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Evaluation of in Vitro Antiviral Activity of Centaurea Kotschyi var. Decumbens Extracts Against Herpes Simplex Virus Type 1 (HSV-1)

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Abstract

Centaurea kotschyi var. *decumbens*, some kind of the species in Centaurea L. (Asteraceae) genus have been used for the treatment to the herpes infection around children's lips in Turkish folk medicine, is an endemic species in Turkey. The methanol and aqueous extracts of this plant were tested as *in vitro* anti-HSV-1 activity to obtain new antiviral agents using natural sources. The colorimetric XTT test was used to assess the maximum non-toxic concentration of the extracts and evaluation of their antiherpetic activity in the experiments. 50% cytotoxic concentrations of the extract (CC_{50}) and the concentration of 50% protection (EC_{50}) against to the cytopathic effect caused by the virus were calculated with nonlinear regression statistical analysis by using the program of GraphPad Prism's. After 72 h incubation in Vero cells, CC_{50} values were determined as 504.30 µg/mL for methanol extract and 880.90 µg/mL for aqueous extract. Methanol extract of *C. kotschyi* var. *decumbens* showed a moderate antiviral activity with 3.63 selective index (SI= the ratio of CC_{50} to EC_{50}), while aqueous extract showed a weak antiherpetic activity with 1.25 SI value.

Keywords: *Centaurea kotschyi* var. *decumbens*, Methanolic and aqueous extracts, Herpes simplex virus type 1, Antiviral activity

1. Introduction

Human herpes simplex virus type 1 (HSV-1) is one of the eight isolated herpesvirus. HSV-1 infections are common and the most widely known symptoms are cold sore (or fever blister) and gingivostomatitis (Villarreal 2001). HSV-1 infections, although they can be self-limiting in most cases and usually doesn't require antiviral therapy, can create problems on patients having adequate immunity. In contrast infection with HSV-1 in individuals with suppressed immune system, which may be life-threatening and require immediate treatment with antiviral agents, may develop severe viral diseases. Furthermore, after primary infection by HSV-1, it makes a latent infection in neurons. Latent HSV-1 reactivation is very common during the inadequate immunity and it causes a repeating herpetic infection. In rare cases, the virus is spread lead to life-threatening encephalitis in the central nervous system (Whitley et al., 1998). To date, all drugs which have been approved for treatment of HSV infections target viral DNA polymerase and they are mostly synthetic nucleoside analogues as acyclovir (ACV) (Villarreal 2001). However the effect of these drugs, as reflected by new increase in virus resistance and recurrence of latent viruses, seems to be limited (Field 2001). Therefore, it is necessary to develop effective and reliable alternative drugs for the treatment of HSV infection. Natural products have been proved to be a major source of precursor

molecules, and many plant extracts and compounds have been reported having antiviral activity (Kitazato et al., 2007). Large varieties of plants growing in Turkey offer interesting opportunities for finding specific antiviral compounds from natural sources.

Centaurea L. (Asteraceae) species spread over the whole Anatolian peninsula, 61% of which are endemic, and includes about 181 species of plant flora in Turkey and they are extremely promising for the pharmacological aspects (Wagenits 1975, Duran and Duman, 2002).

Centaurea species in the popular medicines are used alone or with other plants for the purposes of antidiabetic, antidiarrhetic, antirheumatic, anti-inflammatory, colagog, choleretic, digestive, stomachic, diuretic, menstrual, astringent, hypotensive, antipyretic, cytotoxic and antibacterial (Barrero et al., 1997, Farrag et al., 1993, Gürkan et al., 1998, Kaij-A-Kamb et al., 1992, Orallo et al., 1998). It is reported that *C. pulchella, C. drabifolia* and *C. solstitialis* are used for the treatment of abcesses, hemorrhoids, peptic ulcers, common cold and herpes infections around the lips of children in Turkish folk medicine (Honda et al., 1996, Sezik et al., 2001, Fujita et al., 1995). As a result of the researches conducted on the chemical composition of *Centaurea* species, it was found that their ingredients are very rich for flavonoids and sesquiterpene lactones. In addition to that it is possible to find some different structures such as anthocyanins, aromatic acids, phytosterols and alkaloids (Farrag et al., 1993, Kaij-A-Kamb et al., 1992, Aslan and Öksüz, 1999, Marco et al., 1992, Negrete et al., 1984, Sarker et al., 1997, Tešević et al., 1994). Different ratios of sesquiterpenes, such as germacrene D, β-caryophyllene and β-cedrene were determined in essential oils obtained from flowers on two endemic *Centaurea* species in Turkey (*C. kotschyi* var. *kotschyi* and *C. kotschyi* var. *decumbens*) (Ertugrul et al., 2003). In studies conducted previously, the extract and compounds obtained from *Centaurea* has shown the activities of antiviral (Kaij-A-Kamb et al., 1991, Özçelik et al., 2009, Koca and Özçelik, 2009, Karagöz et al., 2002), antibacterial (Yeşilada et al., 1999, Güven et al., 2005, Karamenderes et al., 2006) and antifungal (Skaltsa et al., 2000, Panagouleas et al., 2003).

