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SHORT COMMUNICATION

Antiviral activity of *Ribes uva-crispa* L. extracts *in vitro*

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Abstract: There is currently no approved vaccine or a useful antiviral drug against respiratory syncytial virus (RSV) that causes viral infection worldwide. Crude plant extracts can be an important resource for the development of new anti-RSV agents. In this study, cytotoxic and anti-RSV effect of the extracts *Ribes uva-crispa*, which has been known as "gooseberry" in Turkey and fruits used in the treatment of the various disorders, were evaluated by colorimetric XTT method. Results were expressed as 50% cytotoxicity (CC_{50}), 50% effective concentration (EC_{50}) and selectivity index (SI: CC_{50} / EC_{50}). Of the tested extracts, the highest antiviral activity was found to be 96.90µg/mL EC_{50} and 11.70 SI from fruit aqueous extract; it was followed by leaf methanol extract (EC_{50}: 2527.41µg/mL, SI: 6.55), leaf aqueous extract (EC_{50}: 1093.37µg/mL, SI: 1.40) and fruit methanol extract (EC_{50}: 11262.35µg/mL, SI: 0.56), respectively. As a result, we can say that these extracts, especially *Ribes uva-crispa* fruit aqueous and leaf methanol extracts, are worthy of further studies for the development of new and unique anti-RSV drugs.

Keywords: *Ribes uva-crispa*, aqueous extract, methanol extract, anti-RSV activity.

INTRODUCTION

Human respiratory syncytial virus (RSV) is one of the viruses that infect people of all ages, especially children. Although the most common clinical finding in RSV infection is upper respiratory tract infection, it can also cause bronchiolitis and rarely pneumonia in young children (Di Giallonardo *et al.,* 2018). Reinfections depending on the nature of RSV and the mode of infection is a very common phenomenon showing that acquired immunity does not provide long-term protection. This has made it impossible to develop an effective vaccine for now. At present, the Food and Drug Administration (FDA) has approved prophylactic drugs for RSV including palivizumab and ribavirin that were used with symptomatic and supportive treatment. Palivizumab, a humanized monoclonal antibody directed against the RSV F protein, effectively prevents RSV infection, while it is expensive and ineffective in the treatment of an existing RSV infection. Ribavirin has been shown to have potent activity against RSV *in vivo* and in vitro; however, the comparison of the results of both animal and cell tests in humans has not yet been completed. Furthermore, the use of ribavirin is limited due to its adverse effects (Lin *et al.,* 2016). For all these reasons, there is an urgent need to develop new and effective anti-RSV drugs for the treatment of RSV infections. Plant extracts can be an important resource in the development of these drugs (Duman *et al.,* 2018).

Turkey, which contains about 12000 plant taxa has a very rich flora (Erik and Tarıkahya, 2004). 234 of these taxa are foreign origin and cultivated plants, and the remaining species are naturally distributed in Turkey (Ekim *et al.,* 1989; Erik and Tarıkahya, 2004). Turkey is also rich in plant endemism. The total number of endemic taxa in all European countries is approximately 2750, while the number of endemic taxa in Turkey is 3778 (Erik and Tarıkahya, 2004). As a result of different studies, the number of plant species in Turkey is increasing with each passing day by the identified new species.

Many of the taxa being used as berries grow naturally in Turkey. These fruits are rich in vitamins and minerals, they are also important in terms of human health, and their usage is increasing in food sector (fruit juice, fruit yoghurt, ice cream, canned food, jam, etc.) (Karaer and Adak, 2006). Grossulariaceae is one of the families with ripe berries. This family plants are mainly distributed in the northern temperate zone. The family is limited to the genus *Ribes* and contains about 200 species (Heywood *et al.,* 2007). 8 species (*R. biebersteinii* Berl. ex. DC., *R. nigrum* L., *R. uva-crispa* L., *R. alpinum* L., *R. orientale* Desf., *R. multiflorum* Kit. ex Romer & Schultes, *R. anatolica* Behçet) of the genus *Ribes* in Turkey grow as naturally, and one (*R. rubrum* L.) is cultivated (Behçet, 2001; Chamberlain, 1972; Chamberlain, 1988).

