RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2026.030

ScienceAsia 52 (2): 2026: ID 2026030: **1–9**

The anti-hepatitis B virus efficacy of *Gymnema sylvestre* and *Hemidesmus indicus*: Cell culture and HPLC validations

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Received 9 Sep 2025, Accepted 6 Mar 2026
Available online 16 Apr 2026

ABSTRACT: Despite the availability of effective antiviral therapeutics, drug resistance in HBV still remains a clinical challenge. Nonetheless, a wide range of anti-HBV natural products comprising plant preparations or isolated compounds have been identified. Of these, some of the globally used herbal products have been reported to cause serious adverse effects, or hepatotoxicity. Here, we pre-tested the non-hepatotoxicity (dimethylthiazolyl-diphenyltetrazolium bromide; MTT assay) of *Gymnema sylvestre* (GM) and *Hemidesmus indicus* (HI) total ethanol extract (Ext) and fractions (hexane: Hex, chloroform: Chl, ethyl acetate: EtAc, and ethanol: EtOH) in HepG2 cells before their anti-HBV assessment (HBV surface antigen/HBsAg and precore antigen/HBeAg ELISA) in HepG2.2.15 cells. Because all GM and HI preparations showed no sign of liver toxicity at the maximal dose (200 µg/ml), the 50 µg/ml dose was selected as the optimal inhibitory dose for testing time-course HBV activities. Of the tested preparations, GM-EtOH (HBsAg ∼41.8%, HBeAg ∼40%) and HI-EtOH (HBsAg ∼39.6%, HBeAg ∼38%) showed moderate activities, whereas GM-Hex (HBsAg ∼54.3%, HBeAg ∼52%), HI-Hex (HBsAg ∼50.2%, HBeAg ∼48.7%), GM-Chl (HBsAg ∼47.2%, HBeAg ∼45.7%), and HI-Chl (HBsAg ∼48.8%, HBeAg ∼46.8%) exhibited high activities on day 5. Further quantitative RP-HPLC analysis identified known anti-HBV flavanols and polyphenols in GM/HI extract (rutin: 48.28, 29.07; quercetin: 36.36, 16.35; kaempferol: 3.65, 6.50; caffeic acid: 56.59, 64.13; and chlorogenic acid: 8.36, 22.62 µg/10 mg), attributed to their anti-HBV activities. Our data, therefore, convincingly and strongly warrant preclinical or clinical studies of the anti-HBV therapeutic potential of *G. sylvestre* and *H. indicus*.

KEYWORDS: anti-hepatitis B virus, *Gymnema sylvestre*, *Hemidesmus indicus*, HepG2.2.15 cells, flavanols, polyphenols

INTRODUCTION

Chronic infectious diseases caused by pathogenic viruses have significantly contributed to a substantial number of morbidity and mortality, worldwide [1]. Of these, the hepatitis B virus (HBV) remains an important pathogen of chronic liver disease in ∼300 million individuals, leading to cirrhosis or carcinoma with substantial death rates [2]. Notably, over 50% of the global population lives in high HBV-endemic countries of Asia, Africa, and the Middle East [3, 4]. HBV is primarily a hepatotropic DNA virus that uniquely replicates via an RNA intermediate, mimicking retroviruses, like human immunodeficiency virus (HIV) and herpes simplex virus (HSV). Therefore, almost all nucleos(t)ide analog-based anti-retroviral drugs have also shown effectiveness against HBV [5].

Despite vaccinations and the availability of effective antiviral drugs, the emergence of vaccine-escape and drug-resistant HBV variants in a proportion of the infected population still remains a clinical bottleneck in completely eradicating the virus [6]. Alternatively, a wide range of anti-HBV-active herbal formulations, mainly traditional Chinese medicine (TCM), and several classes of isolated phytochemicals have been identified in experimental, preclinical, or clinical settings [7, 8]. However, some of the popular anti-HBV TCM formulations have also been reported to cause serious adverse effects, including liver toxicity in clinical cases [9]. However, recently, Imide cantharidins [10] and AZD8055 [11] have been reported for significantly inhibiting HBV antigens without affecting cell viability [10, 11]. To address this, we have previously reported several non-hepatotoxic plant preparations as well as isolated flavonols, catechins, anthraquinones, and polyphenols with potent anti-HBV activities in HepG2.2.15 cell-culture model [12–14].

Gymnema sylvestre and *Hemidesmus indicus* are one of the most popular medicinal plants traditionally used in Asia, Africa, and other parts of the world. *G. sylvestre* and *H. indicus* are known to have hypoglycemic, antioxidant, anti-inflammatory, gastro-hepatoprotective, anticancer, antimicrobial, and antiviral properties [16, 17]. In an *in vitro* study, *H. indicus* hydroalcoholic extract has been shown to inhibit the early step of HSV replication [18]. However, the cell-culture based experimental study on anti-HBV potential of *G. sylvestre* and *H. indicus* still remains

obscure.

