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Anti-HBV Efficacy of *Ficus* Plant's Leaf Extract against the Most Dominant HBV Genotypes in the Nile Delta Region of Egypt

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HEPATITIS B virus (HBV) infects millions of people worldwide annually, causing chronic liver diseases such as cirrhosis, fibrosis, and hepatocellular carcinoma. HBV has nine known genotypes (A, B, C, D, E, F, G, H, and I) and a tentative tenth genotype (J). Identifying HBV genotyping is crucial since different genotypes and sub-genotypes can influence treatment outcomes and responses to antiviral therapy. Therefore, we employed conventional polymerase chain reaction (PCR) to determine the HBV genotypes in three Egyptian governorates: Dakahlia (DK), Gharbia (GHR), and El-Sharkia (SHR). Specific primers for various HBV genotypes (A, B, C, D, E, and F) were used for this purpose. Out of 38 samples, three samples (7.8%) were found to have the D genotype, and four samples (10.5%) were identified as genotype C. The genotypes that were discovered underwent deep sequencing, which revealed two bands in the sequence results that were 99% identical to genotype D. The remaining bands could not be sequenced or gave unclear and incorrect sequence data. We evaluated the leaf extracts of three *Ficus* species (*F. mysorensis, F. deltoidei, and sycomorus*) as antiviral substances against HBV infection using an MTT assay. The results showed promising antiviral effects of these extracts, which could potentially aid in stopping the progression of HBV infection. Our findings indicate that conventional PCR with deep sequencing can accurately identify HBV genotypes, and Ficus plants may serve as an excellent supplement to standard treatment for HBV infections.

Keywords: Anti-HBV, Conventional PCR, *Ficus mysorensis, Ficus deltoidei, Ficus sycomorus*, Natural plant extract.

Introduction

Hepatitis B virus (HBV) is considered one of the leading causes of chronic hepatitis, liver cirrhosis, and liver cancer (hepatocellular carcinoma [HCC]). Egypt had the highest age‑standardized death rate due to cirrhosis from 1990 to 2017 (Sepanlou et al., 2020). The prevalence rate of HBV (1.3%–1.5%) has declined after national infantile immunization (Elbahrawy, 2015). HBV belongs to the family Hepadnavirideae (Charre et al., 2019). It is a partially double-stranded (ds) DNA virus with a genome size of around 3.2 kbp encoding four genes (Akrami et al., 2022). HBV can result in asymptomatic infection, acute self-limiting hepatitis, chronic hepatitis, or fulminant hepatitis, leading to cirrhosis or hepatocellular carcinoma (HCC) (Terrault et al., 2018). In most severe liver transplantation cases, chronic infection complication is closely associated with the viral replication rate (Li et al., 2020).

McNaughton et al., (2020) reported that ten HBV genotypes are designated from A to J. In case studying the association between disease outcomes and viral sequences expands, HBV genotyping becomes increasingly essential (Revill et al., 2019). For instance, the HBV

genotype A is associated with inactive carrier status (Reuter et al., 2022). HBV genotype D is the most frequent genotype and is linked to active viral infection and hepatocellular carcinoma (Alfaiate et al., 2020). Long-term HBV chronic infection indicated a mix of A and D genotypes (Lampertico et al., 2017). To correctly choose a patient for antiviral medication, the HBV virus should be genotyped before treatment begins, and all genotyping data should be conjugated with a liver biopsy (Wu et al., 2021) because several studies have shown that various genotypes and sub-genotypes have different epidemiological and virological properties (Reuter et al., 2022). Moreover, the different genotypes and sub-genotypes would determine the course of the disease treatment (Schaefer, 2007), and affect the response to antiviral therapy (Stein & Loomba, 2009).

Understanding the diverse range of HBV strains circulating globally and their associations with the disease will allow us to move towards a more specific approach to analysis. Ten different HBV genotyping methods have been developed with variable sensitivity and specificity; among those essays, three are commercialized: DNA sequencing-based assay (TRUGENE HBV Genotyping assay; Siemens Healthcare Diagnostics Inc., Deerfield, IL), reverse hybridization-based assay (INNO LiPA HBV Genotyping; Innogenetics, N.V., Ghent, Belgium) and an enzyme immune-based assay (IMMUNIS HBV Genotype EIA; Cosmo Bio, Japan) (Guirgis et al., 2010). TRUGENE HBV Genotyping assay is a direct sequencing assay that amplifies the S gene (s101-s237) from HBV DNA, followed by phylogenetic analysis to detect the HBV genotype. The detection limit for this assay is 2000 copies/ mL (400 IU/mL) (Guirgis et al., 2010). INNO-LiPA assay is a reverse hybridization method by amplification of S gene (s101-s213) from HBV DNA followed by hybridization of polymerase chain reaction (PCR) to probes on nitrocellulose strips to determine genotype; however, the assay is less sensitive for patients with low HBV viral load, and the sensitivity is only 10% for samples with HBV DNA level of 10 IU/mL (Gintowt et al., 2005). The enzyme-linked immunosorbent assay (ELIZA) is designed to determine genotypes A, B, C, and D by detecting genotype-specific epitopes in the PreS2 region (Yang et al., 2007). In addition, it is used to detect HBV surface antigen (HBsAg), envelope antigen (HBeAg), HBV surface antibody (anti-HBs), HBV core antibody (anti-HBc), and the antibody of the HBV envelope (anti-HBe) (Wu et al., 2021). It can diagnose and distinguish between acute and chronic illnesses but has poor accuracy (Indolfi et al., 2019). Therefore, none of the commercialized assays are suitable for HBV DNA-suppressed patients (Liu et al., 2020).

