

# Antiviral Activity of Indonesian Medicinal Plants against Hepatitis B Virus

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## ABSTRACT

**Background:** Hepatitis B virus (HBV) infects more than 300 million people globally and is a common cause of liver cancer. Current therapy using reverse transcriptase inhibitors require long-term treatment and the potential risk of development of drug-resistant viruses remains an important issue to be considered. Hence, the development of new drugs is critical. Traditional medicinal plants used for the treatment of infectious diseases may provide a viable option for the discovery of anti-HBV drug candidates. **Objective:** This study examined anti-HBV activity of 31 kinds of Indonesian plants. **Materials and Methods:** Crude extracts of various part of plants, leaves and stem, were obtained using dichloromethane and ethanol solvent. The effect on viral entry was examined by determining levels of HBsAg expression in the supernatants of HBV-infected HepG2-NTCP cells by ELISA. The effect on HBV replication was determined by measuring HBV DNAs amounts in Hep38.7-Tet cells by quantitative real-time PCR. **Results:** The extracts of *Phyllanthus niruri* leaves and *Curcuma xanthorrhiza* showed reduction of strong HBsAg production from HepG2-NTCP cells with IC<sub>50</sub> values of 170.48 and 270.51 µg/mL, respectively. Treatment of HepAD38.7-Tet cells with *P. niruri* and *C. xanthorrhiza* at the highest concentration while avoiding cytotoxicity reduced extracellular HBV DNA levels by 70% and 30 % of the untreated control respectively. **Conclusion:** *P. niruri* inhibited both the entry and HBV replication, thus *P. niruri* is a promising candidate for anti-HBV drug development.

**Key words:** Hepatitis B Virus, *Phyllanthus niruri*, *Curcuma xanthorrhiza*, Medicinal plants.

## INTRODUCTION

Hepatitis B Virus (HBV), a member of the *Hepadnaviridae* family, is an enveloped virus with partially double-stranded circular DNA genome that attacks the liver and causes both acute and chronic HBV infections. Chronic HBV infections result in liver cirrhosis and hepatocellular carcinoma (HCC).<sup>1,2</sup> It has been estimated that approximately 257 million people are living with chronic hepatitis B infection resulting in circa 887 000 deaths annually.<sup>3</sup> Transmission of HBV infection occurs when an uninfected person comes into contact with infected blood and/or body fluids (e.g., semen, vaginal secretions, etc) through sexual activity, sharing of personal items, contaminated medical equipment and mother to child transmission during birth.<sup>4</sup>

To support HBV prevention, a HBV vaccine has been developed, which is safe, and effective as a routine prophylactic by inducing the appropriate immune response in the host.<sup>5</sup> In contrast, once an infection has occurred, the current antiviral treatments for the suppression of HBV replication are used. Interferon (IFNs) and nucleos(t)ide analogues (NAs) have been used and demonstrated to prevent cirrhosis, liver failure and HCC which is caused by HBV infection.<sup>6</sup> However, the long-term treatment with (NAs) and interferons is associated with an increased risk of side effects and potential drug resistance.<sup>7</sup> Therefore, it is necessary

to develop a new therapy or alternative medicine against HBV infection.

Medicinal plants are rich in various secondary metabolites that have been used to cure several kinds of human disease. It is interesting that about 80% of the active ingredient which have been isolated from plants indicate a positive correlation between modern and traditional therapeutic uses.<sup>8</sup> Some of these plants, such as *Curcuma longa* Linn,<sup>9</sup> *Ganoderma lucidum*,<sup>10</sup> *Acanthus ilicifolius* L,<sup>11</sup> and *Oenanthe javanica*<sup>12</sup> have been reported to exhibit anti-HBV activity. Several compounds (periglucines A–D, norruffescine, (–)-8-oxotetrahydropalmitine) isolated from *Pericampylus glaucus*, showed anti-HBV activity on HepG2.2.15 cell line *in vitro*. Compounds of 8-oxotetrahydropalmitine could inhibit HBsAg secretion with an 50% inhibition concentration (IC<sub>50</sub>) value of 0.14 mM (SI = 22.4). It has also been reported that piperine and guineesine, isolated compounds of *Piper longum*, exhibit inhibitory activity suppressing the secretion of HBsAg and HBeAg with IC<sub>50</sub> values of 0.15 and 0.05 mM for HBsAg, and 0.14 and 0.05 mM for HBeAg, respectively.<sup>13</sup> Our previous study was reported *Cananga odorata* possessed a strong anti-HBV activity both in HepG2-NTCP and Hep38.7-Tet cells which indicated the action of extract in the entry and post entry steps (replication) of HBV life cycle.<sup>14</sup> In this study, we screened 31 samples of Indonesian medicinal plants for anti-HBV activity by two *in-vitro* assays to assess the effect on the viral entry and replication.

