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

Keywords
hepatitis B virus, polyphenols, flavonoids, Ilex paraguariensis, anti-HBV, HepG2.2.15 cells

Abstract
Introduction
Antiviral natural products have shown promising alternative for conventional drug-resistance in hepatitis B virus (HBV).

Material and methods
We evaluated the in vitro hepatocytotoxicity (HepG2 cells; MTT assay) followed by anti-HBV efficacy (HepG2.2.15 cell; HBV-antigens ELISA) of Ilex paraguariensis leaves total-ethanol extract (IP-Ext) and fractionated preparations in n-hexane (IP-Hex), chloroform (IP-Chl), ethyl acetate (IP-EtAc) and ethanol (IP-EtOH).

Results
All tested samples showed non-cytotoxicity except IP-EtAc having mild toxic effect at 200 μg/ml. Their anti-HBV assessment showed dose-dependent inhibitions of HBV antigens (HBs/HBe). At the selected optimal dose (50 μg/ml), while IP-Ext showed mild (HBsAg: 24.2% & HBeAg: 20.6%) and IP-Chl showed moderate (HBsAg: 42.3% & 40.1%) activities, IP-Hex (HBsAg: 55.6% & HBeAg: 52.4%) and IP-EtOH (HBsAg: 53.2% & HBeAg: 50.2%) exhibited high activities. High-performance liquid chromatography (HPLC) validation identified 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 estimated anti-HBV activities at 10 μg/ml dose were: Quercetin (HBsAg: 67.8 % & HBeAg: 64.4%), Kaempferol (HBsAg: 63.5 % & HBeAg: 61.6%), Chlorogenic acid (HBsAg: 55.2% & HBeAg: 53.8%), Rutin (HBsAg: 51.2% & HBeAg: 48.4%) and Caffeic acid (HBsAg: 42.2% & HBeAg: 39.5%). Notably, while our previous molecular docking studies had shown strong interactions of these flavonoids with HBV polymerase active-residues, here, we demonstrated good binding-affinities of the polyphenols with drug-sensitive (wild-type) and drug-resistant (mutant) polymerases.

Conclusions
Taken together, this is the first study suggesting the anti-HBV therapeutic efficacy of I. paraguariensis attributed to its antiviral phytochemicals.
Antiviral flavonoids and polyphenols driven novel anti-HBV efficacy of *Ilex paraguariensis*

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**Short title:** Flavonoids-polyphenols driven anti-HBV efficacy of *I. paraguariensis*

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Abstract

**Introduction:** Antiviral natural products have shown promising alternative for conventional drug-resistance in hepatitis B virus (HBV). Abundance of bioactive flavonoids and polyphenolic contents in *Ilex paraguariensis* (Yerba Mate) leaves warrants assessment of its anti-HBV activity.

**Materials and methods:** *I. paraguariensis* leaves total ethanol-extract (IP-Ext), including its *n*-hexane (IP-Hex), chloroform (IP-Chl), ethyl acetate (IP-EtAc) and ethanol (IP-EtOH) fractions were tested for non-hepatocytotoxicity on HepG2 (MTT assay) and anti-HBV efficacies 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 possible mechanism of anti-HBV activities 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 & HBeAg). At the selected optimal dose (50 µg/ml), while IP-Ext showed mild (HBsAg: 24.2% & HBeAg: 20.6%) and IP-Chl showed moderate (HBsAg: 42.3% & 40.1%) activities, IP-Hex (HBsAg: 55.6% & HBeAg: 52.4%) and IP-EtOH (HBsAg: 53.2% & HBeAg: 50.2%) exhibited high activities. 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) anti-HBV activities were: Quercetin (HBsAg: 67.8 % & HBeAg: 64.4%), Kaempferol (HBsAg: 63.5 % & HBeAg: 61.6%), Chlorogenic acid (HBsAg: 55.2% & HBeAg: 53.8%), Rutin (HBsAg: 51.2% & HBeAg: 48.4%) and Caffeic acid (HBsAg: 42.2% & HBeAg: 39.5%). Notably, while our previous molecular docking had shown strong interactions of these flavonoids with HBV polymerase, here, we demonstrated good binding-affinities of the polyphenols with drug-sensitive (wild-type) and drug-resistant (mutant) polymerases. **Conclusions:** Taken together, this is the first study suggesting the anti-HBV therapeutic efficacy of *I. paraguariensis* attributed to its antiviral flavonoids and polyphenols.