Many scientists have recently focused their attention on the antiviral properties of various plant components (Ma & Yao, 2020). The Moraceae family includes around 800 evergreen trees, shrubs, and vines. They are flowering plants comprising 40 genera and over 1000 species (El-Beltagi et al., 2018). They contain different teams of biologically active compounds that measure chargeable for the biological activity (Mawa et al., 2013). For instance, the phytochemical compound screened by the GC-MS method revealed that 12 bioactive compounds were detected in the fruits and 29 in the leaf extract of the plants belonging to this family. These different active phytochemicals possess a good variety of activities, facilitating the protection against incurable diseases (El-Beltagi et al., 2018). Most species of this family are Fig trees (*Ficus* sp.), native to tropical and subtropical climates (Abdel-Hameed, 2009). They are rich sources of phenolic acid and flavonoids, which make them able to protect against oxidative stress disorders (Mawa et al., 2013). In addition, their fruits can aid with various ailments (Sokkar et al., 2013; Abd El-Fattah et al., 2017; El-hawary et al., 2019). For example, the extract of these plants has been reported to treat diabetes effectively, stomachache, piles, ulcers, dysentery, inflammation, oxidative stress, and cancer (Joseph & Raj, 2010). Moreover, their ethnopharmacological uses showed a possible source of phytomedicine (Mousa et al., 1994). Many *Ficus* species have been used to treat cardiovascular diseases, liver diseases, respiratory problems, menopausal symptoms, and cancer (Zingue et al., 2016; Zhang et al., 2018). Traditional herbal treatments have employed *Ficuscarica*-latex to treat warts, skin ulcers, and wounds (Lansky et al., 2008). They could be used as a novel strategic approach for treating cholestatic liver illnesses (El-hawary et al., 2019). It has been reported that these plants have antiviral properties against various human viruses (Aref, 2011). Many countries employed these plants as a possible source of phytomedicine. For

example, fruit, timber, and occasionally twigs are abundantly depicted in the early, middle, and late Kingdoms (Mon et al., 2020). The stem bark of *F. sycomorus* is traditionally used in Northern Nigeria to cure fungal infections like jaundice and dysentery (Hassan et al., 2007; Adoum, 2012).

This study aimed to detect the HBV dominant genotypes in some Egyptian governorates (Dakahlia, Gharbia, and El Sharkkia) located in Egypt's Nile delta by type-specific conventional PCR, and by confirming the expected results with deep DNA sequencing-based assay. We also aimed to determine whether the leaf extracts of *Ficus species* (*F. mysorensis, F. deltoidei, and F. sycomorus*) have a hepatoprotective effect against the HBV infection. According to our findings, conventional PCR can properly detect HBV genotypes, and we believe that the tested *Ficus* plant species are an excellent supplement to the usual treatment for HBV infections.

Materials and Methods

Patients and blood samples
Blood samples collection and serological tests

Seventy blood samples, 56 (80%) males and 14 (20%) females were collected from three different governorates in Egypt; Urology and Nephrology Center in Mansoura, French eye house in El Gharbia, and private hospitals and medical centers in Zagazig, El Sharkia. The Ethical Committee Review Board of the Faculty of Medicine at Tanta University has approved the study protocol, with the code "33124/05/19". The selected blood samples were positive for Hepatitis B virus (HBV) surface antigen (HBsAg). The blood samples were centrifuged at 3000 rpm for 10min and the plasma was separated and stored at -20℃. The separated plasma was tested for the presence of HBe antigen (HBeAg) and HBV core total antibodies (HBcAb) using CAT ELISA assay kits (Roche, Swiss). The samples were divided into three groups: Group One, 61 samples, 87.1% Chronic carrier group with anti-E seroconversion. HBsAg and HBV core antibodies were positive, while HBe-Ag was negative. Group Two, nine samples, 12.8% Chronic carrier group without anti-E seroconversion, HBsAg, HBeAg, and HBV core antibodies were positive. Group Three, ten samples, 14.2% recent infection group in which HBeAg and HBV core antibodies were negative, HBeAg was positive, and HBV core antibodies were negative.

HBV DNA extraction, and amplification using PCR

According to manufacturing protocol, the DNA of HBV was extracted from 70 samples using the QIAamp® DSP Virus Spin Kit (QIAGEN, Netherlands). Twenty-one samples were neglected as they have low DNA concentrations. The extracted DNA was examined to evaluate the HBV genotype using a conventional PCR kit (Thermo Scientific DreamTaq Green PCR Master Mix), and specific primers for each genotype (A, B, C, D, E, and F) were used by Kirschberg et al. (2004), and Yoosefi et al. (2016) (Table S1). The cycling conditions were as follows; an initial pre-denaturation at 95ºC for 15min, subsequent denaturation at 94ºC for 1min, annealing at 60ºC for 1min, an extension at 72ºC for 1min for 40 cycles besides a final extension at 72ºC for 10min. Once PCR cycles were completed, the product was subjected to electrophoresis on 1.5% agarose gel. The gel was stained with ethidium bromide, and the results adjacent to the DNA ladder (50 bp) were investigated under U.V. light.

Deep DNA sequencing-based assay

The samples that gave clear bands and reproducible PCR products were sequenced. The DNA sequencing was processed via SolGent (Solution for Genetic Technologies, South Korea) Sequencing Company. The nucleotide sequences were viewed using the Chromas program version 2.5. Each sample's electropherograms were initially recognized, evaluated, and data sequence ambiguities were resolved by comparing the findings of forward and reverse primers with HBV sequences retrieved from the NCBI database. The sequence results were then entered to BLAST (an online tool available at http://www.ncbi.nlm.nih.gov/BLAST/) as query sequences to determine their similarity to the reported databases (Stasik et al., 2018). Then, the HBV full-length sequences for different genotypes were derived from the same website. These full-length sequences were then aligned with the partial sequence results of our samples to generate a phylogenetic tree and an alignment report using the lasergen program version 7.0 (DNA stare lasergen program, Madison, Wisconsin, USA).

