

Antiviral flavonoids- and polyphenols-driven novel anti-HBV efficacy of *Ilex paraguariensis*

Sakina Niyazi¹, Mohammad K. Parvez^{2*}, Tanzeel Ahmed^{1*}, Omar A. Basudan²,
Md Tabish Rehman², Mohammed S. Al-Dosari², Rashed N. Herqash², Saleh I. Alqasoumi²

¹School of Biotechnology, IFTM University, Moradabad, India

²Department of Pharmacognosy, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia

Submitted: 11 January 2024; Accepted: 16 May 2024

Online publication: 6 June 2024

Arch Med Sci 2025; 21 (3): 919–929

DOI: <https://doi.org/10.5114/aoms/188809>

Copyright © 2024 Termedia & Banach

*Corresponding authors:

Prof. Mohammad K. Parvez

Department of

Pharmacognosy

College of Pharmacy

King Saud University

Riyadh 11451, Saudi Arabia

E-mail: mohkhalid@ksu.edu.

sa;

E-mail: khalid_parvez@yahoo.

com

Prof. Tanzeel Ahmed

School of Biotechnology, IFTM

University

Moradabad 244102, India

E-mail: ahmed.tanzeel@

gmail.com

Abstract

Introduction: Antiviral natural products have shown great promise as alternatives to conventional therapies to address drug resistance in hepatitis B virus (HBV). The abundance of bioactive flavonoids and polyphenolic contents in leaves of *Ilex paraguariensis* (yerba mate) warrants assessment of its anti-HBV activity.

Material and methods: The total ethanol extract of *I. paraguariensis* leaves (IP-Ext), including its *n*-hexane (IP-Hex), chloroform (IP-Chl), ethyl acetate (IP-EtAc) and ethanol (IP-EtOH) fractions, was tested for non-hepatocytotoxicity on HepG2 cells (MTT assay) and anti-HBV efficacy on HepG2.2.15 cells (ELISA). Further, quantitative high-performance liquid chromatography (HPLC) was employed to identify the antiviral compounds in the extract. In addition, *in silico* molecular docking was performed to delineate the possible mechanism of anti-HBV activity of the identified compounds.

Results: All samples showed non-cytotoxicity except IP-EtAc, with mild toxicity at 200 µg/ml. Their anti-HBV assessment showed dose-dependent inhibitions of HBV antigens (HBsAg and HBeAg). At the selected optimal dose (50 µg/ml), while IP-Ext showed mild (HBsAg: 24.2% and HBeAg: 20.6%) and IP-Chl showed moderate (HBsAg: 42.3% and 40.1%) activity, IP-Hex (HBsAg: 55.6% and HBeAg: 52.4%) and IP-EtOH (HBsAg: 53.2% and HBeAg: 50.2%) exhibited high activity. HPLC revealed known anti-HBV flavonoids (rutin: 18.98, quercetin: 6.52 and kaempferol: 9.10 µg/g) and polyphenols (caffeic acid: 11.43 and chlorogenic acid: 3.22 µg/g) in the extract. Their (10 µg/ml, each) anti-HBV activities were: quercetin (HBsAg: 67.8% and HBeAg: 64.4%), kaempferol (HBsAg: 63.5% and HBeAg: 61.6%), chlorogenic acid (HBsAg: 55.2% and HBeAg: 53.8%), rutin (HBsAg: 51.2% and HBeAg: 48.4%) and caffeic acid (HBsAg: 42.2% and HBeAg: 39.5%). Notably, while our previous molecular docking had shown strong interactions of these flavonoids with HBV polymerase, here, we observed good binding affinity of the polyphenols with both drug-sensitive (wild-type) and drug-resistant (mutant) polymerases.

Conclusions: This is the first study suggesting the anti-HBV therapeutic efficacy of *I. paraguariensis* attributed to its antiviral flavonoids and polyphenols.

Key words: hepatitis B virus, anti-HBV, *Ilex paraguariensis*, yerba mate, flavonoids, polyphenols, HepG2.2.15 cells.

Introduction

Globally, hepatitis B virus (HBV) chronic infection of the liver is a major health issue with about 3 million cases that may progress to fulminant liver failure, cirrhosis or hepatocellular carcinoma, accounting for over 850,000 deaths, annually [1, 2]. About two-thirds of the world population lives in high-endemic regions in Asia, Africa and the Middle East, including Saudi Arabia [1]. Though there are highly protective vaccines and efficacious direct-acting nucleoside analogs, the emergence of vaccine escape and drug-resistant HBV mutants remains a clinical challenge [3, 4]. In the last couple of decades, a range of herbal formulations and bioactive phytochemicals, such as alkaloids, flavonoids, polyphenols, terpenoids, terpenes, lignans, coumarins, saponins, xanthins and anthraquinones, have been reported to have potential activity against various genetically diverse viruses, including HBV, with no issue of drug resistance [5–8]. In line with this, we have also reported various anti-HBV medicinal plant extracts and isolated compounds for their promising activity *in vitro* [9–15].

Ilex paraguariensis (family: Aquifoliaceae) dry leaves known as yerba mate is popularly consumed as a health-protective brewed herbal tea in Brazil, Argentina, Paraguay and Uruguay [16, 17]. Mate tea is also prepared in cold water, called *tereré*, which is mainly consumed in Paraguay. Mate has been reported for its antioxidant [18–20], hepatoprotective and hypocholesterolemic [17], cardiovascular protective [21], and anti-obesity [22] effects *in vitro* and *in vivo*. Further pre-clinical studies have expanded its antioxidant [23] and hypocholesterolemic [24] properties. Moreover, *I. paraguariensis* extract has been shown to have antifungal activity in humans [25]. Of the various bioactive compound identified in *I. paraguariensis* green extracts, the most abundant are caffeic acid, chlorogenic acid, dicaffeoylquinic acid, caffeine, theobromine, rutin, quercetin, and kaempferol [26]. Notably, in recent years, we have reported antiviral rutin, quercetin and kaempferol and their derivatives for their *in vitro* anti-HBV potential [12–15]. In this study therefore, we investigated the *in vitro* anti-HBV efficacy of *I. paraguariensis* and its bioactive phytochemicals in a HBV-reporter cell culture model, validated by high performance liquid chromatography and molecular docking.

Material and methods

Sample collection and preparation of extract and fractions

I. paraguariensis dried leaves (yerba mate) were obtained as a commercially packaged product (La Tranquera, Barcode: 17790480000371) from Argentina. All analytical grade solvents for

extraction and fractionation were procured from Sigma-Aldrich (Merck KGaA, Germany). A portion (2.0 g) of powdered leaves (350.0 g) was extracted with 90% ethanol at room temperature (RT) and concentrated under reduced pressure to yield total ethanol extract (IP-Ext; 129.5 mg). The remaining portion (348.0 g) was further extracted in different solvents to furnish *n*-hexane (IP-Hex; 293.0 mg), chloroform (IP-Chl; 7.0 g), ethyl acetate (IP-EtAc; 10.0 mg) and ethanol (IP-EtOH; 13.5 mg) fractions by distillation under reduced pressure.

