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Antiviral Effect of *Phyllanthus nanus* Ethanolic Extract Against Hepatitis B Virus (HBV) by Expression Microarray Analysis

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Abstract Ethanolic extract of *Phyllanthus nanus* (*P. nanus*) treatment exhibited potent antiviral activity against Hepatitis B virus (HBV). The effects of these extracts on HBV in the HBV genome integrated cell lines—Alexander cells and HepG2 2.2.15 cells were examined. Experimental results showed that the ethanolic extract of *P. nanus* produced suppressive effect on HBsAg secretion and HBsAg mRNA expression. The extract also inhibited HBV replication as measured by HBV DNA level in vitro. In addition, using a duck HBV (DHBV) primary culture model, the *P. nanus* ethanolic extract suppressed viral replication of DHBV in DHBV infected primary duck hepatocytes. The gene expression pattern in Alexander cells that had been treated with the ethanolic extract of *P. nanus* was also revealed by microarray techniques. The microarray results indicated that there was up-regulation of expression of several genes, including annexin A7 (*Axn7*). The subcellular localization of Axn7 and anti-HBV effect of Axn7 over-expression in Alexander cells were also investigated. Results showed that expression of Axn7–GFP fusion protein are localized around the secretory vesicles and could cause a decrease in HBsAg secretion in Alexander cells. Axn7 protein might play an important role in the medicinal effect of the active principle(s) of *P. nanus*. J. Cell. Biochem. 97: 795–812, 2006.

Key words: antiviral agent; hepatitis B virus; *Phyllanthus nanus*; inhibition of HBsAg secretion; microarray; annexin A7 subcellular localization

Hepatitis B virus (HBV) is a major pathogen of human viral hepatitis. It has been estimated that 350 million people are chronic carriers of HBV throughout the world. Increasing evidence indicates that persistent viral infection of the liver is associated with cirrhosis and hepatocellular carcinoma [Chisari, 2000]. HBV belongs to a family of DNA viruses called hepadnaviruses. The current treatment of hepatitis B using interferon or lamivudine has several disadvantages, and there appears to be much room for improvement in terms of medical treatment [Lambiase and Davis, 1992; Allen et al., 1998].

The plants of the genus *Phyllanthus* are widely distributed in most tropical and subtropical countries. A number of recent research studies have been done on different species of *Phyllanthus* [Venkateswaran et al., 1987; Santos et al., 1995; Calixto et al., 1998; Catapan et al., 2000; Cai and Liang, 2003], which has long been used for the treatment of liver disease in China and India [Liu et al., 2001].

Phytochemical studies carried out on these plants led to the isolation and characterization of different classes of compounds, including alkaloids, flavonoids, lignans, phenols, and

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terpenes. These compounds were reported to be responsible for the pharmacological actions of these plants [Blumberg, 1998]. Many studies suggest that most species of the genus *Phyllanthus* have a beneficial effect against HBV, possibly through inhibition of polymerase activity, mRNA transcription, and replication [Venkateswaran et al., 1987; Chang et al., 1995; Lee et al., 1996; Ott et al., 1997].

In 1988, it was reported that an aqueous extract of *P. amarus* exhibit anti-HBV effects [Thyagarajan et al., 1988] in a clinical study. Specifically, Thyagarajan et al. [2002] found that the HBV surface antigen (HBsAg) level of 59% of HBV carriers became negative after 9-month treatments with *P. amarus* collected from India, compared with only 4% in the placebo group [Thyagarajan et al., 1988]. However, another study failed to reproduce this result, finding no difference in HBsAg and HBV e antigen (HBeAg) seroconversion [Leelarasamee et al., 1990]. Subsequently, *P. urinaria* L., which belongs to the same genus, has also been reported to exhibit anti-HBV effects [Liu et al., 1997]. It was shown that *P. urinaria* L. grown in different areas in mainland China exhibited different anti-duck HBV (anti-DHBV) effects, with the plants grown in the Yunnan area showing suppression of DHBV DNA ($P < 0.05$), while those grown in the Chongqing area showed no effect at all [Chen et al., 1995]. The different clinical effects could be due to compositional variations in plants and different constituents produced by plants of different *Phyllanthus* species that were grown in different areas.

Encouraged by these early findings on the anti-HBV effect of *Phyllanthus* species, we studied a species of *Phyllanthus* that were not widely reported to have anti-HBV effect previously—*P. nanus*. We also employed microarray techniques to explore the complicated mechanism of action involved in the anti-HBV effect of *P. nanus*.

MATERIALS AND METHODS

P. nanus

P. nanus was identified according to their morphological appearance by Prof. Li Pingt'ao of the Universitas Agriculturae Austro Sinicae based on the definition described in Flora of British India [Hooker, 1885; Punt, 1987].

Ethanolic extract of *P. nanus* was prepared by boiling the pulverized herbs with tenfold volume of 95% ethanol for 2 h and extraction was repeated for another 2 h after adding the same amount of 95% ethanol again [Huang et al., 2003]. The extract was filtered with filter paper and concentrated under vacuum at 40°C to give the ethanolic extract (syrup) using a rotary evaporator.

Cell Culture

PLC/PRF/5 (Alexander) cell line (ATCC CRL-8024) was human epithelial-like hepatoma cells which contained an integrated HBV genome so that the cells could secrete HBsAg continuously into the culture medium. However, this cell line does not secrete virion particles. The cell line was maintained in Dulbecco's modified Eagle medium (DMEM) (Gibco Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (PS) under a humidified atmosphere at 37°C containing 5% CO_2. During cell differentiation, the medium was changed every 3 days and the drug treatment experiments were performed with cells in passage number between 2 and 20 [Knowles et al., 1980; Park et al., 1995; Gripon et al., 2002].

HepG2 2.2.15 cell line was a gift from Prof. Fong Wang Fun of City University of Hong Kong. This cell line was human epithelial-like hepatoma cells which contained an integrated HBV genome so that the cells could secrete live HBV virions particles, HBsAg as well as HBeAg continuously into the culture medium [Ou and Rutter, 1985; Sureau et al., 1986; Ueda et al., 1989]. This cell line was also maintained in DMEM (Gibco Life Technologies) supplemented with 5% heat-inactivated FBS and 1% PS under a humidified atmosphere at 37°C containing 5% CO_2. During cell differentiation, the medium was changed every 3 days and the cells were kept at 80% confluent. Three hundred thirty micrograms per milliliter G418 (Geneticin^{TM}, Gibco Life Technologies-BRL) was used to "reselect" for G418-resistance after every 3–4 passages because the 2.2.15 cell line was transfected with a vector that conferred resistance to the neomycin analog, G418. The drug treatment experiments were performed with cells with passage number between 2 and 15 [Sells et al., 1987, 1988; Paran et al., 2001].

Cell viability was additionally tested by determining use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye (MTT) (Sigma, St. Louis, MO) after exposing

cells to 1 mg/ml MTT in phenol red-free medium for 3 h at 37°C. The blue formazan product was solubilized in DMSO and optical density measured at 540 nm [Denizot and Lang, 1986].

Antiviral Assay in Cell Culture System

For the cell lines PLC/PRF/5 (Alexander cells) or HepG2 2.2.15 cells, 1 day before drug treatment, confluent culture in 6-well, 24-well, 96-well microplates were seeded [Visweswaram et al., 1994]. The cells were then treated with different concentrations of herbal extracts in DMEM medium with 10% FBS and 1% PS. For the ethanolic extract, 1% methanol was used for dissolving the extract before dissolving into the culture medium. After 48 h treatment (for Alexander cells) or 7 days treatment (for HepG2 2.2.15 cells), the culture medium was collected and assayed for the levels of surface antigen (HBsAg) [Chiang et al., 2003]. Since Alexander cells can only produce HBsAg and do not secrete live HBV into the medium, therefore, shorter incubation time was enough for the extract to take effect in this cell line in order to affect the HBsAg synthesis. However, the HepG2 2.2.15 cells can support the replication of live HBV. Therefore, longer incubation time was allowed for the extract to take effect in the HepG2 2.2.15 cells in order to affect the HBV replication. The determination of the length of incubation time was done in previous studies (data not shown). The cells were then used for cytotoxicity measurement by using MTT assay or for the determination of the mRNA expression level of the *Pre-S* or *S* gene, i.e., *HBsAg* gene, or for the determination of the level of intracellular or extracellular HBV DNA. Inhibitory concentration 50% (IC_{50}) was used to assess the cytotoxicity of the extracts and the percentage of suppression of Pre-S or S mRNA expression level or HBV DNA level were used to assess the antiviral efficacy of the extracts. All virus-handling protocols were reviewed and approved by the safety office of The Chinese University of Hong Kong.

Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Firstly, Alexander cells were treated with *P. nanus* ethanolic extract for 48 h. Total RNA was then isolated from control cells and *P. nanus* ethanolic extract treated cells using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Specific primers for the appropriate genes were designed for performing RT-PCR using the software Genetools™. Then 10 μg of DNase I treated RNA from each sample was incubated for 5 min at 65°C and then cooled on ice for 1 min. Subsequently, the RNA was reverse-transcribed for 1 h at 42°C in a 50 μl reaction mix containing 5× reverse transcription (RT) buffer, 0.3 mM $MgCl_2$, 0.5 mM dNTPs (Invitrogen), 0.4 mM polyA-oligodT 25 primer (Invitrogen), 50 U of $RNaseH^-$ M-MULV reverse transcriptase and 40 U RNaseOUT™ RNase inhibitor (Invitrogen). The reaction was terminated at 70°C for 15 min. Then 2 U RNase H (Invitrogen) was added to the sample to remove the RNA template by incubating at 37°C for 20 min. The cDNA synthesized was then stored at −20°C for PCR. For semi-quantitative RT-PCR, after the cDNA from each of the samples were synthesized, the amounts of cDNA templates were normalized using the internal housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), i.e., the amount of 1st strand cDNA of each sample was adjusted based on the GAPDH concentration. To quantify the expression level of the selected genes, same amounts of cDNA templates, obtained after normalization (GAPDH and *Pre-S* and *S* genes were reverse-transcripted in the same tube for each of the sample) [Kim et al., 2005], was subjected to PCR in the presence of 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 300 μM of each dNTP, 2.5 U *Taq* Polymerase and 10 μM of each primer (*S* gene forward and reverse primers (−5′-CCCAATACCACATCATCC-3′; +5′-GGATTGGGGACCCTGCGC-3′), *Pre-S* gene forward and reverse primers (−5′-GTCCTAGGAATCCTGATG-3′; +5′-GGGTCACCATATTCTTGG-3′), GAPDH forward and reverse primers) in a final volume of 50 μl. A master mix would be used for the PCR reaction of the different primers. It was assumed that the amplification efficiency between the different reactions by using the reactants from the same master mix would be the same. The PCR reaction was subjected to 36 s denaturation at 95°C, followed by 25 cycles of amplification (95°C, 36 s; 60°C, 36 s; 72°C, 90 s) and a final extension at 72°C for 10 min. Each experiment was repeated at least three times. The PCR product was then analyzed on a 1% agarose gel with ethidium bromide (EtBr) and then run in 1× TAE buffer. The semi-quantitative results obtained could represent the difference in expression of selected gene(s).

HBsAg Detection

A diagnostic kit for HBsAg (ELISA) SRB (Shanghai Rongsheng Biotech Co., Shanghai, China) was used as an in vitro enzyme immunoassay for the detection of HBsAg in culture medium. The steps for the assays were according to the manufacturer's protocol. Finally, the absorbance was measured at 450 nm against the blank for determining the about of HBsAg present in the samples.

Real-Time PCR for Detecting HBV DNA

PCR primers (forward primer HBV1F: 5′ CCG TCT GTG CCT TCT CAT CTG; reverse primer HBV1R: 5′ AGT CCA AGA GTY CTC TTA TGY AAG ACC TT) and fluorescent probe (HBV1TAQ: 5′ CCG TGT GCA CTT CGC TTC ACC TCT GC) were designed against a conserved region of the HBV genome overlapping the genes encoding the X-protein and DNA polymerase, defining an amplicon corresponding to bases 1,549–1,653 of the HBV genome. The real-time PCR was done with the ABI PRISM 7000 model. The cycling program was: 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 20 s, 58°C for 1 min. Forty microliters of reaction mixture was prepared for each reaction in a 0.2 ml microcentrifuge tube or each well of a 96-well PCR plate. Platinum Quantitative PCR SuperMix-UDG 25 μl (Invitrogen), ROX reference dye 1 μl, forward primer, 10 μM, reverse primer, 10 μM, fluorogenic probe, 10 μM and autoclaved distilled water. Then 10 μl of sample template (100 pg to 1 μg of genomic DNA or cDNA) was added to each reaction vessel. All components were mixed thoroughly in the reaction vessel. The vessels were placed in the programmed thermal cycler. Data were collected and analyzed as described previously [Nedjar et al., 1994; Nagata et al., 1999; Pas et al., 2000; Chen et al., 2001a; He et al., 2002; Zanella et al., 2002].

Preparation of Intracellular DNA

The media of 1×10^6 cells in 6-well plates were discarded and the cells were washed once with PBS. Then the cells were trypsinized off the culture plates. Cells were counted and washed once with PBS again. Then 400 μl lysis buffer and 20 μl of 10 mg/ml of proteinase K were added and vortexed until no cell debris was left. The cells were incubated at 37°C for 2 h in a 1.5 ml microtube. And then, 150 μl saturated NaCl was added into the microtube and was mixed by vigorously shaking the microtube. The microtube was then centrifuged at 5,000*g* for 15 min at room temperature. The supernatant was transferred into a new tube. Then, 1 ml of cold absolute EtOH was added and mixed by inverting the tube. Centrifugation was carried out at 14,000*g* at 4°C for 20 min. The pellet was washed with 70% EtOH once. The DNA pellet was then briefly dried in air. In order to digest the RNA contaminant, 20 μl TE buffer containing 0.2 mg/ml of RNase A was added and incubated at 37°C for 90 min. The DNA was then stored at −80°C.

Preparation of Extracellular DNA

Cell culture supernatant was collected and clarified by centrifugation for 10 min at 2,000*g* and 1 volume of 50% PEG 8000 in 500 mM of NaCl was added to 4 volume of cell culture supernatant (final concentrations, 10% and 100 mM, respectively) and was kept at 4°C overnight. HBV virions were pelleted by centrifugation at 15,000*g* for 30 min. Then the steps were followed for the preparation of intracellular DNA was repeated here. The DNA was then stored at −80°C.

Primary Culture of Duck Hepatocytes (PDH)

PDH culture was performed using a modification method of Tuttleman et al. [1986]. Experimental cherry-valley ducklings (*Anas domesticus*) were hatched under about 50% humidity in an incubator at 37°C. One-day-old ducklings were intravenously infected with a 3×10^9 viral genome equivalent (VGE, $1\text{VGE} = 3.3 \times 10^{-6}$ pg) of DHBV as described by Jilbert et al. [1992]. Treatments and all procedures were performed in accordance with the guidelines of The Chinese University of Hong Kong's Animal Ethics Committee [Zoulim et al., 1996]. Hepatocytes were obtained from 5 to 12 days' old ducks using a modified method of Tuttleman and co-authors [Tuttleman et al., 1986]. A perfusion buffer was prepared with 6 mM KCl, 2 mM Na_2HPO_4 140 mM NaCl, 10 mM HEPES, 6 mg phenol red, 0.5 mM EGTA, 4 mM $NaHCO_3$, 1 mM Glucose, and ddH_2O 1,000 ml, stored at 2–8°C. A digesting buffer was prepared with 140 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM glucose, 0.5 mM EDTA, 0.5 g collagenase (Sigma), and ddH_2O 1,000 ml. After the ducks were anesthetized with approximately 0.4 ml of 5% sodium pentobarbital, their

livers were perfused via the portal vein with 200 ml perfusion buffer followed by 50 ml of digesting buffer. After perfusion, the livers were removed and cells were dispersed in L-15 medium (Gibco Life Technologies) containing 5% fetal bovine serum (FBS), 15 mM HEPES, 100 IU/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml insulin (Sigma), 1.5 μg/ml glucose, 10^{-8} M dexamesathasone and nystatin (10 μg/ml). Cells were filtered through cell dissociation sieve (Sigma) and centrifuged at 50*g* for 4 min. The cell pellet was washed three times with 5% FBS L-15 medium. Cells were counted in a hemacytometer, and seeded with 1×10^6 cells/ml/well in 24 well plates and 1×10^5 cells/0.1 ml/well in 96 well plates in 5% FBS L-15 medium. Cultures were incubated at 37°C, and the medium was replaced with that containing testing samples at 24 h and the supernatants were centrifuged at 6,000*g* for 10 min 2 days later and stored at −20°C for dot blot detection [Arndeson et al., 1997; Colledge et al., 2000].