Effect of leaves powder extract of F.mysorensis, F. deltoidei, and F. sycomorus on HepG2 cell line

The plant material "leaf powders" were kindly provided by Natural Products Unite, Chemistry Department, National Research Centre. A human liver cancer cell (HepG2) monolayer from the Microbiology Department, Faculty of Medicine, Al Azhar University, was grown on a Dulbecco's Modified Eagle Medium (DMEM; Gibco; Thermo-Fisher Scientific; Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin and seeded in 96-well microtiter plates at 0.1 X 10^6/well (Dhama et al., 2018). The growth medium was decanted after a confluent sheet of the cells was developed. The cells were washed twice with wash media. Different concentrations (10000, 5000, 2500, 1250, 625, 312.5, 156.25, and 78.12µg/mL) from the tested leaf extracts of three different species of Ficus plant (*F. mysorensis, F. deltoidei, and F. sycomorus*) were prepared in DMEM. 0.1 ml of each dilution was tested in different wells, leaving three wells with only a maintenance medium as control. The plate was incubated at 37°C and examined for up to 2 days. Cells were checked for any physical signs of toxicity, for example, partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) solution was prepared (5 mg/ml in PBS) (BIO BASIC CANADA INC). Twenty microliter MTT solution was added to each well. The plate was placed on a shaker (VWR® Symphony™, Radnor, Pennsylvania) at 150 rpm for 5min to mix the MTT into the media thoroughly. The plate was then incubated at 37°C, 5% CO_2 for 4h to metabolize the MTT. The media was dumped off, and then the plate was dried on paper towels to remove residue if necessary. MTT metabolic product (formazan) was resuspended in 200µL dimethyl sulfoxide (DMSO). The microtiter plate was then placed on a shaking table at 150 rpm for 5min to thoroughly mix the formazan into the solvent. The optical density was measured at 560nm and subtracted background at 620nm using a spectrophotometer (Shimadzu, Japan). Optical density should be directly correlated with cell quantity. Each extract's maximum non-toxic concentration (MNTC), which gave 90% or more live non-affected cell population, was determined and used for further biological studies (Akbarizare et al., 2020).

Antiviral assay

The antiviral activity of the studied leaf extracts has been evaluated according to MTT Assay (Mosmann, 1983; Ghasemi et al., 2023). Briefly, a 96-well plate, 10,000 cells in 200µL medium were plated per well, with three wells left empty for blank controls. The plate was then incubated overnight at 37°C with 5% CO_2 to allow the cells to adhere to the wells. Various dilutions of the tested material were prepared (0.61, 1.22, 2.44, 4.9, 9.8, 19.5, 39.1, 78.12µg/mL) from the minimum non-toxic concentration (MNTC). These dilutions were then mixed with the patient's serum, separately including the two different HBV genotypes in a 1:1 ratio (v/v), and incubated for one hour. Subsequently, each well that included 10,000 cells received 100µL of viral/leaf extract suspension**.** The plate was then shaken for 5 minutes at 150 rpm on a thermos-shaker (BioSan, TS-100, Germany), then incubated at 37°C with 5% CO_2 for several days to allow the extract to show its effect on the HBV genotypes C, and D. Two milliliters or more of MTT solution in PBS (5mg/mL) were prepared. Each well received 20 µl of MTT solution, then mixed with the media by shaking at 150 rpm for 5min. The plate was then incubated for 1-5h at 37°C with 5% CO_2 to metabolize the MTT. The medium was removed and, if necessary, paper towels might be used to dry the plate and to remove any residue. In each well, 200µL of formazan dissolved in DMSO was resuspended. The plate was shaken at 150 rpm for 5min to mix the formazan with the solvent thoroughly. The optical density was measured at 560nm using a spectrophotometer (Shimadzu, Japan), and the background was removed at 620nm. Optical density should be directly connected with cell amount (Akbarizare et al., 2020). To calculate the viral inhibition percentage, the following formula was used: Viral inhibition percentage = $[(OD_{560}$ control $- OD_{560}$ treated)/ OD_{560} control$]$ x 100. Where: OD control = the absorbance value of the untreated virus-infected cells (control), OD treated = the absorbance value of the virus-infected cells treated with the leaf extract. A lower percentage indicates a stronger antiviral effect.

Microscopic examination for the cytopathic effect of HBV and the tested leaf extracts

To observe the cytotoxic effect of the HBV or different plant extracts, a microscopic examination was performed to monitor changes in cell morphology and viability. The cells were

first cultured, as previously mentioned, and then incubated with serum including the virus, or a mixture of different leaf extracts and the serum including the virus for several days. After incubation, the cells were washed and fixed with a 10% formalin solution.

The fixed cells were then stained with trypan blue (0.4% trypan blue powder in phosphate-buffered saline (PBS) at a ratio of 1:1), which stains dead cells blue while live cells appear unstained. The stained cells were observed under a microscope, and the number and percentage of dead and live cells were counted and recorded. Additionally, morphological changes such as shrinkage, rounding, detachment, and formation of membrane blebs were observed under the optical microscope (Olympus Corporation, Shinjuku, Tokyo, Japan). The results of the microscopic examination were used to indicate the cytotoxic effect of the virus or different plant extracts on the cells. A higher number of dead cells and significant morphological changes indicated a higher cytotoxic effect (Akbarizare et al., 2020).

Statistical analysis
Data were represented using a computer program Graph Pad Prism version 9.0.1 (161) as mean ± S.D. and analyzed using one-way variance analysis (ANOVA) followed by the Dunnet test. The P value P<0.05 or P<0.01 were statistically significant.

Results

Serological tests
Before we proceeded with PCR, 70 samples that were positive for HBsAg were tested for the presence of HBVe-Ag and total core antibodies. Out of the 70 samples, only 8 samples (11.4%) tested positive for HBe-Ag, while 62 samples (88%) showed the presence of total core antibodies (Table 1).

