

Original Research Article

Determination of antioxidant activity, phenolic contents and antiviral potential of methanol extract of *Euphorbia spinidens* Bornm (Euphorbiaceae)

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Abstract

Purpose: This study was aimed to evaluate the antioxidant activity of the methanol extract of *Euphorbia spinidens* Bornm (Euphorbiaceae) and its effect on Herpes simplex virus type-1 (HSV-1) replication.

Methods: The methanol extract of aerial parts of *E. spinidens* collected from Khorasan State in North-Eastern part of Iran was used in this study. Total phenolic, flavonoid contents and the antioxidant activity were evaluated using Folin-Ciocalteu method, aluminum chloride colorimetric method and β -carotene-linoleate model system, respectively. Both the cytotoxic and antiviral effects of the crude extract on Vero cell line were determined by quantifying the viability of Vero cells using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium (MTS) assay.

Results: Total phenolic and flavonoid contents of *E. spinidens* were 70 ± 1 mg of gallic acid equivalent/g of dry extract (mg GAE/g extract) and 49.66 ± 1.00 mg rutin equivalent/g of dry extract (mg RTN/g extract), respectively. Antioxidant activity was 44 ± 1 % compared with the standard, butylated hydroxytoluene (BHT). The 50 % cytotoxic concentration (CC_{50}) of the extract on Vero cells was 5.072 ± 0.063 mg/ml and its antiviral concentration of 50 % effectiveness (EC_{50}) value was 0.34 ± 0.003 mg/ml.

Conclusion: The findings of this study show that the methanol extract of *E. spinidens* has high content of phenolic and flavonoid compounds with good antioxidant activity. Furthermore, this extract has significant antiviral effect on HSV-1 probably due to the inhibition of viral replication.

Keywords: Antioxidant, Antiviral, *Euphorbia spinidens* Bornm, Herpes simplex virus

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INTRODUCTION

Herpes simplex viruses (HSV) are endemic in through human populations and pathogenic to human. Nucleoside derivative drugs such as acyclovir (AVC), gancyclovir (GCV) and pencyclovir (PCV) have been widely authorized drugs for the treatment of HSV infections [1].

However, the increased and protracted use of these compounds in clinical setting, especially for the therapy of immunocompromised patients, has led to the emergence of viral resistance against most of these drugs [2]. In order to tackle the problem of viral resistance, development of new antiviral products with various mechanisms of action are very much required.

Medicinal plants have been used for many years for the treatment of human diseases [3] and a number of herbal medicines have been developed into therapeutic agents or have had promising results [4-7]. 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 drugs [8-10]. The compounds with natural origin, especially herbal medicines, have been shown to be reliable source of new drugs [11-13].

The Euphorbia is the largest genus in the plant family Euphorbiaceae, containing about 2,000 known species [14]. The plants synthesize large number of diverse secondary metabolites such as terpenoids [15], polyphenols, flavonoids and tannins [16]. Some species of the genus Euphorbia have been exploited as medicinal plants for the treatment of skin diseases, migraine, gonorrhoea and intestinal parasites and as wart cures [17]. Researchers have shown that Euphorbia species have antiproliferative activity, cytotoxicity, antipyretic-analgesic activity and also inhibitory activity on the HIV-1 infection [18]. Therefore, this study was aimed to evaluate both antioxidant and anti-HSV-1 activities of the methanol extract of aerial parts of *E. spinidens*, grown in Iran.

EXPERIMENTAL

Plant material

Aerial parts of *E. spinidens* were collected in June 2011 from the wild populations growing in Northern Khorasan Province of Iran. The plant material was authenticated by Dr Ghorayshol Hosseini of Herbaceous Science Research Center at Ferdowsi University, Mashhad, Iran and a voucher specimen (no. 43033) was deposited at the center's herbarium.

Preparation of plant extract

Air-dried plant material (3 kg) was macerated with 15 L methanol (96 %) at room temperature (RT) for 5 days. The extract was filtered and concentrated under reduced pressure to yield 134 g crude extract.

Determination of total phenolic, flavonoid contents and antioxidant activity of the extract

The total phenolic content of the extract was estimated by Folin Ciocalteu method [19], with

some modifications. Briefly, 0.01 g of dried extracts was dissolved in 10 mL of 60 % methanol. Thereafter, 0.1 ml of extract solution was added followed by 0.5 ml of 10% Folin Ciocalteu reagent and after 4 to 8 min were mixed with 0.4 ml of 7.5 % aqueous sodium bicarbonate. The mixture was kept for 30 min at RT and then the total phenols were determined by spectrophotometrically at 765 nm (Unico UV-2100, USA). The results are expressed as mg of gallic acid equivalents/g of extract (GAEs).

