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## Anti-SARS coronavirus 3C-like protease effects of *Isatis indigotica* root and plant-derived phenolic compounds

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### Abstract

The 3C-like protease (3CL<sup>pro</sup>) of SARS-coronavirus mediates the proteolytic processing of replicase polypeptides 1a and 1ab into functional proteins, becoming an important target for the drug development. In this study, *Isatis indigotica* root extract, five major compounds of *I. indigotica* root, and seven plant-derived phenolic compounds were tested for anti-SARS-CoV 3CL<sup>pro</sup> effects using cell-free and cell-based cleavage assays. Cleavage assays with the 3CL<sup>pro</sup> demonstrated that IC<sub>50</sub> values were in micromolar ranges for *I. indigotica* root extract, indigo, sinigrin, aloe emodin and hesperetin. Sinigrin (IC<sub>50</sub>: 217 μM) was more efficient in blocking the cleavage processing of the 3CL<sup>pro</sup> than indigo (IC<sub>50</sub>: 752 μM) and beta-sitosterol (IC<sub>50</sub>: 1210 μM) in the cell-based assay. Only two phenolic compounds aloe emodin and hesperetin dose-dependently inhibited cleavage activity of the 3CL<sup>pro</sup>, in which the IC<sub>50</sub> was 366 μM for aloe emodin and 8.3 μM for hesperetin in the cell-based assay.

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**Keywords:** SARS-coronavirus; 3C-like protease; *Isatis indigotica* root; Phenolic compounds

Severe acute respiratory syndrome (SARS) was reported in 8447 cases with 811 deaths worldwide from February to June 2003 (Poutanen et al., 2003; Peiris et al., 2003; Drosten et al., 2003). A novel coronavirus, SARS-coronavirus (SARS-CoV) was identified as the etiological agent of the disease (Ksiazek et al., 2003; Lee et al., 2003; Tsang et al., 2003; Hsueh et al., 2003). SARS-CoV particle contains a single positive-stranded RNA genome encoding for replicase, spike, envelope, membrane, and nucleocapsid (Lai, 2003; Enjuanes et al., 2001; Holmes, 2003). The SARS-CoV 3CL<sup>pro</sup> mediates the proteolytic processing of replicase polypeptides into functional proteins, playing an important role in viral replication. Therefore, the SARS-CoV 3CL<sup>pro</sup>

can be considered an attractive target for developing effective drugs against SARS. Several potential 3CL<sup>pro</sup> inhibitors with a 50% inhibitory concentration (IC<sub>50</sub>) below 10 μM were identified from the large number of the structurally diverse small molecules (Kao et al., 2004; Hsu et al., 2004).

*Isatis indigotica* root and phenolic Chinese herbs were frequently used for the prevention of SARS during the SARS outbreaks in China, Hong Kong, and Taiwan. *I. indigotica* root (*Radix isatidis*), belonging to the family Cruciferae, is native to China. Antiviral effects of *I. indigotica* root were found against influenza, hepatitis A and Japanese encephalitis (Qin and Xu, 1998; Wu et al., 1997). *I. indigotica* root contains indigo, indirubin, indican (indoxyl-β-D-glucoside), β-sitosterol, γ-sitosterol, sinigrin, etc. (Gilbert et al., 2004). Indigo and indirubin were identified as the promiscuous chymotrypsin inhibitors (McGovern and Shoichet, 2003).

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Recently, an anti-influenza virus effect of indirubin has been demonstrated (Mak et al., 2004). In addition, several herb-derived phenolics aloemodin, hesperetin, quercetin, and naringenin have been accredited with antiviral effects against poliovirus, vesicular stomatitis virus, Sindbis virus, herpes simplex virus types 1 and 2, parainfluenza virus, and vaccinia virus (Semple et al., 2001; Andersen et al., 1991; Paredes et al., 2003; Kim et al., 2001).

In this study, we characterized the anti-SARS-CoV 3CL<sup>pro</sup> effect of the water extract of *I. indigotica* root, *I. indigotica* root-derived compounds, and herb-derived phenolics using a cell-free cleavage and cell-based cleavage assay.

