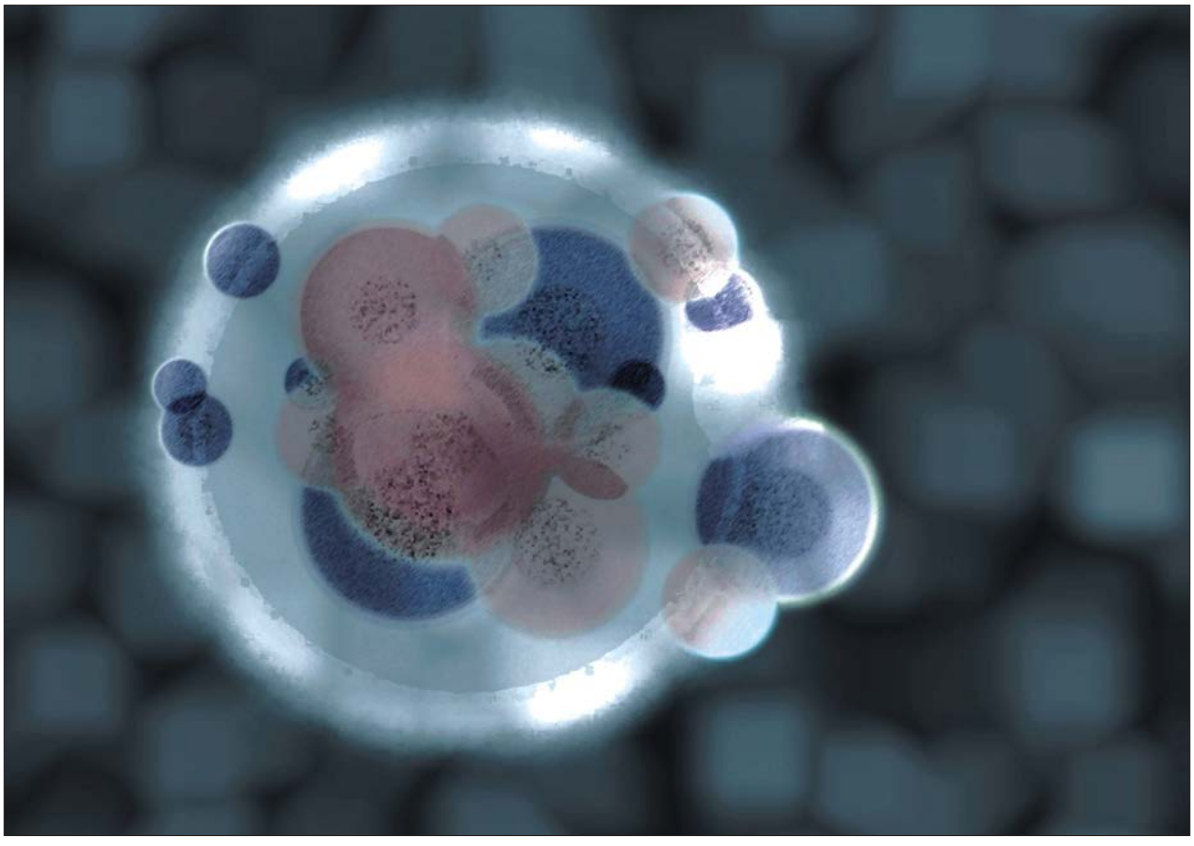

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 8 (4): 809-813, 2008
ISSN 1727-3048

Antiviral Activity of *Euphorbia helioscopia* Extract

[1]M. Ramezani, [2]J. Behravan, [1]M. Arab and [1]S. Amel Farzad
[1]Pharmaceutical Research Center, Bu-Ali Research Institute and School of Pharmacy,
Mashhad University of Medical Sciences, P.O. Box 91775-1365, Mashhad, Iran
[2]Biotechnology Research Center, Bu-Ali Research Institute and School of Pharmacy,
Mashhad University of Medical Sciences, P.O. Box 91775-1365, Mashhad, Iran