This study investigates the potential inhibitory effect of *C. kotschyi* var. *decumbens* against HSV-1 (strain HF). Methanol and aqueous extracts of the aerial parts of the plant have been prepared and these extracts were tested for cytotoxicity and antiviral activity.

2. Material and Method

2.1. Plant material and extraction

The aerial parts of *C. kotschyi* var. *decumbens* were collected in Turkey, C4 Konya, 9 km from Taskent to Ermenek, stony slopes, 1800 m above sea level, on 12 August 2014. Samples were identified by the Department of Biology, Faculty of Science, University of Selçuk, Turkey. A voucher sample of the plant (No. 1414) was deposited in the herbarium, Department of Biology, Faculty of Science, Selçuk University, Konya, Turkey. The aerial parts of the plant specimens were carefully cleaned and dried in the dark at the room temperature in one week. Then they were pulverized using a mill.

Each powdered 25 g samples were separately mixed with 100 ml methanol and 100 ml sterile distilled water, and they were incubated in an orbital shaker with 150 rpm in 48 h at the room temperature. Obtained extracts were filtered in Whatman No: 1, then they were evaporated in a rotary evaporator until to obtain 5-10 mL liquid extract under reduced pressure at 40°C. Finally, they were lyophilized in order to remove moisture completely (Jayaraman et al., 2008).

All extracts and ACV (Sigma-A4669) was dissolved in DMEM, and stock solutions were prepared as 100 mg/mL for extracts and 1 mg/mL for ACV.

2.2. Cell culture and virus

Cercopithecus aethiops African green monkey kidney cells (Vero cell line ATCC CCL-81) and HSV-1 (strain HF) were obtained from the Department of Virology, Faculty of Veterinary Medicine, University of Selçuk (Konya, Turkey). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Biological Industries, Israel) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biological Industries, Israel), 10,000 U/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B (Sigma-Aldrich, USA) in a CO_2 incubator. HSV-1 strain HF was propagated in Vero cells. Viral stock was prepared as described previously (Barardi et al., 1998, Simões et al., 1999) and the supernatant fluid was harvested, titrated and stored at –80ºC until used. HSV-1 titer was obtained by the limit-dilution method and expressed as 50% tissue culture infectious dose per mL ($TCID_{50}$/mL) (Reed and Muench, 1938).

2.3. Evaluation of cytotoxicity

The cytotoxic effect of *C. kotschyi* var. *decumbens* crude extracts on Vero cells was measured by XTT Cell Proliferation Kit (Biological Industries, Israel) in 96 well flat bottomed microtiter plates according to the instruction (Kars et al., 2012, Betancur-Galvis et al., 1999). Briefly, first well was filled with 150 µL, and all wells except the cell control column (second) were filled with 100 µL DMEM. 200 µL of the working solution prepared by using DMEM from stock solution of plant extracts was added in to the third column, and the extract was diluted horizontally by taking 100 µL portion of extract solution (3125 µg/mL) from the third column and putting in to the next column. Finally, the cells were seeded in to 96-well microtiter plates (5×10^3 cells/well) and incubated for 72 h in medium containing horizontal dilutions of extract (except for medium control wells). Then, a mixture of 0.1 mL PMS (*N-methyl dibenzopyrazine methyl sulfate*) and 5 mL XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide) was added to each well in a volume of 50 µL to form a soluble dye. After incubation at 37ºC for 4 h, the dissolution of formazan crystals that were produced by mitochondrial enzymes of the living cells occurred and then the optical density of chromogenic product was measured at 500 nm with a 96-well plate reader (BioTek microplate reader). The percentage of cytotoxicity was calculated as (A-B)/A × 100, where A is the mean optical density of cell control wells and B is the optical density of wells with plant extracts. The 50% cytotoxic concentration (CC_{50}) corresponded to the concentration required to kill 50% of the Vero cells for each extract was calculated by nonlinear regression analysis using GraphPad Prism software. Additionally the maximum non-cytotoxic concentrations (MNCCs) were determined as the maximal concentration of the extracts that did not exert a toxic effect in comparison with cell controls.
Dilutions starting from determined this MNCC (MNCC, MNCC/2, MNCC/4, MNCC/8, MNCC/16, MNCC/32) were used to determine the antiviral activity of the extract. All assays were carried out in triplicate.

