ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES Knowledge to Innovation

Vol 7, Issue 1, 2014

ISSN - 0974-2441

Research Article

# *IN VITRO* ANTIVIRAL SCREENING OF *PSIDIUM GUJAVA* L. AGAINST HSV-1 AND HSV-2 BY CPE INHIBITION ASSAY

**ANJANA.A.K*[1], PRASANTH FRANCIS[1], GOMATHY.S[1], SOUMYA.K.VIJAYAN[2], MANAL MOHAMMED[3], M.J.N CHANDRASEKAR[1]**

**[1] Department of Pharmaceutical chemistry, JSS College of Pharmacy, Ooty, Tamilnadu., [2]Department of Pharmaceutics, Academy of Pharmaceutical sciences, Kannur, Kerala., [3]Department of Pharmaceutical chemistry, Jamia Salafiya Pharmacy college, Malappuram.**
**Email:** anjana.sujith08@gmail.com

***Received: 23 October 2013, Revised and Accepted: 16 November 2013***

**ABSTRACT**

Objective: Herpes simplex virus (HSV) type 1 & 2 are most infectious pathogens for humans, especially in the case of highly susceptible adults. After establishing latency, HSV can reactivate, causing frequent recurrent infections in some patients, while most people experience few recurrences. The increase in viral infections and the prevalent resistance of virions to chemotherapeutic agents urges us to search for an efficient and novel antiviral strategy. Traditionally leaf paste of *Psidium gujava* L. was used to treat acne, ulcers, cholera, nephritis etc. *Psidium gujava* L. has been reported for its antimicrobial, antioxidant and antidiabetic potentials.

Methods: Plant leaves collected in and around Malappuram District of Kerala, India, were extracted by continuous extraction and cold maceration techniques. The selection of extracts made according to the data obtained from phytochemical screening. Cytotoxicity assays such as MTT and SRB were performed to find out the cytotoxic tolerance limits for dose calculation. The plant extracts were screened for its antiviral property by CPE inhibition assay against various virus challenge doses such as $2TCID_{50}$ and $10TCID_{50}$.

Results: Pet. ether extract showed significant activity in lower doses such as 50 µg/ml, 100 µg/ml ($2TCID_{50}$) and 100 µg/ml ($10TCID_{50}$). The hydro alcoholic (Soxhlet extraction & maceration) extracts also showed partial activity.

Conclusion: All the extracts were showing its potential to inhibit both HSV-1 and HSV-2 proliferation in Vero cells. Further research is needed to elucidate the active constituents of this plant which may be useful in the development of new and effective antiviral agents.

**Keywords:** *Psidium Gujava* L., Herpes simplex virus, CPE Inhibition assay, Cytotoxicity.

## INTRODUCTION

HSV-1 and HSV-2 are two kinds of ubiquitous pathogens that may cause serious morbidity in humans. HSV is an enveloped virus which causes a variety of infections in humans. After primary infection, HSV establishes latency in sensory and autonomic neurons innervating the mucosal tissues, where primary infection takes place, and is reactivated by the proper stimulus to cause recurrence. The period of recurrence is regular. Immuno compromised individuals and those with cancer are in danger of recurrent HSV infections. The recipients of organ transplantation are at high risk for increased severity of HSV infections. Infection with HSV-1 can lead to life threatening encephalitis and ocular infections that result in corneal inflammation and scarification. This scarification is the major cause of blindness in developing countries [1, 2].

The development of effective antiviral drugs is an important biomedical scientific achievement of the late 20th century. Viruses that maintain latency (herpes viruses) or persistence (HIV and Hepatitis B virus) are not specifically cleared from the body by these drugs, but their replication can be effectively suppressed [3].More over many of the virus exhibits drug resistance due to specific mutations in the viral genome which leads to alterations in the viral target protein or viral drug activator. Nucleoside analogues such as acyclovir and penciclovir etc. are the only approved drugs for the treatment of HSV infections. However, the widespread use of nucleoside based drug had led to the emergence of resistance in HSV especially among immuno compromised patients [4].

The herbal drugs have been used throughout the world have received greater attention in recent times; because of its diversity of curing diseases, safety and well tolerated remedies compared to the conventional medicines. In contrast to many publications on antibacterial and antifungal screening of plant extracts which have appeared in the last decades; much fewer antiviral screening studies of plant extracts have been reported.

