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ANTI-VIRAL ACTIVITY OF INDIAN MEDICINAL PLANT *JUSTICIA ADHATODA* AGAINST HERPES SIMPLEX VIRUS: AN *IN-VITRO* STUDY

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ABSTRACT

The Herpesviruses are important human pathogens that can cause mild to severe lifelong infections with high morbidity. Alternate drugs such as ayurvedic drugs are now a day's used for management of HSV infections due to the emergence of resistant strains.The aqueous and methanol extract from leaves of *Justicia adhatoda*, were used to study the cytotoxicity effect on Vero cell line by using MTT assay. The methanolic extract at 10mg/ml significantly inhibited formation of plaques in Vero cells infected with 100 pfu of HSV1 and 2 by 100%. Similarly, the aqueous extract at 10mg/ml inhibited the plaque formation by 100% and 86% for HSV1 and 2.These results suggest that this herbal extract has potent anti-viral agents against herpes simplex viruses that can be exploited for development of an alternative remedy for HSV infections.

KEYWORDS: ***Justicia adhatoda*, Herpes simplex virus, anti-viral activity.**

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INTRODUCTION

Medicinal plants play a key role in human health care system. About 80% of world populations rely on traditional medicine which is predominantly based on plant materials [1].It is a myth if assumed, that infectious diseases are prominent in developing nations only, they are well equally deep routed in developed nations as well [2].The herbal drugs popularity increased and used widespread. The research is still lagging behind to get the efficacy of plant derived medicines on microorganisms which induce pathogenesis in human beings and other animals. Various medicinal plant reports collected from years are used for efficient treatment with minimized side effects. There were several reports on the antimicrobial activity of different herbal extracts in different regions of the world [3]. Hence in the current scenario, phytomedicine is a promising approach against intractable infectious diseases [4]. A special feature of higher plants is their capacity to produce a large number of secondary metabolites [5].Herpes simplex virus (HSV) is important viral pathogen in human and is relatively widespread in both developed and developing countries around the world [6]. HSV is a member of family Herpesviridae, subfamily Alphaherpesvirinae an can be divided into two subtypes HSV-1 and HSV-2, causing oral herpes lesions (HSV-1), genital lesion (HSV-2), meningitis and encephalitis [7]. Primary infection within the genital tract, followed by an established latency phase gives rise to lifelong infection in humans [8]. Treatment of herpes infections is thus cause of major concern owing to difficulty in eliminating it from the ganglion, high cost of treatment, increasing drug resistance and association with HIV-1 [9, 10].Several studies have indicated that the frequency of genital herpes as a high recurrence could be around 60% [11, 12]. Anti-herpes virus drugs such as acyclovir (ACV) have been remarkably successful in HSV treatment. However, side effects and drug resistant strains, which affect about 5% of immune-compromised patients receiving long-term prophylactic treatment with ACV, may lead to ineffective therapy [13].

The resistance to ACV has been reported mainly among the immune compromised patients [14]. In a study on persons attending sexually transmitted disease (STD) and human immunodeficiency virus (HIV) clinics in the United States, 226 patients were reported as being HIV-positive. Out of this HIV-positive group, 5.3% yielded resistant HSV isolates to oral and topical acyclovir [15]. Since HSV infection and recurrence is expected to increase in frequency and (e.g. in sub-Saharan Africa) the level of resistance to the available drugs is also expected to increase. Therefore need of the hour is to develop new therapeutic method for the management of HSV infections. *Justicia adhatoda*(L.) *Nees* (family *Acanthaceae*) is a shrub widespread throughout the tropical regions of Southeast Asia [16]. A variety of phytochemical research have been conducted on *Justicia adhatoda* and some of the reported activities of the plant include anti-diabetic [17], anti-phlogistic, anti-allergic [18], anti-ulcer [19], antioxidant, anti-genotoxicity [20] and many more [21, 22, 23].The medicinal value of the plants lies in the bioactive phytochemical compounds that produce normal physiological action on the human body [24]. Some of the most valuable bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, phenolic compounds and many more compounds [25]. The alkaloids from Justica adhatoda have reported excellent anti-bacterial activity against the most resistant bacteria such as *S. aureus*, *P. aeruginosa* and the highly pathogenic bacteria like *S. typhi* [26]. These natural bioactive compounds form the foundations of the modern prescription drugs as we know today.In the present study, we have chosen *Justicia adhatoda* as herbal medicine to determine their anti-HSV property. Limited studies have been conducted on the anti-HSV activity of this plant. This study looks into the in-vitro antiviral activity of methanolic and aqueous extract of this plant.

