Indian J Med Res 120, July 2004, pp 24-29

Antiviral activity of medicinal plants of Nilgiris

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Received August 29, 2003

***Background & objectives*: Medicinal plants have been traditionally used for different kinds of ailments including infectious diseases. There is an increasing need for substances with antiviral activity since the treatment of viral infections with the available antiviral drugs often leads to the problem of viral resistance. Herpes simplex virus (HSV) causes a variety of life threatening diseases. Since the chemotherapeutic agents available for HSV infections are either low in quality or limited in efficiency, there is a need to search for new and more effective antiviral agents for HSV infections. Therefore in the present study 18 plants with ethnomedical background from different families were screened for antiviral activity against HSV-1.**

***Methods*: Different parts of the plants collected from in and around Ootacamund, Tamil Nadu were extracted with different solvents to obtain crude extracts. These extracts were screened for their cytotoxicity against Vero cell line by assay microculture tetrazolium (MTT) trypan blue dye exclusion, proteins estimation and ^{3}H labeling. Antiviral properties of the plant extracts were determined by cytopathic effect inhibition assay and virus yield reduction assay.**

***Results*: Three plant extracts *Hypericum mysorense, Hypericum hookerianum* and *Usnea complanta* exhibited significant antiviral activity at a concentration non toxic to the cell line used. The extracts of *Melia dubia, Cryptostegia grandiflora* and essential oil of *Rosmarinus officinalis* showed partial activity at higher concentrations.**

***Interpretation & conclusion*: Some of the medicinal plants have shown antiviral activity. Further research is needed to elucidate the active constituents of these plants which may be useful in the development of new and effective antiviral agents.**

Key words Cytopathic effect inhibition - cytotoxicity - herpes simplex virus type-I - virus yield reduction

Plants have been used as folk remedies and ethnobotanical literature has described the usage of plant extracts, infusions and powders for centuries for diseases now known to be of viral origin[1]. There is an increasing need for search of new compounds with antiviral activity as the treatment of viral infections with the available antiviral drugs is often unsatisfactory due to the problem of viral resistance[2] coupled with the problem of viral latency and conflicting efficacy in recurrent infection in immunocompromised patients[3]. Ethnopharmacology provides an alternative approach for the discovery of antiviral agents, namely the study of medicinal plants with a history of traditional use as a potential source of substances with significant pharmacological and biological activities[4]. The Indian subcontinent is endowed with rich and diverse local health tradition, which is equally matched with rich and diverse plant genetic source[5]. A detailed investigation and documentation of plants used in local health traditions and ethnopharmacological evaluation to verify their efficacy and safety can lead to the development of invaluable herbal drugs or isolation of compounds of therapeutic value.

A number of compounds extracted from various species of higher plants have shown antiviral activity[6].

Examples included tannins[7], flavones[8], alkaloids[9], that displayed *in vitro* activity against numerous viruses. It has been suggested that selection of plant on the basis of ethnomedical considerations gives a higher hit rate than screening programmes of general synthetic products[10]. *Bacopa monneri* has been used in conditions like epilepsy, insanity, nervous disorders[11], *Hypercicum hookerianum* in anxiety and inflammation[11], *Usnea complanta* and *Tagetes minuta* for bacterial infections[11-13], *Santolina chamaecyparissus* as a stimulant, vermifuge and a stomachic[14].

A number of plant extracts reported in traditional medicine to have antiinfective properties were studied in our laboratory[15-19] and were also screened for antiviral activity.

Herpes simplex viruses (HSV) are ubiquitous agents which cause a variety of diseases ranging in severity from mild to severe, and in certain cases, these may even become life threatenings, especially in immunocompromised patients. After primary infection, HSV persists in the host for the lifetime. HSV infection is thus considered lifelong infection. Nucleoside analogues such as aciclovir (ACV), penciclovir *etc*., are the only approved drugs for the treatment of HSV infections. However, the widespread use of nucleoside based drugs has led to the emergence of resistance in HSV especially among immunocompromised patients[3]. In a recent survey from Taiwan, the incidence of ACV-resistant HSV strains was found to be around 5 per cent among immunocompromised patients and 14 per cent among bone marrow transplant recipients[20]. This indicates the need for search of newer antiviral agents to treat such infections.

The present study was undertaken to test the extracts of 18 plants for their antiviral activity against herpes simplex virus type I (HSV-1, a DNA virus).

Material & Methods

Plant materials, reagents, cell line and virus: The plant materials were collected from in and around Ootacamund, Tamil Nadu, India and were authenticated by the Botanical Survey of India, Government Arts College, Ootacamund where sample specimens were deposited. Extracts of different plants were prepared by using Soxhlet extraction unit (Borosil, Mumbai) as per the standard procedure[21]. The essential oils from different parts of plants were isolated by water distillation using Clavenges apparatus (Borosil, Mumbai)[22].

