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Assessment of anti-hepatitis B virus activity of endemic medicinal plants from Socotra Island

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Hepatitis B virus (HBV) 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 10 endemic medicinal plants from Socotra Island which represents a distinctive region of Yemen. Socotra Island is renowned for its biodiversity with significant flora with globally important plants. The methanolic extracts of the selected plants were first assessed for cytotoxicity on HepG2.2.15 cells and cytotoxicity concentration (CC_{50}) values were resolved. The methanolic extracts of the plants were 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 (IC_{50}) and therapeutic index (TI) values. Out of ten plants only five plants exhibit inhibition of HBsAg production in a dose and time dependent manner. These five plants are *Acacia pennivenia, Boswellia discorea, B. socotrana, Hypoestes pubescens and Dracaena cinnabari* with IC_{50} 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.

Key words: Antiviral, Hepatitis B, medicinal plants; Socotra, Yemen.

INTRODUCTION

Hepatitis B virus infection remains a global health problem. About two billion people worldwide have serologic evidence of infection, and approximately 250-360 million are chronically infected and at risk for liver diseases (Torres et al., 2011; Huang et al., 2019). 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 (Andre, 2006; Shepard et al., 2006; Wu et al., 2018). Both preventive and therapeutic approaches that are currently licensed, including HBV vaccines, interferons and nucleotide-nucleoside analogs 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

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(Torresi, 2008). 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 [Deng and Tang, 2011; Gupta et al., 2014). 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 (Arbab et al., 2017; Huang et al., 2019; Parvez et al., 2016, 2019, 2020; Wei et al., 2014).

Socotra Island, which represents a unique region of Yemen in the Arab sea, is of global significance for conservation of biodeversity, owing to its extraordinary level of biodiversity and endemism in many terrestrial and marine classes including flora. Socotra is particularliy important for its diversity of plants and has 825 plant species of which 307 (37%) are endemic (Mothana et al., 2009). 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 plants were gathered from various locations on Socotra Island and identified at the Pharmacognosy Department, Faculty of Pharmacy, Sana'a University. Voucher specimens were stored at the Pharmacognosy Department, Faculty of Pharmacy, Sana'a University. Each 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 (a kind gift from Dr. S. Jameel, International Center for Genetic Engineering & Biotechnology, New Delhi, India) 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 (1×10^4 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 (0, 6.25, 12.5, 25, 50 and 100 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 h. Upon appearance of purple color, detergent solution (100 µL) was added to each well and further incubated for 1 h. 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 (CC_{50}):

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

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 ($0.5\text{x } 10^4$/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 analyzed 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 (IC_{50}). Non-linear regression analysis was performed:

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

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 (DIASource, Belgium) as per the manufacturer's manual.

Phytochemical screening

The methodology proposed by Fransworth (1966), Marini-Bettolo et al. (1981) and Wagner (1996) 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, borntraeger 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 for all plants except *H. pubescens* (25 µg/ mL) in the subsequent antiviral studies.

Dose and time dependent inhibition of HBsAg

Different non-cytotoxic concentrations of the ten plants 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, five plants showed inhibition of HBsAg production in a dose and time dependent manner. These were *A. pennivenia, B. discorea, B. socotrana, H. pubescens and D. cinnabari* with IC_{50} values 21.15, 24.51, 118.94, 17.65, 20.93 µg/mL, respectively (Figure 1). The active extracts cytotoxicity (CC_{50}), anti-HBV activity (IC_{50}) 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, *A. pennivenia, B. discorea, B. socotrana, H. pubescens* and *D. cinnabari* down-regulated HBV replication by 57.5, 49.9, 23.3, 56.9, 44.7% 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 from *A. pennivenia* (80 µg/mL) and *H. pubescens* (25 µg/mL) 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 and tannins (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 (Wu, 2016; Hantz and Zoulim 2004; Thung et al., 1981). 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 (Doong et al., 1991). In the present study, we report for the first time the *in vitro* anti-HBV activities of ten plants extracts from the Island of Socotra were 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 ten plant extracts were 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 4 plant extracts with significant inhibition on the expression of HBsAg in a dose and time dependent manner at non cytotoxic concentrations; these were *A. pennivenia, B. discorea, , H. pubescens* and *D. cinnabari* with IC_{50} values from 17.65 to 24.51 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 IC_{50} ranging between 0.3 and 12.5 g/mL (Mothana et al.,

