EVALUATION OF ANTI PHAGE CP51 EFFECT OF *EUPHORBIA MYRCINITES* EXTRACT BY A PLAQUE REDUCTION ASSAY

Javad Behravan[1*], Mohammad Ramezani[1], Hassan Alidoost[1] and Melika Ehtesham Gharaee[1]

[1]Biotechnology and Pharmaceutical Research Centers, Department of Pharmaceutical Biotechnology, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

***Corresponding Author**:

Dr. J. Behravan

Dept. of Biotechnology, School of Pharmacy

Mashhad University of Medical Sciences

Mashhad, Iran.

P. O. Box: 91775-1365

Tel: +98-511-8823255-66, Fax: +98-511-8823251

E-mail: behravanj@mums.ac.ir

Summary

In this study, the *Euphorbia myrsinites* extract was tested for antiviral activity using plaque reduction assay. After collecting, plant was powdered and extracted using Soxhlet apparatus. After applying several enriching stages of phage CP51, phage titration was performed to determine the phage concentration in phage lysate to specify the dilution of suspention to be used as negative control for the next working stages. Then IC_{50} of trifluridine for phage CP51 was determined and used as a positive control. In order to examine the effects of extract on transcription process, *Bacillus cereus*, phage CP51 and extract were incubated all together. In this stage, the growth and reproduction of phage were inhibited (>50%) at concentrations of above 12.5 mg mL^{-1}. To determine whether the extract has the ability to inhibit the adsorption of virus to host cell, it was pre-incubated with phage CP51 for 30 min at 25°C. In this stage, the growth and reproduction of phage were inhibited (>50%) at concentrations of above 11 mg mL^{-1}. These results demonstrated that the extract of *E. myrsinites* has a mild antiviral activity in a dose-dependent manner.

Keywords: *Euphorbia myrsinites;* antiviral activity; anti phage; plaque reduction assay

Introduction

Lack of effective curative measures against some severe and deadly viral diseases is of serious concern to health practitioners all over the world. Antiviral drugs are available to treat only a few viral diseases. Those currently available, like most other drugs, are bedeviled to have either side effects and/or loss of efficacy due to the development of resistance to them by these infectious agents (1). There is little likelihood that available orthodox antiviral drugs can eliminate all or even most viral diseases (2). So, There is a need to search for new and more effective antiviral agents. Medicinal plants have been traditionally-used to obtain different kinds of ailments for treatment of infectious diseases (3). Therefore , the development of new medicinal plant products is vital in controlling the threats posed by some pathogenic viruses (2).

Euphorbia species have been the source of a large number of biologically active compounds (4).Several studies have been shown various biological activities of some *Euphorbia* species such as Cytotoxicity (5), skin irritant (6, 7), Dermatitis and conjunctivitis (8), inflammatory (9), antioxidant (4), Tumor promoting (6), antileishmanial (10) and modulatory of multidrug resistance (11). Antiviral activity of Some *Euphorbia* species has been reported by Akihisa *et al*. (12), Mucsi *et al*. (5), Semple *et al*. (13), Yang *et al*. (14) and Khan *et al*. (15). Therefore, the antiviral effects of *Euphorbia myrsinites* which grows wild in different parts of Iran was investigated for its possible antiviral activity. This is the first report on antiviral activity of *E. myrsinites*.

Materials and Methods

Plant material

Euphorbia myrsinites was collected from vicinity of Mashhad (Khorasan Province, Iran). The plant was identified in the Herbarium of Ferdowsi University (Mashhad, Iran).A voucher specimen was preserved for reference at the Herbarium of the Mashhad School of Pharmacy (Iran) with reference number: 108-0513-1. The aerial part of the plant (leaves and stems) was dried in shade for three days and then was powdered.

Phytochemical screening

Several flavonoid glycosides (kaempferol-3-α-rhamnopyranoside (1), kaempferol-3-β-glucoside (2), kaempferol-3-β-galactoside (3) and the three corresponding glycosides of rhamnetin (4)) were isolated and identificated from *Euphorbia myrsinites* by Dumkow (16) (Fig 1).