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## MATERIALS AND METHODS

### Plant material

A total of 37 plants were collected from the National Botanical Garden of Balikpapan Kalimantan Timur and Malang, East Java, Indonesia (Table 1). Plants were identified by an experienced Botanist at the National Botanical Garden, Malang and all leaves were dried at room temperature inside away from direct sunlight.

### Cell lines and virus

HepG2-NTCP cells<sup>15</sup> were used to assess the antiviral effect on the viral entry. These cells are known to express sodium taurocholate co-transporting polypeptide (NTCP) that has been identified as a functional receptor for HBV. Post-entry inhibition was analyzed using Hep38.7-Tet cells that expressed viral HBV pgRNA under tetracycline provision. The viruses that were produced from Hep38.7-Tet were used to infect HepG2-NTCP cells. To enhance the infection process, PEG was added during virus infection. The HepG2-NTCP cells were cultivated using Dulbecco's Modified Eagle Medium/Nutrient Mixture F12 with GlutaMAX (Thermo Fisher Scientific, Carlsbad, CA), supplemented with 10% FBS, 10 mM HEPES, 5 µg/ml insulin (FujiFilm Wako), 100 IU/ml penicillin/streptomycin, and 1 mg/ml G418 (Nacalai Tesque), while the Hep38.7-Tet was grown in basal culture medium with a

composition similar to that used for the HepG2-NTCP cells, but with the addition of 400 µg/ml G418 and 400 ng/ml tetracycline (FujiFilm Wako). All cells were maintained at 37°C and 5% CO<sub>2</sub> atmosphere in a humidified incubator.

### Plant extraction

Several different solvents (ethanol 80%, hexane, and dichloromethane) were used to extract from the leaf and stem samples. The leaves were extracted using n-hexane and ethanol 80%. Meanwhile, the residue from the n-hexane extract underwent a further extraction process using dichloromethane (DCM). In this study we used dichloromethane extracts from samples collected in Balikpapan, Indonesia while ethanol 80% extract was used for the East Java collection. All extracts were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 mg/ml and then stored at -30°C.

### HBV entry inhibition screening

The HepG2-NTCP were seeded in a 24 well plate for 24 hours before the assay was performed. Cells were inoculated with HBV in the presence of a single concentration of plant extract (100 µg/mL) for the screening assay. The activity of plant extract was examined at different concentrations (400; 200; 100; and 50 µg/mL) to obtain the IC<sub>50</sub> value. The mixture of HBV and extract were inoculated onto HepG2-NTCP for 16 hours. Cells were subsequently washed to remove the extract and excess virus. The cells growth medium was replaced every 2 day until day 8. Supernatants were then collected to perform ELISA HBsAg screening (Abnova, HBsAg ELISA kit). Briefly, samples and control treatments at a concentration of 50 µl were added into well plates of the kit, then 50 µl of anti-HBsAg-peroxidase solution was added. The plate was incubated at 37°C for 30 minute and washed with PBS 10 times. To mix the tetramethylbenzidine (TMB) substrate, solution A and B were added by equal volume of 100 µl/well and then incubated for 30 minutes. To stop the reaction, 100µl of 2N H<sub>2</sub>SO<sub>4</sub> were added in each well and absorbance was determined within 30 minutes at 450/650 nm.<sup>14</sup>