The root of *I. indigotica* was purchased from Sun Ten Pharmaceutical Corporation (Taiwan). The plant root of *Isatidis indigotica* was extracted twice with 10 volumes of distilled boiling water for 1 h. The aqueous extract was concentrated under the reduced pressure at 50 °C, passed through 0.22- $\mu$ m filters for sterilization, and diluted in culture medium to make a stock concentration of 10 mg/ml. Indigo and indirubin were kindly provided by Dr. Yuan-Shiun Chang, professor for Institute of Chinese Pharmaceutical Sciences, China Medical University. Indican (indoxyl- $\beta$ -D-glucoside),  $\beta$ -sitosterol, sinigrin, aloe emodin, hesperetin, quercetin, naringenin, daidzein, emodin, and chrysophanol were purchased from Sigma Chemical.

To examine the *trans*-cleavage of SARS-CoV 3CL<sup>pro</sup> in the cell-free assay, recombinant 3CL<sup>pro</sup> was expressed in *E. coli* and purified using the HisTrap Kit (Amersham) as described in our previous report (Lin et al., 2004). Coomassie Blue-staining revealed that recombinant 3CL<sup>pro</sup> contained a major 34-kDa band for the monomer and a minor 68-kDa band for the dimer (Fig. 1A, lane 2). The cleavage substrate (TVRLQAGNATE) was generated as the substrate fusion protein with the N-terminal S-Tag and the C-terminal HSV-Tag. In the cell-free cleavage assay, the substrate fusion protein that was captured by anti-HSV-Tag antibodies in wells incubated with soluble 3CL<sup>pro</sup> for 3 h at 37 °C. The non-cleavage substrate protein was detected by an Enzyme Linked Immunosorbent Assay (ELISA) using peroxidase-conjugated S protein. ELISA showed that cell-free proteolytic activity correlated, in concentration-dependent manner, with the serial twofold dilution of recombinant 3CL<sup>pro</sup> protein in the range from 15  $\mu$ g/ml to 240  $\mu$ g/ml (Fig. 1B). Subsequently, the anti-3CL<sup>pro</sup> effect by the extract of *I. indigotica* root was evaluated using the cell-free cleavage assay. The cell-free cleavage assay indicated that the extract of *I. indigotica* root had a dose-dependent anti-3CL<sup>pro</sup> effect with an IC<sub>50</sub> of  $53.8 \pm 4.2$   $\mu$ g/ml (Fig. 2; Table 1).

The cell-based cleavage assay of 3CL<sup>pro</sup> for screening inhibitors does not require purification of the active 3CL<sup>pro</sup>,

