PHARMACEUTICAL COMPOSITION FOR THE TREATMENT OF HEPATITIS B COMPRISING EXTRACT OF PHYLLANTHUS USSURIENSIS AND/OR PHYLLANTHUS URINARIA

FIELD OF THE INVENTION

The present invention relates to an extract of Phyllanthus ussuriensis and/or Phyllanthus urinaria, which shows a therapeutic activity for hepatitis B and a pharmaceutical composition for the treatment of hepatitis B comprising the extract.

BACKGROUND OF THE INVENTION

Hepatitis B virus("HBV") is a kind of Hepadnaviridae which infects humans. Those infected by HBV may go through a latency period ranging from 60 to 110 days, followed by varying degrees of symptoms. Although 90 to 95% of the infected may eventually recover, some of them may develop chronic active hepatitis, cirrhosis or hepatocellular carcinoma. Chronic hepatitis B frequently causes chronic viral infection, lymphoma and/or chronic nephropathy, leading to the possible death of the patient.

It has been reported that there are approximately 200 million HBV carriers worldwide, creating a potentially serious hygienic havoc of a global scope.

Since a HBV vaccine was developed in 1981, vaccination against HBV has been performed on those who are considered to be susceptible including neonates; however, no significant drop in the number of HBV carriers has been witnessed yet. This may be attributable to the fact that vertical transmission of HBV from mother to fetus is a major cause of becoming a HBV carrier(Chung, D. K. et al., J. Catholic Med. Coll., 27, 256-267 (1976); and Chung, W. K. et al., J. Infec. Dis., 151, 280-286(1985)). While babies born from hepatitis B e antigen(HBeAg)-positive carriers are

seldom infected through their placenta, when exposed to HBV during their birth, or through a damaged placenta, or when sensitized by HBeAg through the placenta, they may become immunologically defective against HBV and turn into chronic HBV carriers.

Recently, it has become possible to detect HBV DNA present in the blood of HBV carriers by using the PCR(polymerase chain reaction) technique, outdating the old method which uses HBeAg as a sole HBV marker. For the purpose of lowering the number of HBV carriers, it is essential to develop an effective anti-HBV vaccine for neonates and also to develop a method for efficiently reducing the amount of HBV DNA in blood rather than suppressing the blood HBeAg level in the carriers, particularly in a pregnant woman.

Hitherto, various HBV treating agents have been developed, with limited success, if any. Moreover, the agents presently in use, e.g., Ara-A(Parke, Davis, U.S.A.) and Acyclovir(Wellcome Foundation, England), have problems in that they are highly toxic. Phosphonoformate(Foscarnet; Astra, U.S.A.), Suramin(Bayer, Germany), and Famciclovir (Smith-Kline Beecham, England) are claimed to be potent HBV treating agents, but further tests are required for the verification of their effectiveness.

Venkateswaran et al.(Proc. Natl. Acad. Sci., U.S.A., 84, 274(1987)), Blumberg et al.(Cancer Detection and Prevention, 14, 195(1989)), and Yanagi et al.(Abstracts of Papers Presented at the 1989 Meeting on Hepatitis B Virus, September 25, 1989) have attempted to treat Hepatitis B by employing plants belonging to the genus Phyllanthus (Euphorbiaceae), which have been used in India as folk medicines.

According to the report of Unander, D. W., et al.(Journal of Ethnopharmacology, 34, 97-133(1991)), which presents a comprehensive review on the worldwide use of the plants of Phyllanthus as well as their biochemical analysis results, 7 out of 550 species of Phyllanthus, i.e., P.

niruri, P. airy-shawii, P. amarus, P. fraternus Webster, P. urinaria, P. gasstroemi Muell-Arg and P. thymoides, have been used in the treatment of jaundice or hepatitis. P. amarus and P. niruri have been reported to have medicinal activities against woodchuck hepatitis B(Venkateswaran, P. S. et al., Proc. Natl. Acad. Sci. U.S.A., 84, 274(1987)) and duck hepatitis B(Nis, J. et al., J. of Med. Virol., 32, 212-218(1990)). However, it has also been reported that they do not exhibit medicinal activities in clinical applications(Leelarasamee, A., et al., Lancet, 1, 335(1990)).

Further, Shead, A. et al. have reported that P. thymoides shows an in vitro affinity to HBsAg which is the highest among those of the various plants of Phyllanthus. However, it has been claimed later that it is a specific lectin, not P. thymoides, which binds HBsAg, and that P. thymoides does not show an antiviral activity against duck hepatitis B in vivo(Shead, A. et al., "Neutralization but not Cure of Duck Hepatitis B by Australian Phyllanthus Extracts", Abstract 602, In: Scientific Program and Abstracts Volume, the 1990 International Symposium on Viral Hepatitis and Liver Disease, April 4-8, 1990, Houston, Texas, U.S.A.).

As described above, certain species of Phyllanthus, e.g., P. amarus and P. niruri, have been used in folk remedies for the treatment of jaundice or hepatitis and their medicinal activities against hepatitis B have been claimed based on some in vitro experiments. Despite these developments, however, the efficacy of such Phyllanthus plants for the treatment of hepatitis B in humans has not been fully established; and there has continued to exist a need to develop a treating agent for hepatitis B which is safe, less toxic and does not show an adverse effect when administered to human, particularly pregnant women and children.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide an extract of plants of the genus Phyllanthus, which has a therapeutic efficacy against hepatitis B and is clinically safe.

It is another object of the present invention to provide a pharmaceutical composition comprising the extract for treating hepatitis B patients, without creating side effects.