Abstract: In the present study, the antiviral effects of *Euphorbia helioscopia* extracts were investigated using plaque reduction assay. Plant extracts were prepared using Soxhlet apparatus or by maceration in methanol. After applying several enriching stages of phage CP51, phage titration was performed to determine the phage concentration in phage lysate for specifying the dilution factor of the phage to be used as negative control for the next working stages. Then IC_{50} of trifluridine, as a positive control, for phage CP51 was determined. The MIC of the extracts for *Bacillus cereus* was determined as 1.25 and 0.5 mg mL^{-1} for Soxhlet and maceration extracts, respectively. To determine 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 concentration of 1 and 0.25 mg mL^{-1}, respectively. In order to test the effects of extract on transcription process, *Bacillus cereus*, phage CP51 and extract were incubated together. The growth and reproduction of phage was inhibited by more than 50% at concentration of 0.75 and 0.125 mg mL^{-1} or Soxhlet and macerated extracts, respectively. These results indicated that both extracts of *E. helioscopia* have considerable antiviral activity.

Key words: *Euphorbia helioscopia*, antiviral activity, phage reduction assay

INTRODUCTION

Viral diseases, including emerging and established viruses, are an increasing worldwide health concern. However, the search for antiviral compounds has not led to potent and safe antiviral drugs, while those approved for use often have exhibited bad side-effect profiles and viral resistance is often occurs after a while (Jassim, 2005). Consequently, the discovery of new plant-derived antiviral agents has assumed more urgency than in the past (Jassim and Naji, 2003). It is essential, therefore, to continue the search for useful and novel natural antiviral agents. Euphorbiaceae plants are well known to contain irritant and tumor-promoting constituents (Hecker, 1977). Several *Euphorbia* species have been studied for various biological activities including anti-tumor, antileishmanial and antibacterial. Some report on the antiviral effect of *Euphorbia* species has been reported by Semple *et al.* (1998), Betancur-Galvis *et al.* (2002), Khan *et al.* (2005), Lin *et al.* (2002) and some potent antiviral compounds were also purified (Abdelgaleil *et al.*, 2001; Madureira *et al.*, 2003; Tanaka *et al.*, 2000). In traditional medicine, the extracts of different species of *Euphorbia* have been successfully used for the treatment of skin diseases which could be caused by viruses (Jassim and Naji, 2003). Therefore, the antiviral effects of *Euphorbia helioscopia* which grows wild in different parts of Iran and used by local people for skin disorders was investigated for its possible antiviral activity using plaque reduction assay. This is the first report on antiviral activity of *E. helioscopia*.

MATERIALS AND METHODS

Media: Phage Assay Broth (PA Broth): nutrient broth 13 g L^{-1} (Merck, Germany), NaCl 5 g L^{-1} (Merck, Germany), at pH 5.6-6.0 was used in all the protocols. Phage Assay Agar, consisted of the above with the addition of 15 g L^{-1} agar (Merck, Germany) was used for *Bacillus cereus* culture to produce the phage. Phage assay top agar: for plaque assay, the soft layer agar was used where the agar content was reduced to 7 g L^{-1}. 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}

Corresponding Author: Mohammad Ramezani, Pharmaceutical Research Centre and School of Pharmacy, Mashhad University of Medical Sciences, P.O. Box 91775-1365, Mashhad, Iran
Tel: +98 511 8823255 Fax: +98 511 8823251

$CaCl_2.2H_2O$. 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 15 g L^{-1}.

Bacterial Strain: *Bacillus cereus* ATCC 10876 was used throughout the study. Cultures were stored at -20°C in 15% glycerol (Favrin *et al*., 2003). Prior to investigation a stock culture of the bacteria was maintained on SCDA plate. 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. Then, a few drops of phage suspension were added and the plate was incubated further for 24 h at 25°C.

Bacteriophage: A *Bacillus* phage CP51 was used in this study. The phage stocks were prepared on the host strain, *Bacillus cereus* ATCC 10876, by a plate lysis procedure essentially equivalent to growing bacteriophage Lambda-derived vectors (Atta-ur-Rahman *et al*., 2001). 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. An aliquot (100 μL) of the phage sample (10-fold serially diluted with PA broth) was mixed with 100 μL of an overnight SCDA culture of *Bacillus cereus* ATCC 10876 in a sterile Eppendorf micro-centrifuge tube (polypropylene; 1.5 mL; Sarstedt) 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 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 filter syringe. The filtrate was used as a phage stock solution. Several dilutions of phage solution were made.

