Antiviral Activity of *Isatis indigotica* Extract and Its Derived Indirubin against Japanese Encephalitis Virus

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1. Introduction

*Isatis indigotica* is an herb distributed widely in China and traditionally used in clinical treatment of viral diseases like influenza, hepatitis, and encephalitis [1, 2]. Accumulated experimental evidence indicates it and related components as associated with antiviral activity against influenza A, SARS-coronavirus, foot-and-mouth disease, rabies, and human immunodeficiency virus type 1 (HIV-1), among others [1, 3–9]. Among natural compounds identified from *I. indigotica*—for example, indican, isatin, indirubin, and indigotin [9]—indirubin exhibits multiple immunomodulatory and antiviral effects [5, 8].

Japanese encephalitis virus (JEV) belongs to genus *Flavivirus* of the Flaviviridae family, an arthropod-borne microorganism [10]. Vaccines against it are currently available and effective, yet viruses’ zoonotic characteristic and occasional infections cause JEV to rank as a leading cause of high morbidity and mortality rate in Southeast Asia and the Western Pacific region [11]. 30–50% of JE patients develop permanent neuropsychiatric sequelae, while 20–30% of JE cases result in death [12]. Extensive study to develop new therapeutic strategy may be needed. This study rated inhibitory effect of ethyl acetate, methanol, and water extracts of *I. indigotica*, along with its related natural compounds, on JEV replication. We proved that pretreatment of *I. indigotica* extracts, indigo, and indirubin greatly inhibit JEV replication in vitro. These agents blocked JEV attachment, which correlated with a potent virucidal activity.

2. Materials and Methods

2.1. Viruses and Cells. JEV strain T1P1 was used as previously described [13], vero cells for JEV amplification maintained in Dulbecco’s modified Eagle’s medium (DMEM), as well as BHK-21 cells used to determine JEV plaques grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Human promonocytic HL-CZ cells cultured in RPMI-1640 medium served to determine JEV yield in vitro.

2.2. *I. indigotica* Extracts and Related Marker Compounds. Crude extract powder of *I. indigotica* was obtained from Sun Ten Pharmaceutical Co., Ltd., a Taiwanese manufacturer.
of concentrated herbal extracts. For each extract tested, 1 g of powder was dissolved in 40 mL ethyl acetate or methanol, then gently shaken overnight at room temperature. Extract solutions were collected following centrifugation at 12,000 rpm for 20 min, filtered using a Whatman number 1 filter paper, then lyophilized using in a freeze dryer (IWAKI FDR-50P). Each lyophilized extract powder was kept in sterile bottles at −20 °C. Stock extract solutions (1 mg/mL) were dissolved in phosphate-buffered saline (PBS), sterilized using a 0.44 µm syringe filter and stored at −80 °C until used. Marker compounds of *I. indigotica* like adenosine, betulin, indigo, indirubin, tryptanthrin, lupeol, and 2-benzoxazolinone were purchased from Sigma Chemical Co. (St. Louis, MO.). Stock solution of marker compounds (20 mg/mL) was dissolved in dimethyl sulfoxide (DMSO), diluted with PBS. DMSO (0.005%, 0.05%, 0.5%, and 5%) was tested as a solvent control.

2.3. Cell Viability Assay. To calculate cytotoxicity to BHK-21 cells and human promonocytic cells, cells were cultured overnight on 96-well plates. Medium containing DMSO (0.005, 0.05, 0.5, or 5%), *I. indigotica* extracts or marker compounds (0 µg/mL, 0.1 µg/mL, 1 µg/mL, 10 µg/mL, and 100 µg/mL) were added and incubated for another 48 hours. Living cells and total HL-CZ cell count with(out) treatment were measured by staining with 0.4% trypan blue; viability was estimated as ratio of living/total cell counts. Quadruplicate wells were analyzed for each concentration. Cytotoxic concentration showing 50% toxic effect (CC50) was derived by computer program (provided by John Spouge, National Center for Biotechnology Information, National Institutes of Health).

2.4. Quantitative Assay of Virus Yields Using Plaque Assay. To test inhibitory effect of *I. indigotica* on JEV yields in human promonocytic cells, HL-CZ cells infected with JEV at multiplicity of infection of 0.5 and treated with DMSO (0.005, 0.05, or 0.5%), *I. indigotica* extract (1, 10, and 100 µg/mL) or marker compound (0.1, 1, and 10 µg/mL) at the same time. At 24 and 48 h after inoculation, cultured supernatant from (un)treated JEV-infected cells was collected for measuring virus yields by plaque assay. A 10-fold serial dilution of cultured medium was added into the well of BHK-21 cell monolayer at 37 °C for 1 h and overlaid with MEM medium containing 1.1% methylcellulose. Viral plaques were stained with naphthol blue-black dye after three-day incubation.