MATERIALS AND METHODS

i. Plant Material

The plant material, *Justicia adhatoda* was collected from Poddar Ayurvedic College, Mumbai. The plant was authenticated by comparing with corresponding herbarium specimen at Blatter Herbarium, St. Xavier's College, Mumbai **(Blatter Herbaium specimen no.1503 of H.Santapau).** Leaves were washed with distilled water, shade dried and powdered.

ii. Preparation of the extract

The aerial part of the plant that was powdered was used for the extraction process. Thirty grams of the powdered sample was submitted to successive solvent extraction separately with 300 ml each of hexane, dichloromethane, methanol and water at room temperature for 24 hours. The solvent extract thus obtained was evaporated to dryness in a rotary evaporator in a vacuum. The aqueous and methanolic extracts were used for further tests. The extracts were filtered with Whatman No 1 filter paper and concentrated and reconstituted at 100mg/ml in the Minimum Essential Medium[27].

iii. Reagents

All extraction reagents, such as dichloromethane, methanol, n-hexane were Analytical reagent (AR) grade. Reagents for cell culture, such as Minimum Essential Media (MEM), Trypsin- Ethylenediaminetetraacetic acid (EDTA), Sodium Bicarbonate were purchased from Life Technologies.

iv. Cell Line and Viruses

African green monkey kidney cells (Vero cells) were procured from National Centre for Cell Sciences (NCCS, Pune) and were grown and maintained in minimum essential medium (MEM), supplemented with 10% foetal bovine serum (FBS) and 1X non-essential amino-acids (NEAA).The standard strain HSV-1 and HSV-2 were a kind gift from the Department of Microbiology, CMC Vellore. The viruses were grown on Vero Cells and the virus stocks were stored at -80˚C for further use.

v. Phytochemical analysis of the extract using HPTLC

A Camag HPTLC system equipped with sample applicator and a Camag TLC scanner at 254nm and 366nm wavelength and data filtering by Savitsky- Goyal 7 was performed in Anchrom Test Lab P. Ltd. (Mumbai, India). Analytical grade toluene, ethyl acetate, methanol, chloroform, glacial acetic acid, diethyl amine and formic acid were obtained from SD Fine Chem Ltd. (Mumbai, India). The sample application for CAMAG Automatic TLC Sampler 4 conditions were Spray gas: Nitrogen (N2), Sample solvent type: Methanol, Filling speed: 15µl/second. Pre-coated silica gel 60 F254 TLC aluminium plates (10x10 cm, 0.2 mm thick) were obtained from E. Merck Ltd. (Mumbai, India).

Table 1

List of phytochemicals, solvent systems and derivatizing agents used in HPTLC analysis

Sr No.	Phytochemicals	Solvent system	Derivatizing agent
1	**Tannins**	Toluene: ethyl acetate: formic acid (6:4:0.3)	$FeCl_3$
2	**Saponins**	Chloroform: acetic acid: methanol : water (6.4:3.2:1.2:0.8)	Anisaldehyde,H_2SO_4
3	**Flavanoids**	Ethyl acetate: formic acid: Glacial acetic acid: water. 10:0.5:0.5:1.3)	Anisaldehyde solution
4	**Alkaloids**	Toluene: Ethyl acetate:Diethylamine (7:2:1)	Dragendroffs reagent

vi. Cytotoxicity Assessment

The evaluation of cytotoxic activity of plant extracts (CC_{50}) was carried out using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Vero cells were cultured onto 96 well plate at density of

