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Research Article

EVALUATION OF ACTIVE FRACTION FROM PLANT EXTRACTS OF *ALSTONIA SCHOLARIS* FOR ITS IN-VITRO AND IN-VIVO ANTIVIRAL ACTIVITY

MOLLY ANTONY[1], CHANDRA SHEKHAR MISRA[2], THANKAMANI V[*]

[1]Sree Chitra Tirunal Institute for Medical Sciences and Technology Trivandrum- 695011,[2]School of Bio Sciences and Technology, VIT University, Vellore-632014, Tamil Nadu, India.[*]Department of Biotechnology, University of Kerala, Kariavattom, Trivandrum 695 581

Email: dr.thankamani@gmail.com

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ABSTRACT

Objective: The present study aims to investigate the anti-viral activity of solvent extracts of the plant *Alstonia scholaris*.

Methods: In vitro antiviral activity of water and alcohol extract was determined against Coxsackie B2, Polio virus and Herpes Simplex virus by the method of inhibition of cytopathic effect (CPE) and in vivo assays were performed in suckling mice. Antiviral activity against Hepatitis B Virus was determined by Neutralization test.

Results: In-vivo assays performed in the suckling mice against Coxsackie virus B2 showed that .mice infected with virus when treated with extract up to concentration 2856µg survived longer than the pups without being treated with the extract. The anti-viral activity of the plant extract against polio virus and Herpes Simplex Virus (HSV) showed considerable amount of activity against both pathogenic viruses. The result of antiviral activity against Hepatitis B virus showed that ethyl acetate fraction of methanol extract of bark with Relative Fluorescence Value (RFV) value being decreased to 1.53 from 16.64 whereas ethyl-acetate fraction of leaves caused the decrease of RFV value from 17.23 to 2.75 in 72 hours together attributing to a strong antiviral activity of *Alstonia scholaris* fractions.

Conclusion: We are reporting the antiviral activity of various fractions of plant extract of *Alstonia scholaris* for the first time and result showed that almost all studies plant extract possessed potent antiviral activity against the viruses studied.

Keywords: *Alstonia scholaris*; Anti-viral; Cytotoxic; Plant Extract; RFV

INTRODUCTION

Several hundred plant and herb species that have potential as novel antiviral agents have been studied. A wide variety of active phytochemicals, including the flavonoids, terpenoids, lignans, sulphides, polyphenols, coumarins, saponins, furyl compounds, alkaloids, polyenes, thiophenes, proteins and peptides have been identified. Some volatile essential oils of commonly used culinary herbs, spices and herbal teas have also exhibited a high level of antiviral activity [1]. Research interests for antiviral agents started after the Second World War in Europe and in 1952 the Boots drug company at Nottingham, England, examined the action of 288 plants against Influenza A virus in embryonated eggs. They found that 12 of them suppressed virus amplification [2].

Viruses have a capability to utilize the host machinery for their own development and growth and it is because of this close linking with the host cell and intelligently using host machinery for propagation that targeting antiviral agents specifically against viruses alone and simultaneously protecting the host cell becomes all the more difficult. The use of medicinal plants for the treatment of viral infections arguably has been based largely on historical and anecdotal evidence [1].

Medicinal plants have been known since time immemorial to be used as folklore remedies for treating various ailments including those caused by the viruses[3]. There is a constant need of searching for a suitable antiviral agents against these pathogenic entities since they develop resistance to antiviral drugs at an alarming rate [4]. Lot of literature evidences are available related to the viral resistance and viral latency leading to recurrent infections in immune compromised patients [5-7]. Of late, a large number of medicinal plants have been studied and have been reported to show potent antiviral effect [8-10].

This prompted us to look for leads that point to any antiviral activity from extracts of *Alstonia scholaris* which by itself has a lot of bio-active moieties. *Alstonia scholaris* is one of the most studied plants of Apocynaceae family and lots of bioactive agents are well documented in literature [11]. However we are reporting for the first time, the anti-viral activity of plant extracts of *Alstonia scholaris* since no such available literature is available for bio-active agents from this plant against viral infections.

MATERIALS & METHODS

For Tissue culture work all glass ware was absolutely clean, rinsed in tap water for about 10 times and in distilled water at least 3 times. Tissue culture grade plastic ware was used to set up cell culture.

