THE INHIBITORY ACTIONS OF HOUTTUYNIA CORDATA AQUEOUS EXTRACT ON DENGUE VIRUS AND DENGUE-INFECTED CELLS

VIJITTRA LEARDKAMOLKARN¹,⁶, WIPAWAN SIRIGULPANIT¹, CHAYAKOM PHURIMSAK², SUPEECHA KUMKATE³, LUKANA HIMAKOUN⁴ and BUNGORN SRIPANIDKULCHAI⁵

¹Department of Anatomy, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
²Toxicology Graduate Program, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
³Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
⁴Department Pathobiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand
⁵Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand

⁶Corresponding author. TEL: +66-2201-5405; FAX: +66-2354-7168; EMAIL: scvlk@mahidol.ac.th

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ABSTRACT

The antiviral activities of Houttuynia cordata (H. cordata) aqueous extract against dengue virus serotype 2 (DEN-2), strain 16681, were evaluated in this study. The results showed that pre- and post-incubation of H. cordata extract (10–100 mg/mL) with HepG2 cells significantly reduced intracellular DEN-2 RNA production correlating with the decrease in dengue protein expression. In the direct blocking mode, the extract bound with DEN-2 and strongly inhibited the intracellular viral RNA replication with an effective dose (EC₅₀) of 0.8 mg/mL. Concentrations as low as 10–40 mg/mL of H. cordata extract also exhibited protective effect on virion release from infected LLC-MK2 cells. High-performance liquid chromatography analysis of H. cordata extract indicated that hyperoside was the predominant bioactive compound, and was likely to play a role in this inhibition. The extract was also shown to have no genotoxic effect on human blood cells.

PRACTICAL APPLICATIONS

Houttuynia cordata is recognized as medicinal vegetable consumed by people in East and Southeast Asia. It has multilateral activities in health promotion and regulation of inflammatory reactions induced by several pathogens. Dengue viral infection is a global health problem since licensed vaccines or specific antiviral drug treatments are not yet available. The current study provides scientific data to support the phytomedicinal properties of H. cordata aqueous extract on anti-dengue virus in three modes of action (prevention, treatment and virucidal). A major constituent in the extract, hyperoside, seems to be the bioactive compound effectively acting against dengue infection. H. cordata aqueous extract was shown to be nontoxic and hyperoside may be considered a lead compound possessing drug potential for further development.

INTRODUCTION

Houttuynia cordata Thunb (Saururaceae; H. cordata) commonly known as “Pak Kan Thong” in Thai, is a popular side-dish vegetable, which is consumed in the Northern and Eastern regions of Thailand. Its medicinal properties in the treatment of chronic sinusitis and allergy are accepted in several Asian countries including Korea, Taiwan and China (Lu et al. 2006a; Ng et al. 2007; Han et al. 2009). Recent investigations have documented H. cordata for treatment of a number of physiological and immunological disorders, such as anti-allergy (Li et al. 2005; Han et al. 2009), antioxidant (Chen et al. 2003; Park et al. 2005; Kusirisin et al. 2009) and anti-leukemia (Chang et al. 2001). The anti-inflammatory
property of *H. cordata* was also demonstrated in rat-induced severe acute respiratory syndrome (SARS; Lu *et al*. 2006). Previously, *H. cordata* was shown to have inhibitory effect on viral replication in human immunodeficiency virus (HIV-1) and influenza virus, after *in vitro* incubation with *H. cordata* water extract (Hayashi *et al*. 1995). A similar preparation of *H. cordata* extract also directly inactivated herpes simplex virus (HSV-1 and HSV-2; Chiang *et al*. 2003). However, what compound in the extract plays a role in its inhibitory action is unknown, since the overall composition of the compound fractions has never been revealed. It has been reported that the foliage part of *H. cordata* is rich in essential oil (81.4% v/v) containing lipid compounds (Tutupalli and Chaubal 1975). The most abundant components in the essential oil are methyl nonyl ketone (21–40.36%), bornyl acetate (0.4–8.1%), and beta-myrcene (2.58–18.47%) oils. These oils possess antibacterial activity and anti-inflammatory action (Lu *et al*. 2006b). Moreover, flavonoid compounds such as quercetin, hyperin and quercetin-3-O-alpha-L-rhamnopyranosyl-7-O-beta-D-glucopyranoside were also isolated and purified from fresh *H. cordata* (Meng *et al*. 2006).

