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Activities of Selected Plants against Hepatitis B Immunological Marker HBsAg

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ABSTRACT Ten plants namely *Phyllanthus urinaria* (dukong anak), *Andrographis paniculata* (hempedu bumi), *Oenanthe javanica* (selom), *Allium sativum* (bawang putih), *Terminalia catappa* (ketapang), *Mimosa pudica* (semalu), *Curcuma zedoaria* (kunyit putih), *Curcuma aeruginosa* (temu hitam), *Curcuma mangga* (temu pauh) and *Curcuma xanthorhiza* (temu lawak) were screened for capabilities in reducing the quantity of HBsAg in Hepatitis B patients' serum. The water extracts of the plants were tested *in vitro* for effects on HBsAg by using ELISA method. Double immunodiffusion tests were carried out on the plants with positive reactions to confirm the interaction between the plant extracts and the serum. *Terminalia catappa, Mimosa pudica* and *Phyllanthus urinaria* were found to significantly reduce by more than 50% the quantity of HBsAg in the serum. Results from the immunodiffusion technique suggest that *Terminalia catappa, Mimosa pudica* and *Phyllanthus urinaria* extracts have the potential to interact with HBsAg and lower its quantity in the Hepatitis B patient's serum *in vitro*.

ABSTRAK Sebanyak sepuluh tumbuhan iaitu *Phyllanthus urinaria* (dukong anak), *Andrographis paniculata* (hempedu bumi), *Oenanthe javanica* (selom), *Allium sativum* (bawang putih), *Terminalia catappa* (ketapang), *Mimosa pudica* (semalu), *Curcuma zedoaria* (kunyit putih), *Curcuma aeruginosa* (temu hitam), *Curcuma mangga* (temu pauh) dan *Curcuma xanthorhiza* (temu lawak) dikenalpasti bagi ujian keupayaan pengurangan kuantiti HBsAg pada serum pesakit-pesakit Hepatitis B. Bagi penilaian ini, kaedah ELISA diguna sebagai ujikaji *in vitro* di antara HBsAg dan ekstrak tumbuhan. Seterusnya ujian lanjutan telah dilakukan melalui teknik 'double immunodiffusion' terhadap tumbuhan yang terbukti positif terhadap ujikaji ELISA. Tiga tumbuhan, *Terminalia catappa, Mimosa pudica* dan *Phyllanthus urinaria,* didapati berkesan mengurangkan kuantiti HBsAg di dalam serum lebih daripada 50%. Keputusan daripada ujian teknik 'double immunodiffusion' mencadangkan bahawa ekstrak-ekstrak *Terminalia catappa, Mimosa pudica* dan *Phyllanthus urinaria* berkeupayaan berinteraksi dengan HBsAg dan seterusnya menurunkan kuantitinya dalam serum pesakit hepatitis secara *in vitro*.

(Hepatitis B, HBsAg, *Terminalia catappa, Phyllanthus urinaria, Mimosa pudica*)

INTRODUCTION

Hepatitis B virus (HBV), a member of Hepadnaviridae family of viruses, has already infected approximately 350 million individuals throughout the world, making it one of the most common human pathogens. The prevalence of chronic HBV infection continues to be highly variable, ranging over 10% in some Asian and Western Pacific countries to under 0.5% in the United States and northern European countries [1]. HBV infects primarily humans where it is present in the blood, body fluids and liver. Infections by HBV will result in Hepatitis B that may lead to cirrhosis, liver cancer and may also subsequently lead to death. The virus can be transmitted by direct contact with blood or body fluids of an infected person. A person usually gets infected by having unprotected sex with an infected person, using unsterile needles and through blood transfusion (horizontal transmission). On the other hand, a baby can get

HBV from his infected mother during childbirth (vertical transmission).

The presence of HBV infection in any person can be detected by looking for immunological markers in the person's blood. Hepatitis B surface antigen (HBsAg) is one of the markers that appear in the patient's blood. A prospective general population study of 22,707 Chinese men in Taiwan has shown that the incidence of primary hepatocellular carcinoma (PHC) among carriers of HBsAg is much higher than among non-carriers (1158/100,000 vs. 5/100,000 during 75,000 man-years of following) [2].