In accordance with one aspect of the present invention, there is provided an extract of Phyllanthus ussuriensis or Phyllanthus urinaria, which shows an effective therapeutic activity against hepatitis B, and a pharmaceutical composition for the treatment of hepatitis B, which comprises the extract and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, in which:

Fig. 1 shows in vitro inhibitory effects of the extracts of P. ussuriensis and P. urinaria on the activity of HBV DNA polymerase; and

Figs. 2A and 2B reproduce HBV particles observed under an electron microscope before and after the addition of 0.1% aqueous solution of P. urinaria extract.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, it has been found that an extract of P. ussuriensis or P. urinaria comprises an anti-HBsAg antibody(HBsAb)-like substance, possesses the ability to inhibit the activity of HBV DNA

polymerase and causes a serious deformation of HBV virus particles, while exhibiting little toxicity or mitogenicity to mammals.

P. ussuriensis and P. urinaria employed in the present invention are annual herbaceous plants, which belong to the genus Phyllanthus of Euphorbiaceae and are indigenous to southern parts of the Republic of Korea. The botanical characteristics and appearances on the scanning electron microscope(SEM) of P. ussuriensis and P. urinaria are as follows:

Table 1

	P. ussuriensis	P. urinaria
Height	· 15-40 cm · stoops with its growth	· 15-35 cm · grows vertically
Color	green or yellow green	reddish green
Leaves	· alternate two lines · oval · the main stalk has leaves	· alternate two lines · oval · no leaves on the main stalk
Flower/ Fruit	· yellow green flowers bloom in June-July · a stem is present between a fruit and the stalk	· reddish brown flowers bloom in July-August · no stem is present on a fruit and the fruit is directly attached to the stalk
Habitat	Kyungsangbukdo, Korea	Cheju Island, Korea
Appearance on SEM	the surface of a seed is relatively smooth	the surface of a seed is horizontally wrinkled

As can be seen from Table 1, P. urinaria is characteristic in that it has a relatively large amount of red pigment and its seed is horizontally wrinkled. On the

other hand, P. ussuriensis is similar in appearance to P. amarus, which is indigenous to the U.S. and bear seeds having vertical stripes.

An extract of P. ussuriensis or P. urinaria may be prepared in accordance with the following preferred embodiment.

P. ussuriensis or P. urinaria is washed with water, air-dried at room temperature, and then chopped into small pieces. The plant pieces are mixed with 25 to 60-fold, preferably, 50-fold volume of water; and the mixture is heated at a temperature ranging from 60 to 90°C, preferably 80°C, for a period ranging from 3 to 5 hours, preferably 4 hours to obtain an aqueous crude extract. The crude extract is centrifuged, filtered and then lyophilized to obtain an extract powder. The powder is stored at 4°C until use.

Further, an organic crude extract of P. ussuriensis or P. urinaria may be prepared by using 25 to 60-fold, preferably, 50-fold volume of an organic solvent, in accordance with the same procedure as above, except that the extraction is carried out at a temperature ranging from 10 to 40°C, preferably 20 to 30°C, for a period ranging from 12 to 48 hours, preferably 24 hours. Exemplary organic solvents may include dimethyl sulfoxide(DMSO), acetone, methanol and ethanol. A powdery extract may then be obtained from the organic crude extract in accordance with the same procedure as above.

The extract of P. ussuriensis or P. urinaria of the present invention exhibits a high level of ability to bind with HBsAg in a serum or in a hepatitis B vaccine. The extracts exert an antagonistic inhibitory effect on the binding of HBsAg with HBsAb at a concentration of 1.25 mg/mℓ or more.

Further, the extracts of the present invention inhibit the activity of HBV DNA polymerase at a concentration of 34 μg/mℓ or more, the inhibitory effect escalating with an increasing concentration thereof. In particular, the extract of P. urinaria causes a serious deformation of HBV

virus particles at a concentration of 0.1%.

Moreover, in spite of their potent efficacies, the extracts of the present invention show little toxicity or mitogenicity in tests using mice and dogs. More specifically, the extract of P. urinaria exhibits no toxicity when it is intraperitoneally administered to a mouse at a dosage level of 70 mg/kg, which corresponds to an intraperitoneal administration of 3,500 mg of the extract to a person weighing 50 kg. Further, the extract of P. ussuriensis or P. urinaria has no adverse effects on the liver function, judging from the fact that the sGOT, sGPT, ALP(alkaline phosphatase) and bilirubin levels of a mouse remain unchanged when the extract is administered to a normal mouse. In Ames tests using Salmonella typhimurium TA100 and TA98, the extracts of P. ussuriensis and P. urinaria are determined to be negative and, accordingly, they are not mitogenic.

In addition, dogs administered with the extracts of the present invention for 120 days show no abnormalities in their liver function. Further, HBsAb-positive reaction is observed when the extract of P. urinaria is administered to a dog, and this result suggests that an HBsAb-like substance is formed in the serum of the dog.

On the other hand, woodchuck hepatitis virus(WHV), which is similar to human HBV, was discovered in Marmota monax, and it was experimentally demonstrated that the WHV infection in a woodchuck may lead to a chronic hepatitis which is similar to that of human(Summers, J., Smolec, J. M. and Snyder. R., Proc. Natl. Acad. Sci. U.S.A., 75, 4533-4537(1978)). When the extract of P. urinaria of the present invention is administered to an infected woodchuck, the concentration of WHV DNA in the blood of the woodchuck decreases remarkably.