Human liver cell cultures, solvents and drugs

Human hepatoma cells HepG2 and its derivative line HepG2.2.15 (generous gift from Dr. S. Jameel, International Center for Genetic Engineering & Biotechnology, New Delhi, India) were grown in DMEM medium (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and 1x antibiotic-antimycotic mix (HyClone; GE Healthcare Life Sciences, Logan, UT, USA) at 37°C with 5% CO₂. For the experiments, cells (0.5 × 10⁵ cells/100 µl/well) were grown in flat-bottom 96-well culture plates (Corning, USA) overnight. The pure natural compounds (rutin, quercetin, kaempferol, caffeic acid and chlorogenic acid) were procured from Sigma-Aldrich (Merck KGaA, Germany). Dimethyl sulfoxide (0.1% DMSO) acted as a vehicle or negative control, whereas the anti-HBV active *Guiera senegalensis* (GS) dichloromethane fraction [9] and the anti-HBV drug lamivudine (LAM; nucleoside analog) served as positive controls. The experiment was repeated twice for reproducibility.

Hepatocyte viability assay of *I. paraguariensis* preparations

The effect of *I. paraguariensis* extract (IP) and fractions (IP-Hex, IP-Chl, IP-EtAc and IP-EtOH) on the viability or toxicity of HepG2 cells was assessed. The fractions were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Merck KGaA, Germany) and then reconstituted in DMEM to furnish four test concentrations or doses of 25, 50, 100 and 200 µg/ml. The overnight grown HepG2 cells (96-well plate) were replenished with fresh media containing different test doses of the preparations, including a negative control (0.1% DMSO) in triplicate, and incubated at 37°C for 3 days. The established assay utilizing dimethylthiazolyl diphenyltetrazolium bromide (MTT) was employed (TACS MTT Cell Proliferation Assay Kit; Tervigen, MD, USA). Briefly, after treating with MTT and incubation, the optical density (λ = 570 nm) of the samples was recorded using a microplate

reader (ELx800; BioTek Instruments, Inc., VT, USA). A non-linear regression analysis was performed (Excel software 2010; Microsoft Corp., WA, USA) to determine the hepatocytes' viability or toxicity of the preparations in relation to the negative control.

HBV envelop/surface antigen (HBsAg) inhibition assay of *I. paraguariensis* preparations

Dose-dependent inhibition of HBsAg expression by the selected doses of the noncytotoxic preparations (25, 50 and 100 µg/ml; in triplicate) was performed. Briefly, overnight grown HepG2.2.15 cells (96-well plate) were replenished with fresh media with treatment doses, including negative and positive controls and incubated at 37°C until day 2 (single time-point). The HBsAg production in culture supernatants was analyzed quantitatively using the diagnostic ELISA kit (cat. no. 72348; Monolisa HBsAg ULTRA, Bio-Rad Laboratories Inc., CA, USA) following the kit's manual. The optical density ($\lambda = 450$ nm) of the samples was recorded, and analyzed (Excel) in relation to the negative control, and the activity was compared with the positive control. Further, with the determined optimally active dose, a time-course analysis of HBsAg expression was carried out. Briefly, HepG2.2.15 cells were replenished with fresh media containing 50 µg/ml (in triplicate) of each fraction, including controls, and incubated at 37°C. The culture supernatants were collected on days 1, 3 and 5, and analyzed as mentioned above.

HBV pre-core antigen (HBeAg) inhibition assay of *I. paraguariensis* preparations

The preparations (50 µg/ml, in triplicate) were further assessed for their time-course (days 1, 3 and day 5) inhibition of HBeAg production in the collected supernatants using ELISA kit (cat. no. KAPG4BNE3; HBeAg/Anti-HBe Elisa Kit, DIAsource ImmunoAssays; Louvain-la-Neuve, Belgium) following the kit's manual. The optical density ($\lambda = 450$ nm) of the samples was recorded, and analyzed (Excel) as above, and the activity was compared with the positive control.

High-performance liquid chromatography (HPLC) analysis of *I. paraguariensis* extract

The *I. paraguariensis* extract was subjected to quantitative HPLC analysis for the known anti-HBV active compounds on the Alliance chromatographic system, equipped with dual wavelength absorbance detectors (Waters Instruments, Inc., MA, USA). Reverse-phase chromatography was achieved using a C_{18} column (4.6 × 300 mm) under gradient flow (1 ml/min) of the mobile phase (Solution A: formic acid : water; 1 : 99, v/v)

and Solution B: acetonitrile : methanol; 70 : 30, v/v). The formulized gradient was 7% Solution B until 4 min and then modified to obtain 65%, 90%, 100%, and 7% Solution B at 12, 20, 23 and 27 min, respectively, as described elsewhere with minor modifications. Samples (20 µl, each) were membrane-filtered (0.45 µm filter, Millipore, USA) before injecting into the system. The identification and detection of the compounds were monitored ($\lambda = 366$ nm) by comparing their retention times with those of the commercial standards.

Anti-HBV assessment of identified flavonoids and polyphenols

The HPLC identified flavonoids and polyphenols (10 µg/ml, in triplicate) were also tested for optimal suppression of HBsAg and HBeAg production in treated HepG2.2.15 cells at day 5 (single time-point) as mentioned above. LAM (2 µM) and DMSO (0.1%) served as the standard (positive) and negative control, respectively, as described previously [9, 10]. The data were analyzed (Excel) in relation to the negative control and the activity was compared with the positive control.

Molecular docking analysis

Docking of the polyphenols (caffeic acid and chlorogenic acid) with in-house modelled drug-sensitive HBV wild-type polymerase (POLwt; catalytic YMDD motif) as well as drug-resistant mutant polymerase (POLmut; catalytic 'YIDD' motif) proteins [10, 11] was performed using Autodock 4.2. The target proteins were prepared by removing water molecules or any unwanted bound atoms, by adding hydrogen atoms and denoting Kollman charges. The 3D structures of POL were finally energy minimized using MMFF (Merck Molecular Force Field). The 2D structures of caffeic acid (CID_689043), and chlorogenic acid (CID_1794427) were obtained from PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) and prepared for docking by denoting bond orders and angles. Further, Gasteiger partial charges were defined and the energies were minimized using Universal Force Field as described elsewhere [10, 11].

The structure-based virtual docking was done inside a defined grid box by selecting the reported key amino acid residues. For both POLwt and POLmut proteins, grid boxes were adjusted to 27.1 × 26.5 × 27.9 Å, centered at 47.8 × 30.1 × 34.4 Å with 0.375 Å spacing. Lamarck Genetic Algorithm and Solis-Wets methods were employed for the global and local search, respectively, as described elsewhere [10, 11].

For each run, a total of 2.5×10^6 energy calculations was estimated, and a total of 10 docking runs was completed. Further, the population size

(= 150), translational step (= 0.2), quaternions (= 5) and torsions (= 5) were adjusted. The van der Waals' and electrostatic parameters were estimated using distance-dependent dielectric function. Finally, the docking or binding affinity (K_b) of ligands for respective target was estimated from docking or binding energy (ΔG) using the formula $\Delta G = -RT \ln K_b$ (R = universal gas constant; 1.987 cal mol⁻¹ K⁻¹ and T = temperature; 298 K) as mentioned elsewhere [27].

Statistical analysis

Data analysis was performed using SPSS 17.0 (SPSS Inc., Chicago, USA). Triplicated data (mean $\pm$ S.E.M) of test samples and the positive control

in relation to the negative control were analyzed by one-way ANOVA, following Dunnett's test. $P < 0.05$ was considered to indicate a statistically significant difference (sample vs. positive control).