Previously, scientists have done some research on plants with antiviral compounds. Some medicinal plants have shown considerable ability in reducing Hepatitis B viral infection. In a clinical trial, the oral preparation of *Phyllanthus amarus* was shown to increase the clearance rate of HBsAg carrier state in human subjects [3]. These results are consistent with the hypothesis that *P. amarus* specifically suppress the gene expression of HBsAg in human liver cell [4]. In another study, phyllanthin, hypophyllanthin, triacontanal and tricontanol have been isolated from a hexane extract of *Phyllanthus niruri*, which are believed to be the active compounds responsible for its biological activity. Phyllanthin and hypophyllanthin were found to protect against carbon tetrachloride (CCl_4) and galactosamine-induced cytotoxicity (GalN) in primary cultured rat hepatocytes, while triacontanal was protective only against GalN induced toxicity [5]. There are also studies that have been done using ELISA methods. Active fractions of butanol extracts of *Phyllanthus amarus* inhibited the interaction between HBsAg/HBeAg and their corresponding antibodies suggesting anti-HBs, anti-Hobe-like activity and also effect on HBV-DNA [6]. In another study, it was discovered that there is a promising anti-HBsAg like activity in picroliv (active principle from *Picrorrhiza kurroa*) and its major components, and alcoholic extract of *Phyllanthus niruri.* This differed from the classical viral neutralization. Picroliv also inhibited purified HBV antigen (HBeAg and HBsAg) prepared from healthy HBsAg carriers [7]. Besides *Phyllanthus* sp., *Terminalia catappa* (ketapang) leaves have been used as an herbal drug in the treatment of liver related diseases in China. *Terminalia catappa* was found to possess good antihepatotoxic activity and superoxide radical scavenger activity [8].

In this study, the anti-hepatitis effect of various plants against HBsAg was screened using ELISA method. Plants selected for this purpose include *Phyllanthus urinaria* (dukong anak), *Andrographis paniculata* (hempedu bumi), *Curcuma zedoaria* (kunyit putih), *Curcuma aeruginosa* (temu hitam), *Curcuma mangga* (temu pauh) dan *Curcuma xanthorhiza* (temu lawak), *Oenanthe javanica* (selom), *Allium sativum* (bawang putih), *Terminalia catappa* (ketapang) and *Mimosa pudica* (semalu).

MATERIALS AND METHODS

Plant Samples

The plants used in this study namely *Phyllanthus urinaria* (HI 1343), *Andrographis paniculata* (HI 1344), *Oenanthe javanica* (HI 1345), *Allium sativum* (bawang putih), *Terminalia catappa* (HI 1346), *Mimosa pudica* (HI 1347), *Curcuma zedoaria* (HI 1348), *Curcuma aeruginosa* (HI 1349), *Curcuma mangga* (HI 1350) and *Curcuma xanthorhiza* (HI 1351) were authenticated by Prof. Halijah Ibrahim and the voucher specimens were deposited at the Institute of Biological Sciences, University Malaya. The plant samples were collected, washed and extracts were obtained by water extraction.

Serum

Serum samples were obtained from Hepatitis B patients associated with liver diseases that were positively identified to contain HBsAg. The serums were provided by Gribbles Pathology (M) Sdn. Bhd.

Plant extraction

Plant extracts were prepared by stirring 4 g of the powdered plant material in 200 ml of water for 3 hours. The crude extracts obtained were clarified by filtration through filter paper (Whatman No. 1).

Quantitative Test (HBsAg Test)

(I) Reaction in the Control and Test Samples

For the test samples, the various plant extracts were separately mixed with an equal amount of HBsAg positive serum. Similarly, in order to calculate the percentage of reduction of HBsAg, the control consisting of 100 µl of serum added with 100 µl of water was prepared. Both the test samples and the control were incubated for an hour at 37^0C. 50 µl samples and 50 µl controls (4 wells each) were then used in the screening with the ELISA method.