### HBV post-entry inhibition assay

Hep38.7-Tet cells were used to assess the effect of extract on the post-entry stage of HBV infection. Cells were seeded in a 24 well plate for 24 hours before the assay was performed. Sample was added to the cells at four different concentrations (200, 100, 50, 25 µg/mL) without tetracycline provision, while the positive control was amended with tetracycline to suppress HBV production. In conditions free from tetracycline, Hep38.7-Tet will produce HBV pg RNA and released it into the supernatant of the culture. The plate was incubated for 16 hours then medium free extract was used to wash the extract residue after 16hr. The medium extract was added every 3 days until day 7. Supernatant was then collected for DNA purification. Real Time (RT)-qPCR was performed to quantify the amount of extracellular HBV DNA in each sample. Briefly, the HBV DNA was purification by centrifugation column filtering (Promega Midi DNA kit purification). The DNA was amplified for real-time quantitative PCR using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan) with specific primers for the HBV genome: Ayw-F (5'-CTCGTGGTGGACTTCTCTC-3') and Ayw-R (5'-AAGATGAGGCATAGCCAGCA-3').<sup>14</sup>

### Cytotoxicity assay

The cytotoxicity of samples was assessed on selected plants by 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Briefly, cells in 96 well plates were treated with various concentrations of crude extracts for 48 hours. The medium was replaced with MTT containing medium and incubated for 4 hours. Insoluble precipitates were dissolved with DMSO and absorbance was measured using a microplate reader. The cell viability percentages

**Table 1: Medicinal plants tested for anti-HBV activity in HepG2-NTCP cell culture.**

No	Species Name	Code	Family
Dichloromethane extract of leaves (Balikpapan sample)			
1	<i>Melicope glabra</i> (Syn <i>M. pinnata</i> )	BP01	Rutaceae
2	<i>Luvunga scandens</i> (Roxb.) Wight	BP02	Rutaceae
3	<i>Artocarpus sericarpus</i>	BP03	Moraceae
4	<i>Artocarpus dadah</i> Miq.	BP05	Moraceae
5	<i>Scorodocarpus borneensis</i>	BP06	Olacaceae
6	<i>Eusideroxylon zwageri</i>	BP07	Lauraceae
7	<i>Fagraea racemosa</i>	BP08	Loganiaceae
8	<i>Pternandra galeata</i>	BP09	Melastomataceae
9	<i>Fordia splendissima</i>	BP11	Fabaceae
10	<i>Aglaiia lawii</i>	BP13	Meliaceae
11	<i>Gonocaryum littorale</i>	BP15	Icacinaceae
12	<i>Orophea hexandra</i>	BP16	Lauraceae
13	<i>Gymnacranthera farguhariana</i>	BP18	Lauraceae
14	<i>Alseodaphne elmeri</i>	BP19	Lauraceae
15	<i>Neolitsea cassiaefolia</i>	BP20	Lauraceae
16	<i>Vernonia arborea</i>	BP21	Asteraceae
17	<i>Ficus geocariss</i>	BP22	Moraceae
Dichloromethane extract of stem bark (Balikpapan Sample)			
18	<i>Melicope glabra</i> (Syn <i>M. pinnata</i> )	SB01	Rutaceae
19	<i>Luvunga scandens</i> (Roxb.) Wight	SB02	Rutaceae
20	<i>Artocarpus sericarpus</i>	SB03	Moraceae
21	<i>Artocarpus anisophyllus</i>	SB04	Moraceae
22	<i>Scorodocarpus borneensis</i>	SB05	Olacaceae
23	<i>Alstonia angustiloba</i>	SB07	Apocynaceae
24	<i>Alseodaphne elmeri</i>	SB08	Lauraceae
25	<i>Ficus geocariss</i>	SB09	Moraceae
Ethanol extract (East Java Sample)			
26	<i>Ruta angustifolia</i> (leaves)	W1	Rutaceae
27	<i>Phyllanthus niruri</i> (herbs)	W2	Euphorbiaceae
28	<i>Curcuma domestica</i> (Rhizome)	W3	Zingiberaceae
29	<i>Curcuma xanthoriza</i> (Rhizome)	W4	Zingiberaceae
30	<i>Curcuma heyneana</i> (Rhizome)	W5	Zingiberaceae
31	<i>Zingiber officinale</i> (Rhizome)	W6	Zingiberaceae

were compared to the control and facilitation calculation of the 50% cytotoxic concentration (CC<sub>50</sub>) values. In this study, the cytotoxicity assay was performed both in HepG2-NTCP cells and Hep38.7-tet.