Results

Assessment of *in vitro* hepatotoxicity of *I. paraguariensis* preparations

The *I. paraguariensis* preparations IP-Ext, IP-Chl, IP-Hex and IP-EtOH were non-hepatotoxic up to the maximal tested dose (200 μ g/ml). However, IP-EtAc had mild toxicity between 100 and 200 μ g/ml doses (Figure 1 A), and therefore was excluded from antiviral analysis.

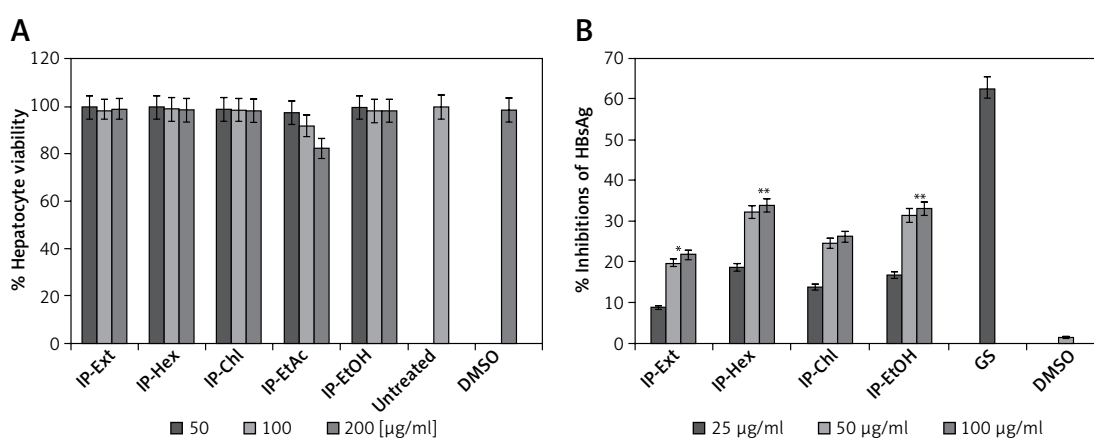


Figure 1. *I. paraguariensis* leaves (yerba mate). **A** – MTT assay showing effects of *I. paraguariensis* extract (IP-Ext) and its hexane (IP-Hex), chloroform (IP-Chl), ethyl acetate (IP-EtAc) and ethanol (IP-EtOH) fractions on HepG2 cell viability in relation to negative control (0.1% DMSO). Data are presented as mean $\pm$ standard error of triplicated values of each sample. ** $P < 0.01$ and *** $p < 0.001$ vs. untreated control (UT). **B** – Anti-HBV assay showing dose-dependent inhibitions of HBV s antigen (HBsAg) by IP-Ext, IP-Hex, IP-Chl, IP-EtAc and IP-EtOH in relation to negative control (0.1% DMSO) in HepG2.2.15 cells at day 2 (single time-point). Data are presented as mean $\pm$ standard error of triplicated values of each sample. ** $P < 0.01$ and *** $p < 0.001$ vs. positive control (GS)

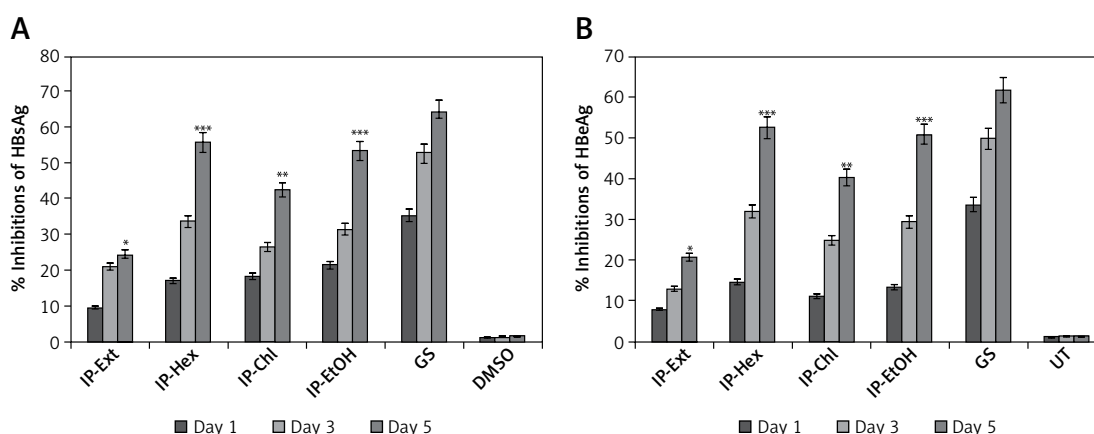


Figure 2. Time-dependent anti-HBV assay of *I. paraguariensis* extract (IP-Ext) and its hexane (IP-Hex), chloroform (IP-Chl), ethanol (IP-EtOH) fractions in HepG2.2.15 cells. **A** – Inhibition of HBV s antigen (HBsAg) in relation to negative/untreated control (DMSO/UT). **B** – Inhibition of HBV e antigen (HBeAg) in relation to negative/untreated control (DMSO/UT). Data are presented as mean $\pm$ standard error of triplicated values of each sample. ** $P < 0.01$ and *** $p < 0.001$ vs. positive control (GS)

Dose- and time-dependent inhibition of HBsAg by *I. paraguariensis* preparations

Of the tested variable doses of *I. paraguariensis* non-cytotoxic preparations, the 50 µg/ml dose maximally inhibited HBsAg production, which was not significantly affected at 100 µg/ml (Figure 1 B). Therefore, 50 µg/ml was selected for further time-course inhibition analysis. Of the tested preparations, IP-Ext and IP-Chl showed mild (24.2%) and moderate (HBsAg: 42.3%) activity, respectively, whereas IP-Hex (55.6%) and IP-EtOH (53.2%) showed high activity at day 5 (Figure 2 A). Because the extended incubation of the treated cultures resulted in cell overgrowth and subsequent apoptosis (data not shown), the experiment was concluded on day 5.

Time-dependent inhibition HBV replication by *I. paraguariensis* preparations

HBeAg, the processed product of HBV pre-core protein co-translated with core/capsid protein by a bicistronic mRNA, is a serological gold marker of HBV DNA replication. In line with HBsAg inhibitory activity, while IP-Ext and IP-Chl had mild (20.6%) and moderate (HBsAg: 40.1%) effects, respectively, both IP-Hex (52.4%) and IP-EtOH (50.2%) had high HBeAg inhibition at day 5 (Figure 2 B). Because the extended incubation of the treated cultures resulted in cell overgrowth and subsequent

apoptosis (data not shown), the study was concluded on day 5.

HPLC validation of anti-HBV active compounds in *I. paraguariensis* extract

Our quantitative HPLC analysis detected known anti-HBV active flavonoids (rutin: 18.98, quercetin: 6.52 µg/g and kaempferol: 9.10 µg/g) and polyphenols (caffeic acid: 11.43 µg/g and chlorogenic acid: 3.22 µg/g) in the extract (Figure 3; Table I).