Dot Blot Hybridization Assay

Seven hundred fifty microliters of the sample's culture supernatants were spotted directly on nitrocellulose filters (Hybond-C; Amersham, Biosciences Corp., Piscataway, NJ) with a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, CA). DNA on the filter was denatured with 0.5 M NaOH at room temperature for 10 min and neutralized first in 1 M Tris-HCl with 0.6 M NaCl (pH 7.5) and then in 1 M Tris-HCl with 1.5 M NaCl (pH 7.5). The filters were exposed to 80°C to fix DNA for 2 h. Hybridization was performed by a modification of the method as previously described [Wang et al., 1991]. The ^{32}P-labeled DHBV probe was prepared by following the nick-translation procedure with a Promega nick translation kit. The plasmid contains a full-length genomic probe of DHBV, which can be tagged with ^{32}P by nick translation reaction and used as a probe [Yang et al., 1992]. Viral DNAs were detected by hybridization with the ^{32}P-labeled probe representing the complete viral genome [Jilbert et al., 1992]. The autoradiographs were quantitated by comparison with a standard control value of known probe loaded in the same batch experiment with an imaging densitometer (model GS690; Bio-Rad, Hercules, CA) with Molecular Analyst computer software. The therapeutic effects were evaluated by the percentage compared to that of the control in pg/ml as observed in the dot blot experiments [Jilbert et al., 1988; Nicoll et al., 1998].

Screening of Differentially Expressed Genes in *Phyllanthus* Treated Cells by Using cDNA Microarray

PLC/PRF/5 (Alexander) cells were used for the screening of differentially expressed genes in *Phyllanthus* treated cells by using cDNA microarray. Alexander cells were seeded at 9×10^6 cells into two 150 ml culture flasks. One flask was the control and the other was treated with the ethanolic extract of *Phyllanthus nanus* at 180 μg/ml for 48 h at 5% CO_2 and 37°C. After incubation, the cells were firstly washed with PBS and then TRIzol reagent (Invitrogen) was added for the isolation of total RNA according to the manufacturer's protocol.

RNA Labeling: Synthesis of Fluorescence Labeled Probe

The cDNA fluorescence labeled probe for microarray hybridization were synthesized by using first-strand cDNA labeling kit. Firstly, 100 μg of total RNA from both control and drug treated cells were used as the template for each reaction. The reaction mixtures of 50 mM Tris-HCl, 75 mM KCl, 10 mM DTT, 2.5 mM $MgCl_2$, 200 μM of each unlabeled dATP, dGTP, dTTP, 200 μM unlabeled dCTP, 100 μM of Cy3-dCTP or Cy5-dCTP (Amersham), 17 U of MMLV reverse-transcriptase in a final volume 19.5 μl were prepared. The reactions were incubated at 42°C for an hour. Eight units of MMLV-RT were added to each reaction as boosters and incubated at 42°C for an additional hour. Twenty microlitres of 0.1 N NaOH and 2 mM EDTA were added to the reactions and incubated at 65°C for 30 min to degrade RNA and to stop the reactions. Twenty microlitres of 0.1 N HCl were then added for neutralization. The labeled probes were purified from unincorporated labeled dCTP by using the AutoSeq ^{TM} G-50 (Amersham) purification columns. After the probes were purified, Amicon Microcon-PCR (Millipore, Bedford, MA) centrifugal filter devices were used to concentrate the cDNA probes to a volume of 20 μl.

Microarray Slide

In our microarray system, the microarray slides that we used were homemade and the target was defined as the product of PCR. The PCR products were prepared from a human

normal liver against hepatoma subtracted cDNA libraries made in our laboratory. The slides were produced by spotting the targets onto DMSO-optimized non-reflective slides (Amersham) by using the Generation III Array Spotter (Amersham). Besides, the target PCR products, Lucidea Microarray ScoreCard control DNA (Amersham) was also spotted onto the slides. The PCR products and control DNA were spotted onto the slides at 25°C and 55% relative humidity. After spotting, the slides were allowed to dry inside the arrayer for 30 min and the spotted DNA was UV-crosslinked under 50 mJ by Spectrolinker™ (Spectronics, Westbury, NY). The spotted slides were stored in a light-tight slides box inside a desiccator at room temperature until hybridization.

Manual Hybridization

Twenty microliters of Microarray hybridization buffer version 2 (Amersham), 16 nM polyA RNA (Sigma), 16 nM Cot 1 human DNA (Invitrogen) were added into 20 μl mixed labeled cDNA probe. After the probe was denatured by incubation at 95°C for 2 min, 40 μl of formamide (Sigma) was added into the denatured probe to a final volume of 80 μl. The slide was placed inside an air chamber and was hybridized at 42°C for 18 h.

Washing the Slides After Hybridization and Array Scanning

The slide was removed from the hybridization chamber and placed into Wash 1A chamber, 2× SSC, 0.03% SDS, 2 min; 2× SSC, 2 min; 1× SSC, 2 min and 0.5× SSC, 2 min. The slide was spun dry by centrifugation in a MSE Mistral 2000 centrifuge at 1,200*g* for 10 min at room temperature to remove excess fluid. Then the slide was scanned by ScanArray® 4000 Microarray Analysis System (Packard BioScience, Meriden, CT; BioChip Technologies, Australia). The microarray images were imported into a software called "Dapple" version 0.86 (http://www.cse.wustl.edu/~jbuhler/research/dapple) for analysis. Dapple was a program for quantitating spots on a two-color DNA microarray image. After analyzing, the image file would be exported as text formatted numerical data file.

Plasmids Construction for Over-Expression and Subcellular Localization of Annexin A7

To clone the annexin A7 (*Axn7*) gene (accession no.: NM_004034) into pcDNA4/HisMax-TOPO (Invitrogen) in order to do the over-expression experiment, the Axn7 ORF was PCR amplified using the following primers: Axn7-hANXA-PC-F: 5′-atgtcatacccaggctatcccccaac-3′ and hANXA-PC-R: 5′-ctactggcccacaatagccagaagaagtc-3′. Reverse transcribed cDNA prepared from Alexander cell line was used as template. The PCR product was then ligated to the linearized pcDNA4/HisMaxTOPO vector according to the manufacturer's protocol (Invitrogen). The recombinant construct was known as pcDNA4-hANXA7. For doing the localization experiment, the recombinant construct pEGFPC1-hANXA7 was made. The Axn7 ORF was PCR amplified using the following primers: ANN-GFPC1-F: 5′-gcgaattctatgtcatacccaggctatccccc-3′ and ANN-GFPC1-R: 5′-gcggatcccctactggcccacaatagccagaag-3′. The PCR product was digested with restriction enzymes (*EcoR*I and *Bam*HI) and ligated to the vector pEGFP-C1 (BD Biosciences Clontech, Mountain View, CA). The correct construction of the two expression vectors were confirmed by DNA sequencing.

Confocal Laser Scanning Microscopy

Alexander cells were transfected with pEGFPC1-hANXA7 or pDAPI-NU (BD Bioscience Clontech) constructs. GFP-tagged Axn7 and DAPI signals were measured using a confocal imaging system (Molecular Dynamics, Sunnyvale, CA) with Nikon objective ×60, n.a. 1.4 at room temperature. For green fluorescence determination, the excitation and emission, the wavelengths used were 488 and 540/30 nm, respectively, and for blue fluorescence determination, the excitation and emission wavelengths used were 358 nm and 461 nm, respectively. Images were processed by the software ImageSpace 3.2 (Molecular Dynamics).