2.5. Plaque Reduction and Time-of-Addition Assay. To gauge inhibitory effect of *I. indigotica* by time of addition on JEV replication in vitro, pretreatment (prior to infection), simultaneous treatment (at the same time as infection), and posttreatment (after entry) experiments were performed. For the pretreatment experiment, BHK-21 cell monolayer was pretreated with/without various DMSO concentrations (0, 0.005, 0.05, or 0.5%), *I. indigotica* extract (0, 1, 10, and 100 µg/mL) or marker compound (0, 0.1, 1, 10 µg/mL) 1 h before infection. BHK-21 cell monolayer was overlaid with MEM medium containing 1.1% methylcellulose 1 h after infection, viral plaques stained with naphthol blue-black dye after three-day incubation. For simultaneous treatment, medium with/without various DMSO concentrations (0, 0.005, 0.05, or 0.5%), *I. indigotica* extract (0, 1, 10, and 100 µg/mL) or marker compound (0, 0.1, 1, 10 µg/mL) was mixed along with JEV at 100 pfu, then forthwith added into the well of BHK-21 cell monolayer at 37 °C for 1 h and overlaid with MEM medium containing 1.1% methylcellulose for viral plaque assays. In posttreatment assay, BHK-21 cell monolayer was infected with JEV at 100 pfu for 1 h, followed by 1 h incubation with drug solutions and overlaid with MEM medium containing 1.1% methylcellulose, as described in plaque assay. Data represent mean ± SD of three independent experiments. Inhibitory concentration showing 50% JEV plaque reduction (IC50) was determined by computer program (John Spouge, National Center for Biotechnology Information, National Institutes of Health).

2.6. Virus Attachment Assay. JEV (120 pfu) was mixed with medium containing various concentrations of DMSO (0, 0.005, 0.05, or 0.5%), *I. indigotica* extract (0, 1, 10, and 100 µg/mL) or marker compound (0.1, 1, and 10 µg/mL), then immediately incubated with BHK-21 cell monolayer at 4 °C to allow attachment. After 1 h incubation, each extract/virus or compound/virus mixture was removed, cell monolayer washed with cold PBS and overlaid with MEM medium containing 1.1% methylcellulose. After 3-day incubation at 37 °C in a 5% CO2 incubator, plaques were stained, as described in plaque assay.

2.7. Virucidal Activity Assay. Virucidal assay was based on prior reports [14, 15]. JEV (105 pfu) was mixed with medium containing DMSO (0.005%, 0.05%, and 0.5%), *I. indigotica* extract (1, 10, and 100 µg/mL) or marker compound (0.1, 1, and 10 µg/mL) and incubated for 60 min at 4 °C. A 1000-fold dilution of each extract/virus or compound/virus mixture was added onto BHK-21 cell monolayer in 6-well plates. After 1 h incubation, mixtures were removed and washed with PBS, while monolayer was overlaid with MEM medium containing 1.1% methylcellulose; residual infectivity and inhibitory concentration showing 50% JEV plaque reduction (IC50) were determined, all as described in plaque assay.

2.8. Mouse Protection Assay. Groups (*n* = 10) of 2-week-old BALB/c mice were intracerebrally infected with 1 × 105 pfu of virulent JEV strain Beijing-1 then underwent three intracerebral treatments with 30 µg/100 µL of *I. indigotica* extract or marker compound (1 mg/kg of body weight) using 100 µL syringes at 2, 24, and 48 h after infection. Two additional groups were infected with JEV and received PBS or DMSO (0.05%) treatment as solvent controls. Survival rates were monitored every day for one week.

2.9. Statistical Analysis. ANOVA using SPSS program (version 10.1, SPSS Inc., IL, USA) or Student *t*-test analyzed data, *P* value less than 0.05 considered statistically significant.
3. Results

3.1. Cytotoxicity of *I. indigotica* Extracts and Related Marker Compounds. To test cytotoxicity, BHK-21 and HL-CZ cells were treated with both *I. indigotica* extract and related marker compounds at concentrations of 0.1–1000 µg/mL. Since diluted solutions of indigo and indirubin contained 0.0005%, 0.005%, 0.05%, 0.5%, and 5% DMSO, cells were also treated with serial dilution of DMSO as solvent control. Cytotoxicity assay indicated extracts of *I. indigotica* by ethyl acetate and methanol less toxic to BHK-21 kidney cells (CC_{50} ≥ 100 µg/mL) than human promonocytic HL-CZ cells (CC_{50} = 49.02 µg/mL). In solvent controls, both cell types had maximum DMSO tolerance under 0.5%; viability of indigo- and indirubin-treated cells was gauged at concentrations of 0.1–100 µg/mL containing less than 0.5% DMSO. CC_{50} values of indigo and indirubin varied from 26.88 µg/mL (BHK-21 cells treated with indigo) to
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57.47 µg/mL (BHK-21 cells treated with indirubin) (Table 1). Other *I. indigotica* related marker compounds like tryptanthrin, adenosine, betulin, lupeol, and 2-benzoxazolinone showed high toxicity to both cell lines (CC₅₀ < 25 µg/mL). Compared to ethyl acetate and methanol extracts, indigo and indirubin manifested low toxicity to such cells, being available for *in vitro* and *in vivo* activity against JEV.