Anti-HBV activity of identified flavonoids and polyphenols

The anti-HBV activity levels of HPLC identified phytochemicals (10 µg/ml, each) on day 5 were as follows: quercetin (HBsAg: 67.8% and HBeAg: 64.4%), kaempferol (HBsAg: 63.5% and HBeAg: 61.6%), chlorogenic acid (HBsAg: 55.2% and HBeAg: 53.8%), rutin (HBsAg: 51.2% and HBeAg: 48.4%), and caffeic acid (HBsAg: 42.2% and HBeAg: 39.5%). LAM (standard) suppressed HBsAg and HBeAg by 83.2 and 74.4%, respectively (Figure 4).

Interaction of polyphenols with POLwt

Because the molecular docking representations of POLwt (YMDD motif) and POLmut (YIDD motif) with lamivudine (standard ligand) have been de-

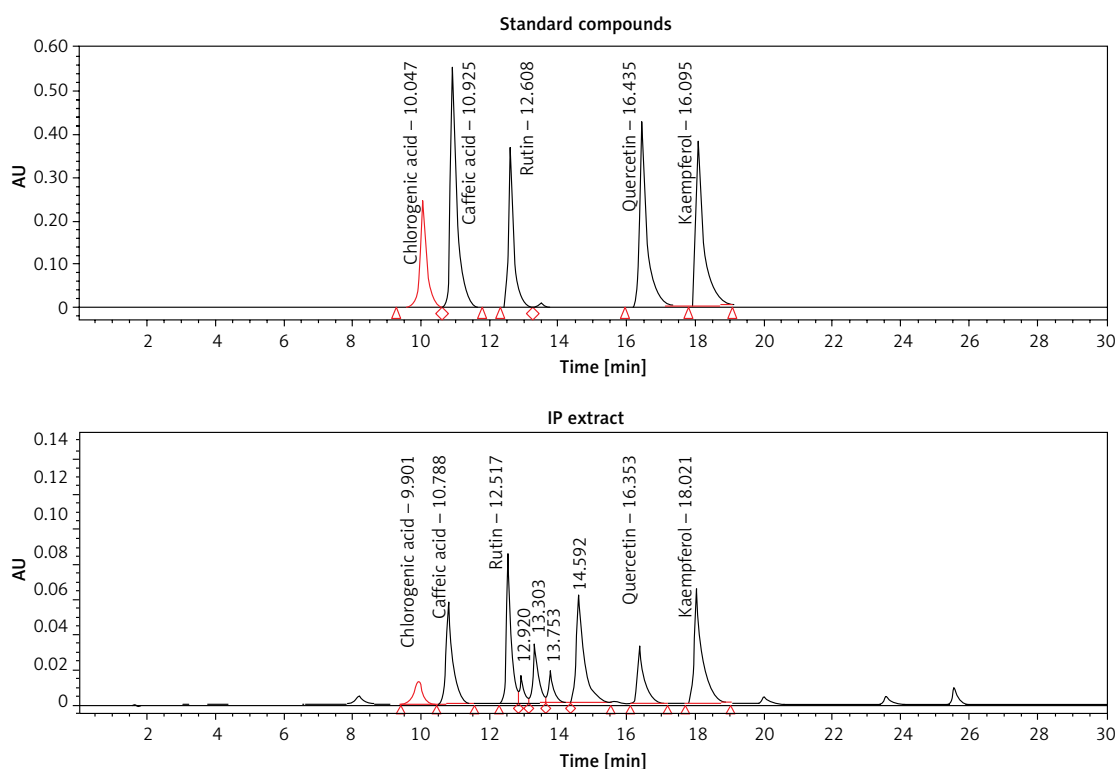


Figure 3. HPLC chromatograms showing standard compounds (rutin, quercetin, kaempferol, caffeic acid and chlorogenic acid) and their identification in *I. paraguariensis* (IP extract)

Table I. HPLC profile of anti-HBV flavonoids and polyphenols in *I. paraguariensis* extract

Compounds	Structures	Retention time	Conc. $\pm$ SD* [mg/g]
Flavonoids	Rutin	12.517	18.98 $\pm$ 3.23
	Kaempferol	18.021	9.10 $\pm$ 4.60
	Quercetin	16.353	6.52 $\pm$ 4.96
Polyphenols	Caffeic acid	10.788	11.43 $\pm$ 4.13
	Chlorogenic acid	9.901	3.22 $\pm$ 5.04

*Data of mean $\pm$ standard deviation (SD) of three determinations.

scribed previously [10, 11], they were not included in the present study. Molecular docking revealed interactions of the polyphenols caffeic acid and chlorogenic acid with drug-sensitive POLwt active-site residues (Figure 5 A; Table I). Caffeic acid formed three conventional hydrogen bonds with GLY151, GLN267, and LYS239 (Figure 5 B; left). In addition, it established electrostatic interactions with LYS241 and ASP83 as well as hydrophobic interactions with LYS241 (Figure 5 B; left). Notably therein, the catalytic YMDD' motif residue ASP206 along with other SER81, LEU82, VAL207, THR240, ASN248, PHE249, MET250, and TYR252

further stabilized the POLwt-caffeic acid complex through van der Waals' interactions. The binding energy and binding affinity of the complex were estimated to be -6.2 kcal/mol and 3.53×10^4 M $^{-1}$, respectively (Table II).

Chlorogenic acid established conventional hydrogen bonds with LYS241, GLY251, and GLN267 as well as the YMDD motif residue ASP206 (Figure 5 B; right). In addition, a carbon-hydrogen bond with ASP206 was also formed. Further, two electrostatic interactions with LYS241 and ASP83 along with a hydrophobic interaction with LYS241 also stabilized the POLwt-chlorogenic acid com-

plex. Moreover, the complex was also stabilized by van der Waals' interactions with YMDD motif residue TYR203 as well as SER81, LEU82, VAL207, LYS239, THR240, ASN248, PHE249, MET250 and TYR252. The binding energy and docking affinity of the complex were estimated to be -7.3 kcal/mol and $2.26 \times 10^5 \text{ M}^{-1}$, respectively (Table II).

Interaction of polyphenols with POLmut

Molecular docking analysis of the polyphenols suggested that both occupied the active site of the drug-resistant POLmut (Figure 6 A; Table II). Caffeic acid primarily formed conventional hydrogen bonds with GLY251, SER81, and LYS239 (Figure 6 B; left). It also had an electrostatic and a hydrophobic interaction with ASP83 and LYS241, respectively. Further, interactions of the mutant YIDD motif ASP206 residue along with LEU82, THR240, ASN248, PHE249 and MET250 through van der

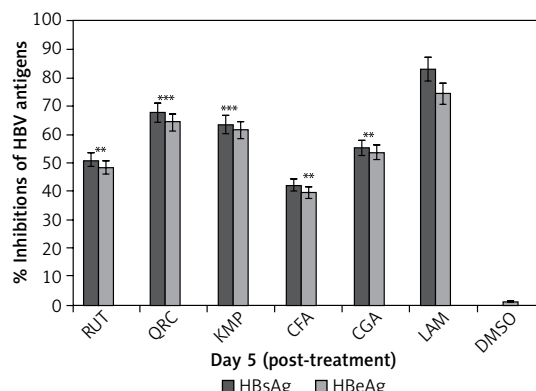


Figure 4. Anti-HBV assay showing optimal inhibition of HBsAg and HBeAg production by *I. paraguariensis* antiviral compounds (10 $\mu\text{g/ml}$, each): rutin (RUT), quercetin (QRC), kaempferol (KMP), caffeic acid (CFA) and chlorogenic acid (CGA) in relation to negative control (DMSO) in HepG2.2.15 cells. Data are presented as the mean $\pm$ standard error of triplicated values of each sample. ** $P < 0.01$ and *** $p < 0.001$ vs. positive control (LAM; 2 μM)