*Psidium gujava* L. is one of the most widely distributed plants of *Myrtaceae* family. It is a small tree or shrub with spreading branches and the leaf paste has been used traditionally to treat infections, diarrhea, decrease BP etc. Besides, there have been some reports on the chemical constituent of this plant includes Phenols, tannins, flavanoids, triterpenes, Saponins etc. [5]. This plant has been reported for various biological activities like antimicrobial, antioxidant and antidiabetic activities but not reported for its anti-viral activity. This encouraged us to investigate anti-HSV effects of *Psidium Gujava* L. Hence the present study is to investigate the *invitro* anti-HSV activities of *Psidium gujava* L.

## MATERIALS AND METHODS

### Plant material and preparation of extracts

Fresh leaves of the plant were collected from Malappuram district of Kerala and authenticated by the Field Botanist, Botanical survey of India, Ootacamund. The collected leaves were dried under shade and coarsely powdered. The powdered leaves then subjected to extraction by continuous extraction (Borosil, Mumbai) and cold maceration [6, 7]. The extract were concentrated by a rotary evaporator (BUCHI Rota vapor) and dried under reduced pressure in an oven at $50^0$C to obtain the solid residues.

### Reagents

Dulbecco's Modified Eagle's medium (DMEM), Trypsin, Streptomycin and Amphotericin B were purchased from HiMedia Labs, Mumbai, India. Acyclovir, 3-(4, 5-dimethyl thiazole-2yl)-2, 5-diphenyl tetrazolium bromide (MTT), SulphorhodamineB (SRB) and Acyclovir were purchased from Sigma, USA. Foetal Bovine Serum (FBS) was procured from PAA Labs, Austria.

**Cell lines**

Vero cells, normal cells of African green monkey kidney, were obtained from Pasteur Institute of India, Coonoor. Vero cells were grown in DMEM supplemented with FBS, 100 IU/ml Penicillin, 100µg/ml streptomycin and 5µg/ml Amphotericin B. The cells were maintained at 37$^0$C in a humidified atmosphere with 5% $CO_2$ and sub cultured twice a week.

**Viruses**

HSV-1 and HSV-2 were procured from Christian Medical College, Vellore and propagated in Vero cell culture and the infective titre of the stock solution was $10^{-7}TCID_{50}$/ml.

**Phyto chemical screening**

The plant extracts were subjected to qualitative phytochemical screening for interpreting various chemical constituents as per the standard chemical tests [6, 7, and 17]. Based on the phytochemical screening petroleum ether extract and hydro alcoholic extracts from both maceration and continuous extraction were selected for further studies.

**Cytotoxicity assays**

Various doses for antiviral screening were calculated based on the cytotoxicity assays such as MTT and SRB. The cytotoxicity assays were carried out using 0.1ml of cell suspension, containing 10,000 cells seeded in each well of a 96-well micro titre plate (Tarsons, India Pvt. Ltd., Kolkata). Fresh medium containing different concentrations of the test sample made in DMSO was added after 24 hr 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 micro titre plates were incubated at 37$^0$C in a humidified incubator with 5 per cent CO2 for a period of 72 h. Sixteen wells were used for each concentration of the test sample. The morphology of the cells were observed daily for microscopically detectable alterations, *i.e.*, loss of monolayer, granulation and vacuolization in the cytoplasm. After confirming the cytotoxic tolerance limits ($CTC_{50}$) of the extract to the cell line under use, different test concentrations were chosen that were below the $CTC_{50}$ limits [8, 9, 10 and 16].

**Cytopathic effect inhibition assay**

The antiviral activity was determined by cytopathic Effect Inhibition Assay. Different nontoxic concentrations of test drugs (lower than CTC50) were checked for antiviral property by cytopathic effect (CPE) inhibition assay against different virus challenge doses of $2TCID_{50}$, $10TCID_{50}$. In CPE inhibition assay, cells were seeded in a 96-well micro titre plate with 10,000 cells per well, incubated at 37$^0$C in a humidified incubator with 5 per cent CO2 for a period of 48 hr. The plates were washed with fresh DMEM and challenged with different virus challenge doses and incubated at 37$^0$C 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 37$^0$C for five days. Every 24 hr, the observation made according to the protection offered to the cell cultures against different virus challenge dose and recorded. Anti-HSV-1 and HSV-2 activity were determined by the inhibition of cytopathic effect compared with control [8, 10].