The biological activities of *Ribes* species (antimicrobial, antioxidant, antitumor, antihypertensive and anti-inflammatory activity studies) have been extensively studied in recent years (Bishayee *et al.,* 2010; Bishayee *et al.,* 2011; Chen *et al.,* 2014; Ehrhardt *et al.,* 2013; Hirano *et al.,* 1997; Ikuta *et al.,* 2012; Kılıç *et al.,* 2008; Knox *et*

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al., 2001; Knox *et al.,* 2003; Lu *et al*., 2002, McCutcheon *et al.,* 1992, McCutcheon *et al.,* 1994, Rauha *et al.,* 2000, Serteser *et al.,* 2009; Stevic *et al.,* 2010; Suzutani *et al.,* 2003; Tabart *et al.,* 2012). The studies on the antiviral activities of *Ribes* species are rather inadequate and mostly focused on *R. nigrum*. This species, which is naturally growing in Turkey, has been shown to have antiviral activity against Herpes Simplex Virus Type 1-2, Bovine Herpes Virus Type 1, Varicella Zoster Virus and Influenza Viruses (Ehrhardt *et al.,* 2013; Duman *et al.,* 2018; Kendir *et al.,* 2016; Knox *et al.,* 2003; Suzutani *et al.,* 2003). Methanol and aqueous exctracts of the leaves and fruits obtained from *Ribes uva-crispa* L. and *Ribes multiflorum* Kit., which are naturally grown in Turkey, were tested for antiviral activity and all of the extracts had been found to have effective against HSV-1 (Duman *et al.,* 2018). Kendir *et al.* (2016), investigated the antiviral activity of methanol and water extract obtained from *Ribes* species, naturally grown in Turkey, against Bovine Herpes Virus Type 1 (BHV-1), and they have found antiviral activity of water extract prepared from the branch of *R. multiflorum* while there was no antiviral activity the extracts of *Ribes uva-crispa*. *R. uva-crispa* fruits are used as fresh, due to their laxative, urine enhancer, stomachic and appetizing effect (Baytop, 1999). *R. uva-crispa* plant is known as "gooseberry" in Turkey and Fructus *Ribis uva-crispa* drugs obtained from its fruits have been used against swelling and inflammation by İbn-i Sina (İbn-i Sina, 2000). *R. orientale* is known as "Çeçem" in Erzurum and its fruits are eaten in this region, it is also known as "grape berry" (Baytop, 1994). *R. biebersteinii* fruits are used fresh or dried against anemia in Erzurum (Özgen and Çoşkun, 2000). In an ethnobotanical study conducted in the villages of Ilıca in the province of Erzurum, it was stated that the fruits of *R. biebersteinii* were consumed fresh or dry (Özgen *et al.,* 2004). In this study, it was aimed to evaluate the anti-HRSV activity of *Ribes uva-crispa*, naturally growing in Turkey, and to contribute to antiviral drug development efforts.

MATERIALS AND METHODS

Plant materials

R. uva-crispa samples were collected from Ankara: Kızılcahamam, Sarayköy, on the road of Çukurören, 3rd km, in stony place, 1137m, 15.05.2015-14.07.2015. Samples were identified Prof. Dr. Muhittin DİNÇ (Necmettin Erbakan University, Biology Department).

Aerial parts of the species were dried in the shade, ground into a fine powder by a mill and stored in sterile black glass jars at room temperature. A voucher sample was kept at Kon Fungarium, Selcuk University, Science Faculty, and Biology Department, Turkey.

Cell and virus

Human larynx epidermoid carcinoma cells [HEp-2; ATCC (the American Type Culture Collection) CCL 23] were used to culture human respiratory syncytial virus (RSV Long strain: ATCC VR-26). Reagents and medium for cell culture were purchased from different companies. Cells were propagated at 37°C in 5% CO_2 in EMEM supplemented with 10% fetal bovine serum (FBS, ATCC-30-2020), 10000U/mL penicillin, 10mg/mL streptomycin and 25µg/mL amphotericin B. Virus was propagated on 90% confluent cell monolayer in EMEM with 2% FBS and antibiotics as described above. Viral titer was determined by 50% tissue culture infectious dose ($TCID_{50}$) method and expressed as $TCID_{50}$ per 0.1mL (Kaerber, 1964). Virus was stored at -80°C until use.