Further, although herbal products have gained much popularity due to their local accessibility, low cost, easy intestinal absorption, and bioavailability, there remain very few experimental or clinical studies reporting on their toxicity or adverse reactions. In addition, because of the possibility of compromised quality or purity of the marketed herbals and their bioactive phytoconstituents, it becomes necessary to assure their claimed quality, efficacy, and safety. For quality control, therefore, selective and highly sensitive spectroscopic (e.g., LC-MS and GC-MS) and chromatographic (e.g., HPTLC and HPLC) methods are popularly used to standardize and authenticate marketed herbal products by quantifying bioactive phytochemicals therein [19]. In this study, we have assessed anti-HBV potential of two non-hepatotoxic medicinal plants, *G. sylvestre* and *H. indicus* in HBV-reporter cells and validated their activities by identifying known anti-HBV active phytoconstituents using HPLC.

MATERIALS AND METHODS

Selection criteria and material collection

Candidate medicinal plants *Gymnema sylvestre* (family: Asclepiadaceae; common names: Australian cowplant or Gurmar) and *Hemidesmus indicus* (family: Apocynaceae; common names: Smilax, Indian sarsapalli, or Ananthamoola) were selected based on their known traditional use for gastro-hepatic disorders or experimentally proven hepatoprotective efficacy or antiviral activities against retroviruses (HIV/HSV) or presence of known anti-retroviral/HBV-active phytochemicals. The crude preparations of *G. sylvestre* (leaves) and *H. indicus* (roots) were purchased from the spice-herbal market of Wayanad, Kerala (India) in July 2022.

Plant extract and fraction preparations

Analytical grade organic solvents (Merck, GmbH, Germany) were used to extract and fractionate plant samples using standard methods. Briefly, the air-dried powder of the plant samples (leaves and roots) was extracted in ethanol (90%) at room temperature (RT), filtered, and concentrated under reduced pressure at 40 °C. The obtained dried total ethanol extract (EtOH; ∼18 g) was further fractionated in n-hexane (Hex), chloroform (Chl), ethyl acetate (EtAc), and ethanol (EtOH) to furnish respective fractions under reduced pressure (40 °C) by distillation.

Human hepatic cell culture and drugs

The human hepatoblastoma cell line HepG2 and its derivative HBV-reporter line HepG2.2.15 (provided by Dr. S. Jameel, ICGEB, New Delhi) were cultured and maintained in DMEM media (Invitrogen, USA) reformulated with 10% bovine calf serum (Invitrogen) and 1x penicillin-streptomycin mix (Invitrogen) at 37 °C with 5% CO_2 supply in a humidified incubator. For the hepatocyte viability/toxicity or anti-HBV assays, cells (0.5×10^5 cells/100 µl/well) were grown overnight in 96-well plates. While DMSO (0.1%) served as the vehicle or negative control, the anti-HBV-active *Guiera senegalensis* dichloromethane extract [10] as well as the anti-HBV drug lamivudine (LAM) acted as positive controls [11].

Hepatocyte viability and toxicity assay

Firstly, the effect of the plant preparations (total ethanol extracts) on hepatocyte viability, if any, was tested on HepG2 cells. The samples were prepared in DMSO (1 g/100 µl) and then reformulated in DMEM to produce four test doses (conc. 25, 50, 100, and 200 µg/ml). The overnight-grown HepG2 cells in 96-well plates were replaced with fresh media containing the test doses of the samples, including the negative control (0.1% DMSO), and incubated at 37 °C for 3 days. Meanwhile, a daily microscopic observation (Optica, Italy, 40× and 100×) was performed to observe any morphological changes or stress in the treated cells. To assess the cell viability and toxicity, the dimethylthiazolyl-diphenyltetrazolium bromide (MTT) assay (TACS MTT Cell Proliferation Assay Kit, USA) was employed. Briefly, following MTT treatment and incubation, the optical density (OD, $\lambda =$ 570 nm) of the samples was recorded in a microplate reader (xMark, Bio-Rad, USA). Further, a non-linear regression (Excel) was performed to determine the hepatocyte viability of the treated cultures in relation to the negative/untreated control. Notably, because all samples tested at 200 µg/ml were non-cytotoxic, their 50% cytotoxic concentration (CC_{50}) were not determined. Plant samples with even milder cytotoxicity at the maximal dose (200 µg/ml) were excluded from the study, and only the nontoxic preparations were analyzed for anti-HBV activities. Further, those with promising activities subjected to fractionation (Hex, Chl, EtAc, and EtOH) were re-tested for cytotoxic effects and anti-HBV activities. Experiments were performed using triplicate samples and repeated for reproducibility.

HBV surface antigen (HBsAg) inhibition assay

To determine the optimal inhibitory dose, firstly, a dose-response (25, 50, and 100 µg/ml, each) study of the selected non-toxic plant preparations on HBsAg expressions was performed at a single time point. Briefly, HepG2.2.15 cultures were replaced with fresh media containing respective treatment doses, including controls, and incubated at 37 °C up to 2 days. Production of HBsAg in culture supernatants was analyzed using ELISA kit (Monolisa HBs Ag ULTRA, Bio-Rad) as per the kit's instructions. Briefly, the OD ($\lambda = 450$ nm) of samples was recorded and analyzed (Excel) in relation to the negative/untreated control. Experiments were

performed using triplicate samples and repeated for reproducibility.