### 3.2. Inhibition of JEV Yield by Indigo and Indirubin

To detect inhibition of virus yield in human promonocytic HL-CZ cells by *I. indigotica*, virus titers in cultured supernatants for JEV-infected HL-CZ cells with or without treatment were measured 24 and 48 hours after infection, using plaque assay (Figure 1). *I. indigotica* extract, indigo and indirubins

Table 1: Cytotoxic effect of *I. indigotica* extracts and its marker components on BHK-21 and HL-CZ cells.

| Component       | BHK-21 (µg/mL) | HL-CZ (µg/mL) |
|-----------------|----------------|---------------|
| Ethyl acetate extract | >100           | 49.02 ± 2.04  |
| Methanol extract    | >100           | >100          |
| Indigo            | 86.88 ± 3.72   | 99.41 ± 0.54  |
| Indirubin         | >100           | 64.89 ± 1.08  |
| Tryptanthrin      | 4.57 ± 0.26    | 1.74 ± 0.34   |
| Adenosine         | 24.10 ± 2.57   | 15.38 ± 1.05  |
| Betulin           | 13.30 ± 2.58   | 2.10 ± 0.78   |
| Lupeol            | 9.53 ± 1.32    | 3.44 ± 1.17   |
| 2-Benzoxazolinone | 19.92 ± 1.57   | 67.33 ± 10.2  |

*a Measured using trypan blue staining.*
of JEV plaques and indirubin showed concentration-dependent inhibition (solvent control). With simultaneous treatment, both indigo and indirubin as well as serial dilution of DMSO for indirubin (Figure 2 and Table 2). Both indigo and

indirubin tallied a therapeutic index (CC_{50}/IC_{50}) of >10. Meanwhile, each dilution of DMSO had no significant effect on plaque reduction (data not shown).

Both pre- and post-treatment plaque reduction assays tested antiviral effect of *I. indigotica* on JEV replication. In pretreatment assay, both indigo and indirubin pretreated before JEV adsorption showed antiviral activity similar to simultaneous treatment assay (Figure 2 and Table 2). However, posttreatment of *I. indigotica* extracts, indigo and indirubin was ineffective in antiviral activity after virus entry. Results demonstrated pretreatment of *I. indigotica* extracts, indigo and indirubin that affects JEV replication in *vivo*.

3.3. Inhibition of JEV Replication by Pretreatment of *I. indigotica* Extracts, Indigo and Indirubin. To ascertain time-of-addition effect of *I. indigotica* on JEV replication, BHK-21 cells were pretreated (prior to infection), simultaneously treated (at the same time as infection), or posttreated (after entry) with various concentrations of *I. indigotica* extracts, indigo and indirubin as well as serial dilution of DMSO (solvent control). With simultaneous treatment, both indigo and indirubin showed concentration-dependent inhibition of JEV plaques *in vitro*: IC_{50} plaque reduction values of 91.57 µg/mL for ethyl acetate extract, 78.47 µg/mL for methanol extract, 37.49 µg/mL for indigo, and 13.68 µg/mL for indirubin (Figure 2 and Table 2). Both indigo and

showed dose-dependent inhibition of JEV replication in HL-CZ promonocytic cells, but no time-dependent inhibitory effect on JEV production *in vitro*. Particularly, indigo (10 µg/mL) and indirubin (10 µg/mL) showed virus yield reduced by approximately 40% after 24 h incubation.

3.4. Inhibition of Virus Attachment by Indigo and Indirubin. To rate inhibitory effect of *I. indigotica* on virus attachment, JEV mixture (120 pfu) with *I. indigotica* extract, indigo, or indirubin (1 mg/kg of body weight) at 2, 24, and 48 h after infection. Two additional groups were infected with JEV and received PBS or DMSO (0.05%) treatment as solvent controls. Survival rates were monitored daily.

3.5. Virucidal Activity of Indigo and Indirubin. To ascertain whether *I. indigotica* has a virucidal action by directly interfering with virus particles, JEV was preincubated with both indigo and indirubin at 4°C for 1 h, each mixture was removed and cell monolayer washed with PBS. Residual infectivity derived by plaque assay yielded IC_{50} values of methanol extracts, indigo and indirubin below pre- and simultaneous treatment (Figure 3, Table 2). However, 0.5% DMSO had no significant effect on virus attachment (data not shown). These results demonstrated a potent inhibitory effect of methanol extracts, indigo and indirubin on JEV attachment.

