

## ***In vitro* anti influenza virus activity, antioxidant potential and total phenolic content of twelve Iranian medicinal plants**

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### **ABSTRACT**

The emergence of medicine resistance strains of influenza A viruses to the chemical drugs lead to the development of alternative herbal compounds that inhibit the virus replication. Therefore, the aim of this research was to investigate *in vitro* anti-influenza A viruses activity, antioxidant potential, total phenolic, and flavonoid content of a total of 12 hydro alcoholic crude extracts obtained from 8 kinds of medicinal plants. Anti-influenza A viruses activity of the extracts was investigated by the using of MDCK cell line and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) method. Both 50% inhibitory concentration ( $IC_{50}$ ) and 50% cytotoxicity concentration ( $CC_{50}$ ) of the extracts were identified using regression analysis. The antioxidant activity, total phenol, and flavonoid content of the extracts were determined using 2,

2-diphenyl-1-picrylhydrazyl (DPPH) assay, Folin-Ciocalteu method, and aluminum chloride colorimetric method, respectively. The results demonstrated that there was high activity against influenza virus for *Peganum harmala* L., *Equisetum arvense* L., and *Punica granatum* L, extracts with  $IC_{50}$  value of 9.1 (CI95%:7.3-11.3), 6.45 (CI95%: 4.5-9.23), and 104.5 (CI95%: 82.8-131.8), respectively. DPPH radical scavenging activity showed that both *Equisetum arvense* L. and *Punica granatum* L. demonstrated the highest antioxidant activity with  $IC_{50}$  value of 6.5, 6.8 and 7.7  $\mu$ g/mL, respectively. According to the results, some of these extracts might be further analyzed to develop effective anti-influenza factors.

**Keywords:** Antiviral activity, medical plant, Influenza virus, Antioxidant potential

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### **1. Introduction**

Among different kinds of viruses, one of the most common human respiratory tract pathogens that have high level of morbidity and death rate is influenza virus [1]. Segmented ribonucleic acid genome and animal reservoir cause genetic reassortment in the virus. The appearance of new human and non-human source of influenza virus with the ability to cross-species barriers creates with high rate of antigenic drift and shift; also it changes to pathogenic type in their new hosts [2, 3].

Currently, in order to treat anti-influenza A virus two main groups of medicines including matrix protein (M2), ion-channel inhibitors (Rimantadine and Amantadine), and neuraminidase inhibitors (Zanamivir, Oseltamivir, and Peramivir) are confirmed. On the one hand, the lack of an effective immunogenic vaccine improved against this virus; and on the other, the constant evolvement of influenza A virus causes the rapid emergence of resistance to current medicines [4, 5]. Therefore, it is essential to make the new

and efficient anti-influenza medicines in order to treat resistant forms of influenza A virus.

For a long time, plants have been used as remedies and have shown the ability to synthesize a wide range of compounds. Therefore, in order to identify the probable sources of herbal medicines several plants are being gathered and examined [6-11]. Besides, different screening experiments have been performed on these plants to isolate the agents with antiviral activity [12-15]. In some cases of these herbal medicines there has been a development into therapeutic agents and they have had promising outcomes.

Iran with rich and diverse local health tradition is endowed valuable plant genetic source. In order to verify their efficacy and safety, more detailed survey and documentation of plants used in local health traditions and ethnopharmacological evaluation might lead to the development of invaluable herbal medicines or isolation of compounds of therapeutic value. Therefore, this research was aimed to investigate 12 species of plants for antiviral activity against influenza A virus.

## 2. Materials and methods

### Plant collection and extraction

Different parts of 12 medicinal plants belonging to 8 different families (Table 1) were tested for their antiviral activity. The selected plants were provided from market of medicinal

plants from Shahrekord, Iran (southwest of Iran) and were identified by the Dr. Lorigooini according to the Flora of Iran. Voucher specimens of the plants were deposited in the herbarium of the Medical Plants Research Center, Shahrekord University of Medical Sciences, Iran (Table 1).