Thiamin, *meta*-tyrosine, triterpenoids (ß-amyrin and taraxerol) (17), diterpene esters (ingenol) (17, 18), esters of diterpene myrsinol (17, 18, 19), cycloartane-type triterpenoids and betulin (19) also were isolated and characterized from *E. myrsinites*.

Preparation of Soxhlet methanolic extract

The plant dried powder (100 g) was extracted with methanol (400 mL) for 12 h using Soxhlet apparatus. The methanol was removed under reduced pressure to yield a viscous extract (20.9 g). The viscous extract was kept in refrigerator until use.

Media and Microorganisms

Phage Assay Broth (PA Broth): nutrient broth 13 g L^{-1}, NaCl 5 g L^{-1}, at pH 5.6-6.0 was used in all the protocols. Phage Assay Agar, consisted of PA Broth with the addition of 15 g L^{-1} agar, 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. 5 mL of the solution consisting of 40 g L^{-1} $Mg(SO_4)$. $7H_2O$, 10 g L^{-1} $MnSO_4.H_2O$ and 30 g L^{-1} $CaCl_2.2H_2O$, was added to per litre of the above medium. Soybean casein digest agar (SCDA): casein enzymatic hydrolysate 15 g L^{-1}, papaic digest of soybean meal 5 g L^{-1}, sodium chloride 5 g L^{-1}, agar 15 g L^{-1}. All reagents and media for culture were purchased from Merck, Germany. In order to investigate antiviral effects of *Euphorbia myrsinites* extract, *Bacillus* phage CP51, and *Bacillus cereus* (ATCC 10876) as the host were used.

Titration of Phage

Bacillus cereus Cultures were stored at -20°C in 15% glycerol (20). In the beginning, a stock culture of the bacteria was maintained on SCDA plate.. The phage stocks were prepared on the host strain (*Bacillus cereus* ATCC 10876) by a plate lysis procedure according to the methods of Atta-ur-Rahman *et al*. (21), for growing bacteriophage lambda-derived vectors. Briefly, One loopful of the *B. cereus* was inoculated into a petri dish containing 15 mL of SCDA and incubated for 24 h at 37°C. 100 µL of an aliquot phage sample (10-fold serially diluted with PA broth) was mixed with 100 µL of *Bacillus cereus* suspention (10^8 cfu ml^{-1}) in a sterile micro-centrifuge tube (polypropylene; 1.5 mL; Eppendorf) and incubated for 15 min at 37°C to facilitate attachment 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 well mixed by swirling, poured over the surface of a plate and allowed to sit for 15 min at room temperature. The plates were incubated for 18 h at 37°C and a plate showing almost confluent plaques was used to prepare a concentrated phage suspension by overlaying with 5 mL of PA broth. The over layer medium containing the phage CP51 was decanted and filtered through a 0.22 µm syringe filter. The filtrate was used as a phage stock solution. Several dilutions of phage solution were made.

Phage inactivation assays

Either pre-incubation or no pre-incubation phage inactivation method was done according to Atta-ur-Rahman *et al.* (21).

Pre-incubation protocol Different concentration of Soxhlet extract of *E. myrsinites* 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 was taken and inoculated into a PA Broth medium (10 mL). The medium was mixed thoroughly and incubated at 37°C for 5 h. 100 µL of phage in proper dilution was added to 500 µL sterile solution of extract and the mixture was incubated at 25°C for 30 min. Then, 500 µL of bacterial suspension and 1.9 mL of PA Top Agar medium were added. This mixture was overlaid onto a PA agar plate and incubated at 25°C for 24 h. The negative control contained all above except the extract solution which was replaced with PA Top Agar. In the positive control plate the extract was replaced with 500 µL trifluridine (Sina Darou Co.) in IC_{50} concentration.

