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Research Article

EVALUATION OF THE ANTIVIRAL ACTIVITY OF *Ballota glandulosissima* Hub.-Mor. & Patzak EXTRACTS AGAINST RESPIRATORY SYNCYTIAL VIRUS (RSV)

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Abstract: In order to find new and effective antiviral agents from natural sources, the antiviral properties of methanol and aqueous extracts of *Ballota glandulosissima* Hub.-Mor. & Patzak were evaluated by Colorimetric XTT test against Respiratory syncytial virus (RSV). The concentration required to provide 50% protection against cytopathic effects caused by the virus was defined as EC_{50}, the selectivity index (SI) was determined from the ratio of CC_{50} (50 % Cytotoxic concentration) to EC_{50}. The results showed that both the methanol (EC_{50} = 12.45 µg/mL; SI = 24.84) and aqueous extracts (EC_{50} = 19.12 µg/mL; SI = 24.59) of *B. glandulosissima* had almost the same strong anti-RSV activity as well as ribavirin, used as a positive control against RSV (EC_{50} = 3.25 µg/mL, SI = 34.89). In conclusion, we can say that *B. glandulosissima* extracts are worthy of further studies in order to develop an alternative to the drugs used in clinical practice against RSV. This is the first report on the anti-RSV activity of *B. glandulosissima*.

Key words: *Ballota glandulosissima*, methanol and aqueous extracts, antiviral activity, respiratory syncytial virus.

Özet: Doğal kaynaklardan yeni ve etkili antiviral etkenler bulmak amacıyla yapılan bu çalışmada, *Ballota glandulosissima* Hub.-Mor. & Patzak'dan elde edilen metanol ve su ekstraktlarının antiviral özellikleri Respiratuvar Sinsityal Virus (RSV)'una karşı kolorimetrik XTT testi ile değerlendirilmiştir. Virüsün neden olduğu sitopatik etkilere karşı %50 koruma sağlaması için gerekli konsantrasyon EC_{50} olarak tanımlanmış, CC_{50} (%50 Sitotoksik Konsantrasyon)'nin EC_{50}'ye oranından da seçicilik indeksi (SI) belirlenmiştir. Araştırma sonucunda, *B. glandulosissima*'nın hem metanol (EC_{50} = 12.45 µg/mL; SI = 24.84) hem de su ekstraktının (EC_{50} = 19.12 µg/mL; SI = 24.59) RSV'ye karşı; pozitif kontrol olarak kullanılan ribavirin (EC_{50} = 3.25 µg/mL, SI = 34.89)'e göre önemli sayılabilecek oranda anti-RSV aktiviteye sahip olduğu tespit edilmiştir. Sonuç olarak, *B. glandulosissima* ekstraktlarının, RSV'ye karşı klinikte kullanılan ilaçlara karşı bir alternatif olarak geliştirilebilmesi için; daha ileri çalışmalara layık olduğunu söyleyebiliriz. Bu çalışma, *B. glandulosissima*'nın anti-RSV aktivitesine yönelik ilk rapordur.

Introduction

Acute respiratory infections caused by viruses are a major reason of morbidity and mortality in children worldwide. Human Respiratory Syncytial Virus (HRSV) is the most important cause of pneumonia and bronchiolitis in infants, young children and adults (Hruska *et al.* 1982, Treanor & Falsey 1999) and can be destructive in immunosuppressed populations (Wyde *et al.* 1998). In addition, recurrent infections are a common phenomenon showing that naturally acquired immunity does not provide long-term protection (Dubovi *et al.* 1981). Although many vaccine development studies have been conducted on RSV, efforts to develop effective vaccines against RSV have been unsuccessful (Chin *et al.* 1969, Kim *et al.* 1969, Wyde *et al.* 1998). Moreover, even if the use of one of the vaccines developed is accepted, this may not be appropriate in some RSV-sensitive populations, e.g., in very young infants and in immunocompromised individuals (Wyde *et al.* 1998). Ribavirin (RBV) and immunoglobulins containing high titer RSV-specific neutralizing antibodies are currently recognized as antivirals for the treatment and prevention of RSV infections (Kneyber *et al.* 2000) although they are expensive and not easy to apply. For instance, RBV has been reported to be myelocytotoxic when administered intravenously, and therefore it is only allowed to be used as small aerosol particles (Smith *et al.* 1991, Lewinsohn *et al.* 1996).

