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The cytotoxicity to leukemia cells and antiviral effects of *Isatis indigotica* extracts on pseudorabies virus

Shih-Ling Hsuan[a], Shih-Chieh Chang[a], Sheng-Yang Wang[b], Tien-Ling Liao[a], Ting-Ting Jong[c], Maw-Sheng Chien[a], Wei-Cheng Lee[a], Shih-Shiung Chen[d], Jiunn-Wang Liao[a,*]

[a] *Graduate Institute of Veterinary Pathobiology and Department of Veterinary Medicine, National Chung-Hsing University, 250, Kuo-Kuang Road, Taichung, 402, Taiwan, ROC*
[b] *Department of Forestry, National Chung-Hsing University, Taichung, 402, Taiwan, ROC*
[c] *Department of Chemistry, National Chung-Hsing University, Taichung, 402, Taiwan, ROC*
[d] *Department of Post Modern Agriculture, Ming Dao University, Changhua, 523, Taiwan, ROC*

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ABSTRACT

Aim of the study: Isatis indigotica (*I. indigotica*), Cruciferae, has been used in Chinese medicine for anti-leukemia and anti-severe acute respiratory syndrome (SARS). The aim of this study was to evaluate the cytotoxicity of *Isatis indigotica* extracts on human leukemia cell line (HL-60) and the antiviral activity on swine pseudorabies virus (PrV) in *in vitro* assays.

Materials and methods: Extracts and derived fractions of *Isatis indigotica* were prepared from root (R) and leaf (L) using methanol (M), ethyl acetate (E) and distilled water (D). The cytotoxic effect of extracts on swine peripheral blood mononuclear cells (PBMCs) and HL-60 was assessed by MTT method. The cytopathic effect (CPE) reduction, plaque reduction and inhibition assays on viral replication, and virucidal activity were further conducted to investigate the anti-PrV activity.

Results: Indirubin, one of the biological active compounds of *Isatis indigotica*, had the most significant cytotoxicity on HL-60 cells and inhibitory effect on PrV replication. Extracts from roots and leaves of *Isatis indigotica* also presented CPE inhibition either before or after infection of PrV on porcine kidney (PK-15) cells. Leaf extracts had better virucidal activity than roots, and ethyl acetate extracts exhibited the highest efficacy among extracts tested.

Conclusion: Isatis indigotica posses a valuable virucidal effect in disease control of pseudorabies virus infection in swine.

1. Introduction

Numerous viral strains are resistant to drugs for therapy in human and animals; therefore, searching for new antiviral substances is in urgent need. During the past few decades, bioactive compounds from numerous plant species have been investigated to show potential antiviral activities, such as anti-herpes virus activity of extracts from the Bulgarian medicinal plant, *Geranium sanguineum* L. (Geraniaceae) (Serkedjieva and Ivancheva, 1999), and extracts of *Phyllanthus urinaria* L. (Euphorbiaceae), which presented potent anti-infection effects against HSV-2 (Yang et al., 2005).

Isatis indigotica has been used in traditional Chinese medicine since ancient times. Its leaves and roots, known as Daqingye and Banlangen, respectively, are frequently used as anti-leukemia, antipyretic, anti-inflammatory and anti-influenza agents (Kunikata et al., 2000; Mak et al., 2004; Liu et al., 2005). Many compounds have been isolated from *Isatis indigotica*, including indigotin, indirubin, isatin, isatan A, isatan B, trytanthrin, purin, isaindigotidione, organic acids and a variety of amino acids (Zou et al., 2005). The biological activity of indigotin and indirubin has been reported, which are used as the markers for quality control due to their unique pharmaceutical activities (Zou et al., 2005; Liau et al., 2007). The *Isatis indigotica* root and phenolic Chinese herbs were frequently used for the prevention of severe acute respiratory syndrome (SARS) during the SARS outbreaks in China, Hong Kong, and Taiwan in 2003 (Lin et al., 2005). Recently, cleavage assays with the 3C-like protease demonstrated that IC_{50} values were in micromolar ranges for *Isatis indigotica* root extract, indigo, sinigrin, aloe emodin and hesperetin (Lin et al., 2005). It has also been shown that *Isatis indigotica* root could promote both humoral and cellular immune responses and would be one of the valuable candidates as a new-type of immunopotentiator on peripheral lymphocyte proliferation and serum antibody titer in chicken vaccinated with Newcastle disease vaccine (Kong et al., 2004). Furthermore, co-injection of the *Isatis indigotica* root extract with food and mouth disease DNA

* Corresponding author. Tel.: +886 4 22840894x406; fax: +886 4 22862073.
E-mail address: jwliao@dragon.nchu.edu.tw (J.-W. Liao).