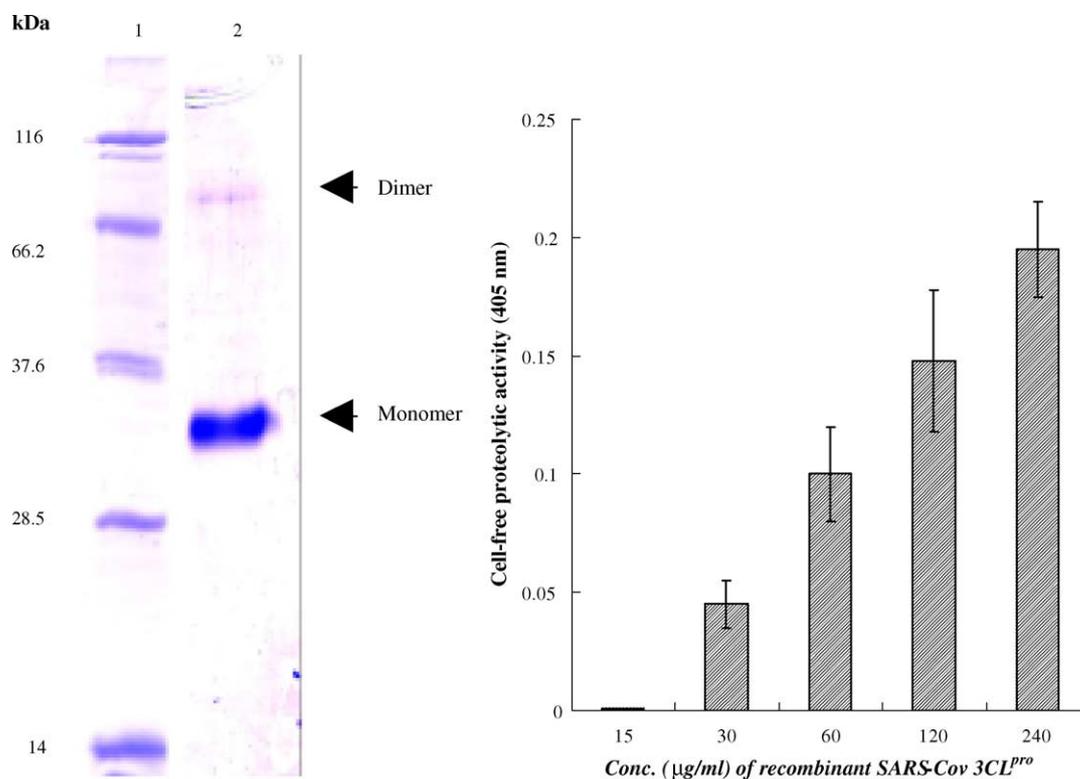
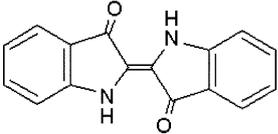
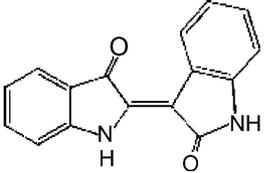
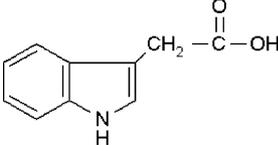
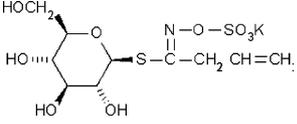
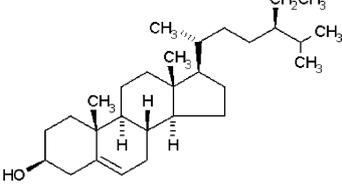
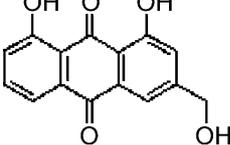
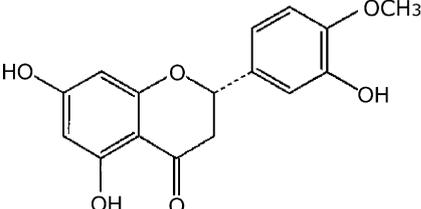
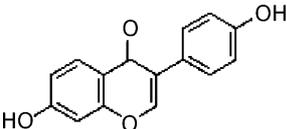


Fig. 1. Cell-free cleavage activity of recombinant SARS-CoV 3CL<sup>pro</sup>. (A) The purified 3CL<sup>pro</sup> recombinant protein at the 1 mg/ml was analyzed by 10% SDS-PAGE with Coomassie blue staining (lane 2). (B) The *trans*-cleavage of the 3CL<sup>pro</sup> with a substrate fusion protein was determined using the ELISA. The substrate fusion protein was captured with anti-HSV mAb, followed by incubation with the serial dilution of the 3CL<sup>pro</sup>. The non-cleavage of the fusion protein was detected using the S protein-HRP conjugate and ABTS/H<sub>2</sub>O<sub>2</sub> substrates. The ELISA product was measured at A405 nm. The relative cell-free cleavage activity was calculated as  $1 - (A405_{3CL^{pro}})/(A405_{no\ 3CL^{pro}})$ .

Table 1  
The inhibitory effect on cell-free and cell-based cleavage activity of the SARS-CoV 3CL<sup>pro</sup>

Compound	Structure	IC <sub>50</sub> <sup>a</sup> of cell-free cleavage (μg/ml)	IC <sub>50</sub> <sup>a</sup> of cell-based cleavage (μg/ml)	CC <sub>50</sub> <sup>b</sup> of cell death (μg/ml)
<i>Isatis indigotica</i> root		53.8 ± 4.2	191.6 ± 8.2	>5000
Indigo		37.3 ± 8.1 (300 μM)	190 ± 2.6 (752 μM)	917 ± 18 (7375 μM)
Indirubin		81.3 ± 5.2 (293 μM)	NS <sup>c</sup>	
Indican		33.1 ± 1.2 (112 μM)	NS <sup>c</sup>	
Sinigrin		50.3 ± 1.5 (121 μM)	90.1 ± 4.2 (217 μM)	>5000 (>10,000 μM)
Beta-sitosterol		47.8 ± 8.6 (115 μM)	502.1 ± 2.9 (1210 μM)	613 ± 9 (1475 μM)
Aloeemodin		35.7 ± 1.5 (132 μM)	99.1 ± 2.1 (366 μM)	3135 ± 9 (11,592 μM)
Hesperetin		18.1 ± 0.6 (60 μM)	2.5 ± 0.8 (8.3 μM)	820 ± 15 (2718 μM)
Daidzein		26.8 ± 1.2 (105 μM)	NS <sup>c</sup>	

<sup>a</sup> IC<sub>50</sub> (50% inhibitory concentration) was the concentration requiring for 50% inhibition on the *cis*-cleavage activity of 3CL<sup>pro</sup>.