The extracts of P. ussuriensis and P. urinaria also exhibit positive results in the clinical tests employing human beings. Specifically, when the extract of P. ussuriensis or P. urinaria was actually administered to

healthy HBV carriers, HBV carriers with putative slight chronic hepatitis, and chronic active hepatitis B patients with complicated hepatocirrhosis in an amount of 50 mg/kg body weight/day, the presence of HBV DNA was no longer detectable after 3 months from the administration thereof.

In case of the test for serum HBsAb, a positive result was obtained after 3 months from the administration of the extract of P. urinaria, but it was determined to be negative by the radioisotope method. This result suggests that the extract of P. urinaria comprises a substance which specifically binds to HBsAg in vivo. Further, the extract of P. urinaria brings about remarkable improvements in the serum GOT and GPT levels of chronic active hepatitis B patients with complicated hepatocirrhosis, after 3 months from its administration.

As described above, the extract of P. ussuriensis or P. urinaria can be used as an effective pharmaceutical agent for the treatment of hepatitis B, which has little toxicity and causes no adverse effect.

Accordingly, the present invention also provides a pharmaceutical composition for the treatment of hepatitis B, which comprises the extract of P. ussuriensis or P. urinaria as an active ingredient, in combination with pharmaceutically acceptable excipients, carriers or diluents.

A pharmaceutical formulation may be prepared by using the composition in accordance with any of the conventional procedures. In preparing the formulation, the active ingredient is preferably admixed or diluted with a carrier, or enclosed within a carrier which may be in the form of a capsule, sachet or other container. When the carrier serves as a diluent, it may be a solid, semi-solid or liquid material acting as a vehicle, excipient or medium for the active ingredient. Thus, the formulations may be in the form of a tablet, pill, powder, sachet, elixir, suspension, emulsion, solution, syrup, aerosol, soft and hard gelatin capsule, sterile injectable solution, sterile packaged

powder and the like.

Examples of suitable carriers, excipients, and diluents are lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, alginates, gelatin, calcium phosphate, calcium silicate, cellulose, methyl cellulose, microcrystalline cellulose, polyvinylpyrrolidone, water, methylhydroxybenzoates, propylhydroxybenzoates, talc, magnesium stearate and mineral oil. The formulations may additionally include fillers, anti-agglutinating agents, lubricating agents, wetting agents, flavoring agents, emulsifiers, preservatives and the like. The compositions of the invention may be formulated so as to provide quick, sustained or delayed release of the active ingredient after their administration to a patient by employing any of the procedures well known in the art.

The pharmaceutical formulations can be administered via various routes including oral, transdermal, subcutaneous, intravenous and intramuscular introduction. A typical daily dose of the active ingredient may range from about 20 to 100 mg/kg body weight, preferably 40 to 80 mg/kg body weight, and can be administered in a single dose or in divided doses. However, it should be understood that the amount of the active ingredient actually administered ought to be determined in light of various relevant factors including the condition to be treated, the chosen route of administration, the age, sex and body weight of the individual patient, and the severity of the patient's symptom; and, therefore, the above dose should not be intended to limit the scope of the invention in any way.

The following Preparation Example and Test Examples are intended to further illustrate the present invention without limiting its scope.

Further, percentages given below for solid in solid mixture, liquid in liquid, and solid in liquid are on a wt/wt, vol/vol and wt/vol basis, respectively, unless specifically indicated otherwise.

Preparation Example: Preparation of Extracts of P. ussuriensis and P. urinaria

P. ussuriensis and P. urinaria were gathered in the fields of Okpo-myun, Talsung-kun, Kyungsangbukdo, Korea and Cheju Island, Korea, respectively, washed with water, dried at room temperature under draft, and then chopped into small pieces. To the pieces of P. ussuriensis or P. urinaria was added distilled water in an amount of 1000 mℓ/20g plant and the mixture was heated at 80°C for 4 hours to obtain an extract. The extract was centrifuged at 3,000 rpm for 30 min. and the supernatant was filtered through Watman No. 2 filter, and then through 0.45 μm Seitz-form filter(Amiccon, U.S.A.). The filtrate was lyophilized to obtain 1.6 g(yield: 8 %) of powdery extract and the extract was stored at 4°C.

Further, an organic extract of P. ussuriensis or P. urinaria was prepared by using various organic solvents listed in Table 2 and a powdery extract was prepared therefrom, in accordance with the same procedure as above. The extracting conditions and yields thereof are shown in Table 2.

Table 2

Solvent	Volume of solvent (mℓ)	Weight of plant (g)	Extracting condition	Yield*1) (g)
DMSO	200	4	at 20-30°C for 24 hours with frequent shaking	0.7
Acetone	200	4		0.5
Methanol	200	4		0.5
Ethanol	200	4		0.45

*1) Yields from P. ussuriensis and P. urinaria are almost equal to each other.

Said powdery extract is highly water-soluble(i.e., more than 20% at 25°C) and, therefore, when it was administered

to human 2 or 3 times a day, a unit dosage form was prepared by dividing the daily dose of 40 mg/kg body weight into 2 or 3 fractions and then dissolving each fraction in 50 to 100 mℓ of distilled water. The daily dose in an animal test was usually about three times that of human, a suitable amount being determined based on the species, size and body weight of the animal.

