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RESEARCH ARTICLE

Anti-adenovirus activity, antioxidant potential, and phenolic content of dried flower buds of *Syzygium aromaticum* extract in HEp2 cell line

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ABSTRACT

The aim of this study is to evaluate *in vitro* anti-adenovirus property, antioxidant potential, and total phenolic content of dried flowers buds of *Syzygium aromaticum* crude extract. The crud extract was prepared and its anti-adenovirus activity was investigated on HEp2 cell line using MTT (3-[4,5-dimethylthiazol–2-yl]-2,5-diphenyltetrazolium bromide) assay. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, Folin-Ciocalteu method and aluminum chloride colorimetric method was used to determine antioxidant activity, total phenol content, flavonoids and flavonols content of the extract, respectively. Based on results, the 50% cytotoxicity concentration (CC_{50}) and the 50% inhibitory concentration (IC_{50}) of the extract were 97.66±11.4 and 4.73±1.6 µg/ml, respectively, with the selectivity index (SI) of 20.64. The crud extract inhibited adenovirus replication in post-adsorption step ($p<0.05$). The extract showed remarkable scavenging activity with IC_{50} values of 10.05±1.93 µg/ml. Total phenolic, flavonoid and flavonol content of the crude extract was 255.8±3.95 mg GAE/g, 63.9±2.35 mg RUT/g and 62±2.35 mg RUT/g, respectively. The results of the present study indicated that *S. aromaticum* crude extract exhibited anti-adenovirus activity with inhibitory effect on adenovirus replication could be considered as a potential anti-adenovirus agents.

Keywords: Antiviral; clove; *Syzygium aromaticum*; Adenovirus; antioxidant activity, Phenolic content

1. Introduction

Human population experience viral diseases frequently around the word and many of these viral infections remained to be treated or pose resistant to antiviral drugs [1-3]. Human adenovirus is associated with a wide range of human diseases including conjunctivitis, gastroenteritis, hepatitis, myocarditis, and pneumonia. This virus causes diseases mostly in children under 5 years of age which are generally self-limiting. However, adenovirus associated severe and life-treating infections occurred particularly in immunocompromised patients [4, 5]. Also, adenoviral conjunctivitis with symptoms such as excess lacrimation, conjunctival injection, and photophobia is a common form of conjunctivitis among adult and children population [6, 7].

There are some anti-viral agents used for treatment of adenovirus infections such as Ribavirin and Cidofovir, with apparently little clinical efficacy and variable outcomes [5, 8, 9]. Cidofovir shows significant nephrotoxicity and limited bioavailability which leads to development of its derivatives. However, the efficacy of these compounds is still being

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studied [10, 11]. Therefore, in light of the growing number of immunosuppressed patients, it is necessary to develop alternative anti-adenovirus treatments.

Herbs and herbal drugs are used to treat human diseases in different countries [12-14]. Moreover, they have been developed to be used for therapeutic purposes or promising results have been obtained about them [15-22].

Clove (*Syzygium aromaticum* (L.) Merr. & L.M.Perry) is the aromatic dried flower buds of a tree in the family *Myrtaceae*. *S. aromaticum* oil is frequently used in perfumery and medicine, but mostly in flavorings. The dried flower buds of *S. aromaticum*, called *Mikhak* in Persian, is a drug of Far East, used as a verminfuge, antibacterial, and toothache-relieving agent [23].

The different species of *S. aromaticum* contain a wide spectrum of important chemical compounds such as sesquiterpenes [24], tannins [25], triterpenoids [26] and a phenolic compound called eugenol (4-allyl-2-methoxyphenol). Eugenol seems to act as antioxidant, anticarminative, antispasmodic, antiseptic, and antimicrobial agent [27, 28]. Also, *S. aromaticum* has been shown to have antimutagenic property. *S. aromaticum* has been shown to have both antiviral and antibacterial effects [29]. To the best of our knowledge, to date, there has been no report on the antiadenovirus activity of dried flower buds of *Syzygium aromaticum* extract and its mine compounds eugenol. The aim of this study is to evaluate *in vitro* anti-adenovirus property, antioxidant potential, and total phenolic content of *S. aromaticum* crude extract.

