EUPHORBIA MICROSCIADIA PERCOLATION AND SOXHLET EXTRACTS EXHIBIT ANTIVIRAL ACTIVITY

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Summary

In the traditional medicine, the extracts of different species of *Euphorbia* have been successfully used for the treatment of skin diseases and some cancers.

Therefore, in this study the antiviral effects of *Euphorbia microsciadia* extracts (EME) were investigated using a plaque reduction assay. After collecting and authentication, aerial parts of the plant were powdered and extracted using percolation and Soxhlet methods. After accomplishing several enriching stages of phage CP51, phage titration was performed to verify the phage concentration in phage lysates to identify the dilution of the phage suspension to be used as negative control for the next working stages. Then IC_{50} of trifluridine for phage CP51 was determined and used as positive control. To study antibacterial activity of extracts, "Broth dilution method" was used to determine MIC. MIC of the extracts for *Bacillus cereus* was determined as 1.5 mg mL^{-1} for both Soxhlet and percolated extracts. To ascertain whether the extracts have the ability to inhibit the adsorption of virus to host cell, it was pre-incubated with phage CP51 for 30 min at 25°C. The growth and reproduction of phage was inhibited by more than 50% at 1 mg mL^{-1}, for soxhlet extract and 1.25 mg mL^{-1} for percolation extract. In order to conclude the effects of extracts on transcription process, *Bacillus cereus*, phage CP51 and extracts were incubated all together. In this stage, growth and reproduction of phage was inhibited by more than 50% at 0.75 mg mL^{-1} for Soxhlet extract and 1 mg mL^{-1} for percolation extract. These results indicated that both extracts of *E. microsciadia* had favorable antiviral activities.

Key words: *Euphorbia microsciadia* extract, antiviral effect, plaque reduction assay

Introduction

Lack of effective curative drugs against some severe viral diseases is a serious concern to health all over the world. Available antiviral drugs frequently lead to the development of viral resistance, characterized by their high mutational rate (1). The search for natural antiviral compounds from plants is a promising approach in the development of new therapeutic agents (2).

A wide variety of active phytochemicals, including the flavonoids, terpenoids, lignans, sulphides, polyphenolics, coumarins, saponins, furyl compounds, alkaloids, polyines, thiophenes, proteins and peptides have been identified. Some volatile essential oils of commonly used culinary herbs, spices and herbal tea have also exhibited a high level of antiviral activity. However, most of the pharmacopoeia of compounds in medicinal plants with antiviral activity is still not known (3).

The plants of the family Euphorbiaceae contain the well-known skin irritating diterpenoids, which have tigliane, ingenane, and daphnane skeletons. Some species of the family are used in folk medicines to cure skin diseases, gonorrhea, migraines, intestinal parasites, and warts (4) and in Iran as a purgative (5). In addition, several macrocyclic diterpenoids with antibacterial, anticancer, PGE2-inhibitory, anti-multidrug- resistant, prolyl endopeptidase inhibitory, antifeedant, anti-HIV, and analgesic activity have recently been isolated from different *Euphorbia s*pecies. They include jatrophane, ingol, and myrsinane diterpenoids (5-7).

In fact, pronounced antiviral activity has been reported from several species of the genus *Euphorbia*, against polio, coxsackie, and rhinoviruses(8). Therefore, in this study, utility of *Euphorbia microsciadia* that grows in Iran and is used in folk medicine for different purposes, was investigated for its potential antiviral effect using plaque reduction assay.

Materials and Methods

Plant collection

Euphorbia microsciadia was collected in October 2004 from near Mashhad (Khorasan Province, Iran). It was identified in the Herbarium of Ferdowsi University (Mashhad, Iran) and voucher specimens were deposited for reference at the Herbarium of Mashhad School of Pharmacy (Iran) with reference number 108-0513-3. The aerial part of the plant was dried in shade for 3 days and then was powdered. This powder was analyzed for the presence of alkaloids, flavonoids, saponins and tannins (9).