Media used for Anti Viral studies

Minimum essential medium (MEM) (Ingredients)

The medium used in our studies was MEM (Hi Media Product no: AT017A) which consisted of Eagle MEM modified for autoclaving with Earl's salts, non essential amino acids and Phenol red.

Trypsin Versene Glucose solution (TPVG)

Sterile Fetal Calf Serum (Sterilized by filtration)

Anti viral assays:

Coxsackie B2 (Entero virus) was obtained from National Institute of Virology, Pune. Cell lines used were Vero cells which were obtained from National Centre for Cell Science, Pune, India. Vero cells were grown on Tissue culture bottles and sub cultured on to flat bottomed Tissue culture plates (96 wells) for virus titrations and for in-vitro antiviral work.

Titration of virus

100 $TCID_{50}$ (Tissue culture infectivity dose) or LD_{50} (Lethal Dose) of the virus was calculated according to Reed and Muench method [12-13].

Reed and Muench formulae:-

$$\text{Index} = \frac{(\%\text{ mortality at dilution next above } 50\%) - (50\%)}{(\%\text{ mortality at dilution next above } 50\%) - (\%\text{mortality at dilution next below})}$$

Titration of Virus in Tissue culture – Vero cell line

Vero cells were sub-cultured on to sterile flat bottomed Tissue culture plates and incubated in a 37^{0}C Carbon dioxide incubator until the cell sheet was complete.

10 fold serial dilutions of the Coxsackie B2 virus was made in test tubes immersed in an ice bath. From each of the dilution 0.25 µl of the virus was added into a set of 6 wells of the Vero cells grown on Tissue culture plates, starting from the highest dilution and using separate micropipettes for each dilution. Uninoculated wells served as cell controls. The plates were incubated in a CO_2 incubator for 2 to 3 days until good cytopathic effect (CPE) was noticed in the virus inoculated wells. Readings were taken using an inverted microscope.

In vitro Antiviral assay

The antiviral activity of the water and alcohol extract was determined by the cytopathic effect. 100 $TCID_{50}$ (10^{-2}) Coxsackie B2 was used with varying concentrations of the extract which was added to the monolayers of Vero cells and incubated for 2- 3 days until CPE was seen in the virus control cells.

Each dilution of the test was repeated in triplicates. The concentration of the extract where viral CPE was inhibited was taken as the concentration showing antiviral activity.

In vivo Anti viral assay

Titration of Coxsackie B2 virus in suckling mice

Coxsackie B2 was titrated in one day old suckling mice as all Entero viruses are pathogenic to suckling mice and not adult mice

One Mice box (Swiss Albino Mice) consisted of one mother mouse and 6 pups which were used for each dilution. 25µl of virus was inoculated into each pup by intra cerebral route using tuberculin syringe. Sickness and paralysis of hind limbs in pups were noticed by 48 hours, post inoculation deaths were recorded from day 3. Final reading was taken on the 5th day.

In vivo Anti viral activity of Alstonia scholaris –alcohol extract of leaf in suckling mice

In vivo anti-Coxsackie viral activity was standardized using viral infection in suckling mice as a model[13]. One day old suckling mice were used and working dilution of Coxsackie B2 virus used was 10^{-4}. Virus was given by I/P route and extract was given orally. Extract used was *Alstonia scholaris* leaf – Alcohol extract. The experiment was so designed that one box of 5 suckling mice were inoculated intra peritoneally with 0.025µl of 10^{-4} virus as control of the working dilution of virus used.

Another 3 sets of suckling mice were given extracts at a concentration of 1354 µg/ml, 2856 µg/ml and 5416 µg/ml orally daily for 15 days as extract controls to look for any toxicity of the extract used. The test set of animals ie 3 sets of suckling mice were inoculated with 10^{-4} of Coxsackie B virus by IP route and extracts at 3 different concentrations – 1354 µg/ml, 2856 µg/ml and 5416 µg/ml were given to the respective lot of 6 suckling mice orally, daily for 15 days. (One set consists of 4 - 6 suckling mice and the mother)

Polio Virus

In vitro Anti viral activity of aqueous extract of Alstonia scholaris- leaf extract against Polio Virus

Cells used for the present study were Vero cell line and the virus strain Polio virus Type I & Type II were obtained from National Institute of Virology, Pune.

