The anti-hepatitis B virus activity of sea buckthorn is attributed to quercetin, kaempferol and isorhamnetin

MOHAMMAD K. PARVEZ, MOHAMMED S. AL-DOSARI, OMAR A. BASUDAN and RASHED N. HERQASH

Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh 11451, Saudi Arabia

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Abstract. The present study assessed the *in vitro* anti-hepatitis B virus (HBV) effects of cold-adapted sea buckthorn (*Hippophae rhamnoides*). Sea buckthorn leaf ethanol extracts subjected to chloroform (SB-Chl), ethyl acetate (SB-Eac), *n*-butanol (SB-But) and aqueous (SB-Aqu) fractionation were first examined (MTT assay) for their toxic effects on HepG2 cells. While SB-Chl (IC_{50}, 32.58 μg/ml) exhibited high cytotoxicity, SB-Eac, SB-But SB-Aqu were non-toxic at up to 150 μg/ml. High performance liquid chromatography analysis led to the identification of the anti-HBV active flavonols, quercetin (93.09 μg/g), kaempferol (44.19 μg/g) and isorhamnetin (138.75 μg/g) in the extract. The analysis of the anti-HBV effects of SB-Eac, SB-But and SB-Aqu (50 μg/ml, each) on HepG2.2.15 cells revealed the marked inhibition of HBsAg and HBeAg expression levels. At the concentration of 10 μg/ml, quercetin and kaempferol exerted potent inhibitory effects on HBsAg (60.5 and 62.3%, respectively) and HBeAg synthesis (64.4 and 60.2%, respectively), as compared to isorhamnetin (30.5 and 28.4%, respectively). The HBV-polymerase inhibitor drug, lamivudine (2 μM), inhibited HBsAg and HBeAg expression by 87.4 and 83.5%, respectively. The data were in good agreement with a previous *in vitro* and *in silico* molecular docking analysis performed by the authors where quercetin, kaempferol and lamivudine had formed stable complexes with HBV-polymerase binding-pocket amino acids. On the whole, to the best of our knowledge, the present study provides the first report of the anti-HBV therapeutic potential of sea buckthorn, attributed to quercetin, kaempferol and isorhamnetin.

Introduction

Sea buckthorn (genus, *Hippophae*; family, Elaeagnaceae) is a cold-adapted thorny shrub native to Asia and Europe (1). Of the three Indian species of sea buckthorn [*Hippophae rhamnoides* (*H. rhamnoides*), *Hippophae salicifolia* and *Hippophae tibetana*], *H. rhamnoides* L. ssp. *turkestanica* is the main one in the high-altitude Himalayan-Karakoram ranges. Sea buckthorn has been traditionally used in Asian, Nordic and Baltic countries for food, medicine, fodder, fuel and fencing (2,3). In traditional Chinese medicine and the Tibetan Amchi system, its leaves and berries are used to treat asthma, skin diseases and gastric ulcers. Further experimental studies have revealed its antioxidant, immune-modulatory, anti-atherogenic, anti-stress and tissue-regenerative properties, as well as its efficacy against jaundice, liver and gastrointestinal disorders (2-8). Phytochemical studied of sea buckthorn have led to identification of a range of bioactive compounds such as β-carotene, lycopene, lutein, zeaxanthin, quercetin, kaempferol, isorhamnetin, β-sitosterol, ursolic acid, oleanolic acid, and palmitic acid etc. in its berries (9-14). In addition, several bioactive flavonols, leucoanthocyanidins, epicatechin, gallocatechin, epigallocatechin, gallic acid, including quercetin and kaempferol derivatives have been reported in its leaf extracts (15,16).

Hepatitis B virus (HBV) the DNA virus, remains a globally crucial hepatovirus, which causes chronic liver diseases, such as cirrhosis and hepatocellular carcinoma in approximately three million individuals worldwide (17). Although there are effective vaccines and potent anti-HBV drugs (polymerase inhibitors) available, the emergence of drug-resistant viral mutants remains an obstacle to its complete eradication (18,19). In recent decades, several herbal formulations and phytochemicals, such as flavonoids, terpenes, saponins, lignans, alkaloids and anthraquinones have been reported as potential antiviral agents against numerous pathogenic RNA and DNA viruses, including HBV (20-24). Quercetin, isorhamnetin and kaempferol are natural flavonoids with various health protective salutations due to their potent anti-inflammatory, antioxidant and anti-carcinogenic properties (25). Notably, quercetin and kaempferol, as well as their glucoside and rhamnoside derivatives have been shown to possess enhanced antiviral activities against human immunodeficiency virus (HIV) and herpes simplex virus (HSV), genetically close to HBV (21). In line with this finding, the authors have recently reported quercetin and kaempferol, including their derivatives, as well as anthraquinones and catechins with promising anti-HBV activities in cell-culture model (26-32).