Plants were dried, powdered, and extracted using maceration method. The plant material was dissolved in 70% ethyl alcohol and kept at room temperature (RT) for 96 h. After that, the mixture was filtered and concentrated under nearly vacuum pressure at 40°C by using rotary evaporator. In order to further use, the extracts were kept in sterile bottles and under refrigerated conditions. In the following, the extracts were suspended at 37°C in dimethylsulphoxide (DMSO) to give a stock solution of 10mg/mL, dissolved in culture medium, filtered and stored (4°C) until used. The little percentage of DMSO present in the wells (maximal 0.2%) has no effect on the results of the experiments [16].

### Determination of the free-radical scavenging activity

In 2,2 diphenyl-1-picrylhydrazyl (DPPH) method described by Moon and Terao with some modifications, the free-radical scavenging activity was measured [17]. Briefly, different amounts of the extracts and methanol were added to a solution of 0.3 mg/mL methanolic solution of DPPH in order to make a total volume of 3.0 mL. After incubation for 15 min at RT, the absorbance was measured at 517 nm using UV-VIS spectrophotometer (UNICO 2100: USA).

**Table 1.** Ethnobotanical data of selected medicinal plants

NO	Family	Plant name	Part used	Local name	Common name	Voucher
1	Apiaceae	<i>Ferula gummosa</i> Boiss	gum	Barigeh	Galbanum	MPSKUMS-650
2	Apiaceae	<i>Ferula assa-foetida</i> L	Leaves	Anghozeh	Asafoetida	MPSKUMS-470
3	Apiaceae	<i>Prangos ferulacea</i> (L.)	gum	Jashir	Ribbed Cachrys	MPSKUMS-320
4	Berberidaceae	<i>Berberis integerrima</i> Bunge	root	Zereshkekoohi	Barberry	MPSKUMS-512
5	Equisetaceae	<i>Equisetum arvense</i> L.	Aerial parts	Domeasb	Horsetail	MPSKUMS-516
6	Lamiaceae	<i>Ziziphora clinopodioides</i> Lam.	Aerial parts	Kakooti	-	MPSKUMS-253
7	Lamiaceae	<i>Thymus daenensis</i> Celak.	Aerial parts	Avishandenaiei	thyme	MPSKUMS-248
8	Lamiaceae	<i>Hyssopus officinalis</i> L.	flowers	Zofa	hyssop	MPSKUMS-177
9	Lythraceae	<i>Punica granatum</i> L.	Peel fruits	Anar	Pomegranate	MPSKUMS-316
10	Pteridaceae	<i>Adiantum capillus-veneris</i> L.	Leaves	Parsiyavash	maidenhair	MPSKUMS-131
11	Rosaceae	<i>Crataegus azarolus</i> L.	Leaves	Zalzalak	Hawthorn	MPSKUMS-499
12	Zygophyllaceae	<i>Peganum harmala</i> L	Seed	Espan	Syrian rue	MPSKUMS-188

High absorbance of the reaction mixture indicated low free radical scavenging activity. Butylated hydroxytoluene (BHT) was used as reference standard. Inhibition of free radical by DPPH was calculated as follows: Antiradical activity (%) =  $(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100$ . The  $IC_{50}$  value was defined as the amount of antioxidant necessary to reduce 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 compounds [18]. The experiment was performed in triplicate and the results had average values.

#### Determination of total phenolic content

In each plant extract, the total phenolic content was determined using Folin-Ciocalteu method with some modifications made by Asadi-Samani and coworkers [19]. 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). Then, amounts of total phenolic were calculated by the application of gallic acid calibration curve. The results were expressed as gallic acid equivalents (GAE) g/g of dry plant matter.