$TCID_{50}$ of Polio Type I was 10^{-6} and working dilution of Polio Type I used was 10^{-4} and (100$TCID_{50}$).

$TCID_{50}$ of Polio Type II 10^{-4} and working dilution of Polio Type II used was 10^{-2} (100 $TCID_{50}$).

Vero cells were grown in MEM (Hi Media) and supplemented with heat inactivated foetal calf serum (5%), sodium bicarbonate, glutamine and Penicillin (200 units/ml, Streptomycin 200µg/ml) on flat bottomed polystyrene 96 well microtitre plates in a humidified 5% CO_2 incubator (Inova make). Virus stocks were prepared in Vero cell line and stored at - 80^{0}C. The virus titre was determined by cytopatheic method applying Reed and Muench formula. Viral suspension was added along with varying concentrations of aqueous extracts of *Alstonia scholaris* leaf extracts to the monolayers of vero cell line grown on flat bottomed tissue culture plates. The cultures were then incubated in 5% CO_2 incubator at 37^{0}C incubator for 3-4 days (until 4+ CPE was seen in the virus control wells). Each test was done in 3 replicates. The virucidal effect of the extract was observed by the degree of CPE produced. Wells with 80% intact cells as compared to virus controls were considered to be positive for antiviral activity. Rounding of cells is the typical CPE for Polio virus.

Herpes Simplex Virus Type I

Anti Viral Activity of Water Extract Of Alstonia Scholaris- Leaf Extract Against HSV I

Cells used were Vero cell line and the virus strain Herpes simplex Type I (HSV) was obtained from National Institute of Virology, Pune.

$TCID_{50}$ of HSV Type I was 10^{-4} and working dilution of HSV Type I used 10^{-2}(100 $TCID_{50}$).

The antiviral activity of the water extract of *Alstonia scholaris* – leaf was determined by the cytopatheic effect. 100 $TCID_{50}$ (10^{-2}) HSV type I was used with varying concentrations of the extract which was added to the monolayers of Vero cells and incubated for 2- 3 days until CPE was seen in the virus control cells.

Each dilution of the test was repeated in triplicate. The concentration of the extract where viral CPE was inhibited was taken as the concentration showing antiviral activity.

Procedure

Vero cells were grown in MEM (Hi Media) and supplemented with heat inactivated foetal calf serum (5%), sodium bicarbonate, glutamine and Penicillin (200 units/ml, Streptomycin 200µg/ml) on flat bottomed polystyrene 96 well microtitre plates in a humidified 5% CO_2 incubator (Inova make). Virus stocks were prepared in Vero cell line and stored at -80^{0}C.

The virus titre was determined by cytopatheic method applying Reed and Muench formula. Viral suspension was added along with varying concentrations of aqueous extracts of *Alstonia scholaris* leaf extracts to the monolayers of vero cell line grown on flat bottomed tissue culture plates. The cultures were then incubated in 5% CO_2 incubator at 37^{0}C incubator for 5 days (until 4+ CPE is seen in the virus control wells). Each test was done in 3 replicates. The virucidal effect of the extract was observed by the degree of CPE produced. Wells with 80% intact cells as compared to virus controls were considered to be positive for ant-viral activity. Syncitium formation was the typical CPE of HSV.

Hepatitis B Virus

HepG2 2.2.15 cell line (HBV producing cell line) used in the present study was supplied by Prof. Bart L. Haagmans (Department of Virology, Dr. Molewaterplein 50, 3015 GD, Erasmus MC Rotterdam, Netherlands). HepG2 human hepatoma cell line transfected with a plasmid containing two head-to-tail dimers of the HBV genome, was used. The HepG2.2.15 cell line released high levels of HBV DNA, HBsAg and HBeAg into the medium during culture [14-15] and this was quantified by ELFA method using VIDAS auto immune-analyser –Biomerieux system.

HepG2.2.15 cell line

HepG2.2.15 cell line was maintained in the lab by sub culturing every ten days. Subculture was done in tissue culture bottles either plastic or glass. MEM (Hi Media) was used with 0.5% Foetal Bovine serum.1 ml of 0.25% collagen prepared in 0.02N HCL was added into the bottles and kept at 37^{0}C for one hour, the collagen solution poured off, allowed to dry for one hour and just before sub culturing the bottles were rinsed in MEM. This prevented the sticky nature of the cell line and allowing cell sheet formation

Hepatitis B Virus Neutralization Test

HepG2.2.15 cells were cultured in MEM (Hi Media) containing 10% fetal calf serum and Gentamycin 20 µg/100 ml medium at 37°C in a humidified incubator gassed with 5% CO_2.

