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Assessment of Anti-Hepatitis B Virus Activity of Leaf Extract of *Morinda Citrifolia*

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Abstract

Hepatitis B virus infection remains a global health problem, it is a leading cause of liver disease and a possible worldwide source of severe morbidity and mortality. The current standard therapy using interferons or antiviral agents is not successful in all cases and is associated with severe side effects. Consequently, the development of new medicines for the treatment of HBV is still relevant. This experimental study was therefore performed to assess the anti-hepatitis B virus (HBV) potential of Leaf extract of *Morinda citrifolia*. The methanolic extracts of the Leaf extract of *Morinda citrifolia* first assessed for cytotoxicity on HepG2.2.15 cells and cytotoxicity concentration (CC50) values were resolved. The methanolic extracts of the Leaf extract of Morinda citrifolia was additionally examined on HepG2.2.15 cells for anti-HBV potential by examining the inhibition of HBsAg and HBeAg production in the culture supernatants, and calculating their half-maximum inhibitory concentration (IC50) and therapeutic index (TI) values. Out of four concentration only three exhibit inhibition of HBsAg production in an increasing dose and time

dependent manner. These four concentrations are 100 µg/mL, 200 µg/mL, 300 µg/mL and 400 µg/mL, with IC50 values of 21.15, 24.51, 118.94, 17.65, 20.93 µg/mL, respectively. Moreover, the presence of terpenoids, tannins, alkaloids and flavonoids that could contribute to antiviral efficacy was validated with a qualitative phytochemical study of active extracts.

Keywords: Assessment, Anti-Hepatitis, Virus, Activity, Leaf, Extract, *Morinda Citrifolia*

INTRODUCTION

Hepatitis B virus infection remains a global health problem. About two billion people worldwide have serologic evidence of infection, and approximately 250360 million are chronically infected and at risk for liver diseases [1]. Approximately 15-40% of HBV infected patients would develop cirrhosis, liver failure, or hepatocellular carcinoma (HCC). Consequently, about 25% of chronically infected adults will die from liver cancer or cirrhosis [2]. Both preventive and therapeutic approaches that are currently licensed, including HBV vaccines, interferons and nucleotide-nucleoside analogues have unfortunately their own limitations. Despite the success of HBV vaccination programs, mutation in HBV genome can lead to viral variants that are able to escape the vaccine [3]. Regarding therapeutic measurers, interferons have restricted effectiveness and a high incidence of adverse effects, and on treatment with nucleotide/nucleoside analogues (NAs) emergence of drug-resistance occurs eventually with long-term treatment [4]. Additionally, for most developing countries the high cost of such anti-HBV drugs is too expensive. Consequently, the production of new antivirals including novel phytoproducts with greater potency and efficacy is urgently needed.

In fact, an ongoing effort is being made to search for new anti-HBV drugs with greater effectiveness and potential from natural sources. Interestingly, it has been documented that up to 80% of the Chinese patients with chronic hepatitis B depend on herbal preparations. In addition, comparison to the treatment of traditional therapies like interferons or lamivudine, meta-analysis of clinical trials indicated that herbal preparations could have comparable or better efficacy than lamivudine in chronic hepatitis B management (Chen and Zhu, 2013). Moreover, there have been studies of several phytocompounds of various phytochemical groups having promising anti-HBV activities [5,6,7].

Socotra Island, which represents a unique region of Yemen in the Arab sea, is of global significance for conservation of biodiversity, owing to its extraordinary level of biodiversity and endemism in many terrestrial and marine classes including flora. Socotra is particularly important for its diversity of plants and has 825 plant species of which 307 (37%) are endemic [8]. In this context, we aimed in the current study to investigate the in vitro anti-hepatitis B virus (HBV) activities of ten plants from the Island of Socotra in human liver cells stably transfected with HBV genome (HepG2.2.15).