Correspondence to: Professor Mohammad K. Parvez, Department of Pharmacognosy, College of Pharmacy, King Saud University, 23 King Khalid Road, Daraiya, Riyadh 11451, Saudi Arabia
E-mail: mohkhalid@ksu.edu.sa; khalid_parvez@yahoo.com

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Sea buckthorn leaf extract has been reported to exhibit anti-dengue virus (DNV) activity (33), and its phenol-rich fraction has been demonstrated to include myricetin, quercetin, kaempferol and isorhamnetin (34). In addition, hiporamin isolated from its leaves has been identified for its potent activities against influenza virus, HIV and HSV (35). However, to the best of our knowledge, the anti-HBV activity of sea buckthorn has not yet been investigated. Thus, the present study assessed the anti-HBV potential of sea buckthorn aerial parts and its bioactive flavonols using a HBV-reporter cell culture model.

Materials and methods

Plant material collection. The fresh aerial parts of sea buckthorn (*H. rhamnoides* L.) were collected from the Karakoram-Indus valley of Pheh, Ladakh (3180 m above sea level), India in late July, 2018 (Fig. 1, upper panel). The plant was identified by a local herbalist-Amchi practitioner (Leh, Ladakh), and further validated by an expert taxonomist, Dr Tariq Husain (National Botanical Research Institute, Lucknow, India).

Extraction and fractionation. Analytical grade solvents (Sigma-Aldrich, Merck KGaA) were used for the extraction and fractionation of sea buckthorn, using standard methods. Briefly, the air-dried powder of the aerial parts (410 g) of sea buckthorn were extracted with 90% ethanol (3x250 ml) at room temperature with continuous shaking till exhaustion. The combined alcoholic-extracts were filtered (Whatman paper no. 1) and concentrated under reduced pressure at 40˚C, using rotary evaporator (Buchi Rotavapor; Model R-215, Thermo Fisher Scientific, Inc.). The obtained dried ethanol-extract (198.7 g) was dissolved in water and successively fractionated with chloroform (SB-Chl; 4.12 g), ethyl acetate (SB-Eac; 2.1 g) and finally with *n*-butanol (SB-But; 62.9 g), along with the aqueous part (SB-Aqu; 122.4 g) to yield the corresponding fractions. For each fraction, solvents were completely evaporated to dryness, and maintained at 4˚C until analysis.

High performance liquid chromatography (HPLC). Based on the published literature, the sea buckthorn extract was subjected to HPLC analysis for the presence of the antiviral flavonoids: Quercetin, kaempferol and isorhamnetin (34,36). The Alliance chromatographic system (Waters Instruments, Inc.) was used for HPLC analysis equipped with built-in quaternary pump, dual wavelength absorbance detectors and autosampler at 25˚C. For separation, an Agilent HC-C18 column (5 μm, 250x4.6 mm) was maintained with the gradient flow rate (1 ml/min) of the mobile phase (A, acidified water:1% acetic acid, v/v; and B, methanol:acetonitrile, 80:20, v/v) and peaks were detected at 370 nm. The gradient was programmed as follows: 0-9 min, 0-35% B; 9-13 min, 35-60% B; 13-18 min, 60-80% B; 18-30 min, 80-90% B. For obtaining the calibration curve, standard stock solutions of quercetin, kaempferol, and isorhamnetin were prepared in methanol (0.5 mg/ml). Furthermore, gradient concentrations (0.01-100.0 μg/ml) of the three compounds were prepared by serial dilution for identifying and quantifying them in the sea buckthorn extract.

Human cell culture and drugs. Two human hepatoma cell lines [HepG2 and its derivative, HepG2.2.15 (HBV-reporter cells)] were generously provided by Dr Shahid Jameel (International Center for Genetic Engineering and Biotechnology, New Delhi, India). The cells were cultured in T25 flasks (BD Biosciences, San Jose, CA, USA) using DMEM reconstituted with 10% bovine calf serum and 1X penicillin-streptomycin mix (all from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37˚C with a 5% CO_2 supply. The anti-HBV drug lamivudine, as well as natural quercetin, kaempferol and isorhamnetin (all from Sigma-Aldrich, Merck KGaA) were procured.

