

In Vitro Anti-adenovirus Activity, Antioxidant Potential and total Phenolic Compounds of *Melissa officinalis* L. (Lemon Balm) Extract

Mohammad-Taghi Moradi¹, Ali Karimi^{2*}, Somayeh Alidadi³, Leila Hashemi⁴

¹Medical Plants Research Center, Shahrekord University of Medical Science, Shahrekord, Iran.

²Medical Plants Research Center; Shahrekord University of Medical Science, Shahrekord, Iran.

³Cellular and molecular Research Center, Shahrekord University of Medical Science, Shahrekord, Iran

⁴Clinical Biochemistry Research Center, Shahrekord University of Medical Science, Shahrekord, Iran

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ABSTRACT

Adenovirus causes a number of diseases in Human, and to date, no specific antiviral therapy approved against this virus. Thus, searching for effective anti-adenovirus agents seems to be required. Many studies showed that components derived from medicinal plants had effective antiviral activity. Therefore, the present was aimed to evaluate activity of *Melissa officinalis* L. extract against adenovirus *in vitro*. In this study, the hydroalcoholic extract of *Melissa officinalis* was prepared and its anti-adenovirus activity was evaluated on HEp2 cell line using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. The 50% inhibitory concentration (IC₅₀) and 50% Cytotoxicity Concentration (CC₅₀) of the extract were determined using regression analysis. Its inhibitory effect on adsorption and/or post-adsorption stages of the virus replication cycle was evaluated. To determine antioxidant activity, total phenol content, and flavonoids content of the extract, the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay; Folin-Ciocalteu method and aluminum chloride colorimetric method was used, respectively. The results revealed that the 50% Cytotoxicity Concentration (CC₅₀) on HEp2 cell line and the 50% inhibitory concentration (IC₅₀) on adenovirus of the extract were 294.9±12.7 and 15±2.7 µg/ml, respectively, with the selectivity index (SI), of 19.66. This extract inhibited adenovirus replication in post-adsorption stage (p<0.05). The IC₅₀ of DPPH radical was 16.8±1.41 µg/ml, compare with butylated hydroxytoluene (BHT), with IC₅₀ of 25.41±1.89 µg/ml. The total phenol and Flavonoid contents were 227.6±4.41 mgGAE/g and 12.5±2.1 mg/g, respectively. We conclude that, having SI value of 19.66 with inhibitory effect on adenovirus replication, particularly during the post-adsorption period, *Melissa officinalis* L. extract could be considered as a potential anti-adenovirus agent.

Keywords: Antioxidant activity, Antiviral, *Melissa officinalis*, Adenovirus.

INTRODUCTION

Human population experience viral diseases frequently around the world and many of these viral infections remained to be treated or pose resistant to antiviral drugs¹⁻³. Adenovirus (ADV) is a non-enveloped virus with a linear double-stranded DNA (dsDNA) which encodes 30-40 proteins. Human Adeno virus, which consists of about 52 serotypes, is associated with a wide range of human diseases including conjunctivitis, gastroenteritis, hepatitis, myocarditis, and pneumonia. Most of the diseases occur in children younger than the age of 5 years and are generally self-limiting illnesses. However, severe and life-threatening adenovirus infections have been reported particularly in immunocompromised patients^{4,5}. Ribavirin and Cidofovir are two anti-viral agents used for treatment of adenovirus infections, with apparently little clinical efficacy and variable outcomes⁵⁻⁷. Cidofovir displays significant nephrotoxicity and limited bioavailability which leads to development of its derivatives. However, the effectiveness of these compounds is still under evaluation^{8,9}. Thus, in view of the growing numbers of immunosuppressed patients, the development of alternative anti-adenovirus