#### Determination of total flavonoid content

With the same previously mentioned method, the total flavonoid content of each plant extract was measured [20]. Independently, 0.5 mL of diluted plant material was 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. Based on the incubation at room temperature (RT) for 40 min, the absorbance of the reaction mixture was read at 415 nm by the application of a UV-VIS spectrophotometer (UNICO 2100: USA). The results were presented in mg of Rutin equivalents of dry plant matter in comparison with the standard curve; which was made in the same condition.

#### Cell and Virus

Madin Darby Canine Kidney (MDCK) cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA), supplemented with 10% fetal bovine serum (FBS; Gibco,

USA) and 1% Pen/Strep (Gibco, USA) at 37°C in a 5% CO<sub>2</sub> atmosphere and humidified incubator.

MDCK cell line and Influenza A virus (A/Iran/12/2014(H1N1)) was obtained from Influenza Unit, Pasteur Institute of Iran. Before the viral infection, MDCK cells were washed twice with phosphate buffered saline (PBS) and cultured in virus growth medium containing DMEM with penicillin/streptomycin, 2µg/mL trypsin TPCK (Sigma-Aldrich, USA), and 0.3% bovine serum albumin (BSA, Gibco, USA).

#### Virus titration

A standard 50% tissue culture infectious doses (TCID<sub>50</sub>) method was applied for virus titration [21]. When, 90% confluent MDCK cells were prepared in 96 well plates, the cell culture medium was aspirated and washed twice with phosphate-buffered saline (PBS). Then, 200µL of a series of 10-fold dilutions of virus in virus growth medium containing DMEM with 0.5µg/mL trypsin TPCK was added into the wells and left to incubate for 2 days. Consequently, 50µL of culture medium were taken from each well and transferred to a U-bottomed 96-well plate for hemagglutination experiment [22]. TCID<sub>50</sub> which was calculated based on the method of Reed and Muench [23].

#### Screening of antiviral activity

The antiviral activity of each plant extract was screened in 96-well culture plates by measuring the reduction of the viral cytopathic effect (CPE) [24]. Briefly, confluent MDCK cell monolayers were infected with 100µL (100TCID<sub>50</sub>) of virus suspension and incubated at 37°C for about one hour to allow virus adsorption and then the virus was removed and the cells were treated with 25, 50 and 100 µg/mL of each extracts [24] supplemented with 2 µg/mL TPCK and 0.3% BSA (200µL/well). The plates were incubated at 37°C in a humidified CO<sub>2</sub> atmosphere until 90% of viral CPE in the control virus was reached. The reduction of viral CPE was determined by measuring cell viability by the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-ol)-2,5-diphenyltetrazoliumbromide], (Sigma, USA)] colorimetric assay.

Results of the screening were expressed as positive (+), reduction of viral CPE was higher than 50% at all three concentrations tested, negative (-) without protection at all three concentrations tested, (++) reduction of viral

CPE was higher than 50% at 100 and 50 µg/mL, and +/-- reduction of viral CPE was higher than 50% only at 100 µg/mL [24].

### Cytotoxicity Experiment

The cytotoxic effect (CPE) of the positive extracts was determined before the investigation of anti-influenza A virus activity. MDCK cells were seeded onto 96-well plates with a concentration of 15000 cells/well with final volume of 100 µL per well. After incubation at 37°C with 5% CO<sub>2</sub> for 24 h, while 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 three positive plant extract (*Punica granatum* L., *Equisetum arvense* L. and *Peganum harmala* L.) (in DMEM), and incubated at 37°C with 5% CO<sub>2</sub> for further 3 days. Cell viability was investigated by the ability of the cells to cleave the tetrazolium salt MTT, by the mitochondrial enzyme succinate dehydrogenase that develops a formazan blue color product, and also by the procedure was followed as described earlier [25]. In sum, the supernatants were removed from the wells in this phase 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 (sigma, USA) was added to the wells to dissolve the MTT crystals. The plates were located on a shaker for 15 min and the absorbance were read on an enzyme-linked immunosorbent experiment (ELISA) reader (STAT FAX 2100, USA) at 492 nm. Data of the study were calculated as the percentage of toxicity by the application of 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 [16]. Therefore, the 50% cytotoxic concentration (CC<sub>50</sub>) was explained as the cytotoxic concentration of the crude extracts by regression Pribit model.