Over-Expression of Recombinant Fusion Protein and Western Blotting Analysis

Approximately 1.7×10^5 cells were transfected with 1 μg of pcDNA4-hANXA7 or control plasmids in a 6-well plate using Lipofectamine PLUS reagent (Invitrogen). At one-day intervals for 5 days, cells were collected, washed with 1× PBS and trypsinized. The cell pellet was then lysed in 100 μl lysis buffer and boiled for 10 min. After centrifugation at 12,000*g* for 20 min, the supernatant was saved for protein quantitation by using BCA protein assay, separated by SDS–PAGE and transferred onto the PVDF membrane (Millipore). The blot was

incubated with primary anti-His-G antibody (Invitrogen) (1:1,000) overnight, and horseradish-peroxidase (HRP)-conjugated secondary antibody (Amersham) (1:1,000) for 1 h. Signals were detected using the Enhanced Chemiluminescence Western Blotting Kit (Amersham).

Transfection of DNA Into Cultured Cells

Cells were seeded in 6-well plates so that they were 50%–80% confluent at the day of transfection. Firstly, 0.4 μg of transfecting DNA was diluted into 25 μl dilution medium without serum. Then, 4 μl of PLUS™ (Invitrogen) reagent was added to dilute DNA and was mixed and incubated at room temperature for 15 min. Then 1 μl of Lipofectamine™ (Invitrogen) reagent was added into 25 μl dilution medium without serum in a second tube and mixed. And then pre-complexed DNA and diluted Lipofectamine reagent were mixed and incubated for 15 min at room temperature. While complexes were forming, the medium was replaced on the cells with 0.2 ml of transfection medium without serum (cell growth medium without serum). Afterwards, the DNA-Plus-Lipofectamine reagent complexes were added into each well of cells containing fresh medium. Complexes were mixed into the medium gently and incubated at 37°C at 5% CO_2 for 3 h. After 3 h, the medium containing the complexes was replaced with fresh, complete medium.

Statistical Analysis

All the assays were done at least in triplicate in independent experiments. Means and standard errors were determined. Control and treated cultures were compared, depending on the type of analyzed experiment by Student's *t*-test.

RESULTS

Cytotoxicity Measurement on Alexander Cells and HepG2 2.2.15 Cells

Prior to the investigation of anti-HBV effects, any putative cytotoxic effects of the extracts were studied by MTT assays. PLC/PRF/5 (Alexander cells) and HepG2 2.2.15 cells were treated with the ethanolic extracts of *P. nanus*. The cells after treatment for 48 h were subjected to MTT assays. The results of the cytotoxicity measurement of the ethanolic extract of *P. nanus* are shown in Figure 1. It shows that in Alexander cells, it had a higher survival rate at relatively low concentrations of *P. nanus* ethanolic extract and the IC_{50} was about 300–400 μg/ml. About 80% of the Alexander cells survived when 150 μg/ml of the extract was used. Thus the effective concentration of the *P. nanus* ethanolic extract was set to be 150 μg/ml in Alexander cells (Fig. 1). In the HepG2 2.2.15 cells experiment, the cells were treated for 7 days with replacement of the culture media on the fourth day, the cells were then subjected to MTT assays. The results of the cytotoxicity effect of the ethanolic extracts of *P. nanus* is also shown in Figure 1. It shows that in HepG2 2.2.15 cells, the IC_{50} was about 100 μg/ml and about 80% of the cells survived when 50 μg/ml of the extract was used.

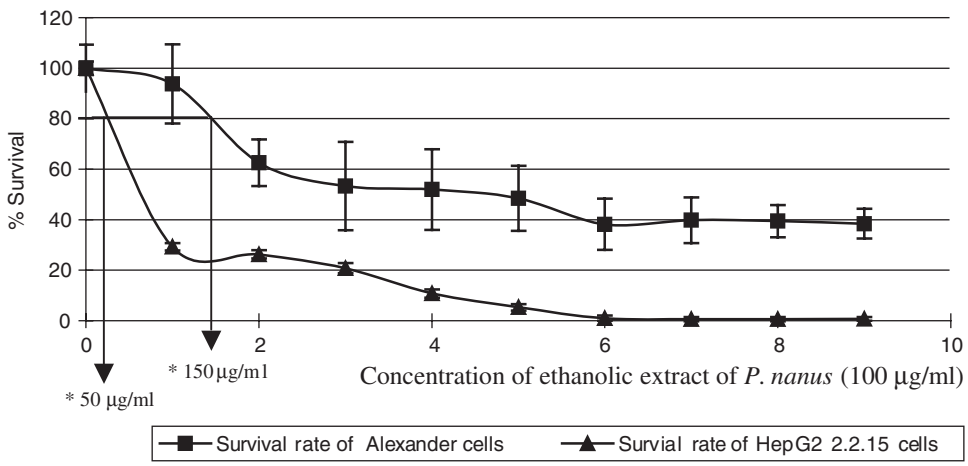

Fig. 1. Survival rate of Alexander cells and HepG2 2.2.15 cells after treatment with different concentrations of ethanolic extract of *Phyllanthus nanus* for 48 h and 7 days respectively. (*, effective concentration of ethanolic extract of *P. nanus* at which 80% of cells survive).

Effect of *P. nanus* Ethanolic Extract on the Suppression of Surface Antigen *Pre-S* and *S* Gene Expression

PLC/PRF/5, Alexander cells were treated with ethanolic extracts of *P. nanus*. The concentration of extract used for each treatment was chosen according to the effective concentration that was measured in the previous MTT assays. After 48 h incubation, the cells were collected and RNA was isolated and semi-quantitative RT-PCR was performed by using the protocols listed in Materials and Methods to determine the level of mRNA for HBsAg expression. In order to study the mRNA expression level of HBsAg, primers were designed to amplify the Pre-S and S regions of the HBV genome, which are encoding the mRNA for HBsAg domains. In addition, the housekeeping gene—glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene, was used as the housekeeping control gene in the semi-quantitative RT-PCR assays. Figure 2 indicates the RT-PCR results of the cells that were treated with the ethanolic extract of *P. nanus*. The results in lanes 1, 2 indicate the suppressive effect on HBsAg expression by the *P. nanus* ethanolic extract at a concentration of 150 μg/ml. The HBsAg level as detected by ELISA was also found to drop by 80% after incubation.

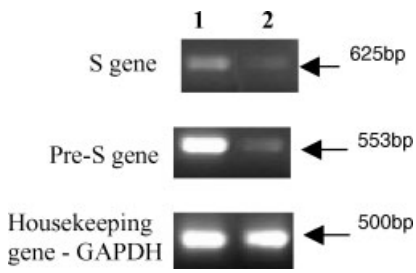

Fig. 2. RT-PCR results showing the effect of *P. nanus* ethanolic extract on the suppression of *Pre-S* and *S* gene expression of Alexander cells. Cells were treated with different concentrations of *P. nanus* extracts as indicated for 48 h and cells were harvested for RNA isolation, cDNA synthesis and PCR analysis. **Lane 1**: cDNA template were prepared from control cells (untreated cells). **Lane 2**: cDNA template were prepared from cells treated with 150 μg/ml of *P. nanus* ethanolic extract. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the housekeeping control gene for RT-PCR analysis. The concentrations of extracts used were determined in cytotoxicity measurements. Representative data from three individual analyses were shown.

Effect of *P. nanus* Ethanolic Extract on the Secretion of Surface Antigen of HepG2 2.2.15 Cells

HepG2 2.2.15 cells were treated with ethanolic extracts of *P. nanus*. The tested concentrations of different extracts used for each treatment were the concentrations that were determined in the previous MTT assays. After 7 days' incubation, the culture medium was collected and assayed for the levels of HBsAg using ELISA method. Figure 3 shows the effects of different concentrations of ethanolic extract of *P. nanus* on the secretion of HBsAg from HepG2 2.2.15 cells. The zero concentration was the control of which 1% solvent (methanol) used. There was about 2% dropped in HBsAg secretion at the concentration of 50 μg/ml when compared with the control.

Effect of *P. nanus* Ethanolic Extract on Inhibiting HBV Multiplication in the HepG2 2.2.15 Cells

HepG2 2.2.15 cells were treated with ethanolic extract of *P. nanus*. The effective concentrations of extract used for each treatment was the concentration, which was determined in the previous MTT assays. After incubation for 7 days, the culture medium and the cells were harvested. Extracellular DNA was prepared from the culture medium and the intracellular DNA was prepared from the harvested cells.