(II) HBsAg screening

The HBsAg was screened by HBsAg ELISA method. The test principle used is the sandwich assay (antibody-antigen-antibody). When anti-HBs coated wells and anti-HBs•HRPO conjugate were incubated at 37°C for 80 minutes with specimens containing HBsAg, (antibody)-(antigen)-(antibody•HRPO) complexes were formed on the wells. After washing, the activity of peroxidase on the wells reflects the presence of HBsAg in the specimen. Then, 100 µl of TMB substrate solution (mixture of equal volume TMB substrate solution A and B) was added into wells and incubated at 37°C for 30 minutes. The magnitude of HBsAg quantity is determined accordingly by the colour changes. The reaction was stopped by addition of 100 µl of Sulphuric acid into wells. The results were read by ELISA Reader to determine the absorbance at 450nm/650 nm. The percentage reduction of HBsAg for each set of screening was calculated by using this formula:

$$\frac{\text{Mean absorbance of Controls - Mean absorbance of Test Sample}}{\text{Mean absorbance of Controls}}$$

Qualitative Test (Double Immunodiffusion Technique)

This technique involves the concept of allowing the antibody to encounter antigen by diffusion. Positive interaction between the antigen (plant extract) and antibody (serum) will result in a clear visible precipitin line upon staining.

(I) Precoating of glass plates

The procedure involves setting a low porosity agar gel onto the plate and then allowing it to dry onto the glass surface. 0.5 g agar was boiled in 100 ml water by using microwave. The agar solution was allowed to cool to 50°C and then 2 ml of it was pipetted onto each microscope slide placed on a levelling table. The agar was then allowed to solidify and then left overnight at room temperature for 4 to 6 hours at 40°C until completely dry.

(II) Preparation of microscope slides

1.0 g agar was boiled in 100 ml water by using microwave to prepare 1% agar solution. The agar solution was allowed to cool to 45°C and then 3.5 ml of it was pipetted onto each precoated slide. The slides were left to solidify at room temperature. Then, four holes were punched out by using a gel puncher (Figure 1).

The plugs of agar were removed from each wells by using a Pasteur pipette attached to a vacuum line. The test was conducted by pipetting 16 µl of serum and 16 µl of plant extracts into the centre well of 1 and 2 respectively followed by incubation overnight at room temperature in a humid chamber. Slides were examined for immunoprecipitin lines.

(III) Staining precipitin lines

Although immunoprecipitin lines produced in agar were often visible, intensification and visualisation of weaker lines can be achieved by staining with Coomassie brilliant blue. Gel was washed in five changes of 100 ml 0.85% NaCl over a period of 48 hours. Then, dried by either leaving it for 16 hours at room temperature or two to six hours at 40°C. The gel was then immersed in stain for 30 minutes to 1 hour until stained bands were visible. The gels were destained in 3 to 4 changes of destaining solution and then left to dry in air at room temperature.

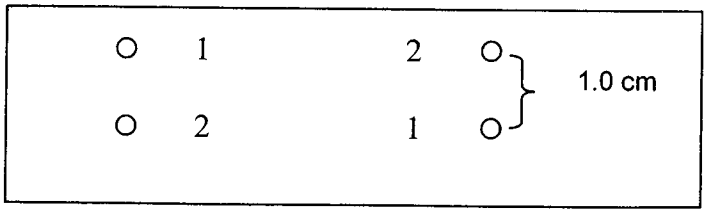


Figure 1. Schematic representation of microslides.

RESULTS

The plants were extracted using water (4g of powdered plant materials in 200 ml of water) and the concentration of the plant extracts estimated by freeze-drying 1ml of each extracts. In total 4 serums from different patients were used in this experiment.

The percentage of reduction of HBsAg in each serum was calculated by using the formula stated earlier. The data represents the mean and standard deviation (S.D.) of HBsAg reduction calculated based on four different HBsAg positive serums used for each plant. *Oenanthe javanica*, *Andrographis paniculata*, and *Curcuma zedoaria* gave negative results in the ELISA screening technique. These plant extracts were considered as having no positive activity against HBsAg and therefore not represented in the graph (Figure 2).

The presence of precipitin line indicated a positive reaction between the plant and the serum. This was seen in all plants with 50% or more reduction of HBsAg in the ELISA method. Plants with positive reactions namely *Mimosa pudica* (whole plant), *Mimosa pudica* (roots only) and *Mimosa pudica* (without roots) failed to give any positive results for the double immunodiffusion technique at low concentrations. However by increasing the concentration of these plants by four fold the precipitin lines were more apparent.