<sup>b</sup> CC<sub>50</sub> (50% cytotoxic concentration) was the concentration giving half the OD<sub>570–630</sub> of mock cells in MTT assay. IC<sub>50</sub> and CC<sub>50</sub> were determined using a computer program based on Fisher's statistical model.

<sup>c</sup> Not significant.

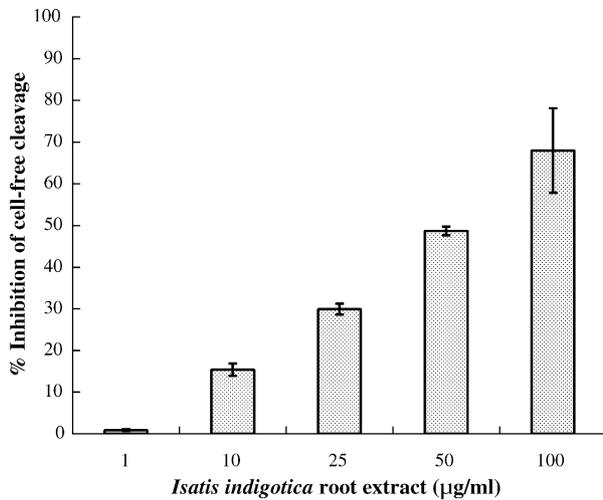


Fig. 2. Inhibition of the cell-free cleavage of the 3CL<sup>pro</sup> by the *Isatis indigotica* root extract. The extract of the *I. indigotica* root was added into the mixture of the substrate fusion protein and the 3CL<sup>pro</sup>, and then incubated at room temperature for 3 h. The non-cleavage of substrate fusion protein was detected using the S protein-HRP conjugate and ABTS/H<sub>2</sub>O<sub>2</sub> substrates. The ELISA product was measured at A405 nm. The relative inhibition of cell-free cleavage activity was calculated as  $1 - (A405_{\text{no 3CL}^{\text{pro}}} - A405_{\text{3CL}^{\text{pro}} \text{ with inhibitor}}) / (A405_{\text{no 3CL}^{\text{pro}}} - A405_{\text{3CL}^{\text{pro}}})$ .

and represents closely the natural physiological state. Therefore, we used the cell-based cleavage assay for examining the inhibitory efficacy of the 3CL<sup>pro</sup> inhibitors. For the cell-based cleavage assay, the in-frame construction of the 3CL<sup>pro</sup>, the substrate, and the luciferase, designed as the plasmid pcDNA3.1-3CL<sup>pro</sup>-S-Luc, was co-transfected with the indicated vector pEGFP-N1 into Vero cells. The stable cell clone for the expression of the 3CL<sup>pro</sup>-substrate-luciferase fusion protein was selected by Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 800 μg/ml of neomycin G418 (Fig. 3A). Since a more than 30 kDa protein fused at the N-terminus of the luciferase resulted in a dramatic decrease of luciferase activity (Joubert et al., 2000), the detection of luciferase activity could be considered as a measure for the *cis*-cleavage by the SARS-CoV 3CL<sup>pro</sup>. Western blotting with the anti-luciferase monoclonal antibody showed a 94-kDa band for the fusion protein 3CL<sup>pro</sup>-S-Luc and a 60-kDa band for the luciferase in Vero cells transfected with the plasmid pcDNA3.1-3CL<sup>pro</sup>-S-Luc (data not shown). The relative luciferase activity in the transfected cells was subsequently measured using the dual Luciferase Reporter Assay System (Fig. 3B). In the cell-based cleavage assay, the extract of *I. indigotica* root significantly inhibited the *cis*-cleavage activity of the SARS-CoV 3CL<sup>pro</sup> with an IC<sub>50</sub>

