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In vitro evaluation of novel antiviral activities of 60 medicinal plants extracts against hepatitis B virus

AHMED HASSAN ARBAB[1,2], MOHAMMAD KHALID PARVEZ[1], MOHAMMED SALEM AL-DOSARI[1] and ADNAN JATHLAN AL-REHAILY[1]

[1]Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh 11451, Kingdom of Saudi Arabia; [2]Department of Pharmacognosy, College of Pharmacy, Omdurman Islamic University, Omdurman 14415, Republic of the Sudan

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Abstract. Currently, >35 Saudi Arabian medicinal plants are traditionally used for various liver disorders without a scientific rationale. This is the first experimental evaluation of the anti-hepatitis B virus (HBV) potential of the total ethanolic and sequential organic extracts of 60 candidate medicinal plants. The extracts were tested for toxicity on HepG2.2.15 cells and cytotoxicity concentration (CC_{50}) values were determined. The extracts were further investigated on HepG2.2.15 cells for anti-HBV activities by analyzing the inhibition of HBsAg and HBeAg production in the culture supernatants, and their half maximal inhibitory concentration (IC_{50}) and therapeutic index (TI) values were determined. Of the screened plants, *Guiera senegalensis* (dichloromethane extract, IC_{50}=10.65), *Pulicaria crispa* (ethyl acetate extract, IC_{50}=14.45), *Coccinea grandis* (total ethanol extract, IC_{50}=31.57), *Fumaria parviflora* (hexane extract, IC_{50}=35.44), *Capparis decidua* (aqueous extract, IC_{50}=66.82), *Corallocarpus epigeus* (total ethanol extract, IC_{50}=71.9), *Indigofera caerulea* (methanol extract, IC_{50}=73.21), *Abutilon figarianum* (dichloromethane extract, IC_{50}=99.76) and *Acacia oerfota* (total ethanol extract, IC_{50}=101.46) demonstrated novel anti-HBV activities in a time- and dose-dependent manner. Further qualitative phytochemical analysis of the active extracts revealed the presence of alkaloids, tannins, flavonoids and saponins, which are attributed to antiviral efficacies. In conclusion, *P. crispa, G. senegalensis* and *F. parviflora* had the most promising anti-HBV potentials, including those of *C. decidua*, *C. epigeus, A. figarianum*, *A. oerfota* and *I. caerulea* with marked activities. However, a detailed phytochemical study of these extracts is essential to isolate the active principle(s) responsible for their novel anti-HBV potential.

Correspondence to: Dr Mohammad Khalid Parvez, Department of Pharmacognosy, College of Pharmacy, King Saud University, PO Box 2457, Riyadh 11451, Kingdom of Saudi Arabia
E-mail: khalid_parvez@yahoo.com; mohkhalid@ksu.edu.sa

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Introduction

Hepatitis B virus (HBV) is responsible for ~2 billion cases of liver infection worldwide, including ~40% of chronic carriers at the risk of developing fulminant hepatitis, cirrhosis and hepatocellular carcinoma (1,2). In total, >50% of the worldwide population lives in areas where HBV infection is highly endemic and >75% of this occurs in Asia, Africa and the Middle East, including the Kingdom of Saudi Arabia (1,3,4). Unfortunately, despite their high efficacies, all currently approved drugs against chronic hepatitis B have their own limitations. While long-term treatment with nucleot(s)ide analogues lead to the emergence of drug-resistance, chemotherapy with interferon-α is associated with a high incidence of adverse effects (3,4). Despite the global success of HBV vaccination programs, vaccine-escape mutants of the virus present another bottleneck in the preventive measures (1,2). In addition, the marketed anti-HBV agents are too expensive for the majority of developing countries. Therefore, there is an urgent need to search for novel anti-HBV agents with greater efficacy and safety.

Currently, there is an ongoing effort to identify anti-HBV products from a variety of plants and natural sources. Notably, it has been estimated that ~80% of Chinese patients with chronic hepatitis B (CHB) rely on traditional herbal remedies. Compared with the treatment of conventional drugs, such as interferons or lamivudine, a meta-analysis of clinical trials suggested that herbal preparations from *Phyllanthus urinaria* and *Scutellaria baicalensis* alone may have equivalent or better effects than lamivudine in the treatment of CHB (5). Additionally, many phytocompounds of different chemical classes have been reported to have promising anti-HBV activities (6-11).

Out of >1,000 species of medicinal plants documented in Saudi Arabia, at least 35 plants are used in Saudi folk medicine for the treatment of liver disorders (12,13). However, this wealth of herbal medicine has not been subjected to sustained scientific evaluations of their anti-HBV potentials to date.

Therefore, the primary aim of the present study was to investigate the *in vitro* anti-HBV activities of 60 candidate plants and to perform a qualitative phytochemical analysis in order to identify the major secondary metabolites.

Materials and methods

Selection criteria. Candidate plants in the present study were selected on the basis of following one or more criteria: i) Claimed efficacies in treating various liver diseases in folk or traditional medicine; ii) reported *in vitro* or *in vivo* hepatoprotective potentials; iii) published antiviral activities against genetically-close human viruses, such as human immunodeficiency virus (HIV) and herpes simplex virus (HSV); and iv) taxonomically related to plants known for their antiviral activities.