MATERIALS AND METHODS

Plant material collection and preparation

The medicinal plant was obtained from various locations on Michika Adamawa State and identified at the Agricultural Department, Faculty of Agriculture and Life sciences, Federal University Wukari. Voucher specimens were stored at the Department, the plant material (10 g of each) was dried, milled and extracted with 200 mL methanol by maceration extraction for 4 days with daily filtration and evaporation by utilizing a rotary evaporator. The dried extracts were kept at -20°C until used.

Cell cultures and drugs

HepG2.2.2.15 was grown in RPMI-1640 medium (Gibco, USA); it was supplemented with 10% heat-inactivated bovine serum (Gibco, USA), 1x penicillin- streptomycin, and 1x sodium pyruvate streptomycin (Hy Clone Laboratories, USA) at 37°C in a humified chamber with 5% CO_2 supply. Lamivudine (Sigma, USA) was used as positive control

Cytotoxicity assessment

On HepG2.2.15 cells, the cytotoxicity of the extracts was evaluated utilizing MTT cell proliferation assay kits (Tervigen, UAS) to determine concentrations that did not affect the cell viability and were utilized in the following tests according to van Meerloo et al. (2011). Cells (1×104 cells/100 µL/well) were seeded in flat bottom 96-well culture plates (Corning, UAS). After 24 h of incubation, the cells were treated (in triplicate) with different extract concentrations (100 µg/mL, 200 µg/mL, 300 µg/mL and 400 µg/mL) which were dissolved in culture media, and incubated again for 48 h. The final concentration of DMSO never exceeded 0.1% in each test and therefore, had no toxic signs. A set of blank (only media) and untreated/negative (0.1% DMSO in media) controls were also included. Cells

were treated with MTT reagent (10 µL/well) and further incubated for 3-4 hr. Upon appearance of purple colour, detergent solution (100 µL) was added to each well and further incubated for 1 hr. The optical density (OD) was recorded at 570 nm in a microplate reader (BioRad, xMark). Non-linear regression test was carried out utilizing the following equation in Excel software to assess the concentration resulting in 50% cytotoxicity (CC50):

$$\text{Survival fraction} = \frac{OD[\text{s}] - OD[b]}{OD[c] - OD[b]}$$

Where, OD[s], OD[b] and OD[c] are the absorbance of sample, blank and negative control, respectively. Cytotoxicity was also calculated as percent of cell viability relative to untreated negative control and the extract was considered cytotoxic if the cell viability is lower than 80%.

Microscopic investigation

At 1, 3- and 5-days post-treatment, cells were visually examined under an inverted microscope (Optica, Italy) with 200 × magnification for morphological changes like cell membrane lesions and the compactness of cytoplasmic components.

Dose and time dependent investigation of HBsAg expression in treated cells

HepG2.2.15 cells were seeded in 96 well plates (0.5x 104/well) and incubated at 37. Next day, the culture media was replaced with fresh media (160 µL, each in triplicate) containing different concentrations (0-80 µg/mL) of extracts and controls, and incubated. On Days 1, 3, and 5 after treatment the cultivated supernatant of each extract was harvested and stored at -20 ° C. In the cultivated supernatants, the secreted HBsAg was analysed utilizing enzyme immunoassays (ELISA) kit (Monolisa HBsAg ULTRA, BioRad, USA) in accordance with the manufacturer's instructions. A microplate reader was used to record the absorbance (OD) at 450 nm. The Excel program was used to assess the concentration (dose) of 50% inhibition (IC50). Non-linear regression analysis was performed

$$Inhibition\ \% = \left(\frac{OD[C] - OD[T]}{OD[C]}\right) X\ 100$$

Where, OD[C] and OD [T] indicated the absorbance of control and the test sample, respectively.