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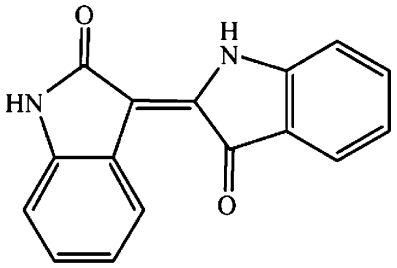

Indirubin

Fig. 1. The chemical structure of indirubin (cited from Liau et al., 2007).

vaccine has adjuvant effect on the immune response against viral pathogens in swine (Chen et al., 2005).

Pesudorabies virus (PrV) is classified as an *Alphaherpes virus* of the family *Herpesviridae* that causes a fetal disease called Aujeszky's disease in swine (Pejsak and Truszczy'nsk, 2006). Similar to other herpes virus, PrV shows latent infection under normal condition in various nerve tissues of pigs (Yoon et al., 2006). However, there is very little literature available on the effects of *Isatis indigotica* on the anti-PrV of swine. In this study, a series of experiments including cytopathic effect (CPE) reduction, plaque reduction assay (PRA) and inhibition assay on viral replication, and virucidal activity assay were conducted to investigate the anti-PrV activities of *Isatis indigotica* root and leaves extracts prepared with different solvents. The cytotoxic effect of extracts on swine peripheral blood mononuclear cells (PBMCs) and human leukemia cell line (HL-60) was also evaluated.

2. Materials and methods

2.1. Plant materials

The roots and leaves of *Isatis indigotica* (Banlangen and Daqingye) were obtained from the Department of Agronomy, National Chung-Hsing University (NCHU), Taichung, Taiwan, ROC. Herb identification was confirmed by macroscopic and microscopic analysis according to the Chinese pharmacopoeia (National Pharmacy and Committee, 2005; Liau et al., 2007). The dried samples were finely pulverized and then stored at room temperature for 3 days before extraction.

2.2. Preparation of plant extracts

The preparation of plant extracts was performed according to our previous study (Liau et al., 2007). Briefly, 2.5 kg of air-dried roots (R) and 1 kg of leaves (L) of *Isatis indigotica* were dipped in 1000 ml of 95% methanol (M) for one week at room temperature. Supernatants were filtrated on Whatman filter paper and concentrated by rotary evaporation at 40 °C and the final extracts were 8.5 and 1.8 g, respectively. These extracts were coded as RM and LM, respectively. The crude RM and LM were further fractionated with ethyl acetate (E) to obtain the soluble and insoluble parts and then concentrated by rotary evaporator at 40 °C. The soluble and insoluble parts of the crude RM were coded as RE (8 g) and RD (6.5 g). The soluble and insoluble parts of the crude LM were coded as LE (2.1 g) and LD (3.2 g). These extracts were diluted with DMSO to be 100 mg/ml and filtrated by 0.22 μm filter, then stored at −20 °C before use. Indirubin (Fig. 1) was isolated from the leave extract of *Isatis indigotica* in our laboratory, and its purity and structure were confirmed by HPLC, and ESI-mass spectrometry as our previous report (Liau et al., 2007).

2.3. Porcine peripheral blood mononuclear cells and HL-60 cells culture

Blood collected from superior vena cava of pigs was placed into heparinized tube (Becton Dickson, NJ, USA) and mixed with 2% dextran solution at a ratio of 2:1 for 15 min to allow the red blood cells to aggregate and precipitate. The supernatant was then transferred onto 2 ml of Ficoll-paque® (Pharmacia Biotech), followed by centrifugation at 840 × *g* for 20 min. The resulting interface layer of peripheral blood mononuclear cells were collected and washed three times with 0.5 mM EDTA (Sigma, MO, USA) in PBS at 4 °C. PBMCs were resuspended in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin G/streptomycin, and 2 mM L-glutamine) to a density of 1×10^7 cells/ml, and cultured at 37 °C overnight in a 5% CO_2 humidified incubator.

HL-60 cells, a human promyeloblastic leukemic cell line (BCRC 60027), were obtained from the Culture Collection and Research Center, Food Industry Research and Development Institution, Taiwan. These cells were grown in Iscove's Modified Dulbecco's Medium (IMDM, Sigma, MO, USA) supplemented with 10% FBS, 100 units/ml penicillin G/streptomycin, and 2 mM L-glutamine at 37 °C in a 5% CO_2 humidified incubator.

