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Research Articles

Effects of *Phyllanthus* plant extracts on duck hepatitis B virus in vitro and in vivo

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Summary

The effects of extracts of five Australian *Phyllanthus* species (*P. hirtellus, P. gunnii, P. gasstroemii, P. similis* and *P. tenellus*), other plant extracts and the antiviral drug foscarnet on duck hepatitis B virus (DHBV) endogenous DNA polymerase (DNAp) activity were compared. All 5 *Phyllanthus* species caused 50% inhibition at concentrations of dry weight between 350–800 μg/ml, which is comparable with the effect described for *P. amarus* on the DNAp of human and woodchuck hepatitis B viruses. Incubation of *P. hirtellus* with 100 ID_{50} DHBV neutralized infection. However, neither *P. gasstroemi* extract, given by intraperitoneal injection (i.p.) at a dose of 20 mg/kg 3 times per week to ducklings early in the incubation period, or *P. hirtellus* extract, given to established DHBV carrier ducklings, prevented or eliminated infection.

Phyllanthus; Duck hepatitis B; DNA polymerase; Neutralization

Introduction

The clinical manifestations of human hepatitis B virus (HBV) infection are varied and range from asymptomatic carriage to fulminant hepatitis, cirrhosis and primary hepatocellular carcinoma (HCC). No consistently effective antiviral agent against HBV infection has ever been found, although many have been tried and some have given a measure of clinical improvement. Since

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acute HBV infection in adults is generally self-limiting, most anti-viral strategies have been formed with chronic infection in mind. Worldwide there is an estimated 200 million people who are HBV surface antigen (HBsAg) carriers (Szmuness, 1979), and even after universal implementation of neonatal vaccination there will still remain the many existing carriers requiring ongoing treatment.

Plants of the *Phyllanthus* genus, family *Euphorbiaceae* have been used medicinally for thousands of years in Asia, Africa, and South and Central America (Thyagarajan et al., 1988). There are about 600 species worldwide, of which about 60 are found in Australia. The Indian species *P. amarus* in particular has been studied for pharmacologically active substances and for its therapeutic effects. It has yielded alkaloids (Joshi et al., 1986), flavonoids, lignans, and an angiotensin converting enzyme inhibitor (Ueno et al., 1988). The traditional diuretic action has been confirmed, and the inhibition of induced liver injury reported (Syamasunder et al, 1985; Ueno et al., 1988). Recently, interest has focused on the plant's activity against viral hepatitis.

Thyagarajan et al. (1976) tested extracts of *P. amarus* against HBsAg. They observed inactivation of the DNAP activity of HBsAg positive sera which was maximal at 37°C and retarded at 4°C. A dose dependent inhibition of both surface antigen binding and the polymerase activity has been obtained in vitro for woodchuck hepatitis B (WHV) and HBV utilising an aqueous extract of *P. amarus* (Venkateswaran et al., 1987). These encouraging results led to in vivo trials being conducted for both WHV and HBV infections.

In recently infected woodchucks weekly intraperitoneal administration of *Phyllanthus* extract caused a significant drop in WHBsAg titre and DNAp activity, 3 out of 4 woodchucks cleared the surface antigen permanently. Treatment of chronic carrier animals was ineffective in clearing the WHBsAg although the level of hepatitis was decreased in all the animals treated. These results pointed to an inhibition of WHV replication in vivo by the *Phyllanthus* extract in addition to its general hepatoprotective function (Venkateswaran et al., 1987).

In man an antiviral effect was reported following oral administration of *Phyllanthus* to chronic HBV carriers. Thyagarajan et al. (1987, 1988) found that HBsAg was lost from the circulation in 59% of treated subjects compared with 4% of placebo-treated subjects. In contrast to the findings in woodchucks, human HBeAg positive carriers were much less likely to clear virus than those with less active viral replication who lacked HBeAg. None of the patients who cleared HBsAg was known to have become positive again after the cessation of treatment but only 10 of the 37 in this group were followed up for over 6 months. Problems in interpreting these results include the loss of greater numbers of the control group, when compared with the loss in numbers of the treated group and lack of knowledge of HBe status of the control group. Both these factors imply selection bias (Brook, 1988). In addition the short follow up period was a cause of concern as spontaneous clearance of virus can occur (Fattovich, 1986). Despite the criticisms, these results encourage further serious

study of the *Phyllanthus* species to clarify the clinical factors predicting a response, and to define the most suitable regime of treatment.