Preparation of the extracts

Each 30g sample in powder form was placed separately in 400 mL of methanol and 400mL of sterile distilled water, and extracted for 1 hour with an ultrasonicator at 37°C. Plant extracts were filtered through Whatman No: 1 filter paper, and then the solvents used were completely evaporated at 40°C under reduced pressure in a rotary evaporator (Heidolph Laborota 4000, Germany). After evaporation, the plant extracts were lyophilized at -110°C under reduced pressure in the lyophilizer (Labconco, USA). Each 1000mg of the lyophilized methanol and aqueous extract were dissolved in 10mL of EMEM (serum-free) and stock solutions were prepared at a concentration of 100mg/mL. The stock solutions were sterilized by trough in 0.22µm Millipore filter, then stored in 2mL tubes at a rate of 1mL concentrations and stored at +4°C until use. Ribavirin (RBV, R9644-10 mg, Sigma, USA), a drug approved for the treatment of RSV infections in humans, was purchased. 10 mg ribavirin was dissolved in 10mL of EMEM (serum-free). This 1mg/mL (1000µg/mL) stock concentration were filtered by trough in 0.22 µm Millipore filter, then they were stored at -80°C or +4°C (When stored at + 4°C, it was used within 1 week).

Cytotoxicity assay

The cytotoxicity of the extracts and positive control anti-RSV drug RBV on HEp-2 cells was assessed by XTT method, as previously described by Ho *et al.* (2010), with some modifications. Briefly, the stock solutions of the extracts and RBV were diluted as 2-fold using EMEM (growth medium, GM) with 10% FBS to obtain appropriate concentrations. Thus, the dilutions of the extracts with the concentrations of 100000, 50000, 25000, 12500, 6250, 3125, 1563, 781, 391, 195µg/mL, and the concentrations for RBV as 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95µg/mL were prepared.

HEp-2 cells (0.625×10^4 cells) in 100µL GM were seeded in 96-well plates and incubated at 37°C in 5% CO_2 for 24 h. Then, 100µL of two-fold diluted extracts were added to wells. 200µL of GM was put to each cell control well without extracts. Plates were incubated for 48 h. Then the

medium in each well was removed, and 150µL GM and a mixture of 50µL from 0.1mL PMS (N-methyl dibenzopyrazine methyl sulfate) and 5mg/5mL XTT (sodium 3mL - [1- (phenylaminocarbonyl) -3,4-tetrazolium] - bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) were added to each well. The plates were further incubated for 2h to allow XTT formazan production. The optical densities were determined with the ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 450nm and a reference wavelength of 690 nm. The tests were performed in triplicate and the results were shown as the ratio of the average cytotoxicity to the cell control.

To calculate the percentage cytotoxicity of the sample tested, the following formula was used where A represents the OD of cell control, B represents the OD of cells treated with extracts or RBV:

$$\text{Cytotoxicity (\%)} = \frac{(A - B)}{A} \times 100$$

50% Cytotoxic Concentration (CC_{50}), which was defined as the concentration reducing the optical density (OD) of the cells treated with extracts or RBV by up to 50% compared to the cell controls (CCs) was determined. Maximum non-toxic concentration (MNTCs) of the extracts and RBV was determined by comparing with the OD of the cell controls. These MNTCs were used to determine the antiviral activity of the extracts or RBV.

Antiviral assay

Anti-RSV activity of the extracts and RBV were examined by a XTT-based method modified from "Cytopathic Effect (CPE) Reduction Assay" previously described (Ho *et al.,* 2010). 2.5×10^4 HEp-2 cells in 100 µL GM were seeded into each well of 96-well plates and incubated for 24h. Two fold decreasing dilutions of the extracts and RBV by using EMEM (maintenance medium, MM) with 1% serum were prepared from two fold concentrated dilutions which were previously determined MNTC in cytotoxic assay. When the cells were confluent, the GM was removed, and 100µL of RSV suspension at 100 $TCID_{50}$ and 100µL two-fold diluted samples at each dilution were added simultaneously to the treatment wells. For the virus control wells, RSV and the MM without the sample were added. For the cell control wells, 200µL of the MM without the extract and virus were added. The plates were incubated at 37ºC in 5% CO_2 for 3 days (until the presence of maximum syncytium formation in virus control wells).