**Results and Discussion**

Phytochemical screening has been carried out for all the extracts to find out the presence of phytoconstituents which may be biologically active. (Table no: 1)

**Table: 1: Phytochemical screening of the extracts.**

| | Leaf extracts of *Psidium gujava* L. | | | | | | |
|---|---|---|---|---|---|---|---|
| | Soxhlet extraction | | | | | Cold maceration | |
| **Phytoconstituents** | **Pet. ether** | **$CHCl_3$** | **E.A** | **Me. OH** | **50%HA** | **50%HA** | **100% Water** |
| Alkaloids | + | – | + | + | + | + | – |
| Carbohydrates | + | + | | + | + | + | + |
| Terpenoids | + | + | + | – | + | + | – |
| Flavanoids | + | + | – | – | + | + | + |
| Glycosides | – | – | + | + | + | + | – |
| Phytosterols | – | – | – | – | – | – | – |
| Saponins | + | + | + | + | + | + | + |

Based on the phytochemical screening, three extracts were selected for further screening to investigate antiviral activity.

The $CTC_{50}$ value was found to be 112µg/ml, 185µg/ml and 208 µg/ml respectively for Pet. ether, 50%HA (soxhlet extraction) and 50%HA (cold maceration) respectively by MTT assay. In SRB assay, the $CTC_{50}$ values were 128µg/ml, 188 µg/ml, and 190µg/ml respectively for the three extracts.

The Pet.ether extract showed marked antiviral potential against both HSV-1and HSV-2 at lower doses such as 50µg/ml, 100µg/ml ($2TCID_{50}$) and 100µg/ml ($10TCID_{50}$) respectively. The other two extracts also showed partial antiviral activity at comparatively higher doses when compared with pet. ether extracts (Table no: 2 & 3). Comparison between the 50%HA extracts obtained from both soxhlet extraction and cold maceration, the macerated extract showed relatively more activity than the other.

**Table: 2: Inhibition of cytopathic effects of HSV-1 by various extracts after 96hrs**

| **Extracts** | **Conc. µg/ml** | **Time period of microscopic observation** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **24hrs** | | **48hrs** | | **72hrs** | | **96hrs** | |
| | | **$2TCID_{50}$** | **$10TCID_{50}$** | **2 $TCID_{50}$** | **10 $TCID_{50}$** | **2 $TCID_{50}$** | **10 $TCID_{50}$** | **2 $TCID_{50}$** | **10 $TCID_{50}$** |
| Pet. Ether | 100 | √ | √ | √ | √ | √ | √ | √ | √ |
| | 50 | √ | √ | √ | √ | √ | √ | √ | + |
| | 25 | √ | + | √ | + | + | + | + | + |
| 50%HA | 150 | √ | √ | √ | + | √ | + | √ | + |
| | 100 | √ | + | √ | + | + | + | + | + |
| | 75 | √ | + | √ | + | + | + | + | + |
| 50%HA | 150 | √ | √ | √ | √ | √ | + | + | + |
| (Maceration) | 100 | √ | √ | √ | √ | √ | + | + | + |
| | 75 | √ | + | √ | + | + | + | + | + |
| Control (Acyclovir) | 10mg | √ | √ | √ | √ | √ | √ | √ | √ |

**Table: 3: Inhibition of cytopathic effects of HSV-2 by various extracts after 96hrs**

| Extracts | Conc.(µg/ml) | Time period of microscopic observation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 24hrs | | 48hrs | | 72hrs | | 96hrs | |
| | | 2TCID$_{50}$ | 10TCID$_{50}$ | 2 TCID$_{50}$ | 10 TCID$_{50}$ | 2 TCID$_{50}$ | 10 TCID$_{50}$ | 2 TCID$_{50}$ | 10 TCID$_{50}$ |
| Pet.ether | 100 | √ | √ | √ | √ | √ | √ | √ | √ |
| | 50 | √ | √ | √ | √ | √ | + | √ | + |
| | 25 | √ | + | + | + | + | + | + | + |
| 50%HA | 150 | √ | √ | √ | + | √ | + | √ | + |
| | 100 | √ | + | √ | + | + | + | + | + |
| | 75 | + | + | + | + | + | + | + | + |
| 50%HA | 150 | √ | √ | √ | √ | √ | + | √ | + |
| (Maceration) | 100 | √ | √ | √ | + | + | + | + | + |
| | 75 | √ | + | + | + | + | + | + | + |
| Control (Acyclovir) | 10mg | √ | √ | √ | √ | √ | √ | √ | √ |

**(√- Protection, + - No protection)**

## CONCLUSION

Plants have been used in the treatment of various diseases from the time immemorial. Because of the drawbacks of nucleoside based antiviral therapeutics and the increase of HSV infections over the past decade, there is an urgent need to develop new anti herpetic drugs, especially those with different mechanism of action than foregoing drugs.