1.0 x 10^5 cells/ml. Different concentrations (100mg/ml – 0.001mg/ml) of aqueous and methanolic crude extract were added to each culture wells at a final volume of 100µl, in triplicate, adding DMSO as a negative control. After incubation at 37˚C with 5% CO_2 for 16-18 hours, 10% of 5mg/ml MTT (100µl) was added to each well. After 4 hours of incubation at 37˚C, the formazan was solubilised by adding DMSO to each well and the absorbance was read at 550*nm* by an ELISA reader [28].

vii. Plaque Inhibition Assay

The herbal extract was examined for extent of inhibition of plaque formation on HSV infected tissue culture as an indication of anti-viral activity in vitro. Briefly, Vero cells were cultured on to 24 well plate to a confluent monolayer in MEM supplemented with 10% FBS in 5% CO_2 at 37˚C. Different dilutions of plant extracts were incubated with equal volume 100 plaque forming units of HSV-1 and HSV-2 for 1hour at 37˚C. Hundred microliters of respective dilutions were then placed in each well of 24 wells plate. The plates were incubated for 2 hours in incubator at 37˚C and with intermediate shaking after every 10 minutes to allow proper viral infection.The infected cells were then overlaid with 2% carboxymethyl cellulose (CMC) and 2X MEM and further incubated at 37˚C in 5% CO_2 for 3 days. After incubation, the CMC with 2X MEM was removed and washed with MEM and stained with amido black solution. The plaques, appearing as transparent dots, were counted using a dissecting microscope and the percent plaque inhibition calculated [29]. The effective concentration inhibiting formation of plaques by 50% was determined by the plaque inhibition assay for the extract at various concentrations (10, 5, 1, 0.5, 0.1, 0.01 and 0.001mg/ml).

STAISTICAL ANALYSIS

Sampling proceeds on three independent replications (n=3) for each tests. Data was subjected to Graph Pad Prism *v*5.04 and *v*6.0 [30] and the percent plaque inhibition was calculated by two-tailed t-test with $P < 0.05$ as significance.

RESULTS

i. Phtyochemical analysis

Table 2

Comprehensive results of the HPTLC qualitative tests showing the phytochemicals found in Aqueous and Methanolic extracts of Justicia adhatoda.

Phytochemical	Dervatizing Agent	Test samples	
		Aqueous	Methanol
Tannins	$FeCl_3$	✓	✓
Saponins	Anisaldehyde,H_2SO_4	✓	-
Flavanoids	Anisaldehyde solution	✓	✓
Alkaloids	Dragendroffs reagent	✓	✓

Figure 1

Figure(a)- Screening for Tannins: The plate after derivatization with ferric chloride viewed under UV light at 366nm. Figure(b)- Screening for flavonoids: The plate after derivatization with anisaldehyde solution and viewed under UV light at 366nm.