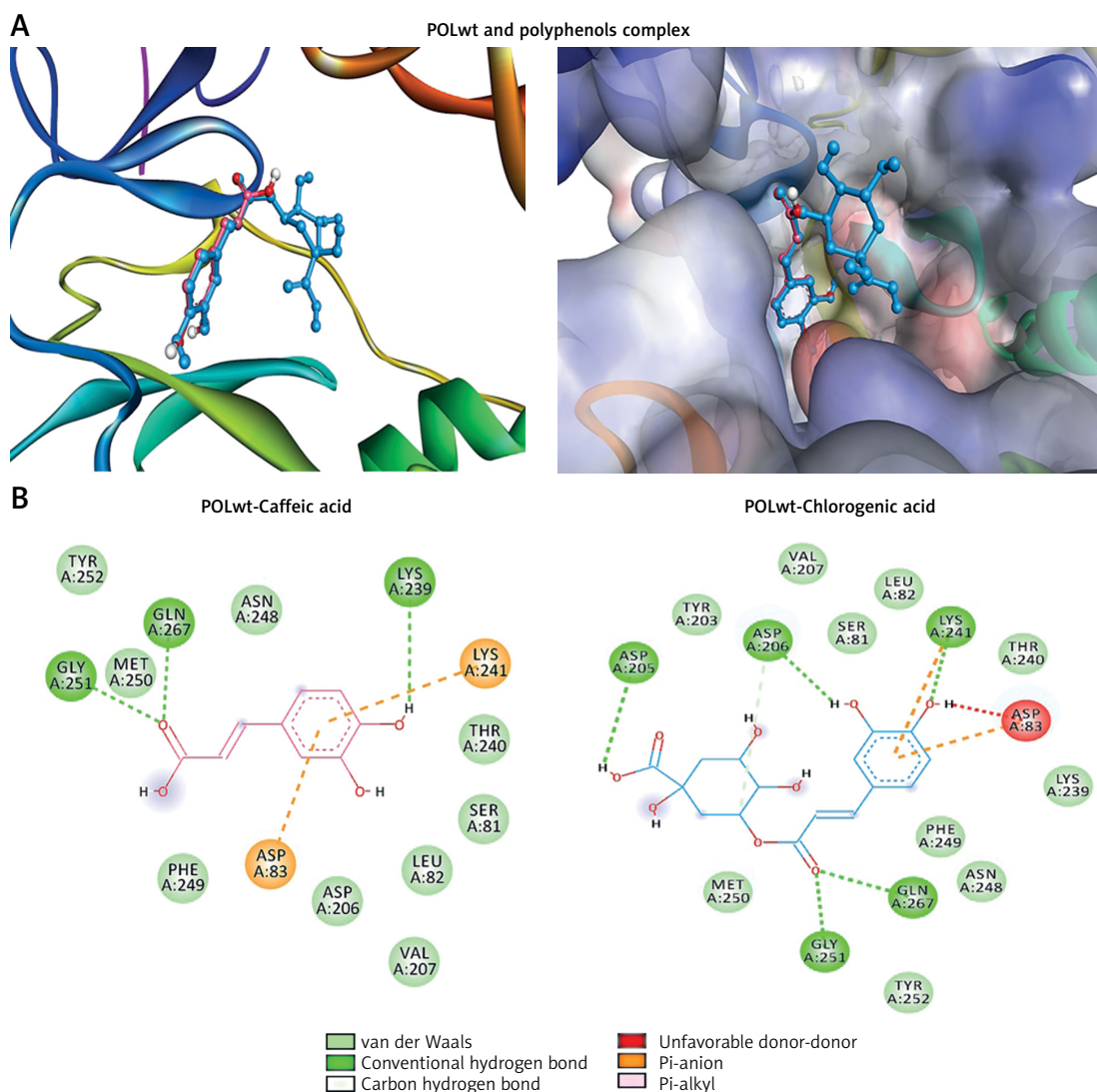


Figure 5. Molecular docking of antiviral polyphenols with drug-sensitive HBV polymerase (POLwt) active residues. **A** – 3D representation caffeic acid (pink) and chlorogenic acid (blue) interactions. **B** – 2D representation caffeic acid (pink) and chlorogenic acid (blue) interactions

Table II. Molecular interaction of HBV polymerases (wild-type and mutant) with *I. paraguariensis* caffeic acid and chlorogenic acid

	Donor-acceptor pair	Distance [Å]	Mode of interaction	Docking energy [kcal/mol]	
POLwt	Caffeic acid complex				
	GLY251:HN – LIG:O	2.2523	Conventional Hydrogen Bond	−6.2	
	GLN267:HE21 – LIG:O	2.1429	Conventional Hydrogen Bond		
	LIG:H – LYS239:O	2.8881	Conventional Hydrogen Bond		
	LYS241:NZ – LIG	4.8940	Electrostatic (Pi-Cation)		
	ASP83:OD2 – LIG	4.4867	Electrostatic (Pi-Anion)		
	LIG – LYS241	4.4755	Hydrophobic (Pi-Alkyl)		
	Chlorogenic acid complex				
	LYS241:HN – LIG:O	2.4288	Conventional Hydrogen Bond	−7.3	
	GLY251:HN – LIG:O	2.1639	Conventional Hydrogen Bond		
	GLN267:HE21 – LIG:O	2.2074	Conventional Hydrogen Bond		
	LIG:H – ASP206:OD1	2.2891	Conventional Hydrogen Bond		
	LIG:H – ASP205:OD1	2.5961	Conventional Hydrogen Bond		
	LIG:C – ASP206:OD2	3.7876	Carbon Hydrogen Bond		
	LYS241:NZ – LIG	4.9866	Pi-Cation		
	ASP83:OD2 – LIG	4.3767	Pi-Anion		
	LIG – LYS241	4.5773	Pi-Alkyl		
	POLmut	Caffeic acid complex			
		GLY251:HN – LIG:O	1.9251	Conventional Hydrogen Bond	−5.6
		LIG:H – SER81:OG	2.5030	Conventional Hydrogen Bond	
LIG:H – LYS239:O		2.2201	Conventional Hydrogen Bond		
ASP83:OD2 – LIG		4.1558	Electrostatic (Pi-Anion)		
LIG – LYS241		4.9489	Hydrophobic (Pi-Alkyl)		
Chlorogenic acid complex					
LYS32:HZ1 – LIG:O		2.4275	Conventional Hydrogen Bond	−6.7	
LYS32:HZ2 – LIG:O		2.3564	Conventional Hydrogen Bond		
ASN36:HD21 – LIG:O		2.6968	Conventional Hydrogen Bond		
ASN36:HD22 – LIG:O		2.7616	Conventional Hydrogen Bond		
ASN36:HD22 – LIG:O		2.8840	Conventional Hydrogen Bond		
LYS239:HZ3 – LIG:O		2.4847	Conventional Hydrogen Bond		
LIG:H – LYS239:O		2.2148	Conventional Hydrogen Bond		
LIG:H – ASP83:OD1		2.7156	Conventional Hydrogen Bond		
SER85:CB – LIG:O		3.3871	Carbon Hydrogen Bond		
ASP83:OD2 – LIG		3.7258	Electrostatic (Pi-Anion)		
LIG – LYS241		5.3531	Hydrophobic (Pi-Alkyl)		

Waals' stabilized the POLmut-caffeic acid complex. Notably therein, GLN262 interacted unfavorably with caffeic acid. The binding energy and binding affinity of the complex were calculated to be –5.6 kcal/mol and $1.28 \times 10^4 \text{ M}^{-1}$, respectively (Table II).