2. Materials and Methods

Preparation of hydroalcoholic extract

The dried flower buds of *Syzygium aromaticum* was purchased from a local market. Then, genus and species of the plant were identified and confirmed in Herbarium of Medical Plants Research Center of Shahrekord University of Medical Sciences, Iran (Herbarium number: 425). Afterwards, 100 g of *S. aromaticum* powder was dissolved in 70% ethyl alcohol (400mL), kept for 96 hr at room temperature (RT) and then, filtered, concentrated using rotary evaporator under nearly vacuum pressure at 40°C, and kept in the sterile bottles at cold temperature. Then, the extract was suspended in dimethyl sulphoxide (DMSO) and Phosphate-Buffered Saline (PBS) at 37°, filtered (Millipore® 0.22 µm) and the prepared stock solution (25mg/mL) was kept at 4°C until to be used in the next experiments. The remaining DMSO (maximal 0.2%) has no cytotoxic effect on the cells [30].

Measuring free radical scavenging activity

The extract's free radical scavenging activity was investigated according to the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method with certain modifications [31]. Briefly, different amounts of the extract and methanol were introduced into 0.3 mg/mL methanolic DPPH solution to a final volume of 3.0 mL and incubated for 15 min at RT. Next, the solution absorbance was read at 517 nm by a UV–VIS spectrophotometer (UNICO 2100: USA). Low absorbance of the mixture represented high free radical scavenging activity. Butylated hydroxytoluene (BHT) was considered to be positive control. DPPH-induced inhibition of free radical was measured according to this protocol: Antiradical activity (%) = [(A control – A sample) / A control] ×100. The IC_{50} value was defined as the antioxidant concentration required to scavenge the initial DPPH concentration by 50%, and calculated according to linear regression of plots of the antiradical activity percentage against the tested compounds' concentrations. Each experiment was performed in triplicate.

Determination of total phenolic, flavonoid and flovonoid content

The *S. aromaticum* extract total phenolic content was measured by Folin-Ciocalteu method [32, 33]. Briefly, 0.2 mL of the diluted sample was added to 1 mL of 10% (v/v) Folin–Ciocalteu reagent and kept for 3-8 min at RT. Subsequently, 0.8 mL of 7.5% (w/v) sodium carbonate solution added to the mixture and 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 milligrams of Gallic acid equivalents per gram of extract powder (mg GAE/g).

The total flavonoid content of the extract was measured as previously described [32]. In summary, 0.2mL of diluted plant material was separately mixed with 1.2mL of 5% (w/v) potassium acetate and 0.2 mL of 2% (w/v) aluminum chloride. After incubation at RT for 40 min, the reaction mixture absorbance was read at 415 nm by a UV–VIS spectrophotometer (UNICO 2100: USA). The results were expressed in milligrams of Rutin equivalents per gram of extract powder (mg RUT/g) with reference to the standard curve, which was plotted under the same conditions.

The total Flavonols content of the extract was measured as previously reported method (21). Briefly, 0.2 ml of diluted plant material (1mg/ml in methanol 60%), 0.2ml of 2% (w/v) aluminum chloride and 1.2 ml of 5% sodium acetate. Following incubation at room temperature (RT) for 2.5 hours, the absorbance of the reaction mixture was read at 440 nm using a UV–VIS spectrophotometer (UNICO 2100: USA). The results were expressed in milligrams of Rutin equivalents per gram of extract powder (mg RUT/g) by comparison with the standard curve, which was made in the same condition.