Preparation of plant extract

Plant material: *Euphorbia helioscopia* was collected in August 2005 from near Mashhad (Khorasan Province, Iran). The aerial part of the plant was dried in shade for three days and then was powdered. It was identified in the Herbarium of Ferdowsi University (Mashhad, Iran) and voucher samples were preserved for reference at the Herbarium of the Mashhad School of Pharmacy (Iran) with reference number: 190.

Phytochemical screening: The extracts were screened for alkaloids, flavonoids, anthocyanins, tannins (Chhabra *et al*., 1984) and saponins (Brain and Turner, 1975).

Preparation of extract

Soxhlet methanolic extract: The plant powder (50 g) was extracted with methanol (200 mL) for 12 h using Soxhlet apparatus. The methanol was removed under reduced pressure to yield a viscous brownish extract (9.5 g) and was kept in refrigerator until use.

Macerated ethanolic extract: The powdered plant (100 g) was extracted with ethanol (1000 mL, 80%, v/v) by maceration. The extract was collected every 24 h for three days. The combined extracts were dried under reduced pressure to yield a brownish extract (14.2 g). The viscous extract was kept in refrigerator for further testing.

Determination of Minimum Inhibitory Concentration (MIC): The MIC of the test extract was determined (Zgoda and Porter, 2001) for test *Bacillus cereus* as follows: Sterile Eppendorf micro-centrifuge tubes (polypropylene; 1.5 mL; Sarstedt) were used for this purpose. Each tube contained colony-forming units (cfu) $mL^{-1}$of *B. cereus* ATCC 10876 cultures, serially diluted *Euphorbia helioscopia* extract in distilled water (62.5, 125, 250, 375, 500, 750, 1000 and 1250 μg mL^{-1}) and respective growth medium (Muller Hinton Broth). Triplicate samples were performed for each test concentration. The controls included bacterial host cell growth medium and (2) bacterial host cell growth medium incubated with erythromycin. All tubes were incubated at 37°C for 24 h. 0.5 mL of MTT solution (2 mg mL^{-1}) was added to each well and the plate was incubated at 37°C for 30 min. The color change was observed and the concentration at which no color change was first observed was taken as MIC of the plant extract against *B. cereus*.

Phage inactivation assays: Either pre-incubation or no pre-incubation phage inactivation method was done according to Atta-ur-Rahman *et al.* (2001).

Pre-incubation protocol: Five hundred concentration of Soxhlet and macerated extracts of *E. helioscopia* in distilled water were prepared and filter sterilized. 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. To a 500 μL sterile solution of extracts, 100 μL of phage in proper dilution was added and the mixture was incubated at 25°C for 30 min. Then, 500 μL of bacterial suspension

and 3 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 for the replacement of bacterial suspension with PA Top Agar medium. In the positive control plate the extract was replaced with 500 μL trifluridine.

No pre-incubation protocol: Different concentration of Soxhlet and macerated extracts of *E. helioscopia* in distilled water were prepared and filter sterilized. 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. To a 500 μL sterile solution of extracts, 100 μL of phage in proper dilution, 500 μL of bacterial suspension and 1.8 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 for the extract. In the positive control plate, the extract was replaced with 500 μL trifluridine.

Statistical analysis: Experiments were performed in triplicate. Bacteriophage titrations and assays were performed in triplicate. The arithmetic mean±standard error of the mean (SEM) of control and experimental results were calculated using the Student's t-test. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Preliminary phytochemical screening of *E. helioscopia* extracts indicated the presence of flavonoids and tannins.

The MIC of the *E. helioscopia* against *B. cereus* was determined. The MIC for Soxhlet and macerated extracts were 1.25 and 0.5 mg mL^{-1}, respectively.