Plant materials. A total of 60 medicinal plants were collected from different regions of the Kingdom of Saudi Arabia (n=57) as well as Sudan (n=3) (Table I). Plants were identified by an experienced plant taxonomist at the College of Pharmacy, King Saud University, (Riyadh, Saudi Arabia) and voucher specimens (Table I) were deposited at the college herbarium.

Preparation of the plants extracts. Dried plant materials were ground to a coarse powder using a mortar and pestle, extracted with 80% ethanol for 3 days followed by filtering with Whatman Grade 1 paper and were concentrated using a rotary evaporator (Buchi Labortechnik AG, Flawil, Switzerland) under reduced pressure at 4˚C. Plants extracts showing anti-HBV activity were further extracted sequentially with different organic solvents of increasing polarity: Hexane, ethyl acetate, dichloromethane, methanol (all from Merck, Darmstadt, Germany), including the aqueous phase. Briefly, 100 g of each plant powder was soaked in a suitable volume of hexane with intermittent shaking for 24 h, and filtered using Whatman Grade 1 paper. Each of the residues were further extracted twice with fresh solvent, and the filtrates were pooled together. The residue was air-dried followed by sequential extractions with dichloromethane, ethyl acetate, methanol and double-distilled water similar to the procedure performed for hexane. Finally, solvents were removed under reduced pressure at 4˚C using a rotary evaporator (Buchi Labortechnik AG). Following complete drying, the yield percentage of each extract was calculated (Table II). For biological screening, each extract was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Merck KGaA), and the stocks (100 mg/ml) were stored at -20˚C until subsequent use.

Cell culture and drug. The HBV reporter cell line (HepG2.2.15) was a generous gift from Dr Shahid Jameel (International Center for Genetic Engineering & Biotechnology, New Delhi, India). HepG2.2.15 cells were grown in RPMI-1640 medium (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1X penicillin-streptomycin, and 1X sodium pyruvate streptomycin (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37˚C in a humidified chamber (5% CO_2). Lamivudine (Sigma-Aldrich; Merck KGaA), the approved anti-HBV nucleoside analog-based drug was used as a standard.

Cytotoxicity assessment of the plants extracts. Cytotoxicity of extracts was tested on HepG2.2.15 cells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay kit (Tervigen, Gaithersburg, MD, USA) to determine the extract concentrations (doses) that did not affect cell viability, and were used in subsequent assays. MTT assay is based on the metabolic reduction of soluble MTT by mitochondrial enzyme activity of viable cells, into an insoluble colored formazan product which can be measured optically (14). Cells were seeded ($0.5x10^5$ cells/100 μl/well) in flat-bottom 96-well tissue culture plates (Corning Inc., Corning, NY, USA). Following 24 h of incubation, the cells were treated (in triplicate) with various concentrations of test samples (0, 6.25, 12.5, 25, 50 and 100 μg/ml) prepared in culture media, and incubated at 37˚C for 48 h. The final concentration of DMSO never exceeded 0.1% in any of the assays and therefore, had no signs of toxicity. 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 at 37˚C for 3-4 h. Upon appearance of a purple color, the detergent solution (100 μl) from the kit was added to each well and further incubated at 37˚C for 1 h. Optical density (OD) was recorded at 570 nm using a microplate reader (ELx800; BioTek Instruments, Inc., Winooski, VT, USA). Non-linear regression analysis was performed using Excel software (Microsoft Corp., Redmond, WA, USA) to determine the concentration that resulted in a 50% cytotoxicity concentration (CC_{50}) using the following equation:

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

Where OD(s), OD(b) and OD(c) are the absorbances of the sample, blank and negative control, respectively.

Microscopy. At 24 and 48 h post-treatment, cells were visually monitored for morphological changes, such as lesions of the cell membrane and the compactness of cytoplasmic components under an inverted microscope (Bio Optical, Milan, Italy) at a magnification of x200.

Dose-dependent analysis of HBsAg expression in treated cells. HepG2.2.15 cells were seeded in 96-well plates ($0.5x10^5$/well) and incubated at 37˚C. The following day, the culture media was replaced with fresh media (150 μl, each in triplicate) containing various doses (0, 6.25, 12.5, 25 and 50 μg/ml) of the test samples and controls, and incubated at 37˚C for 48 h. The culture supernatants of each sample were collected and stored at -20˚C. The secreted HBsAg in the culture supernatants was analyzed using an ELISA kit (cat. no. 72348; Monolisa HBsAg ULTRA; Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the manufacturer's instructions. OD was recorded using an ELx800 microplate reader and analyzed according to the manufacturer's instructions. Non-linear regression analysis was performed using Excel software to determine the half maximal (50%) inhibitory concentration (IC_{50}) of HBsAg secretion.

Table I. List of medicinal plants (n=60) screened in the present study.