Aluminum chloride colorimetric method was used for flavonoids determination with some modifications [4]. Briefly, 0.01 g of dried extracts was dissolved in 10 mL of 60 % methanol. 1 ml of extract solution was mixed with 1 mL of 2 % aluminum chloride and 6 mL of 5 % potassium acetate. Following incubation for 40 min. at RT, the absorbance of the reaction mixture was measured at 415 nm spectrophotometrically. The amount of 2 % aluminum chloride was substituted by the same amount of distilled water in the blank. Total flavonoids were expressed in terms of rutin equivalent (RE) (mg/g).

Total antioxidant capacity of *E. spinidens* extracts was assayed using the β -carotene and linoleic acid [20]. A stock solution of β -carotene (0.5 mg) in 1 mL of chloroform, linoleic acid (25 μ l), and Tween-40 (200 mg) was prepared and chloroform completely evaporated using a vacuum evaporator at 50 °C. Thereafter, 100 mL of oxygenated water (30 min 100 ml/min) were added under vigorous shaking. 2500 μ l aliquots of this emulsion were added to test tubes and 350 μ l of the extracts prepared at 2g/l concentrations were added and incubated for 48 h at room temperature. The same procedure was repeated with BHT (as positive control) and a blank. The absorbance of the solutions was measured at 490 nm. Antioxidant capacities of the extracts were compared with those of BHT and blank.

Cell culture and virus

African green monkey kidney cell line (Vero cell line, ATCC C102) was kindly provided by Pasteure Institute of Iran. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, USA) supplemented with 10 % of fetal bovine serum (FBS; Gibco, Germany), 100 μ g/mL of streptomycin, 100 UI/ml of penicillin and 0.25 μ g/mL amphotericin B (Gibco, Germany), at 37 °C and 5 % CO₂. The same medium containing 2 % PBS was used for cytotoxicity and antiviral assays. HSV-1 (HSV-1, KOS strain) was kindly provided by Department of virology, University of Tarbiat Modares,

Tehran, Iran. Virus stock was prepared by infection of confluent monolayer Vero 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 HSV-1 in Vero cells and was expressed as the 50 % Tissue Culture Infective Dose (TCID₅₀) per ml using the formula of Spearman–Karber [21].

Cytotoxicity assay

Prior to the investigation of anti-HSV-1 activity, the cytotoxic effect of the extract was determined. Briefly, Vero cells were seeded onto 96-well plates with a concentration of 3.5×10^5 cells/well with final volume of 100 µl per well. After incubation at 37 °C with 5 % CO₂ for 24 h, when the cell monolayer was confluent, the cell culture medium of cells aspirated and washed with PBS. Extracts were serially diluted with the culture medium supplemented with 2 % serum to reach the various concentrations. Negative control dilution of dimethyl sulphoxide (DMSO) at 0.1 % was also included.

Cells were incubated with 100 µL/well of various concentrations of the extract or DMSO (in triplicates). After incubation at 37 °C with 5 % CO₂ for 3 days, MTS [3(4,5 dimethylthiazol2yl)5(3carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium] (Cell Titer 96; Promega, USA) was added to each well with a volume of 20 µL. The trays were further incubated for 2 h to allow MTS production. The absorbances were determined with an ELISA reader (StataFax 2100, USA) at a test wavelength of 490 nm. Data were calculated as the percentage of toxicity using Eq 1.

$$\text{Toxicity (\%)} = \{100 - (At/As)100\} \dots\dots\dots (1)$$

where At and As refer to the absorbance of the test and control, respectively [21]. The 50 % cytotoxic concentration (CC₅₀) was defined as the cytotoxic concentration of the crude extract by regression analysis.