Test Example 1: Sedimentation Reaction between HBsAg-Positive Serum and the Extract of P. ussuriensis or P. urinaria

To each of 0.5 mℓ samples of HBsAg-positive serum(Liver Research Institute, School of Medicine, Kyungpook National University, Korea) were placed in 5 mℓ test tubes, 2% aqueous extract of P. ussuriensis or P. urinaria was added slowly to form a layer over the serum sample, and the formation of sedimentation ring was observed. Both of the extracts of P. ussuriensis and P. urinaria induced the formation of white sedimentation rings showing that they have reacted with HBsAg-positive serum samples.

Test Example 2: Binding Affinity of the Extract of P. ussuriensis or P. urinaria to HBsAg

(1) Determination of HBsAg titer

The binding affinity of the extract of P. ussuriensis or P. urinaria to HBsAg was determined by measuring the inhibitory activity of the extract on the binding of HBsAg and HBsAb, and the HBsAg titer was determined by using an ELISA kit(Enzygnost® HBsAg monoclonal II, Behring Co., U.S.A.) in accordance with the manufacturer's protocol.

(2) Affinity Test Employing HBsAg-positive Serum

The powdery extracts of P. ussuriensis and P. urinaria

prepared in the Preparation Example were dissolved in distilled water to final concentrations of 10, 5, 2.5, 1.25 and 0.63 mg/mℓ, respectively. 400 μℓ of each of the resulting solutions was mixed with 400 μℓ of HBsAg-positive serum taken from a hepatitis B patient and the mixture was reacted at 20 °C for 1 hour. The resultant was centrifuged at 3,000 rpm for 15 min. to obtain a supernatant. Titer of HBsAg in the supernatant was measured in accordance with the same procedure as above (1), and the reduction in HBsAg titer was determined therefrom. The activity of the extracts of P. ussuriensis and P. urinaria in inhibiting the binding of serum HBsAg with HBsAb was represented by percentage relative to the value for a control where no extract was employed. The result is shown in Table 3.

Table 3. Inhibition(%) of binding of serum HBsAg with HBsAb by the extracts of P. ussuriensis or P. urinaria

Plant Extract	Concentration of the extract(mg/mℓ)				
	10	5	2.5	1.25	0.63
P. ussuriensis	96.2	95.2	90.3	89.7	68.4
P. urinaria	98.8	92.3	92.6	90.2	70.3

(3) Affinity Test Employing HBsAg in Hepatitis B Vaccine

The powdery extracts of P. ussuriensis and P. urinaria prepared in the Preparation Example were dissolved in distilled water to a final concentrations of 0.63, 0.31 and 0.16 mg/mℓ, respectively. 400 μℓ of each of the resulting solutions was mixed with 400 μℓ of HBsAg solution, which was prepared by diluting Hepavax®(20 μg; Korea Green Cross Corp., Korea) with a 50-fold volume of distilled water, and the mixture was reacted at 20°C for 1 hour. Titer of HBsAg in the resulting mixture was measured in accordance with the

same procedure as above (1), and the reduction in HBsAg titer was determined therefrom. The activity of the extracts of P. ussuriensis and P. urinaria in inhibiting the binding of HBsAg in a hepatitis B vaccine with HBsAb was represented by percentage relative to the value for a control where no extract was employed. The result is shown in Table 4.

Table 4. Inhibition(%) of binding of HBsAg in a hepatitis B vaccine with HBsAb by the extracts of P. ussuriensis and P. urinaria

Plant Extract	Concentration of the extract(mg/mℓ)		
	0.63	0.31	0.16
P. ussuriensis	97.3	92.8	91.2
P. urinaria	98.2	95.3	91.5

(4) Antagonism between Extract of P. ussuriensis or P. urinaria and HBsAb in binding with HBsAg

The antagonism between the extract of P. ussuriensis or P. urinaria and HBsAb in binding with HBsAg was investigated in accordance with the following procedure.

The powdery extracts of P. ussuriensis and P. urinaria prepared in the Preparation Example were dissolved in distilled water to final concentrations of 10, 5, 2.5 and 1.25 mg/mℓ, respectively. 400 μℓ of each of the resulting solutions was mixed with 400 μℓ of HBsAb-positive serum, and titer of HBsAb in the resulting mixture was measured by using Enzygnost® Anti-HBs micro kit(Behring Co., U.S.A.) in accordance with the manufacturer's protocol. The antagonistic inhibitory activity of the extracts of P. ussuriensis and P. urinaria in inhibiting the binding of HBsAg with HBsAb was represented by percentage relative to

the value for a control where no extract was employed. The result is shown in Table 5.

Table 5. Inhibition(%) of binding of HBsAg with HBsAb by the extracts of P. ussuriensis and P. urinaria

Plant Extract	Concentration of the extract(mg/mℓ)			
	10	5	2.5	1.25
P. ussuriensis	92.2	70.8	54.2	26.4
P. urinaria	96.4	80.3	70.4	56.4

As can be seen from Table 5, the extracts of P. ussuriensis and P. urinaria exhibited significant antagonistic activities at a concentration of 1.25 mg/mℓ.

Test Example 3: Inhibitory Activity of Extracts of P. ussuriensis and P. urinaria on the Activity of HBV DNA Polymerase

The powdery extracts of P. ussuriensis and P. urinaria prepared in the Preparation Example were dissolved in distilled water to final concentrations of 340.6, 68.1, 34.1 and 6.8 mg/mℓ, respectively, the resulting solutions were reacted with HBV particles(Dane particles), and then the degree of inhibition of DNA polymerase was determined as follows.