Based on dose-response study, an optimal inhibitory concentration was determined following the estimation of the selectivity index (SI) of the most active extracts/fractions (SI = CC_{50}/IC_{50}). Further, using the optimal inhibitory dose (50 µg/ml), a time-dependent (day 1, 3, and 5) analysis of HBsAg inhibition was also performed. Briefly, HepG2.2.15 cells were replenished with fresh media containing each test maximal dose, including controls, and incubated at 37 °C. The culture supernatants were collected on day 1, 3, and 5 and analyzed as described above. Experiments were performed using triplicate samples and repeated for reproducibility.

HBV precore/e antigen (HBeAg) inhibition assay

The HBsAg tested plant preparations (50 µg/ml, each) were further assessed for their time-dependent HBeAg inhibition in the supernatants collected at day 1, 3, and 5, using ELISA kit (HBeAg/Anti-HBe ELISA Kit, DIAsource ImmunoAssays, Belgium) as per the kit's instructions. The OD (λ = 450 nm) of samples was recorded and analyzed (Excel) in relation to the negative/untreated control. Experiments were performed using triplicate samples and repeated for reproducibility.

High-performance liquid chromatography (HPLC)

The *in vitro* anti-HBV activities of *G. sylvestre* and *H. indicus* extracts were validated by analyzing known antiviral flavonol (rutin: RUT, quercetin: QRC, kaempferol: KMF, and isorhamnetin: IRM) and polyphenol (caffeic acid: CFA and chlorogenic acid: CLA) contents, using HPLC (Alliance Chromatographic System; Waters Instruments Inc., USA). The reverse-phase (RP) analysis of the standard markers (RUT, QRC, KMF, IRM, CFA, and CLA) was carried out using a HPLC column (Pinnacle II C18: 5 µm) at room temperature. Of the different combinations of solvents optimized for the 'mobile phase', Solvent A (acetic acid:deionized water; 1:99, v/v) and Solvent B (methanol:acetonitrile; 65:35, v/v) were finally selected. The elution order was determined by the hydrophobic properties of the stationary phase and by increasing the concentration of methanol and acetonitrile in the mobile phase.

For the quantitative RP-HPLC analysis, solutions of all samples (10 mg/ml) and standards were prepared in methanol and filtered (0.45 µm; Millipore, USA) to further ensure their purity. Following sample injection (20 µl, each), data were acquired, and peak integration as well as calibrations were performed using Empower (version 3.0). Anti-HBV-active compounds were identified and quantified in the samples in relation to their retention times with those of standard compounds.

Table 1 Estimation of cytotoxic concentration (CC_{50}), HBsAg inhibitory concentration (IC_{50}), and selectivity index (SI) of the tested plants.

Plant	Most-active fraction	Conc. (µg/ml)*		SI
		CC_{50}	IC_{50}	
G. sylvestre	Hexane	1416.82 ± 0.63	48.32 ± 1.02	29.20
	Chlorine	1507.65 ± 0.82	53.75 ± 0.98	28.05
H. indicus	Hexane	1605.26 ± 0.66	50.01 ± 1.06	32.10
	Chlorine	1521.65 ± 0.78	52.86 ± 0.86	28.78

* Data of mean ± standard deviation (SD) of three determinations.

RESULTS

Non-hepatotoxicity of the *G. sylvestre* and *H. indicus* preparations

Analysis of *G. sylvestre* and *H. indicus* extracts (Ext) and their individual fractions showed no liver cytotoxicity at the maximal tested dose (200 µg/ml) with estimated CC_{50} values between 1324.54 and 1626.32 µg/ml. Interestingly therein, at doses of 100 and 200 µg/ml, *G. sylvestre* (GM-Ext) promoted HepG2 cell growth by ∼7 and ∼14%, respectively (Fig. 1; left panel), whereas *H. indicus* (HI-Ext) enhanced cell growth by ∼4 and 11%, respectively (Fig. 1; right panel). All non-cytotoxic preparations were, therefore, subjected to anti-HBV analyses. Notably, because of very low yield, the GM-EtAc and HI-EtAc fractions were excluded from the anti-HBV assays.