No.	Plant name	Family	Part used	Voucher no.	Collection location
1	*Abutilon figarianum*	*Malvaceae*	L	16,082	Riyadh, KSA
2	*Acacia hamulosa*	*Fabaceae*	L + S	16,221	South, KSA
3	*Acacia asak*	*Fabaceae*	L	16,387	South, KSA
4	*Acacia ehrenbergiana*	*Fabaceae*	S	16,385	South, KSA
5	*Acacia laeta*	*Fabaceae*	S	16,390	South, KSA
6	*Acacia oerfota*	*Fabaceae*	S	16,389	South, KSA
7	*Acacia salicina*	*Fabaceae*	L	15,007	South, KSA
8	*Acacia tortilis*	*Fabaceae*	S	14,977	South, KSA
9	*Achyranthes aspera*	*Amaranthaceae*	Aerial parts (S, L, Fr)	16,011	Gabeel, KSA
10	*Albizia procera*	*Fabaceae*	L	16,182	Taif, KSA
11	*Alternanthera pungens*	*Amaranthaceae*	Aerial parts (S, L, Fr, Fl)	16,391	South, KSA
12	*Amaranthus alba*	*Amaranthacea*	Aerial parts (S, L, Fr, Fl)	16,189	Riyadh, KSA
13	*Anagallis arvensis, var. caerulea*	*Primulaceae*	Aerial parts (S, L, Fr, Fl)	16,296	South, KSA
14	*Argemone ochroleuca*	*Papaveraceae*	Aerial parts (S, L, Fr)	16,185	Taif, KSA
15	*Atriplex suberecta*	*Chenopodiaceae*	Aerial parts (S, L, Fr)	16,195	Taif, KSA
16	*Aerva Javanica*	*Amaranthaceae*	Aerial parts (S, L, Fl)	16,196	Taif, KSA
17	*Bacopa monieri*	*Scrophulariaceae*	L + S	16,300	South, KSA
18	*Balanites aegyptiaca*	*Zygophyllaceae*	B	560	Khartoum, Sudan
19	*Boerhavia diffusa*	*Nyctaginaceae*	L	16,184	Taif, KSA
20	*Bougainvillea spectabilis*	*Nyctaginaceae*	L	16,177	Taif ,KSA
21	*Capparis decidua*	*Capparaceae*	S	15,841	Tabouk, KSA
22	*Cassytha filiformis*	*Lauraceae*	S	15,716	Taif, KSA
23	*Chenopodium ambrosioides*	*Chenopodiaceae*	Aerial parts (S, L, Fr)	16,181	Taif, KSA
24	*Chenopodiumg laucum*	*Chenopodiaceae*	L + S	16,197	Taif, KSA
25	*Citrus maxima*	*Rutaceae*	L	16,173	Riyadh, KSA
26	*Cleome droserifolia*	*Crassulaceae*	Aerial parts (S, L, Fl)	15,830	Taif, KSA
27	*Clerodendrum inerme*	*Verbenaceae*	L + S	12,788	Riyadh, KSA
28	*Coccinia grandis*	*Cucurbitaceae*	L + S	16,275	South, KSA
29	*Combretum molle*	*Combretaceae*	B	15,496	South, KSA
30	*Corallocarpus epigeus*	*Cucurbitaceae*	L	16,393	South, KSA
31	*Daturai noxia*	*Solanaceae*	L	15,604	Riyadh, KSA
32	*Delonix elata*	*Fabaceae*	L	16,035	South, KSA
33	*Delonix regia*	*Fabaceae*	L	16,183	Taif, KSA
34	*Dodonea angustifolia*	*Sapindaceae*	L	15,787	South, KSA
35	*Eruca sativa*	*Brassicaceae*	L + S	16,318	South, KSA
36	*Euphorbia tirucalli*	*Euphorbiaceae*	S	16,172	Riyadh, KSA
37	*Euphorbia hirta*	*Euphorbiaceae*	Aerial parts (S, L, Fr)	16,084	Omdurman, Sudan
38	*Ficus benghalensis*	*Moraceae*	L + B	16,080	Riyadh, KSA
39	*Ficus palmata*	*Moraceae*	L	15,448	Tanoma, KSA
40	*Flaveria trineriva*	*Asreraceae*	Arial parts (S, L, Fr)	16,198	Taif, KSA
41	*Fumaria parviflora*	*Fumariaceae*	L + S	16,301	South, KSA
42	*Guiera senegalensis*	*Combretaceae*	L	798	Kordofan, Sudan
43	*Haplophyllum tuberculum*	*Rutaceae*	Aerial parts (S, L, Fl)	16,324	South, KSA
44	*Indigofera caerulea*	*Fabaceae*	Aerial parts (S, L, Fl)	16,392	South, KSA
45	*Ipomoea cairica (L.) sweet*	*Convolvulaceae*	Aerial parts (S, L, Fr)	16,075	Riyadh, KSA
46	*Indigofera tinctoria*	*Fabaceae*	Aerial parts (S, L, Fr)	16,390	South, KSA
47	*Jatropha curcas*	*Euphorbiaceae*	Seeds	15,189	Riyadh, KSA
48	*Juniperus phonicea*	*Cupressaceae*	S + L	16,179	Taif, KSA,
49	*Juniperus procera*	*Cupressaceae*	S + L	16,194	Taif KSA,
50	*Marrubium vulgare*	*Labiatae*	Aerial parts (S, L, Fr)	16,043	Hadah, KSA
51	*Momordica balsamina*	*Cucurbitaceae*	L	16,395	South, KSA

Table I. Continued.