500µl of the MEM medium in which HepG2.2.15 cell line was growing was taken in various tubes and 20 µl of various fractions of *Alstonia scholaris* extracts were added and incubated overnight for neutralization to occur. Each of the tubes was tested for quantifying the Hepatitis B antigen. After 48 hours 20 µl of the respective fractions of the extracts were again added and the test repeated after 72 hours of neutralization.

***In Vitro* Anti Viral Activity against HBV Using Transfected Hepg2.2.15 Cell Line**

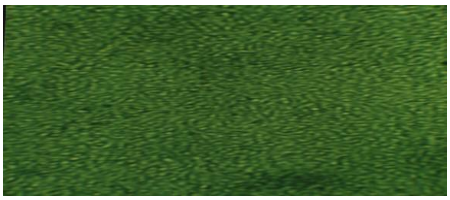
The HepG2.2.15 cells were plated at a density of $1x10^4$ cells per well on 96-well flat bottomed cell culture plates. Complete cell sheet was formed after 48 hours of incubation. Fresh medium was replaced and different concentrations of *Alstonia scholaris* alcohol extract of leaves were added to the wells. After incubation with the extract for 2, 4, 6 and 8 days, 100 µl of the supernatants were collected from test wells (wells containing extracts) and control wells (wells with no extracts) and subjected to HBsAg quantitation by ELFA((Enzyme-linked fluorescent immunoassay)) technology on the VIDAS auto analyser.

The intensity of the fluorescence is proportional to the concentration of antigen present in the sample. At the end of the assay, results are expressed as an index calculated using a standard. The sensitivity of the assay is 0.12 ng/ml.

RESULTS AND DISCUSSION

***In vitro* Antiviral assay**

The cytopathic effect as shown by rounding of cells is shown in Fig. 1. CPE in the case of all Enteroviruses are rounding of cells (Table 1).



A.

B.

Fig. 1: Normal Vero cells(A) and Vero cells showing cytopathic effect(CPE) of Coxsackie B_2 Virus as observed by the rounding of cells(B).

Table 1: Readings obtained on TCID Plate of Coxsackie B_2 Virus

Virus dilution	CPE ratio	Wells showing CPE	Wells showing no CPE	Accumulated values			
				CPE + ve	No CPE	CPE ratio	CPE percentage
10^{-3}	6/6	6	0	17	0	17/17	100
10^{-4}	6/6	6	0	11	0	11/11	100
10^{-5}	6/6	4	2	5	2	5/7	71
10^{-6}	6/6	1	5	1	7	1/8	13
10^{-7}	0/6	0	6	0	13	0/13	0

Applying Reed and Muench formula:

$$\text{Index} = \frac{71 - 50}{71 - 13} = \frac{21}{58} = 0.36$$

The index calculated for the dilution that produced the infection rate immediately above 50% is 0.36 which is adjusted to 0.4 for all practical purposes.

$TCID_{50}$ of Coxsackie B_2 was 10^{-4}

Table 2: *In vitro* Antiviral assay of ASL extract against Coxsackie B_2 Virus

Extract	Antiviral activity (Against Coxsackie B_2) µ g/ ml	Cell cytotoxicity (Vero cells) µ g/ ml
Water extract of *Alstonia scholaris* Leaf	1428	3333
Alcohol extract of *Alstonia scholaris* Leaf	57	100
Water extract of *Alstonia scholaris* Bark	333	>333

Table 3: Titration of Coxsackie $_{B2}$ Virus in Suckling mice

Virus dilution	No: of pups inoculated	Readings – death rate			Interpretation
		3rd	4th day	5th day	
10^{-1}	6	4/6	6/6	6/6	
10^{-2}	6	2/6	5/6	6/6	**$LD_{50} = 10^{-6}$**
10^{-3}	6	3/6	4/6	6/6	**100 $LD^{50} = 10^{-4}$**
10^{-4}	6	3/6	3/6	6/6	
10^{-5}	6	4/6	4/6	5/6	
10^{-6}	6	3/6	4/6	4/6	
10^{-7}	6	0/6	0/6	0/6	

-Lethal dose $_{50}$ (LD_{50}) was calculated applying Reed-Muench formulae.