**Keywords:** hepatitis B virus, anti-HBV, *Ilex paraguariensis*, Yerba mate, flavonoids, polyphenols, HepG2.2.15 cells
INTRODUCTION

Globally, hepatitis B virus (HBV) chronic infection of 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-third of 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, emergences of vaccine-escape and drug-resistant HBV mutants remain clinical challenges [3, 4]. In the last couple of decades, a range of herbal formulations and bioactive phytochemicals, like alkaloids, flavonoids, polyphenols, terpenoids, terpenes, lignans, coumarins, saponins, xanthins and anthraquinones have been reported to have potential activities 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 plants extracts and isolated compounds for their promising activities 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 also prepared in cold water called as ‘Terere’ 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] salutations in vitro and in vivo. Further preclinical studies have expanded its antioxidant [23] and hypocholesterolemic [24] properties. Moreover, *I. paraguariensis* extract has been shown for 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 have investigated the in vitro anti-HBV efficacies of *I. paraguariensis* and its bioactive phytochemicals in HBV-reporter cell culture model, validated by high performance liquid chromatography and molecular docking.
MATERIALS AND METHODS

Sample collection and preparations of extract and fractions

*I. paraguariensis* dried leaves (Yerba Mate) were obtained as commercially packaged product (La Tranquera, Barcode: 17790480000371) from Argentina. All analytical grade solvents were procured from Sigma-Aldrich (Merck KGaA, Germany) extraction and fractionation process. A portion (2.0 g) of powdered dried 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

The 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) supplanted 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 supply. For the experiments, cells (0.5x10⁵ 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 vehicle or negative control, whereas 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.

Hepatocytes 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 negative control (0.1% DMSO) in triplicate, and incubated at 37 °C for 3 days. The established assay utilizing the 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 were recorded using 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 negative control.

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

A dose-dependent inhibition of HBsAg expressions 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 were analyzed quantitatively using the diagnostic ELISA kit (cat. no. 72348; MonolisaHBsAg ULTRA, Bio-Rad Laboratories Inc., CA, USA) following the kit’s manual. The optical density (λ= 450 nm) of the samples was recorded, and analyzed (Excel) in relation to the negative control, and the activity was compared with positive control. Further, with the determined optimally-active dose, a time-course analysis of HBsAg expressions 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 day 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 (day 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 (λ= 450 nm) of the samples was recorded, and analyzed (Excel) as above, and the activity was compared with 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 C$_{18}$ column (4.6 mm × 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 (Szumny and Kucharska. 2015) 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 suppressions of HBsAg and HBeAg productions in treated HepG2.2.15 cells at day 5 (single time-point) as mentioned above. LAM (2 µM) and DMSO (0.1%) served as standard (positive) and negative control, respectively as described previously [9, 10]. The data were analyzed (Excel) in relation to negative control and the activity was compared with 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 omitting water molecules or bound any unwanted atoms, and 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 method were employed for the global and local search, respectively as mentioned elsewhere [10, 11].

For each run, a total of $2.5 \times 10^6$ 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 ($\Delta G$) 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 were performed in SPSS 17.0 (SPSS Inc., Chicago, USA). Triplicated data (mean ±S.E.M) of test samples and positive control in relation to negative control were analyzed by One-way ANOVA, following the Dunnet’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 µg/ml). However, IP-EtAc had mild toxicity between 100 and 200 µg/ml doses (Fig1. A), and therefore, excluded from antiviral analysis.