Eagle's minimum essential medium (EMEM), trypsin, penicillin, streptomycin and amphotericin B were purchased from Hi-media Labs, Mumbai, India. 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and trypan blue dye were purchased from Sigma, USA. New born calf serum (NBCS) was procured from PAA Labs, Austria.

Vero cells (African green monkey kidney cell) were obtained from Pasteur Institute of India, Coonoor. Vero cells were grown in EMEM supplemented with Earle's salts and 10 per cent heat inactivated NBCS, 100 IU/ml penicillin, 100µg/ml streptomycin and 5µg/ml amphotericin B. The cells were maintained at 37ºC in a humidified atmosphere with 5 per cent CO_2 and were subcultured twice a week.

HSV-1 was from the collection of the Christian Medical College and Hospital, Vellore. The virus was propagated in Vero cells and the infective titre of the stock solution was 10^{-7} $TCID_{50}$/ml (50% tissue culture infective dose).

Cytotoxicity assay: Each extract was separately dissolved in 1 ml of distilled dimethyl sulphoxide (DMSO) and volume was made up to 10 ml with maintenance medium to obtain a stock solution of 1 mg/ml concentration, sterilized by filtration and further dilutions were made from the stock. The cytotoxicity assays were carried out using 0.1ml of cell suspension, containing 10,000 cells seeded in each well of a 96-well microtitre plate (Tarsons India Pvt. Ltd., Kolkata). Fresh medium containing different concentrations of the test sample was added after 24 h of seeding. Control cells were incubated without test sample and with DMSO. The little percentage of DMSO present in the wells (maximal 0.2%) was found not to affect the experiment. The microtitre plates were incubated at 37ºC in a humidified incubator with 5 per cent CO_2 for a period of 72 h. Sixteen wells were used for each concentration of the test sample. The morphology of the cells was inspected daily and observed for microscopically detectable alterations, *i.e*., loss of monolayer, granulation and vacuolization in the

cytoplasm. The cytopathogenic effect (CPE) was scored. The 50 per cent cytotoxic concentration (CTC_{50}), was determined by the standard MTT assay[23,24], trypan blue dye exclusion method[25], cell metabolic function by protein estimation[26], and total cellular DNA content by ^3H thymidine labeling[27].

Antiviral assay: Different nontoxic concentrations of test drugs, *i.e.*, lower than CTC_{50} were checked for antiviral property by cytopathic effect (CPE) inhibition assay[28] and virus yield reduction assay[29] against different virus challenge doses of 2, 10 and 100 $TCID_{50}$. In CPE inhibition assay, cells were seeded in a 96-well microtitre plate with 10,000 cells per well, incubated at 37ºC in a humidified incubator with 5 per cent CO_2 for a period of 48 h. The plates were washed with fresh MEM and challenged with different virus challenge doses and incubated at 37ºC for 90 min for adsorption of the virus. The cultures were treated with different dilutions of plant extracts in fresh maintenance medium and incubated at

Table I. Ethnobotanical data of selected medicinal plants

Plant name	Family	Part used	Extracts prepared	Local name	Local uses
Bacopa monnieri Linn.	Scrophulariaceae	Whole plant	Aqueous	Nir brahmi	In epilepsy, insanity, nervous disorders[11]
Solanum trilobatum Linn.	Solanaceae	Leaves	Aqueous	Tuduvalai	In constipation, cough, acute & chronic bronchitis[11,14]
Hibiscus vitifolius Linn.	Malvaceae	Root bark	Aqueous & alcohol	Karupatti	In jaundice, inflammation, diabetes, urease activity[30]
Allium cepa Linn.	Lilliaceae	Bulb	Aqueous	Vengayam	In malaria, asthma, fever, chronic bronchitis[11]
Derris brevipes (Benth) Baker	Papilonaceae	Root	Aqueous		As abortifacient[31]
Hypericum mysorense Weight&Arn.	Hypericaceae	Aerial parts	Methanol	Hypericum	In anxiety, inflammation[11]
Hypericum hookerianum Weight&Arn.	Hypericaceae	Aerial parts	Methanol	Hypericum	In anxiety, inflammation[11]
Berberis tinctoria Lesch	Berberidaceae	Root	Methanol	Oosikala	In stomachache, ulcer, haemor[32]
Mahonia leschenaultia Takeda	Berberidaceae	Root	Methanol	Mulkadambu	In postnatal conditions, jaundice, fever[14]
Usnea complanta Stirt	Usneaceae	Whole plant	Acetone & chloroform	Marappasi	In bacterial infections[13]
Tagetes minuta Linn.	Asteraceae	Whole plant	Essential oil	Stinking rogar	As diuretic, anti inflammatory, stomachic[12]
Leucas lavandulaefolia JE. Sm.	Labiatae	Aerial parts	Methanol	Mosappullu	In sedativeness, nervous disorders, as vermifuge[11,14]
Melia dubia Cav.	Meliaceae	Fruits	Alcohol & ethyl acetate	Malaivanbu	As anthlementic, skin disorders[11,14]
Azadirachta indica A.juss.	Meliaceae	Leaves	Essential oil	Vembu, Vepa	As antibacterial, antihelminthitic[11]
Santolina chamaecyparissus Linn.	Asteraceae	Whole plant	Essential oil	Lavender cotton	As plant stimulant, vermifuge, stomachic[14]
Cryptostegia grandiflora R.Br	Asclepidaceae	Whole plant	Methanol	Palai	Stimulant, in inflammations, antibacterial[18,32]
Daucus carota Linn.	Umbelliferae	Seeds	Essential oil	Karatu	As diuretic, stimulant[11]
*Rosmarinus officinali*s Linn.	Labiatae	Aerial parts	Essential oil	Rusmari	Carminative, diuretic, stimulant, for hair wash[11,14]