Genotyping of HBV in the Nile Delta region of Egypt
The samples containing low HBV DNA were neglected, only 38 from 70 samples that showed a good amount of extracted DNA were tested with type-specific conventional PCR. Two different HBV genotypes have been detected. Three samples (7.8%) of 38 samples were genotype D (samples 9, 10, and 24), and Four samples (10.5%) were given for genotype C (samples 9, 20, 23, and 24). We could not recognize genotype A, B, E, and F (Fig. 1).

Validation of genotypes by sequencing
The genotypes of HBV detected in the present study using conventional PCR were validated through sequencing. The obtained results were primarily aligned with the NCBI website. Two of the sent samples that could be sequenced showed 99% identity to the HBV D genotype. Then the HBV full-length sequences for different HBV genotypes (gt-A, gt-C, C, D1, D3, gt-E, E, G, and H) were derived from the NCBI website. The alignment between the sequenced samples and the different HBV genotypes using the lasergen program version 7.0 reported an identity between them, specifically with genotype D with a sequence distance of 98.5% (Figs. 2, 3).

TABLE 1. Chronic carrier with anti-e seroconversion

| Sample ID no.	Sex	Age	Location	HBs-Ag	HBe-Ag	Total HB-Anti-core
2	M	25	DK	+ve	+ve	+ve
4	M	45	GHR	+ve	+ve	+ve
9	M	24	DK	+ve	+ve	+ve
10	F	24	DK	+ve	+ve	+ve
20	M	49	DK	+ve	+ve	+ve
23	M	50	GHR	+ve	+ve	+ve
24	M	60	SHR	+ve	+ve	+ve
39	M	52	GHR	+ve	+ve	+ve