Preliminary anti-HBV screening and dose determination of test samples

The anti-HBV activities *G. sylvestre* and *H. indicus* preparations were first screened using 25, 50, and 100 µg/ml doses, and the level of HBsAg production in HepG2.2.15 culture was analyzed on day 2. Of the tested doses, HBsAg level was maximally inhibited at 50 µg/ml dose, which was not significantly enhanced at 100 µg/ml. For *G. sylvestre* (50 µg/ml), the inhibitions (in order) were GM-Hex: ∼31.2% > GM-Chl: ∼29% > GM-EtOH: ∼27% > GM-Ext: ∼17% (Fig. 2; left panel). For *H. indicus* (50 µg/ml), the inhibitions (in order) were HI-Hex: ∼29% > HI-Chl: ∼27.4% > HI-EtOH: ∼28.8%) > HI-Ext: ∼17.4% (Fig. 2; right panel). Notably therein, the positive control (*G. senegalensis*) suppressed HBsAg production by ∼62.6%. The 50 µg/ml dose was, therefore, selected as the optimal inhibitory dose for further analyses.

Time-dependent inhibition of HBsAg by the test samples

Of the three time points, *G. sylvestre* and *H. indicus* preparations (50 µg/ml) markedly suppressed HBsAg production on day 5. Notably, due to the cell overgrowth and subsequent apoptosis in the treated cells after extended incubation, the study was concluded

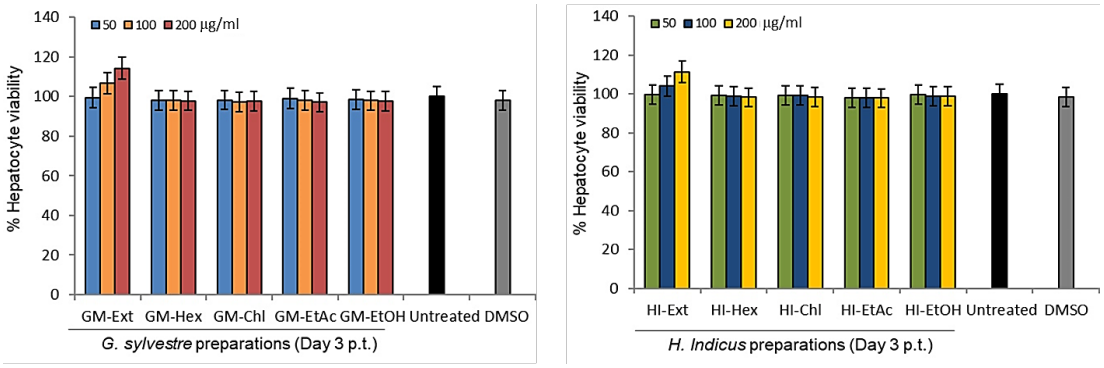

Fig. 1 MTT assay showing the effects of *G. sylvestre* (left panel) and *H. indicus* (right panel) preparations (total ethanol extract: GM-Ext, HI-Ext; hexane fraction: GM-Hex, HI-Hex; chloroform fraction: GM-Chl, HI-Chl; ethyl acetate fraction: GM-EtAc, HI-EtAc; and ethanol fraction: GM-EtOH, HI-EtOH) on HepG2 cell viability in relation to the negative control (0.1% DMSO). Post-treatment: p.t. The data are presented as mean ± standard error (triplicate values) for each sample.

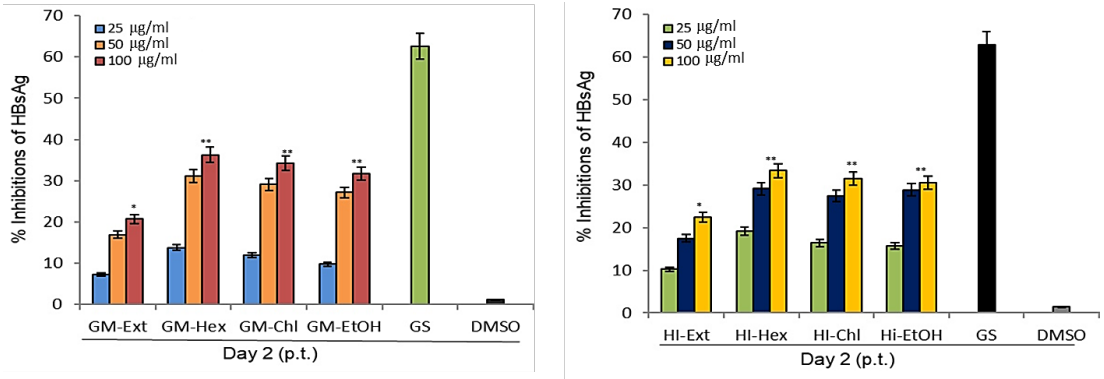

Fig. 2 Dose-dependent inhibition of HBsAg by *G. sylvestre* (left panel) and *H. indicus* (right panel) preparations (total ethanol extract: GM-Ext, HI-Ext; hexane fraction: GM-Hex, HI-Hex; chloroform fraction: GM-Chl, HI-Chl; and ethanol fraction: GM-EtOH, HI-EtOH) in relation to the negative/untreated control (0.1% DMSO) in HepG2.2.15 culture on day 2. Positive control: GS (*G. senegalensis*); Post-treatment: p.t. The data are presented as mean ± standard error (triplicate values) for each sample. * $p < 0.1$, ** $p < 0.01$ vs. GS.