2.4. Treatments and determination of cell viability

According to the pilot study, the PBMCs were treated with various concentrations of RD, RM, LD (1000 μg/ml), indirubin and LE (200–800 μg/ml), and LM, RE (400–1000 μg/ml) of *Isatis indigotica* extracts for 24 h incubation. HL-60 cells (1×10^6 cells/ml) were incubated with *Isatis indigotica* extracts of LE (50–300 μg/ml), RE (200–400 μg/ml), LM (200–1000 μg/ml) and indirubin (40–200 μg/ml) for 24 h, respectively. The cell viability in each treatment was analyzed by the MTT colorimetric assay (Mosmann, 1983; Cory et al., 1991). Briefly, an MTT solution at 2 mg/ml in PBS was added to the 96-well culture plates at 50 μl/well on each time point. Following 3 h of incubation, 100 μl of DMSO (Merck, Darmstadt, Germany) was added to each well and mixed thoroughly to dissolve the dark blue formazen crystals from surviving cells. Thereafter, the plates were read on an ELISA reader

Table 1
Cytotoxic effects of *Isatis indigotica* extracts by different solvents on HL-60 cells.

Group	Concentration (μg/ml)	Viability (% of control)	LC_{50} (μg/ml)
Indirubin	40	90.6 ± 10.3	
	80	69.7 ± 8.7*	
	120	37.8 ± 2.7*	98.1
	160	22.4 ± 0.7*	
	200	24.8 ± 1.7*	
LE	100	95.6 ± 9.7	
	150	86.7 ± 9.7	
	200	63.4 ± 7.0*	226.4
	250	40.8 ± 1.7*	
	300	26.9 ± 2.1*	
RE	240	74.8 ± 1.4*	
	280	58.9 ± 1.2*	
	320	40.9 ± 2.6*	305.9
	360	32.0 ± 1.0*	
	400	24.2 ± 1.8*	
LM	200	73.3 ± 5.5*	
	400	64.0 ± 4.4*	
	600	42.7 ± 8.1*	473.7
	800	35.8 ± 8.5*	
	1000	18.0 ± 0.2*	

R: root; L: leaves; M: MeOH-extracted; E: ethyl acetate-extracted. Data are expressed as mean ± SD of three independent experiments with eight replicates each.

* Indicates significant difference between control and treatment groups at $p < 0.05$.

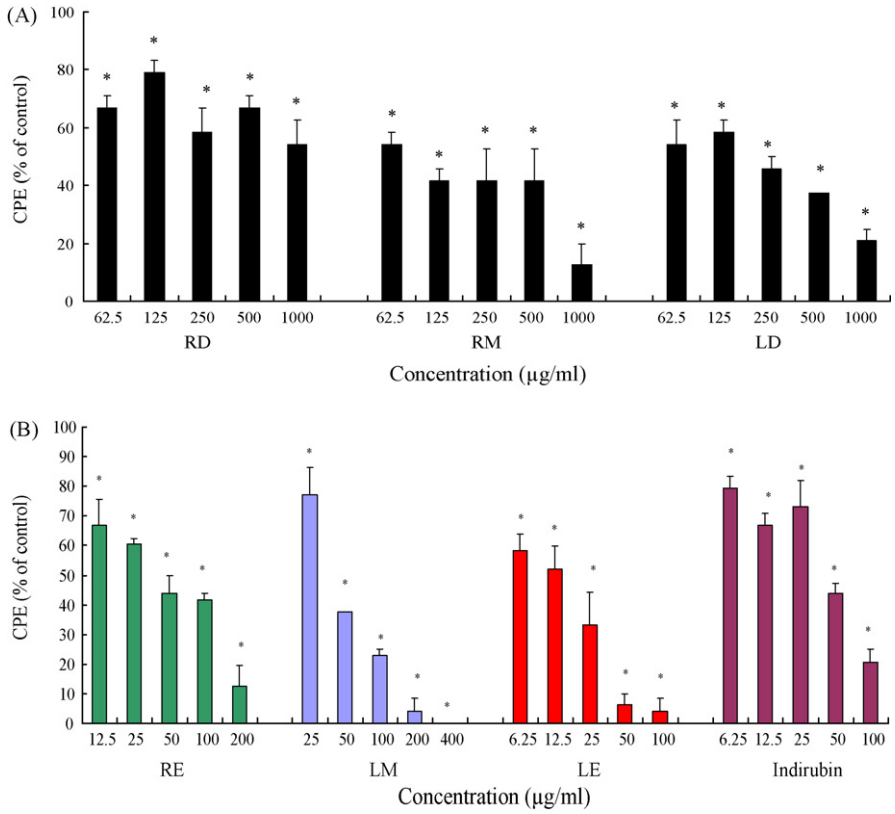

Fig. 2. Effects of *Isatis indigotica* extracts by different solvents on PrV induced cytopathic effect. PK-15 cells were infected with 50 $TCID_{50}$/well PrV and various concentrations of extracts in 96-well plates for 72 h. The inhibition effect (% of control) of RD, RM and LD extracts are shown in (A) and RE, LM, LE and indirubin in (B). Data are expressed as mean ± SD of three independent experiments with eight replicates each. R: root; L: leaves; M: MeOH-extracted; E: ethyl acetate-extracted; D: distilled water-extracted. *Indicates significant difference between control and treatment groups at $p < 0.05$.