Quantitative Test (HBsAg ELISA Test)

Table 1. The mean percentage reduction of HBsAg

Plants	Concentration (mg/ml)	Serum 1	Serum 2	Serum 3	Serum 4	Mean ± S.D.
Phyllanthus urinaria	3.7	52.000%	41.310%	61.362%	55.536%	52.6 ± 8.43
Terminalia catappa	7.3	99.060%	96.496%	99.320%	97.375%	98.1 ± 1.35
Oenanthe javanica	6.5	5.010%	- 3.426%	- 2.836%	- 8.199%	-2.4 ± 5.47
Angdrographis paniculata	5.7	- 0.710%	- 5.185%	- 14.094%	- 2.346%	-5.6 ± 5.97
Allium sativum	4.5	7.040%	11.720%	- 8.340%	16.830%	6.8 ± 10.86
Mimosa pudica (whole plant)	5.7	92.158%	87.305%	98.230%	95.500%	93.3 ± 4.70
Mimosa pudica (without roots)	4.9	97.206%	85.322%	97.205%	90.965%	92.7 ± 5.72
Mimosa pudica (roots only)	2.8	99.448%	99.231%	99.026%	98.221%	98.9 ± 0.54
Curcuma xanthorhiza	3.1	- 2.250%	5.690%	- 2.000%	14.490%	3.9 ± 7.92
Curcuma mangga	8.0	8.860%	2.850%	24.000%	29.050%	16.2 ± 12.36
Curcuma zedoaria	6.3	5.612%	- 5.940%	- 14.640%	7.171%	-1.9 ± 10.29
Curcuma aeruginosa	6.0	6.257%	14.387%	4.863%	7.372%	8.2 ± 4.24

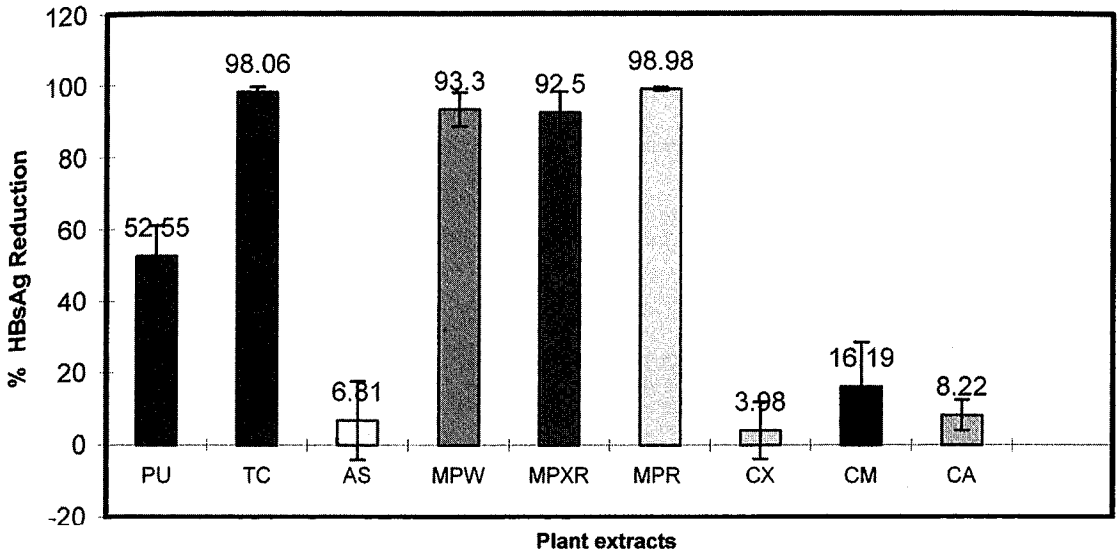


Figure 2. The Mean percentage of HBsAg reduction

Keynote:

PU	*Phyllanthus urinaria*
TC	*Terminalia catappa*
AS	*Allium sativum*
MPW	*Mimosa pudica* (whole plant)
MPXR	*Mimosa pudica* (without roots)
MPR	*Mimosa pudica* (roots)
CX	*Curcuma xanthorhiza*
CM	*Curcuma mangga*
CA	*Curcuma aeruginosa*

Qualitative Test (Double Immunodiffusion Technique)