This paper reports on the testing of extracts from a range of plants of the *Phyllanthus* species in the DHBV model. DHBV employs similar replicative strategies to HBV (Marion, 1988; Mason et al., 1982), and the DHBV DNA polymerase and the HBV DNA polymerase have biochemical features in common (Fourel, 1987). The convenient and relatively cheap DHBV experimental model has been widely adopted to assess the efficacy of single and combination therapies for acute and chronic hepatitis B infection and inactivation of hepatitis B by disinfectants. (Tsiquaye et al., 1985; Sherker et al., 1986; Hirota et al., 1986, 1987; Murray et al., 1990; Civitico et al., 1990).

Materials and Methods

Collection of plants

Ms T. James of the Royal Botanic Gardens' Herbarium, Sydney helped to collect and identify the locally occurring *Phyllanthus* and the other plant species used as controls in these experiments.

P. amarus was obtained from India, through the courtesy of Dr A. Sudasanam of C.M.C. Hospital, Vellore, S. India; and Mr R. Khoury of the National Herbalists' Association of Australia.

Preparation of the plant extracts

The plants were dried, and ground in a hammer mill (Christy and Norris, Chelmsford, England) using a 1.0 mm filter. Twenty grams of plant material was mixed with 200 ml of water and shaken periodically at 60°C for two hours. This mixture was then filtered through nylon mesh, and the filtrate centrifuged at 6000 × *g* for one hour at 20°C. The supernatant was then filtered through a series of Millipore filters ending with a 0.22 μm pore size filter. This filter-sterilized extract was used in all subsequent in vitro and in vivo experiments. The dry weight of the extract was determined by drying 10 ml of extract to constant weight at 65°C.

Determination of DHBV DNA

Genome length DHBV DNA (Mason et al., 1982) subcloned into pACYC 184 was a gift from Dr P. Marion. Excised DHBV DNA was radiolabelled with [^{32}P]dCTP using a multiprime kit (Multiprime DNA, Amersham). Probe specific activities ranged from 0.5–1 × 10^9 dpm/μg DHBV DNA.

The presence of DHBV DNA in serum was detected by dot-blot hybridization. A 25-μl aliquot of serum was denatured by 25 μl 1 M NaOH and spotted onto a nylon membrane. Filters were hybridized with ^{32}P-labelled

DHBV DNA, washed and autoradiographed as described previously (Freiman et al., 1986).

To detect viral DNA in liver, 0.5 cm^3 fragments of tissue were removed immediately upon sacrifice, snap-frozen in liquid nitrogen and stored at −70°C. Following tissue digestion the nucleic acids were phenol/chloroform-extracted and then ethanol-precipitated (Freiman et al., 1986). DNA extracts were examined as for serum by dot-blot hybridization.

DHBV DNA polymerase assay

The assay was adapted from our previous method (Milborrow et al., 1986) of pelleting DHBV particles by ultracentrifugation in a Beckman Airfuge. The pellet was resuspended overnight at 4°C in 50 μl of resuspension mix (150 mM Tris/HCl pH 7.5; 225 mM NaCl; 0.022% Triton X-100 and 0.0015% B-mercaptoethanol) and 25 μl of plant extract in dilutions ranging between 25 and 5000 μg dry wt/ml, or 25 μl of distilled water for the controls.