No.	Plant name	Family	Part used	Voucher no.	Collection location
52	*Pergularia tomentosa*	*Asclepiadaceae*	Aerial parts (S, L, Fr)	16,075	Riyadh, KSA
53	*Psidiumg uajava*	*Myrtaceae*	L	16,085	South, KSA
54	*Pulicaria crispa*	*Asteraceae*	Aerial parts (S, L, Fr)	16,083	Riyadh, KSA
55	*Ricinus communis*	*Euphorbiaceae*	L	14,005	Riyadh, KSA
56	*Rumex dentatus*	*Polygonaceae*	Aerial parts (S, L, Fr)	16,186	Taif, KSA
57	*Senna obtusifolia*	*Fabaceae*	Fr	160,322	South, KSA
58	*Senna occidentalis*	*Fabaceae*	Fr	155,009	South, KSA
59	*Senna alexandrina*	*Fabaceae*	L	16,245	South, KSA
60	*Solanum surrattense*	*Solanaceae*	L	16,386	South, KSA

L, leaves; S, stems; B, bark; Fr, fruits; KSA, Kingdom of Saudi Arabia.

Time-course analysis of HBsAg inhibition. Based on the dose-dependent inhibition results, time-course (day 1, 3 and 5) analyses were performed to further investigate the antiviral potential of the most active extracts. The HBsAg expression study was performed by treating cells with the safest single-dose (50 μg/ml), as determined by the IC_{50} values.

Time-course analysis of HBeAg inhibition. Extracts exhibiting the most promising inhibitory effects on HBsAg secretions were further subjected to time-course (day 1, 3 and 5) analyses of HBeAg expression at a dose of 50 μg/ml. ELISA was performed on the culture media using an HBeAg/anti-HBe ELISA kit (cat. no. KAPG4BNE3; DIAsource ImmunoAssays; SA, Louvain-la-Neuve, Belgium) according to the manufacturer's instructions. OD was recorded using an ELx800 microplate reader, and analyzed following the DIASource manual.

Phytochemical constituent screening. Plants exhibiting the most promising anti-HBV activities were subjected to qualitative phytochemical screening for major secondary metabolites, including alkaloids, flavonoids, anthraquinones, tannins and saponins, following standard procedures (15-17) with minor modifications. Briefly, for alkaloids, the Mayer's test was performed. A total of 0.5 gm of the extract was dissolved in 2% hydrochloric acid (Sigma-Aldrich; Merck KGaA), and filtered. Mayer's reagent was freshly prepared by dissolving 0.68 g of mercuric chloride (Sigma-Aldrich; Merck KGaA) and 2.5 g of potassium iodide (Sigma-Aldrich; Merck KGaA) and made to 50 ml with distilled water. A few drops of the reagent were added to the 3 ml of extract solution in a test tube where formation of a yellowish precipitate indicated the presence of alkaloids. For flavonoids, the sodium hydroxide test was performed. A total of 5 ml of the extract solution was treated with few drops of 20% sodium hydroxide (Sigma-Aldrich; Merck KGaA) in a test tube. Formation of an intense yellow color, which becomes colorless on addition of diluted hydrochloric acid, indicated the presence of flavonoids. For tannins, the ferric chloride test was performed. A total of 0.25 gm of the extract was dissolved in 10 ml of distilled water in a test tube and few drops of 5% ferric chloride (Sigma-Aldrich; Merck KGaA) was added. Appearance of brownish green or blueish-black color indicated the presence of tannins. For saponins, the frothing test was performed. A total of 0.5 gm of the extract was dissolved in 10 ml of distilled water in a test tube and shaken vigorously. Formation of a thick persistent froth that persisted for at least 15 min indicated the presence of saponins. And lastly, for anthraquinone the Borntrager's test was performed. A total 0.5 gm of the extract residue was boiled with 5 ml of dilute hydrochloric acid and filtered while hot. The filtrate was combined with 5 ml of chloroform and shaken. The chloroform layer was transferred into a test tube and 2 ml of dilute ammonia solution (Sigma-Aldrich; Merck KGaA) was added. The appearance of a rose-pink to cherry-red color indicated the presence of anthraquinones.

Results

Effects of plants extracts on cell viability. CC_{50} values of different plants extracts were calculated (Table II), which allowed for the determination of the single optimal dose (50 μg/ml) with no sign of cytotoxicity. This observation was confirmed by microscopic observation of the cell morphology/growth at 24 and 48 h post-treatment with different concentrations of each extract (data not shown). Therefore, extracts at 50 μg/ml were used in the subsequent antiviral assays.