Chlorogenic acid formed conventional hydrogen bonds with POLmut residues LYS32:HZ1, LYS32, ASN36, ASN36, LYS239, LYS239 and ASP83 (Figure 6 B; right). In addition, it also had a C-H bond with SER85, an electrostatic interaction with ASP83, and a hydrophobic link with LYS241. Moreover, van der Waals' interactions were also formed by ASN33, GLU39, ARG41, SER81, LEU82, VAL84, ALA86, ASP206, ASN236, THR240, ASN248, and PHE249 further stabilized the POLmut-chlorogenic acid complex. Notably therein, LYS239 was engaged in an unfavorable interaction. The binding energy and docking affinity of the complex were calculated to be –6.7 kcal/mol and $8.21 \times 10^4 \text{ M}^{-1}$, respectively (Table II).

Discussion

In the recent decades, numerous studies on the pharmacognosy, phytochemistry and pharmacology of natural or herbal products have driven a growing focus on antiviral drug discovery, targeting viral or host factors. Generally, consumption of such therapeutic products is believed to be more effective and safer than conventional or prescription medications. In recent times, however, acute liver failure and adverse clinical effects of some traditional Chinese medicines (TCM) and others used to treat chronic hepatitis B have been widely reported [28, 29]. Of these, herbal formulations such as Chuan Lian Zi (*Meliatoosendan*), Gan Cao (*Glycyrrhiza uralensis*, *Glycyrrhiza glabra*, Gan Cao Zhi, Shen Nong Ben Cao Jing, and Zhi GanCao), Ji Guo Cao (*Abrus cantoniensis*, Ji Gu Cao Wan), Jue Ming Zi (*Cassia obtusifolia*, *Senna obtusifolia*, Cao Jue Ming), Zexie (*Alisma orientalis*), and Zhen Chu

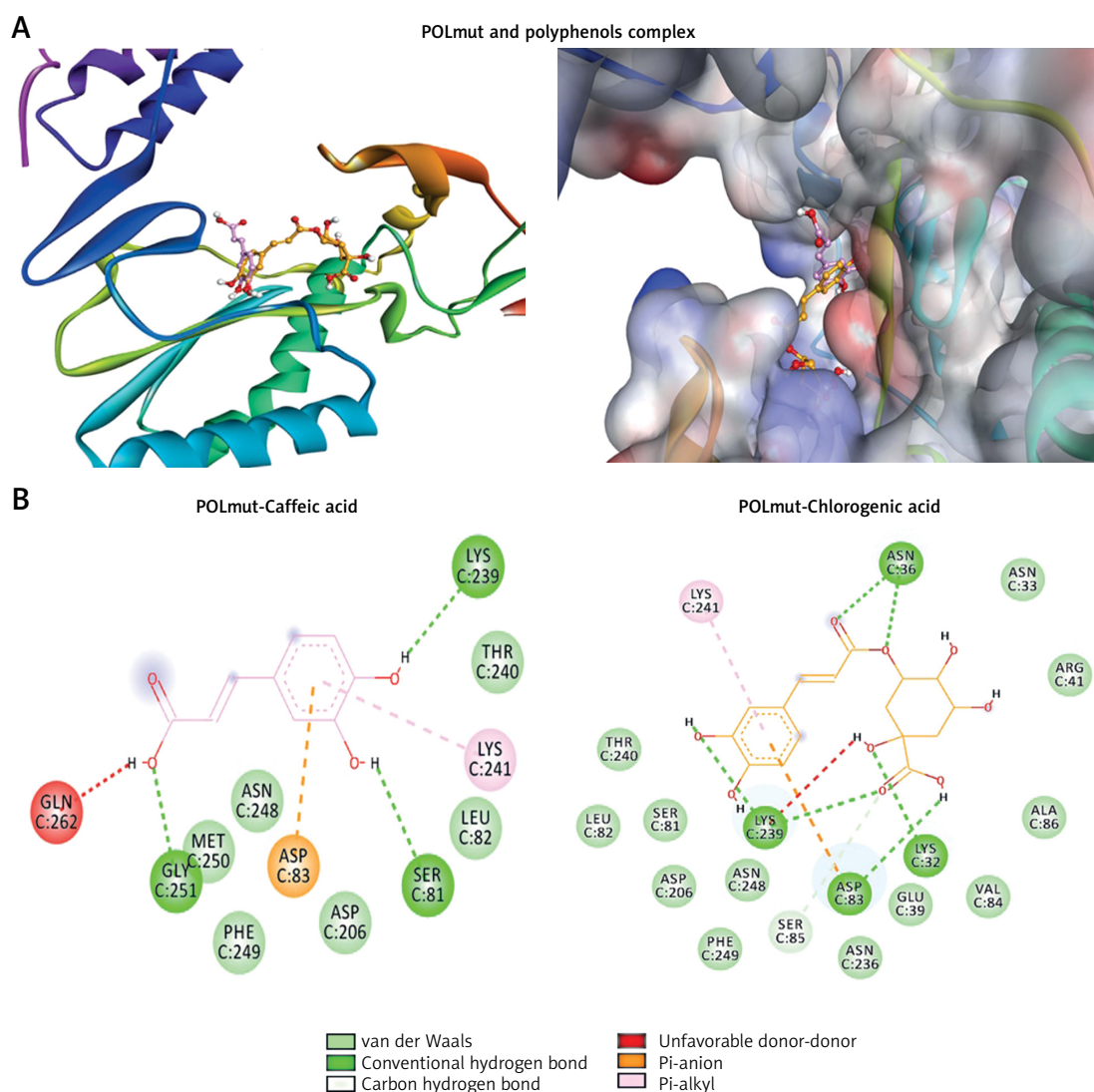


Figure 6. Molecular docking of antiviral polyphenols with drug-resistant HBV polymerase (POLmut) active residues. **A** – 3D representation caffeic acid (pink) and chlorogenic acid (blue) interactions. **B** – 2D representation caffeic acid (pink) and chlorogenic acid (blue) interactions

Cao (*Phyllanthus urinaria*) have shown significant liver toxicity in some individuals [30]. In addition, some hepatitis B patients were also found to be at higher risk for developing liver toxicity or necrosis upon consuming the poly-herbal formulation Long Dan Xie Gan Tang (*Akebia quinata*, *Alisma plantago*, *Angelica sylvestris*, *Bupleurum chinense*, *Gardenia jasminoides*, *Gentiana lutea*, *G. uralensis*, *Rehmannia glutinosa*, and *Scutellaria baicalensis* in China [31], and Bai Fang (*Angelica sinensis*, *Cyperus rotundus*, *Panax ginseng*, *Ligusticum wallichii*, *Paeonia alba*, and *Rehmannia glutinosa*) in the United States [32].