treatment options is required. Medicinal plants have been used for many years for the treatment of human diseases¹⁰⁻¹² and a number of herbal medicines have been developed into therapeutic agents or have had promising results¹³⁻²³. Plants have the ability to synthesize a wide array of compounds and have long been used as remedies, and many are now being collected and examined in an attempt to identify possible sources of drugs²⁴⁻²⁶. *Melissa officinalis* L. (lemon balm) is a member of the Lamiaceae family and is a well-known herbal medicine in the Eastern and Western societies. Its leaves contain polyphenolic compounds such as rosmarinic acid and flavonoids^{27, 28}. The aerial parts of this herb are being used in Iran as a traditional medicine for treatment of depression, bronchitis, asthma, menstrual problems, hypertension, migraines, vertigo, fever, snake bite, eczema, and gout²⁹. Experimental research has indicated the antioxidant^{30,31}, antianxiety³², antiviral, antibacterial, antifungal^{30,33-36}, antihyperlipidemic³⁷, and antitumoral activities^{38,39} of *M. officinalis*. It has been also shown that *M. officinalis* has hepatoprotective³⁷ and neuroprotective effects^{31,40,41}. The antiviral activity of *M. officinalis* extracts against herpes

viruses and HIV has been previously described *in vitro*^{33,34}. However, to the best of our knowledge, there is no published report regarding the Anti-adenoviruses activities of these herbal plants. Therefore, this research was aimed to evaluate both *in vitro* anti-adenoviruses activity and antioxidant properties of *Melissa officinalis* L. extract.

MATERIAL AND METHODS

Preparation of hydroalcoholic extract

Melissa officinalis was purchased from a local market. Then, in Herbarium of Medical Plants Research Center of Shahrekord University of Medical Sciences (Iran), genus and species of the plant were identified and confirmed. The dried Leaves were separately ground to obtain uniform powders. The Leaves powder (100 g) was dissolved in 70% ethyle alcohole (400ml) and kept at RT for 96 h. Subsequently, the mixture was filtered and concentrated under nearly vacuum pressure and at 40°C using rotary evaporator. The extracts were kept in sterile bottles under refrigerated conditions until further use. The extracts were suspended at 37°C in dimethylsulphoxide (DMSO) to give a stock solution of 25mg/mL, dissolved in culture medium, filtered (Millipore® 0.22 µm) and stored (4°C) until use. The remaining DMSO in the wells (maximal 0.2%) did not affect the experiment results⁴².

Determination of the free-radical scavenging activity

The free-radical scavenging activity was measured by the 2,2 diphenyl-1-picrylhydrazyl (DPPH) method described by Moon and Terao, with some modification⁴³. Different amounts of the extract and methanol were added to a solution of 0.3 mg/mL methanolic solution of DPPH to make up a total volume of 3.0 mL. After standing for 15 min at room temperature, the absorbance was measured at 517 nm using UV-Vis spectrophotometer (UNICO 2100: USA). High absorbance of the reaction mixture indicated low free radical scavenging activity. Butylated hydroxytoluene (BHT) was used as positive control. Inhibition of free radical by DPPH was calculated as follows: Antiradical activity (%) = (A control - A sample)/Acontrol×100. The IC₅₀ value, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% and was calculated based on linear regression of plots of the percentage antiradical activity against the concentration of the tested compounds⁴⁴. The experiment was carried out in triplicate and the results are average values.

Determination of total phenolic content

The total phenolic content of the *Melissa officinalis* extract was determined using Folin-Ciocalteu method.⁴⁵ Briefly, 0.1 ml of the diluted sample was added to 0.5 ml of 10% (v/v) Folin-Ciocalteu reagent and kept at room temperature (RT) for 3-8 min. Subsequently, 0.4 ml of 7.5% (w/v) sodium carbonate solution was added to the mixture. After being kept in total darkness for 30 min., the absorbance of the reaction mixture was measured at 765 nm using a UV-Vis spectrophotometer (UNICO 2100: USA). Amounts of total phenolic were calculated using a gallic acid calibration curve. The results were expressed as gallic acid equivalents (GAE) g/g of dry plant matter.

Determination of total flavonoid content

The total flavonoid content of the extract was measured as previously reported method.⁴⁶ Briefly, 0.5 ml of diluted plant material was independently mixed with 1.5 ml of methanol, 0.1 ml of 10% (w/v) aluminum chloride, 0.1 ml of 1M potassium acetate, and 2.8 ml of distilled water. Following incubation at room temperature (RT) for 40 min, the absorbance of the reaction mixture was read at 415 nm using a UV-Vis spectrophotometer (UNICO 2100: USA). The results were expressed in mg of rutin equivalents of dry plant matter by comparison with the standard curve, which was made in the same condition.