When Soxhlet extract of *E. helioscopia* was added to the mixture of phage and *B. cereus*, a significant reduction (>50%) in plaque was observed for concentrations above 0.75 mg mL^{-1} and a 100% reduction was obtained at concentration of 1.0 mg mL^{-1} and higher (Fig. 1).

After pre-incubation of different concentrations of *E. heliscopia* extract with phage CP51 for 30 min, a significant reduction (>50%) in plaque number was observed for concentrations of 1, 1.25, 1.5, 1.75 and 2.0 mg mL^{-1}. While at lower concentration no significant reduction of plaque was observed, at concentration above 2 mg mL^{-1} no plaque was formed indicating a 100% inhibition (Fig. 2).

When macerated extract of *E. helioscopia* was added to the mixture of phage and *B. cereus*, a significant reduction (>50%) in plaque was observed for

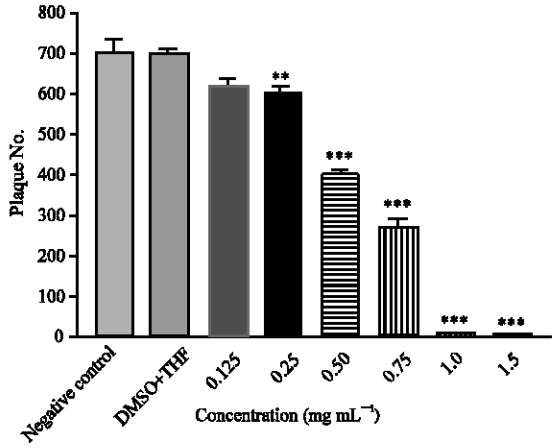

Fig. 1: Effect o f different concentration of *E. helioscopia* soxhlet extract on reduction of phage CP51 using no pre-incubation protocol. Each bar represents the mean±SEM of the number of plaque.**p<0.01, ***p<0.001, Tukey-Kramer test

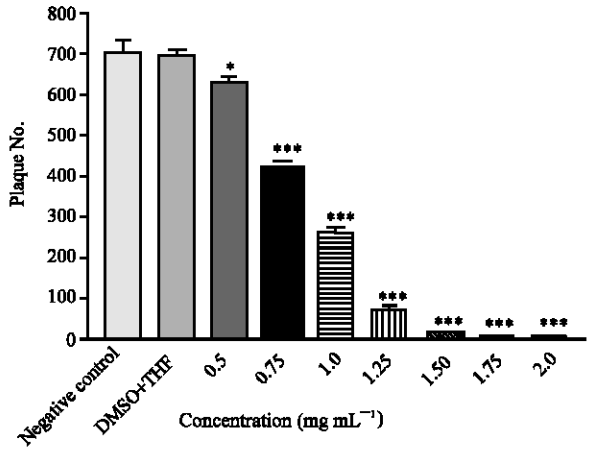

Fig. 2: Effect of different concentration of *E. helioscopia* soxhlet extract on reduction of phage CP51 using pre-incubation protocol. Each bar represents the mean±SEM of the number of plaque. *p<0.05, **p<0.01, ***p<0.001, Tukey-Kramer test

concentrations above 0.125 mg mL^{-1} and a 100% reduction was obtained at concentration of 1.0 mg mL^{-1} and higher (Fig. 3).

After pre-incubation of different concentrations of *E. heliscopia* extract with phage CP51 for 30 min, a significant reduction (>50%) in plaque number was observed for concentrations of 0.25 and 0.5 mg mL^{-1}. While at lower concentration of 0.5 mg mL^{-1} no significant reduction of plaque was observed, at concentration above 2 mg mL^{-1} no plaque was formed indicating a 100% inhibition (Fig. 4).