Dose-dependent inhibition of HBsAg by different extracts. Firstly, the total ethanolic-extracts of 60 medicinal plants were screened for anti-HBV activity by measuring the expression levels of viral HBsAg at 48 h. Of these, 9 plants showed inhibition of HBsAg production in a dose-dependent manner. These were *Abutilon figarianum*, *Acacia oerfota*, *Capparis decidua*, *Coccinea grandis*, *Corallocarpus epigeus*, *Fumaria parviflora*, *Guiera senegalensis*, *Indigofera caerulea* and *Pulicaria crispa* with IC_{50} values of 106.46, 101.46, 76.85, 31.57, 71.90, 79.84, 73.21, 84.62 and 23.10 μg/ml, respectively (Table II). Based on these results, 5 sequential extracts (hexane, dichloromethane, ethyl acetate, methanol and aqueous) of each of the 9 selected plants were prepared, and tested for cytotoxicity. The sequential extracts were further evaluated for dose-dependent

Table II. Determination of cytotoxicity concentration (CC_{50}) and anti-hepatitis B virus activity (inhibition of HBsAg secretion via IC_{50}) and the corresponding TI of the plant extracts.

Plant name	Extraction solvent	Yield (%)	CC_{50} (μg/ml)	IC_{50} (μg/ml)	TI
Abutilon figarianum	EtOH	8.71	366.67	106.46	3.44
	Hex	1.10	1700.00	NA	ND
	DCM	0.64	1375.02	99.76	13.78
	EtAc	0.48	332.30	NA	ND
	MeOH	8.02	284.70	NA	ND
	Aqua	2.21	NC	NA	ND
Acacia oerfota	EtOH	11.13	1375.00	101.46	13.55
	Hex	2.64	1150.10	NA	ND
	DCM	0.19	960.00	NA	ND
	EtAc	0.79	NC	NA	ND
	MeOH	5.54	383.30	118.90	3.22
	Aqua	9.60	422.20	106.84	3.95
Capparis decidua	EtOH	10.31	366.67	76.85	4.77
	Hex	0.27	383.30	NA	ND
	DCM	0.51	400.00	NA	ND
	EtAc	0.18	833.10	NA	ND
	MeOH	3.86	1667.67	NA	ND
	Aqua	6.86	520.00	66.82	7.78
Coccinia grandis	EtOH	8.71	219.44	31.57	6.95
	Hex	1.33	800.00	NA	ND
	DCM	0.86	480.01	57.14	8.40
	EtAc	0.32	NC	NA	ND
	MeOH	7.28	800	NA	ND
	Aqua	5.61	557.14	87.21	6.38
Corallocarpus epigeus	EtOH	7.35	1275.00	71.90	17.73
	Hex	0.86	150.00	NA	ND
	DCM	0.50	112.90	NA	ND
	EtAc	0.31	153.37	NA	ND
	MeOH	0.72	2500.00	NA	ND
	Aqua	2.61	1094.00	70.91	15.42
Fumaria parviflora	EtOH	9.35	NC	79.84	ND
	Hex	0.88	425.00	35.44	11.99
	DCM	0.62	188.10	NA	ND
	EtAc	1.02	221.98	NA	ND
	MeOH	8.10	1950.00	79.19	24.62
	Aqua	2.61	766.67	NA	ND
Guiera senegalensis	EtOH	9.76	1566.00	73.21	21.39
	Hex	0.52	3330.00	NA	ND
	DCM	0.74	200.00	10.65	18.77
	EtAc	0.62	450.10	NA	ND
	MeOH	7.32	1000.06	NA	ND
	Aqua	2.08	370.69	76.67	4.83
Indigofera caerulea	EtOH	8.61	1455.00	84.62	17.19
	Hex	0.62	230.43	NA	ND
	DCM	0.66	208.00	NA	ND
	EtAc	0.42	642.80	NA	ND
	MeOH	2.47	1566	73.21	21.39
	Aqua	4.06	1250.00	125.10	9.99
Pulicaria crispa	EtOH	12.17	686.71	23.10	29.72
	Hex	1.24	160.10	NA	ND

Table II. Continued.

Plant name	Extraction solvent	Yield (%)	CC_{50} (μg/ml)	IC_{50} (μg/ml)	TI
Pulicaria crispa	DCM	1.64	79.31	NA	ND
	EtAc	0.48	603.00	14.45	41.04
	MeOH	7.80	258.33	141.72	1.82
	Aqua	4.41	733.30	NA	ND

CC_{50}, 50% cytotoxicity concentration; IC_{50}, half maximal inhibitory concentration; Hex, hexane; DCM, dichloromethane; EtOAc, ethyl acetate; MeOH, methanol; Aqua, water; NC, non-cytotoxic; NA, not active; TI, therapeutic index; ND, not determined.