0.2 mℓ of serum containing HBV particles was mixed with 2.3 mℓ of solution 1(0.01 M Tris-HCl(pH 7.4), 0.15 M NaCl) and then centrifuged at 4°C, 10,000 rpm for 10 min. The resulting supernatant was placed on 2.5 mℓ of solution 2(30 %(w/v) sucrose, 0.01 M Tris-HCl(pH 7.4), 0.15 M NaCl, 0.001 M EDTA, 0.1 % 2-mercaptoethanol, 1 mg bovine serum albumin(BSA)) and then centrifuged at 4°C, 50,000 rpm for 4 hours. The resulting pellets were suspended in 25 μℓ of solution 3(0.01 M Tris-HCl(pH 7.4), 0.15 M NaCl, 1 % NP-40,

0.1 % 2-mercaptoethanol). To the suspension were added 25 μℓ of each of the extract solutions prepared above and 25 μℓ of polymerase mixture(0.2 M Tris-HCl(pH 7.4), 0.08 M $MgCl_2$, 0.24 M NH_4Cl, 1 mM dATP, 1 mM dTTP, 0.0025 nM ^{3}H-dCTP and 0.0025 nM ^{3}H-dGTP(both of them are inactive and 21 Ci/mM)). The mixture was reacted at 37°C for 3 hours and then spotted on a Watman 3 mm filter. The filter was washed with 15 % trichloroacetic acid and 90 % ethanol and then air-dried.

The amount of radioactivity of each spot was determined by using a densitometer(Biomed. Instrument Inc., U.S.A.), and the degree of inhibition(%) of the DNA polymerase activity was determined based on the activity of DNA polymerase measured in the absence of the extracts of P. ussuriensis and P. urinaria, as 100%. The result is shown in Fig. 1, wherein the activity of HBV DNA polymerase is observed to decrease in proportion with increasing concentration of the extracts at 34.1 μg/mℓ and more.

Test Example 4: Effect of P. urinaria Extract on HBV Particles

A serum sample taken from an HBsAg and HBeAg-positive patient was layered over a 20 % sucrose cushion, centrifuged by an ultracentrifuge(Sorvall®) at 4°C, 100,000xg for 4 hours to obtain HBV particles, and then the pelleted HBV particles were suspended in 20-fold volume of phosphate buffered saline(PBS).

Meanwhile, 2% aqueous solution of the extract of P. urinaria was diluted with distilled water to final concentrations of 0.2% and 0.1%, respectively. Each of the resulting solutions was mixed with the same volume of the HBV particle suspension prepared above and the mixture was left standing at room temperature for 30 min. The resulting mixture was spotted on a grid and negatively stained with 1% phosphotungstic acid. The grid was washed twice with PBS and the moisture remained on the grid was completely removed by Watman No. 1 filter. Then the grid was observed under an

electron microscope(Zeiss) and photographed.

The effect of the P. urinaria extract on the HBV particles was observed and compared with that of a control where distilled water was employed in place of the extract dilutions.

Figs. 2A and 2B show the HBV particles observed under an electron microscope before and after the addition of 0.1% aqueous solution of the extract of P. urinaria. As can be seen from Figs. 2A and 2B, the extract of P. urinaria causes serious deformation of HBV particles at a concentration of 0.1%.

Test Example 5: Acute Toxicity of P. urinaria Extract on Mouse

5 groups of mice, 10 mice(5 males and 5 females) constituting a group, were intraperitoneally administered with P. urinaria extract in 70, 84, 100, 120 and 144 mg/kg body weight doses, respectively. Lethality of mice and abnormalities of the internal organs thereof were observed after 7 days from the administration and the result is shown in Table 6.

Table 6. Acute Toxicity of P. urinaria Extract on Mouse

Group	Sex & Heads	Dose (mg/kg	Dead/ Total	Organ	Anatomical Abnormalities	Frequency	
						A	B
1	M 5	70	0/5	spleen	intumescence	0/0	1/5
	F 5	70	0/5	"	"	0/0	1/5
2	M 5	84	0/5	"	"	0/0	1/5
	F 5	84	0/5	"	"	0/0	1/5
3	M 5	100	0/5	"	"	0/0	2/5
	F 5	100	0/5	"	"	0/0	2/5
4	M 5	120	1/5	"	"	0/1	3/4
					dark red	1/1	0/4
				jejunum	thick foreign substance	1/1	0/4
				cecum	dark red	1/1	0/4
				bladder	dark red	0/1	0/1
	F 5	120	1/5	spleen	intumescence	0/1	3/4
					black	1/1	0/4
5	M 5	144	2/5	spleen	autolysis	1/2	0/3
					intumescence	0/2	3/3
				brain	subdural hemorrhage	1/2	0/3
				eyeball	Harder's gland hemorrhage	1/2	0/3
	F 5	144	2/5	spleen	intumescence	0/2	2/3
					atrophy	1/2	0/3
				mesenterium	dark red	1/2	0/3
					hemorrhage	1/2	0/3

* A = (cases)/(number of dead mice)
* B = (cases)/(number of alive mice)

As can be seen from Table 6, P. urinaria extract exhibited no toxicity when it was intraperitoneally administered at a dose of 70 mg/kg body weight, which corresponds to an intraperitoneal administration of 3,500 mg of the extract to a person weighing 50 kg.

Test Example 6: Effect of P. ussuriensis and P. urinaria Extracts on Hepatic Function of Normal Mouse

Three mice each weighing about 200 g were orally administered with 2% aqueous solution of P. ussuriensis and P. urinaria extracts for 2 days in place of drinking water, and blood samples were taken therefrom. Then, sGOT(serum glutamic oxaloacetic transaminase), sGPT(serum glutamic pyruvic transaminase), serum alkaline phosphatase(ALP) and serum bilirubin activities were determined by using AST/GOT, ALT/GPT, ALP, and serum bilirubin diagnostic kits(Sigma Chemicals Co., U.S.A.) in accordance with the manufacturer's protocols.