Cell and Virus

HEP2 (cervix adenocarcinoma) cells was kindly provided by Pasteur Institute of Iran. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10% of fetal bovine serum (FBS; Gibco, USA), 100 µg/mL of streptomycin, 100 UI/ml of penicillin and 0.25 µg/mL amphotericin B (Gibco, USA), at 37°C and 5% CO₂. The same medium containing 2% PBS was used for cytotoxicity and antiviral assays. Adenovirus (type5) was kindly provided by health faculty, Tehran University of medical sciences, Tehran, Iran. Virus stock was prepared by infection of confluent monolayer HEP2 cells in 75 cm² culture flasks using DMEM medium with 2% FBS, at 37°C in 5% CO₂. Virus titer was determined by cytopathic effect (CPE) of adenovirus in HEP2 cells and was expressed as the 50% Tissue Culture Infective Dose (TCID₅₀) per ml.

Cytotoxicity assay

Prior to the investigation of anti-adenovirus activity, the cytotoxic effect (CPE) of the extract was determined. Briefly, HEP2 cells were seeded onto 96-good plates with a concentration of 10000 cells/well with final volume of 100 µl per well. After incubation at 37°C with 5% CO₂ for 24h, when the cell monolayer was confluent, the cell culture medium of cells aspirated and washed with PBS. Cells were incubated with 100 µL/well of various concentrations of the extract (in triplicates) and incubated at 37°C with 5% CO₂ for further 5 days. Cell viability was examined by ability of the cells to cleave the tetrazolium salt MTT [3-(4, 5-dimethylthiazol-2ol) 2, 5 diphenyltetrazoliumbromide), (Sigma, USA), by the mitochondrial enzyme succinate dehydrogenase which develops a formazan blue color product and the procedure was followed as described earlier⁴⁷. Briefly, the supernatants were removed from the wells and 50 µL of an MTT (Sigma, USA) solution (1mg/mL in PBS) was added to each well. The plates were incubated for 4 h at 37°C, and 100 µL of DMSO (Samchun korea) was added to the wells to dissolve the MTT crystals. The plates were placed on a shaker for 15 min and the absorbance were read on an enzyme-linked immunosorbent assay (ELISA) reader (STATA FAX 2100, USA) at 570 nm. Data were calculated as the percentage of toxicity using the following formula: toxicity (%) = [100 - (At/As) × 100] %, where at and as refer to the absorbance of the test substance and the solvent control, respectively^{42,47}. The 50% cytotoxic concentration (CC₅₀) was defined as the cytotoxic concentration of the crude extract by regression analysis.

Antiviral assay

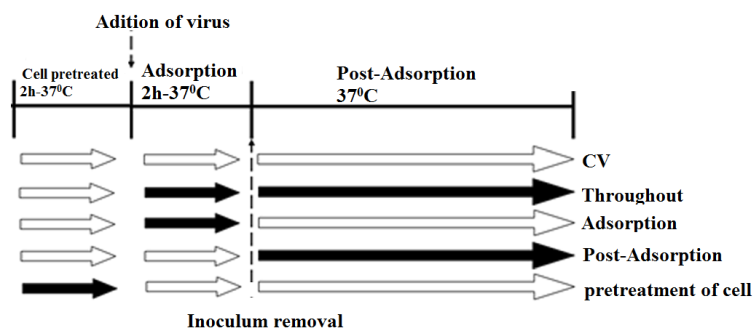


Figure 1: Scheme of addition of extract in the adsorption and post-adsorption stages of the virus.

Table 1: DPPH radical-scavenging activity of the *Melissa officinalis* extract

Sample	Concentration (µg/ml)	Scavenging of DPPH radical activity inhibition (%) (main ± SEM)	DPPH-radical scavenging activity IC ₅₀ / (µg/ml)
<i>Melissa officinalis</i> extract	50	95±2.2	16.8±1.41
	25	85±1.7	
	12.5	47±1.5	
	6.25	20±2.6	
	3.125	17±0.9	
	1.56	8±0.5	
	0.78	1±1	
BHT	50	90.8±1.5	25.41±1.89
	40	78.3±1.2	
	30	55.5±0.7	
	20	40.09±1.7	
	10	22±1.06	

All results are presented as mean ± standard mean error of three assays.

