0	2/1	4/2	6/3
	Effect (%)	0	10	0/0	0/0	20/10	40/20	60/30
2 hours	Inoculated	10	10	10/10	10/10	10/10	10/10	10/10
	Healthy	0	10	0/0	0/0	2/1	3/2	5/2
	Effect (%)	0	10	0/0	0/0	20/10	30/20	50/20

[1]K+: Only WMV-inoculated positive control, [2]K-: only propolis-inoculated negative control.

The results obtained in this study on the inhibitory effect of propolis extract on virus infection are similar to the results of previous studies on the subject in the literature. It was reported that propolis dilutions applied both *in vitro* and *in vivo* conditions to suppress *Cucumber mosaic Cucumovirus* (CMV, Bromoviridae) infections in zucchini plants suppressed CMV by up to 50% in treated plants (Yeşil, 2021b). In a study conducted in Serbia, different propolis concentrations were applied under both *in vitro* and *in vivo* conditions to control infections caused by *Zucchini yellow mosaic Potyvirus* (ZYMV, Potyviridae), which is an important problem in oil pumpkin cultivation areas. According to the results of the application, it was observed that propolis extract prepared at concentrations of 5 and 10% showed inhibitory effects on ZYMV infection both *in vitro* and *in vivo* conditions (Vucurovic et al., 2017). Mohamed and Owayss (2005) showed that different propolis concentrations applied *in vivo* and *in vitro* on *Broadbean mottle Bromovirus* (BBMV, Bromoviridae) reduced virus infection by up to 35-80%. Fahmy and Omar (1989) planted apical meristems and shoots from six different potato cultivars infected with viruses on modified Murashig-Skoog (MS) culture medium supplemented with propolis extract, and it was determined that the presence of Potato S, Y, M and X viruses was significantly reduced in potato plants grown on this propolis-containing medium for about 1 month. In the studies in the literature, it is reported that propolis also has inhibitory effects on viruses that cause disease in animals. Coelho et al. (2014) reported that propolis caused up to 64-fold decrease in *Picornavirus* replication, 32-fold decrease in influenza virus, 8-fold decrease in measles virus and 103-fold decrease in rubella virus replication. In addition, as a result of a study on mice; it was reported that when 5% propolis solution was applied before Influenza virus infection, the infection was completely prevented, while propolis did not show any inhibitory effect when applied after infection (Ghisalberti, 1979).

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