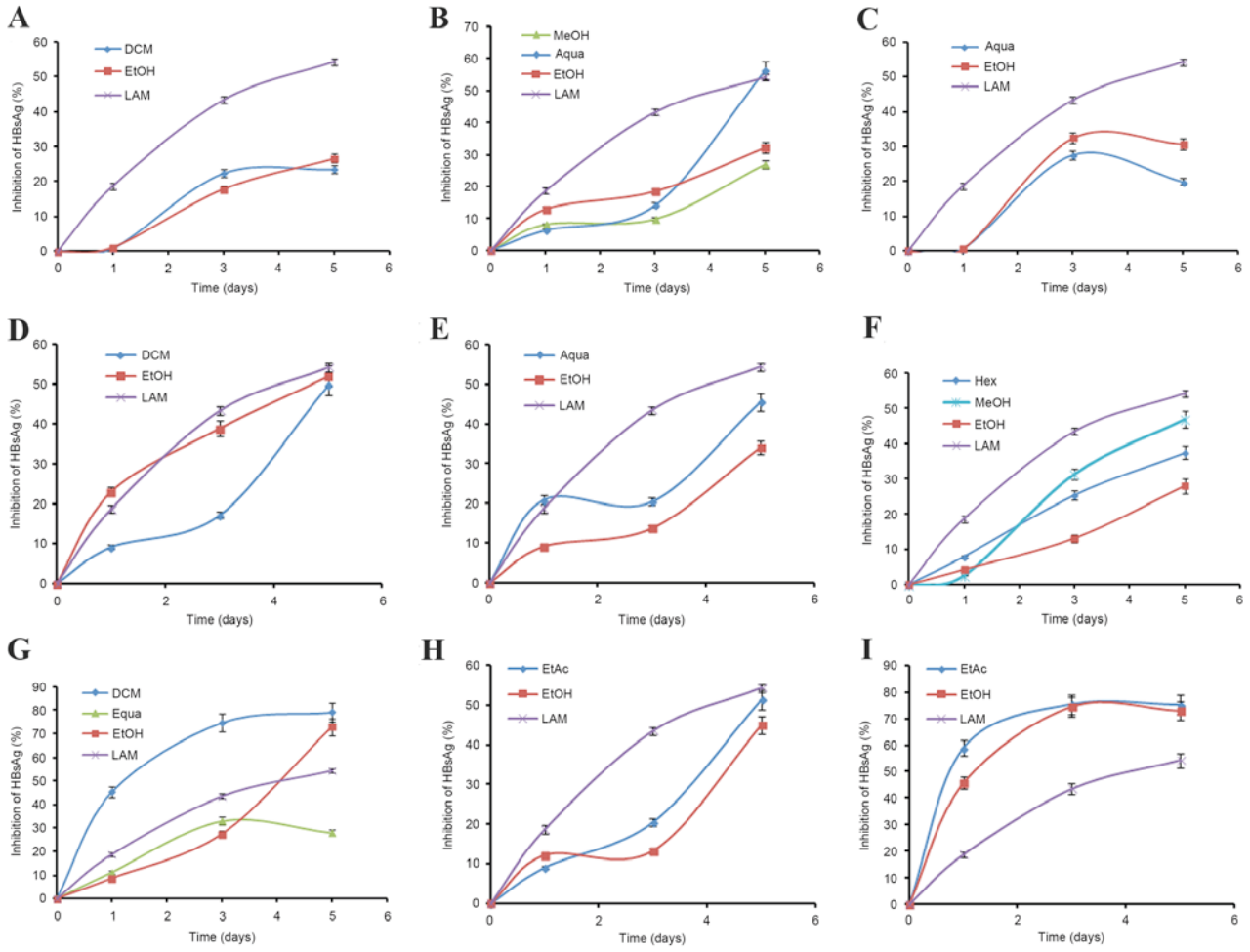

Figure 1. Time-course anti-HBV activity of selected plant extracts (50 μg/ml each). (A) *Abutilon figarianum*; (B) *Acacia oerfota*; (C) *Capparis decidua*; (D) *Co ccinia grandis*; (E) *Corallocarpus epigeus*; (F) *Fumaria parviflora*; (G) *Guiera senegalensis*; (H) *Indigofera caerulea*; (I) *Pulicaria crispa*. ELISA showing inhibitions of HBsAg expression in HepG2.2.15 culture supernatants at days 1, 3 and 5 post-treatment. Lamivudine (2.0 μM) was used as a reference anti-HBV drug. Values (y-axis): means of three determinations. HBV, hepatitis B virus; Aqua, aqueous; DCM, dichloromethane; EtAc, ethyl acetate; EtOH, ethanol; Hex, hexane; MeOH, methanol; LAM, lamivudine.

HBsAg inhibition. The extraction yield percentage, IC_{50}, CC_{50} and their corresponding TI values were calculated (Table II). Of these, 24 different extracts (from the 9 selected plants) that showed marked HBsAg inhibition were evaluated in a time-course study.