The results of the above tests are shown in Table 7.

Table 7

Plant	sGOT (KU/mℓ)		sGPT (KU/mℓ)		ALP (KAU/mℓ)		Bilirubin level(mg/ml)	
	B.A.	A.A.	B.A.	A.A.	B.A.	A.A.	B.A.	A.A.
P. ussuriensis	38.6	36.2	16.4	18.2	97.7	92.5	0.3	0.1
P. Urinaria	38.2	29.3	15.4	12.4	102.3	98.2	0.4	0.2

* B.A. = before administration
* A.A. = after administration

As can be seen from Table 7, it was confirmed that the extracts of P. ussuriensis and P. urinaria do not alter the sGOT, sGPT, ALP and bilirubin levels, which are the markers for the hepatic function, and, therefore, they do not adversely affect the hepatic function of a normal mouse.

Test Example 7: Mutagenicity of Extracts of P. ussuriensis and P. urinaria(Ames test)

The mitogenicity of extracts of P. ussuriensis and P. urinaria was determined in accordance with the methods

described by Ames, B. N., et al.(Proc. Natl. Acad. Sci. U.S.A., 72, 2433-2427(1975)) and Nagao, M., et al.(Ann. Rev. Genet., 12, 117-159(1978)).

Salmonella typhimurium TA100 and TA98 are histidine auxotrophs developed by Ames et al., which turn into histidine non-requiring strains upon contact with an environmental mitogen or a carcinogen, and, accordingly, are used for searching a mitogen.

The extract of P. ussuriensis or P. urinaria was dissolved in distilled water to a concentration of 2000 mg/100 mℓ, and then, 5, 10, 50 and 100 μℓ thereof were mixed with dimethyl sulfoxide(DMSO) to a final volume of 100 μℓ, respectively.

To 100 g of mouse liver was added 10 mℓ of 0.15 M KCl and the liver was scissored to homogeneity by using a Potter-Elvejhem type homogenizer. The homogenate was centrifuged at 9,000xg for 10 min. to obtain a microsome fraction, which was designated S9 fraction. The S9 fraction was used for the activation of a mitogen or a carcinogen, and the amount of protein therein was adjusted to 4 mg%.

0.5 mℓ of each of the extract solutions prepared above were placed in test tubes in order that two test tubes were allotted to one extract solution. The test tubes were divided into two groups, and then, 0.5 mℓ of S9 fraction and 0.5 mℓ of 50 μmol phosphate buffer(pH 7.4) were added to the test tubes of one group, while nothing was added to the test tubes of the other group. To each of the tubes was added 0.5 ml of phosphate buffer(pH 7.4) containing 2 μM NADH, 2 μmol NADPH, 2.5 μmol glucose-6-phosphate, 0.25 unit of glucose-6-phosphate dehydrogenase, 4 μM $MgCl_2$ and 16.5 μM KCl to prepare 1 mℓ of microorganism activation solution.

0.1 mℓ of a culture containing 2 x 10^8 cells of TA100 or TA98 strains was added to the tubes and the tubes were left standing at 37°C for 20 min. The contents of each of the tubes were mixed with 2 mℓ of agar solution(0.7% agar, 0.6% NaCl) at 45°C, and the mixture was immediately smeared on an agar plate. The plates were incubated at 37°C for 2

days and then examined for the occurrence of histidine non-requiring strains.

As a comparative test, the same procedure as above was repeated by using 100 μℓ(0.01 μg/μℓ) of 2-(2-puryl)-3-(5-nitro-2-puryl)acrolamide("AF-2") in DMSO in place of the extract solution. AF-2 is a known carcinogen(Sugimura, T. and Nagao, M., Modification of Mutagenic Activity, in: F.J. de Serres and A. Hollaender(Eds.), Chemical Mutagens, Principles and Methods for their Detection, Vol. 6, Plenum, New York, pp 41-60, 1980). Further, a control test was carried out without using the extract or AF-2.

The extracts were to be judged mitogenic if the number of colonies formed were more than twice that of the control group.

The tests were repeated twice and the mitogenicity of extracts was determined on the basis of the mean of the two tests. The result is shown in Table 8.

Table 8. Number of S. typhimurium Mutant Colonies

Administered Substances	Dose (μℓ)	TA100		TA98	
		+S9	-S9	+S9	-S9
P. ussuriensis extracts	5	122	146	21	22
	20	162	155	32	34
	50	191	178	52	41
	100	179	128	27	20
P. Urinaria extracts	5	112	120	32	18
	20	116	172	28	10
	50	148	122	30	15
	100	101	128	34	11
AF-2	1	1824	1987	1120	512
Control	-	161	123	32	11
	-	112	125	29	15

As can be seen from Table 8, the extracts of P. ussuriensis and P. urinaria exhibited negative results in the Ames test and, therefore, it was concluded that the extracts are not mitogenic.

Test Example 8: Toxicity Test for Extracts of P. ussuriensis and P. urinaria using a Dog

Two groups of korean mongrels each comprising one male and one female each weighing about 12 kg were orally administered with the extracts of P. ussuriensis and P. urinaria, which were prepared in accordance with the Preparation Example, twice a day in a dose of 200 mg/kg body weight for 120 days. Then, a blood sample was taken from each of the dogs and various hepatic functions listed in Table 8 were examined by using Autochemical Analyzer(Dupont, U.S.A., Dimension 380). Averaged values for two animals are shown in Table 9.