Antiviral assay

The virus induced cytopathic effect (CPE) was defined by MTS method. Approximately, 5×10^5 Vero cells/well were seeded onto 96-well plates with final volume of 100 µL per well and cultured for 24 h at 37 °C. When the cell monolayers were confluent, the medium was removed from the wells and 100 µL of test virus (100TCID₅₀) was added and incubated for another 2 h. Various non-cytotoxic concentrations (\leq CC₅₀ values) of the crude extract were then added to culture wells in triplicate test. The maximum

concentration of DMSO (0.1 %) was used as a negative control. Aciclovir (9-(2-hydroxyethoxymethyl); Sigma, USA) was used as a positive control for HSV-1. After incubation at 37 °C with 5 % CO₂ for 72 h, MTS was added to each well with a volume of 20 µL. The trays were renewed incubated for 2 h to allow MTS production. The absorbances were determined with an enzyme-linked immunosorbent assay (ELISA) reader (Stata Fax 2100, USA) at a test wavelength of 490 nm. Viral inhibition rate (V) was estimated as in Eq 2 [13].

$$V = (Atv - Acv)/(Acd - Acv)100 \dots\dots\dots(2)$$

where Atv is the absorbance of the test compounds with virus infected cells. Acv and Acd are the absorbance of virus control and of cell control, respectively. The procedure was carried out three times. The 50 % effectiveness concentration (EC₅₀) was determined from a curve relating inhibition to the concentration of the extract by regression analysis. Selectivity index (SI), as a marker of antiviral activity, was determined as the ratio of CC₅₀ to EC₅₀.

To analyze the dose-dependent effect of the test drugs on infected Vero cells, different concentrations of the extract was added to HSV-1 infected Vero cell culture in triplicate. After 2–3 days MTT assay was carried out to determine the inhibition of infection caused by the HSV, as described previously [22].

Time-course anti-virus analysis

Time-course analysis was carried out to investigate the period of time (stage) during which, the extract inhibits HSV-1 replication. This experiment, in which HSV-1 infected cells were treated with different doses of the extract at 1 h before and 24 h after the infection, was carried out according to previously described procedures with minor modifications [21]. In brief, approximately, 5×10^5 Vero cells/well were seeded onto 96-well plates with final volume of 100 µL per well and cultured for 24 h at 37 °C. When the cell monolayers were confluent, the medium was removed from the wells and 100 µL of virus (100 TCID₅₀ per well) was added and incubated for another 2 h. Various non-cytotoxic concentrations (\leq CC₅₀ values) of crude extract were added to culture cells in triplicate at various times pre-infection (–1 h), co-infection (0 h) or post-infection (2, 4, 8 and 24 h). HSV-1(100 TCID₅₀ per well) was inoculated onto confluent monolayers of Vero cells for 2 h. After 72 h, MTS test and antiviral activity were carried out as previously explained.

Statistical analysis

All experiments were carried out in triplicate. EC₅₀ and CC₅₀ values were calculated using dose-response analyses and related models with linear regression.

RESULTS

Total phenolic and total flavonoid contents of crude methanolic extract of *E. spinidens* were 70 ± 1 mg GAE/g extract and 49.66 ± 0.996 mg RTN/g extract respectively. Also, the crude methanolic extract of *E. spinidens* had lower antioxidant activity than BHT as reference antioxidant (Table 1).

MTS assay was used to determine both cytotoxicity and antiviral activity of the tested agents. The results demonstrated that the CC₅₀

and EC₅₀ of crude methanolic extract of *E. spinidens* were 5.072 ± 0.063 and 0.34 ± 0.003 mg/ml respectively. Moreover, the obtained results based on EC₅₀ value and selectivity index (SI) revealed that the crude methanolic extract inhibited HSV-1 (Table 2).

Time course analysis was carried out with crude methanolic extract to investigate the mechanism of antiviral activity. Inhibition was evaluated by MTS assay at 1 h before to 24 h after virus infection and of treatment with different doses of crude extract and represented as percentage inhibition. The results revealed that crude methanolic extract of *E. spinidens* at 5 mg/ml concentration exhibited the highest inhibition against HSV-1 infection within 2 h post-infection, which was during the early period of virus replication (Figure 1).

Table 1: Antioxidant activity and total phenolic and flavonoid contents of *E. spinidens* methanolic extract.