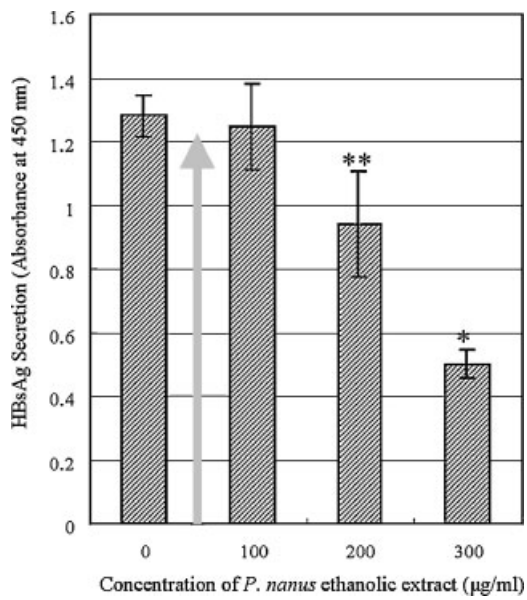

Fig. 3. Effect of different concentrations of ethanolic extract of *P. nanus* on the secretion of HBsAg from HepG2 2.2.15 after 7 days of incubation. Statistical significance was determined using Student's *t*-test. The asterisk (*, **) represented significantly different value from control,*, $P < 0.06$; **, $P < 0.08$.

TABLE I. Effect on HBV Replication in HepG2 2.2.15 Cells by Treatment With the Positive Control: Lamivudine (3TC) and the Ethanolic Extracts of *Phyllanthus nanus* for 7 Days

Extracts	Effective concentration (μg/ml)	Intracellular HBV DNA level (% changes in HBV DNA copies)	Extracellular HBV DNA level (% changes in HBV DNA copies)
Lamivudine (3TC) (positive control)	150	↓74.0	↓100
P. nanus ethanolic extract	50	↑5.2	↓33.4

↑, increase; ↓, decrease.

The levels of extracellular and intracellular HBV DNA were then measured by using real-time PCR techniques. Results are shown in Table I. The positive control drug used in this experiment was lamivudine (3TC). The result shows that *P. nanus* ethanolic extract would cause a decrease in extracellular HBV DNA level and an increase in intracellular HBV DNA level. The explanation for the phenomena of the relative changes in intracellular and extracellular HBV DNA level may be due to the fact that: the *P. nanus* ethanolic extract could suppress the release of HBV into the medium, i.e., the extracellular HBV DNA level would decrease and it may cause an accumulation of HBV inside the cells, i.e., the intracellular HBV DNA level would increase. It follows that the *P. nanus* ethanolic extracts would have an suppression effect on the release of HBV into the medium. Furthermore, the percentage changes in intra- or extra-cellular HBV DNA level were not in proportion. *P. nanus* ethanolic extract could cause 33.4% decrease in extracellular HBV DNA level but only 5.2% increase in intracellular HBV level. These data showing that *P. nanus* ethanolic extract could cause a great drop in extracellular HBV DNA level and the out of proportional changes in HBV level inside and outside the cells might hint that *P. nanus* ethanolic extract not only could suppress the release of HBV but also suppress the replication of HBV inside the cells, i.e., causing less HBV to be accumulated. These results should be reliable because the technique of real-time PCR was adopted and this technique could accurately quantify the amount HBV DNA present inside and outside the cells. However, the half-life of HBV DNA in the medium might be different from that inside the cells.

Cytotoxicity Measurement on DHBV Infected PDH

The DHBV infected primary cultures of duck hepatocytes were then seeded at 1×10^4 cells onto 96-well plates for cytotoxicity measurement of *P. nanus* ethanolic extracts using MTT assay and cell morphology and cell number were also observed in parallel. Approximately 1×10^6 cells were seeded onto 24-well plates for studying the antiviral effect of *P. nanus* ethanolic extract. Figure 4 shows the results of the cytotoxicity measurements of the *P. nanus* ethanolic extract on the primary culture of the DHBV infected duck hepatocytes. From the result, it shows that the *P. nanus* ethanolic extracts did not cause any toxic effect on the primary duck hepatocytes. Even at higher concentrations, the extract showed slight stimulatory effect on cell growth. This might be due to the fact that some compounds present in the extract, which could stimulate the growth of the primary duck hepatocytes.

Effect of P. nanus Ethanolic Extract on DHBV Infected PDH

After measuring the cytotoxicity of *P. nanus* ethanolic extract on the primary duck hepatocytes, the antiviral effect of the *P. nanus* ethanolic extract was studied. Figure 5A shows the result of the effect of different concentrations of *P. nanus* ethanolic extracts on suppression of DHBV replication in the primary culture of DHBV infected duck hepatocytes. Figure 5A

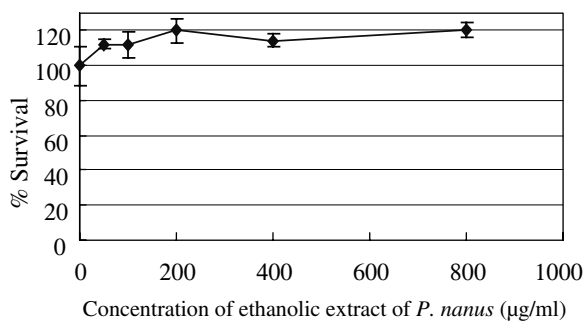

Fig. 4. Graph showing the survival rate of DHBV (duck hepatitis B virus) infected primary duck hepatocytes after treatment with different concentrations of ethanolic extract of *P. nanus* for 48 h.