**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 (Fig.
1B). 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%) activities, respectively, whereas IP-Hex (55.6%) and IP-EtOH (53.2%) showed high activities at day 5 (Fig. 2A). Because the extended incubation of the treated cultures resulted in cell overgrowth and subsequent apoptosis (data not shown), the experiment was concluded at 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 activities, 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 (Fig. 2B). Because the extended incubation of the treated cultures resulted in cell overgrowth and subsequent apoptosis (data not shown), the study was concluded at 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 (Fig. 3; Table I).

**Anti-HBV activities of identified flavonoids and polyphenols**

The anti-HBV activities of HPLC identified phytochemicals (10 µg/ml, each) on day 5 were (in the order): Quercetin (HBsAg: 67.8 % & HBeAg: 64.4%), Kaempferol (HBsAg: 63.5 % & HBeAg: 61.6%), Chlorogenic acid (HBsAg: 55.2% & HBeAg: 53.8%), Rutin (HBsAg: 51.2% & HBeAg: 48.4%), and Caffeic acid (HBsAg: 42.2% & HBeAg: 39.5%). LAM (standard) suppressed HBsAg and HBsAg by 83.2 and 74.4%, respectively (Fig. 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 described 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 (Fig. 5A; Table 1).
Caffeic acid formed three conventional hydrogen bonds with GLY151, GLN267, and LYS239 (Fig. 5B; left). In addition, it established electrostatic interactions with LYS241 and ASP83 as well as hydrophobic interactions with LYS241 (Fig. 5B; 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\(^{-1}\) and 3.53 \(\times\) 10\(^4\) M\(^{-1}\), respectively (Table 1).

Chlorogenic acid established conventional hydrogen bonds with LYS241, GLY251, and GLN267 as well as the ‘YMDD’ motif residue ASP206 (Fig. 5B; 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 complex. 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\(^{-1}\) and 2.26 \(\times\) 10\(^5\) 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 (Fig. 6A; Table 2). Caffeic acid primarily formed conventional hydrogen bonds with GLY251, SER81, and LYS239 (Fig. 6B; 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 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\(^{-1}\) and 1.28 \(\times\) 10\(^4\) M\(^{-1}\), respectively (Table 2).

Chlorogenic acid formed conventional hydrogen bonds with POLmut residues LYS32:HZ1, LYS32, ASN36, ASN36, LYS239, LYS239 and ASP83 (Fig. 6B; 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 binging-energy and docking-affinity of the complex were calculated to be -6.7 kcal mol\(^{-1}\) and 8.21 \(\times 10^4\) M\(^{-1}\), respectively (Table 1).

**DISCUSSION**

In the recent decades, considerable studies on the pharmacognosy, phytochemistry and pharmacology of natural or herbal products have motivated to focus to antiviral drug discovery, by 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 formulation like, Chuan Lian Zi (Melia toosendan), 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), and Yue Ming Zi (Cassia obtusifolia, Senna obtusifolia, Cao Yue Ming), Zexie (Alisma orientalis), Zhen Chu 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 chinese, 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 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 have first tested the hepatotoxicity of *I. paraguariensis* preparations in cultured HepG2 cells. Though the *I. paraguariensis* total ethanol-extract and organic fractions were nontoxic, the ethyl acetate fraction showed moderate hepatotoxicity at the maximal dose [200 \(\mu g/ml\)]. 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 identify known anti-HBV active flavonoids: 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 for strong anti-HBV salutations in HepG2.2.15 cells [10, 12-15]. The polyphenol Caffeic acid has been widely reported for its strong activities 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 activities against HIV [44], adenovirus [45], 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].

