ORIGINAL RESEARCH PAPER

Biological Science

ANTIVIRAL ACTIVITY OF LYOPHILIZED AQUEOUS AND ETHANOLIC EXTRACTS OF TERMINALIA CHEBULA RETZ., ON HEPATITIS B VIRUS

KEY WORDS: HBV DNA polymerase, Terminalia chebula, HBsAg binding Assay, MTT Assay.

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ABSTRACT

The present study is aimed at investigating the antiviral activity of lyophilized aqueous and ethanolic extracts of *Terminalia chebula* on Hepatitis B Virus. *In vitro* cytotoxicity was studied and both aqueous and ethanolic extracts and ethanolic extracts were found to be non toxic at the maximum concentrations of 500µg/ml. HBs antigen binding assay and HBV DNA polymerase inhibition assay were performed in which both the extracts were found to be effective in par with lamivudine against Hepatitis B virus.

INTRODUCTION

India is called as botanical Garden of the World in which large numbers of medicinal plants are available (1). Novel plant drugs were used effectively for the treatment of various ailments. Herbal treatments are available in the Indian system of medicine as early as 1900 BC to treat liver disease (2). Some of the well known plants possess hepatoprotective activity (3). Terminalia chebula of Combretaceae, popularly known as chebulic myrobalan tree, is called king of medicine in Tibet due to the presence of a large number of different types of phytoconstituents. Deciduous tree grows up to 30m in height. The fruits are drupe like, 2 - 4cm long, blackish with white five longitudinal white ridges, small, ribbed and nut like fruits.

Kim *et al.,* reported reduction in HBs Ag and HBV DNA polymerase production by lyophilized aqueous extracts of Terminalia chebula in HepG2 2.2.15 cell cultures (4). Mohan *et al.,* studied and found that 90% of Terminalia chebula ethanolic extracts inhibited on HBs antigen binding assay and HBV DNA polymerase inhibition assay (5). *In silico* analysis of potent compounds from *Terminalia chebula* proved to be effective against Hepatitis B virus (6).

MATERIALS AND METHODS

The Dried fruits of *Terminalia chebula* was collected from Kalrayan Hills, Villupuram district (fig 1.1). The plant was identified and authenticated by the Department of Plant Biology and Plant Biotechnology at Presidency College, Chennai. The collected plant parts were surface sterilized with alcohol, dried in shade and the fruit rind was ground in to powder and sieved. The fine powder obtained was stored in a sterile container aseptically.

Fig1.1 Dried fruits of *Terminalia chebula*

Aqueous Extracts Preparation

Twenty gram of fine powder were weighed and immersed in 100ml of Double distilled water and kept at overnight at low temperature at 4°C. Then it was filtered and centrifuged to obtain the clarified extracts. The clarified extracts were filtered using 0.22 µm Millipore filters (7).

Ethanolic Extracts Preparation

Twenty gram of fine powder were weighed and immersed in 100ml of ethanol and kept at overnight at low temperature at 4°C. Then it was filtered and centrifuged to obtain the clarified extracts. The clarified extracts were filtered using 0.22 Millipore filter.

Lyophilization

The Collected aqueous and ethanolic extracts were further subjected to Lyophilization. In this procedure, the sterile extracts were transferred aseptically into the Lyophilization flask and kept frozen at -80°C in deep freezer. The frozen extract was taken out and connected to lyophilization chamber having temperature -70°C under a deep vacuum pressure allowing the ice to sublimate directly from solid phase to gaseous phase without reaching the liquid phase. These lyophilized extracts were obtained and the physical appearance of the powder was as shown in the **figure (1.2) and (1.3)** and stored at -20°C until use.

Fig 1.2 *T.chebula* (Aq)

Fig 1.3 *T.chebula* (Eth)

In vitro cytotoxicity Assay (MTT Assay)

This assay was carried out on cell lines by Colorimetric method (8). 20µl of MTT (5mg/ml) was dispensed into each well including solvent control and incubated at 37°C for 4hours. After incubation, the culture medium was removed by gentle aspiration. 150 µl of DMSO (Dimethylsulfoxide) was added into each well to dissolve formazan crystals. The colour intensity of crystals were proportional to the number of viable cells present. Absorbance of lysates were recorded by microplate reader at a wavelength of 570nm having 650nm as reference wavelength. Cell viability was calculated by according to the formulae.

$$\% \text{ of cell viability} = \frac{\text{Mean OD of extracts treated cells x100}}{\text{Mean OD of solvent treated cells (control)}}$$

Serum Samples Collection

Positive serum samples were collected from Med Lab, Voluntary health Services, Chennai and stored at -113°C.

HBs Antigen Binding Assay

Principle

This ELISA test works under the principle of direct sandwich method (HEPALISA). The micro wells coated antibody specific for HBsAg was taken. The test samples were added on to the well and then enzyme conjugate antibodies linked to horse radish peroxidase were added. If the sample contains HBsAg, it binds to the monoclonal antibodies, from a direct sandwich conjugate. When substrate was added to it, a blue colour was developed. The intensity of the blue colour is proportional to the concentration of HBsAg present in the sample. Stop solution was added to stop the reaction and the absorbance was read at 450nm spectrometrically.