-LD_{50} of Coxsackie B_2 was 10^{-6}

Table 4: Survival rate of suckling mice challenged with Coxsackie B_2 virus and treated with *Alstonia scholaris* leaf – Alcohol extract

Dose given	Days post inoculation															
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Virus Control 10^{-4}	IP	5/5	2	0												
Virus +Extract 1354 µgm	IP Oral	5/5	5	5	2	0										
1354 µg Extract Control	Oral	4/4	4	4	4	4	2	2	2	2	2	2	2	2	2	2
Virus +Extract 2856 µg	IP Oral	6/6	6	6	5	5	4	4	4	4	4	2	2	2	2	2
2856 µg Extract Control	Oral	6/6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Virus +Extract 5416 µg	Oral IP	5/5	5	4	0											
5416 µg Extract Control	Oral	4/4	4	4	4	4	2	2	2	2	2	2	2	2	2	2

The results of in-vitro antiviral assays showed that antiviral moieties are present in Alstonia scholaris leaf and bark extracts. It can be noted that anti viral activity is at half the dose of the toxic level which is promising for further development as antiviral analogues. Alcohol extract seems to be more potent as anti viral moieties were detected at lower concentrations (Table 2).

Results of in vivo Antiviral assay

The results of *in vivo* anti-viral assay is presented in Table 3.

Result showed that all the pups (5) in the virus control at 10^{-4} dilution died by the 3rd day. At an extract concentration of 1354 µg, when challenged with 10^{-4} virus, 5 pups survived up to day 3 and 2 pups survived up to day 4. However, when the extract concentration was increased to 2856 µg from1354 µg and challenged with the same dose of virus, 50% of the pups survived beyond 15 days post inoculation. There was no toxicity of the extract at 2856 µg concentration as all the pups (6) inoculated with the extract by intra peritoneal route survived beyond 15 days in good health with no weight loss or any behavioral changes. Mild toxicity of the extract was seen at the next higher dilution of 5416 µg.

The in vivo experiment therefore clearly proves that Alstonia scholaris alcohol extract of leaf has anti-viral activity as the one day old pups which were challenged with 10^{-4} dilution of the virus by an intra peritoneal route thereby causing a systemic infection and when the extracts at a concentration of 2856 µg when given orally the extract was absorbed by the alimentary canal and could arrest the infection at the systemic level in 50% of the pups (Table 4). The suckling mice which survived the infection had mild weakness of the hind limbs which was also an indication that the pups had undergone a full onslaught of the Coxsackie B viral infection and survived due to the antiviral moiety of Alstonia scholaris extract (Fig. 2).

A.

B.

C.

D.

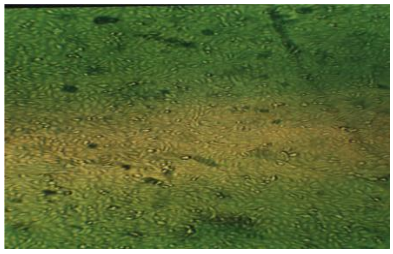
Fig. 2: Mice inoculated with Coxsackie B_2 Virus by IP route and treated with alcohol extract of ASL orally for 15 days: (A) Mice after treatment, (B) Mice after successful treatment and left Leg paralyzed is shown protruding out, (C & D) Hind legs where paralysis had occurred.

To our knowledge this is the first time that anti-viral activity of *Alstonia scholaris* extracts has been demonstrated in-vitro and simultaneously proved by in-vivo experiments by inducing hind limb paralysis by Coxsackie B viral infection and successfully treating it for 15 days orally with *Alstonia scholaris* alcohol extract of leaf. It has been suggested by Vanden and Vlietinek, 1991 that selection of plant on the basis of ethanomedical considerations gives a higher hit rate than screening programmes of general synthetic products[16]. Coxsackie B viruses cause myocarditis and this virus infection is a serious medical problem, especially in new borns[17-18]Gear et al 1973 & Jack et al 1980]. It has been shown by Parida et al in 1997 that ethanol extract of *Ocimum sanctum* inhibits polio virus replication in Vero cells[19]. Antony et al in 2011 has reported an active (butanol) fraction of alcohol extract from *Ocimum sanctum* leaf to have anti viral activity against Coxsackie B virus both in vitro and in vivo[20]. It should be noted that in vitro screening of plants have been carried out in India [21, 9], but a follow up of in vivo studies are rare to substantiate the in vitro results. Khalil et al in 2010 has reported that ethanol extract of *Ocimum basilicum* – leaves had anti viral activity against Coxsackie B4 at a concentration of 0.9µg/ml and against Hepatitis A virus at a concentration of 0.6µg/ml[22]. In 2005 Subramoniam et al has demonstrated in vitro anti viral activity against Coxsackie B virus in vero cells with water extract of *Rhinacanthus communis* –leaf at a concentration of 50µg/ml[23].