Time-course analysis of down-regulation of HBV replication

Based on the HBsAg inhibition results, extracts showing the promising inhibitory effects were further subjected to time-course (days 1, 3 and 5) analysis of downregulation of HBV replication by measuring HBeAg expression at the 80 µg/mL dose. The ELISA was carried out on culture media, utilizing HBeAg/Anti-HBe Elisa Kit (DIA Source, Belgium) as per the manufacturer's manual.

Phytochemical screening

The methodology proposed by [9,10,11] was used for identifying the chemical constituents of the methanol extracts using chemical methods and TLC. Multiple chemical reagents were utilized to perform the detection briefly, vanillin/HCl test for terpenoids, Dragendorf's reagent for alkaloids, sodium hydroxide test for flavonoids, ferric chloride test for tannins, born Traeger reagent for anthraquinones and frothing test for saponins.

RESULTS

Effect of plants extracts on cell viability

The cytotoxicity (CC_{50}) of different plant extracts was evaluated to determine the appropriate concentration without cytotoxicity. The microscopic observation of cell morphology / growth post treatment with various concentrations of each extract (data not shown) supported this result. Extracts of 80 µg/mL were thus used in the subsequent antiviral studies.

Dose and time dependent inhibition of HBsAg

Different non-cytotoxic concentrations of the extracts were screened for anti-HBV activities by measuring the expression levels of viral HBsAg at day 1, 3, and 5 post treatments. Of these, four of the concentration showed inhibition of HBsAg production in a dose and time dependent manner. These are 100 µg/mL, 200 µg/mL, 300 µg/mL and 400 µg/mL with IC50 values 18.44, 20.16, 21.43, 38.37 µg/mL, respectively (Figure 1). The active extracts cytotoxicity (CC50), anti-HBV activity (IC50) and their corresponding therapeutic index (TI) values were demonstrated in Table 1.

Time-course down-regulation of HBV replication

The active extracts were analyzed for time-course effect on HBeAg production, a marker of active viral DNA replication. In agreement with Anti-HBsAg activity, HBV DNA replication was inhibited in a time-dependent manner. At day 5 post-treatment, 100 μg/mL, 200 μg/mL, 300 μg/mL and 400 μg/mL down-regulated HBV replication by 0.7, 10.2, 20.8, 32.3% respectively (Figure 2). The study was terminated at day 5 by the cell overgrowth. Surprisingly, the effects on inhibition of HBV replication of plant extracts are comparable to that of the current anti HBV drug, lamivudine (2.0 μM).

Phytochemical screening

Table 2 displays the findings of the phytochemical screening. The screening of the active plants suggested the presence of various active components including flavonoids, terpenoids, alkaloid Anthraquinones and Saponins (Table 2).

DISCUSSION

Until recently, the production of anti-HBV therapies was impeded by the absence of appropriate in vitro and in vivo cell culture and animal models [12,13]. Cell lines stably transfected with HBV genome were developed to identify potential therapeutics against HBV replication. Hep G2.2.15 cell line represents one of the useful models for evaluation of anti-HBV activities of plants extracts and pure compounds by measuring expressions of HBV surface antigen (HBsAg), a marker of viral infection and HBeAg, a marker of viral DNA active replication (Lin and Kao, 2016; Arbab et al., 2015; Bonino et al., 1981). The application of this model included the original discovery of the use of lamivudine for the treatment of patients infected with HBV [14]. In the present study, we report for the first time the in vitro anti-HBV activities of of leaf extract of Morinda Citrifolia from Michika was investigated for the first time. As the cell culture model assesses the reduction of HBV antigens expression in each treatment, it does not discriminate whether the reduction is due to actual antiviral activity against viral antigens or cytotoxicity. Therefore, all the concentration of extracts was first tested for their cytotoxicity to determine the safe dose for anti-HBV studies by MTT assay. Investigation of the inhibitory effect of the plants extract on HBsAg expression resulted in identification of the plant concentration extracts with significant inhibition on the expression of HBsAg as a dose and time dependent manner at non-cytotoxic concentrations; These four concentrations are 100 μg/mL, 200 μg/mL, 300 μg/mL and 400 μg/mL with IC_{50} values from 18.44 to 38.37 μg/mL). Our

results are also in agreement with a previous study which investigated the anti-viral activity against influenza virus type A and herpes simplex virus type 1. It was indicated that D. cinnabari and two Boswellia species possessed a remarkable antiviral activity against both viral types with IC50 ranging between 0.3 and 12.5 g/mL [8].