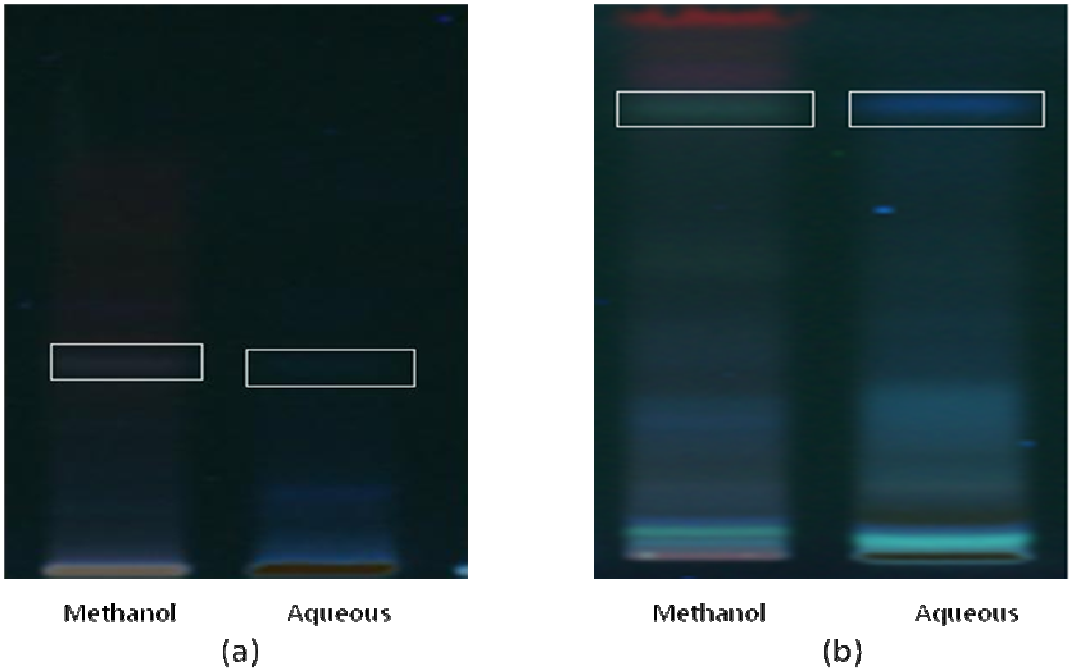
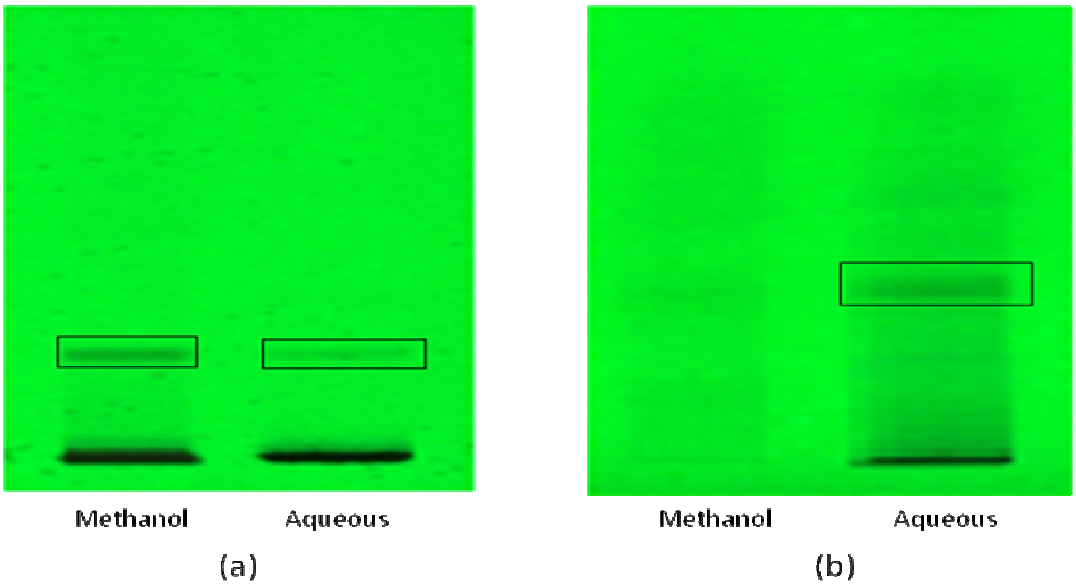


Figure 2

Figure(a)- Screening for Alkaloid: The plate after derivatization with Dragendroffs reagent viewed under UV light at 254nm. Figure(b)- Screening for Saponin:The plate after derivatization with Anisaldehyde, H_2SO_4 solution and viewed under UV light at 254nm.



ii. Cytotoxicity

Cell cytotoxicity was performed by MTT method on Vero Cells. Percent cytotoxicity was calculated using following formula:

Percent Cytotoxicity = 100 – Percent Cell Survival.