Extract preparation

Soxhlet ethanolic extract: 43.5 g of plant powder was extracted with 300 mL of ethanol for 12h using Soxhlet apparatus. The methanol was removed under reduced pressure and the viscose brownish resulted extract (5.87 g) was stored in the refrigerator at 4°C, until used.

Percolated ethanolic extract: 50 g of plant powder was extracted with ethanol by percolation. The extract was evaporated under reduced pressure and viscose resulted extract (3.76 g) was kept in the refrigerator at 4 °c, until use.

Media

Phage Assay Broth (PA Broth): nutrient broth 13 gL^{-1} (Merck, Germany), NaCl 5 gL^{-1} (Merck, Germany), at pH 5.6-6.0 was used in all the protocols. Phage Assay Agar, consisted of PA Broth with the addition of 15 gL^{-1} agar (Merck, Germany) was used for *Bacillus cereus* culture to

produce the phage. Phage assay top agar (PA Top Agar): consisted of PA Broth with the addition of 7 g L^{-1} agar was used for plaque assay as the soft layer agar. All media contained 5 mL of the solution consisting of 40 g L^{-1} Mg $(SO_4)_2$. $7H_2O$, 10 g L^{-1} $MnSO_4.H_2O$, 30 g L^{-1} $CaCl_2.2H_2O$ to increase adsorption of phage on the host cells. Soybean casein digest agar (SCDA): casein enzymatic hydrolysate 15 L^{-1}, papaic digest of soybean meal 5 g L^{-1}, sodium chloride 5 g L^{-1}, agar 15gL^{-1}.

Bacterial Strain

Bacillus cereus (ATCC 10876) was used in this study. Cultures were stored at-20°C in 15% glycerol (Favrin *et al* , 2003). Before verification, a stock culture of the bacteria was maintained on SCDA plate and incubated for 24 h at 37° C.

Bacteriophage

A *Bacillus* phage CP51 was used in this research. The phage stocks were arranged on the host strain, by a plate lysis process, according to the method of Atta-ur-Rahman *et al*. (2001), to growing bacteriophage Lambda-derived vectors. In brief, 100 µL of the phage suspension was mixed with 100 µL of *Bacillus cereus* suspension (10^8 cfu ml^{-1}) in a sterile Eppendorf micro-centrifuge tube (polypropylene; 1.5 mL; Sarstedt) and incubated for 15 min at 25°C to expedite adsorption of the phage to the host cells. The mixture was transferred from the Eppendorf micro-centrifuge tube to a 5 mL Bijou bottle and then 2.3 mL of soft layer agar (PA Top Agar) was added which had been melted and cooled to 40° C in a water bath. The contents of each bottle were then mixed by stirring and poured over the surface of a plate. After 15 minute, plate was incubated for 24 h at 25° C. When plate showing almost confluent plaques, it was used for preparing a concentrated phage suspension by overlaying with 5 mL of PA broth. The over layer medium containing the phage CP51 was decanted and filtered with a 0.22 µM filter syringe. The filtrate was used as a phage stock solution. Several dilutions of phage solution were fabricated.

Determination of Minimum Inhibitory Concentration (MIC)

To study antibacterial activity of extracts, "Broth dilution method "was used to determine MIC (10). Sterile Eppendorf micro tubes were used in this stage. Each micro tube had 1 mL of *B. cereus* suspension (10^6 cfu ml^{-1}) and 1 mL of serially diluted *Euphorbia microsciadia* extracts (EME) in distilled water (375,750, 1000, 1250, 1500, 1750, 2000 and 2250µg mL^{-1}). Triplicate samples were carried out for each concentration. The negative control tube, contained 1 mL of bacterial suspension and 1 mL Muller Hinton Broth medium and positive control tube contained 1 mL bacterial host cell suspension with 1 mL erythromycin (200µgmL^{-1}). All tubes were incubated at 37°C for 24h. Then 0.5 mL of TTC solution (triphenyl tetrazolium chloride) (2 mg mL^{-1}) was added to each microtube and incubated at 37°C for 30 min. After that, color changes was observed and the first concentration at which color did not change, determined as MIC of the plant extracts for *B. cereus*.