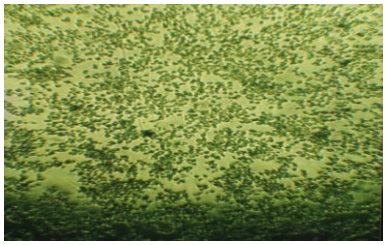
Mice(a) and (b) are shown in fairly good health after treatment with the ASL alcohol extract and in pictures (b), (c) (d) the hind limbs are shown distinctly where paralysis has occurred which was due to a full blown systemic infection caused by Coxsackie B virus injection – depicting the tell tale features of Coxsackie B virus infection(Fig. 2). Dose of Coxsackie B_2 virus given was 10^{-4} intra peritoneal route (0.25µl). Alcohol extract of *Alstonia scholaris* leaf (2856µg/ml) given orally. Coxsackie B virus infection in suckling mice is 100% fatal in untreated cases.

Results of *in vitro* Antiviral assay using Polio virus

Antiviral activity was detected at 233µg concentration of *Alstonia scholaris* water extract of leaf for Polio Virus Type I and II. The morphology of normal Vero cells forming a uniform complete sheet is shown in Fig. 3A and the typical cytoppatheic effect caused by Polio virus is seen(Fig. 3B) where in the spindle shaped cells become rounded.



A.



B.

Fig. 3: (A): The morphology of normal Vero cells forming a uniform complete sheet and (B): The typical cytopathic effect caused by Polio virus is seen where in the spindle shaped cells become rounded.

Subramoniam et al in 2005 reported that water extracts of few plants from the western ghats viz *Aegeria nervosa*-root, *Pellionia heyneana-leaf, Rhinacanthus communis*-leaf and alcohol extract of *Ochreinauclea missionis* –stem bark had anti viral activity against Polio type I virus at concentrations ranging from 250µg to 2500µg/ml[23].

In vitro Antiviral assay using HSV virus

Antiviral activity was detected at 233µg concentration of *Alstonia scholaris* water extract of leaf for Herpes simplex Virus Type I.

The typical syncitium formation caused by Herpes simplex virus is shown in Fig. 4. Syncitium formation is characteristic of Herpes virus CPE.

Vijayan et al in 2004 reported methanol extracts of aerial parts of two plants – *Hypericum mysorense* and *Hypericum hookerianum* belonging to Hypericaceae family to have anti viral activity against HSV I at a concentration of 100µg/ml [9].

Alcohol extract of *Ocimum sanctum* leaves have been reported to have anti viral activity against HSV I in Vero cells at a concentration of 440µg/ml while cell toxicity level was at a very much higher concentration of 3000µg/ml [20].

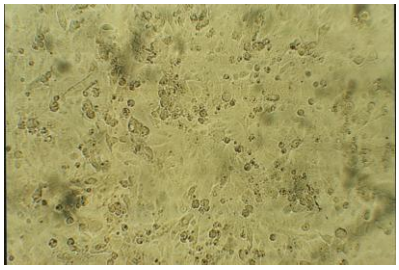


Fig. 4: CPE of Herpes simplex virus Type I (Syncitium formation)

Antiviral activity against Hepatitis B virus by Neutralization test

The Hep G 2.2.15 cell line is shown in Fig. 5.

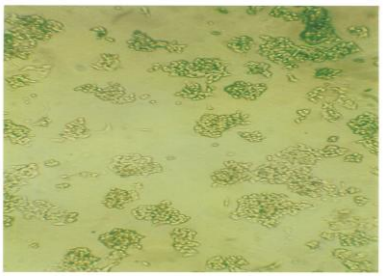


Fig. 5: Hep G 2.2.15 cell line grown in MEM with 0.5% FCS on 0.25% collagen coated tissue culture bottles.