## RESULTS

### Anti-HBV screening of entry stage inhibition and cytotoxicity assay of selected active extracts

Among 31 plant extracts evaluated for anti-HBV activity on the viral entry step, two extracts exhibited strong reduction of HBs production in HepG2-NTCP cells (Figure 1). The extract of *P. niruri* at a concentration of 100 µg/mL showed 48.2% inhibition, while the extract of *C. xanthorrhiza* exhibited 29.0 % inhibition. *P. niruri* had the strongest antiviral activity with IC<sub>50</sub> values of 170.48 µg/mL and CC<sub>50</sub> values of >400 µg/mL. In contrast, *C. xanthorrhiza* extracts showed an IC<sub>50</sub> value of 270.51 µg/mL and CC<sub>50</sub> of >206.69 µg/mL (Figure 2).

### Antiviral HBV screening of post-entry stage inhibition and cytotoxicity assay

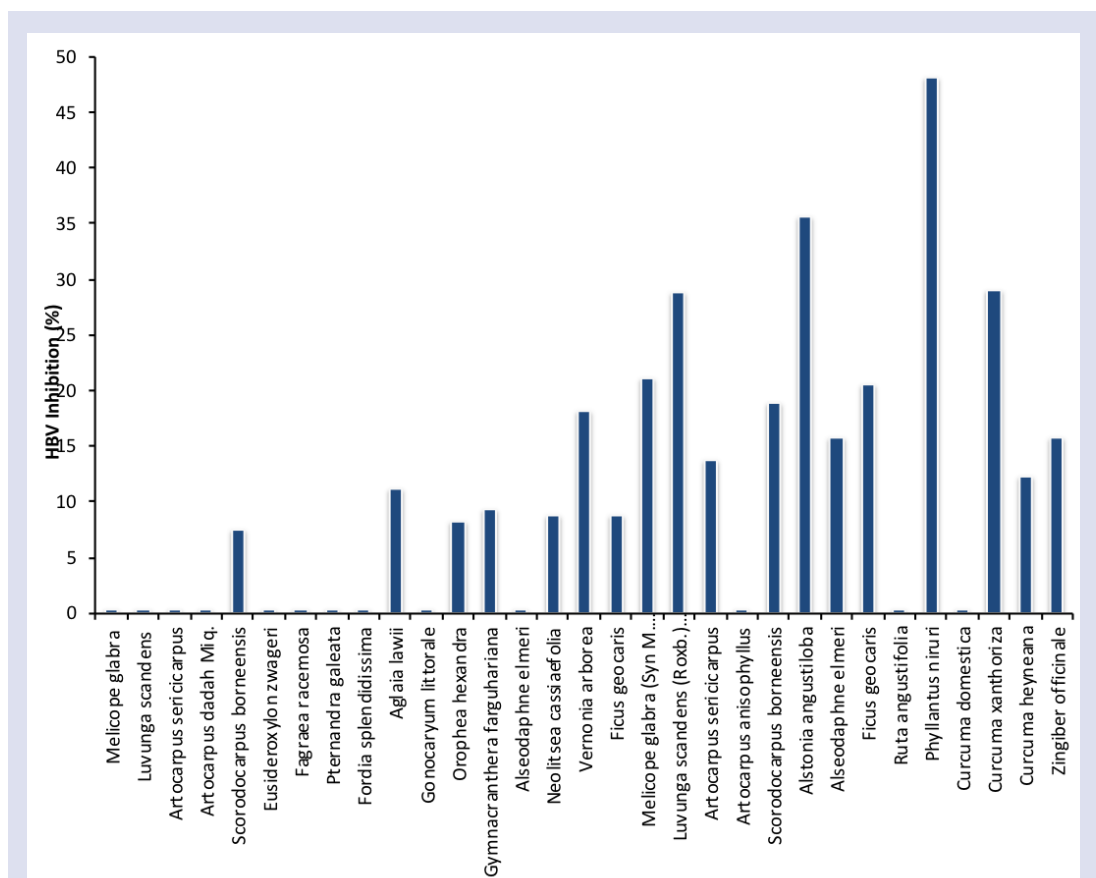
To evaluate the active extract in the post-entry step, further analysis was conducted via Hep38.7-Tet cells culture. As can be seen in Figure 3, the post-entry inhibition assay reflected the ability of *P. niruri* to dramatically reduce extracellular HBV DNA levels. Various concentrations were tested and this showed a dose dependent antiviral effect in the post-entry step without any toxicity developing at a dose of 100 µg/mL. This result indicated that *P. niruri* may inhibit HBV replication, assembly, and virion release. The cytotoxicity of *P. niruri* extract in Hep38.7-tet showed did not suppress the viability of cells