2.4. Evaluation of antiviral activity

Anti-HSV-1 activity was investigated with the colorimetric XTT assay by using 96 well flat bottomed microtiter plates as previously described with minor modifications (Özçelik et al., 2005). Dilutions containing the determined maximum non-cytotoxic concentration of the extract were prepared from the stock solution (100 mg/mL). Each 100 µL extract solutions prepared in MNCC were put in to microtiter plates in the 8 wells of the column 5. Each 50 µL DMEM were put into the rest of other 7 column (viz 6, 7, 8, 9, 10, 11 and 12. column). Serial dilutions with 50 µL were performed from 5th column to 12th column. According to the base of $\log_2$ starting from MNCC, dilution of the extract (MNCC, MNCC/2, MNCC/4, MNCC/8, MNCC/16, MNCC/32, MNCC/64, MNCC/128 µg/mL) was prepared. First column of the microplate was used as medium control, and each 150 µL DMEM were put in to all wells separately. Second column of the microplate was used as cell control and each 100 µL DMEM were put in to all wells separately. Third column of the microplate was used as virus control and each 50 µL DMEM were put in to all wells separately. 4th column of the microplate was used as ACV (Sigma-A4669) control and each 50 µL acyclovir solution (prepared in 10 µg/mL) were put in to all wells separately. Each 50 µL vırus (diluted in the ratio of 100 $DK\dot{I}D_{50}$ in DMEM media) were put in to wells from third to 12th columns separately. Microplate was closed and incubated in a CO_2 incubator at 37ºC for 2 h. After this 2 h incubation time, each 50 µL cell suspension (3×10^5 cells/mL) containing 5% FBS in DMEM was placed in to all wells of microplate (except first well) and incubated in a 5% CO_2 incubator at 37ºC for 72 h (till, 95 % CPE was seen in the virus control wells). Then, each 50 µL mixed solution with 5 mL XTT and 0.1 mL PMS activator was added on to all wells. Plates were gently shaken to disperse homogeneously of the paint in the wells at 150 rpm for 1 min. Plates were incubated to product the formation of XTT formazan further 4 h. Absorbance at 540 nm wavelength in an ELISA reader (BioTek microplate reader) were read and average absorbance values obtained from the 8 wells were recorded. The percent protection rate as spectrophotometrically was calculated from the following formula:

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

A = Average absorbance for the concentration of each extract in every 8 wells.
B = Virus control absorbance (the average of the absorbance values in 8 wells)
C = Cell control absorbance (the average of the absorbance values in 8 wells)

EC_{50} is defined as the concentration that the extracts protect 50% of infected cells.

EC_{50} value, named an extract concentration that protects 50% of infected cells, was determined with the help of % protection against extract concentrations, by non-linear regression analysis using the GraphPad Prism statistics software. Selectivity index of the extracts (SI) was determined the ratio of CC_{50} / EC_{50}. Furthermore all tests were compared with positive control (acyclovir) tested simultaneously under identical conditions.

3. Results and Discussion

In this study, methanol and aqueous extract of *C. kotschyi* var. *decumbens* was investigated for anti-HSV-1 activity as well as in terms of cellular toxicity. Due to crude extracts to be examined for the antiviral effects and the compounds isolated from extracts of different chemical structure can show toxic effects on the cells, these results may indicate a counterfeit antiviral activity. Therefore, to be able to talk about a real antiviral activity, and the important thing for the reliability of the test for antiviral activity is firstly to determine the toxic effects in their host cell system (Dargan and Subak-Sharpe, 1985). Therefore MNCC and CC_{50} values of methanol and aqueous extract prepared from *C. kotschyii* var. *decumbens* were firstly determined, and then serial dilutions prepared in Log2 base starting from MNCC were subjected to colorimetric XTT assay to determine the antiviral activity of extracts.
As shown in Table 1, the MNCC and CC_{50} values of methanol extract of *C. kotschyii* var. *decumbens* were identified as 97.66 µg/mL and 504.30 µg/mL respectively, while the MNCC and CC_{50} values of aqueous extract were found as 390.63 µg/mL and 880.90 µg/mL respectively (Table 1, Figure 1 and 2). The MNCC and CC_{50} values of ACV used as positive control against HSV-1 were also determined as 15.63 µg/mL and 617.20 respectively (Table 1).