HPLC validation, the noncytotoxic preparations of *I. paraguariensis* were tested for their anti-HBV activities in HepG2.2.15. Notably, HepG2.2.15 line is stably-transfected HepG2 cells with HBV genome that allows its DNA replication and gene [e.g. surface, polymerase, capsid, pre-core etc.] expressions, and therefore, 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 activities. 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 suggested the antiviral activities of Rutin, Quercetin and Kaempferol through binding with HBV polymerase and capsid proteins [12,15]. In the present study, we have further shown the interactions of Caffeic acid and Chlorogenic acid with HBV polymerase with good docking affinities.
CONCLUSIONS

Taken together, the *in vitro* anti-HBV activity of *I. paraguariensis* 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 HBV-reporter cell culture model, validated by high performance liquid chromatography and molecular docking analysis. Our findings therefore, warrants further experimental and pharmacological studies on *I. paraguariensis*.

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CONFLICT OF INTERESTS

The authors declare that they have no known competing financial interests.

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Legends

**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-Eac) and ethanol (IP-EtOH) fractions on HepG2 cells viability in relation to negative control (.1% DMSO). Data are presented as the mean ± 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-Eac and IP-EtOH in relation to negative control (1% DMSO) in HepG2.2.15 cells at day 2 (single time-point). Data are presented as the mean ± 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), ethyl acetate (IP-Eac) and ethanol (IP-EtOH) fractions in HepG2.2.15 cells. (A) Inhibition of HBV ‘s’ antigen (HBsAg) in relation to negative control (DMSO). (B) Inhibition of HBV ‘e’ antigen [HBsAg] in relation to negative control (DMSO). Data are presented as the mean ± standard error of triplicated values of each sample. **P<0.01 and ***P<0.001 vs. positive control (GS).

**Figure 3.** HPLC chromatograms showing standard compounds (Rutin, Quercetin, Kaempferol, Caffeic acid and Chlorogenic acid) and their identification in *I. paraguariensis* (IP-extract).

**Figure 4.** The anti-HBV assay showing optimal inhibitions of HBsAg and HBsAg productions by *I. paraguariensis* antiviral compounds 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 ± standard error of triplicated values of each sample. **P<0.01 and ***P<0.001 vs. positive control (LAM).
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.

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.

Table 1. HPLC profile of anti-HBV flavonoids and polyphenols in *I. paraguariensis* extract.

Table 2. Molecular interaction of HBV polymerases (wild-type and mutant) with *I. paraguariensis* Caffeic acid and Chlorogenic acid.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structures</th>
<th>Retention Time</th>
<th>Conc. (mg/g) ± SD*</th>
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<tr>
<td><strong>Flavonoids</strong></td>
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<tr>
<td>Rutin</td>
<td><img src="image" alt="Rutin Structure" /></td>
<td>12.517</td>
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<td><strong>Polyphenols</strong></td>
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<tr>
<td>Caffeic acid</td>
<td><img src="image" alt="Caffeic acid Structure" /></td>
<td>10.788</td>
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<td>Chlorogenic acid</td>
<td><img src="image" alt="Chlorogenic acid Structure" /></td>
<td>9.901</td>
<td>3.22 ± 5.04</td>
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*Data of mean ± standard deviation (SD) of three determinations*
Table II.

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<th>Donor-Acceptor pair</th>
<th>Distance (Å)</th>
<th>Mode of interaction</th>
<th>Docking energy (kcal mol(^{-1}))</th>
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Figure 1

**A.**

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<th>IP-Hex</th>
<th>IP-Chl</th>
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**B.**

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<th>IP-Hex</th>
<th>IP-Chl</th>
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% Hepatocyte viability

% Inhibitions of HBsAg
Figure 2
Figure 3
Figure 4

% Inhibitions of HBV antigens

Day 5 (post-treatment)

RUT  QRC  KMP  CFA  CGA  LAM  DMSO

HBsAg  HBeAg

***  **  **  **  ***  **  **
Figure 5

A. POLwt and polyphenol complex

B. POLwt-Caffeic acid

POLwt-Chlorogenic acid

Figure 5
Figure 6

A. POLmut and polyphenols complex

B. 

- POLmut-Caffeic
- POLmut-Chlorogenic

Figure 6