Cell viability or cytotoxicity assay. The four sea buckthorn fractions (SB-But, SB-Chl, SB-Eac and SB-Aqu; 3 mg/ml each) were dissolved in 100 μl dimethyl sulfoxide (DMSO; Sigma-Aldrich, Merck KGaA) and reconstituted in DMEM to yield various working concentrations (50, 100 and 150 μg/ml). Likewise, isorhamnetin, quercetin and kaempferol (0.5 mg, each) were first dissolved in 50 μl DMSO and reconstituted in DMEM (1 mg/ml, final). HepG2 cells ($0.5x10^5$/100 μl/well) in a 96-well cell culture plate (BD Biosciences) were grown overnight and treated with the sea buckthorn fractions (in triplicate) the following day after an incubation period of 72 at 37˚C h. DMSO (0.1%) served as a vehicle or untreated control. The cytotoxicity induced by the sea buckthorn fractions, if any, was evaluated using MTT assay (TACS MTT Cell Proliferation and Viability Assay kit, Tervigen; Thermo Fisher Scientific, Inc.) according to instructions provided with the kit. The optical density (OD) of each sample was recorded using a microplate reader (ELx800; BioTek Instruments, Inc.). Non-linear regression analysis was performed using Excel software (2010; Microsoft Corporation) to estimate the 50% maximal cytotoxic concentration (CC_{50}) in relation to the untreated control. The experiment was repeated twice under the same conditions for reproducibility.

HBV surface protein (HBsAg) inhibition assay. To assess the inhibitory effects of the sea buckthorn fractions on HBsAg expression levels, the HepG2.2.15 cells were seeded ($0.5x10^5$ cells/100 μl/well) in a 96-well plate and grown overnight. The cells were treated with the non-cytotoxic SB-But, SB-Eac and SB-Aqu fractions (25, 50 and 100 μg/ml, each) as well as with isorhamnetin (10, 20 and 50 μg/ml), quercetin (10 μg/ml) and kaempferol (10 μg/ml). Lamivudine (2 μM) served as the standard, whereas DMSO (0.1%) functioned as a negative or untreated control. Following incubation at 37˚C, culture supernatants were collected on days 1, 3 and 5, and stored at -20˚C for further analysis. HBsAg production was quantified using the Monolisa HBsAg ULTRA Elisa kit (Bio-Rad Laboratories, Inc.) according to the instructions provided with the kit. The OD (λ=570 nm) of the samples was recorded and analyzed in relation to the untreated control, and non-linear regression was performed using Excel software (2010; Microsoft Corporation) to estimate percent inhibitions. All samples were tested in triplicate and the experiment was repeated twice.

HBV pre-core protein (HBeAg) inhibition assay. Based on the HBsAg inhibition data, the SB-But, SB-Eac and SB-Aqu