The antiviral activity of various fraction of alcoholic extract of *Alstonia scholaris* are given in Table 5. The results of the neutralization test show that Anti hepatitis B activity has been spread out in the Butanol and Ethyl acetate fractions of Methanol extract. From a value of 16.64 RFV it was reduced to 1.53 RFV in 72 hours which was the least value obtained in the experiment done to look for neutralizing effect of *Alstonia scholaris* extracts. This least value was obtained from the Ethyl acetate fraction of Bark extract. Correspondingly the value of Ethyl acetate fraction of leaf extract also dropped from 17.23 to 2.75 RFV in 72 hours. There was only a marginal drop in values in the cell control and solvent control tubes which could be expected due to non specific reactions after 72 hours incubation therefore the drop in values when treated with plant extracts could be attributed to the anti viral moieties of *Alstonia scholaris* fractions (Table 5). A known synthetic drug Lamividine was also run and at 100µl concentration the RFV value decreased to 0.46 while the control was at 19.98RFV. The known antiviral drug was run along with the test as a positive control for anti HBV screening. In India the three major systems of traditional medicine, namely the Ayurvedic, Siddha and Unani systems have standard treatments for clinical jaundice. The three treatments consist of oral administration of one or more dried plant extracts, in the form of

tablets or capsules. Other cultures in different parts of the globe also used plant extracts – root extracts of *Glycyrhiza glabra* in China [24]. The most common ingredients in the Indian systems are extracts of the genus *Phyllanthus* of the Euphorbiaceae family. Thyagarajan et al in 1982 had done a similar neutralization test with *Phyllanthus niruri* and *Eclipta alba Hassk* extracts and HBsAg positive sera from chronic hepatitis B carriers and showed that HBsAg was inactivated at 37^{0}C[25].

His group in 1988 and 1990 had also reported that dried milled *Phyllanthus amarus* was successful in clearing hepatitis B surface antigen from blood positive carriers in Madras[26-27].

Table 5: Neutralizing activity of various fractions of Methanol extract of *Alstonia scholaris* against Hepatitis B virus

Medium from bottles in which Hep G2.2.15 cell line was growing and *Alstonia scholaris* methanol extract-fractions	RFV values of HBsAg 24 hours	RFV values of HBsAg 48 hours	RFV values of HBsAg 72 hours
Medium of HepG2.2.15 – cell control	19.19	17.55	17.17
Medium of HepG2.2.15 + 20 µl of DMSO	19.2	17.51	16.6
Medium of HepG2.2.15 + 20 µl of Butanol	18.2	17.83	13.13
Medium of HepG2.2.15 + 20 µl of Ethyl Acetate	19.52	17.74	17.49
Medium of HepG2.2.15 + 20µl of AS Leaf Hexane fraction (5 mg)	18.38	16.61	6.87
Medium of HepG2.2.15 + 20µl of AS Bark Hexane fraction (30 mg)	16.18	15.62	10.23
Medium of HepG2.2.15 + 20µl of AS Leaf Chloroform fraction (2 mg)	18.31	16.63	13.35
Medium of HepG2.2.15 + 20µl of AS Bark Chloroform fraction (25 mg)	17.21	17.09	13.35
Medium of HepG2.2.15 + 20µl of AS Leaf Butanol fraction (1 mg)	14.41	14.98	2.53
Medium of HepG2.2.15 + 20µl of AS Bark Butanol fraction (6 mg)	14.84	14.49	4.97
Medium of HepG2.2.15 + 20µl of AS Leaf Ethyl Acetate fraction (1 mg)	17.23	17.01	2.75
HepG2.2.15 + 20µlitres of AS Bark Ethyl Acetate fraction (4 mg)	16.64	15.57	1.53
Medium of HepG2.2.15 + 20µl of AS Leaf Water fraction (7 mg)	18.23	17.75	14.04
Medium of HepG2.2.15 + 20µl of AS Bark Water fraction (2 mg)	17.97	17.34	17.23

-Values are expressed as RFV- Relative Fluorescence Values

However literature is rife with contradictory conclusions of anti-Hepatitis activity obtained from a variety of crude extracts of *Phyllanthus amarus* and this may have much to do with the extract standardization, species used and location harvested that resulted in different levels of active constituents in samples [28]. Clinical trials in HBsAg carriers done by Hudson in 1990 with extracts of *Phyllanthus niruri* had shown that within 3 months two-thirds of the tested individuals had cleared the Hepatitis B antigen[29]. Experiments on the same lines were repeated by Venkateswaran et al in 1987 using wood chunk Hepatitis B virus and water extract of *Phyllanthus niruri* and the extract were found to inhibit the binding of wood chunk Hepatits virus surface antigens to their corresponding antibodies[30].