Dengue virus (DEN) is a positive single stranded-RNA virus that causes dengue fever, dengue hemorrhagic fever and dengue shock syndrome. The virus is transmitted to human by mosquito vectors (*Aedes aegypti* and *Aedes albopictus*), which are distributed globally. Immunologically, the virus is divided into four serotypes: dengue virus serotype 1 (DEN-1), dengue virus serotype 2 (DEN-2), dengue virus serotype 3 (DEN-3) and dengue virus serotype 4 (DEN-4). Infection by one serotype of dengue virus induces protective antibody against that particular serotype, but not for the other serotypes. Secondary infection with another serotype may cause more severity in patients whose neutralizing antibody from the primary infection enhances the second infection (Halstead 2007). At present, a protective vaccine or an effective drug is still not available against dengue viruses. Therefore, the search for and development of anti-dengue agents are urgently in demanded.

In the present study, we assessed the antiviral activities of *H. cordata* aqueous extract against DEN-2 virus in human hepatocarcinoma cell line (HepG2) and monkey kidney cell line (LLC-MK2) in three strategic ways including protective, treatment and direct blocking modes.

**MATERIALS AND METHODS**

**Preparation of *H. cordata* Extract and HPLC Analysis**

The aerial stems and leaves of *H. cordata*, cultivated in the Northern part of Thailand, were collected and air dried. Then, they were smashed into fine powder-like particles by passing through a 60-mesh sieve. The powder *H. cordata* was prepared following the previous protocol (Ling-Shang *et al*. 2009) with minor modifications. The sample solution was filtered with a 0.22 μm membrane before it was analyzed with high-performance liquid chromatography (HPLC). The bioactive constituents in the extract were analyzed using chlorogenic acid, hyperoside, quercetin and quercitrin as standard compounds. The standard flavonoids, chlorogenic acid (1.27 mg/mL) and hyperoside (0.102 mg/mL) were prepared in 80% methanol, while *H. cordata* aqueous extract was prepared at 5.92 mg/mL concentration. Separation was performed on an Agilent-1200 HPLC system, using an Agilent hypersil ODS column (Agilent Technologies, Santa Clara, CA) (5 μm, 4.6 × 250 mm), eluted with a mobile phase gradient containing absolute methanol (5% ~ 70%) and 0.1% phosphoric acid (95% ~ 30%) within 45 min. The injection volume was 10 μL. The wavelength monitored was 360 nm. The column temperature was 30°C.

For antiviral assay, dried powder of the *H. cordata* aqueous extract was dissolved in serum-free Dulbecco’s Modified Eagle’s Medium (DMEM) and prepared as a stock solution at 100 mg/mL. The extract was filtered through a 0.22 μm membrane before using.

**Cytotoxicity (MTT) Assay**

Cells were seeded in 96-well plates (1 × 10⁴ cells per well) and incubated at 37°C in a 5% CO₂ incubator for 24 h (HepG2 cells) or 48 h (LLC-MK2 cells). The culture medium was removed and the cells were treated with 100 μL of DMEM containing *H. cordata* aqueous extract at various concentrations. After certain exposure periods, the cells were washed with phosphate buffer saline and the 3-(4,5-dimethylthizaol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed according to the manufacturer’s instruction (Roche Applied Science, Mannheim, Germany). The color reaction was measured by automatic VersaMax™ microplate reader (Sunnyvale, CA) at the test wavelength of 570 nm and the reference wavelength of 620 nm. Percent cell viability was compared between the treated and the untreated control group.

**Antiviral Assay**

The inhibitory action of *H. cordata* aqueous extract was performed in three different modes: (a) protective (b) treatment and (c) direct blocking in both HepG2 and LLC-MK2 cells. For (a) and (b) activities, HepG2 cells (1 × 10⁴ cells per well) were cultured for 24 h at 37°C in CO₂ incubator. After pre- or post-incubation with 10 and 100 μg/mL of *H. cordata* extract for 3 h, the cells were inoculated with DEN2 at a multiplicity of infection of approximately 0.01 plaque forming unit (p.f.u.) per cell for 1.5 h. For (c) activity, DEN-2 (5 × 10⁴ p.f.u./mL) was premixed with 10 and 100 μg/mL of
H. cordata extract and incubated at 37°C for 1 h. The mixture was then added into HepG2 cell culture and incubated at 37°C for 1.5 h. The inoculum was removed and the cells were further cultured for 3 days.