Time-course inhibition of HBsAg by selected extracts. The selected extracts (of 9 plants) that showed marked HBsAg inhibition were evaluated in a time-course study, using 50 μg/ml doses for 5 days (Fig. 1). While prolonged treatment beyond day 5 did not show any notable inhibitory effect, further continuation of the culture resulted in cell overgrowth and death (data not shown). The optimal antiviral activities, in order, on day 5 post-treatment were: *G. senegalensis* (dichloromethane extract; IC_{50}=10.65), *P. crispa* (ethyl acetate extract; IC_{50}=14.45), *C. gardis* (total ethanol extract; IC_{50}=31.57), *F. parviflora* (hexane extract; IC_{50}=35.44), *C. decidua* (aqueous extract; IC_{50}=66.82), *C. epigeus* (total ethanol extract; IC_{50}=71.9), *I. caerulea* (methanol extract; IC_{50}=73.21),

Table III. Qualitative phytochemical screening of plants with anti-hepatitis B virus activities.

	Phytochemicals				
Active plant extracts	Alkaloids	Flavonoids	Tannins	Saponins	Anthraquinones
Abutilon figarianum	+	-	-	-	-
Acacia oerfota	+	+	+	-	-
Capparis deciduas	+	+	+	+	-
Coccinea grandis	+	+	+	+	-
Corallocarpus epigeus	+	+	+	-	-
Fumaria parviflora	+	+	+	+	-
Guiera senegalensis	+	+	+	+	-
Indigofera caerulea	+	+	+	+	-
Pulicaria crispa	+	+	+	-	-

+, detected; -, not detected.

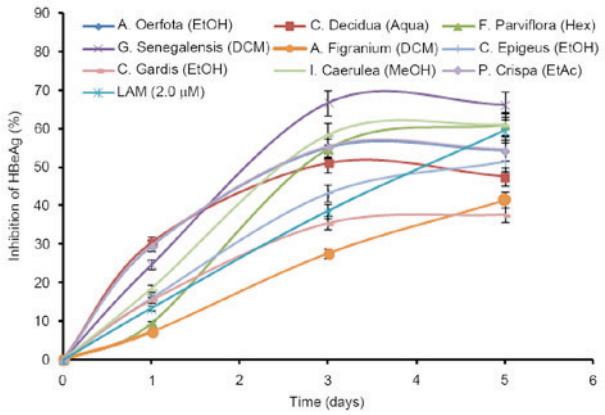

Figure 2. Time-course anti-HBV activity of the plants extracts with anti-HBV potential (50 μg/ml each). ELISA showing inhibitions of HBeAg expression in HepG2.2.15 culture supernatants at days 1, 3 and 5 post-treatment. Lamivudine (2.0 μM) was used as a reference anti-HBV drug. Values (y-axis): means of three determinations. HBV, hepatitis B virus; Aqua, aqueous; DCM, dichloromethane; EtAc, ethyl acetate; EtOH, ethanol; Hex, hexane; MeOH, methanol; LAM, lamivudine.

A. figarianum (dichloromethane extract; IC_{50}=99.76) and *A. oerfota* (total ethanol extract; IC_{50}=101.46) (Table II).

Time-course downregulation of HBV replication by the active extracts. The HBeAg is a processed product of the 'pre-Core' gene that is co-translated with the 'Core' gene by bicistronic subgenomic-RNA. Therefore, in a natural infection, seropositivity of HBeAg is a hallmark of active viral DNA replication. Notably, this is analogous to the HIV 'p24' antigen where ELISA is a valid tool to monitor retroviral RNA replication. Therefore, the most promising active extracts that greatly suppressed HBsAg synthesis (Fig. 1), were analyzed for time-course effect on HBeAg production in the culture supernatants. While HBeAg secretion was inhibited maximally at day 3, there were no further improvements in antiviral activities at day 5 (Fig. 2). Limited by cell overgrowth and death, and unaffected virus replication (inhibition of HBeAg), the study was terminated at day 5. Furthermore, the antiviral activities on downregulating virus replication, as measured by HBeAg production, were: *G. senegalensis* (dichloromethane extract; 66%); *I. caerulea* (methanol extract; 58.60%); *P. crispa* (ethyl acetate extract; 55.30%); *F. parviflora* (hexane-extract; 54.68%); *C. decidua* (aqueous extract; 51.52%); *C. epigeus* (total ethanol extract; 43.31%); *C. grandis* (total ethanol extract; 35.51%); *A. oerfota* (total ethanol extract; 35.28%); and *A. figarianum* (dichloro methane extract; 27.56%) compared to the untreated control (Fig. 2). It is noteworthy that, with the exception of *C. grandis* and *A. figarianum*, all plant extracts (50 μg/ml) showed greater antiviral activity than lamivudine (2 μM).

Phytochemical screening. Plants that exhibited anti-HBV activity showed the presence of alkaloids, flavonoids, tannins, saponins and anthraquinones (Table III), which have been previously reported for their antiviral activities (7,8).

Discussion

Development of anti-HBV therapies has been impeded until recently by the lack of suitable *in vitro* and *in vivo* experimental models that were able mimic natural chronic hepatitis B (18-21). Several lines of hepatoma cells stably transfected with HBV genome have been developed as an *in vitro* model to screen and identify potential antiviral therapeutic agents. Of these, in the present study, the widely used Hep G2.2.15 cells were used to evaluate the anti-HBV potential of candidate plants by measuring the expression of HBsAg (serological marker of viral infection) and HBeAg (serological marker of active DNA replication), respectively (22,23). A total of 60 medicinal plants were investigated for the first time, based on information on either their use in traditional medicine for curing liver diseases or experimental evidence of hepatoprotective or anti-retroviral potentials (12,13,24-37). The preliminary cell viability assay of the plants' total ethanolic extracts in the present study showed no cytotoxicity at concentrations up to 50 μg/ml. Further initial screening (dose-dependent HBsAg inhibition) identified 9 plants with notable anti-HBV activity that were therefore selected for sequential extractions (organic and aqueous phase) and subsequent screening.