A 25-μl aliquot of base mix (110 mM Mg acetate; 1.6 M KCl; 220 mM Tris/HCl pH 7.5; 15 mM dATP, dGTP and dTTP plus 3.5 μl [^{32}P]dCTP/ml of base mix) were added to each sample and incubated at 37°C for 4 h prior to spotting on Whatman No. 3 filter paper and overnight elution with 5% TCA. The DHBV DNA polymerase activity as a measure of the incorporation of the radionucleotide into the viral DNA was counted in a Rackbeta (LKB) Scintillation counter.

Experimental animals

Pekin-Aylesbury cross-bred ducks were obtained from a commercial supplier. The experimental work was performed with approval from the University of Sydney Animal Ethics Committee. All ducklings used in the experiments were obtained from DHBV negative flocks. In addition, serum samples obtained immediately prior to experimental inoculations were shown to be free of DHBV DNA by dot-blot hybridization.

Experimental protocol

In vitro inhibition of DHBV DNA polymerase

Aqueous extracts were prepared of 5 local *Phyllanthus* species (*P. gasstroemii, P. gunnii, P. hirtellus, P. similis* and *P. tenellus*) and of the Indian herb *P. amarus*. A series of other plants, both closely related and unrelated to the *Phyllanthus* genus, were also tested. The closely related plant was *Breynia oblongifolia* which belongs to the same family *Euphorbiaceae* as *Phyllanthus*. The unrelated plants were *Solanum nodiflorum* and *Cestrum pargui* both belonging to the *Solanaceae* family and leaves from the Elm which belongs to the *Ulmaceae* family.

Seven serial dilutions of each extract were made in sterile distilled water, so

that they would range between 25 and 5000 μg dry wt/ml of assay mix when added to the DNA polymerase assay. Each dilution was tested in duplicate. A positive control of DHBV positive sera and a negative control of DHBV negative sera were included. There were two additional controls included. Foscarnet (Foscavir, Astra), a known HBV DNAp inhibitor in vitro, was tested over a range of concentrations (1×10^{-3} to 1×10^{-6} M) known to inhibit HBV polymerase (Lis and Sharon, 1983). Acyclovir (Zovirax, Wellcome) which has no in vitro effect on DNA polymerase was tested at equimolar concentrations.

A prior experiment had shown no difference if plant extract was added at the time of the resuspension mix or if the plant extract was added with the base mix (results not shown). Subsequently, the plant extract was added with the resuspension mix.

One further experiment sought evidence of specific localization of activity within the plant. In this assay, *P. similis* was divided into stem and root for one sample, and leaf and fruit for the other sample.

Because of seasonal conditions insufficient *P. hirtellus* material was available for all the planned experiments. *P. hirtellus* and *P. gasstroemii* had very similar activity in inhibiting the DNAP assay; so both species were used as detailed below.

In vivo trials

Neutralization of DHBV infectivity

One hundred μl of *P. hirtellus* extract (25 mg dry wt/ml) were incubated for 1 h at room temperature with 100 μl DHBV positive sera containing approximately 100 ID_{50} dose of DHBV. Residual infectivity was determined by i.p. injection of the mixture. Seven control ducklings were injected with the same dose of virus incubated with sterile water. Serial weekly serum samples were examined for the presence of DHBV DNA. Liver tissue from non-viremic birds was also assayed for viral DNA.

Post-exposure treatment of DHBV infection

Twenty-five DHBV negative day-old ducklings were inoculated with 200 μl of DHBV DNA positive serum containing 1×10^8 virions/ml (a dose of approximately 3000 ID_{50}). The ducks were divided into 3 groups. Eight ducks began treatment 2 days post-infection, 8 ducks began treatment 4 days post-infection, and the 9 control ducks received an equivalent volume of sterile water i.p. from day 2. Treatment consisted of i.p. injections of 20 mg/kg *P. gasstroemii* three times a week. The ducks were weighed prior to each injection for dosage calculation.

Serial weekly serum samples were examined for the presence of DHBV DNA. Liver tissue from non-viremic birds was assayed for viral DNA.