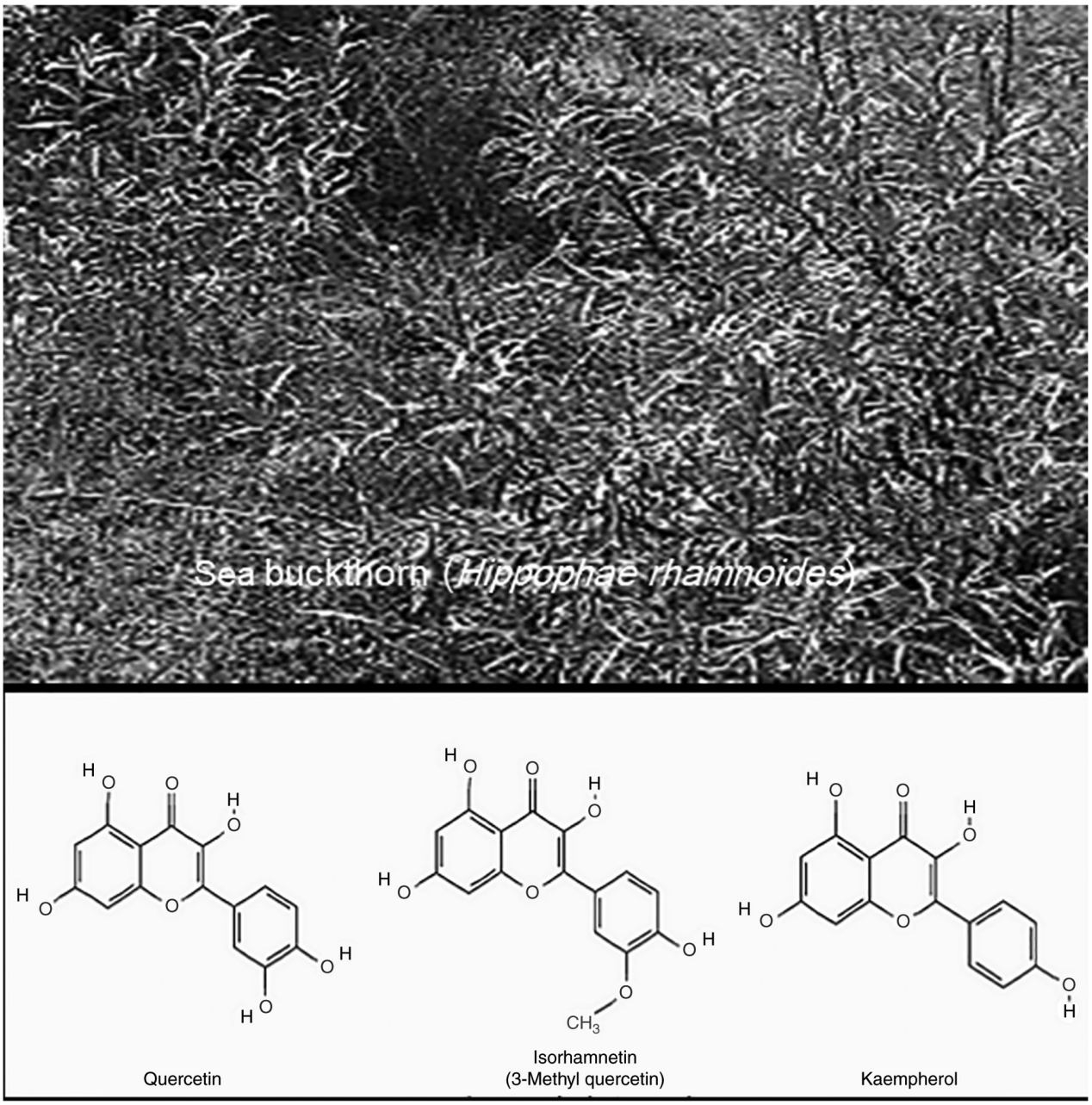

Figure 1. Sea buckthorn (*Hippophae rhamnoides* L.) whole plant collected from Ladakh, India (upper panel) and the chemical structures of its bioactive flavonols: Quecrcetin, isorhamnetin and kaempherol (lower panel).

fractions (50 μg/ml, each) as well as IRM, QRC and KMP (10 μg/ml, each) were further tested for their time-course inhibitory effects on HBeAg synthesis in HepG2.2.15 cells. The secretion of HBeAg was analyzed using the HBeAg/Anti-HBe Elisa kit (DIAsource ImmunoAssays, Belgium) according to the instructions provided with the kit. The OD (λ=570 nm) of the samples was recorded and analyzed in relation to the untreated control, and non-linear regression was performed to determine percent inhibitions. All samples were tested in triplicate and the experiment was repeated twice.

Statistical analysis. All data from all the analyzed samples (in triplicate) are expressed as the mean ± SEM. In a set of data, the determination of total variation was performed using one-way analysis of variance (ANOVA), following the Dunnett's test. Statistical analyses were performed using Excel software (2010; Microsoft Corporation). A value of $P<0.01$ was considered to indicate a statistically significant difference.

Results and discussion

Quantification of antiviral flavonoids in sea buckthorn extract. HPLC analysis of the sea buckthorn extract identified three antiviral flavonols isorhamnetin, quercetin and kaempferol (Fig. 2), and were quantified to be 138.75, 93.09 and 44.19 μg/g, respectively. This was in line with the previous demonstration of these flavonoids in the phenol-rich fraction of sea buckthorn leaves (34). Flavonols are natural flavonoids with variations in their heterocyclic (C_6-C_3-C_6) carbon ring (36). The detected