All the extracts of *Psidium gujava* L. have shown antiviral activity. Further research is needed to elucidate the active constituents of this plant which may be useful in the development of new and effective antiviral agents.

## ACKNOWLEDGEMENT

We acknowledge Dr. P. Vijayan, Professor Emeritus, JSS College of pharmacy, Ooty, and Mr. Ashish Wadwani, CSIR Senior research fellow, JSS College of pharmacy, Ooty for their constant help and support during the course of work.

## REFERENCES

1. Kuo YC, Lin.LC and Tsai WJ. Samarangenin-B from *Liminium sinense* Suppresses the Herpes simplex virus Type-1 replication in Vero cells by regulation of viral macromolecular synthesis. Antimicrobial agents and chemotherapy.2001, 46: 2854-2864
2. Kaize He, Fen Liu and Y Lin. Structure of polysaccharide from *Polygonatum cyrtenoma* Hua and the antiherpetic activity of its hydrolyzed fragments. Antiviral research.2004, 63: 183-189
3. Larry Mckane and Judy Kandel. Microbiology: Essentials and applications,1996, 2nd edn, vol 1; 320-350.
4. Field AK and Biron KK. The end of innocerice revisited: resistance of herpes virus to antiviral drugs. *Clin Microbiol Rev.*1994, *7:* 1-13.
5. Udayan PS and Indira Balachandran. Medicinal plants of Arya Vaidya Sala Herb Garden, 2009 1st edn, Kozhikkode: Dept. Of publications, Kottakkal, 2009; 294.
6. Kokate C.K, A.P. Purohit, Gokhale SB. Pharmacognosy, 1997, 7th edn, Niraliprakashan; 105-144.
7. Kokate.C.K: Practical Pharmacognosy, 2nd edn, Vallabh Prakashan, 1994; 107-111.
8. Vijayan P, Raghu C and Ashok G. Antiviral activity of medicinal plants of Nilgiris. INDIAN J MED RES, 2004, 120: 24-29.
9. Raghu C, Ashok G and Vijayan P. Invitro cytotoxic properties of *Lantana camara* Linn. INDIAN J PHARMACOLOGY, 2004, 36:93-95.
10. Ian Freshneys R. Culture of animal cells- A manual of basic techniques. 2005, 5th edn, 199-240, 365-369.
11. Hudson JB, Anani K and Lee MK. Further investigations on the Antiviral activities of Medicinal plants of Togo, Pharmaceutical biology, 2000, 38:46-50.
12. Selvarani Vimalanathan, Linda Kang and Hudson J. *Echinacea purpurea* aerial parts contain multiple antiviral compounds, Pharmaceutical Biology, 2005, 43: 740-745.
13. Niedermeyer THJ, Mentel R and Lalk M. Antiviral terpenoid constituent of *Ganoderma pfeifferi*, Journal of natural products, 2005, 68: 1728-1731
14. Vanicha Vichai and Kanyawim Kirtikara. Sulphorhodamine B colorimetric assay for cytotoxic screening, Natural Protocols, 2006, 1:1112-1116.
15. Lopez A, Hudson JB and Tower JHN. Antiviral and antimicrobial activities of Colombian medicinal plants, Pharmaceutical Biology, 2001, 77:189-196.
16. Prabavathy D and Valli Nachiyar C. Cytotoxic potential and phytochemical analysis of Justicia beddomei and its endophytic Aspergillus spp. Asian Journal of Pharmaceutical and clinical research.2013, vol 6, suppl 5, 159-161.
17. Sreedevi Nangalingam, Changam Sheela sasikumar and Kotturathu Mammen Cherian. Extraction and phytochemical screening of active compounds in Morinda citrifolia Fruit. Asian Journal of Pharmaceutical and clinical research.2012, vol 5, suppl 2, 179-181.