Therefore, potential medicinal plant extracts, formulations or isolated compounds need in-depth studies for their efficacy, safety, purity, standardization and therapeutic validation. In view of this, to rule out the known organ or liver toxicity associated with some herbal products, we

first tested the hepatotoxicity of *I. paraguariensis* preparations in cultured HepG2 cells. Although the *I. paraguariensis* total ethanol extract and organic fractions were nontoxic, the ethyl acetate fraction showed moderate hepatotoxicity at the maximal dose (200 µg/ml). This *in vitro* observation, therefore, offers a cautionary warning on the high consumption of yerba mate *in vivo*. Further, *I. paraguariensis* extract subjected to HPLC analysis led to identification of known anti-HBV active flavonoids, i.e. rutin, quercetin, kaempferol, caffeic acid and chlorogenic acid, in line with previous studies [12–15, 33].

In addition to the health-protective or therapeutic bioactive natural products commonly consumed as dietary supplements, notably flavonoids, to compensate nutritional deficiencies [34], physical exercise and weight control are widely recommended to rejuvenate and recover

compromised liver health [35, 36]. Of the known bioactive phytochemical classes, flavonoids and polyphenols constitute the largest source of promising antiviral compounds against several viruses, including HBV [6, 37–39]. The flavonoids rutin, quercetin, myricetin and kaempferol as well as some of their derivatives have been recently demonstrated to have strong anti-HBV activity in HepG2.2.15 cells [10, 12–15]. The polyphenol caffeic acid has been widely reported for its strong activity against herpes simplex virus, influenza virus, poliovirus, and hepatitis C virus, vaccinia virus and severe fever with thrombocytopenia syndrome virus [40–43]. Its derivative, chlorogenic acid, has been also shown to have broad-spectrum antiviral activity against HIV [44], adenovirus [45], and HSV [46, 47]. However, the anti-HBV activity of caffeic acid or chlorogenic acid remains underreported. In a single published study, they are demonstrated to effectively suppress HBV antigens and DNA in HepG2.2.15 as well as in duck HBV (DHBV) models [33].

In HPLC validation, the noncytotoxic preparations of *I. paraguariensis* were tested for their anti-HBV activity in HepG2.2.15. Notably, the HepG2.2.15 line comprises stably-transfected HepG2 cells with the HBV genome that allows its DNA replication and gene (surface, polymerase, capsid, pre-core, etc.) expression, and therefore is universally used to test anti-HBV agents. Of the tested noncytotoxic preparations (50 µg/ml), the chloroform fraction showed moderate (40–42.3%) whereas hexane and ethanol fractions exhibited high (52–55.6%) anti-HBV activity. Very convincingly therefore, our data on *in vitro* anti-HBV activity of *I. paraguariensis* could be attributed to rutin, quercetin, kaempferol, caffeic acid and chlorogenic acid, as validated by HPLC. Furthermore, based on our *in silico* molecular docking analysis, we have previously reported evidence for antiviral activity of rutin, quercetin and kaempferol through binding with HBV polymerase and capsid proteins [12, 15]. In the present study, we have further documented the interactions of caffeic acid and chlorogenic acid with HBV polymerase with good docking affinities.

In conclusion, the *in vitro* anti-HBV activity of *I. paraguariensis* is strongly supported by HPLC validation and molecular docking. In conclusion, we, for the first time, demonstrated the HBV inhibitory activity of *I. paraguariensis* attributed to its bioactive phytochemicals rutin, quercetin, kaempferol, caffeic acid and chlorogenic acid in a HBV-reporter cell culture model, validated by high performance liquid chromatography and molecular docking analysis. Our findings, therefore, warrant further experimental and pharmacological studies on *I. paraguariensis*.

Funding

The authors thankfully acknowledge the Researchers Supporting Project (no. RSP2024R379), King Saud University, Riyadh, Saudi Arabia for supporting this work.

Ethical approval

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

References

1. WHO2022. <https://www.who.int/news-room/fact-sheets/detail/hepatitis-b>.
2. Jeng WJ, Papatheodoridis GV, Lok ASF. Hepatitis B. *Lancet* 2023; 401: 1039-52.
3. Yardeni D, Ghany MG. Review article: hepatitis B-current and emerging therapies. *Aliment Pharmacol Ther* 2022; 55: 805-19.
4. Masetti C, Pugliese N, Aghemo A, Viganò M. Safety of current antiviral drugs for chronic hepatitis B. *Expert Opin Drug Saf* 2022; 21: 939-45.
5. Wang G, Zhang L, Bonkovsky HL. Chinese medicine for treatment of chronic hepatitis B. *Chin J Integr Med* 2012; 18: 53-5.
6. Parvez MK, Arbab AH, Al-Dosari MS. An update on natural or herbal drugs against hepatitis B virus. In *Hepatitis B: Diagnosis, Prevention and Treatment*, NOVA Science Publishers, USA, 2021; 159-84.
7. Kapoor R, Sharma B, Kanwar SS. Antiviral phytochemicals: an overview. *Biochem Physiol* 2017; 6: 1000220.
8. Perera WPRT, Liyanage JA, Dissanayake KGC, et al. Antiviral potential of selected medicinal herbs and their isolated natural products. *Biomed Res Int* 2021; 2021: 7872406.
9. Arbab AH, Parvez MK, Al-Dosari MS, Al-Rehaily AJ. *In vitro* evaluation of novel antiviral activities of 60 medicinal plants extract against hepatitis B virus. *Exp Ther Med* 2017; 14: 626-34.
10. Parvez MK, Rehman MT, Alam P, Al-Dosari MS, Alqasoumi SI, Alajmi MF. Plant-derived antiviral drugs as novel hepatitis B virus inhibitors: cell culture and molecular docking study. *Saudi Pharm J* 2017; 27: 89-400.
11. Parvez MK, Al-Dosari MS, Rehman MT, Al-Rehaily AJ, Alqahtani AS, Alajmi MF. The anti-hepatitis B virus and anti-hepatotoxic efficacies of solanopubamine, a rare alkaloid from *Solanum schimperianum*. *Saudi Pharm J* 2022; 30: 359-68.
12. Parvez MK, Al-Dosari MS, Arbab AH, Al-Rehaily AJ, Abdelwahid MAS. Bioassay-guided isolation of anti-hepatitis B virus flavonoid myricetin-3-O-rhamnoside along with quercetin from *Guiera senegalensis* leaves. *Saudi Pharm J* 2020; 28: 550-9.
13. Parvez MK, Ahmed S, Al-Dosari MS, et al. Novel anti-hepatitis B virus activity of *Euphorbia schimperi* and its quercetin and kaempferol derivatives. *ACS Omega* 2021; 6: 29100-10.
14. Parvez MK, Al-Dosari MS, Abdelwahid MAS, Alqahtani AS, Alanzi AR. Novel anti-hepatitis B virus-active catechin and epicatechin from *Rhus tripartita*. *Exp Ther Med* 2022; 23: 398.