### Antiviral Experiment

In positive extracts the antiviral activity was evaluated by inhibitory activity experiment using MTT method that described previously [16]. To test the ability of extracts to inhibit CPE of influenza virus in tissue culture, non-cytotoxic concentrations of each extracts (below the CC<sub>50</sub> value) were applied. Confluent MDCK cells monolayer were infected with 100 µL (100TCID<sub>50</sub>) virus suspension and incubated at 37°C for about one hour to allow virus adsorption. Then, the virus was removed and the cells were treated with serial two

fold dilutions from nontoxic concentration of three positive plants extract (*Punica granatum* L., *Equisetum arvense* L. and *Peganum harmala* L.) in DMEM supplemented with 2 µg/mL TPCK, and 0.3% BSA (200 µl/well). Medium with 0.1% DMSO and oseltamivir (Sigma, USA) were used as negative and positive controls, respectively. The plates were incubated at 37°C in a humidified CO<sub>2</sub> atmosphere for 48 h. Also, cell viability was determined by the application of previously described MTT experiment; and the percentage of protection were calculated as the percentage of inhibition according to the following formula: Antiviral activity (%)=(Atv-Acv)/(Acd-Acv) ×100%, where Atv, Acv, and Acd are the absorbance of the test compounds in virus infected cells, the absorbance of the virus control, and the absorbance of the cell control, respectively[16]. The procedure was performed in triplicate and the 50% inhibitory concentration (IC<sub>50</sub>) was determined by regression Pribit model. As a marker of antiviral activity, selectivity index was determined as the ratio of CC<sub>50</sub> to IC<sub>50</sub>.

### Statistical analysis

All the experiments were carried out in triplicate. The spearman's correlation test was used to determine whether the total phenolic contents and the free radical scavenging property were correlated or not. The IC<sub>50</sub> and CC<sub>50</sub> values were calculated by the application of dose-response analyses and related models with probit procedure using SPSS software.

## 3. Results

### DPPH radical scavenging activity, Total phenolic and flavonoid compounds

Different parts of 12 medicinal plants belonging to 8 different families (Table I) were used traditionally for treatment of diseases and were tested for their antiviral activity. Their DPPH radical scavenging activity, total phenolic, and flavonoid contents were measured. DPPH radical scavenging activity demonstrated that the *Equisetum arvense* L. and *Punica granatum* L. has shown the highest IC<sub>50</sub> value (6.5, 6.8 and 7.7 µg/mL, respectively) in comparison with the other extracts under the investigation. The results are presented in relation to the butylated hydroxytoluene (BHT), a reference standard that has IC<sub>50</sub> of 25.41±1.89 µg/mL. Besides, results demonstrated that among 12 medicinal plant extract, the *Punica granatum* L. (282.9), *Equisetum arvense* L. (262.1), and *Crataegus azarolus* L. (214.2) had the highest amount of

total phenolic compounds. There was negatively significant correlation between total phenolic content and IC<sub>50</sub> of DPPH scavenging activity values ( $r=-0.8472$ ,  $p<0.001$ ). The highest amount of flavonoid (57.2) compound was observed in *Punica granatum* L. (57.6) and *Adiantum capillus-veneris* L. (Table 2).

### Screening of antiviral activity

Three out of 12 tested plants extract were reported to have antiviral activity at a nontoxic concentration to the used cell line against influenza virus (MDCK) (Table 2).