with a CC<sub>50</sub> value exceeding 274.51 µg/mL. In contrast, the extract of *C. xanthorrhiza* inhibited post-entry stage infection with a 30% reduction of HBV DNA levels at a concentration of 50 µg/mL. Furthermore, *C. xanthorrhiza* exhibits greater toxicity than *P. niruri* extract (Figure 3)

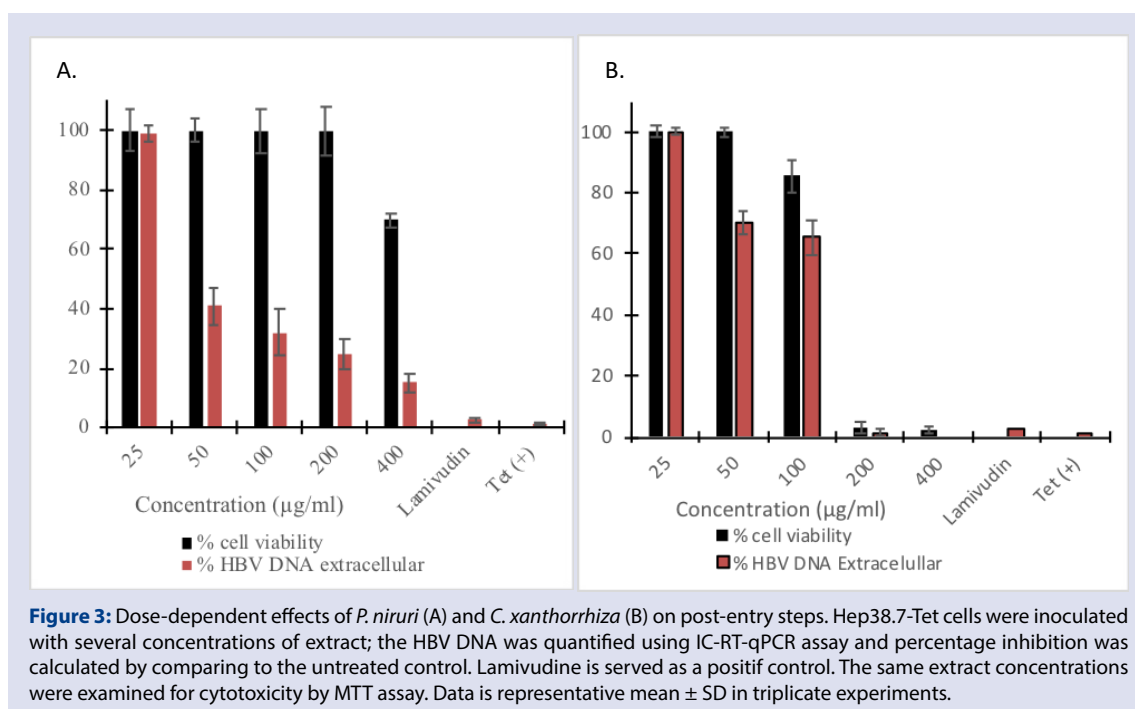
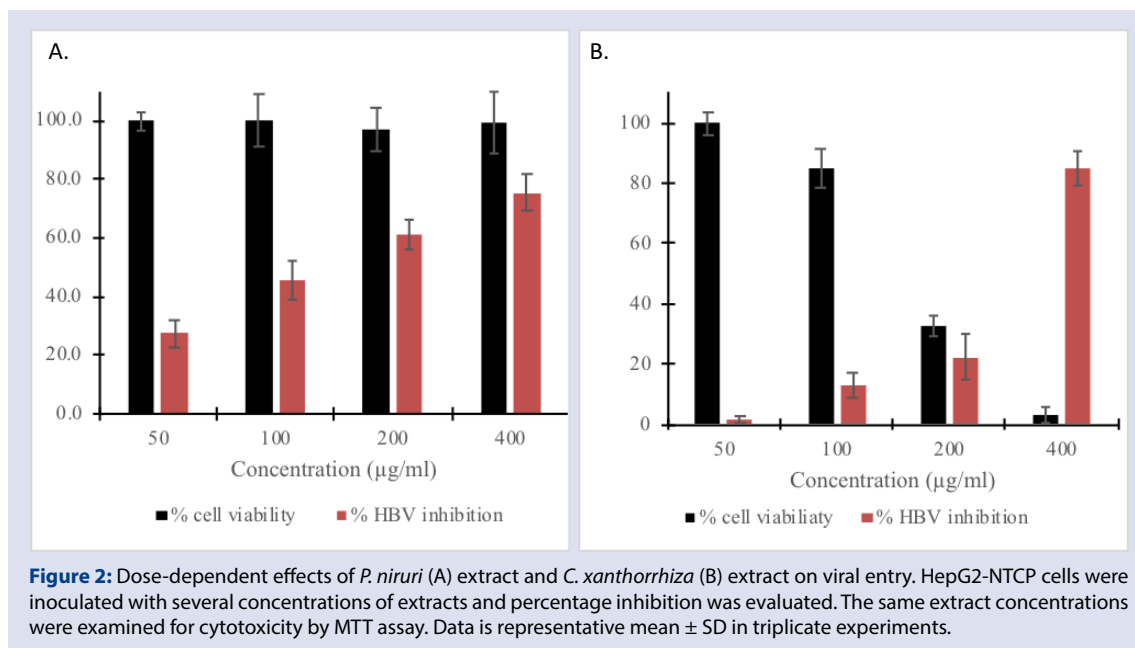
## DISCUSSION

Medicinal plants are promising candidates for drug discovery due to their interesting chemical metabolites. It has been previously reported that various compounds isolated from these plants display promising bioactivity.<sup>16</sup> A number of medicinal plants from various countries have been tested for anti-HBV to find potential candidates for anti-HBV drug development. The goal of HBV therapy is to prevent the progression of chronic hepatitis B to cirrhosis, end-stage liver disease, hepatocellular carcinoma and death.<sup>17</sup> It has also been postulated that some medicinal plants used in Traditional Chinese Medicine exhibit anti-HBV activity.<sup>13</sup> Several plants native to the Arabian Peninsula: *Guiera senegalensis*, *Pulicia crispa*, *Coccinea grandis*, *Fumaria parviflora*, *Capparis decidua*, *Corallocarpus epigeus*, *Indigofera caerulea*, *Abutilon figarianum* and *Acacia oerfota* have also been found to possess anti-HBV activity by inhibiting hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in the cell culture supernatant.<sup>18</sup> Our previous study also reported that *Cananga odorata* exhibited strong inhibition of the hepatitis B virus in Hep38.7-Tet and HepG2-NTCP cells.<sup>14</sup>

In this study we evaluated a number of medicinal plants for anti-HBV which have been reported to show anti-viral activity. The plants were selected based on empirical used and preclinical empirical studies. We examined the abilities of 31 Indonesian medicinal plants to inhibit the entry step of HBV into HepG2-NTCP and the levels of extracellular



**Figure 1:** Anti-HBV activity of Indonesian medicinal plant extracts-tested. HepG2-NTCP cell was treated with plant extracts at a concentration of 100 µg/mL. HBsAg level was examined by ELISA kit and the HBV inhibition was calculated by comparing to the untreated cells.