Aerosol administration of drugs to patients, especially babies and children, is difficult to control and use at home, and therefore patients should go to hospitals for chemotherapy. The absence of a vaccine available to

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prevent RSV and the presence of RBV as the single antiviral agent used only in severe infections are still a problem in pediatric medicine. Therefore, it is necessary to develop specific anti-RSV drugs that can be administered orally or parentally (Ma *et al.* 2002).

Ballota L. species are perennial herbaceous plants belonging to the Lamiaceae family. The genus *Ballota* is represented by 35 species and 14 subspecies in the world of which 12 species and 8 subspecies are present in Turkey. The rate of endemism in high with 8 species in Turkey (Davis 1982, Greuter & Raus 1998, Patzak 1958, Patzak 1960). The genus is well known in Europe due to its spasmolytic and sedative effects (Garnier *et al.* 1961), and its members are called in different regions of Turkey with the local names şalba, çalba, balotu, ballık otu, nemnem otu, ısırgan, gezgez otu, köpek otu, karayer pırasası, elkurtaran, pat pat otu, leylim kara, somruk and karınca somurcağı. Some species are used by people in the treatment of cough, asthma, headache, nausea, haemorrhoid and wound (Baytop 1984, Eryaşar & Tuzlacı 1998, Meriçli *et al.* 1988, Tolon Fenercioğlu & Tuzlacı 1998, Tuzlacı & Tolon 2000, Yeşilada *et al.* 1993, Yeşilada *et al.* 1995). The main components of *Ballota* species are flavonoids, labdan diterpenoids and phenyl propanoids. Diterpenoids (hispanolone, ballonigrine, dehydrohispanolone) and 14 flavonoids (kumatakenin (jaranol), pachypodol, 5-hydroxy-7,3',4' trimethoxy flavone, velutin, velutol, salvigenol, korimbosol, retusol, corymbosine, 5-hydroxy 3,7,4'trimethoxyflavone, retusin, 5-hydroxy 7, 4'dimethoxy flavone, flindulatine, ladanein) were isolated from different *Ballota* species and chemically characterized and analyzed by HPLC (Çitoğlu *et al.* 1998, Çitoğlu *et al.* 1999, Saltan Çitoğlu *et al.* 2003a, Saltan Çitoğlu *et al.* 2003b). Pachypodol (Ro 09-0179) was first isolated from *Plectranthus cylindraceus*, *Pogostemon cablin*, *Heterotheca grandiflora*, *Aglaia andamanica*, *Euodia glabra* and *Larrea tridentata* (Valesi *et al.* 1972, Fraser & Lewis 1973, Sakakibara *et al.* 1976, Wollenweber *et al.* 1985, Miyazawa *et al.* 2000, Orabi *et al.* 2000) has antiviral effect against RNA virus such as poliovirus and rhinovirus. This antiviral effect was shown by inhibiting RNA polymerase in viral RNA synthesis and inhibiting viral replication (Arita *et al.* 2015, Ishitsuka *et al.* 1986, Ninomiya *et al.* 1985, Pérez & Carrasco 1992). Some of these flavonoids isolated from *B. glandulosissima,* a species naturally growing in southern Anatolia, has also been reported to have antimicrobial activity (Saltan Çitoğlu *et al.* 2003a, Saltan Çitoğlu *et al.* 2003b).

Although it is well reported that *Ballota* species have antinociceptive, anti-inflammatory, hepatoprotective, antilisterial, antiproliferative and antioxidant activities (Çitoğlu *et al.* 1998, Çitoğlu *et al.* 1999, Saltan Çitoğlu *et al.* 2003a, Saltan Çitoğlu *et al.* 2003b, Erdoğan-Orhan *et al.* 2010, Özbek *et al.* 2004, Rigano *et al.* 2016, Saltan Çitoğlu *et al.* 2004a, Saltan Çitoğlu *et al.* 2004b, Saltan Çitoğlu *et al.* 2004c, Sever Yılmaz *et al.* 2005, Sever Yılmaz *et al.* 2006), researches on the antiviral activities of *Ballota* species with different biological activities are limited only to picornaviruses. *Ballota* species, which have different components one of which is pachypodol, have not been searched for antiviral activity against HRSV.