(MRX, Dynatech Medical Products, Guernsey, Channel Island, Great Britain) at the wavelength of 540 nm using absorbance at 630 nm as a reference. Data were expressed as a percentage of control (ΔOD) = [(OD value of treated group)/(OD value of medium control)] × 100. The median lethal concentration (LC_{50}) was further calculated as described by Mosmann (1983) and Scudiero et al. (1988).

2.5. Preparation of swine pseudorabies virus

Porcine kidney cells (PK-15, BCRC 60057) were cultured and maintained in Dulbecco's Modified Eagles Medium (DMEM) containing 10% FBS and 100 units/ml penicillin G/streptomycin in a 5% CO_2 humidified incubator at 37 °C. Pseudorabies virus (TNL strain) was obtained from the Research Institute for Animal Health, Taiwan. For the viral infection, a maintenance medium was used in which the concentration of FBS was reduced to 5%. The PK-15 cells were infected with 0.05 multiplicity of infection (MOI) of PrV at 37 °C for 1.5 h, then washed with HBSS three times and maintained in maintenance medium. Virus harvested when the number of cytopathic effect formation reached 80–90% of infected PK-15 cells was stored at −70 °C for further use.

2.6. Determination of 50% tissue culture infectious dose ($TCID_{50}$) of pseudorabies virus

PK-15 cells were seeded into flat bottomed, 96-well microtiter trays at 2×10^4 cells/well and incubated at 37 °C in 5% CO_2 for 1 day. Virus stock solution was diluted with FBS free DMEM in serial dilutions and incubated with PK-15 cells for 72 h. The CPE formation was counted and the proportional distances (PD) were calculated following the method of Reed and Muench (1938). PD = (>50% infectious − 50% infectious)/(>50% infectious − <50% infectious). The $TCID_{50}$ was calculated by using the log expression and one $TCID_{50}$ indicated that 50% of cells were infected.

2.7. Determination of plaque formation unit (pfu) of pseudorabies virus

PK-15 cells were seeded into 24-well tissue culture plates at a density of 1.6×10^5 cells per well and incubated at 37 °C in 5% CO_2 for 1 day. Virus stock solution was diluted with 2% FBS/DMEM in a series dilution and incubated with PK-15 cells with rocking for 1 h. The medium was discarded after which 1 ml of 1% methylcellulose (containing 2% FBS DMEM) was added to the plates. The plates were incubated at 37 °C in 5% CO_2 for 48 h. The infected cells were fixed with methanol and stained with 0.5% crystal violet solution for 30 min, and the number of plaques was counted and expressed as plaque forming unit/ml.

2.8. Cytopathic effect reduction assay

Cytopathic effect reduction assay was performed according to the method described by Serkedjieva and Ivancheva (1999). Briefly, monolayer of PK-15 cells was grown on 96-well culture plates. Fifty $TCID_{50}$ of the PrV were incubated with various concentrations of extracts under 5% CO_2 at 37 °C for 72 h. After incubation, the infected cells were stained with 0.5% crystal violet solution for 30 min, and the CPE was observed under a light microscope. The CPE was graded as follows: 0 = 0% CPE, 1 = 0–25% CPE, 2 = 26–50% CPE, 3 = 51–75% CPE, 4 = 76–100% CPE (Serkedjieva and Ivancheva, 1999). The inhibited percentage (%) of CPE was calculated: % of control = $CPE_{exp}/CPE_{virus\ control} \times 100$, and the values of EC_{50} (50% effective concentration) were calculated.