Table 9. Hepatic Function of Dogs Administered with the Extracts of P. ussuriensis and P. urinaria

Items	P. ussuriensis extract		P. urinaria extract	
	B.A.	A.A.	B.A.	A.A.
Total Protein(g/100 mg)	5.6	6.1	6.1	6.5
Albumin(g/100 mg)	1.2	1.5	1.6	1.7
Total Bilirubin(Mmol/ℓ)	1.6	1.3	1.0	1.0
Direct Bilirubin(Mmol/ℓ)	1.8	1.5	1.4	1.3
Cholesterol(Mmol/ℓ)	3.7	3.2	2.6	2.2
Alkaline phosphatase(U/ℓ)	113	136	142	136
GOT(U/ℓ)	28	24	21	26
GPT(U/ℓ)	25	26	30	30

* B.A. = before administration
* A.A. = after administration

As can be seen from Table 9, the extracts of P. ussuriensis and P. urinaria caused no abnormalities in the hepatic function of dogs treated therewith for 120 days.

Also, after administering 200 mℓ of the extracts of P. ussuriensis and P. urinaria to dogs, blood samples were taken therefrom at 30, 60 and 120 min. and the appearance of

HBsAb was measured with an HBsAb test kit(Abbott, U.S.A.). The result is shown in Table 10.

Table 10. Appearance of HBsAb after Administration of Extract of P. ussuriensis and P. urinaria

Administered plant extract	Time after the Administration		
	30 min.	60 min.	120 min.
P. ussuriensis extract	-	-	-
P. urinaria extract	+	+	+

As can be seen from Table 10, HBsAb-positive reactions were observed when the P. urinaria extract was administered to a dog, while HBsAb-negative reactions were observed when P. ussuriensis extract was administered. Further, a dog showed an HBsAb-positive reaction when it was administered with P. ussuriensis extract followed by P. urinaria extract, while it showed no HBsAb-positive reaction when it was administered with P. urinaria extract followed by P. ussuriensis extract. Accordingly, it was concluded that P. urinaria extract comprises a component which shows an HBsAb-like activity in a blood in vivo.

Test Example 9: Activity of P. ussuriensis and P. urinaria Extracts in Woodchuck Models

6 woodchucks infected with WHV(woodchuck hepatitis virus), which is similar to human HBV, were administered with P. urinaria extract in a dose of 600 mg/kg body weight/day for 12 weeks and the serum WHV DNA concentration (pg/mℓ) was determined at a predetermined time. The changes in the serum WHV DNA concentration of the woodchucks before and after the administration are shown in Table 11.

Table 11

Group		5 WK B.A.	1 WK B.A.	J.B.A (Time 0)	1 WK A.A	4 WK A.A	8 WK A.A.	12 WK A.A.
Test Group	1	2900	3300	2200	3600	2800	3610	110
	2	3100	5700	4400	2900	3300	32	7
	3	8200	11000	9100	14000	13000	250	18
	4	5200	4800	4300	5500	7000	1800	220
	5	4100	3200	2300	1300	2100	2600	2300
	6	3000	1300	2200	1400	2200	2400	1900
Control Group	1	1300	1600	1300	1300	1400	1300	1400
	2	1500	1600	1400	1400	1500	1800	1300
	3	1400	1700	1800	1500	1800	1700	1600
	4	8800	7800	13000	5300	3200	2300	4400
	5	6600	4400	5300	3100	2100	2800	2700
	6	12000	9200	11000	8800	8900	9600	11000

* B.A. = before the administration
* J.B.A. = Just before the administration
* A.A. = after the administration
* WK = week(s)

As can be seen from Table 11, WHV DNA concentrations of woodchucks 5 weeks before the administration were 8,200 - 2,900 pg/mℓ for the test group and 12,000 - 1,300 pg/mℓ for the control group; and just before the administration, 9,100 - 2,200 pg/mℓ for the test group and 13,000 - 1,300 pg/mℓ for the control group. However, after 12 weeks from the administration of P. urinaria extract, WHV DNA concentration of the control group remained high, i.e., 11,000 - 1,300 pg/mℓ, while that of the test group decreased to a remarkably low level of 220 - 7 pg/mℓ in 4 of the 6 woodchuck groups tested.

Test Example 10: Clinical Effects of P. ussuriensis and P. urinaria Extracts

The clinical effects of the P. ussuriensis and P. urinaria extracts were investigated employing a healthy HBV carrier, a HBV carrier with putative slight chronic

hepatitis, and a chronic active hepatitis B patient with complicated hepatocirrhosis as test subjects, as follows.

(1) Healthy HBV Carrier

A healthy HBV carrier, who showed relatively normal results in a hepatic function test except that the subject was HBsAg(+) HBsAb(-) HBeAg(+) HBeAb(-), was administered three times a day with the extract of P. urinaria in a dose of 40 mg/kg body weight/day for 24 months. Each unit dose, i.e., one-thirds of the daily dose, was dissolved in 50 mℓ of water and then administered 1 hour after meal.

The alteration of the serological idices during the treatment is shown in Table 12.