After the maximum syncytium presence was observed in the virus control wells, the supernatant in the wells was removed and the wells were filled with 150µL EMEM (serum free). Then a mixture of 50µL from 0.1mL PMS and 5 mg/5mL XTT were put in to each well. The plates were gently shaken to homogeneously distribute the dye into the wells. The plates were incubated for further 3 h to form the XTT formazan product. ODs were read by an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 450 nm and a reference wavelength of 690 nm, and ODs averages from the wells were recorded. The percentages of protection of different extracts and RBV concentrations were calculated spectrophotometrically as [(AB) / (C-B) × 100], where A, B and C indicate the absorbance (optical densities) of the extracts or RBV, virus and cell controls, respectively.

Fig. 1: A view of uninfected HEp-2 cells (Original).

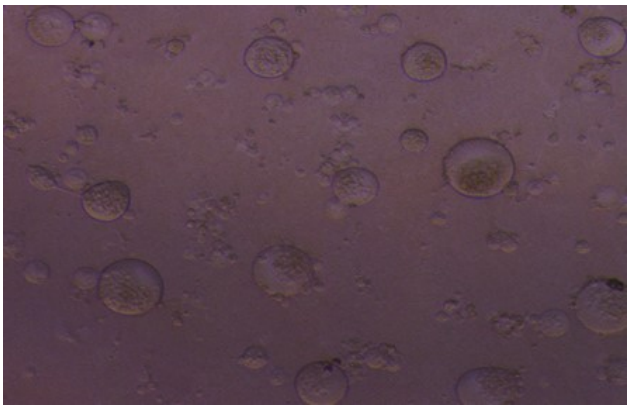

Fig. 2: CPE view of RSV in HEp-2 cells (Original).

EC_{50} values (defined as extract or RBV concentration providing protection in 50% of infected cells) by using % protection ratio observed against extract and RBV concentrations were determined. The selectivity index of the extracts and RBV (SIs) were calculated from the CC_{50} / EC_{50} ratios.

STATISTICAL ANALYSIS

To determine CC_{50} and EC_{50} values of the extracts and Ribavirin, non-linear regression analysis was done in the GraphPad Prism Version 5.03 statistical program.

RESULTS

Virus titration

In the titration of RSV in HEp-2 cell culture by the microtitration method, the power of infectiousness was

Table: Cytotoxicity and antiviral activity assays of methanol and aqueous extracts prepared from fruits and leaves of *Ribes uva-crispa*.

Plant name	Extract type	Cytotoxicity		Anti-HRSV activity	
		MNTC (µg/mL)	CC_{50} (µg/mL)	EC_{50} (µg/mL)	SI
Ribes uva-crispa	Fruit methanol	3125	6293.56	11262.35	0.56
	Fruit aqueous	3125	16552.91	2527.41	6.55
	Leaf methanol	195	1134.19	96.90	11.70
	Leaf aqueous	391	1526.77	1093.37	1.40
Ribavirin (RBV)		0.98	117.00	4.19	27.92

determined as $DCID_{50} = 10^{-4.5}$/0.1mL at the end of the 5th day. The CPEs of the virus in HEp-2 cells, and the appearance of uninfected HEp-2 cells (HEp-2 Control) are shown in figs. 1 and 2.

Cytotoxicity and antiviral results

The cytotoxicity rates and antiviral results calculated in order to determine the MNTC and CC_{50} value of Ribavirin and the different extracts of *R.* uva *crispa* were given in table. The MNTC of the ribavirin was determined as 0.98µg/mL, and its CC_{50} value was 117 µg/mL. MNTC of *R. uva-crispa* fruit methanol and aqueous extracts were determined as 3125µg/mL and CC_{50} values were 6293.56µg/mL and 16552.91µg/mL, respectively. MNTC of *R. uva-crispa* leaf methanol and aqueous extracts were determined as 195µg/mL and 391 µg/mL, and CC_{50} values were 1134.19µg/mL and 1526.77µg/mL, respectively. The EC_{50} value of RBV was determined as 4.19µg/mL and SI was 27.92. EC_{50} value of *R. uva-crispa* fruit methanol extract were determined as 11262.35µg/mL and SI were 0.56. EC_{50} value of *R. uva-crispa* fruit aqueous extract were determined as 2527.41µg/mL and SI was 6.55. EC_{50} value of *R. uva-crispa* were determined as 96.90µg/mL and SI was 11.70. EC_{50} value of *R. uva-crispa* leaf aqueous was 1093.37µg/mL and SI was 1.40.