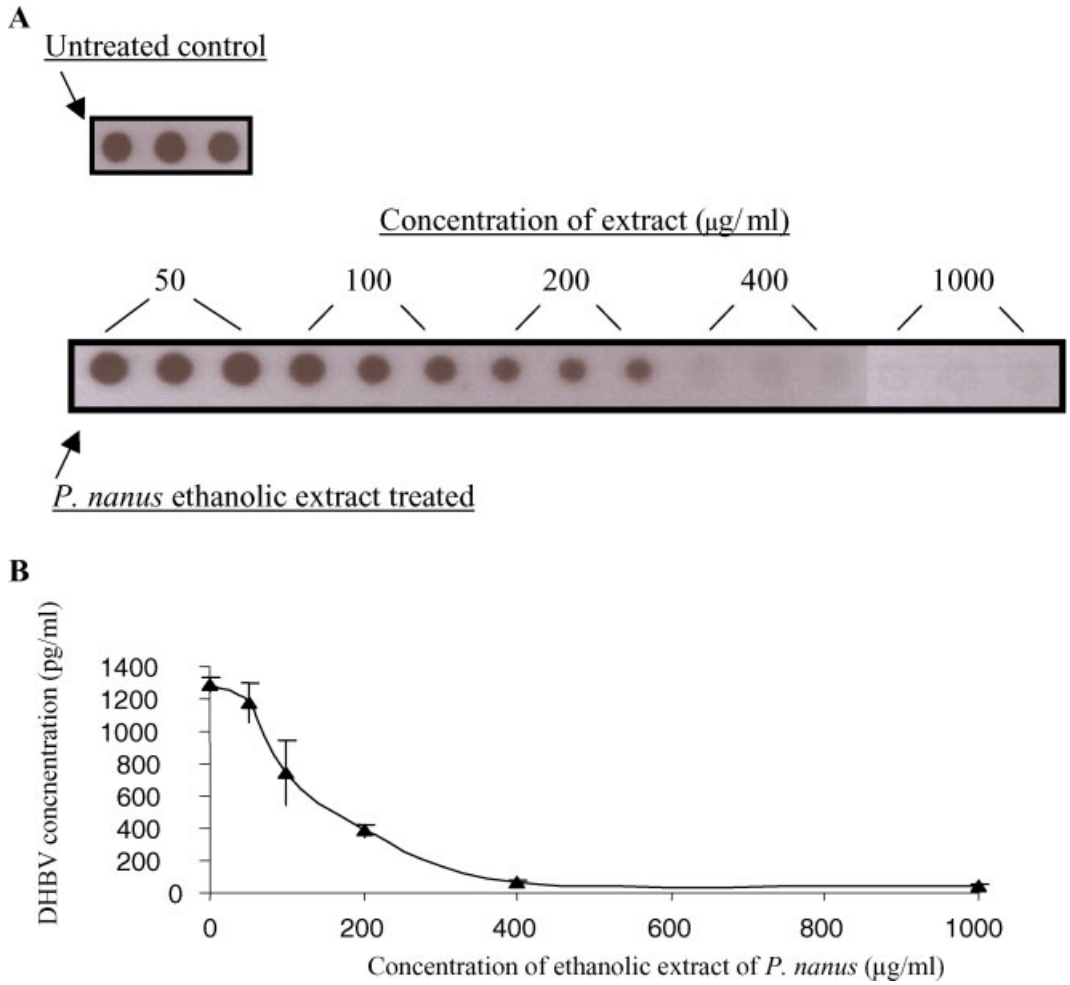

Fig. 5. **A**: Evaluation of DHBV DNA by dot blot hybridization, from cell culture supernatants of DHBV infected primary duck hepatocytes after 48 h of incubation in the presence of various concentrations of *P. nanus* ethanolic extracts. The cell cultures were exposed to extracts at 0 (Untreated control), 50, 100, 200, 400, 1,000 μg/ml. Untreated control group—1% methanol was added in culture medium. The experiment was performed in triplicate (arranged horizontally for each concentration). Viral DNA on nitrocellulose medium was hybridized with ^{32}P-labeled probes as described in Materials and Methods. **B**: Graphical presentation of the level of duck HBV (DHBV) DNA present in the medium after the DHBV infected primary duck hepatocytes were treated with different concentrations of *P. nanus* ethanolic extracts for 48 h.

is the dot-blot analysis result. In the dot-blot analysis result, an ethanolic untreated control group was shown because 1% solvent (Methanol) was used to dissolve the ethanolic extract for treating the cells. Figure 5B is a graphical presentation of the dot-blot analysis after the autoradiographs were quantified with an imaging densitometer. From the graph (Fig. 5B), it shows that all the tested *P. nanus* ethanolic extract could suppress DHBV replication in the cell culture system. The result shows that *P. nanus* ethanolic extract efficiently suppress the replication of DHBV in primary duck hepatocytes starting from a concentration of 200 μg/ml. And at concentration of 400 μg/ml, *P. nanus* ethanolic extract showed 98% suppression of DHBV. That was more efficient than the control drug—2,3-dideoxycytidine 5-triphosphate (ddC) which showed 93% suppression of DHBV in our primary culture of DHBV infected duck hepatocytes system (data not shown).

The results obtained in the primary culture of DHBV infected duck hepatocytes system were consistent with that of the HepG2 2.2.15 and Alexander cell line models.

Microarray Experiments

The microarray technique was used to investigate the effect and mechanism of *P. nanus* ethanolic extract at the gene level. *P. nanus* ethanolic was selected for use in this experiment, because it shows the highest antiviral

potency than the other species in the previous in vitro assays. By understanding the performance of the control spots, the variation of intensity of Cy3 and Cy5 could be normalized and therefore, the quality of the experiment was assured.

Due to differences in labeling efficiency, intensity and half-life between the two dyes Cy3 and Cy5, and different amounts of starting materials, the measured intensities must be normalized before the data can be analyzed. A scattered plot of logCy3 against log Cy5 before and after the Cy3 signal was normalized showed that the spots shift closer to the zero line after the Cy3 signals were normalized, i.e., the plot had a log ratio equal to 0.

Differential Gene Expression Identified by Using Microarray Technique

After analysis of the data from the microarray experiment, a set of up-regulated or down-regulated genes were shortlisted. Six genes were selected from the microarray results for further studied (Table II). This was because the microarray slides that we had used containing only 1,472 spots of 800 human cDNA clones that were obtained from a HBV integrated hepatocellular carcinoma (HCC) subtracted cDNA library, i.e., the genes on the slides were already HBV or liver cancer related genes. Therefore, the results coming out from the experiment, as expected, contain less genes with greater than threefold up or down-regulation than that of a slide spotted with the whole array of the normal human genes. Furthermore, the experiment was also repeated by reverse dye labeling to increase the reliability of the results.

Confirmation of the Differential Expression by Semi-Quantitative RT-PCR

Six genes were selected for confirmation of their changes in gene expression. RT-PCR using gene specific primers targeted for the up- or down-regulated genes obtained from the microarray results was used as a reconfirmation experiment (data not shown except for Axn7). Among all the genes tested, Axn7 showed about threefold up-regulated expression in *P. nanus* ethanolic extract treated Alexander cells (Fig. 6). The results obtained from RT-PCR agreed with that of the microarray experiment.

Effect of Over-Expression of Axn7 on HBsAg Secretion in Alexander Cells

Over-expression experiment was performed to study the effect of over-expression of Axn7 on the secretion of HBsAg from Alexander cells for a longer time courses of incubation after transfection (2–5 days). During this period, the accumulation of HBsAg in the medium was measured by MEIA method. Figure 7A shows the over-expression results as detected by Western blotting analysis. The Western blotting results indicated that Axn7 protein was continuously over-expressed during incubation from day 2 to day 5 after transfection. Figure 7B shows the results of HBsAg measurement during the Axn7 over-expression period. These results show that over-expression of Axn7 could result in lower HBsAg secretion in the medium of the Alexander cells as compared with the control. The effect was more prominent on the second day after transfection. This might be due to the fact that over-expressed Axn7 protein would mediate endocytosis, so that HBsAg in the medium might bind to Axn7 and forming vesicles which would be taken up into the cells for degradation.

Subcellular Localization of Axn7 in Alexander Cells

Axn7 was cloned into the pEGFP-C1 vector for transfection into Alexander cells. The *Axn7* gene cloned into the vector would be expressed as fusions to the C terminus of EGFP retain the fluorescent properties of the native protein allowing the localization of the fusion protein in vivo detected by confocal laser scanning

TABLE II. List of Up-Regulated and Down-Regulated Genes Found in the Microarray Experiment

Name of up-regulated gene	Fold of up-regulation
H. sapiens annexin A7 (Axn7)	3
H. sapiens mRNA for C-reactive protein	2
H. sapiens mRNA for leucine-zipper protein	2
H. sapiens hH3.3B gene for histone H3.3	2
Name of down-regulated gene	Fold of down-regulation
Human mRNA for elongation factor 1-alpha	3
Human aldehyde dehydrogenase 1 mRNA	2

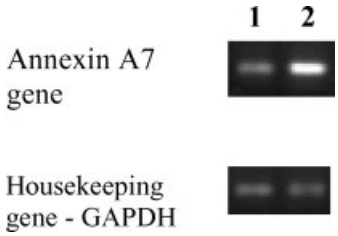

Fig. 6. RT-PCR result of Annexin A7 (*Axn7*) gene. The cDNA template used for PCR was normalized by using *GAPDH* housekeeping gene. **Lane 1**: cDNA prepared from control cells (i.e., not drug treated cells). **Lane 2**: cDNA prepared from *P. nanus* ethanolic extract (150 μg/ml) treated cells. (*Axn7* gene: accession no. NM004034, Axn7 forward primer sequence 5′-cggaaaactcacggctcacac-3′, Axn7 reverse primer sequence 5′-gggttttgggacagatgagca-3′).

microscopy. The localization results are shown in Figure 8. The cells were fixed and stained for the GFP expressed protein and the nucleus with DAPI, respectively. Merged images showed that Axn7 would be localized in the cytoplasm and form circular "spots" which were surrounding the exocytotic or endocytotic vesicles inside the cells, for example, vesicles for secreting or after engulfing HBsAg. From this result, it is suspected that Axn7 might be involved in the secretion of other viral proteins, such as HBeAg or even the mature virus itself.