This study aimed to find new and reliable antiviral agents against RSV. For this purpose, crude extracts obtained from *B. glandulosissima* were used to evaluate the antiviral activity against RSV.

Materials and Methods

Plant materials

Ballota glandulosissima specimens were collected from Antalya in 2016 during flowering period (in July). The samples were identified by Prof. Dr. Osman TUGAY from Pharmacy Faculty of Selçuk University. Aerial parts of the specimens 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 is kept at KNYA Herbarium, Selcuk University, Science Faculty, Biology Department, Konya, Turkey.

Preparation of the extracts

20 g powder samples were placed separately in 400 ml of methanol and 400 ml of sterile distilled water and extracted for 1 hour with an ultrasonicator at 37°C. The extracts were filtered through Whatman No: 1 filter paper, and the solvents used were completely evaporated at 40°C under reduced pressure in a rotary evaporator (Heidolph Laborota 4000, Germany). After evaporation, the extracts were lyophilized at -110°C under reduced pressure in a lyophilizer (Labconco, USA). Each 1000 mg of the lyophilized methanol and aqueous extracts were dissolved in 10 mL of EMEM (serum-free) and stock solutions were prepared at a concentration of 100 mg/mL. The stock solutions were sterilized with 0.22 µm Millipore filter, put in 2 mL tubes at a rate of 1 mL concentrations and stored at +4°C until use. Dilutions of the extracts for cytotoxicity and antiviral activity tests were prepared from these stocks. Ribavirin (RBV, R9644-10 mg, Sigma, USA), a drug approved for the treatment of RSV infections in humans, was purchased and used as positive control. 10 mg RBV was dissolved in 5 mL of EMEM (serum-free). This 2 mg/mL stock concentration were filtered with a 0.22 µm Millipore filter and stored at -80°C or +4°C (When stored at + 4° C, it was used within 1 week).

Cells and the virus

Human larynx epidermoid carcinoma cells [HEp-2; ATCC (the American Type Culture Collection) CCL 23] were used to culture HRSV (RSV Long strain: ATCC VR-26). Reagents and medium for cell culture were purchased from different companies. Cells were propagated at 37°C under 5% CO_2 in EMEM supplemented with 10% fetal bovine serum (FBS, ATCC-30-2020), 10000 U/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B. The 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 0.1 mL (Kaerber 1964). The virus was stored at -80°C until use.

Cytotoxicity assay

The method described by Eskiocak *et al.* (2008) was used to determine the cytotoxic effects of RBV and methanol and aqueous extracts *of B. glandulosissima* on HEp-2 cells.

The cytotoxicity test was carried out as follows;

First column of a 96-well microplate was used as the medium control (MC) and 150 µL EMEM (serum-free) was added in each of the 8 wells contained in this column. Except for the 3rd column, 100 µL EMEM (serum-free) was put in the remaining 10 columns (viz, 2, 4, 5, 6, 7, 8, 9, 10, 11 and 12 column). An experiment solution of 2 mg/mL (2000 µg/mL) was prepared using EMEM (serum-free) from stock solution of the extracts (100 mg/mL).

200 µL from experiment solution of the extracts were added in each of the 8 wells in the 3rd column and serial two fold dilutions according to log2 base (2000, 1000, 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 µg/mL) were prepared. The second column of the microplate was used as cell control (CC).

The same procedures were performed for RBV using another microplate. First column of the microplate was used as MC and 150 µL EMEM (serum-free) were put in each of the 8 wells. Except for the 3rd column, 100 µL EMEM (serum-free) was put in the remaining 10 columns (viz, 2, 4, 5, 6, 7, 8, 9, 10, 11 and 12 column). An experiment solution of 750 µg/mL was prepared by using serum-free EMEM from stock solution of RBV (2000 µg/mL). 200 µL RBV from 750 µg/mL experiment solution was placed in each of the 8 wells. Serial two fold dilutions were made from the 3rd to the 12th columns (750, 375, 187.5, 93.75, 46.88, 23.44, 11.72, 5.86, 2.93 and 1.46 µg/mL).