Treatment of an established DHBV infection

Fifteen day-old ducklings were inoculated i.p. with 200 μl of DHBV-positive serum (9×10^8 virions/ml). Blood was taken before inoculation on day 1, then on day 5, and weekly from then on. Treatment began on day 21 when all ducks were positive for DHBV DNA in the serum. The 15 ducks were divided into 4 dosage groups. Three ducks were given 20 mg/kg, 4 ducks were given 5 mg/kg and 3 ducks were given 2 mg/kg of *P. hirtellus* extract i.p. The remaining 5 ducks formed the control group, and were given i.p. injections of sterile distilled water of equivalent volume to the injections received by the 20 mg/kg treatment group. All ducks were injected three times each week after being weighed group by group to determine the required dosage.

Results

In vitro inhibition of DHBV DNA polymerase

The inhibition of the DHBV DNA polymerase by the individual *Phyllanthus* plant extracts are shown in Fig. 1. Table 1 lists the 50% inhibitory concentration of each individual plant extract.

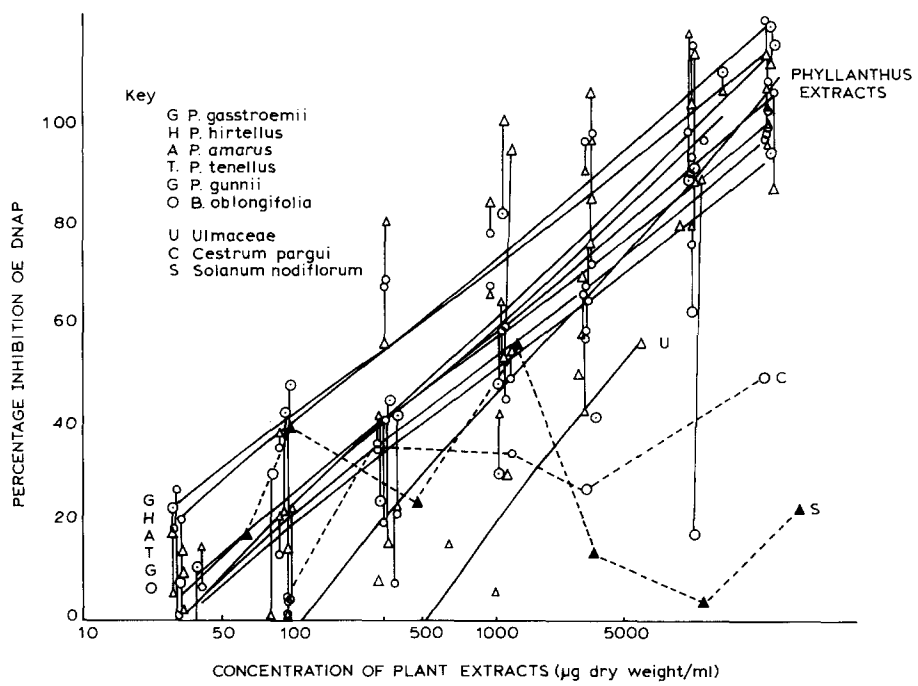

Fig. 1. The inhibition of the endogenous DNA polymerase activity of duck hepatitis B virus produced by crude aqueous extracts of different *Phyllanthus* species and unrelated control plants.

TABLE 1

Concentration of different plant extracts producing 50% inhibition of DHBV endogenous DNA polymerase activity

Species	μg/ml dry weight
P. amarus	500
P. tenellus	600
P. gunnii	800
P. gasstroemii	350
P. similis	500
P. hirtellus	350
Breynia oblongifolia	500
Solanum nodiflorum	>5000
Cestrum pargui	>5000
Ulmaceae	>5000

The extracts from the five Australian *Phyllanthus* species, *P. amarus* and the closely related plant *Breynia oblongifolia* exhibit a similar dose-dependent inhibition of DHBV DNA polymerase.