HBV DNA on HepAD38.2 cells. In the HBV life cycle, after infecting hepatocyte cells, the virus will undergo several steps, including, attachment, endocytosis, and release of capsid. Following this, the relaxed circular HBV DNA genome is converted into covalently closed circular DNA (cccDNA). The replication process includes transcription, translation, encapsidation, reverse transcription, DNA synthesis, budding the virion and release. NTCP is a receptor for HBV entry, which is overexpressed in the HepG2-NTCP cell line used to evaluate anti-HBV activity in the entry step<sup>15</sup>. The other cell line, Hep38.7-Tet, was a tetracycline inducible hepatitis B virus expressing cell line. Replication of the virus can be induced in this cell line and, therefore, it is suitable for detecting inhibition in the post-entry step as well as replication of virus.<sup>19</sup> Our research demonstrated that the extracts of *P. niruri* and *C. xanthorrhiza* exhibited anti-HBV activities of a greater magnitude than the other plant extracts tested. *P. niruri* inhibited viral entry with an IC<sub>50</sub>

value of 170.5 µg/ml and CC<sub>50</sub> value of >400 µg/mL and inhibited post-entry steps (extracellular HBV DNA was reduced reduced) without exhibiting cytotoxicity. In contrast, *C. xanthorrhiza* showed a toxic effect in HepG2-NTCP and Hep38.7-Tet cells at a concentration of 200 µg/ml and did not inhibit HBV replication in Hep38.7-Tet cells.

*P. niruri* is a plant that is found in many areas across tropical and subtropical regions. The genus of Phyllanthus (Euphorbiaceae) has been used for various traditional medicines in many countries, including in Indonesia. It has been reported to exhibit varied and interesting biological activities, including stomachic, diuretic, febrifuge, deobstruent, and antiseptic activity and has used in effective remedies for hepatopathy.<sup>20</sup> The various species of the Phyllanthus family, such as the *P. amarus*, *P. niruri*, *P. urinaria*, and *P. orbicularis*, have been reported to inhibit a broad spectrum of viruses. Previous work showed that these plants may



inhibit human immunodeficiency virus (HIV) and herpes simplex virus (HSV).<sup>21,22</sup> It has been reported that extracts from *Phyllanthus* species have a positive effect on the antiviral activity and serology response of HBsAg in HBV carriers.<sup>23,24</sup> It was also demonstrated that the extract of *Phyllanthus amarus* suppresses hepatitis B virus by interrupting the interaction between HBV enhancer I and cellular transcription factors, resulting in an inhibition of polymerase activity and mRNA transcription.<sup>25,26</sup> Our findings demonstrate a different mechanism of anti-HBV activity for *P. niruri*.

*P. niruri* contains numerous compounds, including alkaloids, flavonoids, lignans, phenols, and terpenes, which display equally varied biological activities including antiviral activity. Phyllanthin and hypophyllanthin are the lignin compounds that were found to be major compounds of *P. niruri*.<sup>27</sup> Phyllanthin was predicted to interact with the entry receptors of the hepatitis virus and exhibit hepatoprotective activities. Niranthin and hinokinin isolated from *P. amarus* strongly inhibit hepatitis B activity while Corilagin was reported to block HCV NS3 protease and NS5B RNA-dependent-RNA-polymerase.<sup>28,29</sup>

In contrast to *P. niruri*, *C. xanthorrhiza* was widely used in many traditional medicines as an anticancer, anti-microbial, anti-inflammatory, anti-oxidant, anti-hyperglycemic, anti-hypertensive, anti-platelet and nephroprotective.<sup>30</sup> The ethanol extracts of *C. xanthorrhiza* have been demonstrated to reduce fatty liver symptom and inhibit alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and protein content.<sup>31</sup> The isolated compound from the fresh rhizome, xanthorrhizol, expresses antimicrobial activity against pathogenic bacteria and fungi.<sup>31-33</sup> Extracts of *C. xanthorrhiza* have previously been demonstrated to exhibit anti-hepatitis C virus activity.<sup>34</sup> Our study showed *C. xanthorrhiza* has marginal inhibitory effect on HBV entry step.