M; male, F; female
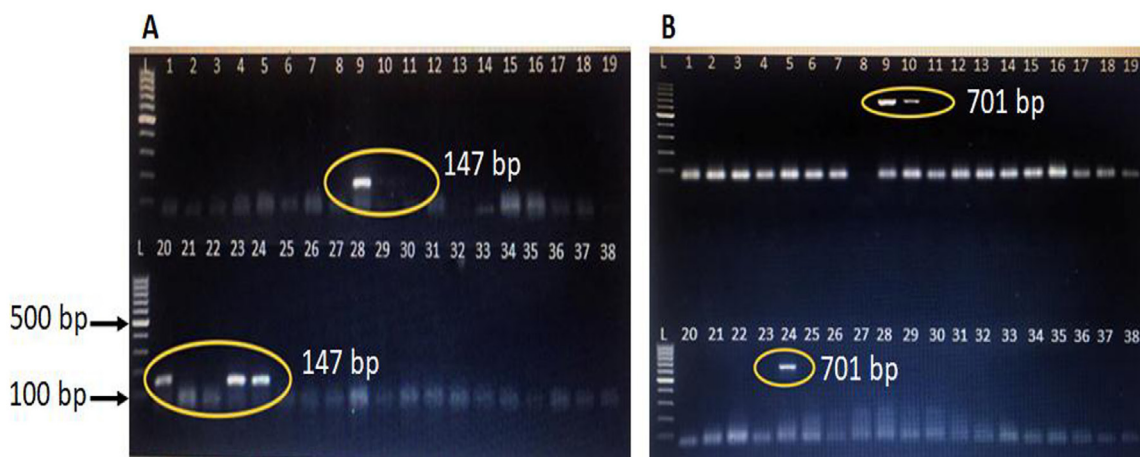
Fig. 1. Agarose gel electrophoresis (2.5% agarose) of PCR amplified products using specific primers for each HBV genotypes (A, B, C, D, E, and F) [A. Lanes 1–38 are examined HBV using specific primer for C genotype. B. Lanes 1-38 are examined HBV using specific primer for D genotype. We could not recognize genotype A, B, E, and F. Lane L, 1 kb DNA size marker]
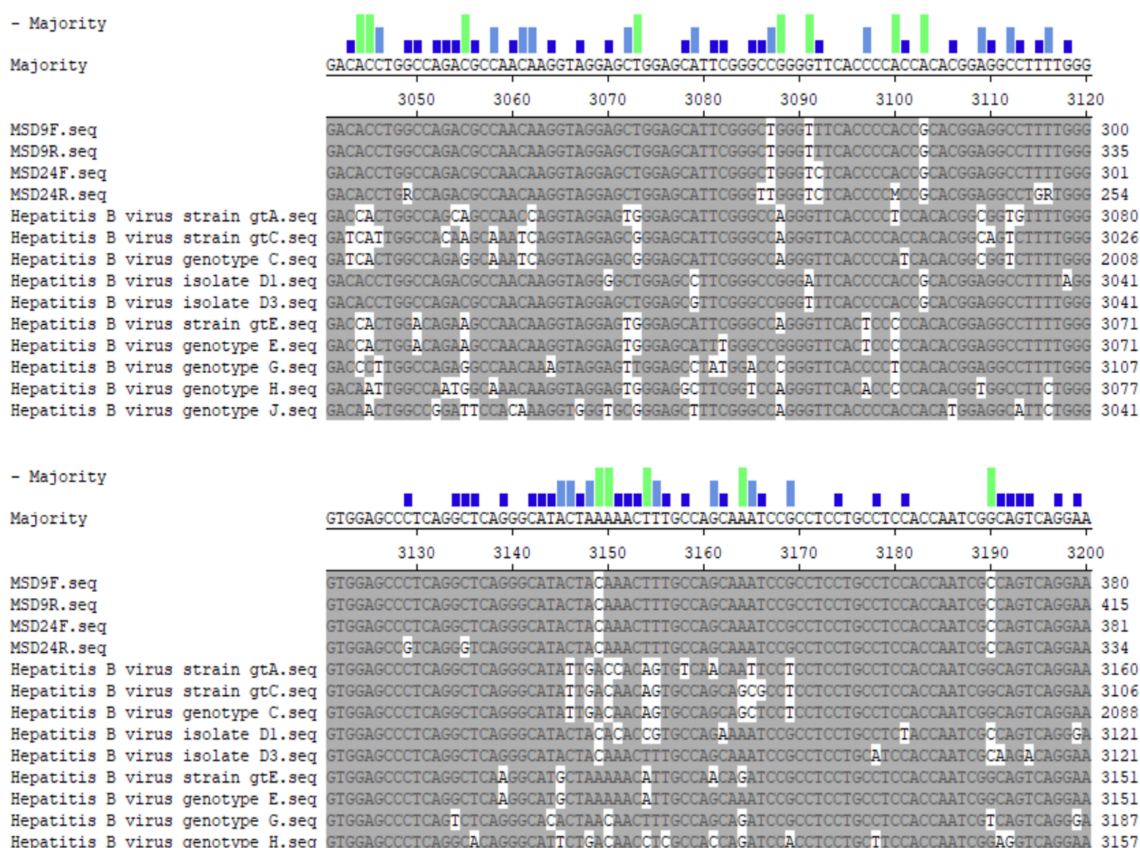
Fig. 2. Nucleotide sequence alignment of the two sequenced bands; both forward (F) and reverse (R) primers were used to confirm the sequence results [The samples were labeled MSDF, MSDR, MSD2F, and MSD2R. As shown, the sequenced result is identical to the different genotypes of HBV (gt-A, gt-C, C, D1, D3, gt-E, E, G, and H) specifically with genotype D. The sequence result was primarily aligned in the NCBI website, that showed 99% identity to HBV D genotype, then the HBV full length sequences for different genotypes were derived from the same website. The alignment report was done using lasergen program version 7.0]
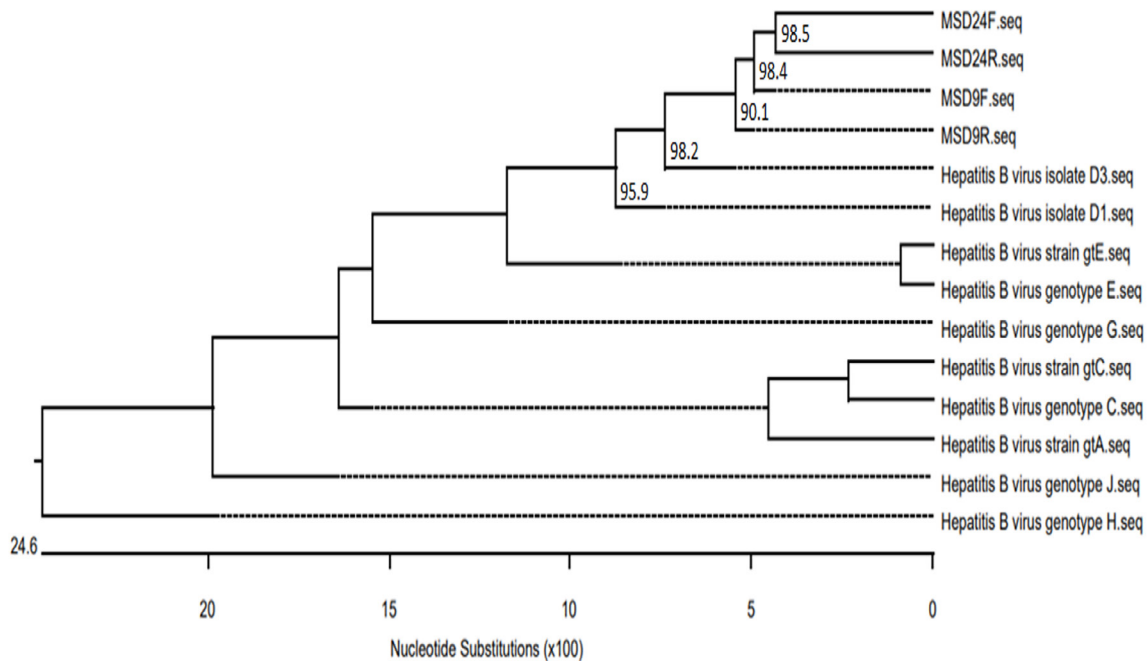
Fig. 3. The phylogenetic tree between the sequenced bands and the different HBV genotypes (gt-A, gt-C, C, D1, D3, gt-E, E, G, and H) [The lower amino acid substitution per 100 residues, the higher similarity between the corresponding genotypes. The percentage identity and the divergence between the different genotypes and the sequenced bands was shown as percentage number]

Cytotoxic effect of the leaf extract of F. mysorensis, F. sycomorus, and F. deltoidei against HepG2 cell viability and determination of the maximum non-toxic concentration

The cytotoxicity of the studied leaf extracts has been evaluated according to Atta et al. (2023), and the obtained results are illustrated in Figs. 4, 5. The maximum non-toxic concentration (MNTC) of each extract which gave 90% or more viable, non-affected HePG2 cell population was detected as (78.12μg/mL) for the three species of *Ficus* plants (Table S2). As a result, extracts at 78.12μg/mL were employed in the following antiviral experiments.

Anti-HBV effect of Ficus sp. leaf extract

Different dilutions from each extract's maximum non-toxic concentration (78.12 μg/ml) were tested for antiviral activity against HBV- genotype C and HBV- genotype D (Table S3, and Table S4). Our findings showed that combining the viral suspension (1:1, v/v) with the specific dilution from each extract increased antiviral activity gradually from the lowest concentration of the MNTC for each extract to the highest one (Fig. 5). Microscopic

assessment of cell morphology/growth was evaluated repeatedly every 24 hours after treatment for about 4 days. The observed changes supported this finding, where around 90% of HePG2 cells were viable without any morphological changes at the MNTC from each extract, compared to both healthy control cells and the cells treated with the viral suspension only (Fig. 4). No significant differences were observed between the effect of these extracts on the two HBV genotypes.