The inhibition of the DNA polymerase is fairly constant within the *Phyllanthus* species, with a 50% inhibition being obtained by plant extract concentrations of between 350–800 μg/ml. Similarly, the maximum inhibition produced was between 74.4 and 88% for plant extract concentrations of between 4200–4700 μg/ml, except for *P. amarus* whose maximal concentration of 2900 μg/ml gave 82% inhibition. The species *Solanum nodiflorum* and *Cestrum pargui* showed an erratic inhibitory pattern that never reached 50% and was unrelated to extract concentration. The elm leaf extract did show some inhibition, but only at 10 times the concentration of *Phyllanthus* and *Breynia*.

Division of *P. similis* into stem and root for one sample and leaf and fruit for the other sample produced no difference in the inhibition of the DNAP assay indicating that the active principle was not limited to one particular part of the plant.

The inhibition of DHBV DNAP by *Phyllanthus* was compared with that of the antiviral drug foscarnet which was active at much lower concentrations. The 50% end point for foscarnet in our system was between 1×10^{-6} and 5×10^{-6} M in different experiments. As expected acyclovir showed no inhibition.

In vivo trials

Neutralization of DHBV infectivity

P. hirtellus completely neutralised the infectivity of DHBV positive serum in the seven test ducks. None of the test ducks were viremic at any stage and all liver samples were negative for DHBV DNA at sacrifice. All seven control birds injected with the same viral dose were viremic within two weeks post-inoculation and remained positive for the experimental period (Table 2).

TABLE 2

Neutralization of DHBV infectivity by *P. hirtellus* extract

	Blood					Liver
Day	0	7	14	21	28	28
Treated[a]	0/7	0/7	0/7	0/7	0/7	0/7
Controls[a]	0/7	6/7	7/7	7/7	7/7	7/7

100 μl *P. hirtellus* extract (25 mg dry weight) was mixed with 100 ID_{50} DHBV and this mixture allowed to stand at room temperature for 1 h before inoculation. Controls were inoculated with the same dose of virus which had been incubated with distilled water.

[a] Number of ducks DHBV-DNA-positive/number tested.

TABLE 3

Effect of post-exposure treatment with 20 mg/kg *P. gasstroemii* i.p. three times a week[a]

Treatment group	Serum							PM liver
Day	0	4	7	14	21	28	34	
Controls	0/9	0/9	1/9	3/9	4/7[b]	7/7	7/7	7/7
Treatment started 2 days after infection	0/8	0/8	1/8	7/8	7/8	6/7[a]	6/7	7/7
Treatment started 4 days after infection	0/8	0/8	2/8	6/8	6/8	0/8	6/8	8/8

[a] Number of ducklings DHBV DNA positive/number negative.

[b] 3 ducks died during the course of the experiment.

TABLE 4

Effect of 3 × weekly treatment of recent DHBV infection with 'high' and 'low' doses of *P. hirtellus* extract given i.p.[a]

Treatment	Day										
	0	8	24	31	38	46	52	59	66	73	80
Controls	0/5	5/5	5/5	5/5	5/5	5/5	5/5	4/5	4/5	4/5	4/5
20 mg/kg	0/3	3/3	3/3	3/3	3/3	2/3	3/3	3/3	2/3	3/3	3/3
5 mg/kg	0/4	3/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
2 mg/kg	0/3	3/3	3/3	3/3	3/3	2/3	2/3	2/3	2/3	2/3	2/3

[a]Number of ducklings DHBV DNA positive/number tested.

Post-exposure treatment of DHBV infection

Commencing treatment with *P. gasstroemii* 2 or 4 days after inoculation (that is in control) prior to viremia had no effect on either the level of viremia achieved or the number of ducks that ultimately became infected as judged by DHBV DNA detection in the liver at the end of the study period (Table 3).

Efficacy against established DHBV infection

None of the three dosage regimes begun 21 days after inoculation affected the course of DHBV infection (Table 4).

