Tropical Journal of Pharmaceutical Research April 2016; 15 (4): 759-764
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v15i4.13

Original Research Article

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

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Received: 16 March 2015 *Revised accepted: 3 February 2016*

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, buthylated hydroxytuloene (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*

Tropical Journal of Pharmaceutical Research is indexed by Science Citation Index (SciSearch), Scopus, International Pharmaceutical Abstract, Chemical Abstracts, Embase, Index Copernicus, EBSCO, African Index Medicus, JournalSeek, Journal Citation Reports/Science Edition, Directory of Open Access Journals (DOAJ), African Journal Online, Bioline International, Open-J-Gate and Pharmacy Abstracts

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, gonorrhea 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 µl), and Tween-40 (200 mg) was prepared and chloroform completely evaporated using a vacuum evaporator at 50 ºC. Thereafter, 100 mL of oxygenated water (30 min 100 ml/min) were added under vigorous shaking. 2500 µl aliquots of this emulsion were added to test tubes and 350 µl 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 µg/mL of streptomycin, 100 UI/ml of penicillin and 0.25 µg/mL amphotericin B (Gibco, Germany), at 37 °C and 5 % CO_2. 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 cm^2 culture flasks using DMEM medium with 2 % FBS, at 37 °C in 5 % CO_2. Virus titer was determined by cytopathic effect (CPE) of HSV-1 in Vero cells and was expressed as the 50 % Tissue Culture Infective Dose ($TCID_{50}$) 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 % CO_2 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 % CO_2 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.

Toxicity (%) = {100 – (At/As)100} …….. (1)

where At and As refer to the absorbance of the test and control, respectively [21]. The 50 % cytotoxic concentration (CC_{50}) 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 ($100TCID_{50}$) was added and incubated for another 2 h. Various non-cytotoxic concentrations (≤ CC_{50} 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 % CO_2 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 ……(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 (EC_{50}) 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 CC_{50} to EC_{50}.

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 monolayer's were confluent, the medium was removed from the wells and 100 µL of virus (100 $TCID_{50}$ per well) was added and incubated for another 2 h. Various non-cytotoxic concentrations (≤ CC_{50} 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 $TCID_{50}$ 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. EC_{50} and CC_{50} 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 cytoxicity and antiviral activity of the tested agents. The results demonstrated that the CC_{50} and EC_{50} 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 EC_{50} 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: Buthylated Hydroxytuloene; [a]*Values are given as mean ± SD (n=3);* [b]β-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

[a]Values are given as mean ± S.D (n=3); [b]Selectivity index (SI) = CC_{50}/EC_{50}. CC_{50}:50% cytotoxic concentration on Vero cells; EC_{50}: 50% effectiveness concentration

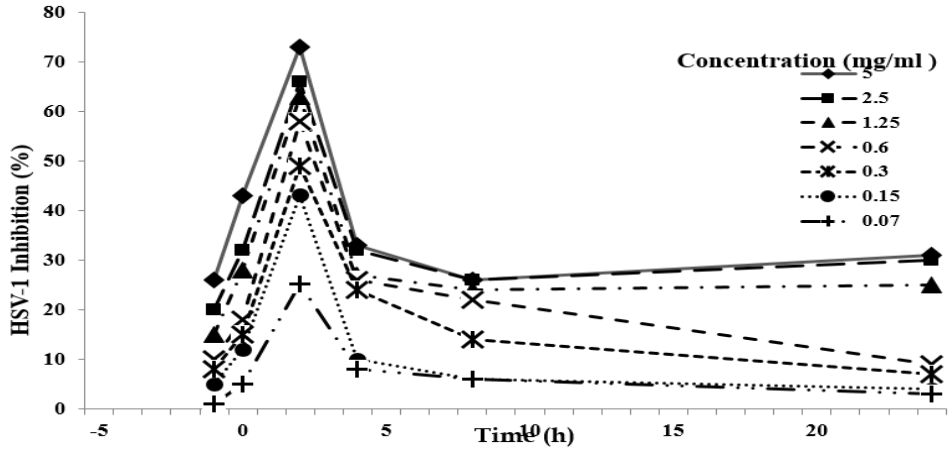

Figure 1: Inhibitory effect of adding methanol extract of *E. spinidens* at various times of herpes virus (HSV-1) to Vero cells. Various concentrations of E. spinidens methanolic extract were added with the HSV-1 infected Vero cells at various times period like pre-infection (-1 h), co-infection (0 h) 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

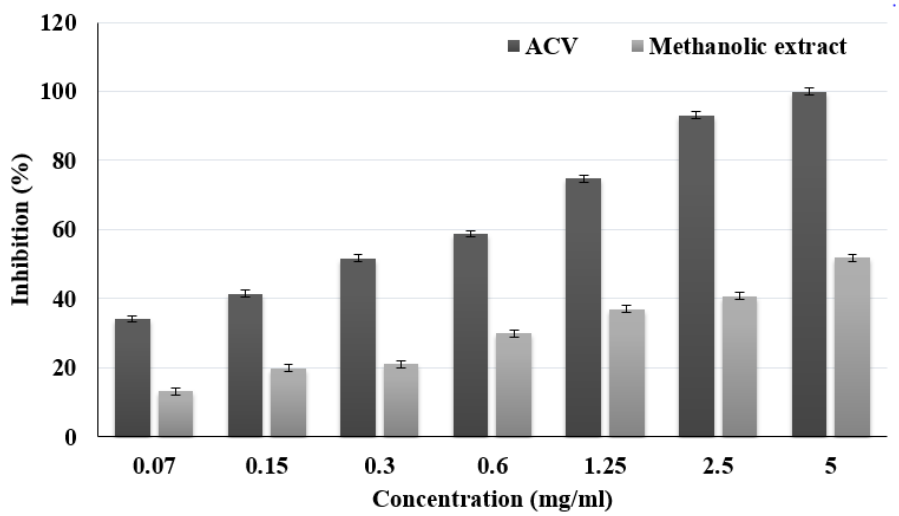

Figure 2: Dose-dependent effect of antiviral activity induced by *E. spinidens* extract**.** Various concentrations of *E. spinidens* methanolic extract (black bars) or acyclovir (gray bars) were added to HSV-1 infected Vero cells after 1 h of infection. Inhibition percentage was evaluated by MTS assay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

ACKNOWLEDGEMENT

This work is derived, in part, from the MSc thesis of the second-named author and financially supported by the Research Deputy of Shahrekord, Iran. Special thanks go to Dr Teymori of Virology Sciences, Department of Virology, Tarbiat Modares University, Tehran, Iran) for his help, including provision of HSV-1 (Strain KOS).

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