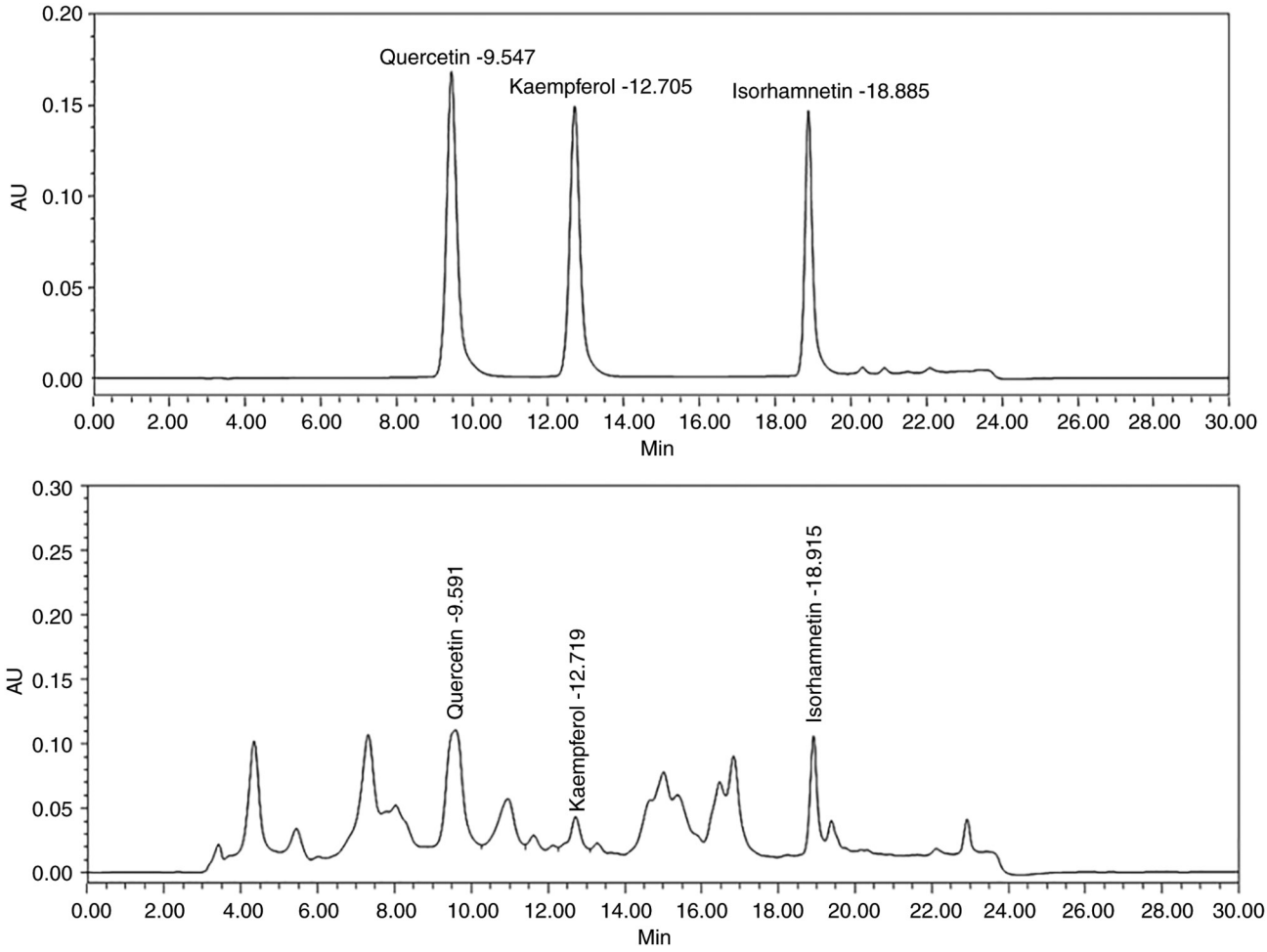

Figure 2. High performance liquid chromatography chromatograms of standard flavonols: quercetin, kaempferol and isorhamnetin (upper panel) and sea buckthorn extract (lower panel).

flavonols are structurally similar in having hydroxyl groups at same positions on their A and B rings; however, isorhamnetin has an additional 3'-methyl group on the B ring as compared to quercetin and kaempferol (Fig. 1, lower panel).

Cytotoxic assessment of sea buckthorn fractions. The present study first examined the cytotoxic effects of the SB-But, SB-Chl, SB-Eac and SB-Aqu fractions on HepG2 cells. Of these, while SB-Chl exhibited significantly high cytotoxicity (CC_{50}, 32.58 μg/ml; P<0.01), SB-Eac, SB-But and SB-Aqu exhibited non-toxicity at concentrations of up to 150 μg/ml dose as compared to the untreated control (Fig. 3). Notably, HepG2 and HepG2.2.15 cells are biologically and physiologically the same, apart from the fact that HepG2.2.15 cells allow for HBV DNA replication and gene expression. In addition, while the HepG2 cells are widely distributed and easily available, the accessibility of HepG2.2.15 cells, even commercially, is very limited worldwide (37). In view of this, in the present study, MTT assay was performed on HepG2 cells, whereas the HepG2.2.15 cells were only used for anti-HBV assays.

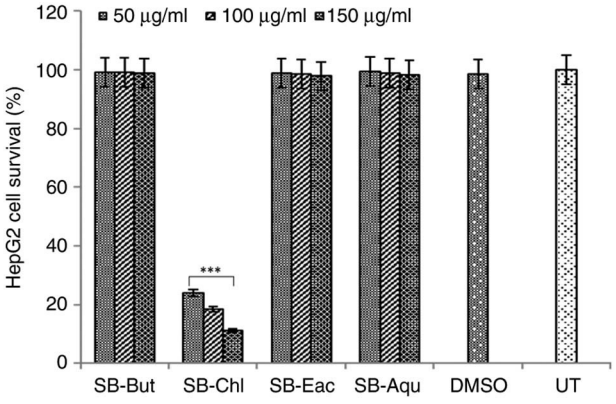

Figure 3. Cell viability or cytotoxicity assay of HepG2 cells treated with various concentrations of the sea buckthorn SB-But, SB-Chl, SB-Eac and SB-Aqu fractions, as well as DMSO (0.1%). The untreated control served as a negative control. Data are presented as the mean ± SEM (n=3); ***P<0.001 vs. UT. UT, untreated control; SB-But, n-butanol fraction; SB-Chl, chloroform fraction; SB-Eac, ethyl acetate fraction; SB-Aqu, aqueous fraction.