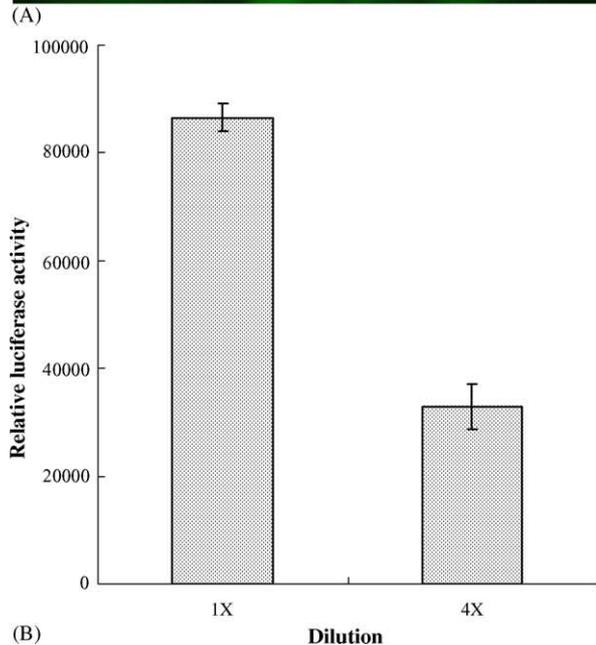
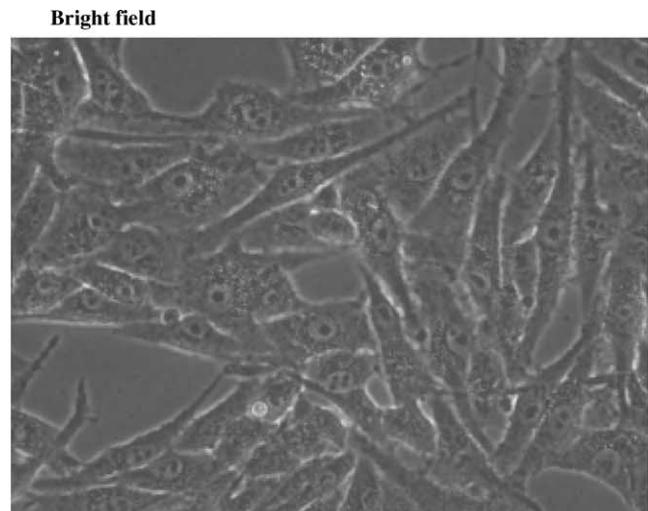


Fig. 3. Cell-based cleavage assay of the 3CL<sup>pro</sup> in Vero cells. (A) Vero cells were transfected with the plasmid containing the 3CL<sup>pro</sup>-substrate-luciferase in-frame gene plus the indicated vector pEGFP-N1. (B) Relative Luc activity in the dilution of transfected cell lysates was determined using the dual Luciferase Reporter Assay System and the Lumimeter TROPIX TR-717.

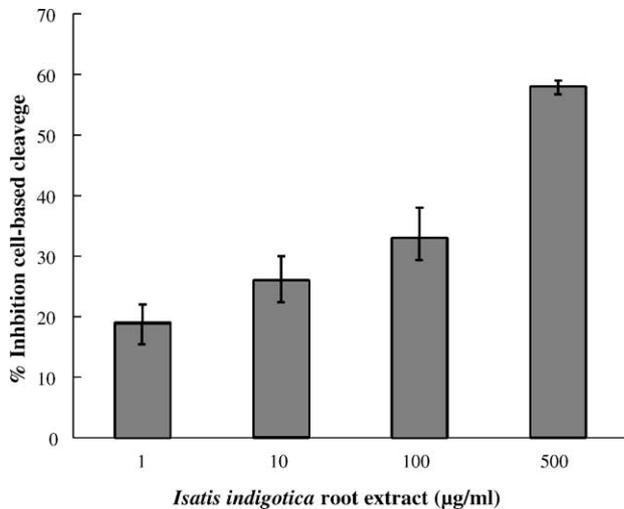


Fig. 4. Inhibition of the cell-based cleavage of the 3CL<sup>PRO</sup> by the *Isatis indigotica* root extract. Vero cells generating the 3CL<sup>PRO</sup>-substrate-luciferase fusion protein were treated with the indicated concentration of the *I. indigotica* root extract. Equal amounts (100 μg) of cell lysates were used to determine the luciferase activity (LUC) using the dual Luciferase Reporter Assay System. The relative inhibition of cell-based cleavage activity was calculated as  $1 - (LUC_{\text{with inhibitor}})/(LUC_{\text{without inhibitor}})$ .

of  $191.6 \pm 8.2$  μg/ml (Fig. 4; Table 1). The IC<sub>50</sub> value from cell-based assay by the *I. indigotica* root extract was twofold higher than the IC<sub>50</sub> value from the cell-free assay. The reason may be that the *I. indigotica* root extract could contain some inhibitory compounds that cannot permeate cellular membranes to reach intracellular SARS-CoV 3CL<sup>PRO</sup>. The results of cell-free and cell-based cleavage assays demonstrated that the *I. indigotica* root extract might contain potent anti-SARS-CoV 3CL<sup>PRO</sup> compounds.