## CONCLUSION

Among 31 plant extracts, *P. niruri* exhibited the greatest anti-HBV activity on both the viral entry step and viral replication. This plant is promising candidate for anti-HBV drug development.

## CONFLICTS OF INTEREST

None.

## ACKNOWLEDGEMENTS

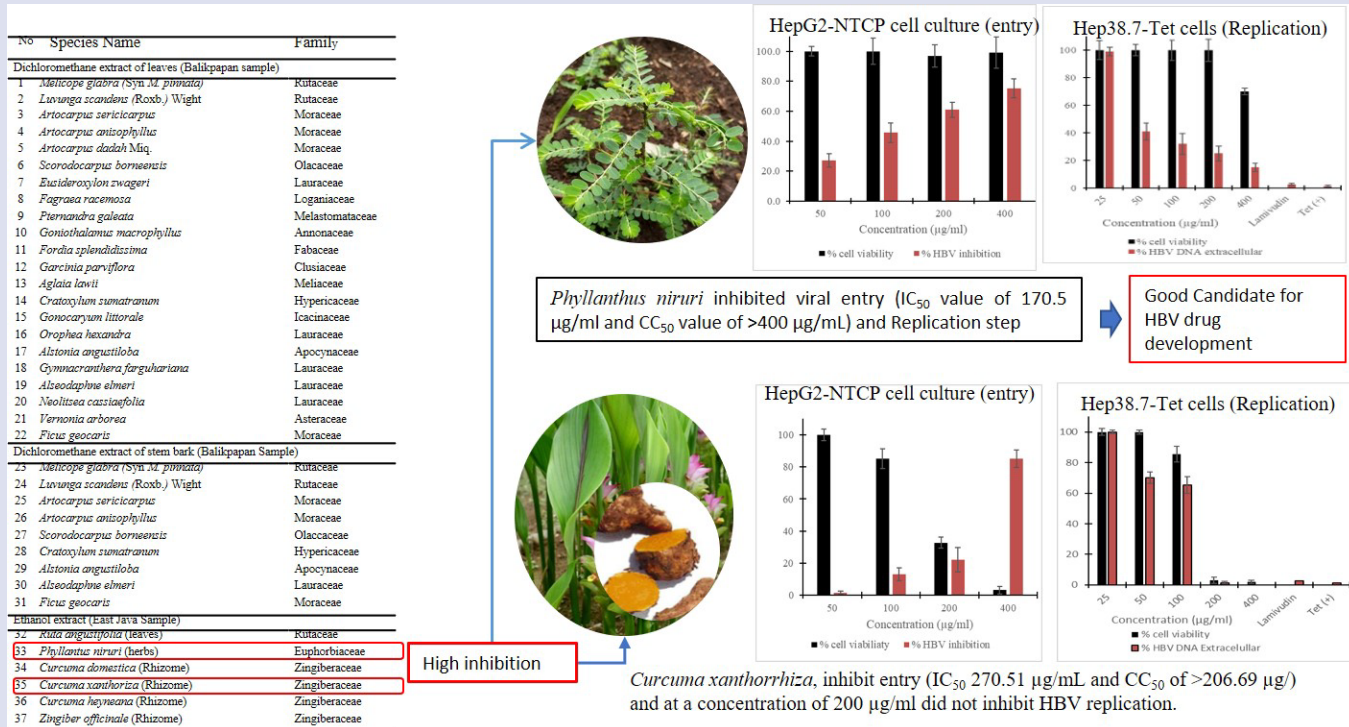
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## GRAPHICAL ABSTRACT



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