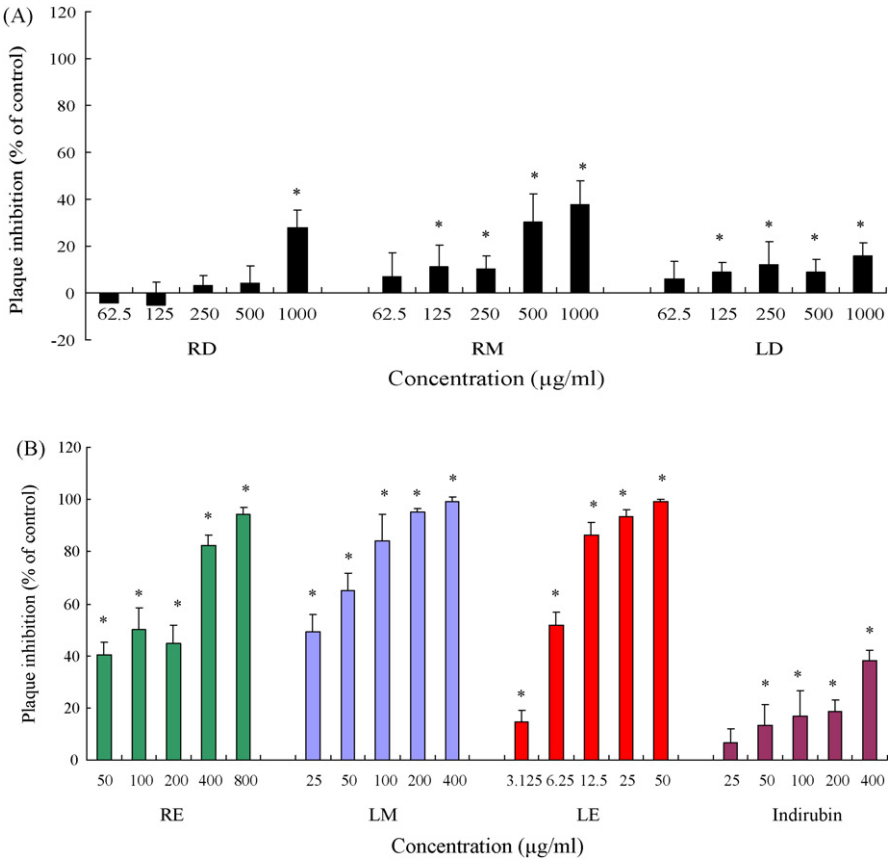

Fig. 3. Effects of *Isatis indigotica* extracts by different solvents on PrV plaque reduction. PK-15 cells were firstly infected with 100 pfu/well and various concentrations of extracts in 24-well plates for 1.5 h, washed and then incubated for 48 h. The plaque inhibition effect (% of control) of different extracts of RD, RM and LD are shown in (A) and RE, LM, LE and indirubin in (B). Data are expressed as mean ± SD of three independent experiments with eight replicates each. R: root; L: leaves; M: MeOH-extracted; E: ethyl acetate-extracted; D: distilled water-extracted. *Indicates significant difference between control and treatment groups at $p < 0.05$.

2.9. Plaque reduction assay and inhibition assay on PrV viral replication

Plaque reduction assay was conducted according to the method described by Zhu et al. (2006). Briefly, monolayer of PK-15 cells was grown on 24-well culture plates. For the PRA, various concentrations of extracts and 100 pfu of PrV were simultaneously added to PK-15 cells and incubated at room temperature for 1.5 h. The virus/extract mixture was discarded after which 1 ml of 1% methylcellulose (in DMEM containing 2% FBS) was added to the well plates. Plates were incubated under 5% CO_2 at 37 °C for 48 h. The infected cells were fixed with methanol and stained with 0.5% crystal violet solution for 30 min, and the number of plaques was counted. The 50% inhibited concentration (IC_{50}) of plaques was calculated as follow: $IC_{50} = [1 - (pfu_{exp}/pfu_{virus\ control})] \times 100$ (Yang et al., 2005).

Inhibition assay was performed by incubation of 100 pfu of PrV with monolayer PK-15 cells at room temperature for 1.5 h. Subsequently, the viral solution was replaced with 1 ml of 1% methylcellulose supplemented with various concentrations of extract. Cells were incubated under 5% CO_2 at 37 °C for 48 h, fixed, stained with crystal violet, and the IC_{50} calculated as described in PRA.

2.10. Determination of extracellular virucidal assay

The extracellular virucidal activity of extracts on PrV was performed according to the method of Carlucci et al. (1999). Firstly, the 5×10^6 pfu of PrV was mixed with various concentrations of extracts and incubated 25 °C for 6 h, followed by a tenfold serial dilutions. One hundred microliter of viral/extract was mixed with an equal amount of 2% FBS/DMEM that was used to infect PK-15 cells at room temperature for 1.5 h. Subsequently, the viral/extract mixture was replaced with 1 ml of 1% methylcellulose in 2% FBS/DMEM and incubated under 5% CO_2 at 37 °C for 48 h. The plaques were fixed with methanol and stained with 0.5% crystal violet solution for 30 min, and the number of plaques was counted. The residual virus infectivity was determined by plaque formation as pfu = plaques × (1/viral inoculation) × (1/diluted fold). The IC_{50} of plaques was calculated as follow: $IC_{50} = (pfu_{exp}/pfu_{virus\ control}) \times 100$.

2.11. Statistical analysis

Data expressed as mean ± SD were obtained from at least three independent experiments, each with quintuplicate determinations. Mean values were compared by Least Significant Difference (LSD) using the SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). The LC_{50}, EC_{50} and IC_{50} values were calculated by log expression using the software of Microsoft Excel 2003. The difference was considered statistically significant when $p < 0.05$.