50 µL HEp-2 cell suspensions containing 1×10^5 cells per mL were added to each well in columns 2 to 12 making the final concentrations of the extracts in the wells as 1333.33, 666.67, 333.33, 166.67, 83.33, 41.67, 20.83, 10.42, 5.21 and 2.60 µg/mL, while the final concentrations of RBV in the wells were 500.00, 250.00, 125.00, 62.50, 31.25, 15.63, 7.81, 3.91, 1.95 and 0.98 µg/mL. The plates were incubated in 5% CO_2 humidified incubator at 37°C for 3 days. Then 50 µL suspensions mixed with 5 mL XTT reagent and 0.1 mL PMS activator were placed in each well. The plates were incubated for a further 3 hours to form the XTT formazan product. Optical densities (OD) were recorded in an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 490 nm and a reference wavelength of 630 nm to record OD averages from 8 wells. The test was performed in triplicate and the results were shown as the ratio of the average cytotoxicity to the cell control.

The following formula was used to calculate the percentage of cytotoxicity of the extracts on HEp-2 cells and RBV.

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

A: The optical density of cell control

B: The optical density for the cells treated with extracts or RBV.

The cytotoxicity percentages calculated were plotted according to the corresponding concentrations of the tested samples (extracts and RBV). 50% cytotoxic concentration (CC_{50}), defined as the concentration reducing the OD values of the cells treated with extracts or RBV by up to 50% compared with CC, was determined with non-linear regression analysis run in the GraphPad Prism Version 5.03 statistical program. The maximum non-toxic concentrations (MNTCs) of the extracts and RBV were also determined by comparing OD with CC. These MNTCs were used to determine the antiviral activity of the extracts and RBV.

Antiviral activity assay

After MNTCs were determined against HEp-2 cells, 10 times more concentrated dilutions of the extracts and RBV were prepared. The dilutions were diluted according to Log_2 base and their antiviral activities against RSV (diluted at 100 $DKID_{50}$ dose) were tested by XTT method (Chiang *et al.* 2002). The method was applied as described below;

Suspensions were prepared such that the HEp-2 cells were at a concentration of 1.43×10^5 cells / mL using 2% FBS-containing EMEM (cell maintenance medium). From these cell suspensions, the wells of 96-well culture plates (except for 8 wells in the 1st column of the plate used as MC) were seeded at a volume of 70 µL per well (~ 10^4 cells / well). 100 µL aliquots of cell maintenance medium were placed in 8 wells used as MC and incubated in 5% CO_2 at 37ºC for 4 hours. Then, 20 µL of RSV suspension diluted in 100 $TCID_{50}$/0.1 mL using the maintenance medium were put in the wells (except for 8 wells in the 1st column used for MC and the 2nd column used for CC). 8 wells in the 3rd column of the microplates were used as Virus Control (VC). 20 µL aliquots of the maintenance medium were placed in 8 wells in the 2nd column used as CC and the plate was incubated for 2 hours. 10 × MNTC dilutions containing 2% FBS were prepared from stock solutions (100 mg / mL) of the extracts. Subsequently, serial two fold dilutions [(10×MNTC)/2, (10×MNTC)/4, (10×MNTC)/8, (10×MNTC)/16, (10×MNTC)/32, (10×MNTC)/64, (10×MNTC)/128, (10×MNTC)/256] using the maintenance medium were prepared from the extract solutions of 10 × MNTC concentrations. After incubation for 2 hours, 10 µL of the dilutions prepared 10×MNTC

concentration were put in 8 wells of the 4th column. In the remaining 8 columns of the microplates (viz, wells on columns 5, 6, 7, 8, 9, 10, 11 and 12), 10 µL of the extract dilutions [(10×MNTC)/2, (10×MNTC)/4, (10×MNTC)/8, (10×MNTC)/16, (10×MNTC)/32, (10×MNTC)/64, (10×MNTC)/128, (10×MNTC)/256] were added to the each well.10 µL cell maintenance medium was placed in the wells of the microplates used as CC and VC wells. The same procedures were also performed for RBV using another microplate. A dilution of 10×MNTC containing 2% FBS was prepared from the stock solution (1000 µg/mL) of RBV. Subsequently, two fold dilutions [(10×MNTC)/2, (10×MNTC)/4, (10×MNTC)/8, (10×MNTC)/16, (10×MNTC)/32, (10×MNTC)/64, (10×MNTC)/128, (10×MNTC)/256] using the maintenance medium were prepared from the RBV solution in 10 × MNTC. Next, the steps in determining the antiviral activity of the extracts were followed. The plates were incubated in 5% CO_2 at 37°C for 3 days. Then, 5 mL of the XTT reagent was mixed with 0.1 mL of the PMS activator and 50 µL was added to each well. The plates were gently shaken to homogeneously distribute the dye into the wells. The plates were incubated for a 3 hours to form the XTT formazan product. OD values were read at 490 nm test and 630 nm reference wavelengths in an ELISA reader, and OD averages from 8 wells were recorded. The protection percentages of extracts or RBV concentrations against viruses were calculated from the following formula where A, B and C indicate the absorbance of the extracts or RBV, the virus and the cell controls, respectively (Andrighetti-Fröhner *et al.* 2003):