### Cytotoxicity and Antiviral Experiment

In the present study, *in vitro* anti influenza activity of hydroalcoholic extracts were evaluated using MDCK cell line. According to MTT analysis and probit analysis, the CC<sub>50</sub> value of *Punica granatum* L., *Equisetum arvense* L. and *Peganum harmala* L. crud extracts on MDCK cells were 55.66 (48.4-64), 639.3 (592-689.9), 122.9 (107.8-140) µg/mL, respectively. The analysis demonstrated that the extract

concentration was significantly related to the cell death ( $P<0.01$ , Figure 1).

The antiviral activity of the most promising extracts detected during the screening procedure was more evaluated in detail by the application of MTT method. MDCK cells were inoculated with 100 TCID<sub>50</sub> (100 µL/well) of influenza virus for 1h and were treated with various doses of *Punica granatum* L., *Equisetum arvense* L., and *Peganum harmala* L. crude extracts. Findings of this study showed that the more extract concentration, the more cytopathic effect (CPE) inhibition ( $P<0.05$ , Figure 1). According to Probit analysis, the extracts of *Peganum harmala* L., *Punica granatum* L., and *Equisetum arvense* L. had high activity against influenza virus with IC<sub>50</sub> values of 9.1 (CI95%:7.3-11.3), 6.45 (CI95%: 4.5-9.23), and 104.5 (CI95%: 82.8-131.8), respectively (Table 3). Most of these extracts had partial activity at the applied low concentration. The results demonstrated that *Punica granatum* L., *Equisetum arvense* L., and *Peganum harmala* L. exhibited the highest SI values (8.63, 6.12 and 13.5, respectively) against Influenza virus (Table 3).

**Table 2.** DPPH-radical scavenging activity (IC<sub>50</sub>), total phenolics and flavonoid contents and Screening of antiviral activity against influenza A virus of plants extract

NO	Plant name	DPPH-radical scavenging activity IC <sub>50</sub> (µg/ml)	Total phenolics (mg GAE/g)	Flavonoid content (mgRUT/g)	Anti influenza virus activity
1	<i>Peganum harmala</i> L	125.8	111.16	46.2	+
2	<i>Ferula gummosa</i> Boiss	>1000	33.26	-	-
3	<i>Ferula assa-foetida</i> L	396.6	110.4	51.2	-
4	<i>Prangos ferulacea</i> (L.)	>1000	43.17	-	-
5	<i>Equisetum arvense</i> L.	22.7	136.2	25.25	+/-
6	<i>Punica granatum</i> L.	10.33	174.9	60.6	+
7	<i>Crataegus azarolus</i> L.	231	171.38	69.9	-
8	<i>Berberis integerrima</i> Bunge	163.2	100.42	25.21	-
9	<i>Ziziphora clinopodioides</i> Lam.	31.8	165.88	109.2	-
10	<i>Thymus daenensis</i> Celak.	51.9	114.83	92.45	-
11	<i>Adiantum capillus-veneris</i> L.	235.9	110.3	51.2	-
12	<i>Hyssopus officinalis</i> L.	522.2	58.77	27.14	-

The antiviral activity was tested by the reduction of viral cytopathic effect (CPE) assays.

(+) positive: reduction of viral CPE higher than 50% at all three concentrations tested.