Discussion

Different HBV genotypes are found worldwide; however, their virulence and pathogenicity vary significantly. HBV genotype is crucial for determining the virus's infection route, severity, pattern of serological activity, virus replication, and treatment response. In this context, patients with genotype C have a lower response to treatment than the other genotypes (Iqbal et al., 2016). Moreover, Precore mutates are frequently associated with genotype D, which raises the risk of cirrhosis and hepatocellular carcinoma (Hamida et al., 2021).
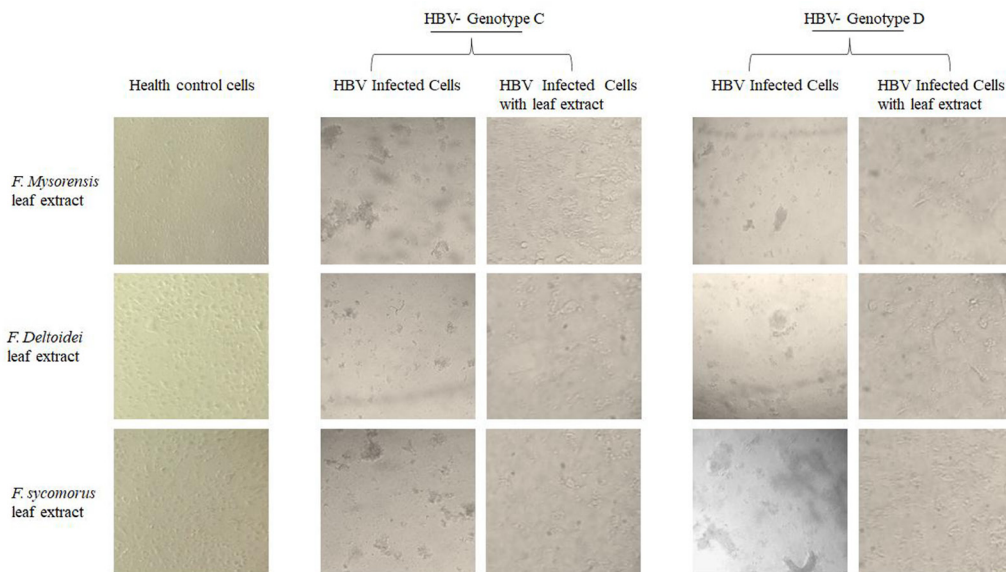
Fig. 4. Changes in the morphology of HepG2 cells following treatment with HBV genotypes C and D, and with HBV genotypes mixing with the MNTC of leaf extracts of *F. mysorensis*, *F. deltoidei*, and *F. sycomorus*. [HepG2 cells, plated at 10,000 cells in 200μL medium per well in a 96-well microtiter plates. The plates were cultured overnight at 37 OC, 5% CO2 with the different treatments as shown in the photos. The health control cells without any treatment were used as negative control]
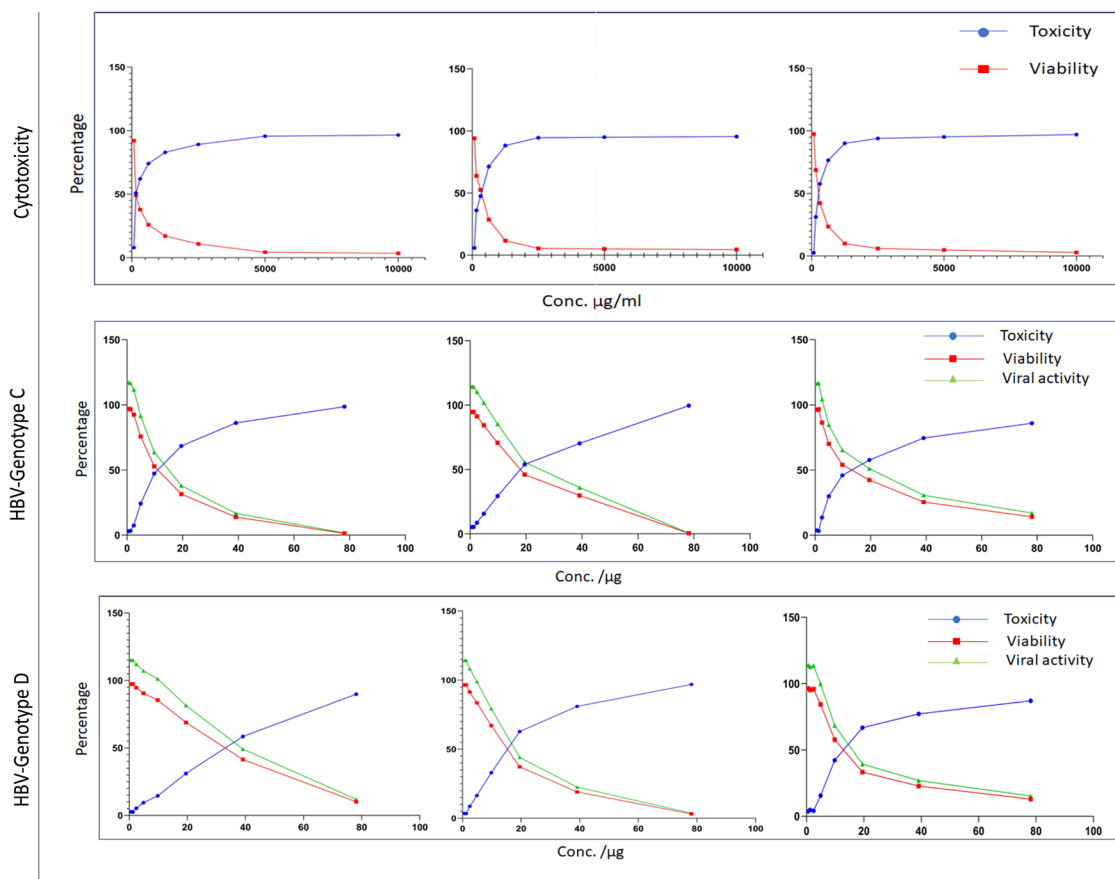
Fig. 5. Cytotoxic effect of the leaf extract of *F. mysorensis*, *F. sycomorus*, and *F. deltoidei* against HepG2 cell viability, and determination their antiviral activity against both HBV-C, and HBV-D genotypes