No Pre-incubation protocol One loopful of *B. cereus* from overnight culture was taken and inoculated into a PA Broth medium (10 mL). The medium was mixed thoroughly and incubated at 37°C for 5 h. Different concentration of Soxhlet extract of *E. myrsinites* in distilled water were prepared and filter sterilized. To a 500 µL sterile solution of extracts, 100 µL of phage in proper dilution, 500 µL of bacterial suspension and 1.9 mL of PA Top agar were added and the mixture was overlaid onto a PA agar plate and incubated at 25°C for 24 h. The negative control contained all above except the extract solution which was replaced with PA Top Agar. In the positive control plate, the extract was replaced with 500 µL trifluridine in IC_{50} concentration.

Statistical analysis

Results were expressed as the mean±SD for three independent experiments. The Student`s t-test was used to calculate *P* values for means between control and experimental results. $p < 0.05$ was considered statistically significant.

Results

The effect of different concentrations of trifluridin (TFT) on reduction of phage CP51 was investigated. IC_{50} for the Pre-incubation method was 138 µg mL^{-1} and for the No Pre-incubation protocol was 264 µg mL^{-1} (Fig. 2,3). After examination of the solvents (5% DMSO + 5% THF), no significant reduction of plaques was observed in both protocols.

When Soxhlet extract of *E. myrsinites* was added to the mixture of phage and *B. cereus*, the only high concentrations of the extract were able to significantly reduce (>50%) plaque number (above 12.5 mg mL^{-1}) . At lower concentrations (8, 9, 10, 11 and 12 mg mL^{-1}) still significant reduction of plaques numbers was observed and there was no difference between samples and negative control in plaque number (0% reduction) at concentrations below 8 mg mL^{-1} (Fig 4).

After pre-incubation of different concentrations of *E. myrsinites* extract with phage CP51 for 30 min, a significant reduction (>50%) in plaque number was observed for concentrations of 11, 12, 13, 14 and 15 mg mL^{-1} . At lower concentrations (6-11 mg mL^{-1}), still significant reduction of plaque was observed and at concentrations of 5 mg mL^{-1} and lower, 0% reduction was obtained indicating no inhibition (Fig 5).