Anti-HBV activities of SB fractions and flavonols. The concentration-dependent analysis of the SB-Eac, SB-But and SB-Aqu fractions revealed marked inhibitory effects on HBsAg production at 50 and 100 μg/ml (P<0.01) in relation to the untreated control on 48 h (Fig. 4, left panel). As no further considerable enhanced effect was observed at 100 μg/ml when compared to 50 μg/ml, the 50 μg/ml concentration was selected for time-course analysis. In the time-course assay of

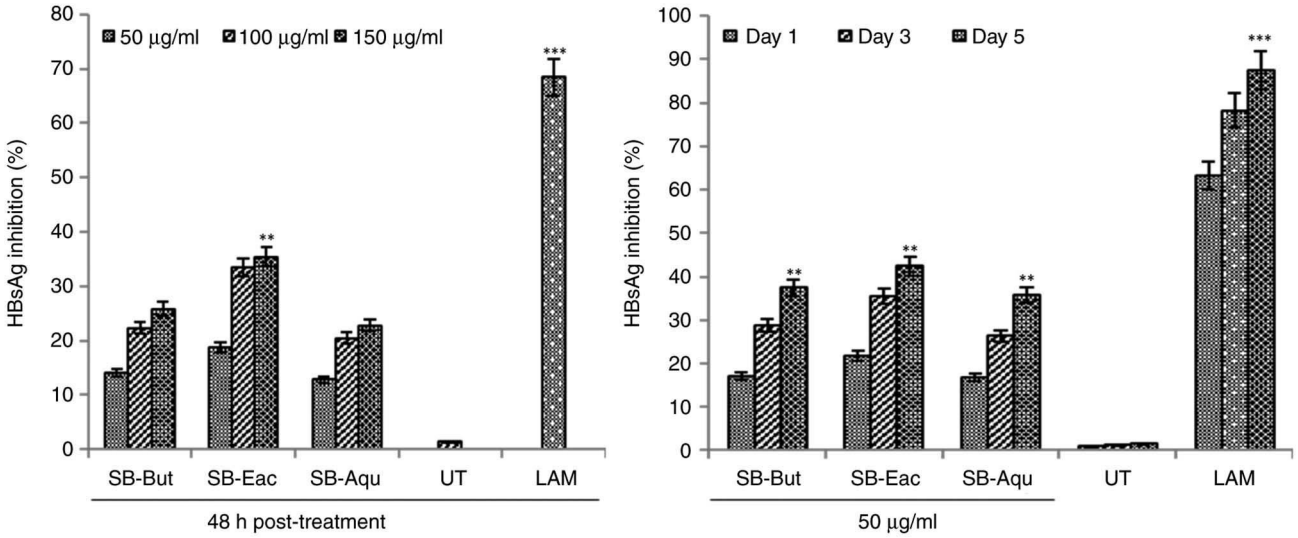

Figure 4. The anti-HBV assay showing concentration-dependent (left panel) and time-dependent (right panel) inhibitions of HBV surface or 's' antigen (HBsAg) by sea buckthorn SB-But, SB-Eac and SB-Aqu fractions in HepG2.2.15 cells. Lamivudine (2 μM) served as a reference or positive control, while DMSO (0.1%) functioned as the untreated control. Data are presented as the mean ± SEM (n=3); **P<0.01 and ***P<0.001 vs. UT. UT, untreated control; SB-But, n-butanol fraction; SB-Eac, ethyl acetate fraction; SB-Aqu, aqueous fraction; LAM, lamivudine.

the fractions (50 μg/ml), the optimal inhibition of HBsAg was observed by SB-Eac (~42.6%; P<0.01) followed by SB-But (~37.5%) and AB-Aqu (~36.2%) in relation to the untreated control on day 5 (Fig. 4, right panel). The concentration- and time-dependent analysis of isorhamnetin revealed its optimal, but comparative HBsAg inhibitory activity at the 10 and 20 μg/ml concentrations on day 5 (Fig. 5). At the selected 10 μg/ml concentration, isorhamnetin suppressed HBsAg levels by ~30.5% as compared to the high activities of quercetin (~67.5%; P<0.01) and kaempferol (~62.3%; P<0.01) in relation to the untreated control on day 5 (Fig. 5). The reference drug lamivudine (2 mM) suppressed HBsAg synthesis by ~87.4% (P<0.001) in relation to the untreated control on day 5.