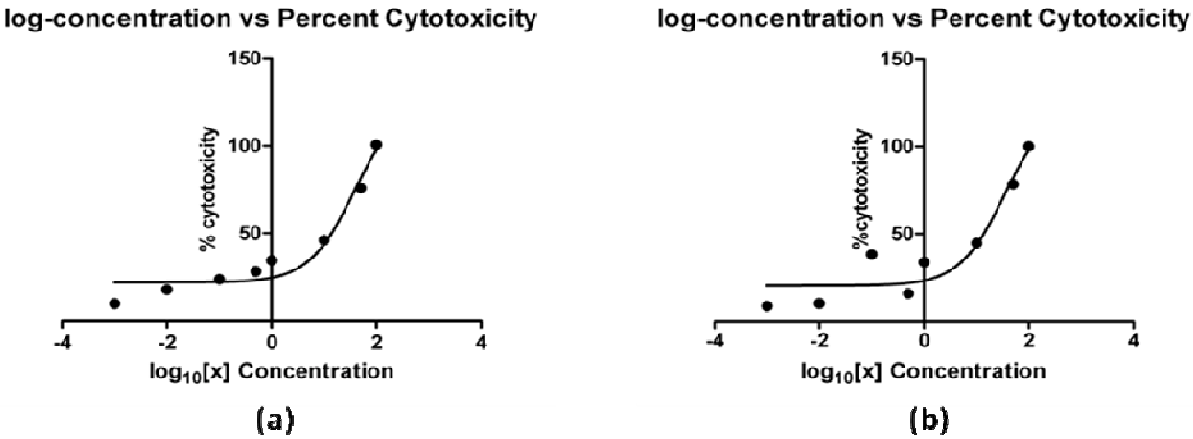
Percent Cell Survival = {(At- Ab) / (Ac-Ab)} x 100

Where,

Absorbance value of test compound - At
Absorbance value of blank - Ab
Absorbance value of control - Ac

Figure 3

Log concentration vs Percent cytotoxicity for Methanolic extract (a) and Aqueous extract (b)

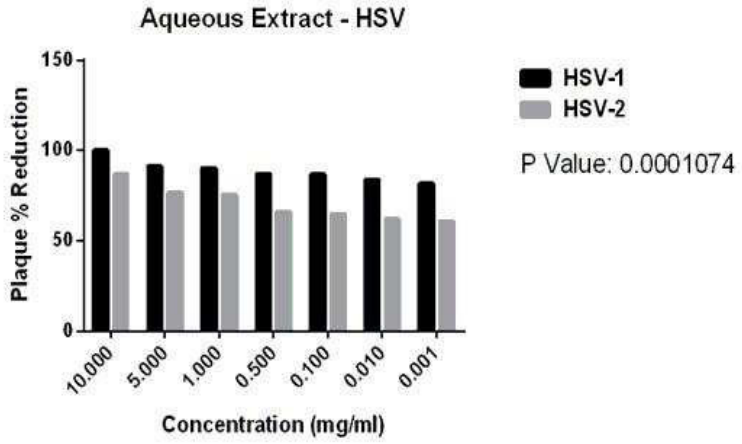


The CC50 value for Methanolic extract was found out to be 38.08mg/ml and for aqueous extract it was 35.27mg/ml

iii. Plaque Inhibition Assay

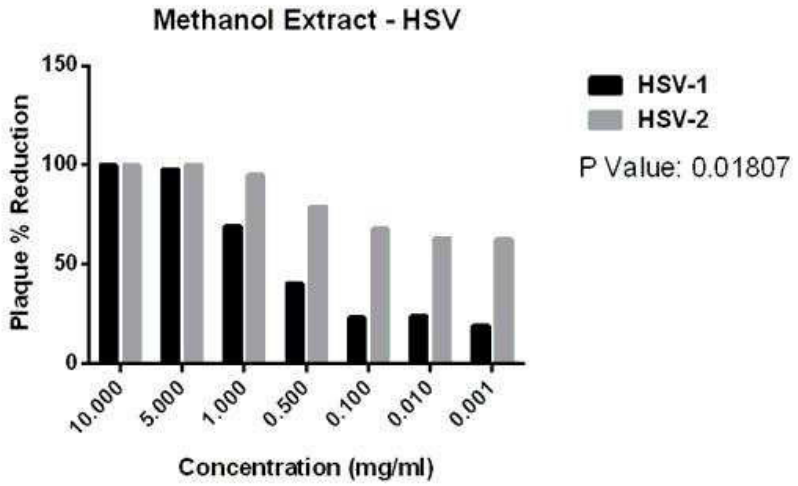
Figure 4

Plaque percent reduction vs the concentration of the aqueous extracts



The above graph indicating Plaque percent reduction vs the concentration of the aqueous extract of Justicia adhatoda on HSV-1 and HSV- 2.

Figure 5
Plaque percent reduction vs the concentration of the methanolic extracts



The above graph indicating Plaque percent reduction vs the concentration of the methanolic extracts of Justicia adhatoda on HSV-1 and HSV- 2.