37ºC for five days. Every 24 h the observation was made and cytopathic effects were recorded. Anti-HSV-1 activity was determined by the inhibition of cytopathic effect compared with control, *i.e.*, the protection offered by the test samples to the cells was scored. In virus yield assay, reduction in the yield of virus when cells were treated with the plant extracts was determined.

Results

Different parts of 18 medicinal plants belonging to 14 different families (Table I) used in the traditional system of medicine collected from Nilgiris were tested for their antiviral activity. Seven plant extracts from six different families were found to have antiviral activity against HSV-1, at a concentration non toxic to the cell line (Vero) used (Table II). Most of these extracts have partial activity at the low concentration used. The methanol extracts of the aerial parts of *Hypericum mysorense* and *Hypericum hookerianum*, exhibited detectable antiviral effect towards HSV-1 with an inhibitory concentration for 50 per cent (IC_{50}) of 100 and 50µg/ml respectively. The acetone extract of *Usnea complanta* also showed antiviral activity at an IC_{50} value of 100 µg/ml. The extracts of *Melia dubia*, *Cryptostegia grandiflora* and essential oil of *Rosmarinus officinalis* exhibited a partial activity at higher concentrations. Other plant extracts failed to show significant antiviral property. From the 21 extracts tested, four plants *Usnea complanta*, *Berberis tinctoria*, *Mahonia leschenaultii* and *Togetes minuta* showed significant cytotoxicity against Vero cells, the IC_{50} value ranging between 37-49 µg/ml.

The results obtained by both CPE inhibition assay and virus yield assay were comparable. The extracts of *Hypericum mysorense* and *Hypericum hookerianum* exhibited virus inhibitory activity by both the assays. But the remaining plant extracts failed to reduce the virus yield in comparison to the yield obtained in the virus controls and the virus yield reduction was found to be less than 0.5 log.

Discussion

The results from this preliminary investigation provide evidence of the importance of ethnopharmacology as a guide to the screening of biologically active plant materials[33]. We used 100 per cent inactivation to define an extract with antiviral activity, but many extracts had partial antiviral activity.

Table II. Cytotoxicity and antiviral activity of selected plant extracts

Name of the plant	Extract	Cytotoxicity µg/ml	Concentration tested (µg/ml) IC_{50}	CPE inhibition assay		
				2 $TCID_{50}$	10 $TCID_{50}$	100 $TCID_{50}$
Hypericum mysorense	Methanol	123	100	++++	++++	++++
			50	++++	+++	++
Hypericum hookerianum	Methanol	122	100	++++	++++	++++
			50	+++	++	+
Usnea complanta	Acetone	123	100	++++	++++	++
			50	++++	+++	+
Cryptostegia grandiflora	Methanol	182	150	++	++	+
			100	++	+	+
Rosmarinus officinalis	essential oil	349	300	++	+	+
			200	+	+	+

0, no potection; +, 25% protection; ++, 50 protection; +++, 75% protection; ++++, 100% protection

CPE, cytopathic effect

IC_{50}, inhibitory concentration for 50 per cent of viruses

Of the 18 plant extracts tested, three *(H. mysorense, H. hookerianum* and *U. complanta*) were found to exhibit potent antiviral activity. *H. mysorense* and *H. hookerianum* are used in the treatment for anxiety and inflammation traditionally[11]. *Hypericum perforatum* from the same species is reported for its antiviral activity against human immunodeficiency virus (HIV)[34] and hepatitis C virus[35]. Three plant species *Hypericum*s *connatum, Hypericum caprifoliatum* and *Hypericum polyanthemum* (Guttiferae), growing in Southern Brazil were chemically investigated and tested for their antiviral activity against feline immunodeficiency virus (FIV)[36]. Our results showed that *H. mysorense* and *H. hookerianum* suppressed HSV-1 infection. These extracts may have compounds that are true antiviral, but are present at quantities insufficient to inactivate all infectious virus particles. It is possible that the elucidation of active constituents in these plants may provide useful lead to the development of new and effective antiviral agents.

Acknowledgment

The authors acknowledge the Department of Biotechnology, Government of India, New Delhi for financial support.

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