Phage inactivation assays

Both pre-incubation and no pre-incubation phage inactivation protocol was performed according to Atta-ur-Rahman *et al*. (2001).

Pre-incubation protocol: Different concentrations of extracts of *E. microsciadia* in distilled water were prepared and filter sterilized (stock solution was 15 mg mL^{-1} and contained 5% DMSO and 5% THF). One loopful of *B. cereus* from overnight culture (10^8 cfu ml^{-1}) was inoculated into a PA Broth medium (10 mL), mixed and then incubated at 37°C for 5 h. 100 µL of phage in proper dilution (containing 800 PFU) was added to 500 µL sterile solution of extracts and then this mixture was incubated at 25°C for 30 min. After that, 500 µL of bacterial suspension and 1.9 mL of melted PA Top Agar medium were added. This mixture poured over the PA agar plate and incubated at 25°C for 24 h. The negative control included all above except the extract solution. In positive control plate, the extract was replaced with 500 µL trifluridine in IC_{50} concentration 204.32(µg.ml^{-1}).

No pre-incubation protocol: One loopful of *B. cereus* from overnight culture (10^8 cfu ml^{-1}) was inoculated into a PA Broth medium (10 mL), mixed and incubated at 37°C for 5h. Different concentrations of *E. microsciadia* extracts in distilled water were prepared and filter sterilized. 500 µL sterile solutions of extracts, 100 µL of phage in proper dilution (containing 800 PFU) and 500 µL of bacterial suspension were mixed together and then 1.9 mL of PA Top agar was added and the mixture was poured onto a PA agar plate and incubated at 25° C for 24 h. The negative control included all above except the extract solution. Positive control plate contained 500 µL trifluridine (adjusted at the IC_{50} concentration, 204.32(µg.ml^{-1}).

Investigation of effect of solvents (5% DMSO and 5% THF) on the phage and bacteria

Different percentages for these mixed solvents were prepared. Then, 100 µL of phage in proper dilution (containing 800 PFU) was added to 500 µL of solvents and incubated at 25° C for 30 minutes. After that, 500 µL of bacterial suspension (10^8cfu ml^{-1}) and 1.9 mL of PA Top Agar medium were added. This mixture poured over the PA agar plates and incubated at 25° C for 24 h. Triplicate samples were performed for each percentage of solvents.

Statistical analysis

Experiments were accomplished in triplicate. The arithmetic mean± standard error of the mean (SEM) of control and experimental results were estimated using the Student`s t-test. $p< 0.05$ was regarded statistically significant.

Results

Primary phytochemical screening results of *E. microsciadia* extracts is shown in Table1 which indicate the presence of flavonoids and tannins.

The MIC of the *E. microsciadia* Soxhlet and percolated extracts for *B. cereus* were 1.5 mg mL^{-1}. After examination of the solvents (5% DMSO + 5% THF), no significant reduction of plaque number was observed in both protocols.

After addition of Soxhlet extracts of *E. microsciadia* to the mixture of phage and *B. cereus*, a great reduction (>50%) in plaque was detected from concentration 0.75 mg mL^{-1} and 100% reduction was resulted at concentration of 2.0 mg mL^{-1} (Fig. 1).