3. Results

3.1. Cytotoxic effect of Isatis indigotica extracts on PBMCs and HL-60 cells

The cytotoxic effect of *Isatis indigotica* extracts on porcine PBMCs was determined by MTT assay after 24 h co-incubation. Results revealed that only a slight cytotoxic effect (27%) of RE group was

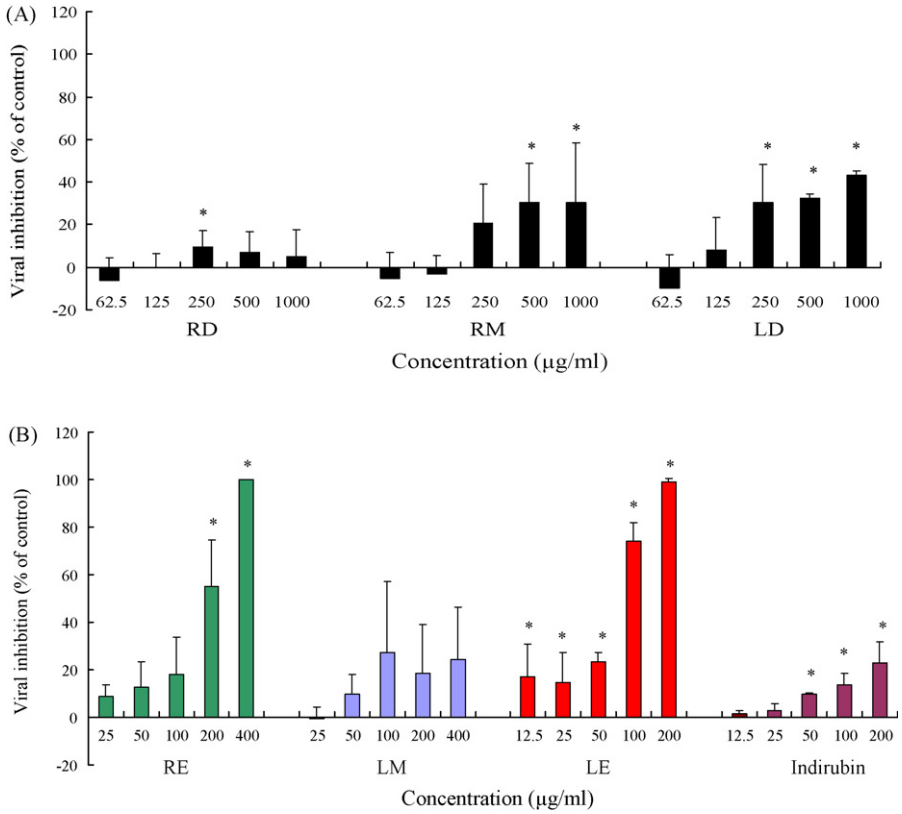

Fig. 4. Effects of *Isatis indigotica* extracts by different solvents on PrV replication. PK-15 cells were firstly preinfected with 100 pfu/well for 1.5 h, washed, and then incubated with various concentrations of extracts in 24-well plates for 48 h. The viral inhibition (% of control) of RD, RM and LD extracts are shown in (A) and RE, LM, LE and indirubin in (B). Data are expressed as mean ± SD of three independent experiments with eight replicates each. R: root; L: leaves; M: MeOH-extracted; E: ethyl acetate-extracted; D: distilled water-extracted. *Indicates significant difference between control and treatment groups at $p < 0.05$.

found at the highest tested concentration of 1000 μg/ml. The cytotoxicity of *Isatis indigotica* extracts of RD, RM, LD, LE and LM, and indirubin in PBMCs was ranging from 0% to 11%, suggesting none or insignificant cytotoxicity toward normal porcine PBMCs.

For the cytotoxic effect on HL-60 cells, cells were incubated with *Isatis indigotica* extracts of indirubin (40–200 μg/ml), LE (50–300 μg/ml), RE (200–400 μg/ml), and LM (200–1000 μg/ml) for 24 h and then evaluated by MTT assay. Results showed that HL-60 cells became pyknosis, condensation and even lyses after treated with LE, RE and LM extracts in a dose-dependent manner. The LC_{50s} of indirubin, LE, RE and LM in HL-60 cells were 98, 226, 305 and 473 μg/ml, respectively (Table 1). However, the RM, RD and LD extracts revealed no cytotoxic effect on HL-60 cells (data not shown).

3.2. Effects of Isatis indigotica extracts on cytopathic effect of PrV

The activities of *Isatis indigotica* extracts against the PrV were evaluated using CPE reduction assay. PK cells were infected with PrV that were pre-incubated with various concentrations of extracts and the CPE reduction calculated. Except for the RD group, all other *Isatis indigotica* extracts exhibited dose-dependent inhibitions of CPE formation. The EC_{50s} of LD, RM, RE, LM, LE and indirubin on CPE reduction were 147, 95, 38, 45, 11 and 35 μg/ml, respectively (Fig. 2).