Table 1. Cytotoxicity and anti-HSV-1 activity results of C. kotschyi var. decumbens extracts

Extract	**Cytotoxicity**		**Anti-HSV-1 activity**	
	MNCC[b] (µg/mL)	**CC_{50}**[c] (µg/mL)	**EC_{50}**[d] (µg/mL)	**SI**[e]
Methanolic	97.66	504.30	139.00	3.63
Aqueous	390.63	880.90	702.00	1.25
Acyclovir[a]	15.63	617.20	0.20	3086.00

Note: [a]ACV positive control for HSV-1 infection; [b]MNCC maximum non-cytotoxic concentration; [c]CC_{50} 50% cytotoxic concentration; [d]EC_{50} 50% inhibitory concentration of the viral effect; [e]SI selectivity index

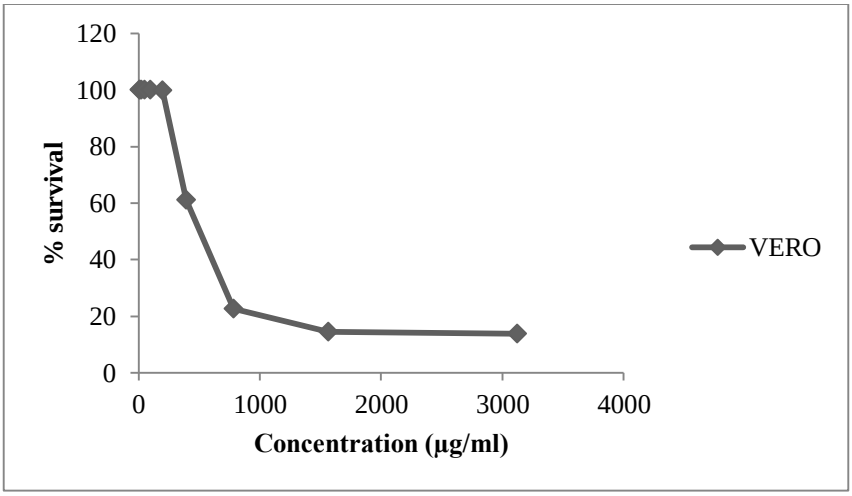

Figure 1. Cytotoxic activity on Vero cells of the methanolic extract of *Centaurea kotschyi* var. *decumbens* (MNCC: 97.66 µg/ml; CC_{50}: 504.30 µg/ml)

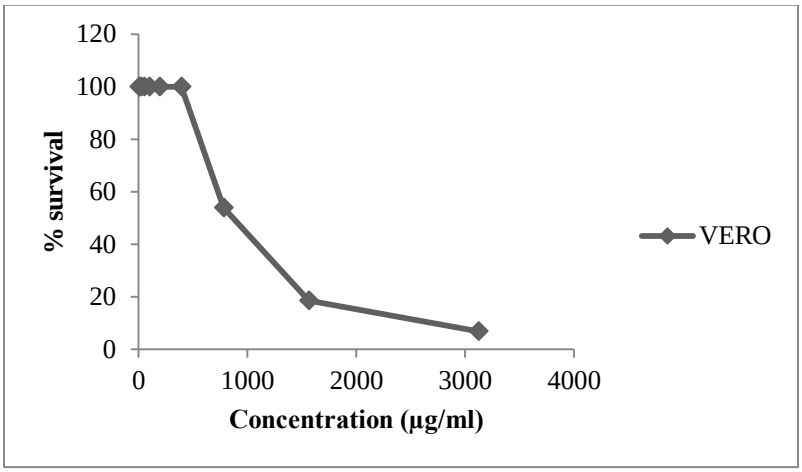

Figure 2. Cytotoxic activity on Vero cells of the aqueous extract of Centaurea kotschyi var. decumbens (MNCC: 390.63 µg/ml; CC50: 880.90 µg/ml)