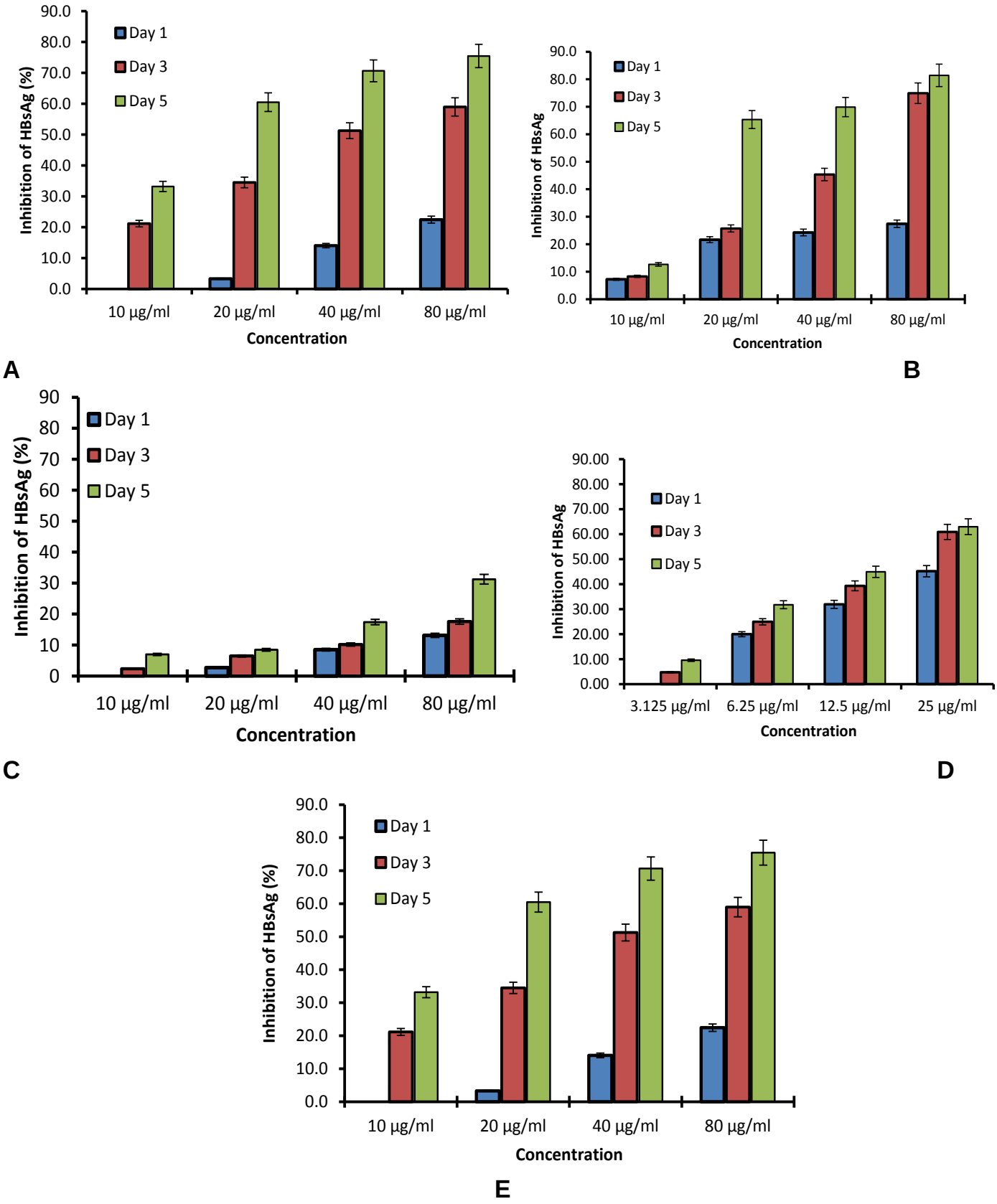

Figure 1. Dose and time dependent anti-HBV activities measured by ELISA showing inhibitions of HBsAg expression by (A) *A. pennivenia,* (B) *B. discorea,* (C) *B. socotrana,* (D) *H. pubescens* and (E) *D. cinnabari*. Values are means of 3 determinations.

Table 1. Results of cytotoxicity (CC_{50}), anti-HBV activity (IC_{50}) and their corresponding therapeutic index (TI) of the extracts.

Plant species	Family	Part used	Voucher No.	CC_{50} (µg/mL)	IC_{50} (µg/mL)	TI
Acacia pennivenia Balf. f.	Mimosaceae	L, S	Mo-Sq28	292.35	21.15	13.82
Boswellia dioscorea Thulin & Gifri	Burseraceae	B	Mo-Sq26	361.54	24.51	14.75
Boswellia socotrana Balf. f.	Burseraceae	B	Mo-Sq24	282.30	118.94	2.37
Commiphora ornifolia J. B. Gillett	Burseraceae	B	Mo-Sq23	-	-	-
Croton socotranus Balf. f.	Euphorbiaceae	L, T	Mo-Sq4	-	-	-
Dracaena cinnabari Balf. f	Agavaceae	R	Mo-Sq25	288.24	20.93	13.77
Euphorbia socotrana Balf. f.	Euphorbiaceae	L	Mo-Sq5	-	-	-
Hypoestes pubescens Balf f.	Acanthaceae	L	Mo-Sq12	172.69	17.65	9.78
Lycium sokotranum Wagner & Vierh.	Solanaceae	L, S	Mo-Sq20	-	-	-
Teucrium sokotranum Vierh.	Labiatae	F, L	Mo-Sq22	-	-	-

B, Bark; F, Flower; L, Leaves; R, Resin; S, Stems; T, Fruits; -, No measurable effect.