3.3. Plaque reduction assay

For the PRA, one hundred pfu/well of the PrV was incubated with various concentrations of extracts with PK-15 cells rocking for 1.5 h and then mixed with 1 ml of 1% methylcellulose for 48 h. Results revealed that RD, RM, LD and indirubin groups had a slight inhibition on the plaque formation; however, RE, LM and LE groups presented significant inhibition on the PrV-infected plaque formation at concentrations of 800, 400 and 50 μg/ml, respectively. The IC_{50s} of RE, LM and LE extracts on plaque reduction were 150, 30 and 5 μg/ml, respectively (Fig. 3).

3.4. Inhibition assay on the viral replication

To evaluate the effects of various extracts on PrV replication, PK-15 cells were firstly infected with PrV, followed by treatment of various concentrations of extracts and inhibition of viral replication determined. Results exhibited that the inhibition rate of viral replication of RM, RD, LD, LM and indirubin was less than 50%; however, RE and LE extracts showed 99–100% inhibition on the viral replication at concentrations of 400 and 200 μg/ml. The IC_{50s} of LE and RE extracts on PrV replication were 99 and 156 μg/ml, respectively (Fig. 4).

3.5. Virucidal activity

To determine whether the inhibition of PrV replication by various extracts of *Isatis indigotica* was a result of direct virus inactivation, extracts were pre-incubated with PrV and residual infectivity of virus analyzed. Results showed that the extracellular virucidal activity of RD, RM and LD extracts had a slight inhibition on PrV with range of 20–50% at 1000 μg/ml. The RE, LM, LE, and indirubin resulted in dose-dependent reductions of remaining infectivity of the PrV when compared with the control. The IC_{50} of RE, LM, LE, and indirubin on plaque formation were 299, 36, 39, and 100 μg/ml, respectively (Fig. 5).

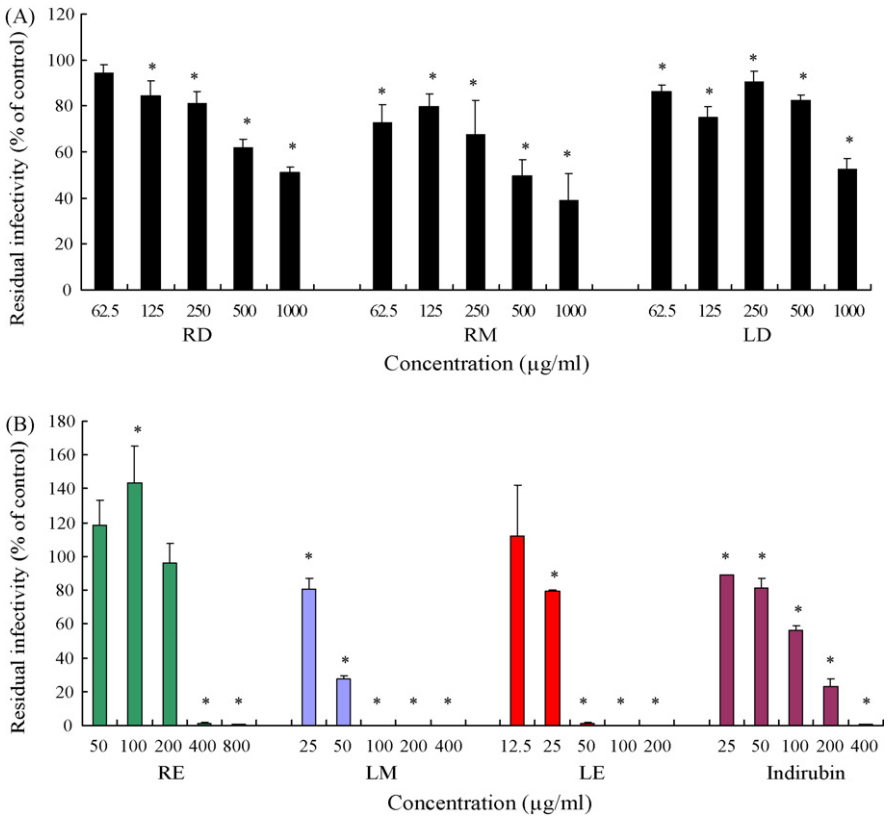

Fig. 5. Virucidal effects of *Isatis indigotica* extracts by different solvents on PrV. Different concentrations of extracts and indirubin were premixed with 5×10^6 pfu PR virus 25 °C for 6 h, and then incubated with PK-15 cells in 24-well plates for 48 h. The residual infectivity of PrV (% of control) after treatment of RD, RM and LD extracts are shown in (A) and RE, LM, LE and indirubin groups in (B). Data are expressed as mean ± SD of three independent experiments with eight replicates each. R: root; L: leaves; M: MeOH-extracted; E: ethyl acetate-extracted; D: distilled water-extracted. *Indicates significant difference between control and treatment groups at $p < 0.05$.