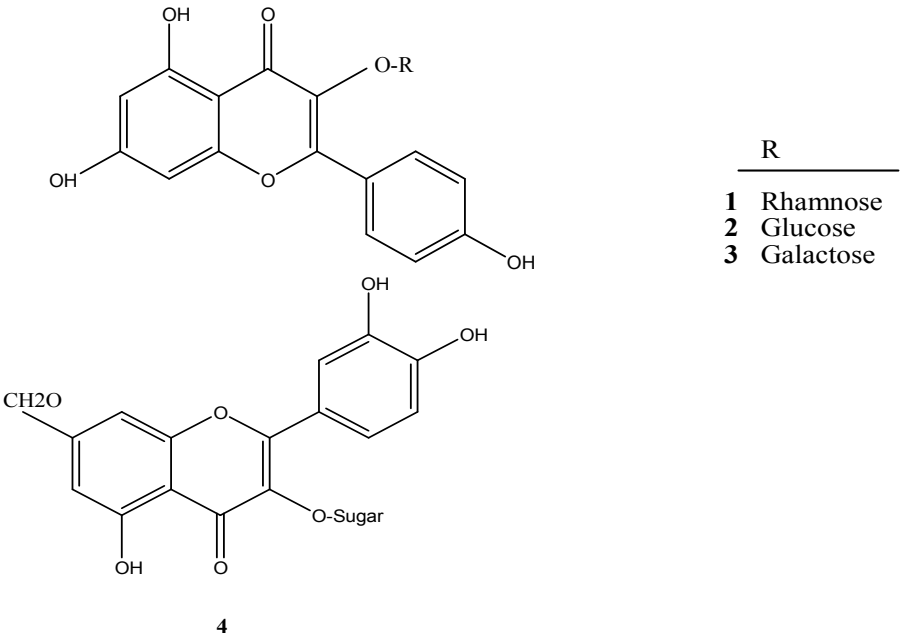

Fig. 1 The Structures of flavonoid glycoside Compounds 1 – 4

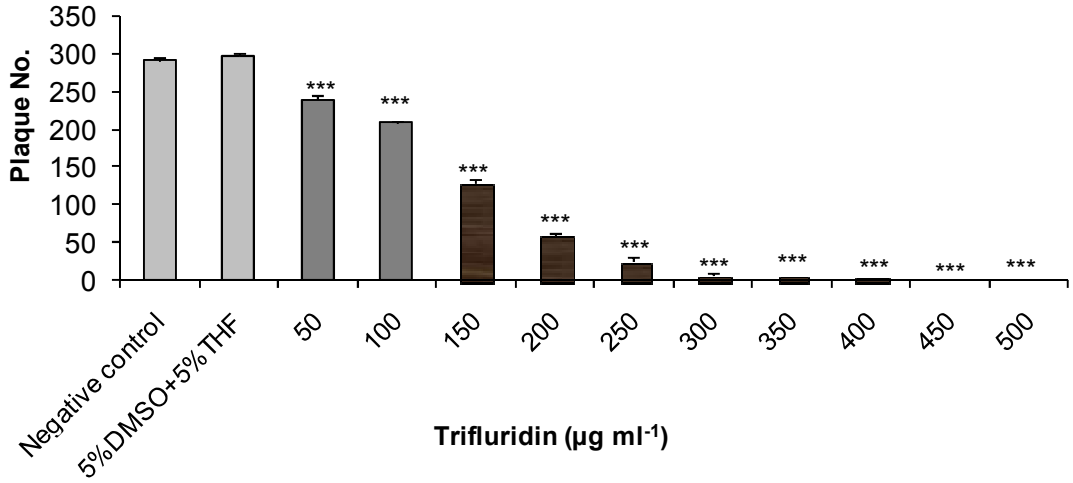

Fig. 2 Effect of different concentration of trifluridin (TFT) on reduction of phage CP51 using pre-incubation protocol. Data are mean±SD of three independent experiments.***p<0.001, Tukey-Kramer test

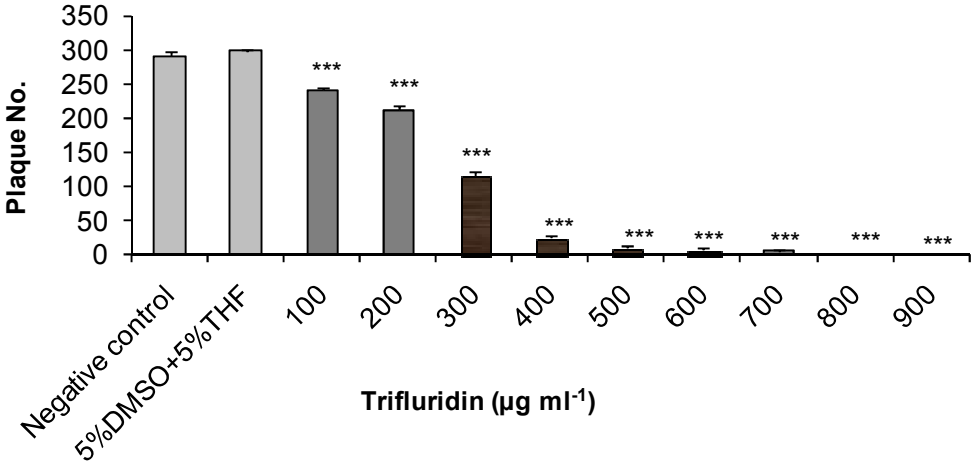

Fig. 3 Effect of different concentration of trifluridin (TFT) on reduction of phage CP51 using no pre-incubation protocol. Data are mean±SD of three independent experiments.***p<0.001, Tukey-Kramer test