As HBeAg production is a serological hallmark of active viral DNA replication (18), the present study further tested the three fractions (50 μg/ml) and the flavonoids (10 μg/ml) for their effects on HBeAg synthesis. SB-Eac suppressed HBeAg production by ~43.2% in relation to the untreated control on day 5. In addition, SB-But suppressed this by ~35.5% and AB-Aqu by ~32.5% in relation to the untreated control on day 5 (Fig. 6). Isorhamnetin decreased HBeAg production by ~28.4%, whereas quercetin and kaempferol inhibited this by ~64.4% (P<0.01) and ~60.2% (P<0.01), respectively in relation to the untreated control (Fig. 6). Lamivudine (2 mM) suppressed HBsAg production by ~83.5% (P<0.001) in relation to the untreated control on day 5. In a previous study by the authors, the assessment of the synergistic effects of quercetin with other flavonoids, such as rutin and hesperidin revealed further suppressions of HBsAg and HBeAg by ~10% in HepG2.2.15 cells (28). Thus, the present study did not examine the synergistic effects of quercetin with kaempferol or isorhamnetin.

Of note, although isorhamnetin was quantified as the most abundant flavonol in the sea buckthorn extract, it exhibited low anti-HBV activity, as compared to that observed for quercetin and kaempferol (Figs. 5 and 6). As isorhamnetin has an additional 3'-methyl group on its B ring as compared to quercetin

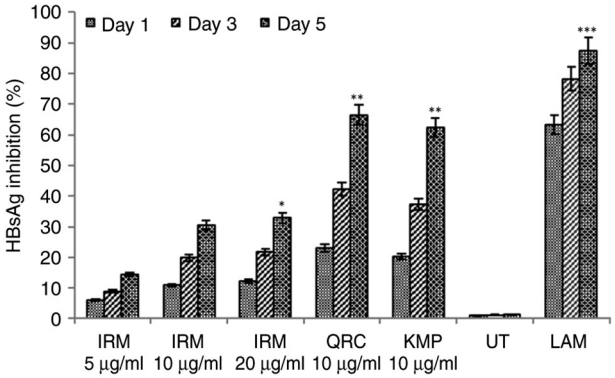

Figure 5. The anti-HBV assay showing time-course inhibitions of HBV surface or 's' antigen (HBsAg) by sea buckthorn-derived isorhamnetin (5, 10 and 20 μg/ml), quercetin (10 μg/ml) and kaempferol (10 μg/ml) in HepG2.2.15 cells. Lamivudine (2 μM) served as a reference or positive control, while DMSO (0.1%) functioned as the untreated control. Data are presented as the mean ± SEM (n=3); *P<0.05, **P<0.01 and ***P<0.001 vs. UT. UT, untreated control; IRM, isorhamnetin; QRC, quercetin; LAM, lamivudine.

and kaempferol (Fig. 1, lower panel), the structure-activity association may be attributed to its weak antiviral activity. In contrast to the present data, in a previous comparative antiviral study of structurally-related flavonoids, isorhamnetin was reported to have the most potent activity against influenza virus, which is an RNA virus (38). Notably, HBV is a DNA virus that has different replication mechanisms than RNA viruses. Nonetheless, HBV has a unique replication mechanism similar to HSV and HIV, the retroviruses. Therefore, the majority of the anti-HSV and anti-HIV drugs are also potent anti-HBV drugs. In previous studies, while sea buckthorn leaf extract was reported for anti-DNV activity (32), the isolated compound, hiporamin, was found to exert potent antiviral effects against influenza virus, HSV and HIV (34). Of the three antiviral flavonoids identified in its leaves (34), quercetin and kaempferol, as well as their derivatives have been