4. Discussion and conclusion

In the present study, we found that HL-60 cells appeared pyknosis, condensation and even lysis after treatment of indirubin and some of the *Isatis indigotica* extracts. Indirubin is one of the major ingredients of *Isatis indigotica* that inhibits cell proliferation and cyclin-dependent kinase on various cells, including promyelocytic HL-60 cells and induces apoptosis via G1-phase arrest (Hoessel et al., 1999). Although the RM, RD and LD groups revealed no cytotoxic effect on HL-60 cells, LE, RE and LM extracts displayed a dose-dependent cytotoxicity. Indirubin had the most significant cytotoxicity on HL-60 cells as compared to the ethyl acetate and methanol extracts. The anti-leukemia effect of *Isatis indigotica* extracts prepared from ethyl acetate was stronger than that of methanol. These results are comparable to the previous report showing that indirubin and some of *Isatis indigotica* extracts posed as reliable anti-leukemia medicine (Hoessel et al., 1999) without toxic to the normal swine PBMCs in this study.

Numerous plant extracts displayed inhibitory or inactivating effect on virus replication, including antiviral properties of *Opuntia streptacantha* extracts on DNA and RNA virus replication (Ahmad et al., 1996). Also, *Phyllanthus urinaria* inhibits herpes simplex virus infection through disturbing the early stages of virus infection (Yang et al., 2005). One of the components in *Isatis indigotica*, indirubin has been shown to inhibit RANTES expression in influenza virus-infected human bronchial epithelial cells via the intervention of virus-induced p38 MAP kinase activation and NF-κB translocation (Mak et al., 2004). In this study, the antiviral activities of indirubin were assessed by four methods of cytopathic effect, plaque reduction, viral replication and virucidal activity of PrV on PK-15 cells. It was found that indirubin inhibited CPE formation and virucidal activity in a dose related manner. The IC_{50} of indirubin in CPE formation and virucidal activity assay were 35 and 100 μg/ml, respectively; however, less effective in inhibiting of plaque formation and viral replication.

In this study, the antiviral activities of *Isatis indigotica* with different extracts of distilled water, methanol and ethyl acetate obtained from roots and leaves presented significant inhibition on PrV-infected PK-15 cells. For the viral replication, PK-15 cells were infected with 50 $TCID_{50}$/well PrV and *Isatis indigotica* extracts at various concentrations for 72 h. The inhibition effect (% of control) of *Isatis indigotica* extracts showed dose-dependency on PrV-infected CPE formation. The extracts of RD, RM and LD had weaker inhibition effect than that of RE, LM, and LE (Fig. 2). The effect of *Isatis indigotica* extracts on plaque reduction was similar to the CPE formation assay. The LE group showed the most significant inhibition effect on the plaque formation and plaque reduction among the all extracts, and the IC_{50s} of LE were 11 and 5 μg/ml. In comparison of the effects of CPE formation and viral replication, less effect of CPE formation than that of viral replication on LE-treated group might be due to less PrV inoculation (50 $TCID_{50}$/well vs. 100 pfu/well) or the longer incubation time (72 h vs. 1.5 h) of PrV on viral replication assay.

Moreover, to understand the antiviral activities of extracts on the infected cell, the PK-15 cells were first infected with PrV, washed and then incubated with *Isatis indigotica* extracts. The extracts posed less protective effect on cells than that of extracts co-incubated with PrV during infection. This result indicated that extracts of LE and RE had better inhibition on the initial stage of PrV infection before the attachment of the virus to the cells. Furthermore, LM and LE extracts displayed significantly virucidal activities by reducing the residual virus infectivity to cells.

Although traditional Chinese medicines are commonly decocted by boiling with water; however, the concentrations of most of the components extracted from *Isatis indigotica* roots were found to be lower in the aqueous extract than in the methanol extract (Zou et al., 2005). Some low polarity components were not found in the chromatogram of the aqueous extract. For this, the virucidal effect of LM and LE extracts might be better than that of water extracts of *Isatis indigotica* and indirubin. In our recent study, there was an 85% decrease for indigo content, which is perhaps due to the isomerization of indigo to indirubin during drying (Liau et al., 2007). In contrast, more indirubin was found and increased fourfolds in the dried leaves than in the fresh leaves. The findings from our research indicated that the amounts of the three components of tryptanthrin, indigo and indirubin in the roots Banlangen are much smaller than those in the leaves Daqingye (Liau et al., 2007).

In conclusion, the LE extract showed the most significant inhibition on the anti-PrV infectivity among all other extracts from roots. The antiviral effects of LE extracts are consistent with the active ingredients from chemical analysis (Liau et al., 2007). Nevertheless, further studies are needed to elucidate the mechanism of action of the leaf extracts in inhibiting PrV infection.

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