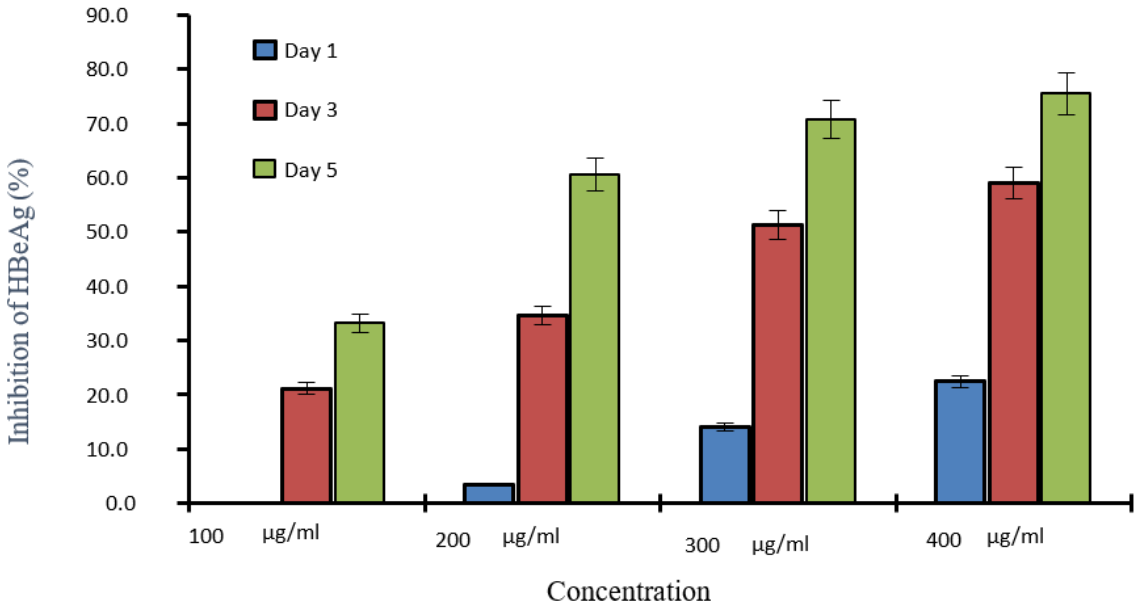

Figure 1. Dose and time dependent anti-HBV activities measured by ELISA showing inhibitions of HBsAg expression at different concentration. Values are means of 3 determinations.

Table 1. Results of cytotoxicity (CC50), anti-HBV activity (IC50) and their corresponding therapeutic index (TI) of the extracts concentration.

Extract Concentration	CC_{50} (µg/mL)	IC_{50} (µg/mL)	TI
100 µg/mL,	172.69	18.44	9.78
200 µg/mL	292.35	20.16	13.82
300 µg/mL	361.54	21.43	14.75
400 µg/mL	282.30	38.37	16.37