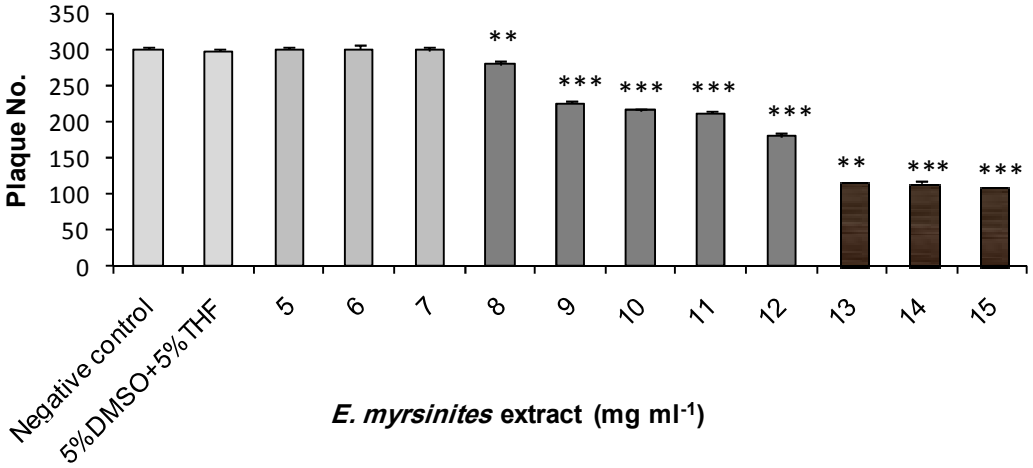

Fig. 4 Effect of different concentration of *E. myrsinites* extract on reduction of phage CP51 using no pre-incubation protocol. Data are mean±SD of three independent experiments.***p<0.001, Tukey-Kramer test

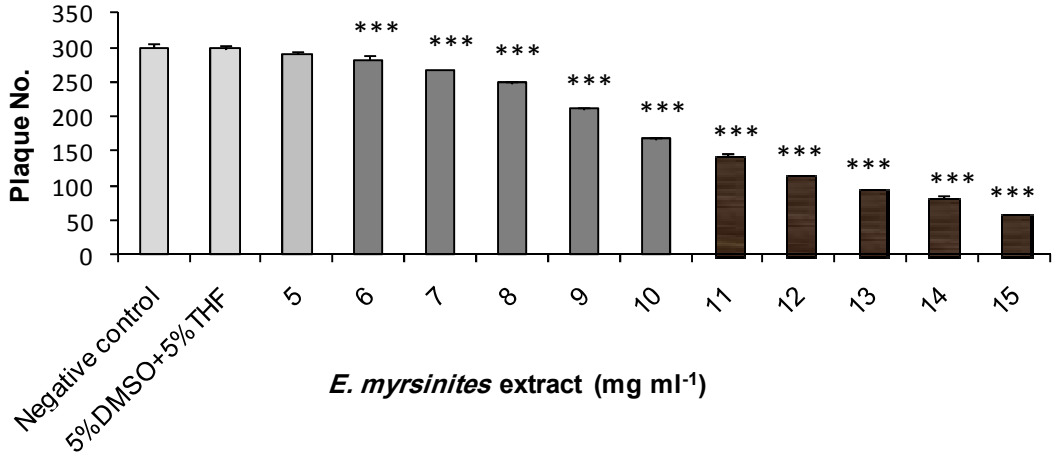

Fig. 5 Effect of different concentration of *E. myrsinites* extract on reduction of phage CP51 using pre-incubation protocol. Data are mean±SD of three independent experiments.***p<0.001, Tukey-Kramer test

Discussion

Some of the *Euphorbia* species have shown antiviral activity. The extract of *Euphorbia australis* was the most active against HCMV (13). *Euphorbia antiquorum* showed potent inhibitory effect on Epstein-Barr virus activation (12) and the extract of *E. thymifolia* was concluded to inhibit HSV-2 multiplication (14). In a study the extracts from *E. cotinifolia* and *E. tirucalli*using among 10 species of the genus *Euphorbia*, showed high anti-herpetic activity (15). Several compounds derived from *Euphorbia* species have been shown to have antiviral activity,for example diterpenes isolated from *Euphorbia* species exhibited a pronounced or moderate anti-herpes virus effect (5), lupenone (a triterpene isolated from *Euphorbia segetalis*) exhibited strong viral plaque inhibitory effect against HSV-1 and -2 (22) and putranjivain A (a diterpene isolated from *Euphorbia jolkini*) reported to have anti-HSV-2 activity (23). Therefore, the present study was carried out to investigate the antiviral activity of *E. myrsinites* 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.