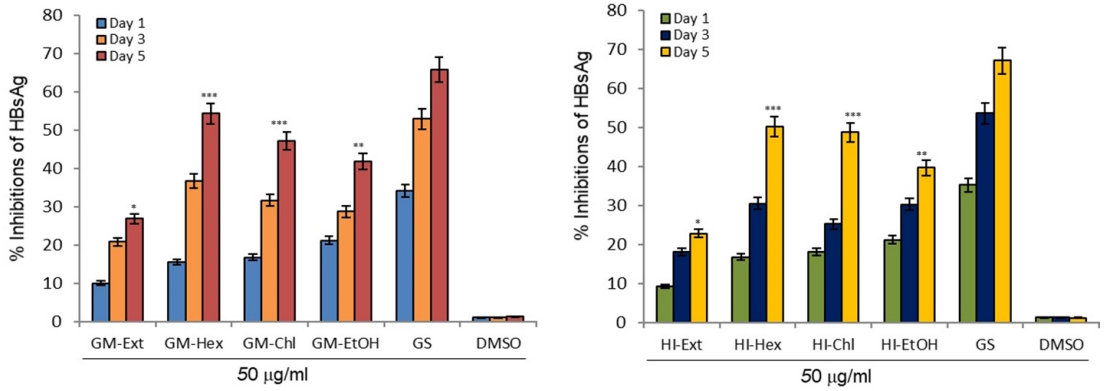

Fig. 3 Time-dependent HBsAg inhibition assay of *G. sylvestre* (left panel) and *H. indicus* (right panel) preparations (total ethanol-extract: GM-Ext, HI-Ext; hexane fraction: GM-Hex, HI-Hex; chloroform fraction: GM-Chl, HI-Chl; and ethanol fraction: GM-EtOH, HI-EtOH) in HepG2.2.15 cells in relation to the negative/untreated control (0.1% DMSO). Positive control: GS (*G. senegalensis*). The data are presented as mean ± standard error (triplicate values) for each sample. * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$ vs. GS.

Table 2 HPLC profiles of anti-HBV flavonoid and polyphenol compounds in *G. sylvestre* and *H. indicus* extracts.

Anti-HBV Compound	Retention Time	Conc. (µg/10 mg)*	
		G. sylvestre	*H. indicus*
Rutin	12.51	48.28 ± 1.03	29.07 ± 0.76
Quercetin	16.35	36.36 ± 0.59	16.35 ± 0.38
Kaempferol	18.02	3.65 ± 29	6.50 ± 1.19
Caffeic acid	10.78	56.59 ± 0.33	64.13 ± 1.05
Chlorogenic acid	9.90	8.36 ± 0.72	22.62 ± 0.35

* Data of mean ± standard deviation (SD) of three determinations.

on day 5. For *G. sylvestre*, GM-Ext and GM-EtOH showed mild (∼26.8%) and moderate (∼41.8%) activities, respectively, whereas GM-Hex (∼54.3%) and GM-Chl (∼47.2%) showed high activities (Fig. 3; left panel). For *H. indicus*, HI-Ext and HI-EtOH showed mild (∼22.8%) and moderate (∼39.6%) activities, respectively, whereas HI-Hex (∼50.2%) and HI-Chl (∼48.8%) showed high activities (Fig. 3; right panel). Comparatively, the positive control (*G. senegalensis*) suppressed the HBsAg production by ∼65–67%. Moreover, the selectivity index (SI) of the most active extracts/fractions (50 µg/ml) was determined (Table 1).

Time-dependent inhibition of HBeAg by the test samples

In line with HBsAg inhibitory activities, the *G. sylvestre* and *H. indicus* preparations (50 µg/ml) efficiently suppressed HBeAg production on day 5. For *G. sylvestre*, GM-Ext and GM-EtOH showed mild (∼24%) and moderate (∼40%) activities, respectively, whereas GM-Hex (∼52%) and GM-Chl (∼45.7%) showed high activities (Fig. 4; left panel). For *H. indicus*, HI-Ext and HI-EtOH showed mild (∼21%) and moderate (∼38%) activities, respectively, whereas HI-Hex (∼48.7%) and HI-Chl (∼46.8%) showed high activities (Fig. 4; right panel). Comparatively, the positive control (*G. senegalensis*) suppressed the HBeAg production by ∼62–64%.

RP-HPLC validations of anti-HBV-active *G. sylvestre* and *H. indicus* extracts

The RP-HPLC method utilized a selective mobile-phase based on the diverse polarities of each anti-HBV-active compounds RUT, QRC, KMF, IRM, CFA, and CLA (standards), a gradient elution system, and the optimized UV-absorbing properties of the compounds. Following the profile, a gradient flow rate of 0.8 ml/min was achieved (data not shown), and all peaks were detected ($\lambda = 280$ nm). The analysis involved comparing the retention parameters of detected compounds with their corresponding standards and assessing their peak purity using UV-visible spectral reference data. This method efficiently separated, identified, and quantified RUT, QRC, KMF, IRM, CFA, and CLA in the anti-HBV-active *G. sylvestre* (Fig. 5; Table 2) and *H. indicus* (Fig. 6; Table 2) extracts.