$$\text{Protection percentage} = [\ (A-B) / (C-B) \times 100\ ]$$

EC_{50} value, defined as the concentration of the extracts or RBV that provides protection in 50% of the infected cells, was determined using nonlinear regression analysis in GraphPad Prism Version 5.03 taking into account the % protection rates determined with extract or RBV concentrations. The selectivity index (SI) of the extracts and RBV was calculated from the CC_{50} / EC_{50} ratio. The experiments were done in triplicate.

Results

Virus titration

Titres of RSV in HEp-2 cells were determined as $TCID_{50} = 10^{-4}$ / 0.1 mL at the end of the 3rd day.

Cytotoxicity assay

In order to determine the MNTC and CC_{50} values of *B. glandulosissima* methanol and aqueous extracts against HEp-2 cells, the obtained cytotoxicity rates are shown in Table 1 and Fig. 1, respectively. The CC_{50} value of *B. glandulosissima* methanol extract was determined as 309.30 µg/mL and the MNTC as 10.42 µg/mL. The CC_{50} and MNTC of *B. glandulosissima* aqueous extract were determined as 470.10 47 µg/mL and 2.60 µg/mL (Table 1 and Fig. 1). The MNTC and CC_{50} values (113.40 µg/mL and 0.98 µg/mL, respectively) of RBV are shown in Table 1 and Fig. 1.

Table 1. The results of cytotoxicity and antiviral activity assays for RBV and methanol and aqueous extracts of *B. glandulosissima*.

	Extract type	Cytotoxicity		Antiviral activity	
		MNTC (µg/mL)	CC_{50} (µg/mL)	EC_{50} (µg/mL)	SI
B. glandulosissima	Methanol	10.42	309.30	12.45	24.84
	Aqueous	2.60	470.10	19.12	24.59
Ribavirin (RBV)	--	0.98	113.40	3.25	34.89

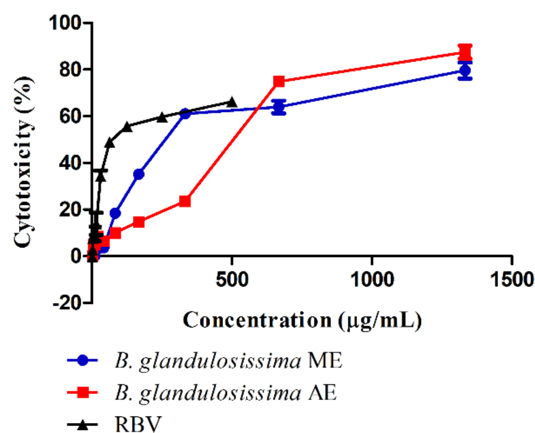

Fig. 1. Cytotoxic activities of methanol and aqueous extracts of *B. glandulosissima* and RBV.

Antiviral activity assay

Percentage protection rates of methanol and aqueous extracts starting from MNTC dilutions against RSV are shown in Table 1. The EC_{50} values of methanol and aqueous extracts were determined as 12.45 µg/mL and 19.12 µg/mL, respectively (Fig. 2). SI (CC_{50}/EC_{50}) values of methanol and aqueous extracts are 24.84 and 24.59, respectively (Table 1).

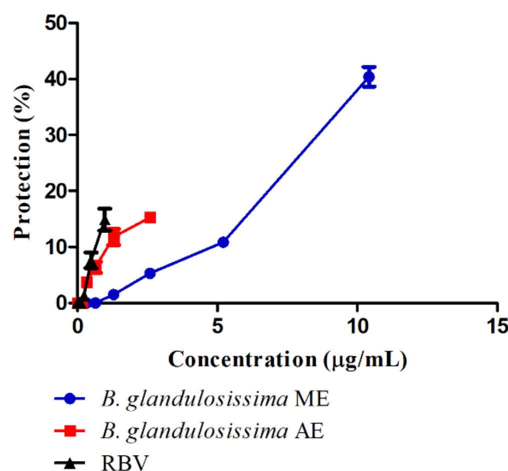

Fig. 2. Antiviral activities of methanol and aqueous extracts of *B. glandulosissima* and RBV.