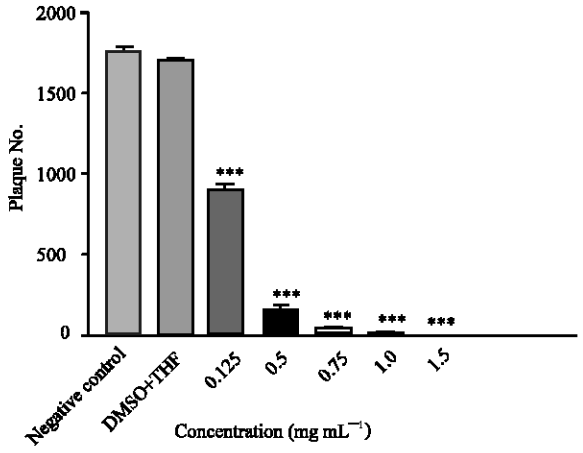

Fig. 3: Effect of different concentration of *E. helioscopia* macerated soxhlet extract on reduction of phage CP51 using no pre-incubation protocol. Each bar represents the mean ±SEM of the number of plaque.***p<0.001, Tukey-Kramer test

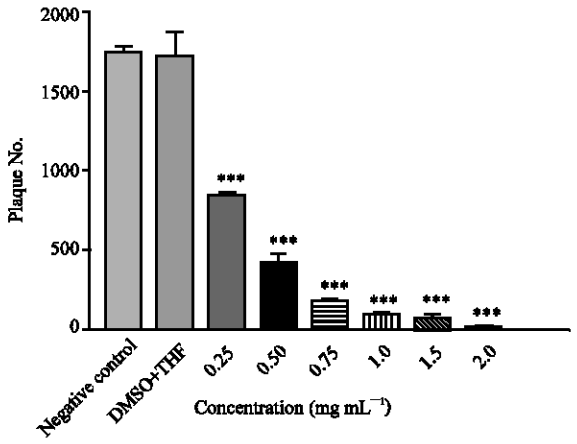

Fig. 4: Effect of different concentration of *E. helioscopia* macerated extract on reduction of phage CP51 using pre-incubation protocol. Each bar represents the mean±SEM of the number of plaque.***p<0.001, Tukey-Kramer test

Several species of *Euphorbia* have been tested for their antiviral activity. Among 10 species tested, 3 showed potent antiviral activity (Betancur-Galvis *et al.,* 2002). Several plant-derived compounds have been shown to have antiviral activity (Abdelgaleil *et al.*, 2001; Madureira *et al.*, 2003; Tanaka *et al.*, 2000). Putranjivain A, a diterpene isolated form *E. jolkini*, has shown potent antiviral activity (Cheng *et al.*, 2004). Since no phytochemical investigation has been carried out on *E. helioscopia*, it is hard to attribute the antiviral activity of *E. helioscopia* to any specific type of compound(s). Therefore, the present study was carried out to investigate the antiviral activity of *E. helioscopia* so that the justification for the isolation of its antiviral component(s) could be achieved.

Most extracts or pure compounds obtained from plants act as antiviral via two mechanisms including exhibiting their effects on viral particles prior to attachment to host cell or after the virus enters the host cell. The bioactive component(s), namely, protein and some derived polyphenolic compounds such as polysaccharides, lignins and bioflavonoids, were reported to act principally by binding to the protein coat and thus arrest absorption of the virus (Jassim and Mazen, 2007). To differentiate between these two mechanisms, we performed our experiment using 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 indicated that the extract exerted its antiviral activity in either method suggesting that the extract has no significant effects on the attachment of phage to the host cells or on phage itself.

In phage reduction assay, the antiviral activity should be distinguished from antibacterial activity. If an extract or compound reduces the number of plaques, it could be attributed to either its antibacterial or antiviral activity. Thus, the MIC determination against the host bacterium should be performed. MICs of both Soxhlet and macerated extracts against *B. cereus* were above the concentrations at which significant plaque reductions were observed indicating that the antiviral activity of the extracts were due to the action of the extracts on phage itself rather than killing the bacteria and that the antibacterial activity of these two extracts did not significantly contribute to the observed antiviral activity.

Two different common extraction protocols (Soxhlet vs maceration) were used to test the effect of heat and solvent on the antiviral constituents of *E. helioscopia*. The results showed that the macerated extract had a higher activity in reduction of the number of plaques suggesting that the heat used in Soxhlet method might destroy the active constituents of the extract.