Table 1. Major non-volatile components of aerial parts of the plant

Chemical components	Presence
Alkaloids	_
Flavonoids	+
Saponins	_
Tannins	+

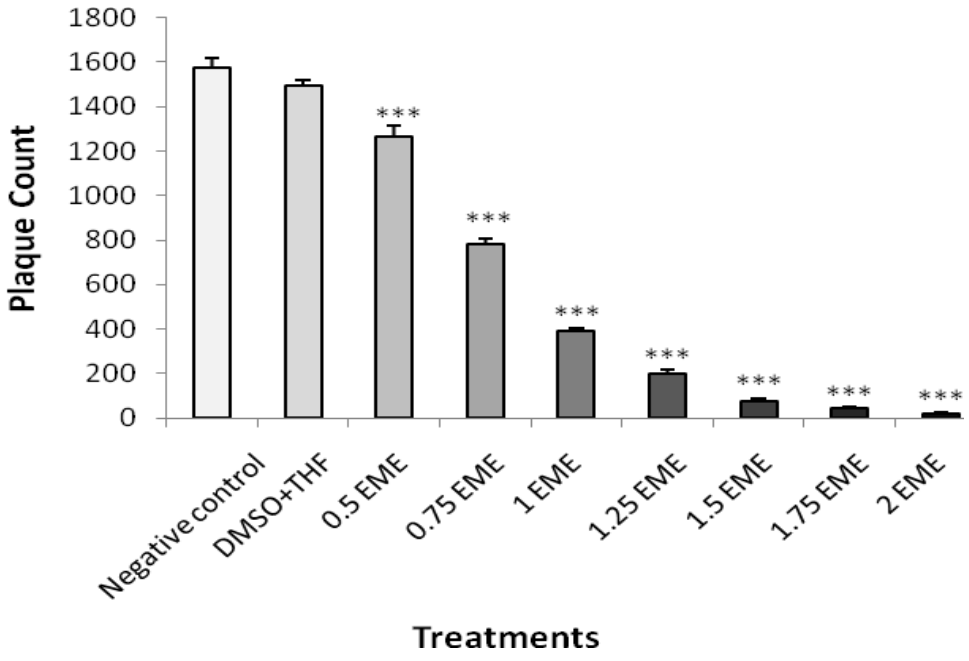

Fig. 1 Effect of different concentration of *E. microsciadia* soxhlet extract on reduction of phage CP51 using no pre-incubation protocol. The negative control included 100 µL of phage in proper dilution (containing 800 PFU), 500 µL of bacterial suspension and 1.9 mL of PA Top agar. DMSO5% and THF5% is as extract solvent. Each bar presents the mean±SD of the number of plaque. *** $p<0.001$, Tukey-Kramer test.

After pre-incubation of *E. microsciadia* Soxhlet extracts with phage CP51 at 25 °C for 30 min, a significant reduction (>50%) in plaque number was resulted from concentrations of 1 mg mL^{-1}, and at concentration of 2.75 mg mL^{-1},100% inhibition was obtained (Fig. 2).

When percolated extracts of *E. microsciadia*, suspension of *B. cereus* and phage CP51 were mixed together without pre-incubation, a great reduction (>50%) in plaque number was detected from concentration of 1 mg mL^{-1} and 100% reduction was gained at concentration of 2.5 mg mL^{-1}.

After pre-incubation of *E. microsciadia* percolated extracts with phage CP51 at 25°C for 30 min, a significant reduction (>50%) in plaque was resulted from concentrations of 1.25 mg mL^{-1} and at concentration of 3.75 mg mL^{-1} ,100% inhibition was observed.