To assess the anti-DEN activities in HepG2 cells, intracellular RNA synthesis and protein production were investigated by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and flow cytometry analysis, respectively. For RT-PCR, total RNA was extracted from DEN-2 infected HepG2 cells. Real-time RT-PCR was performed using iScript™ One-Step RT-PCR Kit with SYBR® Green (Bio-Rad Laboratories, CA) and DEN2 specific primers (forward primer 5′ AAGGTGAGATGAAAGCTG TAGTCTC 3′ and reverse primer 5′ CATTCCATT'TCTG GCGTTCT 3′). The cycling condition was as recommended by manufacturer. The numbers of intracellular viral RNA copies were calculated using the reference standard curve established from known amounts (ranging from 9 × 10^2 to 9 × 10^6 copies) of in vitro RNA transcripts which were transcribed from pD2/IC-30P-A (Kinney et al. 1997). For flow cytometry, DEN-2 infected-HepG2 cells were examined for DEN-2 protein expression after (a) and (b) modes assays (above). The cells were stained with mouse monoclonal antibody against DEN envelope (E) protein (clone DE1; Abcam, Cambridge, U.K.) and goat-anti mouse immunoglobulin conjugated with fluorescein isothiocyanate (Jackson Immunology, PA). The level of viral E expression in single cell was analyzed by FACScanto equipped with FACSDiva software (BD Biosciences, San Jose, CA). Similar experimental procedures were used for antiviral activities in LLC-MK2 cells as performed in HepG2 cells. The culture supernatant from (a) and (b) experimental modes were collected and the virus released from cells was determined by the standard plaque titration assay (Miller and Mitchell 1986; Huang et al. 2000). For (c) activity, the plaque assay was performed directly after incubation between the virus and the extract. The percent reduction in plaque formation was compared between treated and untreated control.

**Chromosome Aberration Assay**

Following the protocol guided by Mateuca et al. (2006), human peripheral blood lymphocytes in culture were treated with different concentrations (1–10 mg/mL) of H. cordata extract, 0.2% of dimethyl sulfoxide (negative control) or 1.5 μg/mL of mitomycin C (positive control) for 70 h in cultures. The cultures were added with 50 μL of colchicine (25 μg/mL) for 24 h to arrest mitotic cell division and examined for aberration of metaphase chromosome.

**Statistics**

Data was compared as arithmetic means ± SE using Student’s t-test with a 95% confidence interval (⁎ P < 0.05). Unless otherwise stated, data shown were representative of two to four experimental repeats.

**RESULTS AND DISCUSSION**

H. cordata aqueous extract showed inhibitory activity to DEN2 RNA production in HepG2 cells in all experimental modes of action, although the greatest inhibitory effect was in the protective mode. The concentration of 100 μg/mL was effective to protect the cell from DEN virus infection (protective mode, Fig. 1A), as well as to decrease the intracellular viral RNA synthesis (treatment mode, Fig. 1B) and to inactivate the virus (direct blocking mode, Fig. 1C). The decrease in DEN-2 E proteins expression in HepG2 cells (Table 1) correlated well with the copy number of viral RNA products. In LLC-MK2 cells, H. cordata extract showed a concentration dependent effect on inhibition of the virus. Pre- and post-incubation of the cells with the extract (10–40 μg/mL) for 1 h (Fig. 2A,C) and 3 h (Fig. 2B,D) significantly decreased DEN2 virus release into the culture medium. Although low concentrations (10–40 μg/mL) of H. cordata extract were tested here, the effect tended to increase with the higher doses. The most potent effect of H. cordata aqueous extract in LLC-MK2 cells was shown in the direct blocking mode, where incubation of DEN-2 virus with 0.2–1 μg/mL of the extract significantly reduced virus released from the cells. The 50% effective concentration (EC50) that caused 50% reduction of plaque formation by the virus in this mode was 0.8 μg/mL (data not shown). Moreover, the extract exhibited a very low to non-toxic effect on both cells (50% cytotoxic concentration (CC50) = 1.24 mg/mL and 1.99 mg/mL in LLC-MK2 cells and HepG2 cells, respectively) and also caused no chromosomal damage to lymphocytes (data not shown). Thus, compounds in the extract were considered safe for consumers.

In order to identify the bioactive contents in H. cordata aqueous extract, four flavonoid-based compounds including quercetin, chlorogenic acid, quercitrin and hyperoside were quantified by HPLC. Of these compounds, the amount of hyperoside was higher than other related compounds (Fig. 3A), indicating that the active compound from H. cordata extract showed a concentration dependent effect on inhibition of the virus. Pre- and post-incubation of the cells with the extract (10–40 μg/mL) for 1 h (Fig. 2A,C) and 3 h (Fig. 2B,D) significantly decreased DEN2 virus release into the culture medium. Although low concentrations (10–40 μg/mL) of H. cordata extract were tested here, the effect tended to increase with the higher doses. The most potent effect of H. cordata aqueous extract in LLC-MK2 cells was shown in the direct blocking mode, where incubation of DEN-2 virus with 0.2–1 μg/mL of the extract significantly reduced virus released from the cells. The 50% effective concentration (EC50) that caused 50% reduction of plaque formation by the virus in this mode was 0.8 μg/mL (data not shown). Moreover, the extract exhibited a very low to non-toxic effect on both cells (50% cytotoxic concentration (CC50) = 1.24 mg/mL and 1.99 mg/mL in LLC-MK2 cells and HepG2 cells, respectively) and also caused no chromosomal damage to lymphocytes (data not shown). Thus, compounds in the extract were considered safe for consumers.