Discussion

Antiviral strategies have tended to focus on one of two areas: the autogenous immune response to infection on one hand, and the replication cycle of the virus on the other, but combination therapies are also often suggested. In human HBV infection viremia can resolve spontaneously even after many years and this encourages the continued search for an effective antiviral strategy. However no treatment in current use produces a 'complete response', that is, causes irreversible inhibition of HBV replication followed by seroconversion to anti-HBe and anti-HBs (Thomas, 1985). A number of drugs inhibit viral replication during treatment but a rebound of viremia to previous or higher levels occurs after the drug is withdrawn. The mechanism of the rebound is not perfectly understood, but the role of the pool of HBV-specific CCC (covalently closed circular) DNA in hepatocytes appears important. This pool is unaffected by any agents known to inhibit the replication cycle of the virus, and can act as a transcriptional template for new viral production when therapy ceases (Sherker et al., 1986; Tuttleman et al., 1986; Hirota et al., 1987; Summers, 1988).

All hepadnaviruses have a virus encoded DNA polymerase associated with the viral core (Kaplan, 1973; Gust, 1986). Inhibition of this enzyme has been one of the main targets for antiviral research, since it is involved in the unique reverse transcription stage of the viral replication process. Drugs like suramin (Tsiquaye, 1985) and the thymidine analogues (Tao et al., 1988) have been shown to inhibit DHBV DNA polymerase in vitro. Some traditional medicinal plants have also been shown to inhibit DNAp in vitro (Yanagi, 1989).

In our experiments, the in vitro effect of the extracts on DHBV polymerase activity was quite comparable with that reported against WHV and HBV DNA polymerase. Similar dose dependent inhibition was found with a closely related species of plant (*B. oblongifolia*). Inhibition of the DNAp also occurred at very high concentrations of some non-related plant extracts, but this effect was not dose-dependent and could be due to nonspecific action of tannins or other plant components affecting the assay. Yanagi (1989) reported that variation of the dNTPs and DNA concentrations did not affect the inhibitory potential of *P. amarus*. He concluded that the inhibitory effect was a direct interaction between the extract and the enzyme.

A mechanism that is often associated with the antiviral effects of plant products is the nonspecific action of lectins (Lin et al., 1973). Lectins are proteins found in plants and animals which mimic specific antibody actions, classically effecting agglutination and precipitation (Stedman, 1972). In

addition, lectins have been shown to bind to membranes of virus-infected cells inhibiting viral entry and exit (Poste et al., 1973). Inhibition of the polymerase enzyme by *Phyllanthus* is unlikely to be due to lectin activity as the inhibition obtained was unrelated to whether the extract was added before or after the liberation of the enzyme.

However, the 'lectin' activity might account for the neutralization of infectivity of the DHBV inoculum by *Phyllanthus* extract which occurs when the two are mixed in vitro before inoculation into susceptible ducklings. The extract may bind to the viral surface receptor which mediates attachment to the hepatocyte thus preventing viral attachment. This experiment is an exact parallel, as regards conditions and virus dose, with our published conventional tests for virus neutralization by rabbit and duck antisera. (Vickery et al., 1989).

The *P. gasstroemii* extract failed to prevent viremia when given soon after inoculation of day-old ducklings and prior to a high viral load developing. The *P. hirtellus* extract was ineffective in treating established DHBV infection when given to three-week old ducks infected on the day of hatching. The doses of *Phyllanthus* chosen were significantly higher on a body weight basis than those successfully used in humans and woodchucks. We elected to employ roughly double the dose used in mammals to take account of the higher metabolic rates of birds. It is possible that more frequent dosing, a higher dose rate or indeed an alternative route of dosage may be more effective because the effect in mammals could be due to a metabolite not produced in birds given the extract by i.p. inoculation. However, our results give little hope that *Phyllanthus* extracts will be useful in treating either established carrier or patients such as new infants of carrier mothers who are in the incubation period of hepatitis B.

Our findings also suggest that while inhibition of viral enzymes by putative therapeutic agents is a convenient screening test it is not an infallible indicator of either an effect on viral replication or of therapeutic value.

Acknowledgements

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References

Berk, L., deMan, R.A., Schalm, S.W., Cabadia, K.P. and Heijtinic, R. (1991) Beneficial effects of *Phyllanthus amarus* for chronic hepatitis B, not confirmed. J. Hepatol. 17, 405.