The in vitro cytotoxicity profile of the *I. indigotica* root extract was examined using Vero cells. Vero cells in MEM medium with 10% FBS were plated in 96-well plates ( $5 \times 10^4$  cells/well) and then treated with the indicated compounds. After the treatment for 20 h, 25 μl of a MTT solution at 5 mg/ml was added to each well and incubated at 37 °C in 5% CO<sub>2</sub> for 3 h. After a three-time washing of phosphate buffer saline, 100 μl DMSO was then added into the plates for dissolving the formazan crystals. OD<sub>570–630</sub> in each well was then measured with a micro-ELISA reader. The result indicated that the 50% cytotoxic concentration (CC<sub>50</sub>) was greater than 5000 μg/ml.

Five major compounds of the *I. indigotica* root, including indigo, indirubin, indican, sinigrin, and beta-sitosterol were further tested for anti-SARS-CoV 3CL<sup>PRO</sup> action (Fig. 5, Table 1). Of the five compounds, sinigrin, beta-sitosterol and indigo dose-dependently inhibited cleavage activities of the 3CL<sup>PRO</sup> in cell-free and cell-based assays (Fig. 5, Table 1). The IC<sub>50</sub> in the cell-free assays was 115 μM for beta-sitosterol, 121 μM for sinigrin, and 300 μM for indigo. The cell-based assay indicated that sinigrin (IC<sub>50</sub>: 217 μM) was more efficient in blocking the cleavage processing of the 3CL<sup>PRO</sup> than indigo (IC<sub>50</sub>: 752 μM) and beta-sitosterol (IC<sub>50</sub>:

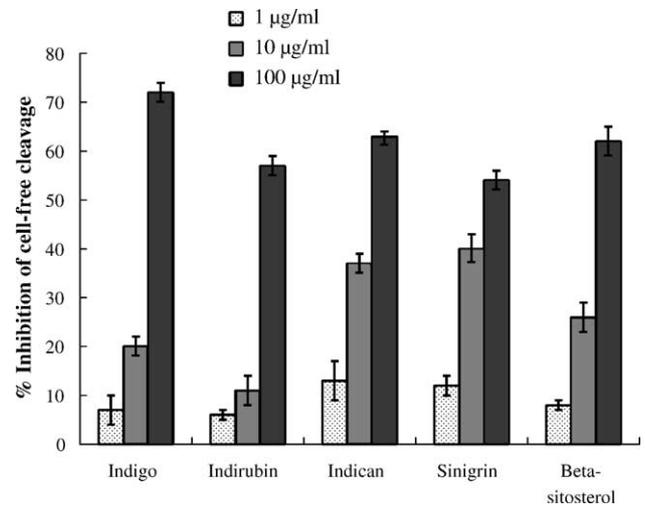


Fig. 5. Inhibition of the cell-free cleavage of the 3CL<sup>PRO</sup> by the compounds derived from *Isatis indigotica* root. The indicated compound was added into the mixture of the substrate fusion protein and the 3CL<sup>PRO</sup>, and then the uncleaved substrate was detected using the S protein-HRP conjugate and ABTS/H<sub>2</sub>O<sub>2</sub> substrates. The ELISA product was measured at A405 nm. The relative inhibition of cell-free cleavage activity was calculated as  $1 - (A405_{\text{no 3CL}^{\text{PRO}}} - A405_{\text{3CL}^{\text{PRO}} \text{ with inhibitor}})/(A405_{\text{no 3CL}^{\text{PRO}}} - A405_{\text{3CL}^{\text{PRO}}})$ .