DISCUSSION

Effects of *P. nanus* Ethanolic Extract on HBV Cell Line Models

One rational approach to the development of drugs for the treatment of HBV is to identify those compounds that specifically inhibit the HBV viral proteins expression, secretion, and to study the HBV DNA replication in in vitro models. Our study showed that the *P. nanus* ethanolic extract did not produce cytotoxic

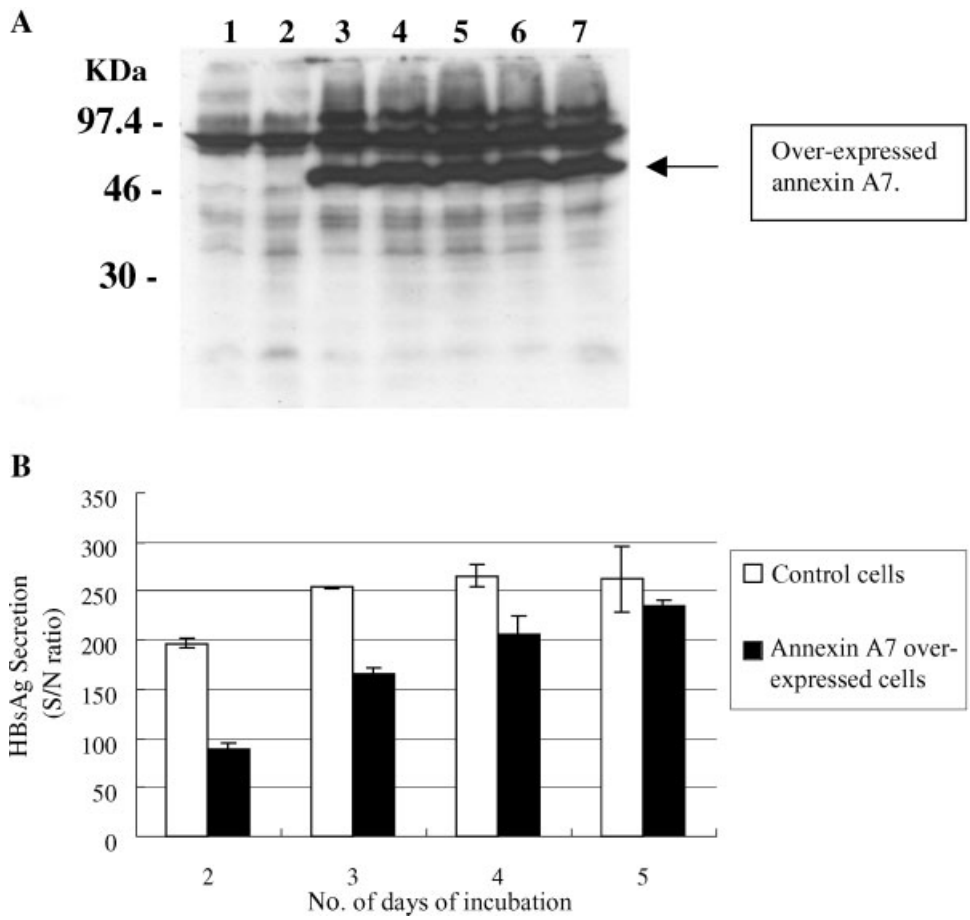

Fig. 7. **A**: Western blotting analysis showing the over-expression of Axn7 in Alexander cells. **Lane 1**: Alexander cells positive control (only plasmid without annexin A7 cDNA insert), (**lane 2**) Alexander cells negative control (no transfection), 3–6: day 2, day 3, day 4, and day 5 of incubation, respectively after transfection. **B**: The effect of over-expression of Axn7 on the secretion of HBsAg from Alexander cells at different time courses of incubation after transfection. Control cells: Cells that were transfected with plasmid carrying no Axn7 cDNA insert. (S/N ratio is the ratio of sample to HBsAg calibrator ratio.)

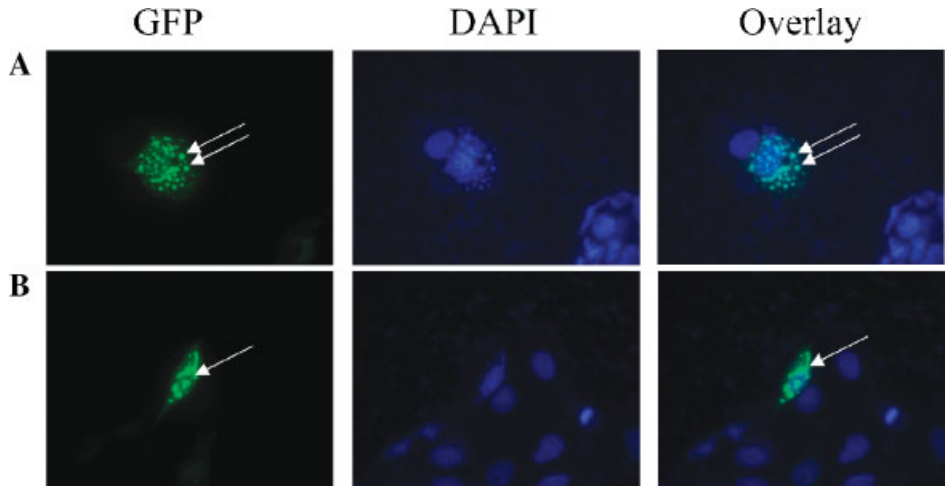

Fig. 8. Subcellular localization pattern of Annexin A7 expressed in Alexander cells (PLC/PRF/5). The cells were transfected with control and annexin A7 cloned GFP vectors. The cells were fixed and stained for the nucleus with DAPI. **A**, **B**: Two regions in the cell culture were observed, magnification 600×.

effect on HBV integrated cells within a reasonable dose range. This result suggested that the antiviral effect of *P. nanus* ethanolic extract might be more specific to the HBV itself instead of killing the HBV infected cells.

The PLC/PRF/5 (Alexander cells) were firstly used for studying the effect of *P. nanus* ethanolic extract on HBV. The cells could secrete HBsAg continuously into the culture medium [Cattaneo et al., 1983; Ou and Rutter, 1985]. As a result, after the treatment with the *P. nanus* ethanolic extract, the expression level of HBsAg mRNA by the cells, and in turn the antiviral effect of the extract, could be estimated to a certain extent [Jayaram and Thyagarajan, 1996]. The Alexander cells are suitable for the study of the effect of drugs on HBsAg expression and secretion, such as studying the effect of drugs for signals regulating HBsAg transcription [Cattaneo et al., 1983]. Furthermore, this cell line does not produce infectious virion particles, and it is safe to handle this cell line under biosafety level 2 containment. However, there are some limitations on the use of this cell line model in studying the anti-HBV effect of the herbs. The effect on HBV replication cannot be easily reflected in this model, such as HBV DNA levels or HBeAg levels.

Thus we also studied another cell model, HepG2 2.2.15 cells. The HepG2 2.2.15 cell line was the second cell line used to evaluate the effect of antiviral drugs against HBV. The 2.2.15 cell line was a clonal derivative from HepG2 (ATCC HB8065) human hepatocellular carcinoma cell. It was obtained by stable transfection with a plasmid containing head-to-tail HBV dimer and was selected in culture medium containing G418. The cells were human epithelial-like hepatoma cells, which could secrete live HBV virions particles, HBsAg, as well as HBeAg continuously into the culture medium [Sells et al., 1987]. Therefore, by measuring the amount of intracellular HBV DNA in the cells and extracellular HBV DNA present in the medium, the effect of the extracts on HBV replication could be determined [Yeh et al., 1993].

In HepG2 2.2.15 cells, *P. nanus* ethanolic extract showed antiviral effect on HBV because it had high suppression level of viral replication of 33.4% in terms of suppression of HBV DNA levels. In studies reporting HBV DNA inhibition, it was shown that another *Phyllanthus* species—*P. amarus* could cause a 21.6% drop in HBV DNA level in an in vitro HBV model [Mehrotra et al., 1991]. Therefore, in view of the fact that our *P. nanus* ethanolic extract showed a higher inhibition effect on HBV DNA replication than that of *P. amarus*, our results further support that *P. nanus* has an antiviral effect.

Effects of *P. nanus* Ethanolic Extract on an In Vitro Duck Model of HBV

DHBV has a similar molecular organization compared to HBV in human and belongs to the same viral family. DHBV infection of the Pekin ducks (*Anas domesticus*) [Mason et al., 1980] represents a useful animal model for in vitro and in vivo evaluation of candidate inhibitors of HBV replication [Zuckerman, 1987].