Although Restriction Fragment Length Polymorphism (RFLP) direct sequence analysis and enzyme-linked immunosorbent assay (ELISA) are standard gold methods for determining different HBV genotypes, they are not suitable for finding mixed genotypes (Wang et al., 2007). This study aimed firstly, to find out HBV dominant genotypes in Egypt using conventional PCR and then to detect the effect of different plant extracts on the activity of such strains. Specific primers pair were constructed based on nucleotide sequences between S and pre-SI genes. These specific primers were developed to find genotypes A to F and sub-genotypes (Liu et al., 2020). Depending on this approach, our results showed clear bands with genotype D at 701 bp and genotype C at 147 bp. Genotype D was the most predominant strain among all-identified genotypes. The study indicated that sequenced samples with genotype D had the same sequence as the online database of the NCBI website. These findings are consistent with two additional Egyptian investigations (Zekri et al., 2007; Elbahrawy, 2015). They showed that the most common HBV genotype in Egypt was genotype D. In another investigation, HBV genotypes were determined by sequencing in 150 serum samples from Egyptian carriers, and HBV genotype D was shown to be the most prevalent (Abdel-Maksoud et al., 2019).

The development of anti-HBV drugs was impeded by a lack of appropriate in *vitro* and in *vivo* experimental models that could imitate natural chronic hepatitis B. Many hepatoma cell lines, such as Hep-G2 cells, were transfected with the HBV genome and developed as an *in vitro* model to evaluate and find potential antiviral therapy medicine (Watashi & Wakita, 2015). In this work, the Hep-G2 cells were employed to assess the anti-HBV capability of three *Ficus* plant species (*F. mysorensis*, *F. deltoidei*, and *F. sycomorus*) using a cell viability assay. A preliminary cell viability experiment of leaf extracts of the three *Ficus* plant species revealed each extract's maximum non-toxic concentration (MNTC) at doses of 78.12μg/mL with no evidence of cytotoxicity.

The capacity of the three *Ficus* plant extracts to protect Hep-G2 cells from the cytopathic effect of HBV reported that combining the viral suspension (1:1, v/v) with the specific dilutions from MNTC of each extract increased antiviral activity gradually from the lowest to the highest

dilution. According to our knowledge, this is the first study that showed the antiviral activity of these plants against HBV. Previous investigations proved that *Ficus* leaves have antihypertensive, anti-hypercholesterolemic, and hepatoprotective properties (El-hawary et al., 2019). These properties might be the reason that helps protect the Hep-G2 cells from the cytopathic effect of HBV. For instance, chlorogenic acid, an active component from the *Ficus* plant, protects the liver from the damage caused by pentachlorophenol, a pollutant found in the environment (Wang et al., 2022). Its antioxidant properties, downregulation of hepatic glucose-6-phosphatase expression, and reduction of hepatic steatosis were responsible for this impact. Moreover, *In-vivo* clinical investigations have shown that chlorogenic acid possesses antioxidant action against metabolic syndrome caused by oxidative stress (Miao & Xiang, 2020). In addition, Rutin, another component from these plant species, is a flavonoid glycoside that has been shown to have hepatoprotective properties against carbon tetrachloride-induced liver damage by increasing endogenous hepatic antioxidant enzymes and suppressing inflammatory cytokines at the gene level. Therefore, the damage that occurred by HBV infection might be inhibited by these properties.

Moreover, several studies utilized gas chromatographic-mass spectrum analysis (GC-MS) for the leaf extracts of these plants and tested their possible antimicrobial activity. For instance, Abbass et al. (2015) explore the phytochemical compounds and biological activities of *Ficus mysorensis*. Their study reveals that the leaf extract of *Ficus mysorensis* contains a diverse range of bioactive compounds, including phenolic compounds, flavonoids, alkaloids, terpenoids, tannins, and sterols. These compounds have potential health benefits such as antioxidant, antimicrobial, and anti-cancer properties. The study also identified fatty acids and sterols which exhibit an inhibiting activity of 5-alpha reductase and anti-inflammatory effects. Using GC-MS analysis six major phytochemical compounds were identified in *Ficus mysorensis*; Friedelin, β-friedelinol, Lupeol, β-sitosterol, Erythrodiol, and Benzyl-O-β-D-glucopyranoside.

Friedelin and β-friedelinol are triterpenoids that represent various biological activities, including anti-inflammatory, antidiabetic, and anticancer

properties. Lupeol is a pentacyclic triterpene that possesses anti-inflammatory, antioxidant, and anticancer activities. β-sitosterol is a phytosterol that is reported to have cholesterol-lowering effects and anti-inflammatory properties. Erythrodiol is a triterpenoid that is shown to possess antitumor and anti-inflammatory activities. Benzyl-O-β-D-glucopyranoside is a glycoside that has been reported to exhibit antioxidant activity. Furthermore, the study also recorded the cytotoxic activity of *Ficus mysorensis* against human colon carcinoma (HCT-116) and hepatocellular carcinoma (HEP-G2) cell lines.