15. Parvez MK, Al-Dosari MS, Basudan OA, Herqash RN. The anti-hepatitis B virus activity of sea buckthorn is attributed to quercetin, kaempferol and isorhamnetin. *Biomed Rep* 2022; 17: 89-94.
16. Heck CI, DeMejia EG. Yerba mate tea (*Ilex paraguariensis*). A comprehensive review on chemistry, health implications, and technological considerations. *J Food Sci* 2009; 72: 138-51.
17. Bracesco N, Sanchez AG, Contreras V, Menini T, Gugliucci A. Recent advances on *Ilex paraguariensis* research: minireview. *J Ethnopharmacol* 2011; 136: 378-84.
18. Filip R, Lotito SB, Ferraro G, Fraga CG. Antioxidant activity of *Ilex paraguariensis* and related species. *Nutr Res* 2000; 20: 1437-46.
19. Schinella GR, Troiani G, Davila V, de Buschiazzi PM, Tournier HA. Antioxidant effects of an aqueous extract of *Ilex paraguariensis*. *Biochem Biophys Res Commun* 2002; 269: 357-60.
20. Vanderjagt TJ, Ghattas R, Vanderjagt DJ, Crossey M, Glew RH. Comparison of the total antioxidant content of 30 widely used medicinal plants of New Mexico. *Life Sci* 2022; 70: 1035-40.
21. Schinella G, Fantinelli JC, Mosca SM. Cardioprotective effects of *Ilex paraguariensis* extract: evidence for a nitricoxide-dependent mechanism. *Clin Nutr* 2005; 24: 360-6.
22. Opala T, Rzymiski P, Pischel I, Wilczak M, Wozniak J. Efficacy of 12 weeks supplementation of a botanical extract-based weight loss formula on bodyweight, body composition and blood chemistry in healthy, overweight subjects – a randomized double-blind placebo-controlled clinical trial. *Eur J Med Res* 2006; 11: 343-50.
23. Paganini Stein FL, Schmidt B, Furlong EB, et al. Vascular responses to extractable fractions of *Ilex paraguariensis* in rats fed standard and high-cholesterol diets. *Biol Res Nurs* 2005; 7: 146-56.
24. Martins F, Noso TM, Porto VB, et al. Mate tea inhibits in vitro pancreatic lipase activity and has hypolipidemic effect on high-fat diet-induced obese mice. *Obesity (Silver Spring)* 2010; 18: 42-7.
25. Filip R, Davicino R, Anesini C. Antifungal activity of the aqueous extract of *Ilex paraguariensis* against *Malassezia furfur*. *Phytother Res* 2010; 24: 715-9.
26. Pomilio AB, Trajtemberg S, Vitale AA. High-performance capillary electrophoresis analysis of Mate infusions prepared from stems and leaves of *Ilex paraguariensis* using automated micellar electrokinetic capillary chromatography. *Phytochem Anal* 2002; 13: 235-41.
27. Rehman MT, Ahmed S, Khan AU. Interaction of meropenem with 'N' and 'B' isoforms of human serum albumin: a spectroscopic and molecular docking study. *J Biomol Struct Dyn* 2016; 34: 1849-64.
28. Parvez MK, Rishi V. Herb-drug interactions and hepatotoxicity. *Curr Drug Metab* 2019; 20: 275-82.
29. Parvez MK, Anjum N. Liver diseases and adverse reactions induced by medicinal herbs and herb-drug interactions. *Gastroenterol Hepatol Lett* 2022; 4: 25-9.
30. Yuen MF, Tam S, Fung J, Wong DK, Wong BC, Lai CL. Traditional Chinese medicine causing hepatotoxicity in patients with chronic hepatitis B infection: a 1-year prospective study. *Aliment Pharmacol Ther* 2006; 24: 1179-86.
31. Lee CH, Wang JD, Chen PC. Risk of liver injury associated with Chinese herbal products containing radix bupleuri in 639,779 patients with hepatitis B virus infection. *PLoS One* 2011; 6: e16064.
32. Estes JD, Stolpman D, Olyaei A, et al. High prevalence of potentially hepatotoxic herbal supplement use in patients with fulminant hepatic failure. *Arch Surg* 2003; 138: 852-8.
33. Wang GF, Shi LP, Ren YD, et al. Anti-hepatitis B virus activity of chlorogenic acid, quinic acid and caffeic acid in vivo and in vitro. *Antiviral Res* 2009; 83: 186-90.
34. Ahmed S, Parvez T, Parvez MK. Dietary or nutritional supplements associated adverse effects and toxicity. *Medp Nutr Food Sci* 2022; 1: 202203002.
35. Ismail AMA, El Gressy NSA, Hegazy MD, Elfahl AMH, Ahmed OSM. Randomized controlled effect of treadmill walking exercise on liver enzymes, psychological burden, and erectile dysfunction in men with hepatitis C. *Gastroenterol Rev* 2024; 19: 263-70.
36. Ismail AMA, Tolba AMN, Felaya ESS. Effect of aerobic exercise training on leptin and liver enzymes in non-diabetic overweight hepatitis C men. *Adv Rehab* 2021; 35: 17-23.
37. Zakaryan H, Arabyan E, Oo A, Zandi K. Flavonoids: promising natural compounds against viral infections. *Arch Virol* 2017; 162: 2539-51.
38. Wang L, Song J, Liu A, et al. Research progress of the antiviral bioactivities of natural flavonoids. *Nat Prod Bioprospect* 2020; 10: 271-83.
39. Badshah SL, Faisal S, Muhammad A, Poulson BG, Emwas AH, Jaremko M. Antiviral activities of flavonoids. *Biomed Pharmacother* 2021; 140: 11596.
40. Ikeda K, Tsujimoto K, Uozaki M, et al. Inhibition of multiplication of herpes simplex virus by caffeic acid. *Int J Mol Med* 2011; 28: 595-8.
41. Utsunomiya H, Ichinosei M, Ikeda K, et al. Inhibition by caffeic acid of the influenza A virus multiplication in vitro. *Int J Mol Med* 2014; 34: 1020-4.
42. Shen J, Wang GF, Zuo JP. Caffeic acid inhibits HCV replication via induction of IFN alpha antiviral response through p62-mediated Keap1/Nrf2 signaling pathway. *Antivir Res* 2018; 154: 166-73.
43. Langland J, Jacobs B, Wagner CE, Ruiz G, Cahill TM. Antiviral activity of metal chelates of caffeic acid and similar compounds towards herpes simplex, VSV-Ebola pseudotyped and vaccinia viruses. *Antivir Res* 2018; 160: 143-50.
44. Ogawa M, Shirasago Y, Ando S, Shimojima M, Saijo M, Fukasawa M. Caffeic acid, a coffee-related organic acid, inhibits infection by severe fever with thrombocytopenia syndrome virus in vitro. *J Infect Chemother* 2018; 24: 597-601.
45. McDougall B, King PJ, Wu BW, Hostomsky Z, Reinecke MG, Robinson WE. Dicafeoylquinic and dicafeoyltartaric acids are selective inhibitors of human immunodeficiency virus type 1 integrase. *Antimicrob Agents Chemother* 1998; 42: 140-6.
46. Chiang LC, Chiang W, Chang MY, Ng LT, Lin CC. Antiviral activity of *Plantago* major extracts and related compounds in vitro. *Antiviral Res* 2002; 55: 53-62.
47. Khan MT, Ather A, Thompson KD, Gambari R. Extracts and molecules from medicinal plants against herpes simplex viruses. *Antiviral Res* 2005; 67: 107-19.