(+/-) reduction of viral CPE higher than 50% at 100 and 50 µg/mL

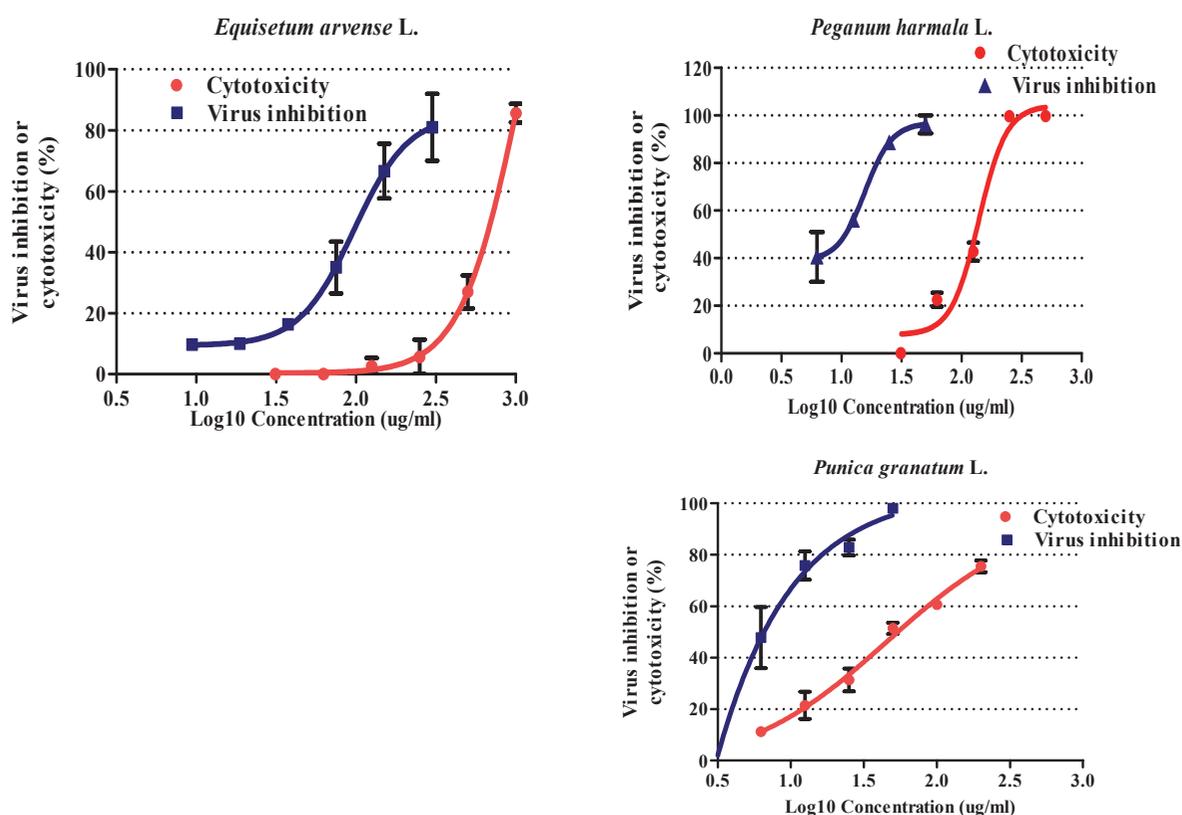
(+/-) positive/negative: reduction of viral CPE only at 100 µg/mL.

(-) negative: without protection at 25, 50 and at 100 µg/mL.

**Table 3.** Assessment of cytotoxicity and Anti influenza virus activity of selected plant extracts by MTT assay in MDCK cell line

Plant name	CC <sub>50</sub> <sup>a</sup> µg/ml (CI95%)	IC <sub>50</sub> <sup>b</sup> µg/ml (CI95%)	SI <sup>c</sup>
<i>Punica granatum</i> L.	55.66 (48.4-64)	6.45 (4.5-9.23)	8.63
<i>Equisetum arvense</i> L.	639.3 (592-689.9)	104.5 (82.8-131.8)	6.12
<i>Peganum harmala</i> L.	122.9 (107.8-140)	9.1 (7.3-11.3)	13.5
Oseltamivir(µmol)*	539.4 (378.9-768.5)	0.873 (0.55-1.37)	617.8

<sup>a</sup>CC<sub>50</sub>: 50% cytotoxic concentration, <sup>b</sup>IC<sub>50</sub>: 50% inhibitory concentration; <sup>c</sup>SI: Selectivity index, is the ratio of CC<sub>50</sub> to IC<sub>50</sub>; CI95%: 95% Confidence Interval; \*Oseltamivir was used as positive controls.