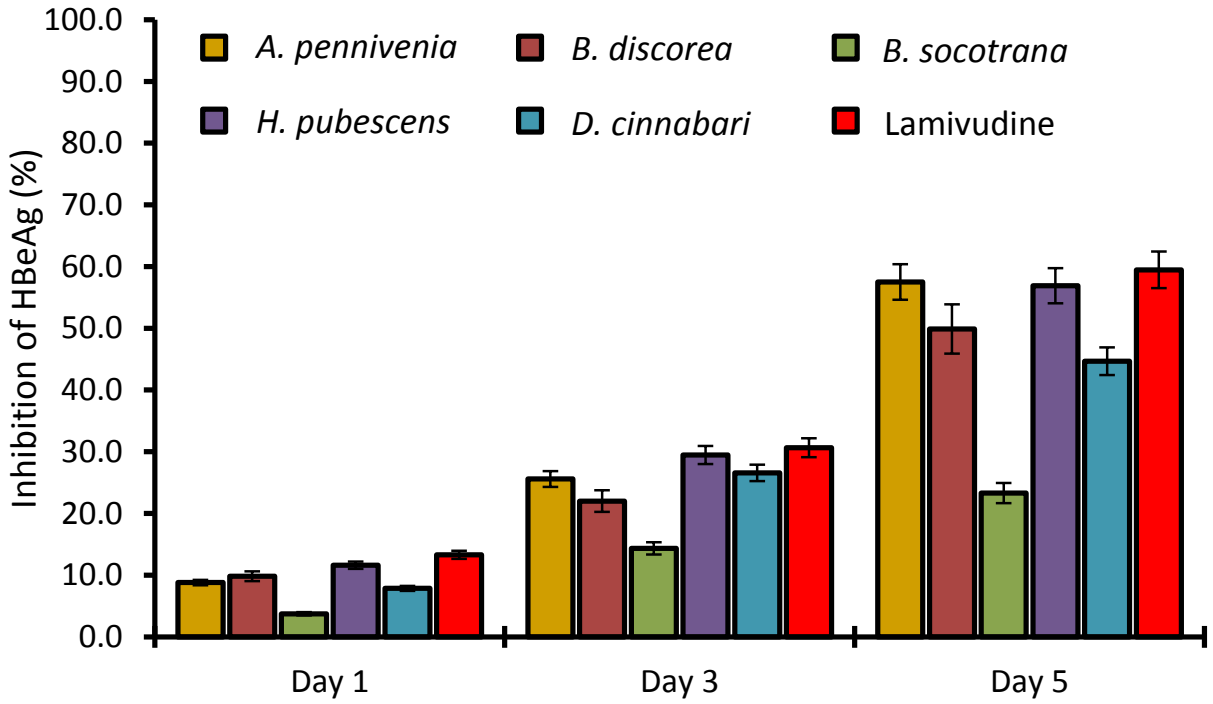

Figure 2. Effect of promising plants extracts on time-course downregulation of HBeAg expression in HepG2.2.15 culture supernatants at days 1, 3, and 5 measured by ELISA. Lamivudine (2.0 μM) was used as reference anti-HBV drug. Values are means of 3 determinations.

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

Plant species	Phytochemicals					
	Terpenoids	Alkaloids	Flavonoids	Tannins	Anthraquinones	Saponins
A. pennivenia	+	-	+	+	-	+
B. dioscorea	+	-	+	+	-	-
B. socotrana	+	-	+	+	-	-
D. cinnabari	+	-	+	+	-	-
H. pubescens	+	+	+	-	-	-

+, detected; -, not detected

2006).

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 (Jia et al., 2019; Schüpbach et al., 2001; Yahaya et al., 2006). 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 Socotra Island. Findings of this investigation support the idea that medicinal plants can still be favorable source of potential antiviral agents. Our study has shown that *A. pennivenia, B. discorea, B. socotrana, H. pubescens* and *D. cinnabari* had the most interesting anti-HBV potentials. Further work on the isolation and characterization of the anti-HBV active compounds from the active plants is in progress.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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