Characterization of the active ingredients in plant extracts that have been shown antiviral effects, reveal useful compounds belonging to a wide range of different structural classes, e.g. coumarins, flavonoids, tannins, alkaloids, lignans, terpenes, naphtho- and anthraquinones, polysaccharides, proteins and peptides. These phytochemicals have complementary and overlapping mechanisms of action, including antiviral effects by either inhibiting the formation of viral DNA or RNA or inhibiting the activity of viral reproduction. So, assay methods to determine antiviral activity including pre- and post-treatment analyses, are needed (2). Therefore, we used either protocol where phage was pre-incubated with the extract prior to its exposure to *B. cereus* or without any pre-incubation with the phage. The results demonstrated that the extract exerted its antiviral activity in either method suggesting that the extract has no significant effects on the viral DNA or RNA replication or on phage itself.

It seems reasonable to conclude that there are probably numerous kinds of antiviral agents in *E. myrsinites* extract. Phytochemical screening of *E. myrsinites* extract has indicated the presence of flavonol glycosides (16), terpen esters (ingenol and myrsinol) (17, 18), thiamin, triterpenoids (ß-amyrin and taraxerol) and *meta*-tyrosine (17). The antiviral activities of bioflavonoids extracted from medicinal plants have been evaluated (24, 25). New flavonol glycosides were isolated from *Barleria prionitis* (two iridoid glycosides) and from the roots of *Markhamia lutea* (three phenylpropanoid glycosides,) shown to have potent *in vitro* activity against RSV (26, 27). Baicalein, a flavonoid compound purified from *Scutellaria baicalensis* Georgi, has been shown to possess anti-inflammatory and anti-HIV-1 activities (28). A new flavonoid glucuronide was isolated from the flowers of *Chrysanthemum morifolium*, showed strong HIV-1 integrase inhibitory activity and anti-HIV activity in a cell culture assay (29). The antiviral activity of seven flavonoids belonging to the kaempferol series against HCMV (30), RNA synthesis inhibition of poliovirus by 3-methylkaempferol (a flavonoid from *Psiadia dentate*) (31) and anti-HIV activity of rhamnetin (a flavonoid compound) (32) have been reported. There are some reports that flavonoids exhibit antiviral effects via inhibiting the RNA synthesis of viruses (2). flavonoids have been shown antiviral activity against RNA (RSV, Pf-3, polio) and DNA (HSV-1) viruses via inhibiting infectivity and/or replication (33).

β amyrin is a triterpenoid that its antiviral activity has been reported (34). Taraxerol is a triterpenoid that has been shown phytotoxic (35), antibacterial and Cytotoxic activity (36). Ingenol is an inflammatory diterpene ester that was found in Inflammatory latices of some Nigerian *Euphorbia* species (9). *Meta*-Tyrosine is an herbicidal nonprotein amino acid that functions as a broad-spectrum phytotoxin (37). With respect to preliminary Phytochemical screening of *E. myrsinites* extract, the antiviral activity of the extract could be attributed to its flavonoids and β amyrin.

It could be concluded from this study that the extract of *E. myrsinites* has a mild antiviral activity in a dose-dependent manner. This finding suggests that further investigation using human viruses and further phytochemical testing including isolation and purification of different fractions of extract especially flavonol glycosides and evaluation antiviral effects of them is merited.

Acknowledgments

The authors are indebted to the Research Council of Mashhad University of Medical Sciences, Iran for approval and financial support of this project.