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Taken together, the overall results implied that H. cordata aqueous extract affected dengue virus as was previously shown for HSV-1, HIV-1 and influenza virus (Hayashi et al. 1995; Chiang et al. 2003). Hyperoside or quercetin-3-O-galactoside was a predominant content flavonoid subclass (Ling-shang et al. 2009) from H. cordata extract that might have played this significant role. Presumably, the mode of inhibitory action of H. cordata aqueous extract was by direct interaction and deactivation of dengue viral particles. This is
similar to the mode of action of the bactericidal effect of flavonoids previously reported against *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains, since hyperoside is one of the four glycoflavonoids (rutin, nicotiflorin, hyperoside and isorhamnetin) isolated from *Tripodanthus acutifolius* leaves (Soberon et al. 2010). Considering the phenolic structure of hyperoside, its antiviral activity might be attributed to both hydroxyl groups and glycosidic side group. The hydroxyl groups of the compound might form polymers and the glycoside could complex with several molecules on the viral cell surface in a similar way to fucoidan, a sulfated polysaccharide from marine alga *Cladosiphon okamuranus*, which could interact directly with the envelope glycoprotein on DEN-2 and inhibit the virus potently (Hidari et al. 2008). Such an interaction complex could subsequently induce conformational changes or destroy viral epitopes, leading to virus inactivation, weakening virus attachment and abolishing virus infectivity (Hung et al. 1999). Meanwhile, the galactoside group might specifically bind with the galactose receptor known to be present on the HepG2 cell surface. This could prevent the virus entry into cells. The findings in this study strengthen the proposed mechanism of antiviral action, since our compound has structural relationship to geraniin, a compound from *Geranium carolinianum*, which contains multiple hydroxyl groups and galactose group and has higher anti-hepatitis B virus activity than the other compounds in *G. carolinianum* extract (Li et al. 2008). Previous biochemical studies have also suggested that certain agents block activity of dengue virus on the cell surface, such as heparin (Lin et al. 2002), and DL-galactan hybrid C2S-3 (Talarico et al. 2005). In addition, other phenolic constituents in *H. cordata* extract present at lower concentration may contribute to this inhibition mechanism. In the treatment mode, hyperoside in the extract probably inhibited intracellular RNA synthesis by interacting with enzymes or proteins in the viral replication complex by a comparable mechanism to quercetin-3-β-galactoside that could interact with SARS-CoV-3-like pro-
FIG. 2. ANTIVIRAL ACTIVITY OF H. CORDATA EXTRACT AGAINST DEN-2 RELEASE IN LLC-MK2 INFECTED CELLS AT DIFFERENT MODES OF ACTION. THE CELLS WERE INCUBATED WITH THE EXTRACT (0–40 μg/mL) FOR PROTECTIVE AT 1 H (A) AND 3 H (B) AND FOR TREATMENT AT 1 H (C) AND 3 H (D). DATA WERE SHOWN AS MEANS ± SE. VALUES WERE REPRESENTATIVE OF TWO DIFFERENT EXPERIMENTS (* P < 0.05)

FIG. 3. HPLC CHROMATOGRAM SHOWING BIOCHEMICAL COMPOSITIONS OF H. CORDATA AQUEOUS EXTRACT (A) AND THE CHEMICAL STRUCTURE OF HYPEROSIDE (B)
tease (3CL^pro) to inhibit the SAR viral replication. The 3CL^pro plays an important role in cleavage of polyprotein precursor from the replicase complex which is essential in SAR viral replication (Chen et al. 2006). In the case of dengue virus, after the binding interaction of the compound with the non-structural proteins (NS3 and NS5 enzymes) involved in RNA replication (Chambers et al. 1990), viral replication would be inhibited. So both proteins would be most interesting drug targets for the development of therapeutics against dengue infection. Detailed study of the inhibitory action of pure hyperoside compound at the molecular structural level would be interesting for chemists and biophysicists, since this may provide interesting information on structure-activity relationships. Further development and phytomedicinal use of this extract would be worthy of future study.

In conclusion, the effective inhibitory action of H. cordata aqueous extract to DEN2 was due to direct inactivation of viral particles before infection of the cells. The extract also protects the cells from viral entry and inhibits virus activities after adsorption. The selective index in inhibiting dengue virus, as determined from the ratio between CC50 and EC50, was very high (= 1,550.36), suggesting potential for antiviral drug development.

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