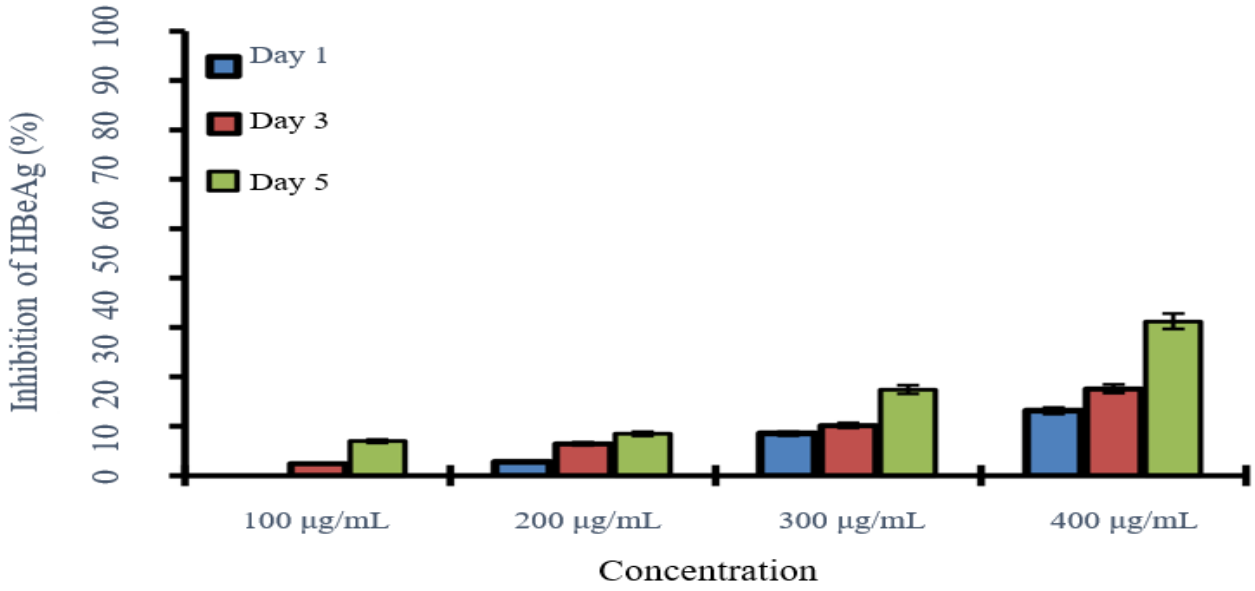

Figure 2. Effect of promising plants extracts on time-course down regulation of HBeAg expression in HepG2.2.15 culture supernatants at days 1, 3, and 5 measured by ELISA. Lamivudine (2.0 µM) was used as reference anti-HBV drug. Values are means of 3 determinations.

Table 2. Results of the phytochemical screening of the plant species showing anti-hepatitis B activity.

Plant Species	Phytochemicals				
	Terpenoids	Alkaloids	Flavonoids	Tannins	Saponins
M. citrifolia L	+	+	+	+	-
+ Detected; _- Not detected					

The HBV early antigen (HBeAg) is a secretory protein of the 'pre-Core' gene. In natural infection, seropositivity of HBeAg is a hallmark of active HBV DNA replication. The lower costs of HBeAg assay offer an opportunity for more affordable HBV DNA replication testing. It is similar to the antigen HIV 'p24' where ELISA is a reliable tool for monitoring retroviral RNA replication [16,17,18]. The most interesting active extracts have been therefore tested for time-course effect on HBeAg expression.

CONCLUSION

The current investigation has been one of the first attempts to thoroughly examine the anti-hepatitis B virus (HBV) potential of endemic medicinal plants from Michika. Findings of this investigation support the idea that medicinal plants can still be favourable source of potential antiviral agents. Our study has shown that Leaf Extract of *Morinda Citrifolia* had the most interesting anti-HBV potentials. Further work on the isolation and characterization of the anti-HBV active compounds from this active plant should be considered.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

[1]. Huang Si-Xin, Mou Jun-Fei, Qin Luo, Mo Qing-Hu, Zhou Xian-Li, Huang Xiao (2019) anti-hepatitis b virus activity of esculetin from Microsorium fortunei in vitro and in vivo. Molecules 24(19):3475.

[2]. Wu C-C, Chen Y-S, Cao L, Chen X-W, Lu M-J (2018). Hepatitis B virus infection: Defective surface antigen expression and pathogenesis. World Journal of Gastroenterology 24(31):3488–3499.