DPPH: 1,1-Diphenyl-2-picrylhydrazyl, BHT; Butylated hydroxytoluene

Antiviral activity of the extract was evaluated by inhibitory activity assay using MTT method, as described previously⁴⁷. Briefly, Non-cytotoxic concentrations of the extract below the CC₅₀ value were used to test the ability of them to inhibit CPE of adenovirus in tissue culture. To confluent HEp2 cell monolayer in a 96-well plate, 100µl (100TCID₅₀) virus suspension was added and incubated at 37°C for about two hour to allow virus adsorption. Thereafter, serial twofold dilutions prepared from non-toxic dose of the extract was added and tested in triplicate. As positive control, cells were infected with the same concentration of virus but without addition of extract. As a negative or cell control, only DMEM and 1.5% FBS was added to the cells. The plates were incubated at 37 °C in a humidified CO₂ atmosphere for 4 days. Cell viability was also determined using previously described MTT assay⁴⁷. Data were calculated as the percentage of inhibition using the following formula: Antiviral activity (%)=(Atv–Acv)/(Acd–Acv) ×100%, where Atv, Acv, and Acd are the absorbance of the test compounds on virus infected cells,

the absorbance of the virus control and the absorbance of the cell control, respectively⁴². The procedure was carried out three times. The 50% inhibitory concentration (IC₅₀) was determined from a curve relating inhibition to the concentration of the extract. Selectivity index (SI), as a marker of antiviral activity, was determined as the ratio of CC₅₀ to IC₅₀.

Mode of antiviral activity

To assess the mode of antiviral action of the extract, confluent cell cultures were treated in four different manners; 1) pretreated prior to infection 2 h at 37 °C (pretreatment of cell); 2) only during the adsorption period (adsorption); 3) after adsorption and until the end of the experiment (post-adsorption); and 4) during and after the adsorption (throughout) (Figure 1). To carry out these experiments, 90% confluent cells were pretreated with the extract prior to infection for 2 h at 37°C (pretreatment of cell) then cells were infected with 100TCID₅₀ (100 µL/well) of the virus in the presence or absence of extract and further incubated at 37°C for 2h allowing only the adsorption step of the viral particles to the cells (Adsorption). Subsequently, the supernatant was removed and the medium and 1.5% PBS with or without the extract was replaced, and incubated for 4 days at 37°C /5% CO₂. Cell viability was also determined using previously described MTT assay. The procedure was carried out three times. The IC₅₀ and SI was determined for each different manner.

Open and black arrows indicate the absence and presence of extract, respectively

Statistical analysis

All experiences were carried out in triplicate. The IC₅₀ and CC₅₀ values were calculated using dose-response analyses and related models with probit procedure using SPSS program. A p-value of less than 0.05 was considered statistically significant

RESULTS

Antioxidant capacity, flavonoids, and phenolic compounds

Total phenolic and flavonoid amounts of *Melissa officinalis* extract was 227.6±4.41 mg/g galic acid equivalent and 12.5±2.11 mg/g, respectively. Our results showed that the scavenging effect of the extract increases as the concentration increases. The crude extract had IC₅₀ values of 16.8±1.41µg/ml. The results are expressed compared with butylated hydroxytoluene (BHT), a reference standard with IC₅₀ of 25.41±1.89 µg/ml (table1).