DISCUSSION

Although consumable medicinal plants or herbal products, particularly TCMs, are generally perceived as more effective and safer than prescription drugs, cases of their adverse effects, including acute hepatotoxicity and deaths, have been reported [9, 20]. Interestingly, while the TCMs contribute to about 50% of anti-HBV medications in China, they also have worldwide popularity. Several clinically used TCM-based herbals, including *Glycyrrhiza uralensis*, *Glycyrrhiza glabra*, *Melia toosendan*, *Phyllanthus urinaria*, *Abrus cantoniensis*, *Cecropia obtusifolia*, *Senna obtusifolia*, *Akebia quinata*, *Plantago arenaria*, *Anthriscus sylvestris*, *Gardenia jasminoides*, *Gentiana lutea*, *Scutellaria baicalensis*, *Angelica sinensis*, *Cyperus rotundus*, *Panax ginseng*, *Ligusticum wallichii*, *Pityriasis alba*, and *Rehmania glutinosa*, have been linked to significant hepatotoxicity in some patients with chronic hepatitis B [20, 21]. Unfortunately, there are very few experimental or clinical studies on herbal pharmacokinetics, leading to their limited evaluation and under-reporting of such cases and the underlying mechanisms. Also, information on their ingredients, phytochemical profiling, purity, and safety remains mostly obscure. Therefore, their experimental validation and quality control, including the disclosure of pharmacologically active ingredients, have become a necessity for assuring their claimed efficacy and safety in the treatment of chronic hepatitis B.

Cell culture-based assays are the first-line experimental evidence for identifying non-toxic and promising antiviral active plant extracts or phytochemicals. We, therefore, used the established HepG2 cells to rule out the hepatotoxicity of *Gymnema sylvestre* and *Hemidesmus indicus* preparations even at a milder level, and then tested their anti-HBV efficacy in the globally recognized HBV-reporter cell line HepG2.2.15. Notably, HepG2.2.15 cells are a derivative of the HepG2 line established with stable transfection of HBV genomic DNA (genotype D; wild-type) that allows expression of HBV proteins/antigens and DNA replication [22]. Nonetheless, because HepG2.2.15 cells harbor a stably integrated HBV genome (episomal DNA) in their chromosomes, this model does not fully recapitulate the complete HBV life cycle and dose not mimic the natural viral infection steps. This limitation, however, poses challenges for screening anti-HBV drug efficacy towards eliminating the viral episomal DNA. Moreover, due to the integrated wild-type DNA, testing new drugs against drug-resistant viral strains (polymerase gene mutants) is restricted.

Analysis of *Gymnema sylvestre* and *Hemidesmus indicus* extracts and fractions showed no sign of liver cytotoxicity at the maximal tested dose (200 µg/ml). Interestingly, both plants mildly enhanced cell growth. Further, of the tested treatment doses of all non-toxic preparations for anti-HBV activities, the 50 µg/ml dose was selected as the optimal inhibitory dose. In

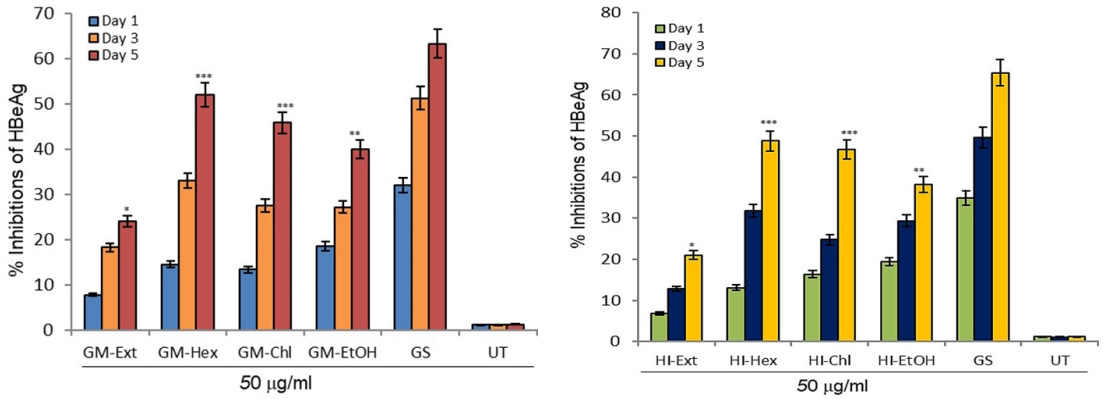

Fig. 4 Time-dependent HBeAg inhibition assay of *G. sylvestre* (left panel) and *H. indicus* (right panel) preparations (total ethanol-extract: GM-Ext, HI-Ext; hexane fraction: GM-Hex, HI-Hex; chloroform fraction: GM-Chl, HI-Chl; and ethanol fraction: GM-EtOH, HI-EtOH) in HepG2.2.15 cells in relation to the negative/untreated control (UT; 0.1% DMSO). Positive control: GS (*G. senegalensis*). The data are presented as mean ± standard error (triplicate values) for each sample. * $p < 0.1$, ** $p < 0.01$, *** $p < 0.001$ vs. GS.