Table 2. Presence or Absence of Precipitin Line

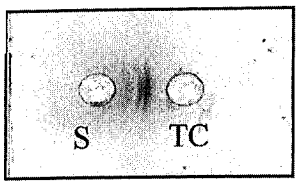
Plants	Precipitin line
Phyllanthus urinaria	+ve
Terminalia catappa	+ve
Oenanthe javanica	-ve
Angdrographis paniculata	-ve
Allium sativum	-ve
Mimosa pudica (whole plant)	+ve
Mimosa pudica (without roots)	+ve
Mimosa pudica (roots only)	+ve
Curcuma xanthorhiza	-ve
Curcuma mangga	-ve
Curcuma zedoaria	-ve
Curcuma aeruginosa	-ve

Keynote:

+ve : visible precipitin line
-ve : no precipitin line

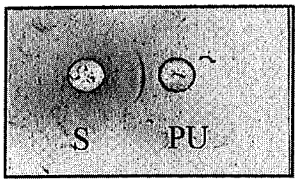
The presence of precipitin line indicated that there was interaction between the serum and the plant extracts.

(a)



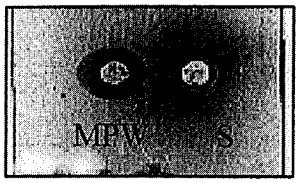
Concentration of extract: 7.3 mg/ml

(b)



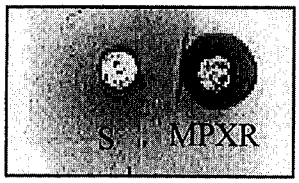
Concentration of extract: 3.7 mg/ml

(c)



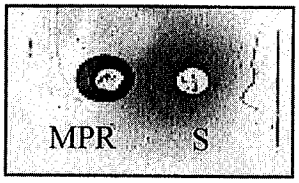
Concentration of extract: 22.8 mg/ml

(d)



Concentration of extract: 19.6 mg/ml

(e)



Concentration of extract: 11.2 mg/ml

Keynote:

S	:	Serum
PU	:	*Phyllanthus urinaria*
TC	:	*Terminalia catappa*
MPW	:	*Mimosa pudica* (whole plant)
MPR	:	*Mimosa pudica* (roots only)
MPXR	:	*Mimosa pudica* (without roots)

Figure 3. Results of the Double Immunodiffusion Technique on 3 plants

DISCUSSION

All the plants tested, except for *Oenanthe javanica*, *Andrographis paniculata* and *Curcuma zedoaria*, gave positive results for ELISA Test or HBsAg Test (Table 1). This indicated that the plants have capabilities to interact and lower the quantity of HBsAg in the serum *in vitro*. However, the percentage of reduction of HBsAg differs from one plant to another. *Terminalia catappa* (Ketapang), *Mimosa pudica* (Semalu) and *Phyllanthus urinaria* (Dukong anak) reduced the quantity of HBsAg in the serum more than fifty percent (50%). On the other hand, *Oenanthe javanica*, *Andrographis paniculata* and *Curcuma zedoaria* gave negative results, which were later concluded as having no positive activity against HBsAg.

These results were further supported by the presence of precipitin line obtained from the double immunodiffusion technique. The immunoprecipitation line will occur if there is an interaction between the HBsAg in the serum and particular compound(s) in the plant extracts. The precipitin line was clearly detected in *Terminalia catappa, Mimosa pudica* (whole plant) and *Phyllanthus urinaria* (Figure 2(a)-(e)). This showed that the three plants interacted with HBsAg, in comparison to the other plants. From the experiment, it was also found that when the concentration of the plant extracts (*i.e.* the plants with positive reactions) were increased, the precipitin line appeared more prominently as compared to those with lower concentration. This was evident for *Mimosa pudica* extract whereby initially as the concentration was only 5.7 mg/ml, no precipitin line was noted. However, as the concentration of the extract was increased to 22.8 mg/ml, the precipitin line was more apparent.

CONCLUSION

The results of this study indicated that the water extracts of *Phyllanthus urinaria, Terminalia catappa* and *Mimosa pudica* reduced the quantity of HBsAg in Hepatitis B serum *in vitro*. Further studies would be carried out as a continuation of current findings to confirm the effectiveness of these three plants against other Hepatitis B markers (*i.e.* HBeAg, HBV-DNA).

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