Brook, M.G. (1988) Effect of *Phyllanthus amarus* on chronic carriers of hepatitis B virus. [letter] Lancet i, 1017–1018.

Civitico, G., Wang, Y.Y., Luscombe, C., Bishop, N., Tachedjian, G., Gust, I. and Locarnini, S. (1990) Antiviral strategies in chronic hepatitis B virus infections. Inhibition of duck hepatitis B virus in vitro using conventional antiviral agents and supercoiled DNA active compounds. J. Med. Virol. 31, 90–97.

Fattovich, G., Rugge, M., Brollo, L. et al. (1986) Clinical, virologic and histologic outcome following seroconversion from HBeAg to anti-HBe in chronic hepatitis type B. Hepatology 6, 167–172.

Fourel, I., Hantz, O., Cova, L., Allaudeen, H.S. and Trepo, Co. (1988b) Inhibitors of DNA polymerase of the duck, human, and woodchuck hepatitis B virus. In: Zuckerman A.J. (ed.), Viral Hepatitis and Liver Disease. pp. 526–529. Alan R. Liss, New York.

Freiman, J.S. and Cossart, Y.E. (1986) Natural hepatitis B virus infection in Australia. Aust. J. Exp. Biol. Med. Sci. 64, 477–484.

Gust, I.D., Burrell, C.J., Coulepis, A.G., Robinson, W.S. and Zuckerman, A.J. (1986) Taxonomic classification of human hepatitis B virus. Intervirology 25, 14–29.

Hirota, K., Sherker, A.H., Omata, M., Yokosuka, O. and Okuda, K. (1987) Effects of adenine arabinoside on serum and intrahepatic replicative forms of duck hepatitis B virus in chronic infection. Hepatology 7, 24–28.

Joshi, B.S., Gawad, D.H., Pelletier, S.W., Kartha, G. and Bhandary, K. (1986) Isolation and structure (X-ray analysis) of ent-norsecurinine, an alkaloid from *Phyllanthus niruri*. J. Natural Prod. 49, 614–620.

Kaplan, H.A., Greenman, R.L., Gerin, J.L., Purcell, R.H. and Robinson, W.S. (1973) DNA polymerase associated with human hepatitis B antigen. J. Virol. 12, 995–1005.

Leelarasamee, A., Trakulsomboon, S., Maunwongyalhi, P. et al. (1990) Failure of *Phyllanthus amarus* to eradicate hepatitis B surface antigen from symptomless carriers. Lancet i, 1600–1601.

Lis, H. and Sharon, N. (1973) The biochemistry of plant lectins (phytohaemagglutinins). Annu. Rev. Biochem. 42, 541–574.

Marion, P.L. (1988) Use of animal models to study hepatitis B virus. Prog. Med. Virol. 36, 829–836.

Mason, W.S., Aldrich, C., Summers, J. and Taylor, J.M. (1982) Asymmetric replication of duck hepatitis B virus DNA in liver cells: free minus-strand DNA. Proc. Natl. Acad. Sci. USA 72, 3997–4001.

Milborrow, H.M., Burnett, L., Lowe, S.B., Nightingale, B.N. and Cossart, Y.E. (1986) Rapid method for the concentration of hepatitis B virus particles using Beckman airfuge. J. Clin. Pathol. 39, 808–809.

Murray, S.M., Freiman, J.S., Vickery, K., Lim, D., Cossart, Y.C. and Whiteley, R.K. (1991) Duck hepatitis B virus: a model to assess efficacy of disinfectants against hepadnavirus infectivity. Epidemiol. Infect. 106, 435-443

Niu, J.Z., Wang, Y.Y., Quao, M. et al. (1991) Effect from *Phyllanthus amarus* on duck hepatitis B replication in vivo. J. Med. Virol. 32, 212–218.

Poste, G., Alexander, D.J., Reeve, P. and Hewlett, G. (1973) Modification of Newcastle disease virus release and cytopathogenicity in cells treated with plant lectins. J. Gen. Virol. 23, 255–270.