3.6. Protection against Lethal Challenge in Mice by Indigo and Indirubin. To investigate *in vivo* protective potential of *I. indigotica*, groups of mice were intracerebrally challenged

| Isatis indigotica | IC_{50} (µg/mL) of pretreatment | IC_{50} (µg/mL) of simultaneous treatment | IC_{50} (µg/mL) of posttreatment | IC_{50} (µg/mL) of virus attachment | IC_{50} (µg/mL) of virucidal assays |
|------------------|-------------------------------|----------------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Ethyl acetate extract | 138.81 ± 3.71 | 91.57 ± 3.54 | >200 | >200 | 65.79 ± 3.54 |
| Methanol extract | 85.46 ± 8.54 | 78.47 ± 3.06 | >200 | 50.57 ± 2.12 | 22.17 ± 3.06 |
| Indigo | 11.79 ± 0.10 | 37.49 ± 3.18 | >50 | 5.15 ± 0.18 | 3.03 ± 3.18 |
| Indirubin | 23.50 ± 0.89 | 13.68 ± 2.83 | >50 | 5.10 ± 1.32 | 0.47 ± 2.83 |

Figure 5: Mouse protection against lethal JEV challenge by *I. indigotica*. Groups of 2-week-old BALB/c mice were intracerebrally infected with JEV at 1×10^7 pfu, then underwent three intracerebral treatments with *I. indigotica* extract, indigo, or indirubin (1 mg/kg of body weight) at 2, 24, and 48 h after infection. Two additional groups were infected with JEV and received PBS or DMSO (0.05%) treatment as solvent controls. Survival rates were monitored daily.
with lethal dose of virulent JEV strain Beijing-1 and treated with extracts, indigo, indirubin, PBS, or 0.5% DMSO at 2, 24, and 48 h after infection. Survival rate of the indirubin-treated group on Day 6 after infection was 70%, starkly higher than others: for example, indigo- (50%) and ethyl acetate extract-treated (20%) (Figure 5). None in the methanol extract-, PBS-, or DMSO-treated groups survived, indicating indirubin as superior to indigo, ethyl acetate extract better than methanol extract in mouse protection against lethal i.c. challenge with JEV.

4. Discussion

This study demonstrated I. indigotica extracts as having low cytotoxicity and concentration-dependent inhibitory effects on JEV replication in vitro: for example, reducing virus yield, blocking virus attachment, and virucidal activity (Figures 1–4, Tables 1 and 2). I. indigotica extract displays multiple antiviral and immunomodulatory activity against foot-and-mouth disease, rabies, HIV-1, influenza A, and SARS-coronavirus [1, 3–9]. Our results indicate antiviral potential of I. indigotica against JEV.

Among related compounds, indirubin manifested potent anti-JEV activities with plaque reduction (IC₅₀ = 13.68 µg/mL via simultaneous treatment), virus attachment inhibition (IC₅₀ = 5.10 µg/mL) and virucidal inactivation (IC₅₀ = 0.47 µg/mL) (Figures 2–4 and Table 2). Indirubin also concentration-dependently reduced virus yield in cell cultures (Figure 1(b)). Indigo effectively inhibited JEV replication in vitro, reduced virus yield and attachment (Figures 1(b) and 3(b)), showing greater virucidal activity (IC₅₀ = 3.03 µg/mL) than I. indigotica extracts. Indirubin and indigo had a potent virucidal activity through directly inactivating virus particles, linking with a better inhibition of JEV replication by pretreatment, and a significant reduction of virus attachment and yield in vitro. Similar antiviral effect of indirubin against pseudorabies virus has been also reported [5]. The inconsistency in anti-JEV abilities among reducing indirubin against pseudorabies virus has been also reported [6]. The inconsistency in anti-JEV abilities among reducing virus yield, virus attachment and virucidal activity could be due to the possibility that cells rapidly uptakes indirubin and indigo, then metabolizes them as inactive production, being supported in a prior report [16].

Indirubin had potent in vivo protection against intracerebral JEV challenge at lethal dose, more than indigo, ethyl acetate extract, or methanol extract (Figure 5). Indirubin likewise regulates immunomodulatory activity on RANTES expression in influenza-infected bronchial epithelial cells [8], showing broad-spectrum antiviral activity and more effective virucidal action against JEV. In sum, I. indigotica contains potential antiviral components against JEV, and so forth through virucidal actions. Among major I. indigotica components, indirubin manifests potential for antiviral activity against JEV infection, which could yield new anti-JEV agents.

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