[3]. Torresi J (2008). Hepatitis B antiviral resistance and vaccine escape: Two sides of the same coin. Antiviral Therapy 13:337-340.

[4]. Gupta N, Goyal M, Wu CH, Wu GY (2014). The Molecular and Structural Basis of HBV-resistance to Nucleos(t)ide Analogs. Journal of Clininical and Translational Hepatology 2:202-211.

[5]. Huang Si-Xin, Mou Jun-Fei, Qin Luo, Mo Qing-Hu, Zhou Xian-Li, Huang Xiao (2019) anti-hepatitis b virus activity of esculetin from *Microsorium fortunei* in vitro and in vivo. Molecules 24(19):3475.

[6]. Parvez MK, Al-Dosari MS, Arbab AH, Niyazi S (2019). The *in vitro* and *in vivo* anti-hepatotoxic, anti-hepatitis B virus and hepatic CYP450 modulating potential of *Cyperus rotundus*. Saudi Pharmaceutical Journal 27(4):558–564.

[7]. Parvez MK, Al-Dosari MS, Arbab AH, Al-Rehaily AJ, Abdelwahid MAS (2020). Bioassay-guided isolation of anti-hepatitis B virus flavonoid myricetin-3-O-rhamnoside along with quercetin from *Guiera senegalensis* leaves. Saudi Pharmaceutical Journal 28(5):550–559.

[8]. Mothana RA, Mentel R, Reiss C, Lindequist U (2006). Phytochemical screening and antiviral activity of some medicinal plants from the island Soqotra. Phytotherapy Research 20(4):298-302.

[9]. Fransworth NR (1966). Biological and phytochemical screening of plants. Journal of Pharmmaceutical Sciences 55:225-276.

[10]. Marini-Bettolo GB, Nicoletti M, Patamia M, Galeffi C, Messana I (1981). Plant screening by chemical and chromatographic procedure under field conditions. Journal of Chromatography 213(1):113-217.

[11]. Wagner H, Bladt S (1996). Plants Drug Analysis: A Thin Layer Chromatography Atlas. 2nd edition. Springer, Berlin pp. 306–364.

[12]. Wu Y-H (2016). Naturally derived anti-hepatitis B virus agents and their mechanism of action. World Journal of Gastroenterology 22(1):188204.

[13]. Hantz O, Zoulim F (2004). Duck hepatitis B virus primary hepatocyte culture model. Methods in Molecular Medicine 96:189-197.

[14]. Doong SL, Tsai CH, Schinazi RF, Liotta DC, Cheng YC (1991). Inhibition of the replication of hepatitis B virus in vitro by 2',3'-dideoxy 3'-thiacytidine and related analogues. Proceedings of the National Academy of Sciences 88:8495-8499.

[15]. Mothana RA, Mentel R, Reiss C, Lindequist U (2006). Phytochemical screening and antiviral activity of some medicinal plants from the island Soqotra. Phytotherapy Research 20(4):298-302.

[16]. Jia W, Zhu MQ, Qi X, Wang T, Wen X, Chen PD, Fan QQ, Zhang W-H, Zhang JM (2019). Serum hepatitis B virus RNA levels as a predictor of HBeAg seroconversion during treatment with peginterferon alfa-2a. Virology Journal 16:61.

[17]. Schüpbach J, Böni J, Flepp M, Tomasik Z, Joller H, Opravil M (2001). Antiretroviral treatment monitoring with an improved HIV-1 p24 antigen test: An inexpensive alternative to tests for viral RNA. Journal of Medical Virology 65:225-32.

[18]. Yahaya A, Yola A, Babashani M (2006) . Comparative study on use of CD4 T cell count and plasma P24 antigen concentration in antiretroviral therapy monitoring for HIV/AIDs patients in Kano, Nigeria. Retrovirology 3(1):67.