The leaves of *Ficus sycomorus* have a high concentration of phytochemical compounds according to the GC-MS analysis that was established by El-Beltagi et al. (2018). These phytochemical compounds included total phenols, anthocyanins, and flavonoids, which have antioxidant properties and potential health benefits such as preventing cancer and mutations. The Leaves extract also contains high amounts of tannins, which inhibit the growth of microorganisms by depriving them of proteins. Moreover, Leaves contain high amounts of Alkaloids that can be toxic to foreign organisms and inhibit certain enzymes. The GC-MS analysis identified some fatty acids including unsaturated fatty acids (linoleic, oleic, and linolenic acid), and sterol (β-sitosterol and campesterol) compounds. Phytosterols have important roles in cell processes and may have inhibitory effects against 5-alpha reductase and provide anti-inflammatory effects. Some common sterols are used for medicinal purposes. Moreover, the plant contains phenolic and flavonoid compounds such as quercetin, kaempferol, benzoic acid, ellagic acid, myricetin, and naringenin. Quercetin may protect DNA and prevent mutations and cancer. Kaempferol and its derivatives have antibacterial activity.

Azizan et al. (2017) utilized GC-MS analysis of the leaf extract of *Ficus deltoidea* and identified some sesquiterpenes, such as β-caryophyllene (36.3%), eugenol (8.1%), α-humulene (5.5%), α-copaene (3.3%), and germacrene D (7.7%) which were the dominant compounds. β-caryophyllene is a common natural compound found in aromatic plants, which is a bicyclic sesquiterpene composed of three isoprene units and a cyclobutane ring. It is often found in a mixture of α-humulene and caryophyllene. α-Humulene, on the other hand, is an isomer of β-caryophyllene, and it is a monocyclic sesquiterpene that occurs naturally and is made of three isoprene units, but it lacks a cyclobutane ring. These compounds represented antimicrobial activity against several pathogenic oral bacteria, namely *Streptococcus mutans*, *Streptococcus sanguinis*, and *Lactobacillus acidophilus*, which are known to cause tooth decay and periodontal diseases. Eugenol can disrupt the membranes and walls of bacterial cells, causing the release of intracellular components. Germacrene D is a sesquiterpene that could damage the morphological structure of bacterial cell membranes, as well.

As far as our knowledge goes, this is the first study to establish the potential antiviral activity of these three plants. Based on our findings, in addition to previous research, further investigations are needed to test the role of each component of these extracts and examine their in vivo cytotoxicity. However, if future studies confirm their efficacy and safety, these extracts could be an excellent supplementary choice for the standard treatment protocol for HBV infection.

Conclusion

The results of this study provide important information for understanding the prevalence and genotypes of HBV in the Nile Delta region of Egypt, which could help in developing effective strategies for the prevention and treatment of HBV infections. The findings also suggest that leaf extracts from Ficus plants may have potential as an antiviral agent against HBV, which could be explored further in future studies. However, the study has a small sample size and is limited to the Nile Delta region of Egypt, so the results may not be generalizable to other regions. Additionally, the study only tested the antiviral activity of leaf extracts from three species of *Ficus* plants and did not explore the mechanisms behind their activity. Further studies with larger sample sizes and more in-depth exploration of the antiviral mechanisms are needed to validate these findings.

Conflict of interests: The authors confirm that there is no conflict of interest to disclose.

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supervision, Y. A. M., G. K. K., M. S. Y. and A. G.; writing—original draft preparation, M.A.E.; writing—review and editing M.A.E.; All authors read and approved the final manuscript.

Ethical approval: This study complies with all relevant ethical regulations. The experimental protocol was established according to the International Ethical Guidelines for Biomedical Research Involving Human Subjects and were approved by The Ethical Committee Review Board, Faculty of Medicine, Tanta University, Tanta, Egypt, granted this cross-sectional study (approval code "33124/05/19").

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فاعلية مستخلص أوراق نبات اللبخ ضد الأنماط الجينية لفيروس التهاب الكبد الوبائي B الأكثر شيوعًا في دلتا النيل بمصر

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فيروس التهاب الكبد الوبائي B (HBV) يصيب ملايين الأشخاص في جميع أنحاء العالم سنويًا، ويسبب أمراض الكبد المزمنة مثل التليف، وسرطان الكبد الخلوي. يعتبر HBV تسعة أنواع معروفة (A، B، C، D، E، F، G، H، و I) ونوعًا عاشرا مؤقتًا (J). يعد تحديد النمط الجيني لـ HBV أمرًا ضروريًا لأن الأنواع المختلفة والسلالات الفرعية يمكن أن تؤثر على نتائج العلاج والاستجابة للعلاج المضاد للفيروسات. لذلك، استخدمنا تفاعل البوليميراز المتسلسل التقليدي (PCR) لتحديد أنواع HBV في ثلاث محافظات مصرية: الدقهلية (DK)، الغربية (GHR)، و الشرقية (SHR). تم استخدام primers محددة لأنواع مختلفة من (A ، B، C، D، E، و F) لهذا الغرض. من بين 38 عينة، تم العثور على ثلاث عينات (7.8٪) لها النمط الجيني D ، وتم تحديد أربع عينات (10.5٪) على أنها النمط الجيني C. خضعت الأنماط الجينية المكتشفة إلى التسلسل العميق، مما كشف عن نوع واحد فقط والذي كان 99٪ مطابقا للنوع D. لم يتم تسلسل الأنماط المتبقية أو أعطت بيانات تسلسل غير واضحة وخاطئة. قمنا بتقييم مستخلصات أوراق ثلاثة أنواع من نبات اللبخ (F. mysorensis ، F. deltoidei، و F. sycomorus) كمواد مضادة للفيروسات ضد عدوى HBV باستخدام اختبار نشاط الخلايا الحية بصبغة MTT. أظهرت النتائج تأثيرات مضادة للفيروسات واعدة لهذه المستخلصات، والتي يمكن أن تساعد في وقف تطور عدوى HBV. تشير نتائجنا إلى أن PCR التقليدي مع التسلسل العميق يمكن أن يحدد أنواع HBV بدقة، وأن الثلاث أنواع المستخدمة من نبات اللبخ يمكن أن تكون مكملًا ممتازًا للعلاج القياسي لالتهابات HBV.