Sample	Total phenolics ^a (mg GAE/g)	total flavonoids ^a (mg RTN/g)	Antioxidant activity ^{ab} (%)
Extract	70.03 ± 1.12	49.66 ± 0.996	44.04 ± 1.02
BHT	-	-	90.06 ± 0.01

mg GAE/g: mg of gallic acid equivalent/g of dry extract, mg RTN/g: mg rutin equivalent/g of dry extract, BHT: Butylated Hydroxytoluene; ^aValues are given as mean ± SD (n=3); ^bβ-carotene-linoleate model

Table 2: Assessment of anti-HSV-1 activity of *E. spinidens* methanolic extract by MTS assay

Test drug	CC ₅₀ ^a (mg/ml)	EC ₅₀ ^a (mg/ml)	Selectivity index (SI) ^{ab}
Extract	5.072 ± 0.063	0.34 ± 0.003	14.917
Acyclovir	>5	0.028 ± 0.001	>178.571

^aValues are given as mean ± S.D (n=3); ^bSelectivity index (SI) = CC₅₀/EC₅₀. CC₅₀:50% cytotoxic concentration on Vero cells; EC₅₀: 50% effectiveness concentration

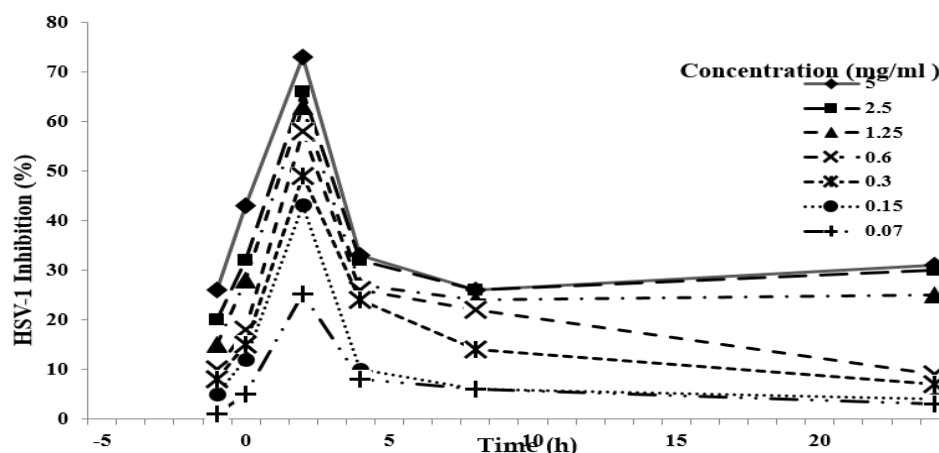


Figure 1: Inhibitory effect of adding methanol extract of *E. spinidens* at various times of herpes virus (HSV-1) to Vero cells. Various concentrations of *E. spinidens* methanolic extract were added with the HSV-1 infected Vero cells at various times period like pre-infection (-1 h), co-infection (0 h) or post-infection (2-24 h). After 3 days of incubation at 37°C, inhibition was evaluated by MTS assay and expressed as the inhibition percentage. Each bar represents the Mean ± SE of three independent experiments

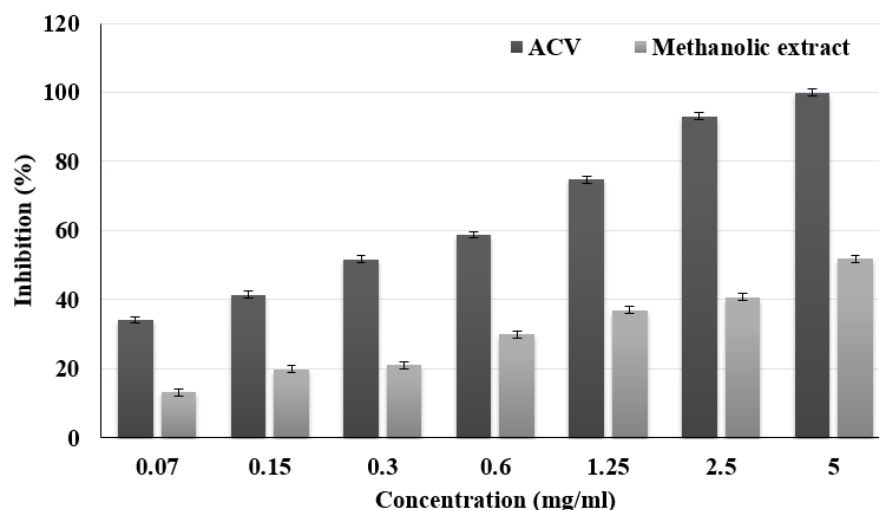


Figure 2: Dose-dependent effect of antiviral activity induced by *E. spinidens* extract. Various concentrations of *E. spinidens* methanolic extract (black bars) or acyclovir (gray bars) were added to HSV-1 infected Vero cells after 1 h of infection. Inhibition percentage was evaluated by MTS assay after 3 days of incubation at 37°C. Each bar represents the Mean \pm SE of three independent experiments