Cell and Virus

HEp2 (Cervical adenocarcinoma; NCBI_Iran Cat# C144) cells was purchased from Pasteur Institute of Iran. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 0.02% (v/v) amphotericin B and 1% (v/v) Pen/Strep (Gibco, USA) under 37°C in 5% CO_2. A similar medium containing 1.5% FBS was used for antiviral assays and cytotoxicity. Adenovirus (type 5) was provided by the Faculty of Health, Tehran University of Medical Sciences. Virus stock was prepared with infection of confluent monolayer HEp2 cells in 75 cm^2 culture flasks by DMEM medium with 1.5% FBS, under 37°C in 5% CO_2. Virus titer was calculated by cytopathic effect (CPE) of adenovirus in HEp2 cell and expressed as the 50% tissue culture infective dose ($TCID_{50}$) per 1 mL [34].

Cytotoxicity assay

Before examination of anti-adenovirus activity, the extract's cytotoxic effect was measured. Briefly, HEp2 were seeded onto 96-well plates with 8000 cells/well concentration with 100 µl/well final volume. Following the incubation at 37°C with 5% CO_2 for 24h, the overlay medium of the confluent monolayer was removed and the cells were incubated with 200 µL/well of various doses of the extract (in triplicate) for 4 days under 37°C with 5% CO_2. Cell viability was determined by the cells' ability to cleave the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-ol)-2,5-diphenyltetrazolium bromide)] (Sigma, USA), by mitochondrial enzyme succinate dehydrogenase which develops a formazan blue color product. The protocol described earlier was followed [30, 34]. Briefly, the supernatants were removed from the wells and 50µL of an MTT (Sigma, USA) solution (1 mg/mL in PBS) was added to each well. The plates were incubated for 4 h under 37°C and then the supernatants were removed and 100µL of DMSO (Samchun, Korea) was added to the wells for dissolving the MTT crystals. The plates were placed on a rotator for 15 min and the absorbance was read on an enzyme-linked immunosorbent assay (ELISA) reader (STATA FAX 2100, USA) at 570 nm wavelength. The data were expressed as toxicity percentage calculated according to the formula below: Toxicity (%)= [100– (At/As)] ×100 %, where At and As refer to the absorbance of the test substance and the solvent control, respectively [30, 35]. The CC_{50} was defined as the cytotoxic concentration of the crude extract according to regression analysis.

Antiviral assay

Antiviral activity of the extract was evaluated by inhibitory activity assay according to MTT method, as it has already been described [30]. Briefly, non-cytotoxic concentrations of the extract under the CC_{50} value were used to investigate their ability to inhibit CPE of adenovirus in tissue culture. 100µL ($100TCID_{50}$) virus suspension was introduced into confluent HEp2 cell monolayer in a 96-well plate and incubated at 37°C for about two hours to absorb virus. Thereafter, serial two-fold dilutions prepared from non-toxic dose of the extract were added and tested in triplicate. For negative or cell control, only DMEM containing 1.5% FBS was added to the cells. As positive control, the cells were infected with an equal concentration of virus but without addition of the extract. The plates were incubated under 37°C in a humidified CO2 atmosphere for 4 days.

Cell viability was calculated using previously described MTT assay [30]. The data were expressed as the percentage of inhibition according to the following formula:

Antiviral activity (%)=[(Atv–Acv)/ (Acd–Acv)] ×100%, where Atv, Acv, and Acd represent the absorbance of the tested compounds on virus infected cells, the absorbance of the virus control, and the absorbance of the cell control, respectively. The experiment was performed in triplicate. The IC_{50} was determined using a curve that related inhibition to the extract concentration. Selectivity index (SI), as a marker of antiviral activity, was determined to be CC_{50}/IC_{50} ratio.