Numerous studies were done to demonstrate the cytotoxic effects of extracts and compounds derived from different *Centaurea* species, and some of the isolated compound has been shown to be responsible for the cytotoxic activity (Barrero et al., 1997, Karagöz et al., 2002, Lonergan et al., 1992, Barrero et al., 1995). Cytotoxic effect of onopordopicrin isolated from the aerial parts of *C. sonchifolia* were studied on human skin cancer cells, and onopordopicrin was determined to have 0.85 µg/mL CC_{50} value, and it has been also reported to be highly cytotoxic effect such as other sesquiterpene lactones containing α-methylene-γ-lactone structure (Lonergan et al., 1992). Cytotoxic activity of nine sesquiterpene lactone isolated from *C. malacitana*, *C. melitensis*, *C. aspera* subsp. *aspera*, *C. aspera* subsp. *scorpiurifolia* and *C. aspera* subsp. *stenophylla* species were investigated against some cancer cells such as P-388 (mouse lymphocytic leukaemia), A-549 (human large cell lung cancer) and HT-29 (human colon cancer). As a result, CC_{50} values of three sesquiterpene lactones, structured in cnicin, onopordopicrin, tulipalin B, monoacetylcnicin, salonytenolide, stenofillolit and elemanolide, were found to have antiviral activity varying between 0.20-10.00 µg/mL that is the same with germacranolides bearing structure of α-methylene-γ-lactone (Barrero et al., 1995). *In vitro* cytotoxic effects of 5,7',4'-trihydroxy-6,3'-dymethoxyflavon and 5,7',4'-trihydroxy-6-methoxyflavon isolated from aerial parts of *C. malacitana* were investigated against cancer cells of P-388, A-549 and HT-29, and 5,7',4'-trihydroxy-6,3'-dymethoxyflavon have demonstrated a high cytotoxic activity ($CC_{50} \leq 5$ µg/mL) (Barrero et al., 1997). Cytotoxic effect on the Vero cells and antiviral effects on the human parainfluenza type 2 virus to (PIV-2) of aqueous extract from plants belonging to different families (*Trachystemon orientalis*, *Stellaria media*, *Ranunculus ficaria*, *Centaurea amasiensis*, *Aristolochia pontica*) were studied, and any cytotoxic effects for aqueous extract prepared from leaf of *C. amasiensis* were determined as 250 µg/mL and higher concentrations (Kitazato et al., 2007). In the present study, compared to the previous researches, the crude extracts have been determined to have a moderate cytotoxic activity (Table 1). Due to chemical compositions of the extract did not detected, it is difficult to say which substance or substances are responsible for the cytotoxic activity.

As for the anti-HSV-1 activities of *C. kotschyii* var. *decumbens* extracts; it was determined that methanol extract has a moderate anti-HSV-1 activity (EC_{50} = 139.00 µg/mL, SI = 3.63), whereas the aqueous extract has a weak anti-HSV-1 activity (EC_{50} = 702.00 µg/mL, SI = 1.25) (Table 1, Figure 3 and 4). The test extracts of SI values which is bigger than three has been reported to be showed a potentially reliable antiviral activity (Chattopadhyay et al., 2009).

There are few studies about antiviral activity of extracts and components, and are especially very little study on the anti-HSV activity from *Centaurea* species (Kaij-A-Kamb et al., 1991, Özçelik et al., 2009, Koca and Özçelik, 2009, Karagöz et al., 2002, Rusak et al., 1997). Antiviral activity of two flavone derivatives (Jasein and centaurein) isolated from *Centaurea nigra* were investigated against HSV-1 as DNA vırus and Sabin tip 2 poliovirus as RNA virus, and results were found to be inactive (Kaij-A-Kamb et al., 1991). Antiviral activity of three sesquiterpene lactones (centaurepensin, chlorojanerin and 13-acetyl solstitialin A) isolated from *C. solstitialis* L. ssp. *solstitialis* (*Asteraceae*) were studied against

Parainfluenza virus (PIV), and 13-acetyl solstitialin A has been determined to have an antiviral activity as 16 and 0.00006 µg/mL maximum and minimum concentrations values which are the same values of reference acyclovir (ACV) (Özçelik et al., 2009). *In vitro* antiviral, antibacterial and antifungal effects of nine extracts obtained from *Centaurea tchihatcheffii* were studied, and aqueous-chloroform interphase, chloroform and ethyl acetate extracts were found to be effective against HSV-1 with the different concentration between 1.00-16.00 µg/mL and parainfluenza type 3 virus (PIV-3) with the different concentration between 1.00-8.00 µg/mL (Koca and Özçelik, 2009). Cytotoxic effects on vero cells and antiviral activity on the human parainfluenza type 2 virus (PIV-2) of aqueous extracts obtained from plant species belonging to different families were investigated, and aqueous extract prepared from the leaves of *C. amasiensis* did not show cytotoxic effect at 250 µg/mL and the higher concentrations but it has been found to have no anti-PIV-2 activity (Karagöz et al., 2002).