To analyze the dose-dependent antiviral activity, HSV-1 infected Vero cells were treated with various concentrations of the crude methanolic extract of *E. spinidens* in DMSO. Acyclovir and DMSO (0.1 %) were used as positive and negative control, respectively. The results revealed that the crude methanolic extract, at 5 mg/mL concentration, exhibited about 55 % inhibition against HSV-1 replication (Figure 2). Also, it was shown that at concentrations between 0.07 to 5 mg/mL, there was a good correlation between the crude extract concentration and HSV-1 inhibition rate.

DISCUSSION

The results of this study showed that the crude methanolic extract of *E. spinidens* possess *in vitro* anti-HSV-1 activity with SI value of 14.917. Our findings of time course analysis experiments also revealed that the extract exhibited the highest inhibition against HSV-1 infection within 2.0 h post-infection. This may indicate that the extract probably interferes with post-absorption and early stages of HSV-1 replication. Similarly, it has been reported that the crude methanol extract of *Mallotus peltatus* leaves and *Cuminum cyminum* L seeds inhibited HSV-1 replication in the early stage and adsorption, respectively [21,22]. These results, together, might indicate that some herbal extracts are able to interact with this virus in the intracellular stages of replication. The results of dose-dependent experiments, carried out in this study, in consistent with the results of some other reports [23,24] may indicate that anti-HSV-1 activity of the crude methanolic extract of *E. spinidens* is dose

dependent. It has been reported that flavonoids, terpenoid, and saponins are the main active components of *Euphorbia* genus which are highly extracted by methanol and that the antiviral effect of this genus is due to these secondary metabolites [14,25-27]. Thus, inhibition of HSV-1 replication seen in the present study may probably be due to the action of these secondary metabolites of *E. spinidens*.

CONCLUSION

Based on our results, crude methanol extract of *E. spinidens* exhibits anti HSV-1 activity. This is probably due to the inhibition of early viral replication and may be dose-dependent.

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REFERENCES

- Vijayan P, Raghu C, Ashok G, Dhanaraj SA, Suresh B. Antiviral activity of medicinal plants of Nilgiris. *Indian J Med Res* 2004; 120: 24-29.
- Villarreal EC. Current and potential therapies for the treatment of herpes virus infections. *Prog Drug Res* 2001; 60: 185-228.