Procedure

Lyophilized plant extracts were solubilised with appropriate solubilising agents were taken. Equal volume of HBsAg positive serum samples and plant extracts were mixed and incubated 37°C for 24 hours. Positive (Lamivudine with positive serum samples) and Negative (PBS) controls were included. This mixture was assayed every day till day 5 using HBsAg ELISA Kit (ERBA). Binding inhibition of the extracts were analysed daily and the percentage of inhibition was calculated.

HBV DNA polymerase inhibition Assay

This assay is determined by the activity of DNA polymerase which plays an important role in replication. The addition of dNTPs to the viral extracts promotes DNA replication and the addition of plant extracts to this mixture interferes with the DNA replication and inhibits the activity of DNA polymerase if antiviral property exists. The efficacy of the plant extracts were directly proportional to the fluorescence measured.

Procedure

100µl reaction mixture consists of dATP, dGTP, dCTP (1mM), dTTP was replaced with Fluorescein - 12 - dUTP - 0.1µM (fluorochrome tagged molecule) Tris HCl - pH 8 (100mM), MgCl2 (20mM) and KCL (200mM). Equal volume of viral extracts, plant extracts, reaction mixture and buffer mixture were added to the well of 96 micro titre plates. Triplicates were made including positive and negative control and incubated at 37°C for 3 hours. Then the reaction was stopped by adding 10µl of 0.2 M EDTA. This test was measured using flourimetry and the readings were recorded and calculated by measuring the inhibition activity.

Calculation

Percentage of inhibition was calculated by the following formulae

$$100-\frac{\text{Mean sample absorbance} \times 100}{\text{Mean control-2 absorbance}}$$

Statistical Analysis

Statistical analysis of the plant extracts were done by using Two way Anova, One way Anova and DMRT(Duncan Multiple Range Test) to know their significance by using IBM SPSS Statistics version 18.

RESULTS AND DISCUSSION

The aqueous and ethanolic lyophilized extracts of *Terminalia chebula* were evaluated to study the antiviral property on Hepatitis B virus. In cytotoxicity assay, after 72 hours of incubation, the intensity of the colour on the viability of the cells was determined by MTT Assay. The results were recorded and safety dose was calculated by comparing the cells treated with the extracts and with that of untreated cells. Both aqueous and ethanolic extracts of *Terminalia chebula* were found to be non toxic at the maximum concentrations of 500µg/ml. The standard drug control Lamivudine showed MNTC (Maximum non toxic concentrations) at the highest dilution of 500µg/ml. In HBs antigen binding assay, the lyophilized ethanolic extracts of *Terminalia chebula* exhibited 60% of maximum inhibition whereas the aqueous extracts showed 55% of inhibition and the control lamivudine exhibited 60% of inhibition.

In HBV DNA polymerase inhibition assay, the lyophilized aqueous extracts registered 74% of inhibition; in the case of ethanolic extracts only 50% of inhibition was recorded. The *in vitro* antiviral activity of lyophilized extracts of *Terminalia chebula* on Hepatitis B virus as mentioned in the **table (1.1)** and the comparative analysis of both the extracts were shown in **Chart (1.1).**

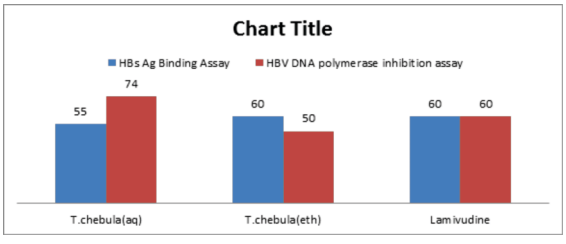
The statistical analysis of the lyophilized aqueous and ethanolic extracts of *Terminalia chebula* adopting ANOVA and DMRT has amply justified its significance at 1% and 5% level.

The aqueous extracts were found to be superior in anti HBV activity than that of the alcoholic extracts and slightly superior to the control lamivudine due to its higher percentage of HBV DNA polymerase inhibition assay. The observation of the anti HBV activity in lyophilized ethanolic extracts which is comparable and slightly superior to lamivudine is new to literature, the observation on anti HBV activity by aqueous extracts of Terminalia chebula on HBs Ag binding assay and HBV DNA polymerase inhibition assay confirm the observation of Kim *et al.,* in cell culture in comparison with lamivudine. Based on the observation, further studies suggested to determine the active HBV compound for drug development and the extracts should be are suggested subjected for separation of compounds for further studies on HBs Antigen binding assay and HBV DNA polymerase inhibition assay.

Table1.1 *In vitro* antiviral assay of *Terminalia chebula* extracts and lamivudine on Hepatitis B virus

S.No	Test Extracts	MTT Assay (MNTC)	Hbs Ag Binding Inhibition in percentage	HBV DNA Polymerase Inhibition in percentage
1.	*Terminalia chebula*(aqueous)	500 µg/ml	55	74
2.	*Terminalia chebula*(ethanolic)	500 µg/ml	60	50
3.	Lamivudine	500 µg/ml	60	60

Chart 1.1 Comparative analyses of HBs antigen binding assay and HBV DNA polymerase inhibition assay on antiviral activity of lyophilized extracts of *Terminalia chebula*



CONCLUSION

The present study has clearly revealed that the lyophilized aqueous and ethanolic extracts possess anti HBV activity comparable to Lamivudine.

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