1210 μM). Sinigrin showed a strong correlation between the effects on cell-free and cell-based cleavage of the SARS-CoV 3CL<sup>PRO</sup>. Moreover, indigo (CC<sub>50</sub>: 7.4 mM) and sinigrin (CC<sub>50</sub>: >10 mM) were not toxic to Vero cells. Sinigrin, an antioxidant, has been reported to possess inhibitory effects on quinine reductase and glutathione S-transferase, antiproliferative effects against cancer cells, and antimicrobial activity against *Bacillus subtilis* and *Saccharomyces cerevisiae* (Brabban and Edwards, 1995; Munday and Munday, 2002; Smith et al., 2004). This study is the first report in that sinigrin significantly blocks the cleavage processing of a viral protease.

Seven phenolic compounds, aloemodin, hesperetin, quercetin, naringenin, daidzein, emodin, and chrysohanol were also tested for their inhibitory effects on the SARS-CoV 3CL<sup>PRO</sup> (Fig. 6; Table 1). Only two of the phenolic compounds, aloemodin and hesperetin dose-dependently inhibited cleavage activity of the 3CL<sup>PRO</sup> in cell-free and cell-based assays (Fig. 6; Table 1). In the cell-free assay, the IC<sub>50</sub> values were 132 μM for aloemodin and 60 μM for hesperetin. Quercetin has been reported to block the entry of SARS-CoV into host cells (Yi et al., 2004). However, no inhibitory effect on SARS-CoV 3CL<sup>PRO</sup> was found for quercetin in the cell-free and cell-based cleavage assays. Interestingly, hesperetin (CC<sub>50</sub>: 2.7 mM) had an IC<sub>50</sub> of 8.3 μM in the cell-based assay (Table 1). Hesperetin is poorly soluble in water; so, hesperetin was less inhibitory in the cell-free assay than in the cell-based assay. The finding of the anti-3CL<sup>PRO</sup> effects of hesperetin at the micromolar range was consistent with a previous report indicating that hesperetin had an inhibitory activity on Sindbis virus infection with an IC<sub>50</sub> of 20.5 μg/ml (about 68 μM) by plaque assay (Paredes

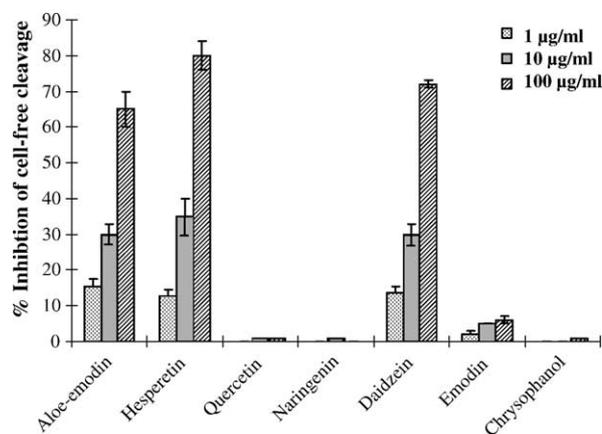


Fig. 6. Inhibition of the cell-free cleavage of the 3CL<sup>pro</sup> by the phenolic compounds. The indicated compound was added into the mixture of the substrate fusion protein and the 3CL<sup>pro</sup>, and then the uncleaved substrate was detected. The relative inhibition of cell-free cleavage activity was calculated as  $1 - (A_{405}_{no\ 3CL^{pro}} - A_{405}_{3CL^{pro}\ with\ inhibitor}) / (A_{405}_{no\ 3CL^{pro}} - A_{405}_{3CL^{pro}})$ .

et al., 2003). Of the compounds tested, hesperetin was the most potent inhibitor of SARS-CoV 3CL<sup>pro</sup> (Table 1).

Our results have demonstrated significantly inhibitory effects on SARS-CoV 3CL<sup>pro</sup> by *I. indigotica* root extract, indigo, sinigrin, aloemodin and hesperetin in the micromolar range. Particularly, the cell-based assay demonstrated that hesperetin (IC<sub>50</sub>: 8.3 µM) and sinigrin (IC<sub>50</sub>: 217 µM) could be potential inhibitors of SARS-CoV 3CL<sup>pro</sup>. In addition, sinigrin and hesperetin with a CC<sub>50</sub> of over 2 mM were considerably less cytotoxic to Vero cells (Table 1). Akin to other reported anti-SARS substances, such as glycyrrhizin (Cinatl et al., 2003a), nelfinavir (Yamamoto et al., 2004), aurintricarboxylic acid (He et al., 2004), and interferon (Cinatl et al., 2003b), the compounds reported here may be considered as potential leads in the development of inhibitors of SARS-CoV 3CL<sup>pro</sup>.

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