References

1. Obi RK, Iroagba II, Ojiako OA. Virucidal potential of some edible Nigerian vegetables. African Journal of Biotechnology 2006; 5(19):1785-1788.
2. Jassim SAA, Naji MA. Novel antiviral agents: a medicinal plant perspective. Journal of Applied Microbiology 2003; 95(3):412-427.
3. Monavari HR, Hamkar R, Norooz-Babaei Z, et al. Antiviral effect assay of twenty five species of various medicinal plants families in Iran**.** IJMM 2007; 1(2):49-59.
4. Barla A, Öztürk M, Kültür S, Öksüz S. Screening of antioxidant activity of three *Euphorbia* species from Turkey. Fitoterapia 2007; 78(6):423-425.

5. Mucsi I, Molnar J, Hohmann J, Redei D. Cytotoxicities and anti-herpes simplex virus activities of diterpenes isolated from *Euphorbia* species. Planta Med 2001; 67(7):672-674.
6. Upadhyay RR, Sater AM, Moinzadeh F, et al. Tumor promoting activity of *Euphorbia striatella* (Boiss) and skin irritant activity of some *Euphorbia* species. Neoplasma 1984; 31(3):347-350.
7. Kinghorn AD, Evans FJ. A biological screen of selected species of the genus *Euphorbia* for skin irritant effects. Planta Med 1975; 28(4):326-335.
8. Eberle MM, Erb C, Flammer J, Meyer P. [Dermatitis and conjunctivitis after contact with *Euphorbia myrsinites* (wolf's milk extract)--a case report]. Klin Monatsbl Augenheilkd 1999; 215(3):203-204.
9. Abo KA. Characterisation of ingenol: an inflammatory diterpene from some Nigerian *Euphorbia* and *Elaeophorbia* species. Afr J Med Med Sci 1994; 23(2):161-163.
10. Duarte N, Kayser O, Abreu P, Ferreira MJU. Antileishmanial activity of piceatannol isolated from *Euphorbia lagascae* seeds. Phytotherapy Research 2007; 22(4):455–457.
11. Hohmann J, Molnar J, Redei D, et al. Discovery and biological evaluation of a new family of potent modulators of multidrug resistance: reversal of multidrug resistance of mouse lymphoma cells by new natural jatrophane diterpenoids isolated from *euphorbia* species**.** J Med Chem 2002; 45(12):2425-2431.
12. Akihisa T, Kithsiri Wijeratne EM, Tokuda H, et al. Eupha-7,9(11),24-trien-3beta-ol ("antiquol C") and other triterpenes from *Euphorbia antiquorum* latex and their inhibitory effects on Epstein-Barr virus activation. J Nat Prod 2002; 65(2):158-162.
13. Semple SJ, Reynolds GD, O'Leary MC, Flower RL. Screening of Australian medicinal plants for antiviral activity. J Ethnopharmacol 1998; 60(2):163-172.
14. Yang CM, Cheng HY, Lin TC, Chiang LC, Lin CC. *Euphorbia thymifolia* suppresses herpes simplex virus-2 infection by directly inactivating virus infectivity. Clinical and Experimental Pharmacology and Physiology 2005; 32(5-6):346-349.
15. Khan MTH, Thompson KD, Ather A. Antiviral activities of three Bangladeshi medicinal plant extracts against herpes simplex viruses. Antiviral Research 2005; 67(2):107-119.
16. Dumkow K. [Flavonolglycosides from *Euphorbia myrsinitis*. Their isolation and identification. 6. Flavonoids of native *Euphorbiacae*]. Planta Med 1971; 19(3):197-202.
17. Aynehchi Y, Mojtabaii M, Yazdizadeh. Chemical examination of *Euphorbia myrsinitis Linn*. J Pharm Sci 1972; 61(2):292-293.
18. Rentzea M, Hecker E, Lotter H. New tetracyclic polyfunctional diterpenes from Euphorbia myrsinites L. crystal structure and stereochemistry of 14-deoxo-14b-hydroxymyrsinols. Tetrahedron Lett 1982; 23:1781–1784.
19. Öksüz S, Gürek F, Gil R.R, et al. Four diterpene esters from Euphorbia myrsinites. Phytochemistry 1995; 38:1457–1462.
20. Favrin SJ, Jassim SAA, Griffiths MW. Application of a novel immunomagnetic separation-bacteriophage assay for the detection of *Salmonella enteritidis* and *Escherichia coli* O157: H7 in food. Int J Food Micribial 2003; 85:63-71.
21. Atta-ur-Rahman, Choudhary MI, Thomsen WJ. Bioassay Techniques for Drug Development. Harwood Academic Publishers: Australia 2001:47.
22. Madureira AM, Ascenso JR, Valdeira L, et al. Evaluation of the antiviral and antimicrobial activities of triterpenes isolated from *Euphorbia segetalis*. Nat. Prod. Res 2003; 17(5):375–380.