3. Rafieian-Kopaei M, Sewell RD. The history and ups and downs of herbal medicines usage. *J HerbMed Pharmacol* 2014; 3(1): 1-3.
4. Karimi A, Moradi M-T. Total phenolic compounds and in vitro antioxidant potential of crude methanol extract and the correspond fractions of *Quercus brantii* L. acorn. *J HerbMed Pharmacol* 2015; 4(1): 35-39.
5. Karimi A, Moradi MT, Saeedi M, Asgari S, Rafieian-Kopaei M. Antiviral activity of *Quercus persica* L.: High efficacy and low toxicity. *Adv Biomed Res* 2013; 2: 36
6. Moradi MT, Rafieian-Koupaei M, Imani-Rastabi R, Nasiri J, Shahrani M, Rabiei Z, et al. Antispasmodic effects of yarrow (*Achillea millefolium* L.) extract in the isolated ileum of rat. *Afr J Tradit Complement Altern Med* 2013; 10(6): 499-503.
7. Moradi M-T, Karimi A, Alidadi S, Gholami-Arjenaki M. In vitro anti-herpes simplex type-1 activity, antioxidant potential and total phenolic compounds of pomegranate (*Punica granatum* L.) peel extract. *J Chem Pharm Res* 2015; 7(8): 82-88.
8. Asgary S, Kelishadi R, Rafieian-Kopaei M, Najafi S, Najafi M, Sahebkar A. Investigation of the lipid-modifying and anti-inflammatory effects of *Cornus mas* L. supplementation on dyslipidemic children and adolescents. *Pediatr Cardiol* 2013; 34(7): 1729-1735.
9. Asgary S, Sahebkar A, Afshani MR, Keshvari M, Haghjooyjavanmard S, Rafieian-Kopaei M. Clinical evaluation of blood pressure lowering, endothelial function improving, hypolipidemic and anti-inflammatory effects of pomegranate juice in hypertensive subjects. *Phytother Res* 2014; 28(2): 193-199.
10. Rafieian-Kopaei M, Asgary S, Adelnia A, Setorki M, Khazaei M, Kazemi S, et al. The effects of cornelian cherry on atherosclerosis and atherogenic factors in hypercholesterolemic rabbits. *J Med Plants Res* 2011; 5(13): 2670-2676.
11. Bahmani M, Zargaran A, Rafieian-Kopaei M, Saki K. Ethnobotanical study of medicinal plants used in the management of diabetes mellitus in the Urmia, Northwest Iran. *Asian Pac J Trop Med* 2014; 7s1: S348-354.
12. Delfan B, Bahmani M, Hassanzadazar H, Saki K, Rafieian-Kopaei M. Identification of medicinal plants affecting on headaches and migraines in Lorestan Province, West of Iran. *Asian Pac J Trop Med* 2014; 7s1: S376-379
13. Nasri H, Shahinfard N, Rafieian M, Rafieian S, Shirzad M, Rafieian M. Turmeric: A spice with multifunctional medicinal properties. *J Herb Med Pharmacol* 2014; 3(1): 5-8.
14. Jassbi AR. Chemistry and biological activity of secondary metabolites in *Euphorbia* from Iran. *Phytochemistry* 2006; 67: 1977-1984.
15. Appendino G, Spagliardi P, Ballero M, Seu G. Macrocyclic diterpenoid from *Euphorbia hyberna*. *Fitoterapia* 2000; 73: 756-762.
16. Yoshida T, Amakura Y, Liu Z, Okuda T. Three new hydrolyzable tannins and a polyphenol glucoside from *Euphorbia humifusa*. *Chem Pharm Bull* 1994; 42: 1803-1807.
17. Singla AK, Pathak K. Topical anti-inflammatory effects of *Euphorbia prostrata* on carrageenan induced footpad oedema in mice. *J Ethnopharmacol* 1990; 29: 291-294.
18. Qing-Wen Shi, Xiao-Hui Su, Hiromasa K. Chemical and pharmacological research of the plants in genus *Euphorbia*. *Chem Rev* 2008; 108: 4295-4327.
19. Folin O, Ciocalteu V. On tyrosine and tryptophane determinations in proteins. *J Biol Chem*. 1927; 73(2): 627-650.
20. Sharafati-chaeshtori R, Rafieian-kopaei R, Mortezaei S, Sharafati-chaeshtori A, Amini E. Antioxidant and antibacterial activity of the extracts of *Echinophora platyloba* D.C. *Afr J Pharm Pharmacol* 2012; 6: 2692-2695.
21. Bag P, Chattopadhyay D, Mukherjee H, Ojha D, Mandal-Mamta N, Sarkar C, Gobardhan T, Sekhar-Chakraborti D. Anti-herpes virus activities of bioactive fraction and isolated pure constituent of *Mallotus peltatus*: an ethnomedicine from Andaman islands. *J Virol* 2012; 9: 98-105.
22. Motamedifar M, Ghafari N, Talezadeh-Shirazi M. The effect of Cumin seed extracts against herpes simplex virus type 1 in Vero cell culture. *Int J Mol Sci* 2010; 35: 304-309.
23. Betancur-Galvis LA, Morales GE, Forero JE, Roldan J. Cytotoxic and antiviral activities of Colombian medicinal plant extracts of the *Euphorbia* genus. *Mem Inst Oswaldo Cruz Rio de Janeiro* 2002; 97: 541-546.
24. Sudhakar M, Rao-Ch V, Rao PM, Raju DB, Venkateswarlu Y. Antimicrobial activity of *Caesalpinia pulcherrima*, *Euphorbia hitra* and *Asystasia gangeticum*. *Fitoterapia* 2006; 77: 378-380.
25. Shi QW, Su XH, Kiyota H. Chemical and pharmacological research of the plants in genus *euphorbia*. *Chem Rev* 2008;108:4295-4327
26. Ghanadian M, Akhavan A, Ayatollahi A, Mohammadi-Kamalabadi M, Abdella O. Triterpenes from *Euphorbia spinidens* with their immunomodulatory activity. *Res Pharm Sci* 2013; 8: 205-210.
27. Mothana R, Lindequist U, Geraenert R, Bednarski P. Studies of the in vitro anticancer antimicrobial and antioxidant potentials of selected Yemeni medicinal plants from the Island Soqatra. *BMC Complement Altern Med* 2009; 9:7-23.