Table 12

Time		Markers for HBV Infection					Transaminase	
		HBsAg	HBsAb	HBeAg	HBeAb	HBV DNA	GOT	GPT
Before Treatment		+	-	-	-	+	21	24
Months after Treatment	2.5	+	-	-	+	-	18	56
	9.5	+	(+)	-	+	-	12	26
	11.5	±	(+)	-	+	ND	20	30
	13	+	(+)	-	+	-	32	37
	17	+	(+)	-	+	-	32	37
	24	+	(+)	-	+	-	22	30

* ND : not determined

As can be seen from Table 12, HBsAb-positive reaction was observed after 9.5 months from the initiation of the treatment and thereafter, while the serum was HBsAb-negative as was determined by using a radioisotope method kit(Sorin Biomedica Co., Italy) in accordance with the manufacturer's protocol. Further, HBeAb and serum HBV DNA were detected as positive and negative, respectively, after 2.5 months of the treatment.

(2) HBV Carrier with Putative Slight Chronic Hepatitis

A HBV carrier, who was presumed to suffer a chronic active hepatitis on the basis of the result of HBsAg(+) HBsAb(-) HBeAg(+) HBeAb(-) anti-HBc(+) in a hepatic function test and showed GOT of 23.9 and GPT of 63.0, which fall in the warning ranges, was administered with P. ussuriensis extract for 11 months and, thereafter, with P. urinaria extract for 17 months. The dose of the extracts and the administration method were the same as in (1) above.

The alterations of the serological indices during the treatment is shown in Table 13.

Table 13

Time		Markers for HBV Infection					Transaminase	
		HBsAg	HBsAb	HBeAg	HBeAb	HBV DNA	GOT	GPT
Before Treatment		+	-	+	-	+	32	60
Administration of P. ussuriensis Extract								
Months after Treatment	1	+	-	+	-	+	28	65
	4	+	-	+	-	-	34	76
	10	+	-	+	-	-	38	65
	11	+	-	+	-	-	32	72
Administration of P. urinaria Extract								
Months after Treatment	3	+	(+)	+	-	-	46	77
	6	+	(+)	+	-	-	38	115
	14	+	(+)	+	-	-	32	65
	17	+	(+)	+	-	-	30	69

As can be seen from Table 13, the markers for HBV infection maintained the state of HBsAg(+) HBsAb(-) HBeAg(+) HBeAb(-) and HBV DNA(+), and the GOT and GPT values remained in the warning range after 1 month from the administration of P. ussuriensis extract. However, HBV DNA was not detected after 4 months treatment of P. ussuriensis extract, while other markers remained unchanged.

When the P. urinaria extract was administered, HBsAb-positive reaction was observed after 3 months treatment, but a result of HBsAb-negative was obtained when a radioisotope method was used. These results suggest that an HBsAb-like substance which specifically binds to HBsAg in vivo is present in the P. urinaria extract. Further, HBsAb and serum HBV DNA were always detected as positive and negative, respectively, during the administration of P. urinaria extract.

(3) Chronic Active Hepatitis B Patient with Complicated Hepatocirrhosis

A chronic active hepatitis B patient with complicated hepatocirrhosis, who showed GOT/GPT=129/184 and HBsAg(+) HBeAg(+) HBsAb(+) HBeAb(-) in a hepatic function test, was administered with the P. urinaria extract for 3 months in accordance with the same procedure as in (1).

The alterations of the serological indices during the treatment is shown in Table 14.

Table 14

Time	Markers of HBV Infection					Transaminase	
	HBsAg	HBsAb	HBeAg	HBeAb	HBV DNA	GOT	GPT
Before the Treatment	+	-	+	-	+	268	339
1 Month after the Treatment	+	-	+	-	+	140	239
3 Months after the Treatment	+	(+)	+	-	-	47	105

As can be seen from Table 14, HBV DNA-positive reaction was observed after 3 months treatment with the P. urinaria extract and the transaminase values were significantly improved. However, the patient suffered from a complicated hepatocirrhosis, and therefore, a continuous observation was

required.

As can be seen from the results of the above Test Examples, the aqueous extracts of P. ussuriensis and P. urinaria may be used as an effective HBV treating agent which has little toxicity and causes no adverse effect.

Formulation Example: Pharmaceutical Formulations comprising Extracts of P. ussuriensis and P. urinaria

(1) Preparation of capsules comprising an extract of P. ussuriensis or P. urinaria

Hard gelatin capsules were prepared using the following ingredients:

	Quantity (mg/capsule)
Active ingredient (Extract of P. ussuriensis or P. urinaria)	800
Starch, dried	1100
Magnesium stearate	100
Total	2000 mg

The above ingredients were mixed and filled into hard gelatin capsules in 2000 mg unit quantities.

(2) Preparation of capsules comprising extracts of P. ussuriensis and P. urinaria

Hard gelatin capsules were prepared using the following ingredients:

	Quantity (mg/capsule)
Active ingredient	
Extract of P. ussuriensis	200
Extract of P. urinaria	600

Starch, dried	1100
Magnesium stearate	100
Total	2000 mg

The above ingredients were mixed and filled into hard gelatin capsules in 2000 mg unit quantities.

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made to the invention by those skilled in the art which also fall within the scope of the invention as defined by the appended claims.

What is claimed is:

1. An extract of Phyllanthus ussuriensis and/or Phyllanthus urinaria which shows a therapeutic activity for hepatitis B.

2. The extract of claim 1, which is prepared by extracting Phyllanthus ussuriensis or Phyllanthus urinaria with water.

3. A pharmaceutical composition for treating hepatitis B, which comprises a therapeutically effective amount of the extract of claim 1 and a pharmaceutically acceptable carrier.