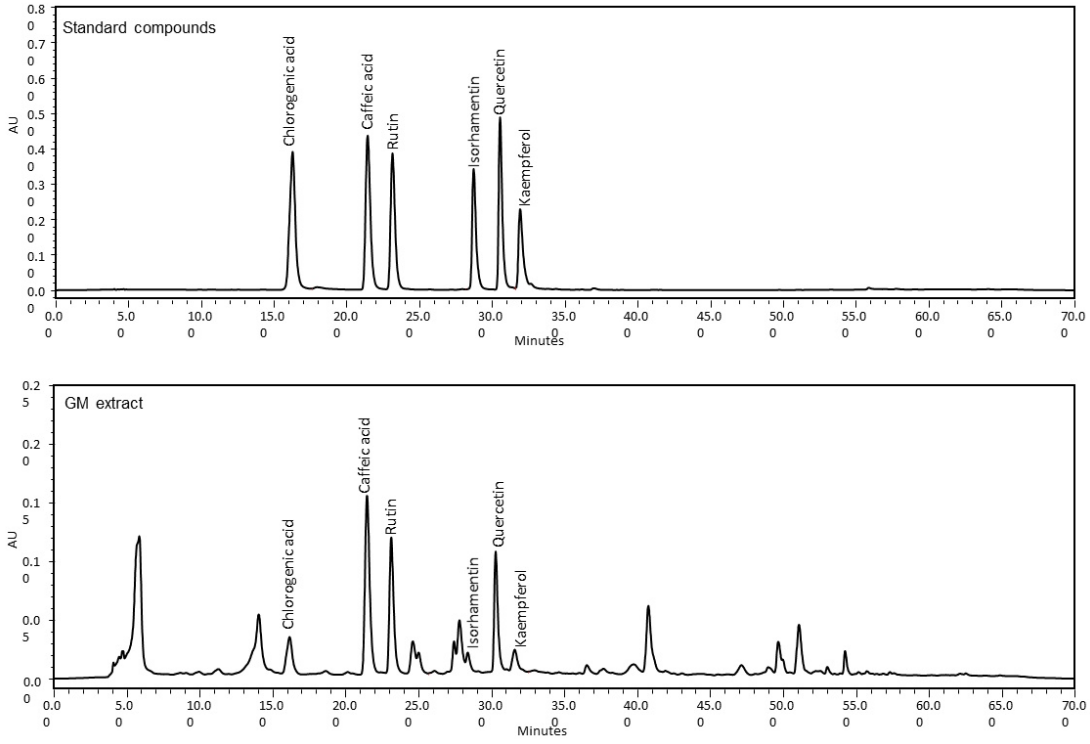

Fig. 5 HPLC chromatograms showing quantitative detection of quercetin, rutin, kaempferol, chlorogenic acid, and caffeic acid in *G. sylvestre* (GM) extract.

the time-dependent analysis, *Gymnema sylvestre* and *Hemidesmus indicus* preparations optimally suppressed HBV antigens (HBsAg and HBeAg) production on day 5. Notably, because both plants had shown cell growth-stimulatory activities, extended treatment time caused cell overgrowth and apoptosis; therefore, the study was terminated on day 5. Therein, for both *Gymnema sylvestre* and *Hemidesmus indicus*, compared to other preparations, their hexane and chloroform fractions demonstrated high activity by efficiently inhibiting the levels of HBV antigens by 47–52%.

Because the data obtained from a single experimental method may not be sufficient to ensure the reliable quality and safety of therapeutic products,