It could be concluded from the present study that the extract of *E. helioscopia* has a mild antiviral activity and it is good candidate for further antiviral testing using human viruses and further phytochemical testing will also be valuable.

REFERENCES

Abdelgaleil, A.A.M., S.M.I. Kassem, M. Doe, M. Baba and M. Nakatani, 2001. Diterpenoids from *Euphorbia paralias*. Phytochemistry, 58: 1135-1139.

Atta-ur-Rahman, M.I. Choudhary and W.J. Thomsen, 2001. Bioassay Techniques for Drug Development. Harwood Academic Publishers, Australia, pp: 47.

Betancur-Galvis, L.A., G.E. Morales, J.E. Forero and J. Roldan, 2002. Cytotoxic and antiviral activities of Colombian medicinal plant extracts of the *Euphorbia* genus. Mem. Inst . Oswaldo Cruz, Rio de Janeiro, 97: 541-546.

Brain, K.R. and T.D. Turner, 1975. The Practical Evaluation of Phytopharmaceuticals. 1st Edn. Wright-Scientechnica, Bristol, London, pp: 153.

Cheng, H.Y., T.C. Lin, C.M. Yang, K.C. Wang, L.T. Lin and C.C. Lin, 2004. Putranjivain A from *Euphorbia jolkini* inhibits both virus entry and late stage replication of herpes simplex virus type 2 *in vitro*. J. Antimicrob. Chemother., 53: 577-583.

Chhabra, S.C., F.C. Uiso and E.N. Mshiu, 1984. Phytochemical screening of Tanzanian medicinal plants. Part 1. J. Ethnopharmacol., 11: 157-179.

Favrin S.J., S.A.A. Jassim and M.W. Griffiths, 2003. Application of a novel immunomagnetic separation-bacteriophage assay for the detection of *Salmonella enteritidis* and *Escherichia coli* O157: H7 in food. Int. J. Food Microbiol., 85: 63-71.

Hecker, E., 1977. New toxic, irritant and cocarcinogenic diterpenoids esters from Euphorbiacea and from Thymelaeaceae. Pure Applied Chem., 49: 1423-1431.

Jassim, S.A.A. and M.A. Naji, 2003. Review/novel antiviral agents: A medicinal plant perspective. J. Applied Microbiol., 95: 412-427.

Jassim, S.A.A., 2005. Review/novel phyto-anti-HIV drugs: A cause for optimism. Biologist, 52: 268-272.

Jassim, S.A.A. and A.N. Mazen, 2007. *In vitro* evaluation of the antiviral activity of an extract of date palm (*Phoenix dactylifera* L.) Pits on a *Pseudomonas* Phage. Evidence-Based Compl. Alter. Med., (eCAM), pp: 1-6. 10.1093/ecam/nem160.

Khan, M.T.H., A. Ather, K.D. Thompson and R. Gambari, 2005. Extracts and molecules from medicinal plants against herpes simplex viruses. Antiviral Res., 67: 107-119.

Lin, C., H. Cheng, C. Yang and T. Lin, 2002. Antioxidant and antiviral activities of *Euphorbia thymifolia* L. J. Biomed. Sci., 9: 656-664.

Madureira, A.M., J. R. Ascenso, L. Valdeira, A. Duarte, J.P. Frade, G. Freitas and M.J.U. Ferreira, 2003. Evaluation of the antiviral and antimicrobial activities of triterpenes isolated from *Euphorbia segetalis*. Nat. Prod. Res., 17: 375-380.

Semple, S.J., G.D. Reynolds, M.C. O'Leary and R.L.P. Flower, 1998. Screening of Australian medicinal plants for antiviral activity. J. Ethnopharmacol., 60: 163-172.

Tanaka, T., K. Kasubuchi, S. Kita, H. Tokuda, H. Nishino and S. Matsunaga, 2000. Bioactive Steroids from the Whole Herb of *Euphorbia chamaesyce*. J. Nat. Prod., 63: 99-103.

Zgoda, J.R. and J.R. Porter, 2001. A convenient microdilution method for screening of natural products against bacteria and fungi. Pharm. Biol., 39: 221-225.