Mode of antiviral activity

To evaluate the mode of antiviral action of the *S. aromaticum* extract, confluent monolayer cells were treated with the extract 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

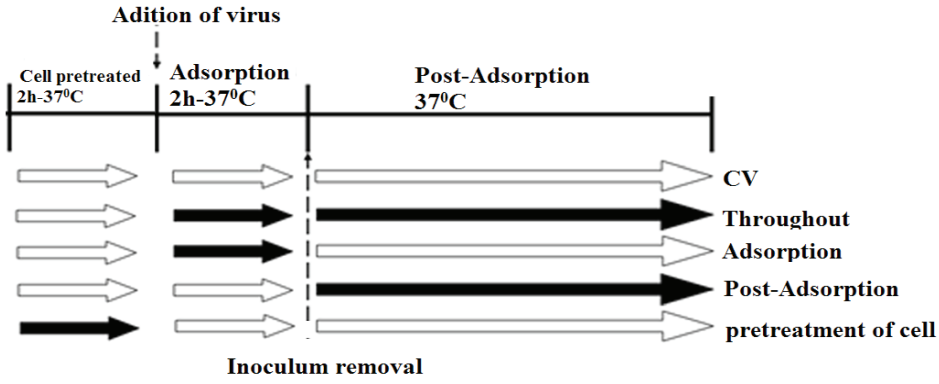

Figure 1. Pattern of extract addition during adsorption and post-adsorption stages of the virus: Open and black arrows represent the absence and presence of extract, respectively. CV: Control Virus

until the completion of the experiment (post-adsorption); and 4) throughout and after the adsorption. To implement these experiments, 90% confluent cells were pretreated with the extract before infection for 2 h at 37°C (pretreatment of cell) then cells were infected with 100 $TCID_{50}$ (100 µL/well) of the virus in the presence or absence of the extract and incubated again at 37°C for about 2 hours for adsorption of the viral particles to the cells (adsorption). Subsequently, the supernatant was separated and the medium by 1.5% FBS with or without the extract was replaced, and incubated at 37°C and 5% CO2 for 4 days. Cell viability was also determined using previously described MTT assay. The experiment was carried out in triplicate and the IC_{50} and SI was determined for any individual experiment.

Statistical analysis

All experiences were conducted in triplicate. The IC_{50} and CC_{50} values were calculated by dose-response analyses and related models with regression probit procedure by SPSS. P values of less than 0.05 were considered statistically significant.

3. Results

Antioxidant capacity, total phenolic, flavonoid and flavonol content

For standardization of the extract, antioxidant capacity, total phenolic, flavonoid and flavonol content in *S. aromaticum* extract were measured. Total phenolic, flavonoid and flavonol content of the crude extract was 255.8±3.95 mg GAE/g, 63.9±2.35 mg RUT/g and 62±2.35 mg RUT/g, respectively. The crude extract had IC_{50} values of 10.05±1.93 µg/mL. The results were expressed with reference to BHT, a reference standard with IC_{50} of 25.41±1.89 µg/mL (Table 1). The results showed that the more concentration of the extract, the more scavenging effect of free radicals.

Table 1. DPPH radical-scavenging activity of the *Syzygium aromaticum* extract

Sample	Concentration (µg/mL)	Scavenging of DPPH radical activity inhibition (%) (main ± SEM)	DPPH-radical scavenging activity IC_{50} (µg/mL)
S.aromaticum extract	80	100±1.8	10.05±1.93
	40	97.25±2.2	
	30	87.2±2.6	
	20	81.7±2.3	
	10	45.7±1.9	
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	

The results were presented as mean (± standard mean error) value of the three assays. BHT: Butylated hydroxytoluene, DPPH: 1,1-Diphenyl-2-picrylhydrazyl.

Cytotoxicity and Anti-adenovirus activity of *S. aromaticum* extracts on HEp2 cells

In the present study, *in vitro* anti-adenovirus activity of *S. aromaticum* hydroalcoholic extract was evaluated using HEp2 cell line. According to MTT analysis and probit analysis, the CC_{50} value of the crude extract on HEp2 cells was 97.66±11.4 µg/mL. The analysis showed that the extract concentration was significantly related to cell death (P<0.01, Figure 2).