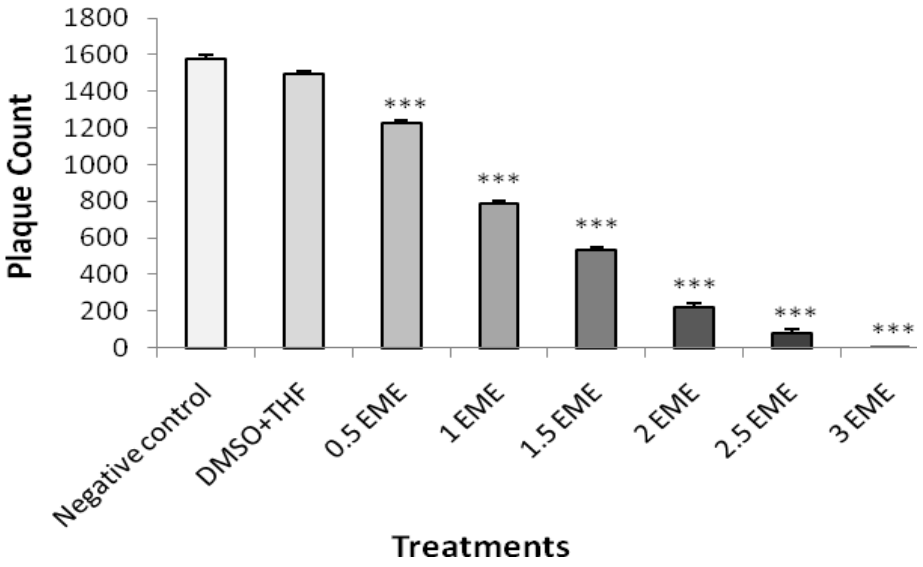

Fig. 2 Effect of different concentration of E. microsciadia soxhlet extract on reduction of phage CP51 using pre-incubation protocol. The negative control contained 100 µL of phage in proper dilution (containing 800 PFU), 500 µL of bacterial suspension and 1.9 mL of PA Top agar. DMSO5% and THF5% is as extract solvent. Each bar presents the mean±SD of the number of plaque. *** $p<0.001$,Tukey-Kramer test.

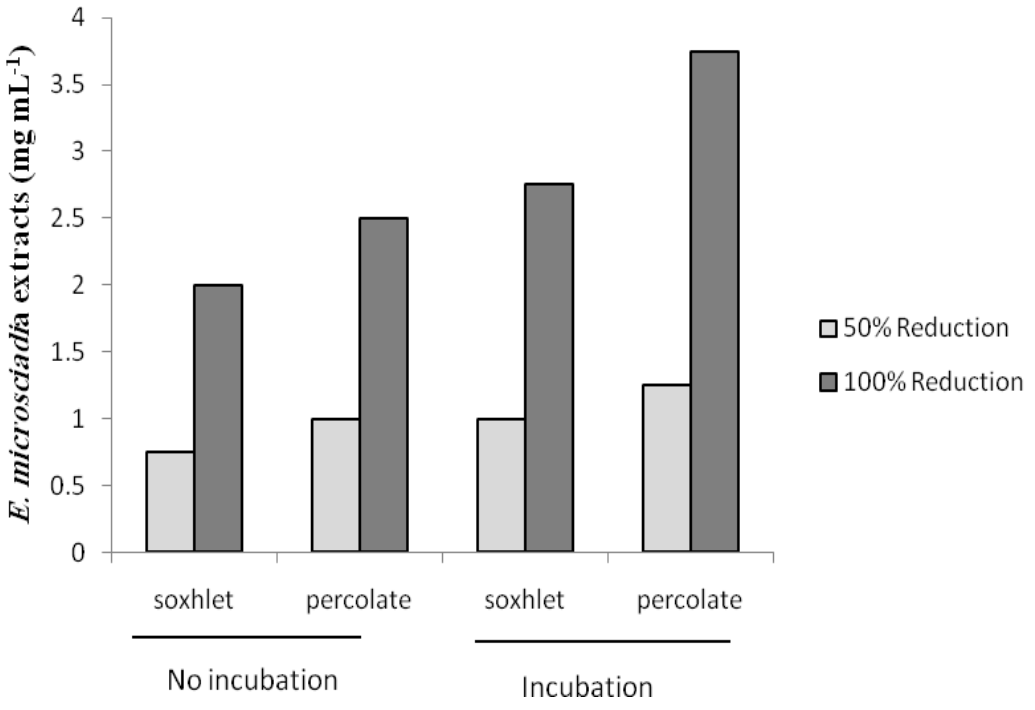

Fig. 3 Comparison of different concentrations of soxhlet and percolated extracts for 50% and 100% reduction of plaques.

Discussion

Several antiviral studies have been performed on the Euphorbiaceae family and some of the species have shown antiviral activity (11). To date, seven compounds with antiviral activity derived from 3-methylethers of quercetin and Kaempferol have been isolated from *E. grantii* (12).