In our study, we used DHBV-infected primary cultures of duck hepatocytes as a model to study the antiviral effect of *P. nanus* ethanolic extract. From our results, the extract that we tested was not toxic to the primary cell culture, and the extract could successfully suppress DHBV replication in DHBV-infected PDHs (approximately 70%) starting from the concentration of 200 μg/ml of extract in a dose dependent manner. This in vitro effect of the *P. nanus* ethanolic extract on DHBV was comparable with that described in a previous report showing viral clearance of up to 50% on DHBV in vitro model by using extracts of five different Australian *Phyllanthus* species [Shead et al., 1992].

Microarray Analysis of the Antiviral Effect of *P. nanus*

Although many compounds have been purified from different *Phyllanthus* species, the active principle(s) of *Phyllanthus* species related to the positive effect of antiviral activities have not yet been identified. The mechanism involved in the antiviral effect of *Phyllanthus* may be mediated via direct antiviral effect, i.e., directly inhibiting viral entry and viral replication [Munshi et al., 1993] or it may be mediated indirectly by changes in the immune response, i.e., the herbs may boost up the hosts' defensive system to fight against the invading viruses, or it may be a combination of both effects [Calixto et al., 1998].

There are some clues of the possible mechanism from recent studies, which showed that the aqueous extract of *Phyllanthus amarus* has the ability to inhibit the reaction between HBsAg and anti-HBsAg possibly due to its property of binding to HBsAg [Venkateswaran et al., 1987]. The binding property of the plant extract to HBsAg may be responsible for the rapid clearance of surface antigen and virus from the serum by rapid phagocytosis and degradation [Munshi et al., 1993].

At the molecular biological level, it was also found that the extract could inhibit HBV polymerase activity, decrease episomal HBV DNA content and suppress virus release by down-regulates HBV mRNA transcription and replication [Lee et al., 1996]. Further studies found that *Phyllanthus* could suppress HBV by interrupting interactions between HBV enhancer I and cellular transcription factors [Ott et al., 1997].

Since the antiviral effect of *Phyllanthus* may occur at a molecular level, (i.e., by altering gene expression level) microarray experiment was performed in order to characterize the active principle(s) of *P. nanus* ethanolic extract at the molecular level [Chen et al., 2001b). This is a relatively new approach to investigate the molecular mechanism involved in the healing effect(s) of traditional herbal extracts. The advantage of using microarray technique is that the effects of the herbal extract on thousands of genes could be screened simultaneously. Then, after specific gene(s) was/were identified, more in-depth molecular characterization could be done. In our study, genes related to the pathogenesis of HBV infected hepatocytes were screened in the first instance.

Possible Role of Axn7 in Suppressing HBV Infection

In the microarray experiment, the human *Axn7* gene was found to have about threefold up-regulation of gene expression. This result was further confirmed by using semi-quantitative RT-PCR with *Axn7* gene specific primers. This is a novel finding that had not been published previously.

Axn7 is one member of the annexins family. Annexins are a family of Ca^{2+}- and phospholipid-binding proteins encoded by at least 12 different genes in mammals and by numerous other genes in invertebrates and plants. They are characterized by a bipartite structure with a variable N-terminal domain and a conserved C-terminal core. The latter is formed by either fourfold or eightfold repeats of approximately 70 amino acids, each repeat (17 amino acid consensus sequence called the endonexin fold [Della et al., 2001]), carrying a Ca^{2+}-binding site. This C-terminal domain is also responsible for phospholipid binding. The unique N-terminal regions are thought to confer functional diversity [Raynal and Pollard, 1994]. Although annexins have been characterized structurally and biochemically, their cellular functions are unclear [Garbuglia et al., 2000; Rand, 2000; Caohuy and Pollard, 2001, 2002; Gerke and Moss, 2002; Chander et al., 2003; Choi et al., 2003; Clemen et al., 2003; Hayes and Moss, 2004; Hayes et al., 2004; Kwon et al., 2005].

Axn7 (also called synexin), was firstly isolated as the agent that mediated aggregation of chromaffin granules and fusion of membranes and phospholipids [Creutz et al., 1978]. It

carries a long N-terminal extension of more than 100 amino acids. Alternative splicing may lead to the inclusion of an extra exon in this region and leads to the generation of two isoforms of 47 and 51 kDa [Selbert et al., 1995; Clemen et al., 1999]. In our experiments, the isoform of 47 kDa was expressed. The function of Axn7 remains unclear. There are reports of it acting as a Ca^{2+} channel and as Ca^{2+}-activated GTPase, supporting Ca^{2+}/GTP-dependent secretion [Caohuy et al., 1996]. Axn7 is found in the vicinity of secretory vesicles, on subcellular membranous structures, and on plasma membranes [Clemen et al., 1999], suggesting a possible role in Ca^{2+}-mediated exocytosis. Besides its role in exocytosis, reports also show that Axn7 may be involved in endocytosis. Axn7 is found to translocate from cytosolic to membrane regions (phagosomes) upon particle ingestion [Pittis and Garcia, 1999; Herr et al., 2003; Kundranda et al., 2004].

In an attempt to better understand the roles of up-regulated Axn7 expression in *Phyllanthus* extracts treated cells, plasmids containing *Axn7* gene were constructed. These plasmids were used for the study of (1) the effect of over-expressed Axn7 on HBsAg secretion in Alexander cells and (2) the localization of the Axn7 protein inside the Alexander cells. The reasons for studying these two aspects were that the relationship between Axn7 and HBV, such as on HBsAg secretion, may play an important role in the life cycle of HBV, for examples, in viral entry, viral assembly or virions release. These steps could become key targets of drug development.

The experimental results also showed that in Axn7 over-expressed Alexander cells, the level of HBsAg in the medium was reduced. This may be explained by: (1) the enhancement of endocytosis properties of Axn7 which helped to remove HBsAg from the medium [Walters et al., 2004], and (2) the over-expressed Axn7 may bind to the intracellular HBsAg and prevented them from secretion as have been reported by de Bruin et al. [1995].

The role of Axn7 involved in endocytosis or exocytosis may depend on the physiological Ca^{2+}/GTP level inside the cells. This may explain why in some of the *Phyllanthus* treatment trials, HBsAg could not be removed from the serum while in some other experiments positive results were shown. In the experiment of subcellular localization of Axn7 in Alexander cells, results showed that Axn7 tended to be localized in the cytoplasm of the Alexander cells. They may arrange in a circular pattern and it was speculated that they were arranged near the exocytotic or endocytotic vesicles, for example, vesicles for secreting HBsAg. It was also proposed that the over-expressed Axn7 might bind to HBsAg, further affecting the packaging and secretion of mature virus [Sodeik, 2000]. These results may account for the lowering of HBV DNA level in the medium of HepG2 2.2.15 cells after the cells were treated with *P. nanus* ethanolic extract.

Our study shows that *P. nanus* ethanolic extract may interfere with steps involved in viral entry, viral assembly, or virions release through induction of Axn7 expression to achieve its antiviral activity. Furthermore, the exact site on the liver cells where the Axn7 exert its function remains unknown. Therefore, studying the effect of the over-expression and localization of Axn7 in HBV-related cell models would allow better understanding of the mechanism involved in HBV control by using *P. nanus* ethanolic extract.

CONCLUSION

In conclusion, we have studied the in vitro anti-HBV effect of the India *Phyllanthus* species—*P. nanus*. The ethanolic extract of *P. nanus* was shown to have anti-HBV effect in the in vitro models. In addition, the extract was found to be more efficient in suppressing HBV viral replication. This extract also demonstrated clear antiviral effect in the in vitro model. Meanwhile, DNA microarray results suggested that one of the active principles of *Phyllanthus* antiviral activities should be associated with the protein expression of Axn7. To date, other studies suggest the following conclusions for the mechanism of action of *Phyllanthus*: (1) *P. amarus* extracts can down-regulated HBsAg mRNA transcription by a specific mechanism involving interactions between HBV enhancer I and CCAAT/enhancer-binding proteins (C/EBPs) [Ott et al., 1997]. (2) *P. urinaria* can suppress the promoter activity of *Pre-S1* gene of the HBsAg protein [Lam, 2002].

Our results show that the mechanism of the action of the herb might be due to induction of Axn7, which in turn might act by enhancement of endocytosis and exocytosis processes, thus helping the clearance of HBsAg or other viral

proteins from the cell culture. This proposed mechanism is a new finding, which has not been reported previously. It is also important to note that Axn7 might be an interesting drug target for HBV infected diseases.

ACKNOWLEDGMENTS

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