23. Cheng HY, Lin TC, Yang CM, et al. Putranjivain A from *Euphorbia jolkini* inhibits both virus entry and late stage replication of herpes simplex virus type 2 in vitro. J. Antimicrob. Chemother 2004; 53(4):577–583.
24. Beladi I, Pusztai R, Mucsi I, Bakay M, Gabor M. Activity of some flavonoids against viruses. Annuals of the New York Academy of Sciences 1997; 284:358–364.
25. Tsuchiya Y, Shimizu M, Hiyama Y, et al. Antiviral activity of natural occurring flavonoids in vitro. Chemical and Pharmaceutical Bulletin (Tokyo) 1985; 33:3881–3886.
26. Chen JL, Blanc P, Stoddart CA, et al. New iridoids from the medicinal plant *Barleria prionitis* with potent activity against respiratory syncytial virus. Journal of Natural Products 1998; 61:1295–1297.
27. Kernan MR, Amarquaye A, Chen JL, et al. Antiviral phenylpropanoid glycosides from the medicinal plant *Markhamia lutea*. Journal of Natural Products 1998; 61:564–570.
28. Li BQ, Fu T, Dongyan Y, Mikovits JA, Ruscetti FW, Wang JM. Flavonoid baicalin inhibits HIV-1 infection at the level of viral entry. Biochemical and Biophysical Research Communications 2000a; 276:534–538.
29. Lee JS, Kim HJ, Lee YS. A new Anti-HIV Flavonoid Glucuronide from Chrysanthemum morifolium. Planta Medica 2003; 69(9):859-861.
30. Mitrocotsa D, Mitaku S, Axarlis S, Harvala C, Malamas M. Evaluation of the antiviral activity of kaempferol and its glycosides against human cytomegalovirus. Planta Med 2000; 66(4):377-379.
31. Robin V, Irurzun A, Amoros M, Boustie J, Carrasco L. Antipoliovirus flavonoids from Psiadia dentate. Antiviral Chemistry and Chemotherapy 2001; 12(5):283-291.
32. Fesen MR, Pommier Y, Leteurtre F, et al. Inhibition of HIV-1 integrase by flavones, caffeic acid phenethyl ester (CAPE) and related compounds. Biochemical Pharmacology 1994; 48(3):595-608.
33. Kaul TN, Middleton Jr E, Ogra PL. Antiviral effect of flavonoids on human viruses. Journal of Medical virology 2005; 15(1):71-79.
34. Subba Rao G, Sinsheimer JE, Cochran KW. Antiviral activity of triterpenoid saponins containing acylated beta-amyrin aglycones. J Pharm Sci 1974; 63(3):471-473.
35. Macías-Rubalcava ML, Hernández-Bautista BE, Jiménez-Estrada M, Cruz-Ortega R, Anaya AL. Pentacyclic Triterpenes with Selective Bioactivity from Sebastiania adenophora Leaves, Euphorbiaceae. J Chem Ecol 2007; 33:147–156.
36. Rabie A. Bioactivity of extracts and components of *Pteleopsis myrtifolia*. Ph.D. Thesis, University of Pretoria, South Africa 2005.
37. Bertin C, Weston LA, Huang T, et al. Grass roots chemistry: *meta*-Tyrosine, an herbicidal nonprotein amino acid. PNAS 2007; 104(43)16964-16969.