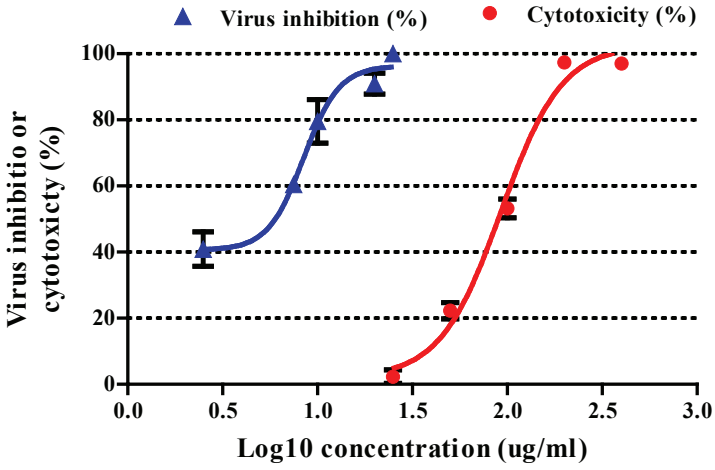

Figure 2. Anti-adenovirus activity and cytotoxicity of *S. aromaticum* extracts on HEp2 cells. Confluent HEp2 cells without virus or after adenovirus infection were exposed to different concentrations of the extract. Cell viability was measured with MTT assay.

HEp2 cells were inoculated with 100 $TCID_{50}$ (100 µL/well) of adenovirus for 2h and were treated with various doses of the crude extract. Our findings showed that the more extract concentration, the more cytopathic effect (CPE) inhibition (P<0.05, Figure 2). According to Probit analysis, IC_{50} of *S. aromaticum* extract on adenovirus was 4.73±1.62 µg/mL. The SI value of the extract on adenovirus was 20.64. Therefore, this extract seems to have noticeable activity against adenovirus.

Confluent HEp2 cells without virus or after adenovirus infection were exposed to different concentrations of the extract for 96h. Cell viability was measured in MTT assay. Experiences were carried out in triplicate.

Mode of antiviral activity

To evaluate the mode of antiviral action of the *S. aromaticum* extract, confluent monolayer cells were treated with the extract 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 completion of the experiment (post-adsorption); and 4) throughout and after of the adsorption (Figure 1). Based on the results, the IC_{50} value of the extract on adenovirus pre-treatment of cell, adsorption, post-adsorption, and throughout was <97.66, 24.42±3.62, 4.73±1.62and 5±1.34 and the selectivity index was <1, 3.99, 20.64 and 19.53 respectively. Our results showed that the inhibitory effect of the extract on adenovirus replication in HEp-2 cell line was greater in post-adsorption and during and after the