DISCUSSION

Justicia adhatoda(L.) Nees are known to be rich in phytochemicals, and of importance are its leaves which contain the ingredients of medical importance. The extraction of the phytochemicals was carried out in methanol and aqueous (water) solvent by using soxhelet extraction system. Qualitative presence of tannins, flavonoids, alkaloids and saponins in the prepared extract was carried out by performing HPTLC (Table No 2). The results confirm the presence of phytochemicals in both the extracts with the exception of saponin in methanolic extract. Previous reports on phytochemical analysis shows that phenols, tannins, alkaloids, anthraquinone, saponins, flavonoids and reducing sugars were found in the leaves of *J.adhatoda* [31, 32].A cytotoxicity assay was performed to check the toxicity levels of the extracts prepared. The 50% cell cytoxicity (CC50) for methanolic extract was found to be 38.08mg/ml as compared to 35.27mg/ml of aqueous extract. Ten milligrams per microliter of extract concentration was prepared and used further for the anti-HSV assay (Figure no 3).Studies on antiviral activities of medicinal plants have been performed using an in-vitro experiment. Among various assay methods, plaque reduction assay has been used for detecting antiviral activities of both synthetic and natural products [33]. In this study, plaque reduction assay showed 100 percent plaque reduction activity in both HSV-1 and HSV-2 at concentration of 10mg/ml and 5mg/ml in methanolic extract. But as the concentration decreased, the percent reduction also decreased to about 19.22% and 62.75% respectively for HSV-1 and HSV-2 at concentration of 0.001mg/ml (Figure No 5). Paired t-test was carried out to compare the difference of methanolic extract on both the viruses and the R^2 value was 0.6338 and $p<0.01$.Similarly, 100 percent reduction for HSV-1 was found to be in aqueous extract at 10mg/ml as compared to 86% reduction for HSV-2. As the concentration decreased the percent reduction was also reduced to 81.73 % and 61.06% at 0.001mg/ml. The statistical analysis for aqueous extract on both viruses was R^2 value of 0.9306 and $p<0.01$.

The pharmacologically most studied chemical component in *J. adhatoda* is a bitter quinazoline alkaloid called vasicine which is present in the leaves, roots and flowers. Besides vasicine, the leaves contain several

alkaloids (Vasicinone, Vasicinol, Adhatodine, Adhatonine, Adhvasinone, Anisotine and Hydroxypeganine) betaine, steroids and alkanes [34, 35]. A study on high performance liquid chromatography was carried out for vasicine and vasicinone using different solvent extraction methods and methanol was found to be a suitable solvent for extraction of maximum vasicine and vasicinone [36]. Thus, our results of HPTLC for the presence of phytochemicals draw an assumption that alkaloids and its derivatives may be present in the methanol and aqueous extract of *J.adhatoda*.

It was observed that acetone, ethanol and methanol extracts inhibit HSV-2 infection by disturbing the early stage of virus infection and by diminishing the virus infectivity in *Phylllanthus urinaria Linn* [37]. On the other hand, aqueous extract of *Swertia chirata* have shown the inhibition of HSV-1 viral dissemination [38] and Putranjivain A, a isolate of *Euphorbia jolkini Bioss* showed late stage inhibition of HSV-2 replication *in-vitro* [39]. A Kenyan plant *Carissa edulis* aqueous extract inhibited efficacy against both *in-vitro* and *in-vivo* models of HSV-1 infection [40].Various solvent extracts having different potent phytochemicals as anti-viral targets against HSV-1 and 2 has been carried out. We report here that the methanolic extract was the most active anti-viral agent against HSV-2 and aqueous extract against HSV-1. These methanolic and aqueous extract may contain alkaloids and its derivatives as potent molecular targets against the Herpes Simplex Virus.

CONCLUSION

This is a preliminary report on antiviral activity of *Justicia adhatoda*, an Indian medicinal plant against Herpes Simplex Viruses. Further studies are required to know the mechanism of action using suitable animal models. We are hoping to identify few molecular targets so that, further strategy for utilization of this plant extract can be directed.

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