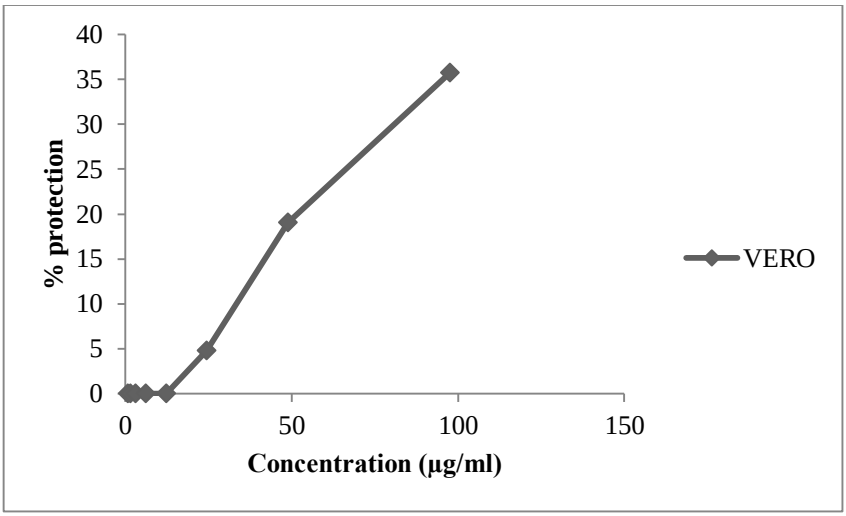

Figure 3. Anti-HSV-1 activity of the methanolic extract of *Centaurea kotschyi* var. *decumbens* (EC_{50}: 139.00 µg/ml; SI: 3.63)

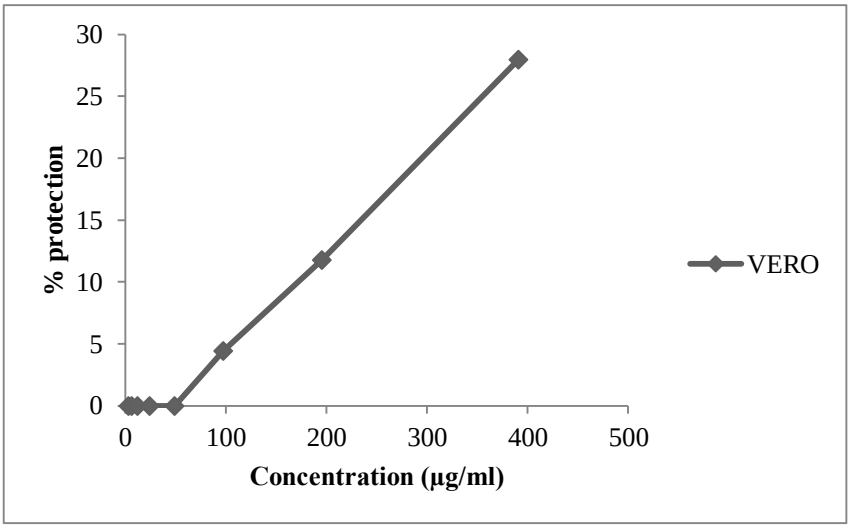

Figure 4. Anti-HSV-1 activity of the aqueous extract of *Centaurea kotschyi* var. *decumbens* (EC_{50}: 702.00 µg/ml; SI: 1.25)

In present study, it was determined that the extract results showed a parallelism with the previously studies (Özçelik et al., 2009, Koca and Özçelik, 2009), but was also determined to have anti-HSV-1 activity, particularly as moderately and weak. However, it is also better to say that the extracts used this research are not pure compounds.
Considering the results obtained, it can be stated that these extracts protect against viral infection, but the mechanism of their antiviral action and the active substances are not yet identified. Further studies are needed in order to verify which compounds could be responsible for this activity and how they exert their antiviral effects.

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