The protection rates of RBV against RSV are given in Table 1. The EC_{50} value of RBV was determined as 3.25 µg/mL (Fig. 2). The selectivity index (SI) of the RBV was determined as 34.89 from the CC_{50}/EC_{50} ratio (Table 1).

Discussion

RSV is the most common cause of acute respiratory infections in infants and children. Although the mortality rate is generally low, it can also cause heart or respiratory failure up to 37-73% and bone marrow transplantation up to 36-45% in infants (Kimura *et al.* 2000, MacDonald *et al.* 1982, Englund *et al.* 1988, Harrington *et al.* 1992). Original anti-RSV compounds with better efficacy and safety than ribavirin have been the target of researches. Natural products may constitute different sources of antiviral agents. For example, pachypodol was isolated from many plant species, in addition to *B. glandulosissima,* and it has been reported to have antiviral activity against RNA viruses such as poliovirus and rhinovirus (Arita *et al.* 2015, Ishitsuka *et al.* 1986, Ninomiya *et al.* 1985, Pérez & Carrasco 1992).

Cytotoxic and antiviral activities of methanol and aqueous extracts prepared from the aerial parts of *B. glandulosissima* and RBV were evaluated by colorimetric XTT test (see Table 1. The results showed that the extracts with CC_{50} values in the range of 309.30–470.10 µg/mL were found to be non-toxic to HEp-2 cells according to the criteria of Rukunga & Simons (2006). According to the classification of Rukunga & Simons (2006), extracts with a CC_{50} value less than 2 µg/mL are cytotoxic, extracts with a CC_{50} value in the range of 2-89 µg/mL are partially (moderately) cytotoxic and extracts with a CC_{50} value greater than 90 µg/mL are non-toxic.

The antiviral activity experiments revealed that methanol and aqueous extracts of *B. glandulosissima* had a significant antiviral activity comparable to RBV. The EC_{50} values of the extracts were determined as 12.45 µg/mL and 19.12 µg/mL, respectively, while SI values were determined as 24.84 and 24.59, respectively. Vanden Berghe & (1993) suggested that the antiviral activity of crude plant extracts should be detectable in at least two consecutive maximum non-toxic dilutions to discriminate between the virus-induced CPE and the CPE resulting from the toxic effect of the extracts. In addition, Cos *et al.* (2006) defined quality standards for basic evaluation of activity screening of natural products. As a standard for the antiviral efficacy of natural products, such as plant extracts, investigators have proposed a mandatory endpoint of EC_{50} values of less than 100 µg/mL. Apart from EC_{50} values, SI values of 10 or greater are generally reported to be considered as indicators of positive antiviral activity (Chattopadhyay *et al.* 2009).

In this study, our results showed a parallelism with the studies of Vanden Berghe *et al.* (1993), Cos *et al.* (2006) and Chattopadhyay *et al.* (2009). Extracts with more than two consecutive maximum non-toxic dilution in the concentration of antiviral effect has been determined, and EC_{50} values less than 100 µg/mL and SI values greater than 10 were obtained. Therefore, it was concluded that the extracts of *B. glandulosissima* had a reliable antiviral activity. Various components may be responsible for this antiviral activity. For instance, the rich flavonoid components identified in this plant can especially be effective on the virus (Saltan Çitoğlu & Sever 2002). Saltan Çitoğlu & Sever (2002) isolated seven flavonoids (kumatakenin (jaranol), pachypodol, 5-hydroxy-7,3',4' trimethoxy flavone, velutin, salvigenin, corymbosin, retusin) from *B. glandulosissima* extracts among which pachypodol has an antiviral effect. This effect is shown by inhibiting RNA polymerase in viral RNA synthesis and inhibiting viral replication (Arita *et al.* 2015, Ishitsuka *et al.* 1986, Ninomiya *et al.* 1985, Pérez & Carrasco 1992).

This is the first study to determine the anti-RSV activities of *B. glandulosissima*. Therefore, no comparison could have been done with other studies.

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