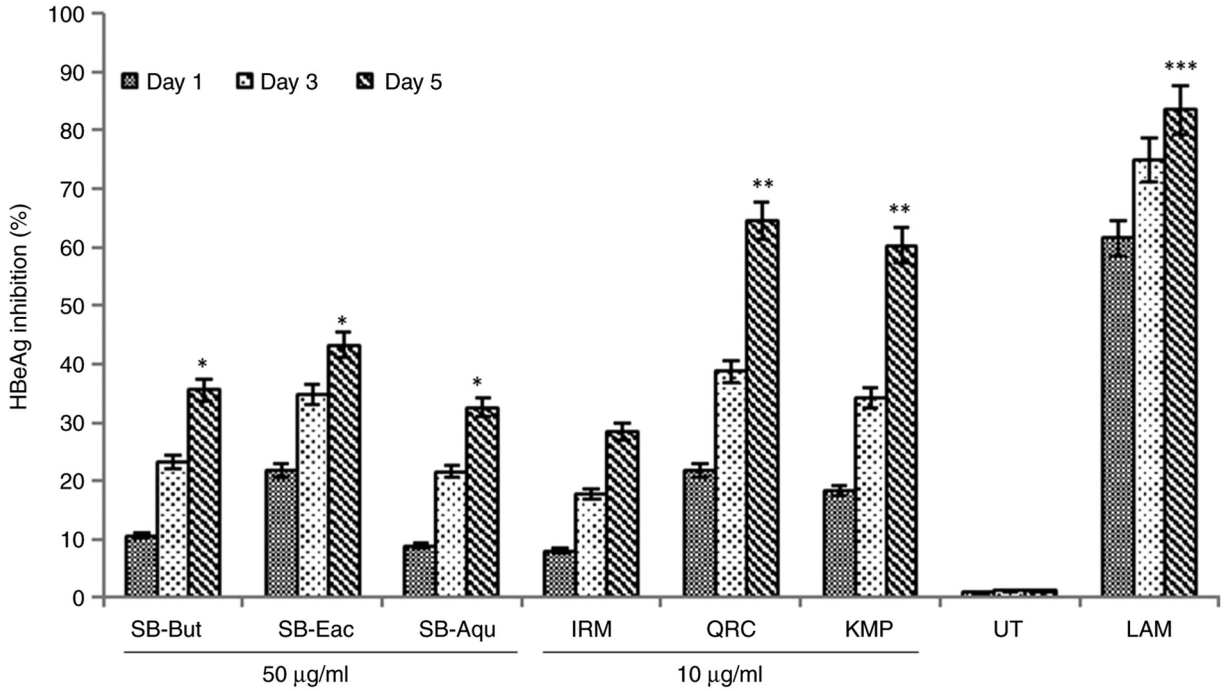

Figure 6. The anti-HBV assay showing time-course inhibitions of HBV pre-core or 'e' antigen (HBeAg) by the sea buckthorn SB-But, SB-Eac and SB-Aqu fractions (50 μg/ml, each), as well as isorhamnetin (10 μg/ml), quercetin (10 μg/ml) and kaempferol (10 μg/ml) in HepG2.2.15 cells. Lamivudine (2 μM) served as a reference or positive control, while DMSO (0.1%) functioned as the untreated control. Data are presented as the mean ± SEM (n=3); *P<0.05, **P<0.01 and ***P<0.001 vs. UT. UT, untreated control; SB-But, n-butanol fraction; SB-Eac, ethyl acetate fraction; SB-Aqu, aqueous fraction; IRM, isorhamnetin; QRC, quercetin; LAM, lamivudine.

demonstrated to have marked HBsAg and HBeAg inhibitory potentials in HepG2.2.15 cells (27-30). Notably, although the broad antiviral potential of isorhamnetin remains unexplored, a previous study reported its *in vitro* activity against the influenza virus (37). Moreover, to further obtain insight into the association between the chemical structures and anti-HBV activities, molecular docking was previously performed for quercetin, kaempferol and lamivudine with HBV-polymerase in previous studies by the authors (28,29). As in the present study, isorhamnetin exhibited a comparatively low anti-HBV activity, molecular docking was not performed.

Notably, based on the safe usages of sea buckthorn in traditional medicine, several clinical studies have revealed the therapeutic effects of sea buckthorn against ulcerative stomatitis (39), chronic cervicitis and (40) and atopic dermatitis (41). In another study, sea buckthorn juice was reported to be beneficial in reducing the risk factors for coronary heart disease, possibly due to its high anti-oxidant property (42), Moreover, when used in cirrhotic patients, sea buckthorn was found to prevent the progression of liver fibrosis (8). In view of this, as well as in the absence of a primate model of chronic HBV infection, these data suggest that it is worthy of further clinical assessment.

In conclusion, the present study demonstrates that the *in vitro* anti-HBV potential of sea buckthorn is attributed to its well-known antiviral flavonols, quercetin and kaempferol. Notably, although with a comparatively minimal effect, isorhamnetin was found to exhibit anti-HBV activity, for the first time, at least to the best of our knowledge. Nonetheless, further molecular and pharmacological studies are warranted in order to validate and develop anti-HBV therapeutics.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MKP and MSAD conceptualized and designed the study, carried out experiments, collected and analyzed data, and wrote the manuscript. OAB processed and prepared the plant sample. RNH performed the HPLC analysis. MKP and MSAD confirm the authenticity of all raw data. All authors have read and approved the final manuscript.

Ethical approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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