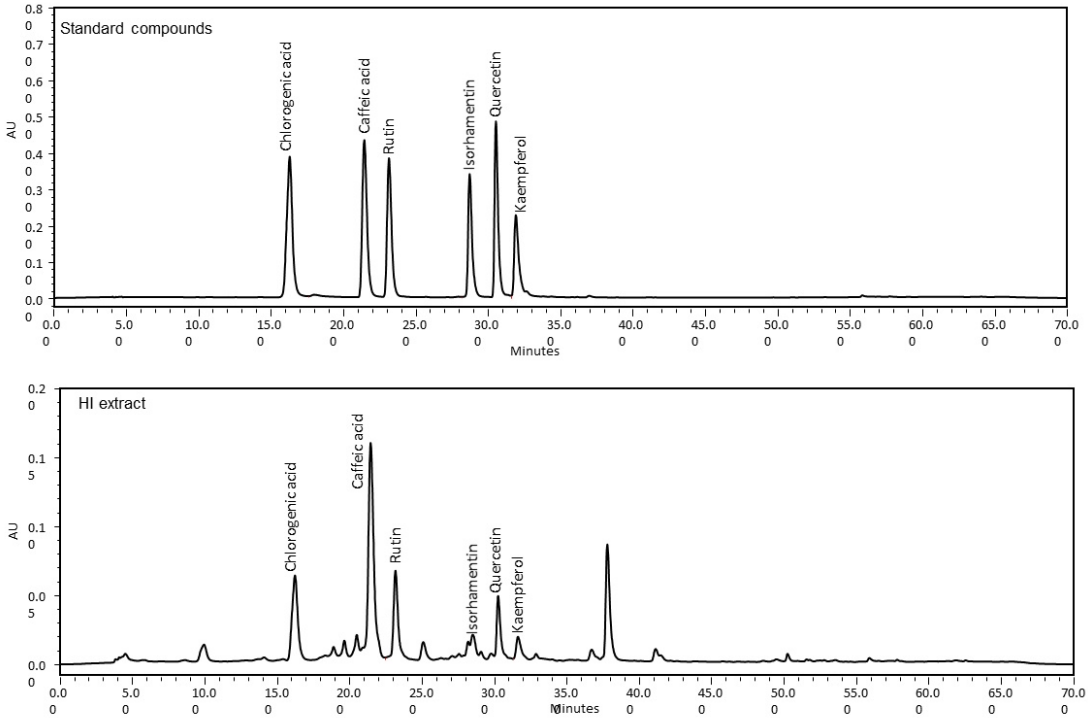

Fig. 6 HPLC chromatograms showing quantitative detection of quercetin, rutin, kaempferol, chlorogenic acid, and caffeic acid in *H. indicus* (HI) extract.

better standardization methods are required for their further clinical studies and approval. Of such methods, with more sophisticated instrumentation and efficient column materials, HPLC has become precisely more reliable tool. In view of this, we have validated the cell culture-proven anti-HBV-active extracts of *Gymnema sylvestre* and *Hemidesmus indicus*, using the RP-HPLC method. Therein, high contents of known anti-HBV flavanols (quercetin, rutin, and kaempferol) and polyphenols (chlorogenic acid and caffeic acid) were detected in both plants.

Natural bioactive flavonoids and polyphenols are the most abundantly produced phytochemicals with known therapeutic potentials against several metabolic and infectious diseases [7, 8]. Rutin is a pharmacologically active flavonol known for its *in vitro* antiviral effects against avian influenza virus (H5N1) and murine norovirus (MNV) [23, 24]. Recently, we have also reported its anti-HBV efficacy in HepG2.2.15 cells [8]. Quercetin, the aglycone derivative of rutin, has been shown to exhibit *in vitro* activities against HIV, HSV, parainfluenza type 3, pseudorabies virus, respiratory syncytial virus (RSV), and Sindbis virus (SNV) [25–28]. In addition to quercetin, its derivatives quercitrin-3-*O*-glucuronide and quercitrin-3-*O*-rhamnoside were recently reported for anti-HBV activities in HepG2.2.15 cells [12]. Kaempferol and its derivatives also possess antiviral activities against influenza virus (INV), enterovirus, Japanese encephalitis virus (JEV), dengue virus (DNV), and HSV [30–32]. Notably, while kaempferol-3,7-bisrhamnoside has been shown to exhibit strong antiviral activity against hepatitis C virus (HCV) [33] and HBV [34], we have further demonstrated the anti-HBV potential of kaempferol-3-*O*-glucuronide in HepG2.2.15 cells [12]. Structurally, isorhamnetin has an additional 3′-methyl group as compared to quercetin and kaempferol. However, although there are few *in vitro* studies on its antiviral activities, it has been shown to suppress INV [34] and HBV activities [14]. Caffeic acid and chlorogenic acid are structurally related bioactive polyphenols. While caffeic acid is known for marked antiviral activities against HSV, INV, HCV, severe fever with thrombocytopenia syndrome virus (SFTV), poliovirus, and vaccinia virus [35–38], chlorogenic acid has been reported for its activities against HIV, HSV, and adenovirus [39]. However, in a single study, both have been demonstrated to have strong anti-HBV potential in HepG2.2.15 cells as well as in a duck model [40].

CONCLUSION

To address the issues of drug resistance in HBV as well as herb-drug interaction-induced serious clinical side effects, including liver toxicity, our data on the non-hepatotoxic extracts and organic fractions of *Gymnema*

sylvestre and *Hemidesmus indicus* have demonstrated their antiviral therapeutic potential against HBV infection in a cell culture model. Further quantitative HPLC identification of known antiviral phytochemicals, including quercetin, rutin, kaempferol, chlorogenic acid, and caffeic acid in both plants validated their significant contributions to suppressing HBV antigens. Our data, therefore, convincingly and strongly warrant preclinical or clinical studies on the anti-HBV therapeutic potential of *Gymnema sylvestre* and *Hemidesmus indicus*.

Acknowledgements: This work was supported by the Ongoing Research Funding program (ORF-2026-379), King Saud University, Riyadh, Saudi Arabia.

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