The highest level of anti-HBV activity was observed in the dichloromethane extract of *G. senegalensis*, known as the 'cure all' medicine in African traditional medicine due to its wide applications (24). It is used to treat venereal, stomach, respiratory, dermatological and microbial diseases (25), including malaria (26). In agreement with its reported anti-HSV potential (27), *G. senegalensis* is likely to have exhibited anti-HBV activity because HSV and HBV are biologically and genetically similar. In addition to this, it has previously been reported that administration of *G. senegalensis* extract to Wistar rats for 6 months did not cause any significant hematological, biochemical or histological toxicity (28), confirming its *in vivo* safety. Assuming there was no synergy among the phytochemical constituents present in the dichloromethane-extract, it can be implied that the active compound(s) could be more potent than the lamivudine used as standard reference drugs.

F. parviflora has traditionally been used in Saudi folk medicine for the treatment of jaundice and hepatobiliary disorders (29). At day 5 post-treatment, hexane and methanol extracts of *F. parviflora* showed the best anti-HBV activities by ~37.45 and 46.86%, respectively. Besides its use in Sudanese traditional medicine to treat fever and jaundice, an aqueous-extract of *C. decidua* has been demonstrated to show hepatoprotective activity in rats (30). Notably, in line with its reported antiviral activity against HIV reverse-transcriptase (31), the aqueous-extract of *C. decidua* exhibited anti-HBV potential in the present study.

Acacia spp. constitute a large variety of medicinal plants worldwide, and of these, *A. catechu* has been previously reported for its anti-HIV activity (23). The present authors have previously demonstrated that *A. mellifera* ethyl acetate, n-butanol and aqueous extracts also have hepatoprotective and anti-HBV effects (32). In the present study, anti-HBV evaluation of *A. oerfota* extracts at a non-cytotoxic dose showed its association with the methanolic and aqueous extracts. Furthermore, the highest anti-HBV activity of *C. grandis* and *C. epigeus* was associated with the crude ethanolic-extract, indicating the possibility of synergy among the antiviral phytochemical constituents of the extract. Synergistic activity of antiviral components of plant extracts that act by different mechanisms has been reported previously (33). Traditionally, crude extracts of *C. grandis* are used to treat coughing, bronchitis, skin diseases, tongue sores and liver disorders (34). In previous studies, *in vivo* antioxidant and hepatoprotective efficacies of *C. grandis* (crude ethanolic-extract) and *C. epigeus* (ethanolic and aqueous extracts) have been demonstrated (35-37).

A variety of active phytochemicals (alkaloids, flavonoids, lignans, tannins, terpenoids, saponins and anthraquinones) of diverse geographic origins have already been reported to be effective against HBV *in vitro* and/or *in vivo* (7,8,38,39). Of these, promising anti-HBV phytoproducts such as picroliv (*Picrorhiza kurroa*), andrographolide (*Andrographis paniculata*), artemisinin (*Artemisia annua*) and silymarin have been reported for a long time (40). Notably, the most potent anti-HBV phytochemicals include isolated niranthin and hinokinin (lignans). From *Phyllanthus spp.* (41-43), helioxanthin from the Chinese *Taiwania cryptomerioides* (44), wogonin, another flavonoid from *Scutellaria radix* (45), the polyphenolic extract from *Geranium carolinianum* L. (46), protostane triterpenes from *Alisma orientalis* (47), dihydrochelerythrine alkaloids from *Corydalis saxicola* (48), Saikosaponin C from *Bupleurum* species (49), extracts from *Rheum palmatum* L. (50), and LPRP from *Liriope platyphylla* (51). Furthermore, the qualitative phytochemical analyses of the selected plant extracts in the present study that showed promising anti-HBV potential also revealed the presence of alkaloids, flavonoids, triterpenoids and tannin, which may have contributed to the antiviral activities observed. However, a detailed phytochemical investigation of these extracts is essential to elucidate the active principle(s) responsible for the anti-HBV potential.

In conclusion, antiviral screening in the present study discovered that extracts of *G. senegalensis*, *F. parviflora* and *P. crispa* had the most promising anti-HBV potential, followed by those of *A. figarianum*, *A. oerfota*, *C. decidua*, *C. grandis*, *C. epigeus* and *I. caerulea* with notable activities. From the results of the present study, it is possible to demonstrate the importance of the application of ethnobotanical information in the search and selection of traditionally used plants, which may provide new opportunities for the treatment of chronic hepatitis B. However, a detailed phytochemical study of these extracts is required in order to elucidate the active principle(s) responsible for their novel anti-HBV potential.

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