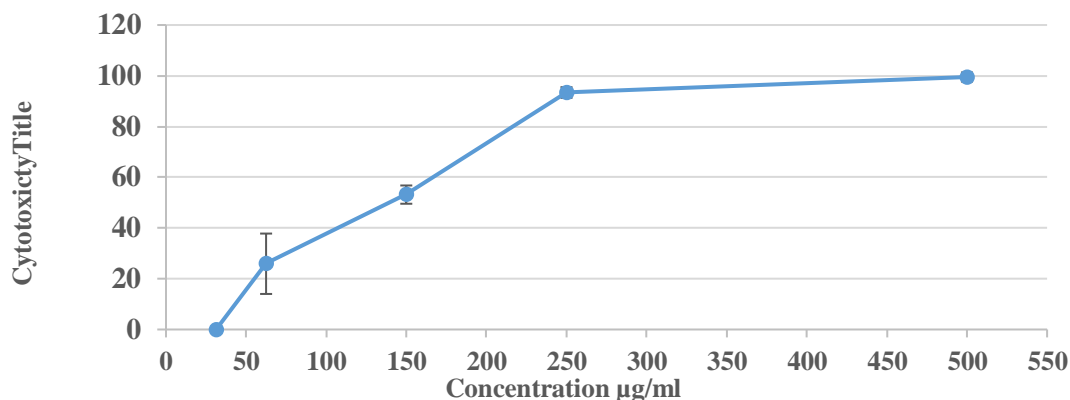


Figure 2: Cytotoxicity of *Melissa officinalis* extracts on HEP2 cells.

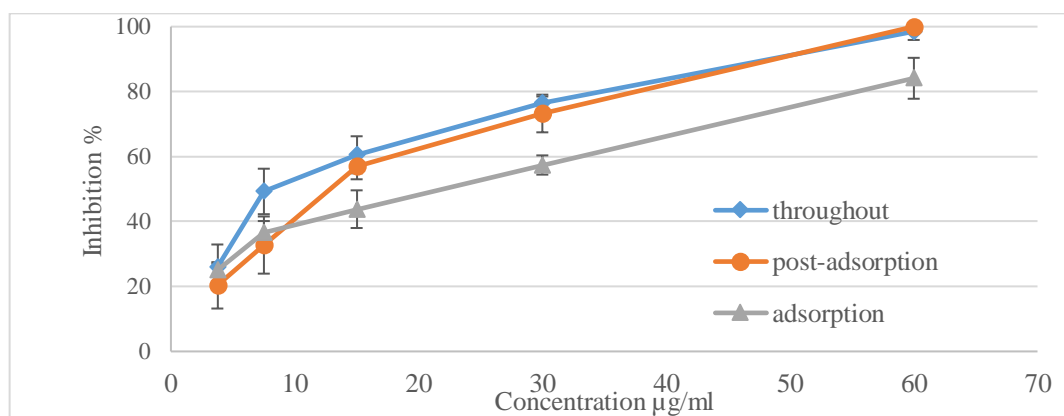


Figure 2: Inhibitory effect of *Melissa officinalis* extract at different steps of adenovirus to Hep2 cells.

Table 2: Cell cytotoxicity, Anti-adenovirus, and selectivity index of the *Melissa officinalis* extract at different steps.

protecol	Cell cytotoxic ^a (CC ₅₀ , µg/ml)	Antiviral activity ^b (IC ₅₀ , µg/ml)	Selectivity index (SI) ^c
adsorption	294.9±12.7	22.11±2.3	4.5
post-adsorption	294.9±12.7	15±2.7	19.66
throughout	294.9±12.7	12.3±3.7	23.97
cell pre-treated	294.9±12.7	>294	<1

Cell cytotoxicity effect and antiviral activity was determined by MTT assay. ^a CC₅₀ was the concentration that showed 50% cellular cytotoxic effect on HEp2 cells. ^b IC₅₀ were the concentrations that inhibited 50% of adenovirus. ^c SI is the ratio of CC₅₀ to IC₅₀.

Cytotoxicity and Anti-adenovirus activity of the extracts on HEp2 cells

Based on analysis of MTT results and probit analysis, the CC₅₀ value of crud extract on HEp2 cells was 294.9±12.7µg/ml. The analysis showed that there was significant relationship between the concentration of the extract and cell death with the more extract concentration, the more cell death (P<0.01, Figure 2). Confluent MDCK cells were exposed to different concentrations of the extract for 48 h. Cytotoxicity was measured in MTT assay. Experiences were carried out in triplicate. Hep2 cells simultaneously were inoculated with 100TCID₅₀ (100 µL/well) of adenovirus and treated with different concentrations of the crude extract. Our results showed that the more extract concentration, the more cytopathic effect (CPE) inhibition (P<0.05). Based on Probit analysis, IC₅₀ of *M. officinalis* extract on adenovirus was 15±2.7µg/ml with SI value of 19.66. To evaluate the mode of action of the extract against adenovirus, we also carried