Sherker, A.H., Hirota, K., Omata, M. and Okuda, K. (1986) Foscarnet decreases serum and liver duck hepatitis B virus DNA in chronically infected ducks. Gastroenterology 91, 818–824.

Stedman's Medical Dictionary, 22nd Edn. (1972) Williams and Wilkins, Baltimore.

Summers, J., Smolec, J.M. and Snyder, R. (1978) A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proc. Natl. Acad. Sci. USA 75, 4533–4537.

Syamasundar, K.V., Singh, B., Thakur, R.S., Husain, A., Kiso, Y. and Hikino, H (1985) Antihepatotoxic principles of *Phyllanthus niruri* herbs. J. Ethnopharmacol. 14, 41–44.

Szmuness, W., Harley, E.J., Ikram, H. and Stevens, C.E. (1978) Sociodemographic aspects of the epidemiology of hepatitis B. In: Vyas, G.N., Cohen, S.N. and Schmid, R. (Eds.), V. Hepat. pp. 297–320, Franklin Institute Press, Philadelphia.

Tao, P-Z., Corley, D.G., Beutler, J.A., Metral, C.J., Yunes, R.A., Giacomozzi, C.A. and Calixto, J.B. (1988) Phyllanthimide, a new alkaloid from *Phyllanthus sellowianus*. J. Natural Prod. 51, 617–618.

Thomas, H.C. and Scully, L.J. (1985) Antiviral therapy in hepatitis B infection. Br. Med. Bull. 41, 374–380.

Thyagarajan, S.P., Thiruneelakantan, K., Subramanian, S. and Sundaravelu, T. (1976) In vitro inactivation of HBsAg by *Eclipta alba* Hassk and *Phyllanthus niruri* Linn. Indian J. Med. Res. 76(suppl.), 124, 130.

Thyagarajan, S.P., Thirunalasundari, T., Subramanian, S., Nammalwar, B.R. and Prabha, V. (1987) Effect of a medicinal plant on hepatitis B surface antigen carriage: a double blind clinical trial. Vir. Inf. Exch. News 4, 9.

Thyagarajan, S.P., Subramanian, S., Thirunalasundari, T., Venkateswaran, P.S. and Blumberg, B.S. (1988) Effect of *Phyllanthus amarus* on chronic carriers of hepatitis B virus. Lancet 764–766.

Thyagarajan, S.P., Jayaram, S., Valliammai, T. et al. (1990) *Phyllanthus amarus* and hepatitis B. Lancet 336, 949–950.

Tsiquaye, K. and Zuckerman, A. (1985) Suramin inhibits duck hepatitis B virus DNA polymerase activity. J. Hepatol. 1, 663–669.

Tuttleman, J.S., Pourcel, C. and Summers, J. (1986) Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell 47, 451–460.

Ueno, H., Horie, S., Nishi, Y., Shogawa, H., Kawasaki, M., Suzuki, S., Hayashi, T, Arisaawa, M., Shimizu, M., Yoshizaki, M. and Morita, N. (1988) Chemical and pharmaceutical studies on medicinal plants in Paraguay. Geraniin, an angiotensin-converting enzyme inhibitor from 'Paraparai mi,' *Phyllanthus niruri*. J. Natural Prod. 51, 357–359.

Venkateswaran, P.S., Millman, I. and Blumberg, B.S. (1987) Effects of an extract from *Phyllanthus niruri* on hepatitis B and woodchuck hepatitis viruses: In vitro and in vivo studies. Proc. Natl. Acad. Sci. USA 84, 274–278.

Vickery, K., Freiman, J.S., Dixon, R.I., Kearney, R., Murray, S. and Cossart, Y.E. (1989) Immunity in Pekin ducks experimentally and naturally infected with Duck hepatitis B virus. J. Med. Virol. 28, 231–236.

Yanagi, M., Unuora, M., Kobayashi, K., Hattori, N. and Murakimi, S. (1989) From unpublished proceedings of the Cold Spring Harbour Symposium on Quantitative Biology.