Results of Antiviral Activity against Hepatitis B Virus using Hepatitis B Virus Transfected .Cell line (HepG2.2.15)

The antiviral activity of alcoholic extract of leaves of *Alstonia scholaris* against HBV is tested in HepG2.2.15 cell line and values are detected by ELFA technology on the Vidas Auto Analyser and are expressed as Relative Fluorescence Value (RFV). The result is presented in Table 6.

Table 6: Anti Hepatitis B with alcoholic extract of leaf of *Alstonia scholaris* and Hepatitis markers (HB_sAg)

Alcohol extract of AS-Leaf µg	HBsAg 2 days	HBsAg 4 days	HBsAg 6 days	HBsAg 8 days
Cell line control	21.5	21.34	16.93	14.92
2	20.8	21.76	17.28	15.89
4	19.96	20.68	16.62	14.67
6	20.44	20.52	16.13	16.36
8	20.5	20.21	13.44	13.93
10	20.7	20.94	14.33	13.48
12	20.3	18.25	8.99	4.01
14	19.5	18.98	9.12	4.4
16	19.07	17,02	6.16	3.45
18	19.72	17.16	7.4	2.96
20	20.32	17.77	6.74	2.62
22	20.15	17.74	6.33	2.1
24	19.43	15.98	4.62	1.5

From the data depicted in Table 6, a tapering effect of RFV can be noted with increasing concentrations of the extract. At a concentration of 12µg the value of Hepatitis B antigen was 4.01 by day 8 and when the concentration was doubled to 24 µg the Hepatitis antigen was reduced to almost a quarter (1.5 by day 8). The toxicity of the same extract was tested on Vero cell line by XTT test and no cell cytotoxicity was noticed up to 100µg/ml (Unpublished Data). In the present study wherein we investigated the anti-HBV activity of ASL in stable HBV-transfected HepG2.2.15 cells, which can continuously produce complete virion particles of HBV and a high level of viral proteins, we found that ASL –alcohol extracts could decrease the secretion of HBsAg in a dose-dependent manner. These results demonstrated for the first time that ASL possesses potent inhibitory activity against HBV replication in vitro. From these experiments in vitro we could draw conclusions that alcohol extracts of ASL seems to be good candidate for further in vivo studies and drug development. Although several pharmacological strategies are currently being implemented to treat affected patients, no 100% satisfactory antiviral therapies against HBV infection have yet been fully developed. Thus it has become urgent to find new and effective anti-HBV drugs to treat patients as well as carriers as a good number of chronic HBV patients can develop Hepatomas subsequently. Ran Pang et al, 2011 has reported that ethanol extracts from *Amelopsis sinica* root extracts (Vitaceae family) exerts anti Hepatitis B virus activity via inhibition of p53 pathway in vitro and that it effectively suppressed the secretion of HBsAg in a dose dependent manner[31]. Xu et al, 2008 have reported that green tea extracts and its major component epigallocatechin gallate inhibits Hepatitis B virus in vitro[32]. Li et al, 2008 could demonstrate in vitro and in vivo anti-Hepatitis B virus activities from plant extracts of *Geranium carolinianum* L[33].

CONCLUSION

We are presenting the anti-viral activity of plant extract of *Alstonia scholaris* for the first time and as the result shows, the various fractions of alcoholic extract of leaf and bark possess potent activity against some of the potential pathogenic strain which might pave the way of isolating important therapeutic isolate from the plant extract for treating diseases like Hepatitis, the cure of which eludes

many in the developing countries. However, techniques need to be devised for more economical means of isolating potential therapeutic drug before which more detailed bioassay guided experiments need to be done in order to establish the scientific authenticity of application of the potential isolate preliminarily described in the present study.

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