An extract of the whole plants of *E. australis* was found to be the most active extract against the Herpes virus, HCMV(13). In addition, several macrocyclic diterpenoids with antibacterial, anticancer, PGE2-inhibitory, anti-multidrug-resistant, prolyl endopeptidase inhibitory, antifeedant, anti-HIV, and analgesic activity have recently been isolated from different Euphorbia species. They include jatrophane, ingol, and myrsinane diterpenoids (5-7) . Since no phytochemical study has been performed on *E. microsciadia,* it is difficult to attribute it's antiviral effect to specific compounds in the extract .Therefore, the present study was accomplished to investigate the antiviral activity of *E. microsciadia* so that the justification for the isolation of its antiviral component(s) could be achieved, which may be useful in the development of new and effective antiviral agents. Chemical constituents of different Iranian Euphorbia was given in Table 2.

Table 2. Chemical constituents of different Iranian Euphorbia

Euphorbia species	Flavonoids and Coumarins	Triterpenoids and Steroids	Other constituents
E. *larica*	*Kaempferol-3-o-glucoside,quercentin-3-0-glucosid*	Lupeol, Lupeol acetate, Lupeone, ginnone	Nonacosane, Octacosyl behenate
E. *virgata*	*Kaempferol*		
E. *petiolata*		Cycloartenol,24-methylenecyloartanol	
E. *falcata* L.		Obtusifoldienol, β-amyrin	Octadecan-2-one, eicosan-2-one
E. *tinctoria*	*Quercentin*, *quercentin-7-glucosid,Kaempferol* rhamnoside	euphorbol	
E. *myrsinites*		taraxerol	

* Jassbi, 2006 (5)

Several methods are available to detect either virucidal or inhibitory (antiviral) plant activity. such as cytopathic effects(3-4) or plaque formation(4, 14-15) or for transformation or proliferative effects on cell lines(4) . Viral replication may be assayed by detection of viral products such as DNA, RNA, or polypeptides. Table 3 lists various in vitro antiviral screening assays.

Table 3. In vitro antiviral screening assays*****

Determination of viral infectivity under two conditions: I. In cultured cells during virus multiplication in the presence of a single compound or a mixture of compounds, e.g., plant extracts. II. After extracellular incubation with a single compound or a mixture of compounds.
Plaque inhibition assay (only for viruses which form plaques in suitable cell systems) Titer determination of a limited number of viruses in the presence of a nontoxic dose of the test substance.

Plaque reduction assay (only for viruses which form plaques in suitable cell systems) Titer determination of residual virus infectivity after extracellular action of test substance(s)
Inhibition of virus-induced cytopathic effect (for viruses that induce cytopathic effect but do not readily form plaques in cell cultures) Determination of virus-induced cytopathic effect in cell monolayers cultured in liquid medium, infected with a limited dose of virus, and treated with a nontoxic dose of the test substance(s)
Virus yield reduction assay Determination of the virus yield in tissues cultures infected with a given amount of virus and treated with a nontoxic dose of the test substance(s) Virus titer determination carried out after virus multiplication by the plaque test or the 50% tissue culture infective dose end point test
End-point titer determination technique Determination of virus titer reduction in the presence of twofold dilutions of test compound(s) This method especially designed for the antiviral screening of crude extracts
Assays based on measurement of specialized functions and viral products (for viruses that do not induce cytopathic effects or form plaques in cell cultures) Determination of virus specific parameters, e.g., hemagglutination and hemadsorption tests (myxoviruses), inhibition of cell transformation (Epstein-Barr virus), immunological tests detecting antiviral antigens in cell cultures (Epstein-Barr virus,HIV, HSV, cytomegalovirus)
Reduction or inhibition of the synthesis of virus specific polypeptides in infected cell cultures, e.g., viral nucleic acids, determination of the uptake of radioactive isotope-labelled precursors or viral genome copy numbers