out pre-treatment of cell, during the treatment (adsorption), after treatment until the end of the experiment (post-adsorption), and during and after the treatment (throughout) as explained in detail in materials and methods section and as illustrated in figure 1. Based on this data, we calculated the CC₅₀, the IC₅₀, and finally the selectivity index (SI = CC₅₀/IC₅₀). Based on the results, the selectivity index of *M. officinalis* extract on adenovirus pre-treatment of cell, adsorption, post-adsorption, and throughout was >294, 13.3, 19.66 and 23.97 respectively (table 2). We found that post-adsorption and during and after the adsorption (throughout) with the extract inhibited viral infection more strongly than the other treatment durations (figure 3). Various concentrations of *Melissa officinalis* extract were added carried out during the treatment (adsorption), after treatment until the end of the experiment (post-adsorption), and during and after the treatment (throughout).

DISCUSSION

In the present study, in vitro anti-adenovirus activity of *M. officinalis* hydroalcoholic extract was evaluated. Based on our results, the CC₅₀ value (using HEP2 cells) and the IC₅₀ value of the extract (on adenovirus) were 294.9±12.7µg/ml and 15±2.7µg/ml, respectively. The SI value of the extract on adenovirus was 19.66. So, this extract seems to have strong activity against adenovirus. The recommended IC₅₀ value, characteristic of herbal extract against infectious diseases is less than 100µg/mL⁴⁸. The extract used in this study revealed an IC₅₀ value of 15±2.7µg/mL which is far below the recommended cut-off. Total phenolic and flavonoid amounts of *M. officinalis* extract was 227.6±4.41 mg/g galic acid equivalent and 12.5±2.11 mg/g, respectively. Phenolic compounds have been shown to have antibacterial and anti-viral activities^{17,49}. The results of this study also showed high level of these compounds in *M. officinalis* extract. Hence, the antiviral activity of this plant might, in part, be attributed to phenolic compounds. To investigate the antiviral mechanism of the extract against the adenovirus, we also carried out pre-treatment of cell, during the adsorption (adsorption), after adsorption and until the end of the experiment (post-adsorption), and during and after the adsorption (throughout). We found that post-adsorption and throughout with the extract inhibited viral infection more strongly than the other treatment durations. Our results showed that the extract does not prevent the entry of adenovirus into the Hep-2 cell, but it acts following penetration of the virus into the cell. Hydroalcoholic extract of *M. officinalis* has been shown to be quite effective against intracellular HSV type 2³⁶. Unlike our results, Astani et al. reported that aqueous extract of *Melissa officinalis* and the corresponding compounds phenolic caffeic acid, p-coumaric acid, and rosmarinic acid significantly inhibit infectivity of Acyclovir-resistant HSV-1 only at early steps (attachment) of virus replication³⁵. It has been reported that the extract of *M. officinalis* may inhibit protein synthesis in a cell-free system using rat liver cells, and that this effect may be due to its caffeic acid-like components⁵⁰. Moreover, it was shown that caffeic acid derivatives such as rosmarinic acid, are potent antioxidants with reputed antiviral activity⁵¹. This extract did not effect on the adenovirus replication, when it was added to the cell culture before infection, thus, it is unlikely that its antiviral activity could be due to direct effects on the host's cell.

CONCLUSION

Melissa officinalis extract with SI value of 21.7 against adenovirus and its ability to inhibit the viral cycle, particularly during the post-adsorption period could be considered as a potential anti-adenovirus agent. Further studies need to be carried out regarding better understanding of the mechanism of action and the bioassay guided fractionation of this extract. Phenolic compounds have been shown to have antibacterial and anti-viral activities. The results of this study also showed high level of these compounds in *M. officinalis* extract. Hence, the antiviral activity of this plant might, in part, be attributed to phenolic compounds. It should be noted that there are a

lot of other plants which have these compounds⁵²⁻⁵⁴ which worth examining.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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