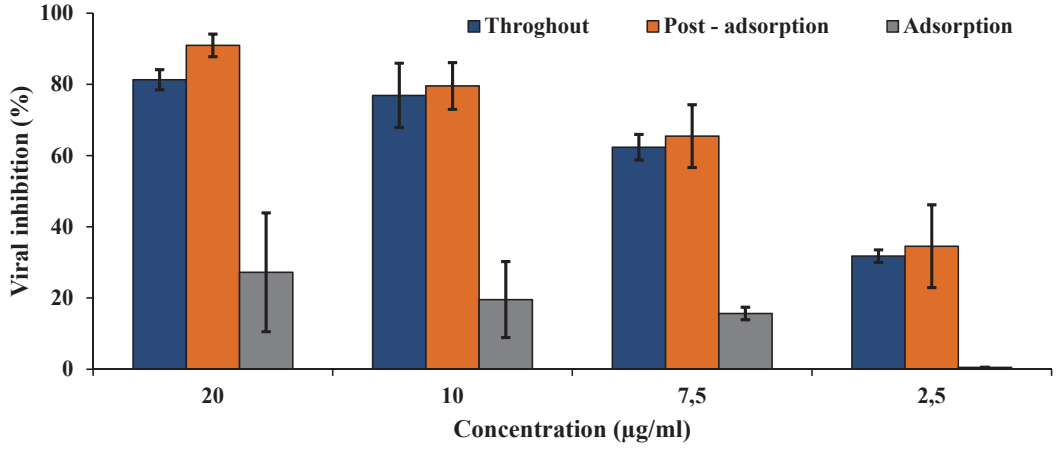

Figure 3. Inhibitory effect of *S. aromaticum* at different steps of adenovirus to HEp2 cells. Various concentrations of *S. aromaticum* extract were added carried out during the adsorption (adsorption), after adsorption until the completion of the experiment (post-adsorption), and during and after the treatment (throughout). After incubated cell viability was determined using MTT assay. The procedure was carried out three times."

adsorption (throughout) than that of the other "treatment measurements" (Figure 3).

4. Discussion

In the present study, in vitro anti-adenovirus activity of *S. aromaticum* hydroalcoholic extract was evaluated using HEp2 cell line. According to our results, the CC50 value and the IC50 value of the extract (on adenovirus) were 99.66±11.4µg/mL and 4.73±1.62µg/mL, respectively. The SI value of the extract on adenovirus was 20.64. So, this extract seems to have noticeable activity against adenovirus. The recommended IC_{50} value, characteristic of herbal extract against infectious diseases is less than 100 µg/mL [36]. The extract used in this study revealed an IC_{50} value of 4.73±1.62µg/mL which is far below the recommended cutoff. Total phenolic and flavonoid amounts of *S. aromaticum* extract was 255.8±3.95 mg GAE/g and 63.9±2.35 mg RUT/g respectively. Phenolic compounds have been shown to have antibacterial and anti-viral activities [34, 37-40]. *S. aromaticum* is one of the richest sources of phenolic compounds such as gallic acid, eugenol and eugenol acetate [41]. One of these compounds, Eugenol (4-allyl-1-hydroxy-2-methoxybenzene) has been shown to have antiviral activity against HSV-1 and HSV-2 virus replication *in vitro* [42, 43]. The results of our study also showed that the *S. aromaticum* extract contained high level of phenolic compounds. Therefore, the anti-adenovirus activity of this extract could be attributed to its phenolic compounds such as eugenol.

To evaluate the antiviral mechanism of the extract against adenovirus, we also carried out pre-treatment of cell, during the adsorption (adsorption), after adsorption and until the completion of the experiment (post-adsorption), and during and after the adsorption (throughout). We observed that the antiviral effect of the *S. aromaticum* extract is more relevant to post-adsorption and during and after the adsorption (throughout) in comparison by other "treatment durations" on HEp-2 cell line. Based on our findings, the extract did not prevent the entry of adenovirus into HEp-2 cell, but it did act following penetration of the virus into the cell. Also, when extract was added to the cell culture before infection, did not effect on the adenovirus replication. Thus, the extract probably had no inhibitory effect on attachment of this virus to HEp-2 cell, but it did following penetration of the virus into this cell line.

The hydroalcoholic extract of *S. aromaticum* buds showed remarkable scavenging activity with IC_{50} values of 10.05±1.93 µg/mL, as compared with synthetic antioxidants BHT with IC_{50} of 25.41±1.89 µg/mL. They depend on phenol derivate compounds that obtained from extraction. Phenol compounds are essential constituent in plants and extract that containing much phenol has free radical scavenging activity is higher than other extracts [32,44-47].

5. Conclusions

Flower buds of *Syzygium aromaticum* L. extract with SI value of 20.64 against adenovirus and its capability of inhibiting the viral cycle, particularly throughout post-adsorption, can be considered a potential anti-adenovirus agent. 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 *S. aromaticum* extract. Hence, the antiviral activity of this plant might, in part, be attributed to phenolic compounds.

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