* Cowan, 1999 (4)

Medicinal plants have a variety of chemical constituents, which have the ability to inhibit the replication cycle of various types of DNA or RNA viruses (1, 3, 13). Other mechanisms of the most active antiviral compounds from medicinal plants are inhibition of viral adsorption processes to host cells(3-4, 16) and blocking DNA or RNA or protein synthesis(3). Wide variety of active phytochemicals, including the flavonoids(4-5, 16), terpenoids, tannins(3-5), lignans, sulphides, polyphenolics(3), coumarins(4), saponins, polysaccharides(16), furyl compounds, alkaloids, polyines, thiophenes, proteins and peptides(3) have been identified that exhibited a high level of antiviral activity. So, assay methods to determine antiviral activity including pre- and post-treatment analyses, are needed(16). Therefore, we used either protocol where phage was pre-incubated with the extract before confronting to *B. cereus* or without pre-incubation with the phage.

To determine whether the extracts have the capability of inhibiting the adsorption of virus to the host cells, it was pre-incubated with phage *CP51* for 30 minutes in temperature of 25°C.In the next step, for examining the effect of extracts on transcription process, *Bacillus cereus,* phage *CP51* and extracts were incubated all together. The results showed that the extracts exhibited antiviral activity in either method implying that the extracts had no considerable effects on the adsorption of phage to the host cells or on direct inactivation of the virus. On the other hand, because “no pre-incubation protocol” was more effective in reducing of plaques, probably extracts may have been effective on the inhibition of replication cycle of virus or viral replication enzymes.

Primary phytochemical screening of *E. microsciadia* extract has indicated the presence of flavonoids and tannins. Also in another study three compounds discovered in *E. microsciadia* extract include cycloclarkeanol**,** β-sitosterol and nonacosane (17). The antiviral effects of bioflavonoids and tannins extracted from medicinal plants have been proved. For example, Baicalein (BA), is a flavonoid compound purified from the medicinal plant *Scutellaria baicalensis Georgi* and has been shown to possess anti-inflammatory and anti-HIV-1 activities(18) . BA may interfere with the interaction of HIV-1 envelope proteins and chemokine co-receptors and block HIV-1 entry of target CD4 cells(18). One stage of viral replication that may be inhibited by flavonoids is viral DNA synthesis(19). Most of the potent anti-HIV flavonoids such as BA, quercentin and myricetin have shown inhibitory activity not only against the virus-associated RT but also against cellular DNA or RNA polymerase (3). Also tannins have shown potential antiviral and antibacterial effects. For example, casuarinin, a hydrolyzable tannin, has been demonstrated to inhibit directly the HSV to attach to and penetrate into the cells; affect the late event(s) of HSV-2 infection; and exhibit viral inactivation activity at high concentrations(20).

In phage reduction assay, the antiviral activity should be differentiated from antibacterial activity. If an extract or compound decreases the number of plaques, it could be because of either its antiviral or antibacterial effect. Consequently, the MIC determination against the host bacterium *(Bacillus cereus*) should be performed. MICs of both Soxhlet and percolated extracts against *B. cereus* were more than the concentrations at which considerable plaque reductions were obtained indicating that the antiviral effect of these extracts were owing to the exploits of the extracts on phage itself rather than against the bacteria.

Two different widespread herbal extraction methods (Soxhlet and percolation) were performed to detect the effect of heat and solvent on the antiviral ingredients of *E. microsciadia*. The results illustrated that the Soxhlet extract had a higher activity in decrease of plaque number indicating that the active ingredients and components of extract were not sensitive to heat that used in Soxhlet method.

It could be deduced from this study that the extracts of *E. microsciadia* had a mild antiviral activity in a dose-depenedent manner. These results suggest that further investigations using human viruses and further phytochemical screening including isolation and purification of different fractions of extract and evaluation antiviral effects of them is beneficial. Also study of cell toxicity of this extracts using cell culture protocols is valuable.

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