DISCUSSION

HRSV can infect the upper respiratory mucosa and replicate in the nasopharynx initially. HRSV is probably rapidly spreading to the lower respiratory tract by aspiration of secretions. HRSV mainly causes morbidity and mortality with pathology of the lower respiratory system. Therefore, management of HRSV infection requires an effective strategy to prevent viral infection of both the upper and lower respiratory tract (Collins and Crowe, 2007).

This study showed that methanol and aqueous extracts obtained from *R. uva-crispa* fruits and leaves were effective in different degrees in the inhibition of HRSV, although they are not as effective as RBV as a standard drug against RSV infections.

The most potent anti-HRSV activity among these extracts were observed from the fruit aqueous (EC_{50}=2527.41 µg/mL; SI=6.55) and the leaves methanol (EC_{50}=96.90 µg/ml; SI=11.70), while the fruit methanol (EC_{50}= 11262.35µg/ml; SI=0.56) and leaf aqueous (EC_{50}= 1093.37µg/ml; SI=1.40) were found to have weak anti-HRSV activity. EC_{50} and SI values of RBV were found as 4.19µg/mL and 27.92, respectively. As shown in Table, the extracts (fruit aqueous and leaf methanol) found to have potent anti-HRSV activity are less toxic to the HEp-2 cells than RBV. It is also noted that CC_{50} values of the extracts and RBV are higher than the EC_{50} values. This is important for the safety of an antiviral agent (Schinazi *et al.,* 2009). Furthermore, Chattopadhyay *et al.* (2009) reported that if SI values are 3 or greater than 3, it should be considered as an indicator of the potentially reliable antiviral activity of the test extracts.

Phenolic compounds, such as simple phenols, flavonoids and phenolic acids, are commonly found in plants (Lule and Xia, 2005). Flavonoids can be divided into 4 main groups based on their molecular structure: flavones, flavanones, catechins and anthocyanidins (Rice-Evans *et al.,* 1996). Several studies have found that flavone derivatives are inhibitors of RSV virus (Barnard *et al.,* 1993; Kaul *et al.,* 1985). Kendir and Koroglu (2015) examined the various *Ribes* species including *R. uva-crispa*, which constitute the material of our research subject, in terms of their morphological, anatomical, chemical and biological activities. In this study, the total phenolic contents of methanol and aqueous extracts prepared from the leaves of *R. uva-crispa* were determined as 273.13 and 341.25mg/g, whereas the total phenolic contents of methanol and aqueous extracts from the branches of the same plant were determined as 247.50 and 333.44mg/g, respectively. Nevertheless, the fruits of the *Ribes* species were not examined for their chemical composition.

As a result, anti-RSV activities of *R. uva-crispa* may be due to have the high or small amount of phenolic compounds [flavonoids (such as flavones, flavanones, catechins and anthocyanidins)] in the their ingreedings and the ability of the solvents to dissolve these compounds.

CONCLUSION

In this study, it was revealed that methanol and aqueous extracts prepared from the leaves and fruits in *R. uva-crispa* tested with colorimetric XTT method for anti-RSV activities have different degrees of anti-RSV activity. It was determined that leaf methanol and fruit aqueous extracts of the plant had a strong activity against RSV, whereas leaf water and fruit methanol extracts were found to have weak antiviral activity. It can be said that *R. uva-crispa* extracts, especially leaf methanol and fruit aqueous extracts, are worthy of further studies (detection of active compound/compounds responsible for anti-HRSV activity) in the fightening against HRSV infection.

Therefore, in the future researches, it is tried to determine the anti-RSV activities of the pure compound or compounds to be obtained from the extracts of the same plant species by removing these deficiencies.

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