**Figure 1.** Anti influenza virus activity and cytotoxicity of *Punica granatum* L., *Equisetum arvense* L. and *Peganum harmala* L. extracts on MDCK cell lines. Confluent MDCK cells without virus or after influenza A virus infection were exposed to different concentrations of the extract for 48h. Cell viability was measured with MTT assay. Results are means  $\pm$  SD from three experiments.

#### 4. Discussion

Initial screening of plants typically begins by using crude aqueous or alcohol extraction in order to explore their active natural products and then will be followed by various organic extraction methods [26-29]. In this study, we investigated 12 plant extracts for their anti-influenza virus activity, antioxidant potential, and total phenolic compounds.

DPPH radical scavenging activity showed that the IC<sub>50</sub> of *Equisetum arvense* L. and *Punica granatum* L. extracts (6.5, 6.8 and 7.7µg/mL, respectively) was less than the other plant extracts under study. These results showed that the radical scavenging activity of these plants extracts were higher than BHT. The antioxidant potential and phenolic diversity in many plants have been previously reported [30, 31]. In present

study, the amount of phenolic compounds was expressed as gallic acid equivalent. Our results showed that among the 12 medicinal plants extract, the *Punica granatum* L. (282.9), *Equisetum arvense* L. (262.1), and *Crataegus azarolus* L. (214.2) had the highest amount of total phenolic compounds. There was a negatively significant correlation between phenolic content and  $IC_{50}$  values ( $r=-0.8472$ ,  $p<0.001$ ). Phenolic compounds by donation of one  $H^+$  cause scavenging free radicals and inhibition of macromolecules damage. In other words, in these extracts, phenolic compounds may be responsible for a part of radical scavenging activity. In other researches, a moderate correlation was found between DPPH radical scavenging and phenolic content in plant extract [32, 33].

According to Probit analysis, crude extracts of *Peganum harmala* L., *Punica granatum* L., and *Equisetum arvense* L. had high activity against influenza virus with  $IC_{50}$  values of 9.1 (CI95%:7.3-11.3), 6.45 (CI95%: 4.5-9.23), and 104.5 (CI95%: 82.8-131.8) respectively. As an  $IC_{50}$  value of a characteristic herbal extract against infectious diseases is less than 100  $\mu\text{g}/\text{mL}$  [33], these extracts should have strong antiviral activity against influenza virus.

The antiviral activity of pomegranate extract may be associated with the presence of hydrolysable tannins and polyphenols, specifically punicalagin and gallagic acid [34].

*Peganum harmala* L. with some alkaloids compounds has been considered from long time ago as a herbal medicine. The pharmacological studies compounds of this herb include several alkaloids,  $\beta$ -carboline (such as harmine, harmaline, harman and harmalol), and the quinazoline derivatives of vasicine and vasicinone [35]. It has been reported that two alkaloid compounds of the  $\beta$ -carboline and the quinazoline have anti-influenza A virus activity [36-38]. Therefore, the anti-influenza A virus activity of *Peganum harmala* L. extract used in this study could be attributed to its alkaloid components.

The *Punica granatum* L. peel is considered as an agro-waste but it can be a potential source of various phenolic and flavonoid compounds which shows antibacterial, antiviral, antioxidant, anti-inflammatory and antineoplastic bioactivities [39]. The anti-influenza A virus activity of this extract could be attributed to its phenolic compounds. The anti-influenza A virus activities of some extracts have been attributed to their phenolic and flavonoid compounds. Therefore, other plants having these compounds [40-44] may also have antiviral activities which worth examining.

## 5. Conclusion

*Equisetum arvense* L. and *Punica granatum* L. extracts were rich in total phenolic compounds and exhibited antioxidant activity higher than the other plants tested. Also, there was a significant correlation between phenolic content and antioxidant activity. Since *Equisetum arvense* L., *Punica granatum* and *Peganium harmala* L. extracts have inhibited influenza virus replication, therefore further research is needed to elucidate the active constituents of these extracts for discovering of the novel anti-influenza activity agents.

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